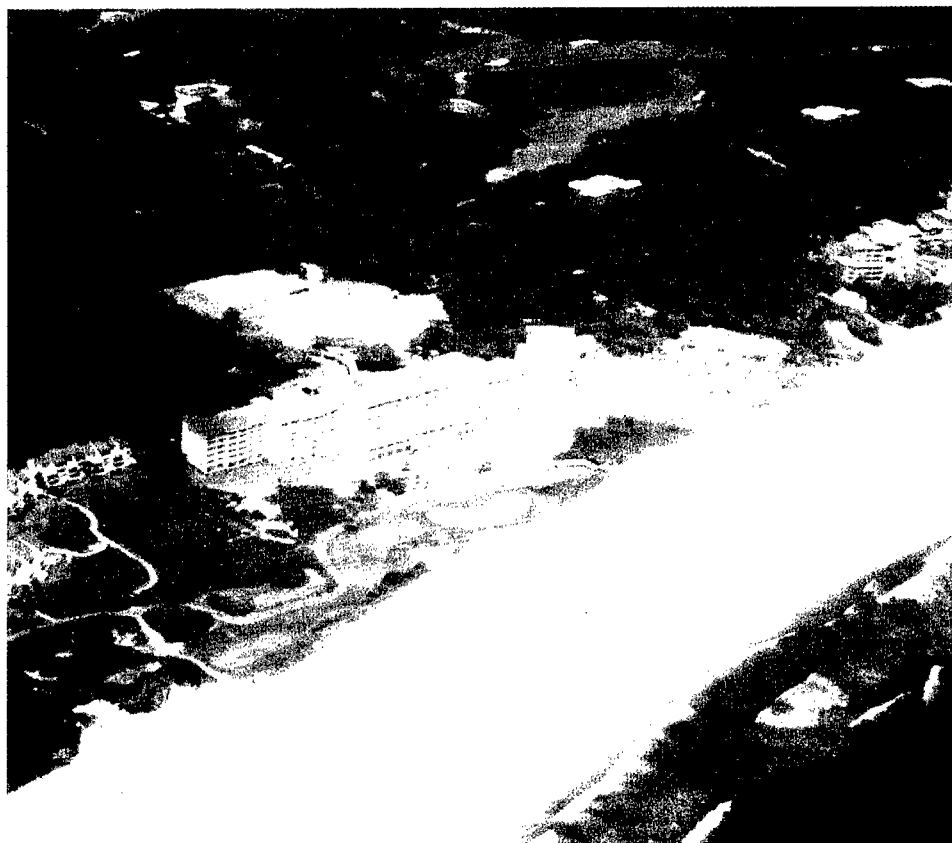


Program and Abstracts

Seventh Meeting
Society for Research on
Biological
 Rhythms



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May 10-13, 2000
Amelia Island Plantation
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Jacksonville, Florida

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13. ABSTRACT (Maximum 200 words) With partial support from AFOSR, the Seventh Meeting of the Society for Research on Biological Rhythms (SRBR) took place in Jacksonville, Florida, May 10-13, 2000. More than 280 scientific papers and symposia were presented. There was strong participation by graduate students, especially in the poster sessions. Review lectures and tutorials were given on the current state of knowledge on topics such as Molecular Approaches to Circadian Clocks, Sleep Methodology, and Chronotherapeutics. A session on The Genetic Basis of Circadian Sleep Regulation was held to inform scientists who are not SRBR members, but whose work draws upon this field. Abstracts from the meeting were published in a 167-page book provided to all registrants. The SRBR executive committee responsible for this meeting was composed of Jay C. Dunlap (Dartmouth), Rae Silver (Columbia), Carl Johnson (Vanderbilt), Terry Page (Vanderbilt), Serge Daan (Rijksuniversiteit, Gronigen), Fred Davis (Northwestern), and Eberhard Gwinner (Max Planck Institut fur Verhaltensphysiologie).			
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List of attendees, SRBR 2000, with type of registration

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Aeschbach, Daniel	Speaker Non SRBR
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Albrecht, Urs	Regular Non-member
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Schwartz, William	Speaker/Regular	
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Shaw, Paul J.	Regular Non-member	
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Tischkau, Shelly	Speaker/Non-member	
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Tosini, Gianluca	Speaker/Regular	
Tsirline, Victor B.	Student Non-member	
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Program and Abstracts

for the
Seventh Meeting
of the
Society for Research on

Biological Rhythms

Amelia Island Plantation Conference Center
Jacksonville, Florida
May 10-13, 2000

The SRBR wishes to thank the following for their contributions:

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General Information

President's Welcome

Well here we are again, and it is a good thing to be able to look forward to another stretch of 4 days when one's prime duties are to think about our science and to meet with old and new friends and colleagues.

The field of Biological Rhythms Research has never been stronger, both in its breadth and depth, and the progress we have made as a field is now serving as a launch pad for insightful work in a variety of related disciplines. The Program Committee under the energetic leadership of Steve Reppert has developed a diverse but well integrated mix of presentations including reviews and tutorials to educate, workshops to discuss, and centered on the symposia, slide, and poster sessions where the cutting edge of chronobiology will be the focus. There is truly something here for everyone, and the Organizing Committee directed through the

untiring efforts of Mary Hemdon has made certain that we'll all enjoy the experience.

We are blessed by being able to work on one of the great scientific problems of the last century and the next, a topic that enjoys consistent and well deserved visibility in both the lay and scientific press. This is a tremendous benefit and also a challenge to us to return to our international communities a measure of what we have been granted. On behalf of the Society, I bid you Welcome and trust that you will enjoy the time here, leaving with renewed enthusiasm for studying clocks and using the knowledge to make Earth a better place for all to live.

Jay C. Dunlap
President, SRBR

Registration

Meeting registration will take place on Wednesday, May 10, from 12:00 until 19:00 at the Amelia Island Plantation Executive Conference Center. Hotel check-in will be facilitated at the Conference Center by Amelia Island staff at the on-site registration desk.

Early Registration: Postmarked by March 20, 2000

Regular SRBR Members	\$185.00
Non-SRBR Members	\$235.00
Student Members	\$135.00
Student Non-Members	\$160.00
Guest Registration	\$85.00

Late Registration: Postmarked after March 20, 2000

Regular SRBR Members	\$210.00
Non-SRBR Members	\$260.00
Student Members	\$160.00
Student Non-Members	\$185.00
Guest Registration	\$105.00

Membership rates apply to individuals who have joined SRBR before the meeting and have paid current dues. Student rates apply to registrants who will not have received their Ph.D. or M.D. degree at the time of the meeting. Registered participants and guests are invited to an opening reception, evening social activities and banquet.

SRBR Information and Message Desk

The Society will maintain an information desk in the Executive Conference Center from 08:00 to 11:00 on May 11-13, and from 14:30 to 16:30 on May 11-12. Late arrivals can register during these times. A message board will be located next to the information desk. Check this board for mail, notes, and telephone messages.

Where to Eat

There are a variety of restaurants available, designed to meet the requirements of any palate. The *Amelia Inn Restaurant*, Amelia's flagship restaurant, features 5-course gourmet dining. Reservations are required for dinner, as well as jackets for gentlemen. The *Amelia Inn Restaurant* is not open for lunch. The *Beach Club Grill* offers poolside casual fare; no reservations or special dress required. The *Verandah*, Amelia's seafood restaurant, is informally styled and located in Racquet Park. The *Coop* and the *Golf Shop Restaurant* offer light fare and take-out options. For more information, and for other restaurant locations, check your "Passport" guide.

Amelia's Village Store supplies most general store items. If you wish to take the 15 minute trip to Fernandina Beach, a variety of grocery and other stores are located there.

To provide timely lunch service for meeting attendees during the relatively short lunch break on Thursday, the Amelia Inn Restaurant will offer a buffet lunch on Thursday afternoon, May 11.

Shuttle Service

Amelia offers an on-property shuttle service at no extra charge. Regularly scheduled shuttles will run between villas and meeting areas before and after each session, as well as social events. During these times the trams will make several tours of the grounds. Anyone wishing transportation should wait by the tram stop, and the bus will stop by to pick you up. If you phone for shuttle service, you must wait at the tram stop for the bus. A brief wait of up to 15 minutes may be needed.

Amelia's Facilities

The whole gamut of outdoor activities, tennis, horseback riding, sailing, excursions, fishing, golf and more are available at Amelia. Wheels 'n Keels has a supply of rental vehicles: bicycles, paddle-boats, island hoppers, and automobiles. The Health and Fitness Center also provides racquetball, an indoor lap pool, sauna, Jacuzzi, and exercise equipment. Programs for children and baby-sitting services are available.

Social Events

The Opening Reception will begin at 19:30 on Wednesday, May 10, at the Beach Club Deck/Pool. A banquet dinner for SRBR meeting attendees and registered guests will be held in the Amelia Ballroom on Saturday, May 13, at 20:00.

Instructions to Presenters

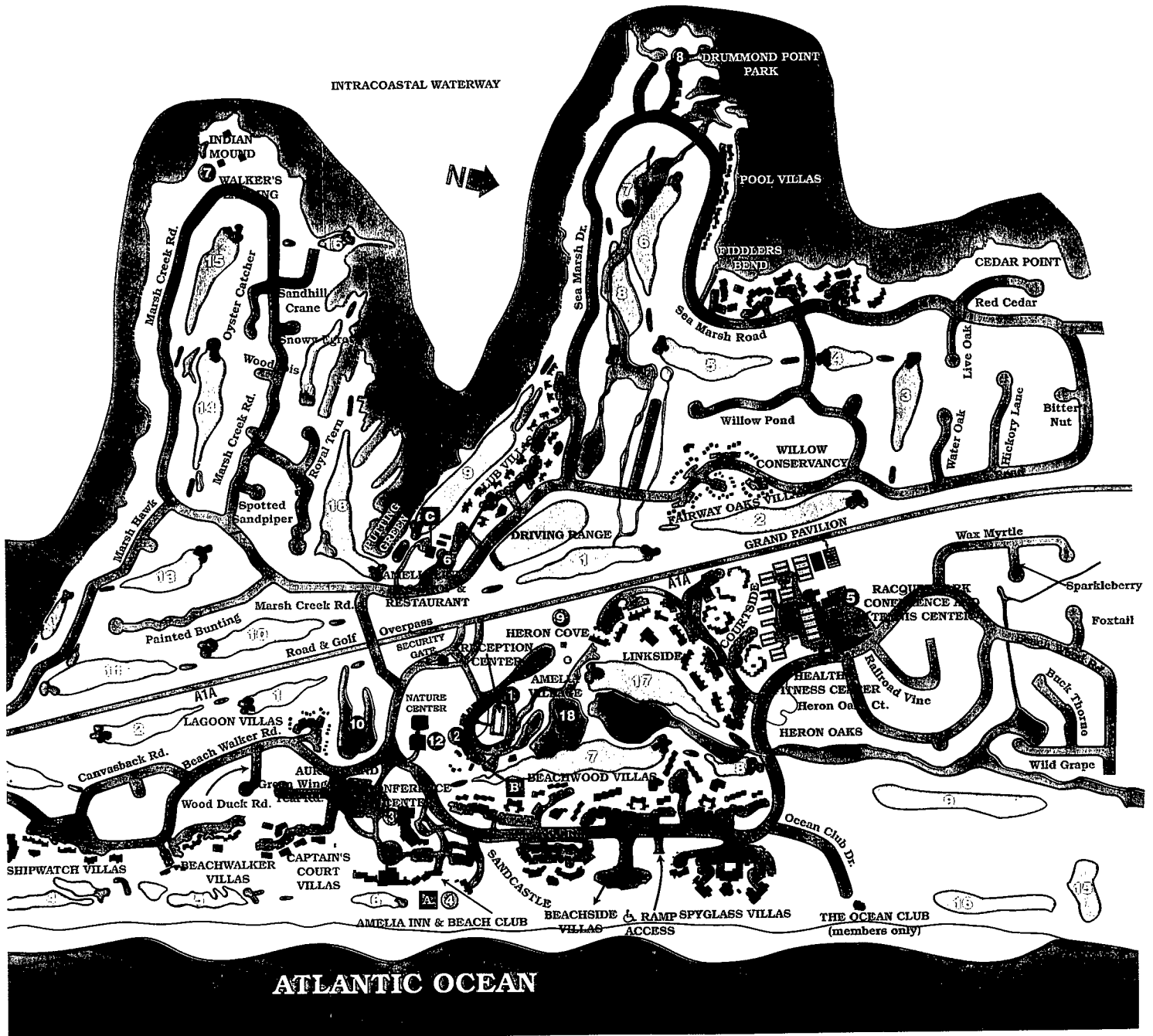
Posters should be assembled between 13:00 and 16:30 on Thursday, May 11 (Group A) or Friday, May 12 (Group B). Authors are requested to attend their posters from 20:00 - 22:00 on Thursday, May 11 (Group A) or Friday, May 12 (Group B). Posters presented by Group A should be removed by 13:00 on Friday. Group B posters should be removed on Saturday by 14:00.

Slide talk presenters should give their slides (clearly numbered and in correct order) to the projectionist 15 minutes before the start of the session.

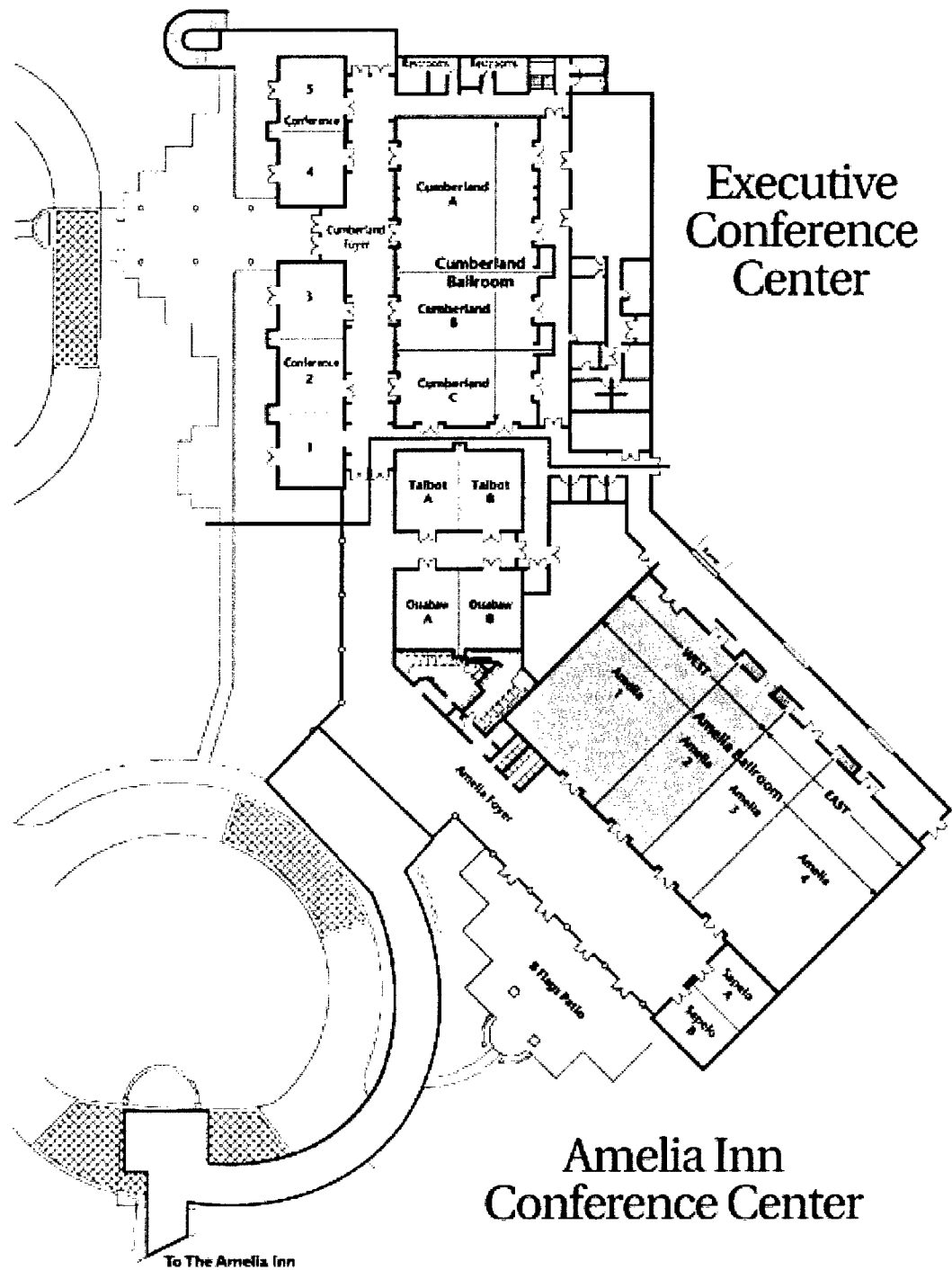
Airport Transportation

Amelia Island Plantation provides professional ground transportation service between Amelia Island Plantation and the Jacksonville International Airport. Cancellations must be made 48 hours in advance. The shuttle to the airport departs the Reception Area 1½ - 2 hours prior to flight time. Call 5920 to confirm your departure time.

Amelia Island



Session Rooms



Program Schedule

WEDNESDAY, MAY 10

12:00-18:00 Registration:
Conference Room 4/5

19:30-21:30 Opening Reception:
Beach Club Deck/Pool

THURSDAY, MAY 11

08:30-10:30 Symposia 1-3:

- 1) *Molecular I*
Cumberland Ballroom
- 2) *Cellular Mechanisms in the SCN*
Talbot Conference Room
- 3) *Complexities of Human Circadian Physiology*
Ossabaw Conference Room

10:30-11:00 Coffee Break - Patio

11:00-13:00 Slide Sessions 1-3:

- 1) *Clock Genes I: Deciphering Molecular Mechanisms*
Cumberland Ballroom
- 2) *Human Rhythms I: Photic Entrainment*
Talbot Conference Room
- 3) *Phase Shifting and Entrainment*
Ossabaw Conference Room

13:00-14:30 Break: put up posters (Group A)
Amelia Ballroom

14:30-16:00 SRBR Tutorials:

- 1) *Molecular Approaches to Circadian Clocks*
Cumberland Ballroom
- 2) *Sleep Methodology*
Talbot Conference Room
- 3) *Grant Writing*
Ossabaw Conference Room

16:30-18:30 Workshops 1-4:

- 1) *Diurnality: Definitions and Underlying Mechanisms*
Cumberland Ballroom
- 2) *Rhythms and Melatonin in Higher Plants*
Cumberland C
- 3) *Analysis of Rhythms: Constant Routines and Purification Methods*
Talbot Conference Room
- 4) *Criteria for and Conservation of Components in Circadian Systems*
Ossabaw Conference Room

20:00-22:00 Poster Presentations (Group A)
Amelia Ballroom

FRIDAY, MAY 12

08:30-10:30 Symposia 4-6:

- 4) *Molecular II*
Cumberland Ballroom
- 5) *Circadian Photoreceptors*
Talbot Conference Room
- 6) *Construction of Circadian Models*
Ossabaw Conference Room

10:30-11:00 Coffee Break - Patio

11:00-13:00 Slide Sessions 4-6:

- 4) *Clock Genes II: Temporal Analysis of Clock Gene Expression*
Cumberland Ballroom
- 5) *Human Rhythms II: Sleep and Jet Lag*
Talbot Conference Room
- 6) *Molecular Mechanisms of Circadian Timing I*
Ossabaw Conference Room

13:00-16:30 Break: Put up posters (Group B)

16:30-18:00 Review Lectures:

- 1) *Photoperiodism*
- 2) *Chronotherapeutics*
Cumberland Ballroom

20:00-22:00 Poster Presentations (Group B)
Amelia Ballroom

SATURDAY, MAY 13

08:30-10:30 Symposia 7-9:

- 7) *Sleepy Genes*
Cumberland Ballroom
- 8) *Control and Regulation of Circadian Outputs*
Talbot Conference Room
- 9) *Photoperiodism and Melatonin*
Ossabaw Conference Room

10:30-11:00 Coffee Break - Patio

11:00-13:00 Slide Sessions 7-9:

- 7) *SCN Physiology*
Cumberland Ballroom
- 8) *Molecular Basis of Circadian Timing II*
Talbot Conference Room
- 9) *Physiological Aspects of Circadian Timing*
Ossabaw Conference Room

13:00-16:30 Break

16:30-18:00 Pittendrigh/Aschoff Lecture
Cumberland Ballroom

18:00-19:00 SRBR Business Meeting
Cumberland Ballroom

20:00-22:00 Banquet Dinner
Amelia Ballroom

Scientific Program

THURSDAY, MAY 11

08:30-10:30 Cumberland Ballroom
Symposium 1
Molecular I

Chair: Michael Young
Rockefeller University

Speakers: Isaac Edery
Rutgers University
The Drosophila circadian clock, a timekeeping device for all seasons

Jay Dunlap
Dartmouth University
PAS proteins and feedbacks in the Neurospora clock

Cheng Chi Lee
Baylor College of Medicine
Circadian clock behavior of mPer1 and mPer2 mutants

Steven Reppert
Harvard Medical School
Interacting molecular loops in the mammalian clock

08:30-10:30 Talbot Conference Room
Symposium 2
Cellular Mechanisms in the SCN

Chair: Martha Gillette
University of Illinois, Urbana-Champaign

Speakers: William Schwartz
University of Massachusetts Medical School
Morning and evening circadian oscillations revealed in the SCN in vitro

Michael Hastings
Cambridge University
Period genes as targets for clock resetting by light and behavioral cues

Karl Obrietan
University of Washington
Circadian regulation of cAMP response element-mediated expression in the SCN

Shelly Tischkau
University of Illinois, Urbana-Champaign
Cellular mechanisms for glutamateric regulation of the SCN

08:30-10:30 Ossabaw Conference Room
Symposium 3
Complexities of Human Circadian Physiology

Chair: Derk-Jan Dijk
University of Surrey

Speakers: Kenneth Wright, Jr.
Harvard Medical School
Entrainment of the human circadian pace-maker to a dim light and rest-activity cycle

Daniel Aeschbach
National Institute of Mental Health
Circadian correlates of short-sleep and long sleep: Inter-individual differences in alpha:rho?

Christian Cajochen
University of Basel
Quality of waking: Measures and mediators of its circadian control

Debra Skene
University of Surrey
Entrainment: Role of ocular light and its spectral composition

10:30-11:00 Coffee Break
Conference Center Patio

11:00-13:00 Cumberland Ballroom
Slide Session 1
Clock Genes I: Deciphering Molecular Mechanisms

Chair: Carla Green
University of Virginia

11:00

1 THE HAMSTER *tau* LOCUS IS ENCODED BY CASEIN KINASE I EPSILON (CKI ϵ) A HOMOLOG OF THE *DROSOPHILA* CIRCADIAN GENE, *double-time*. Phillip L. Lowrey, Neurobiology & Physiology, Northwestern University, Evanston, IL.

11:15

2 POTENTIAL ROLE OF CASEIN KINASE I ϵ IN THE REGULATION OF MAMMALIAN CIRCADIAN RHYTHM. David Virshup, Pediatrics & Oncological Sciences, Huntsman Cancer Institute, University of Utah, Salt Lake City, UT.

11:30

3 A TIMELESS-INDEPENDENT FUNCTION OF PERIOD PROTEINS IN THE *DROSOPHILA* CLOCK. Lino Saez, Laboratory of Genetics, Rockefeller University, New York, NY.

11:45

4 KNOCKOUT OF THE MURINE *TIMELESS* GENE ARRESTS EMBRYONIC DEVELOPMENT. Anthony L. Gotter, Laboratory of Developmental Chronobiology, Massachusetts General Hospital, Boston, MA.

12:00

5 CLONING AND CHARACTERIZATION OF *ZEITLUPE*, A NOVEL COMPONENT OF THE PLANT CIRCADIAN CLOCK SYSTEM. David E. Somers, Plant Biotechnology Center, Ohio State University, Columbus, OH.

- 12:15
6 THE *DROSOPHILA TAKEOUT* GENE IS A NOVEL MOLECULAR LINK BETWEEN CIRCADIAN RHYTHMS AND FEEDING BEHAVIOR. L. Sarov-Blat, Dept of Biology, Brandeis University, Waltham, MA.
- 12:30
7 THE XPER2 GENE IN *XENOPUS LAEVIS* RETINA IS PRIMARILY DRIVEN BY LIGHT. Brooke Steenhard, Cell Biology, Neurobiology & Anatomy, Medical College of Wisconsin, Milwaukee, WI.
- 12:45
8 CHARACTERIZATION OF A NOVEL CIRCADIAN GENE ISOLATED FROM A CHICK PINEAL cDNA LIBRARY. James Olcese, Hormone & Fertility Research, University of Hamburg, Germany.
- 11:00-13:00 Talbot Conference Room
Slide Session 2
Human Rhythms I: Photic Entrainment
Chair: Susan Benloucif
Northwestern University
- 11:00
9 SPECTRAL SENSITIVITY OF MELATONIN SUPPRESSION IN HUMANS. Kavita Thapan, School of Biological Sciences, University of Surrey, Guildford, Surrey, United Kingdom.
- 11:15
10 CIRCADIAN PHOTORECEPTION IN HUMANS: ACTION SPECTRUM FOR MELATONIN SUPPRESSION. George Brainard, Dept of Neurology, Thomas Jefferson University, Philadelphia, PA.
- 11:30
11 ENTRAINMENT OF TOTALLY BLIND SUBJECTS: PHOTIC OR NON-PHOTIC? Steven W. Lockley, School of Biological Sciences, University of Surrey, Guildford, Surrey, United Kingdom.
- 11:45
12 A PHASE RESPONSE CURVE TO SINGLE BRIGHT LIGHT PULSES IN HUMANS. Sat Bir S. Khalsa, Circadian, Neuroendocrine and Sleep Disorders Section, Brigham & Women's Hospital, Boston, MA.
- 12:00
13 EFFECTS OF TIMED ONE HOUR PULSES OF BRIGHT BROAD SPECTRUM WHITE LIGHT AT NIGHT ON PERFORMANCE, ALERTNESS AND MELATONIN SUPPRESSION. Katherine Hoppen, School of Biological Sciences, University of Surrey, Guildford, Surrey, United Kingdom.
- 12:15
14 ASSOCIATION BETWEEN 24-H LIGHT EXPOSURE AND MELATONIN SECRETION IN NIGHT WORKERS. Marie Dumont, Sacre-Coeur Hospital, University of Montreal, Quebec, Canada.
- 12:30
15 EFFECT OF LIGHT HISTORY ON LIGHT SENSITIVITY IN HUMANS. Marc Hebert, Dept of Ophthalmology, Royal Alexandra Hospital, Edmonton, Alberta, Canada.
- 12:45
16 TIMING OF BRIGHT LIGHT TO NORMALISE CIRCADIAN PHASE IN OLDER INDIVIDUALS WITH INSOMNIA AND DEPRESSION. Shawn D. Youngstedt, Dept of Psychiatry, Univ of California San Diego, La Jolla, CA.
- 11:00-13:00 Ossabaw Conference Room
Slide Session 3
Phase Shifting and Entrainment
Chair: Elizabeth Maywood
University of Cambridge
- 11:00
17 GLUCOCORTICOIDS CAN RESET CIRCADIAN TIME IN PERIPHERAL TISSUES BUT NOT IN THE SCN. Aurelio Balsalobre, Dept of Molecular Biology, University of Geneva, Switzerland.
- 11:15
18 RESTRICTED FEEDING ENTRAINS CIRCADIAN RHYTHMICITY IN THE LIVER. Karl-Arne Stokkan, Dept of Biology, University of Virginia, Charlottesville, VA.
- 11:30
19 EFFECTS OF AN ENVIRONMENTAL TOXICANT ON THE BIOLOGICAL CLOCK. Dani L. Binegar, Dept of Pharmacology, Texas Tech University Health Sciences Center, Lubbock, TX.
- 11:45
20 THE EFFECT OF CENTRAL INFUSIONS OF NPY ON THE EXPRESSION OF CLOCK GENES IN THE SUPRACHIASMATIC NUCLEI OF THE MOUSE. Elizabeth S. Maywood, Dept of Anatomy, University of Cambridge, United Kingdom.
- 12:00
21 NEUROPEPTIDE Y APPLIED IN VITRO CAN BLOCK THE PHASE SHIFTS INDUCED BY LIGHT IN VIVO. Mary E. Harrington, Dept of Psychology, Smith College, Northampton, MA.
- 12:15
22 PHOTIC PHASE RESPONSE CURVE AND LIGHT-DARK MASKING OF CIRCADIAN RHYTHMS IN *OCTODON DEGUS*: ASSESSMENT AS A FUNCTION OF ACTIVITY PHASE PREFERENCE. Martien J.H. Kas, Sleep Research Ctr, Stanford University, Palo Alto, CA.
- 12:30
23 CIRCADIAN PHOTIC SENSITIVITY IS SEXUALLY DIMORPHIC IN *OCTODON DEGUS*. Theresa M. Lee, Dept of Psychology, University of Michigan, Ann Arbor, MI.
- 12:45
24 A TWO-COMPONENT MODEL EXPLAINS WHY SIBERIAN HAMSTERS FAIL TO REENTRAIN TO A PHASE-SHIFT OF THE PHOTOCYCLE. Norman F. Ruby, Biological Sciences, Stanford University, CA.
- 13:00-14:30 Break: Put up posters (Group A)
Amelia Ballroom
- 14:30-16:00 Cumberland Ballroom
Tutorial 1
Steve Kay
Scripps Research Institute
Functional Genomics: What is it and why should clock researchers care?
- 14:30-16:00 Talbot Conference Room
Tutorial 2
Rod Hughes
Harvard Medical School
Sleep Methodology

14:30-16:00 Ossabaw Conference Room
Student Seminar
Israel Lederhendler
National Institutes of Health
Writing Grant Proposals

16:00-16:30 Break

16:30-18:30 Cumberland Ballroom
Workshop 1
Diurnality: Definitions and Underlying Mechanisms

Discussion

Leader: Laura Smale
Michigan State University

16:30-18:30 Talbot Conference Room
Workshop 2
Rhythms and Melatonin in Higher Plants

Discussion

Leader: Birgit Piechulla
University of Rostock

16:30-18:30 Ossabaw Conference Room
Workshop 3
Analysis of Rhythms: Constant Routines and Purification Methods

Discussion

Leader: James Waterhouse
Liverpool John Moores University

16:30-18:30 Cumberland C
Workshop 4
Criteria for and Conservation of Components in Circadian Systems

Discussion

Leaders: Carl Johnson
Vanderbilt University
Jennifer Loros
Dartmouth University
W. Otto Friesen
University of Virginia

18:30-20:00 Break

20:00-22:00 Poster Presentations, Group A
Amelia Ballroom

Molecular Biology-Vertebrates

25 STRUCTURAL AND FUNCTIONAL ANALYSIS OF mPER1 : CASEIN KINASE I : mCRY1 INTERACTIONS. Erik Eide, Pediatrics & Oncological Sciences, Huntsman Cancer Institute, University of Utah, Salt Lake City, UT.

26 ASSOCIATION AND NUCLEAR ENTRY OF mPER PROTEINS IN MAMMALIAN CELLS. Hitoshi Okamura, Anatomy & Brain Science, Kobe University School of Medicine, Kobe, Japan.

27 CHARACTERIZATION OF MICE WITH TARGETED DISRUPTION OF THE *mPer3* GENE. Lauren P. Shearman, Developmental Chronobiology, Massachusetts General Hospital, Boston, MA.

28 MOLECULAR INTERACTIONS BETWEEN MAMMALIAN CLOCK PROTEINS. Nobuya Koike, Human Genome Center, University of Tokyo, Japan.

29 IDENTIFICATION OF FUNCTIONAL DOMAINS OF HUMAN PERIOD 1 (PER1) PROTEIN. Yong Guo, Dept of Neuroscience, Aventis Pharmaceuticals, Bridgewater, NJ.

30 FIVE CONSERVED E-BOXES ADDITIVELY CONTRIBUTE TO THE ENHANCEMENT OF *mPer1* TRANSCRIPTION. Akiko Hida, Human Genome Center, University of Tokyo, Japan.

31 CONSTITUTIVE EXPRESSION OF MOUSE *per1* LENGTHENS THE CIRCADIAN PERIOD AND DISRUPTS ENTRAINMENT IN RAT. Rika Numano, Human Genome Center, University of Tokyo, Japan.

32 SIGNAL TRANSDUCTION PATHWAYS INCLUDING RAPID RESPONSE AND CIRCADIAN EXPRESSION OF *Per1* AND *Per2* mRNA IN RAT-1 FIBROBLASTS. Kazuhiro Yagita, Anatomy & Brain Science, Kobe Univ School of Medicine, Kobe, Japan.

33 A RECESSIVE SCREEN FOR MOUSE CIRCADIAN RHYTHM MUTANTS. Sandra M. Siepka, Neurobiology & Physiology, Northwestern University, Evanston, IL.

34 IDENTIFICATION AND CHARACTERIZATION OF A ZEBRAFISH CLOCK MUTANT. Ying Tan, Dept of Biology & Biochemistry, University of Houston, Houston, TX.

35 GENETIC ANALYSIS OF CIRCADIAN RHYTHMICITY IN ZEBRAFISH. Jason DeBruyne, Dept of Biology & Biochemistry, University of Houston, Houston, TX.

36 RESTRICTED DISTRIBUTION AND RHYTHMICITY OF CLOCK-IMMUNOREACTIVITY IN THE SCN: IMPLICATIONS FOR CIRCADIAN OUTPUTS CONTROLLING BEHAVIORAL RHYTHMS. Michael Lehman, Cell Biology, Neurobiology & Anatomy, University of Cincinnati College of Medicine, Cincinnati, OH.

37 TRANSCRIPTIONAL PROFILING OF CENTRAL AND PERIPHERAL MAMMALIAN CIRCADIAN CLOCKS. Giles E. Duffield, Dept of Biochemistry & Genetics, Dartmouth Medical School, Hanover, NH.

38 EXPRESSION AND REGULATION OF PER1 AND PER2 IN THE MAMMALIAN PINEAL GLAND. Chiaki Fukuhara, Neuroscience Institute, Morehouse School of Medicine, Atlanta, GA.

39 CIRCADIAN EXPRESSION AND ADRENERGIC REGULATION OF *Per1* AND *Per2* IN THE RAT PINEAL GLAND. Seiichi Takekida, Anatomy & Brain Science, Kobe University School of Medicine, Japan.

- 40 PER1 IS EXPRESSED IN MOUSE SPERMATOCYTES AT ALL TIMES OF DAY. Eric L. Bittman, Dept of Biology, University of Massachusetts, Amherst, MA.
- 41 MOLECULAR ANALYSIS OF AVIAN CIRCADIAN CLOCK. Takashi Yoshimura, Lab of Biomodelling, Nagoya University, Japan.
- 42 CLONING AND INITIAL CHARACTERIZATION OF CHICKEN CLOCK GENES. Toshiyuki Okano, Dept of Biophysics & Biochemistry, University of Tokyo, Japan.

SCN Anatomy/Pharmacology

- 43 LEPTIN RECEPTOR IMMUNOREACTIVITY IN THE RAT SUPRACHIASMATIC NUCLEUS. Harriet E. Bergeron, Biochemistry, Cell & Molecular Biology, University of Tennessee, Knoxville, TN.
- 44 HYPOCRETIN-LIKE IMMUNOREACTIVITY IN THE SYRIAN HAMSTER CIRCADIAN SYSTEM. H. Elliott Albers, Dept of Biology, Georgia State University, Atlanta, GA.
- 45 THE SUPRACHIASMATIC NUCLEUS DOES NOT CONTAIN THE P-75 NGF RECEPTOR BUT DOES CONTAIN BDNF IN THE DIURNAL RODENT *ARVICANTHIS NILOTICUS*. Heather Ross, Dept of Zoology, Michigan State University, East Lansing, MI.
- 46 DISTRIBUTION OF SUBSTANCE-P AND NEUROKININ-1 RECEPTOR IMMUNOREACTIVITY IN THE SUPRACHIASMATIC NUCLEI AND INTERGENICULATE LEAFLET OF FOUR RODENT SPECIES. Rayna E. Samuels, School of Biological Sciences, University of Manchester, U.K..
- 47 NEUROANATOMICAL STUDY OF THE RETINOHYPOTHALAMIC AFFERENTS IN MICE DURING POSTNATAL DEVELOPMENT. Daniela Lupi, Cerveau et Vision, INSERM U371, Bron, France.
- 48 CIRCADIAN OSCILLATIONS IN MEMORY FUNCTION: CORRESPONDENCE TO CHANGES IN MUSCARINIC CHOLINERGIC RECEPTOR DENSITY IN THE RAT SUPRACHIASMATIC NUCLEUS? B. A. M. Biemans, Zoological Laboratory, University of Groningen, Haren, The Netherlands.
- 49 IGL LESIONS ABOLISH NPY FIBERS IN THE SCN OF *ARVICANTHIS NILOTICUS*. Betty H. Gubik Dept of Psychology, Michigan State University, East Lansing, MI.
- 50 ANGIOTENSIN 1A RECEPTOR NULL MUTATION: EFFECTS ON CIRCADIAN RHYTHMS IN THE MOUSE. R. E. Mistlberger, Dept of Psychology, Simon Fraser University, Burnaby BC, Canada.

In Vitro Analysis of Clock Cellular Biology

- 51 VASOACTIVE INTESTINAL POLYPEPTIDE (VIP) PHASE SHIFTS THE FIRING RATE RHYTHM OF RAT SCN NEURONES *IN VITRO*. Helen E. Reed, School of Biological Sciences, University of Manchester, U.K.

- 52 ANISOMYCIN INHIBITS SEROTONERGIC PHASE ADVANCES OF THE SCN CIRCADIAN CLOCK *IN VITRO*. A. Jovanovska, Biochemistry, Cell & Molecular Biology, University of Tennessee, Knoxville, TN.
- 53 ADENOSINE MODULATION OF GLUTAMATERGIC NEUROTRANSMISSION IN THE SCN. Michael A. Rea, Dept of Biology & Biochem, University of Houston, TX.
- 54 PHASE SHIFTS OF THE CIRCADIAN BIOLOGICAL CLOCK INDUCED BY 5-HT₇ AGONISTS AND INVERSE AGONISTS: RELATIONSHIP TO cAMP PRODUCTION. Jeffrey Sprouse, Pfizer Central Research, Groton, CT.
- 55 THE NEUROPEPTIDE Y Y5 RECEPTOR MEDIATES THE BLOCKADE OF "PHOTIC-LIKE" NMDA-INDUCED PHASE SHIFTS. Paola C. Yannielli, Dept of Psychology, Smith College, Northampton, MA.
- 56 A CODE FOR LIGHT: NOCTURNAL GLUTAMATE-PACAP INTERACTIONS REGULATE SHIFT AMPLITUDE OF THE SUPRACHIASMATIC CIRCADIAN CLOCK. Martha U. Gillette, Cell & Structural Biology, University of Illinois, Urbana, IL.
- 57 ACTIVATION OF PROTEIN KINASE A: A CELLULAR MECHANISM FOR NOCTURNAL GLUTAMATE-PACAP INTERACTIONS IN THE SCN. Shelley Tischkau, Cell & Structural Biology, University of Illinois, Urbana, IL.
- 58 HISTAMINE AND SUBSTANCE-P RESET THE CIRCADIAN CLOCK THROUGH ACTIVATION OF THE NITRIC OXIDE PATHWAY. Jian Ding, Hennepin County Medical Center, University of Minnesota, Minneapolis, MN.
- 59 RHYTHMIC COUPLING AMONG CELLS IN THE SUPRACHIASMATIC NUCLEUS. Christopher S. Colwell, Dept of Psychiatry, NPI, Univ of California, Los Angeles, CA.
- 60 CONSTRUCTING SUPRACHIASMATIC NUCLEUS (SCN) CHIMERAS *IN VIVO*. Masato Mitome, Dept of Neurology, University of Massachusetts Medical School, Worcester, MA.
- 61 BIOLUMINESCENCE IMAGING OF INDIVIDUAL CELLS IN TRANSGENIC MOUSE SCN. Laura Sigworth, Dept of Biological Sciences, Bowling Green State Univ, Bowling Green, OH.
- 62 LIGHT EXPOSURE PRIOR TO BRAIN SLICE PREPARATION CAN INDUCE INCREASED *PER1* AND *PER2* LEVELS MEASURED *IN VITRO*. J. M. Brewer, Dept of Psychology, Smith College, Northampton, MA.
- 63 EFFECT OF NEUROPEPTIDE Y ON *PER1* GENE EXPRESSION IN THE HAMSTER SCN *IN VITRO*. C. Fukuhara, Dept of Psychology, Smith College, Northampton, MA.
- 64 GENE EXPRESSION AND NEURONAL FIRING SIMULTANEOUSLY MONITORED IN SCN NEURONS. Sandra Kuhlman, Dept of Physiology, University of Kentucky Medical Center, Lexington, KY.

- 65 ILLUMINATION OF MOLECULAR CLOCKWORK IN THE SCN: REPORTER GENE ANALYSIS OF CLOCK AND CLOCK-CONTROLLED GENES WITHIN IMMORTALIZED CELLS. David J. Earnest, Human Anatomy & Neurobiology, Texas A&M University, College Station, TX.
- 66 Ca^{2+} -INDUCED EXPRESSION OF CIRCADIAN CLOCK GENE, *MPER1* IN THE MOUSE CEREBELLAR GRANULE CELL CULTURE. Masashi Akiyama, Dept of Pharmacology, Waseda University, Tokorozawa, Saitama, Japan.
- 67 METABOLIC COUPLING IN CULTURED CHICK ASTROCYTES BY RHYTHMIC MELATONIN ADMINISTRATION. Akihito Adachi, Dept of Biology, Texas A&M University, College Station TX.
- 68 INHIBITORS OF MAP AND p38 KINASES INDUCE LIGHT-LIKE AND DARK-LIKE PHASE SHIFTS IN CHICK PINEAL CELLS, BUT DO NOT POINT TO A CLEAR ROLE FOR THESE ENZYMES IN PHOTO-ENTRAINMENT. Lakshmanan, Geetha, LCMR, NIMH, Bethesda, MD.
- 77 PUPILLARY LIGHT REFLEXES IN MICE (C3H *rd/rd* *cl/+*) BEARING LESIONS OF BOTH ROD AND CONE PHOTORECEPTORS. Robert J. Lucas, Integrative & Molecular Neuroscience, Imperial College, Charing Cross Hospital, London, U.K.
- 78 LOCALIZATION OF SITES OF MELATONIN SYNTHESIS IN A MAMMALIAN RETINA. Susan E. Doyle, Dept of Biology, University of Virginia, Charlottesville, VA.
- 79 MELATONIN RECEPTOR RNA RHYTHMICITY AND LOCALIZATION IN THE CHICK RETINA. Arjun Natesan, Dept of Biology, Texas A&M University, College Station TX.
- 80 EFFECTS OF TIMED MELATONIN INJECTIONS ON CIRCADIAN RHYTHMS OF CHICK ELECTRORETINOGRAM. Jennifer L. High, Dept of Biology, Texas A&M University, College Station, TX.
- 81 AN OCULAR CLOCK CONTROLS ROD-CONE DOMINANCE AND SENSITIVITY IN QUAIL RETINA. Mary E. Pierce, Neuroscience & Physiology, SUNY Upstate Medical University, Syracuse, NY.

Retina Clocks

- 69 NOCTURNIN PROTEIN EXPRESSION AND INTRACELLULAR LOCALIZATION IN *XENOPUS LAEVIS* RETINA. Julie E. Baggs, Dept of Biology, University of Virginia, Charlottesville, VA.
- 70 A PORTION OF THE NOCTURNIN PROMOTER CONTAINING AN E BOX-LIKE ELEMENT IS SUFFICIENT TO TARGET SIGNALS TO CLOCK-CONTAINING PHOTORECEPTOR CELLS IN *XENOPUS LAEVIS*. Xiaorong Liu, Dept of Biology, University of Virginia, Charlottesville, VA.
- 71 OVEREXPRESSION OF DOMINANT-NEGATIVE CLOCK CAN ABOLISH MELATONIN RHYTHMICITY IN THE *XENOPUS* RETINA. Naoto Hayasaka, Dept of Biology, University of Virginia, Charlottesville, VA.
- 72 *BMAL1* IS EXPRESSED IN THE RETINA OF *XENOPUS LAEVIS* WITH HIGH AMPLITUDE RHYTHMS. Francesca E. Anderson, Dept of Biology, University of Virginia, Charlottesville, VA.
- 73 THREE CRYPTOCHROMES ARE EXPRESSED IN *XENOPUS LAEVIS* PHOTORECEPTORS. Haisun Zhu, Dept of Biology, University of Virginia, Charlottesville, VA.
- 74 EVEN CHICKS CRY2. Michael J. Bailey, Dept of Biology, Texas A&M University, College Station, TX.
- 75 REGULATION OF THE CIRCADIAN OSCILLATOR BY c-JUN N-TERMINAL KINASES. Minoru Hasegawa, Dept of Biology & Biochem, University of Houston, TX.
- 76 THE CIRCADIAN SYSTEM OF AGING RODLESS + CONELESS MICE: AN ANATOMICAL AND BEHAVIOURAL ANALYSIS. Ma'ayan Semo, Neuroscience & Psychological Medicine, Imperial College, Charing Cross Hospital, London, U.K.
- 82 SEQUENCE, GENOMIC STRUCTURE AND TISSUE EXPRESSION OF CARP VERTEBRATE ANCIENT (VA) OPSIN. Paraskevi Moutsaki, Neuroscience & Psychological Medicine, Imperial College, Charing Cross Hospital, London, U.K.
- 83 A NOVEL ROD-LIKE OPSIN ISOLATED FROM THE EXTRA-RETINAL PHOTORECEPTORS OF TELEOST FISH. Alisdair R. Philp, Neuroscience & Psychological Medicine, Imperial College, Charing Cross Hospital, London, U.K.
- 84 CHARACTERISTICS OF THE CIRCADIAN CLOCKS LOCATED IN THE PINEAL ORGAN OF AYU (*PLECOGLOSSUS ALTIVELIS*). Iigo, Masayuki, School of Medicine, St. Marianna University, Kawasaki, Japan.
- 85 CIRCADIAN REGULATION OF DOPAMINE AND MELATONIN CONTENT IN THE EYE OF THE GREEN IGUANA. Paul A. Bartell, Dept of Biology, University of Virginia, Charlottesville, VA.
- 86 CIRCADIAN RHYTHM OF ERG IN GREEN IGUANA: EFFECT OF MELATONIN AND DOPAMINE. Manuel Miranda-Anaya, Dept of Biology, University of Virginia, Charlottesville, VA.
- 87 REGULATION OF THE RHYTHM OF ApC/EBP IN THE EYE OF *APLYSIA*. Samer S. Hattar, Dept of Biology & Biochemistry, University of Houston, TX.
- 88 COUPLING OF CIRCADIAN PACEMAKER CELLS IN THE MARINE SNAIL *BULLA GOULDIANA*. Corina Ehner, Zoological Inst, University of Leipzig, Germany.
- 89 BRAIN OPSINS ANTISENSE CONSTRUCTS PREVENT ENTRAINMENT OF LOCOMOTOR RHYTHMS IN PINEALECTOMIZED-RETINECTOMIZED LIZARDS. Augusto Foa, Dept of Biology, University of Ferrara, Italy.

Molecular Biology-Insects

- 90 MOLECULAR AND GENETIC ANALYSIS OF *DROSOPHILA* CLOCK. Ravi Allada, Dept of Biology, Brandeis University, Waltham, MA.
- 91 *DROSOPHILA* MALPIGHIAN TUBULES: CIRCADIAN RHYTHMS OF PER AND TIM IN *cry^b* MUTANTS. Maria G. Ivanchenko, Dept of Entomology, Oregon State University, Corvallis, OR.
- 92 mCRY INHIBITS CLOCK:BMAL1-ACTIVATED TRANSCRIPTION INDEPENDENT OF THE mPER AND mTIM PROTEINS. Sriram Sathyanarayanan, Lab of Developmental Chronobiology, Massachusetts General Hospital, Boston, MA.
- 93 EXAMINING THE ROLE OF *dCLK* mRNA CYCLING IN THE CIRCADIAN OSCILLATOR. Nick R. J. Glossop, Dept of Biology & Biochem, University of Houston, TX.
- 94 OVEREXPRESSION OF PER DISRUPTS MOLECULAR RHYTHMS WHILE RESCUING BEHAVIORAL RHYTHMICITY IN *DROSOPHILA*. Lisa Lyons, Dept of Biology & Biochemistry, University of Houston, TX.
- 95 ANALYSIS OF THE *TIMELESS* PROMOTER. Michael J. McDonald, Dept of Biology, Brandeis University, Waltham, MA.
- 96 THE SPLICING FACTOR *DPRP43* IS REQUIRED FOR PROPER CIRCADIAN CLOCK FUNCTION IN *DROSOPHILA MELANOGASTER*. Sebastian Martinek, Laboratory of Genetics, Rockefeller University, New York, NY.
- 97 IDENTIFICATION OF RHYTHMICALLY EXPRESSED GENES IN *DROSOPHILA* BY ENHANCER-TRAP MUTAGENESIS USING A *LUCIFERASE* REPORTER CONSTRUCT. Marion Vogel, Institut fur Zoologie, University of Regensburg, Germany.
- 98 SEXUAL CLOCKS: THE INVOLVEMENT OF CLOCK GENES IN REPRODUCTION OF *DROSOPHILA MELANOGASTER*. Laura M. Beaver, Dept of Entomology, Oregon State University, Corvallis, OR.
- 99 EXPRESSION OF PERIOD GENE IN THE REPRODUCTIVE SYSTEM OF COTTON LEAFWORM, *SPODOPTERA LITTORALIS*. Zdenka Syrova, Dept of Entomology, Oregon State University, Corvallis, OR.
- 100 ANATOMY AND PHYSIOLOGY OF NEURONS OF THE CIRCADIAN PACEMAKER OF AN INSECT. Uwe Homberg, Biology, Animal Physiology, University of Marburg, Germany.
- 101 CRYPTOCHROME-IMMUNOREACTIVITY REVEALS HOMOLOGOUS EXTRARETINAL PHOTORECEPTOR SYSTEMS IN COCKROACHES AND BEETLES. Gerta Fleissner, Zoology Inst, Johann W. Goethe University, Frankfurt/Main, Germany.
- 102 MELATONIN AS A CANDIDATE MESSENGER IN THE OUTPUT PATHWAY OF CIRCADIAN / PHOTO-

PERIODIC CLOCK IN *ANTHERAEA PERMYI*. Naoyuki Ichihara, Graduate School of Science, Kobe University, Kobe, Japan.

Molecular Biology-Fungi and Plants

- 103 A PHASE-SPECIFIC CIRCADIAN RESPONSE ELEMENT. Todd P. Michael, Dept of Biological Sciences, Dartmouth College, Hanover, NH.
- 104 INTERACTION OF THE PHYB AND CRY1 PHOTORECEPTORS AND DETERMINATION OF CIRCADIAN PHASE. Patrice A. Salome, Dept of Biological Sciences, Dartmouth College, Hanover, NH.
- 105 CALCIUM OSCILLATIONS IN PLANT CELLS AND CHLOROPLASTS. Jiqing Sai, Dept of Biology, Vanderbilt University, Nashville, TN.
- 106 MOLECULAR CLONING OF THE *TOC1* LOCUS IN ARABIDOPSIS. Tokitaka Oyama, Dept of Cell Biology, Scripps Research Institute, La Jolla, CA.
- 107 INTERACTION BETWEEN THE BLUE AND RED LIGHT SENSING SYSTEMS IN PLANTS. Paloma Mas, Dept of Cell Biology, Scripps Research Institute, La Jolla, CA.
- 108 EFFECTS OF MELATONIN AND AUXIN ADMINISTRATION ON GROWTH IN ARABIDOPSIS THALIANA AND COLEUS AND ON TESTICULAR DEVELOPMENT IN RICE RATS. Kent E. Edmonds, Dept of Biology, Indiana University, New Albany, IN.
- 109 DISTRIBUTION OF MELATONIN IN THE PLANT KINGDOM. Rolf Dubbels, Dept of Biology, University of Bremen, Germany.
- 110 IDENTIFICATION AND CHARACTERIZATION OF *NEUROSPORA CRASSA* CASEIN KINASE IG, A HOMOLOGUE OF *DROSOPHILA MELANOGASTER* DOUBLE-TIME (DBT). Margit Goerl, Institut fur Physiologische Chemie, University of Munich, Germany.
- 111 LIGHT RESPONSIVE ELEMENTS WITHIN THE *FREQUENCY* PROMOTER AFFECT BOTH PHASE AND RHYTHMICITY OF THE *NEUROSPORA CRASSA* CIRCADIAN CLOCK. Allan Froelich, Dept of Biochemistry, Dartmouth Medical School, Hanover, NH.
- 112 TEMPERATURE-DEPENDENT ALTERNATIVE SPLICING AFFECTS THE TYPE OF FRQ PROTEIN SYNTHESIZED IN *NEUROSPORA*. Hildur V. Colot, Department of Genetics, Dartmouth Medical School, Hanover, NH.
- 113 FLO: CHARACTERIZATION OF THE *FREQUENCY*-INDEPENDENT OSCILLATOR IN *NEUROSPORA CRASSA*. Antonio M. Penguero, Dept of Biochemistry & Genetics, Dartmouth Medical School, Hanover, NH.
- 114 IDENTIFICATION OF FACTORS THAT REGULATE CIRCADIAN RHYTHMICITY OF THE CLOCK-CONTROLLED *eas(ccg-2)* GENE IN *NEUROSPORA CRASSA*. Zachary A. Lewis, Dept of Biology, Texas A&M University, College Station TX.

115 CONTROL OF CONIDIAL DEVELOPMENT BY THE CIRCADIAN CLOCK IN *NEUROSPORA CRASSA*. Alejandro Correa, Dept of Biology, Texas A&M University, College Station, TX.

116 DEMONSTRATION OF CIRCADIAN RHYTHMS OF DEVELOPMENT IN *ASPERGILLUS FLAVUS*. Andrew Greene, Dept of Biology, Texas A&M University, College Station, TX.

Photosynthetic Microorganisms

117 CIRCADIAN RHYTHMS IN ABUNDANCE OF Kai PROTEINS AND PHOSPHORYLATION OF KaiC IN CYANOBACTERIA. Jun Tomita, Graduate School of Science, Nagoya University, Japan.

118 GENETIC AND BIOCHEMICAL CROSSTALK AMONG CLOCK PROTEINS IN CYANOBACTERIA. Hideo Iwasaki, Graduate School of Science, Nagoya University, Japan.

119 REGULATION OF CIRCADIAN TIMEKEEPING IN *SYNECHOCOCCUS* SP. STRAIN PCC7942. Jayna L. Ditty, Dept of Biology, Texas A&M University, College Station, TX.

120 *ARS2* AS A QUANTITATIVE REPORTER AT THE ENZYME ACTIVITY LEVEL IN *CHLAMYDOMONAS* MUTANT SCREENS. Sigrid Jacobshagen, Dept of Biology, Western Kentucky University, Bowling Green, KY.

121 DAILY VARIATIONS IN SURVIVAL FROM ULTRA-VIOLET RADIATION IN *CHLAMYDOMONAS*: THE ESCAPE FROM LIGHT HYPOTHESIS. Selene S. Nikaido, Dept of Biology, Central Missouri State University, Warrensburg, MO.

Modeling

122 ROBUST OSCILLATIONS WITH TWO INTER-LOCKED FEEDBACK MODEL OF *DROSOPHILA* CIRCADIAN RHYTHM. Hiroki Ueda, Dept of Pharmacology, University of Tokyo, Japan.

123 MODELING CLARIFIES THE ROLES OF DELAYS AND FEEDBACK IN CIRCADIAN OSCILLATORS. Paul Smolen, Dept of Neurobiology & Anatomy, University of Texas, Houston Medical School, Houston, TX.

124 COMPUTER SIMULATIONS OF "SPLITTING" OF ACTIVITY RHYTHMS IN HAMSTERS. Gisele Oda, Dept of Biology, University of Virginia, Charlottesville, VA.

125 SLEEP AND TEMPERATURE: SINGLE SITE ASSESSMENT POORLY REFLECTS BODY AND SKIN THERMOREGULATORY PROCESSES. Eus J. W. Van Someren, Netherlands Institute for Brain Research, Amsterdam, The Netherlands.

126 TOWARDS A BIOCHEMICAL MODEL OF THE HUMAN CIRCADIAN PACEMAKER. Daniel B. Forger, Courant Institute of Math Sciences, New York University, NY.

127 THE HUMAN CIRCADIAN SYSTEM OPERATES AS A PHASE LOCKED LOOP. Steven B. Lowen, Developmental Biopsychology Research, McLean Hospital, Belmont, MA.

128 CHRONOBIOLOGY, A COMPREHENSIVE CD-ROM ON THE BIOLOGY OF TIMING WITH GUIDED INTERNET ACCESS TO DATABANKS AND WEBSITES. Gunther Fleissner, Zoology Inst, Johann W. Goethe University, Frankfurt/Main, Germany.

FRIDAY, MAY 12

08:30-10:30 Cumberland Ballroom
Symposium 4
Molecular II

Chair: Paulo Sassone-Corsi
CNRS-INSERM-ULP
Signaling to central and peripheral clocks

Speakers: Paul Hardin
University of Houston
Feedback circuits within the Drosophila circadian oscillator

Takao Kondo
Nagoya University
Toward a molecular mechanism for circadian characteristic in cyanobacteria

Jay Hirsh
University of Virginia
Bugs on drugs: Novel functions for circadian genes

Hitoshi Okamura
Kobe University Medical School
mPer1 oscillation in mammals

08:30-10:30 Talbot Conference Room
Symposium 5
Circadian Photoreceptors

Chair: Anthony Cashmore
University of Pennsylvania

Speakers: James Tepperman
University of California, Berkeley
Photosensory perception and signaling by the phytochromes

Steve Kay
Scripps Research Institute
Circadian Photoreception in Arabidopsis

Russell Foster
Imperial College
Mammalian photoentrainment: Photopigment or photofigment?

Susan Golden
Texas A&M University
CikA, a phytochrome family member important for resetting the cyanobacterial circadian clock

08:30-10:30 Ossabaw Conference Room
Symposium 6
Construction of Circadian Models

Chair: Janis Anderson
Harvard Medical School

Speakers: Megan Jewett
Harvard Medical School
Are circadian models medically useful?

Robert Sack
University of Oregon
What can be learned about the human circadian system by disorders in the totally blind

Eve van Cauter
University of Chicago
Ignoring circadian and sleep signals: The price to pay

Emery Brown
Harvard Medical School
Making inferences from circadian data

10:30-11:00 Coffee Break
Conference Center Patio

11:00-13:00 Cumberland Ballroom
Slide Session 4
Clock Genes II: Temporal Analysis of Clock Gene Expression

Chair: Lauren Shearman
Massachusetts General Hospital

11:00

129 GREEN FLUORESCENT PROTEIN REPORTS REGIONAL REGULATION OF mPER1 ACTIVITY IN THE SCN. Douglas G. McMahon, Dept of Physiology, University of Kentucky, Medical Center, Lexington, KY.

11:15

130 MOUSE *PERIOD1*-DRIVEN GFP GENE EXPRESSION CYCLES IN SCN SLICES AND IN INDIVIDUAL SCN CELLS. Jorge E. Quintero, Dept of Physiology, University of Kentucky Medical Center, Lexington, KY.

11:30

131 GENERATION AND ANALYSIS OF mPer1-LUCIFERASE TRANSGENIC MICE. Lisa D. Wilsbacher, Neurobiology & Physiology, Northwestern University, Evanston, IL.

11:45

132 TRANSGENIC ANALYSIS OF A MAMMALIAN CLOCK GENE, *Per1*. Hajime Tei, Human Genome Center, University of Tokyo, Japan.

12:00

133 RESETTING CENTRAL AND PERIPHERAL CIRCADIAN OSCILLATORS IN TRANSGENIC RATS. Shin Yamazaki, Dept of Biology, University of Virginia, Charlottesville, VA.

12:15

134 DAMPED OSCILLATION OF *PER1-LUC* IN ISOLATED BRAIN REGIONS. Michikazu Abe, Dept of Biology, University of Virginia, Charlottesville, VA.

12:30

135 IDENTIFICATION OF DOWN-STREAM GENES OF mPer2 *IN VIVO* USING cDNA MICROARRAYS. Jens D. Mikkelsen, Display Systems Biotech A/S, Lyngby, Denmark.

- 12:45
136 BEHAVIORAL REVERSION IS ASSOCIATED WITH LOSS OF DAILY BEHAVIORAL RHYTHM AND CHANGES IN *PERIOD* GENE EXPRESSION IN THE BRAIN OF THE HONEY BEE. Buy Bloch, Dept of Entomology, University of Illinois, Urbana, IL.
- 11:00-13:00 Talbot Conference Room
Slide Session 5
Human Rhythms II: Sleep and Jet Lag
Chair: Diane Boivin
Douglas Hospital Research Center
- 11:00
137 EFFECTS OF AGING ON THE TIMING OF SPONTANEOUS DAYTIME SLEEP. Patricia J. Murphy, Lab of Human Chronobiology, Weill Medical College, Cornell University, White Plains, NY 10605.
- 11:15
138 ATTENUATION OF HOMEOSTATIC PROCESS DURING DAYTIME RECOVERY SLEEP IN THE MIDDLE YEARS OF LIFE. Helene Gaudreau, Dept of Psychology, Univ of Montreal, Hospital Sacre-Coeur, Montreal, Quebec, Canada.
- 11:30
139 SLEEP DURING FORCED DESYNCHRONY IN ADOLESCENTS. Mary A. Carskadon, Bradley Hospital Sleep Research Lab, Brown Univ School of Medicine, E. Providence, RI.
- 11:45
140 SLEEP ALTERS HUMAN PHASE RESPONSE TO EXTRAOCULAR LIGHT. Scott S. Campbell, Lab of Human Chronobiology, New York Presbyterian Hospital, White Plains, NY.
- 12:00
141 WHAT IS THE RELATIONSHIP BETWEEN ASSESSMENTS OF JET-LAG AND SOME OF ITS COMPONENTS? James Waterhouse, Sport & Exercise Sciences, Liverpool John Moores University, Henry Cotton Campus, Liverpool, U.K.
- 12:15
142 ORDINARY INDOOR ROOMLIGHT CAN AFFECT THE ADAPTATION TO SIMULATED JET-LAG. Francine James, Douglas Hospital Research Center, McGill University, Verdun, Quebec, Canada.
- 12:30
143 DOES MORNING MELATONIN ADMINISTRATION PHASE DELAY HUMAN CIRCADIAN RHYTHMS? Wirz-Justice, Anna, Chronobiology & Sleep Laboratory, Psychiatric University Clinic, Basel, Switzerland.
- 12:45
144 THE EFFECT OF DAY-TIME EXOGENOUS MELATONIN ADMINISTRATION ON CARDIAC AUTONOMIC ACTIVITY. Helen J. Burgess, Centre for Sleep Research, University of South Australia, Elizabeth Hospital, Woodville, Australia.
- 11:00-13:00 Ossabaw Conference Room
Slide Session 6
Molecular Basis of Circadian Timing I
Chair: Martha Merrow
University of Munich
- 11:00
145 PHOSPHORYLATION OF THE *NEUROSPORA* CLOCK PROTEIN FREQUENCY DETERMINES ITS DEGRADATION RATE AND STRONGLY INFLUENCES THE PERIOD LENGTH OF THE CIRCADIAN CLOCK. Yi Liu, Dept of Physiology, University of Texas Southwestern Medical Center, Dallas, TX.
- 11:15
146 THE INTERPLAY BETWEEN WC-1 AND FRQ IN THE *NEUROSPORA CRASSA* CIRCADIAN CLOCK. Kwangwon Lee, Dept of Biochemistry, Dartmouth Medical School, Hanover, NH.
- 11:30
147 PERIOD LENGTH, TEMPERATURE COMPENSATION AND LIGHT ENTRAINMENT DEFECTS IN THE PAS PROTEIN, WHITE COLLAR-2, A POSITIVE ELEMENT IN A CIRCADIAN CLOCK. Michael A. Collett, Dept of Genetics, Dartmouth Medical School, Hanover, NH.
- 11:45
148 *DROSOPHILA* CRY IS A DEEP-BRAIN CIRCADIAN PHOTORECEPTOR. Patrick Emery, Dept of Biology, Brandeis University, Waltham, MA.
- 12:00
149 THE ROLE OF CRY IN LIGHT-INDUCED DEGRADATION OF TIMELESS. Wei Song, Dept of Neuroscience, University of Pennsylvania, Philadelphia, PA.
- 12:15
150 EFFECTS OF A CRYPTOCHROME MUTANT ON OLFACTORY RESPONSES IN *DROSOPHILA MELANOGASTER*. Balaji Krishnan, Dept of Biology & Biochemistry, University of Houston, TX.
- 12:30
151 PER-CRY INTERACTIONS INTEGRATE LIGHT INFORMATION TO THE CIRCADIAN PACEMAKER IN *DROSOPHILA*. Ezio Rosato, Dept of Genetics, University of Leicester, United Kingdom.
- 12:45
152 ANALYSIS OF CIRCADIAN BEHAVIOR AND SCN CELLULAR COMPOSITION OF *CLOCK* CHIMERIC MICE. Sharon Low-Zeddies, Neurobiology & Physiology, Northwestern University, Evanston, IL.
- 13:00-16:30 Break
Take down Group A posters by 14:00
Put up Group B posters
- 16:30-18:00 Cumberland Ballroom
Review Lectures
Speakers: Brian K. Follett
University of Warwick
Photoperiodism
William Hrushesky
Albany Medical School
Chronotherapeutics

Entrainment

- 153 ACTIVATION OF ADENOSINE RECEPTORS IN THE SCN REGION BLOCKS THE PHASE SHIFTING EFFECTS OF LIGHT AND INHIBITS LOCOMOTOR ACTIVITY IN SYRIAN HAMSTERS. Eric M. Mintz, Dept of Biology, Georgia State University, Atlanta, GA.
- 154 CHARACTERIZATION OF SEROTONERGIC PATHWAYS AND RECEPTOR SUBTYPES IN THE CIRCADIAN SYSTEM OF LABORATORY RATS. Franziska Wollnik, Biology Institute, University of Stuttgart, Germany.
- 155 INTRA-RAPHE INJECTION OF METERGOLINE BLOCKS DRN ELECTRICALLY-STIMULATED 5-HT RELEASE IN THE SCN. Lisa A. DiNardo, Dept of Biological Sciences, Kent State University, Kent, OH.
- 156 THE 5-HT_{1A/7} AGONIST 8-OH-DPAT DOES NOT INHIBIT NMDA-INDUCED PHASE ADVANCES IN SYRIAN HAMSTERS. Karen L. Gamble, Dept of Psychology, Georgia State University, Atlanta, GA.
- 157 TIMED *IN VIVO* MICRODIALYSIS PERFUSION OF THE SCN REGION WITH 5-HT AGONISTS PHASE-SHIFTS THE HAMSTER CIRCADIAN ACTIVITY RHYTHM. J. Christopher Ehlen, Dept of Biological Sciences, Kent State University, Kent, OH.
- 158 PHOTIC STIMULATION OF POLYSIALIC ACID AND ITS NCAM-180 CARRIER IN THE HAMSTER SUPRACHIASMATIC NUCLEI. Lenka Fedorkova, Dept of Biological Sciences, Kent State University, Kent, OH.
- 159 DISTINGUISHING THE ROLES OF POLYSIALIC ACID AND NEURAL CELL ADHESION MOLECULE IN CIRCADIAN TIME-KEEPING FUNCTION IN THE MOUSE. Huaming Shen, Dept of Biological Sciences, Kent State University, Kent, OH.
- 160 THE ROLE OF CALBINDIN-D28K ON THE PHOTO-ENTRAINMENT MECHANISM OF HAMSTER SUPRACHIASMATIC NUCLEUS. Toshiyuki Hamada, Dept of Psychology, Columbia University, New York, NY.
- 161 PARAVENTRICULAR THALAMIC NUCLEUS ENTRAINS CIRCADIAN RHYTHMS AND MODULATES THE EFFECT OF LIGHT PULSES, IN RATS. Alberto Salazar-Juarez, Dept of Neurosciences, University of Mexico, D.F. Mexico.
- 162 PARTICIPATION OF A Rel/NF- κ B TRANSCRIPTION FACTOR IN THE HAMSTER CIRCADIAN SYSTEM. Luciano Marpegan, Dept of Science & Technology, University of Quilmes, Buenos Aires, Argentina.
- 163 RHYTHMICITY OF THE cGMP-RELATED SIGNAL TRANSDUCTION PATHWAY IN THE MAMMALIAN CIRCADIAN SYSTEM. Gabriela A. Ferreyra, Science & Technology, Univ of Quilmes, Buenos Aires, Argentina.
- 164 DIFFERENTIAL EXPRESSION OF PROTEIN KINASE C β I (PKC β I) BUT NOT PKC α AND PKC β II IN THE SUPRACHIASMATIC NUCLEUS OF SELECTED HOUSE MOUSE LINES. Abel Bult, Institute of Arctic Biology, University of Alaska, Fairbanks, AK.
- 165 RAPID RESETTING OF THE FETAL CIRCADIAN PACEMAKER BY MELATONIN. Xiaodong Li, Dept of Biology, Northeastern University, Boston, MA.
- 166 DISSOCIATION OF *mCry* AND *mPer* RHYTHMS IN THE SUPRACHIASMATIC NUCLEI DURING TRANSIENT RESETTING TO LIGHT. Manuel Field, Dept of Anatomy, University of Cambridge, United Kingdom.
- 167 NMDA RECEPTOR-MEDIATED PHOTIC INDUCTION OF PERIOD GENES IN THE HAMSTER SCN. Takahiro Moriya, Advanced Center for Human Sciences, Waseda University, Tokorozawa, Saitama, Japan.
- 168 NON-PHOTIC ENTRAINMENT BY 5-HT_{1A/7} RECEPTOR AGONISTS ACCOMPANIED BY REDUCED *PER1* AND *PER2* MRNA LEVELS IN THE SUPRACHIASMATIC NUCLEI. Kazumasa Horikawa, Dept of Pharmacology, Waseda University, Tokorozawa, Saitama, Japan.
- 169 MELATONIN ADMINISTRATION ENTRAINS CIRCADIAN RHYTHMS OF TEMPERATURE AND ACTIVITY IN B6D2F₁ MICE EXPOSED TO CONSTANT LIGHT. X.-M. Li, Chronobiology Lab, ICIH Hospital Paul Brousse, Villejuif, France.
- 170 DAILY RHYTHM IN THE BODY WEIGHT OF C57BL/6J PUPS DURING PRESENCE AND ABSENCE CYCLES OF THE MOTHER MOUSE. N. Viswanathan, Dept of Biology, Northeastern University, Boston, MA.
- 171 USE OF FOS INDUCTION TO ASSESS SPECTRAL SENSITIVITY OF THE MOUSE CIRCADIAN SYSTEM. C. Rieux, INSERM U-371, Bron, France.
- 172 RETINAL INPUT TO THE VENTROLATERAL PRE-OPTIC AREA (VLPO) AND THE MODULATION BY LIGHT OF FOS EXPRESSION IN THE VLPO OF NOCTURNAL AND DIURNAL RODENTS. Julie A. Harris, Dept of Psychology, Michigan State University, East Lansing, MI.
- 173 WHEEL RUNNING INDUCES ELEVATED FOS EXPRESSION IN NEUROPEPTIDE-Y CELLS OF THE INTERGENICULATE LEAFLET OF *ARVICANTHIS NILOTICUS*. Joshua P. Nixon, Dept of Zoology, Michigan State University, East Lansing, MI.
- 174 LIGHT-INDUCED FOS EXPRESSION IN THE SUPRACHIASMATIC NUCLEUS OF *ARVICANTHIS NILOTICUS*. Megan M. Mahoney, Dept of Zoology, Michigan State University, East Lansing, MI.
- 175 PHASE RESPONSE TO LIGHT PULSES OF RATS BEARING SCN GRAFTS. Ivette Caldelas, Dept of Neurosciences, University of Mexico, D.F. Mexico.

176 RAPID CIRCADIAN CLOCK RESETTING BY SLEEP DEPRIVATION AND ITS INHIBITION BY CAFFEINE. M. C. Antle, Dept of Psychology, Simon Fraser University, Burnaby BC, Canada.

177 14 HOUR PHASE SHIFTS IN *TAU* MUTANT HAMSTER IN RESPONSE TO A DOUBLE LIGHT PULSE. Joseph LeSauter, Dept of Psychology, Columbia University, New York, NY.

Food-Entrainable Oscillators

178 ANTICIPATION OF LIVER METABOLISM IN RATS ENTRAINED BY RESTRICTED FEEDING SCHEDULES. Carolina Escobar, Dept of Anatomy, University of Mexico, D.F. Mexico.

179 PHASE SHIFT OF SERUM CATECHOLAMINES RHYTHM BY RESTRICTED FOOD ACCESS IN RATS. Jose L. Chavez-Juarez, Dept of Neurosciences, University of Mexico, D.F. Mexico.

180 FEEDING-ENTRAINED CIRCADIAN RHYTHMS ARE ATTENUATED BY LESIONS OF THE PARABRACHIAL REGION IN RATS. Alec J. Davidson, Dept of Psychology, Florida State University, Tallahassee, FL.

181 IMMUNOREACTIVITY FOR IMMEDIATE-EARLY GENE PROTEINS AND OREXIN IN MOUSE BRAINS AFTER FOOD RESTRICTION AND REFEEDING. Marleen de Groot, Dept of Psychology, Dalhousie University, Halifax, Nova Scotia, Canada.

182 RESTRICTED FEEDING INDUCES THE ANTICIPATORY EXPRESSIONS OF *MPER* GENES mRNA IN THE CEREBRAL CORTEX AND HIPPOCAMPUS IN MICE. Hisanori Wakamatsu, Dept of Pharmacology, Waseda University, Tokorozawa, Saitama, Japan.

183 IS DAILY ADRENALINE ADMINISTRATION AN ENTRAINMENT SIGNAL FOR BEHAVIORAL CIRCADIAN RHYTHMICITY IN RATS? Jorge Mendoza, Dept of Anatomy, University of Mexico, D. F. Mexico.

184 GASTROINTESTINAL TRACT MELATONIN RELEASE IN FASTED INTACT AND PINEALECTOMIZED ZEBRA FINCHES (*TAENIOPYGIA GUTTATA*). Thomas J. Van't Hof, Research Center for Ornithology, Max-Planck Society, Andechs, Germany.

Aging

185 EVIDENCE OF ARTIFICIALLY ACCELERATED AGING IN THE SUPRACHIASMATIC NUCLEUS OF THE GRAY MOUSE LEMUR (*MIRCOCEBUS MURINUS*). Martine Perret, Lab d'Ecologie Generale, UMR CNRS 8571, Brunoy, France.

186 CIRCADIAN RHYTHMS OF PLASMA DHEAS AND CORTISOL IN YOUNG AND OLD FEMALE RHESUS MACAQUES. Vasilios Garyfallou, Division of Neuroscience, Oregon Regional Primate Ctr, Beaverton, OR.

187 AGING ALTERS CIRCADIAN GENE EXPRESSION IN THE SUPRACHIASMATIC NUCLEI OF MALE SYRIAN HAMSTERS. Daniel E. Kolker, Neurobiology & Physiology, Northwestern University, Evanston, IL.

188 AGE-RELATED ALTERATION OF CLOCK-RELATED GENES EXPRESSION IN RAT. Makoto Asai, Dept of Pharmacology, Waseda University, Tokorozawa, Saitama, Japan.

Physiological Aspects of Biological Timing

189 REGULATION OF WHEEL RUNNING BEHAVIOR IN RODENTS. Michael Pollock, Dept of Psychology, Simon Fraser University, Burnaby BC, Canada.

190 SIMILAR GENETIC CONTROL OF NEONATAL AND ADULT SLEEP IN MICE. Sally Battle, Neurobiology & Physiology, Northwestern University, Evanston, IL.

191 SLEEP DEPRIVATION STIMULATES SEROTONIN RELEASE IN THE HAMSTER SUPRACHIASMATIC NUCLEUS. Gregory H. Grossman, Dept of Biological Sciences, Kent State University, Kent, OH.

192 METHAMPHETAMINE SCHEDULES REINITIALIZE CIRCADIAN MECHANISMS IN RATS IN THE ABSENCE OF OTHER TIME CUES, LOCOMOTION, OR SCN TISSUE. Norman Pecoraro, Dept of Psychology, Indiana University, Bloomington, IN.

193 MORPHINE EFFECTS ON CIRCADIAN NOCICEPTION AND BETA-ENDORPHIN. Natalie A. Rasmussen, College of Nursing, Nebraska Health Science Center, Omaha, NE.

194 CIRCADIAN ORGANIZATION BEFORE, DURING, AND AFTER ALCOHOL ADMINISTRATION IN RAT MODELS OF ALCOHOLISM. William Timberlake, Dept of Psychology, Indiana University, Bloomington, IN.

195 DIFFERENTIAL EFFECTS OF VASOPRESSIN ON ACTIVITY AND TEMPERATURE OSCILLATORS. Helen M. Murphy, Dept of Psychology, John Carroll University, Cleveland, OH.

196 REACTIVENESS TO STRESS PREDICTS THE RATE OF RESYNCHRONIZATION OF THE CIRCADIAN CLOCK IN RATS. Laurence Weibel, BP 27, INRS, Occupational Physiology Lab, Vandoeuvre, France.

197 REST-ACTIVITY AND BODY TEMPERATURE RHYTHMS AFTER SUPRACHIASMATIC NUCLEUS DESTRUCTION IN B6D2F₁ MICE. Elisabeth Filipski, Biological Rhythms Lab, ICI Hospital Paul Brousse, Villejuif, France.

198 DORSAL LATERAL GENICULATE LESIONS PREVENT THE ENHANCEMENT BUT NOT THE SUPPRESSION OF LOCOMOTOR ACTIVITY BY LIGHT IN THE MOUSE. K. Edelstein, Dept of Zoology, University of Toronto, Ontario, Canada.

- 199 EFFECT OF PINEALECTOMY ON MELATONIN RECEPTOR PROTEIN EXPRESSION IN CHICK DIENCEPHALON. Stephen P. Karaganis, Dept of Biology, Texas A&M University, College Station, TX.
- 200 DAILY RHYTHM OF PINEAL MELATONIN CONTENT IN THE DIURNAL MURID RODENT, *ARVICANTHIS NILOTICUS*. Colleen M. Novak, Dept of Biology, Georgia State University, Atlanta, GA.
- 201 ADULT GONADAL HORMONES DETERMINE SOCIAL CUE RESPONSIVENESS IN CIRCADIAN RHYTHMS OF THE DIURNAL RODENT *OCTODONDEGUS*. Tammy J. Jechura, Dept of Psychology, University of Michigan, Ann Arbor, MI.
- 202 LIGHT INTENSITY IN THE AFTERNOON AND DURING A SOLAR ECLIPSE: EFFECTS ON RETREAT UNDERGROUND IN A DIURNAL MAMMAL. Kamiel Spoelstra, Zoological Laboratory, University of Groningen, Haren, The Netherlands.
- 203 PHOTIC AND NON-PHOTIC CIRCADIAN PHASE-SHIFTING RESPONSES IN A DIURNAL MONKEY, THE COMMON MARMOSET. J. David Glass, Dept of Biological Sciences, Kent State University, Kent, OH.
- 204 DOES THE TAU MUTATION IN THE SYRIAN HAMSTER AFFECT THE TIMING OF OTHER PROCESSES, FROM MILLISECONDS TO YEARS? Malgorzata Oklejewicz, Zoological Laboratory, University of Groningen, Haren, The Netherlands.
- 205 THE CRAYFISH *PROCAMBARUS CLARKII* SHOWS CIRCADIAN VARIATIONS IN DIFFERENT PARAMETERS OF THE GSH CYCLE. M. E. Duran-Lizarraga, Lab of Comparative Neurophysiology, University of Mexico, D.F. Mexico.
- 206 APPEARANCE AND ENTRAINMENT OF SPLIT ACTIVITY RHYTHMS IN 4-PHASE LDLD AND SKELETON LIGHT CYCLES. Jeffrey A. Elliott, Dept of Psychiatry, University of California, San Diego, CA.
- 207 THE TIME IS ZT 13: DO YOU KNOW WHERE YOUR HAMSTERS ARE? Patricia J. Sollars, Dept of Anatomy & Neurobiology, Colorado State University, Fort Collins, CO.
- 208 EFFECTS OF LIGHT INTENSITY AND RESTRAINT ON DARK PULSE-INDUCED PHASE SHIFTING DURING EARLY SUBJECTIVE NIGHT IN HAMSTERS. Suzanne M. Dwyer, Dept of Psychology, University of Maine, Orono, ME.
- 209 NON-PHOTIC AND ANTI-PHOTIC MECHANISMS UNDERLYING THE DARK-PULSE PHASE-RESPONSE CURVE (PRC). Alan M. Rosenwasser, Dept of Psychology, University of Maine, Orono, ME.
- 210 DECREASED SAMPLING RATES MAY PROVIDE ACCEPTABLE MELATONIN PHASE, DURATION AND AMPLITUDE ESTIMATES, DEPENDING ON THE PRECISION REQUIRED. Kelly S. Benke, Circadian, Neuroendocrine and Sleep Disorders Section, Brigham & Women's Hospital, Boston, MA.
- 211 A COMPARISON OF TWO CIRCADIAN PHASE MARKERS IN HUMANS. Andrea G. Suhner, Lab of Human Chronobiology, Weill Medical College, Cornell University, White Plains, NY.
- 212 SALIVARY MELATONIN: SAMPLING PROCEDURES AND STABILITY. Jacob Weber, Buhlmann Laboratories, Allschwil, Switzerland.
- 213 CIRCADIAN RHYTHM AND SLEEP DIFFERENCES BETWEEN COMMUNITY DWELLING AND INSTITUTIONALIZED OLDER ADULTS. Claudia Chaperon, College of Nursing, Nebraska Health Science Center, Nebraska Medical Center, Omaha, NE.
- 214 SLEEP DISTURBANCE IN PATIENTS AND THEIR CARE GIVERS FOLLOWING ARTHROPLASTIC SURGERY. Lynn A. Farr, Nebraska Health Science Center, College of Nursing, Omaha, NE.
- 215 TIME COURSE OF SLOW WAVE ACTIVITY DURING EXTENDED SLEEP IN ADOLESCENTS. Michelle U. Urali, Circadian, Neuroendocrine and Sleep Disorders Section, Brigham & Women's Hospital, Boston, MA.
- 216 ARE THERE HANGOVER-EFFECTS ON PHYSICAL PERFORMANCE WHEN MELATONIN IS INGESTED BY ATHLETES BEFORE NOCTURNAL SLEEP? Greg Atkinson, Sport & Exercise Sciences, Liverpool John Moores University, Henry Cotton Campus, Liverpool, U.K.
- 217 FRONTAL TASK PERFORMANCE IN DEMENTED ELDERLY IS RELATED TO THE CIRCADIAN AMPLITUDE IN THE REST-ACTIVITY RHYTHM. R. S. Van Hutten, Netherlands Institute for Brain Research, Amsterdam, The Netherlands.
- 218 MELATONIN AND SLEEP IN DEMENTIA. Emmalee Kennedy, Dept of Neurology, Northwestern University Medical School, Chicago, IL.
- 219 BRIGHT LIGHT AND FIXED SLEEP SCHEDULES COMBINE TO SPEED CIRCADIAN ADAPTATION TO NIGHT WORK. Todd S. Horowitz, Circadian, Neuroendocrine and Sleep Disorders Section, Brigham & Women's Hospital, Boston, MA.
- 220 THE PHASE ANGLE OF ENTRAINMENT TO 10MG MELATONIN IN BLIND PEOPLE DEPENDS ON PRIOR FREE-RUNNING PERIOD. A. J. Lewy, Sleep & Mood Disorders Lab, Oregon Health Sciences University, Portland, OR.
- 221 AGING CAUSES A SLIGHT LENGTHENING OF THE INTRINSIC PERIOD IN THE BLIND. A. R. Kendall, Sleep & Mood Disorders Lab, Oregon Health Sciences University, Portland, OR.

Humans-Methods, Techniques Assessment, Sleep/Wake Cycles, Entrainment

- 222 PHASE SHIFTING RESPONSE TO LIGHT IN YOUNG AND OLDER ADULTS. Susan Benloucif, Molecular Pharmacology, Northwestern University Medical School, Chicago, IL.
- 223 3-CYCLE BRIGHT LIGHT STIMULUS INDUCES TYPE 0 RESETTING IN HUMAN MELATONIN RHYTHM. Megan E. Jewett, Circadian, Neuroendocrine and Sleep Disorders Section, Brigham & Women's Hospital, Boston, MA.
- 224 PHASE-SHIFTING EFFECTIVENESS OF INTERMITTENT LIGHT PULSES: RELATIONSHIP TO MELATONIN SUPPRESSION. Claude Gronfier, Circadian, Neuroendocrine and Sleep Disorders Section, Brigham & Women's Hospital, Boston, MA.
- 225 A SUBSTANCE ANTAGONIST REDUCES THE LIGHT-INDUCED SUPPRESSION OF MELATONIN IN HUMANS. Debra J. Skene, School of Biological Sciences, University of Surrey, Guildford, Surrey, United Kingdom.
- 226 DIURNAL VARIATION OF RETINAL SENSITIVITY IN MORNING AND EVENING TYPES. Marianne Rufange, Sacre-Coeur Hospital, University of Montreal, Quebec, Canada.
- 227 EVENING LIGHT: ASPECTS OF HUMAN ENTRAINMENT. Daniel F. Kripke, Dept of Psychiatry, Univ of California San Diego, La Jolla, CA.
- 234 SCN SIGNAL OF CHANGE OF SEASON IN SEASONAL AFFECTIVE DISORDER. Thomas A. Wehr, Section on Biological Rhythms, NIMH, Bethesda, MD.
- 235 RHYTHMICITY OF LYMPHOCYTES AND MELATONIN IN HUMANS. Park F. Cho, Douglas Hospital Research Center, McGill University, Verdun, Quebec, Canada.
- 236 GENDER DIFFERENCES IN TIME-DEPENDENT POSTPRANDIAL TRIACYLGLYCEROL VARIATIONS. Michelle Sopowski, School of Biological Sciences, University of Surrey, Guildford, Surrey, United Kingdom.
- 237 PAP SMEAR DETECTION OF UTERINE CERVICAL EPITHELIAL CARCINOGENESIS AND PROGRESSION IS CIRCAANNUALLY RHYTHMIC AND APPARENTLY TIED TO RHYTHMS IN HUMAN SEXUALITY. William J.M. Hrushesky, Medical Chronobiology Lab, Stratton VA Medical Center, Albany, NY.
- 238 MEANINGFUL AND REPRODUCIBLE REPRODUCTIVE CYCLE MODULATION OF CANCER BIOLOGY. Patricia A. Wood, Medical Chronobiology Lab, Stratton VA Medical Center, Albany, NY.
- 239 THE NOCTURNAL INCREASE IN ALDOSTERONE IS BLUNTED DURING SLEEP DEPRIVATION. Gabrielle Brandenberger, Institut de Physiologie, Strasbourg, France.
- 240 EFFECT OF REPEATED CAFFEINE ADMINISTRATION ON CORE BODY TEMPERATURE DURING 88 HOURS OF SUSTAINED WAKEFULNESS. Naomi L. Rogers, Experimental Psychiatry, University of Pennsylvania School of Medicine, Philadelphia, PA.

Humans-Circadian Rhythms & Physiology, Disease States

- 228 WEEK-LONG BLOOD PRESSURE MONITORING FOR REFERENCE VALUES AND DIAGNOSIS OF DISEASE RISK SYNDROMES. Katarina T. Borer, Division of Kinesiology, University of Michigan, Ann Arbor, MI.
- 229 CIRCADIAN RHYTHM DISTURBANCES IN ALZHEIMER'S DISEASE. David G. Harper, McLean Hospital, Belmont, MA.
- 230 RHYTHMS OF AGITATION: DOES SUNDOWNING EXIST? Sonia Ancoli-Israel, Dept of Psychiatry, Univ of California, San Diego, CA.
- 231 CORTISOL CIRCADIAN RHYTHMS DURING THE MENSTRUAL CYCLE AND WITH SLEEP DEPRIVATION IN PREMENSTRUAL DYSPHORIC DISORDER AND NORMAL CONTROL SUBJECTS. Barbara L. Parry, Dept of Psychiatry, Univ of California San Diego, La Jolla, CA.
- 232 DUAL EPILEPTIC FOCI IN A SINGLE PATIENT EXPRESS DISTINCT TEMPORAL PATTERNS DEPENDENT ON LIMBIC VERSUS NONLIMBIC BRAIN LOCATION. Mark Quigg, Dept of Neurology, University of Virginia Health Sciences Center, Charlottesville, VA.
- 233 CIRCADIAN VARIATION OF BODY TEMPERATURE IN WINTER DEPRESSED PATIENTS AND MATCHED CONTROLS. K. M. Koorengevel, Dept of Biological Psychiatry, Psychiatric University Clinic, Groningen, Netherlands.
- 241 REFRACTORINESS TO MELATONIN IS BRAIN-SITE SPECIFIC IN SIBERIAN HAMSTERS. David A. Freeman, Dept of Psychology, University of California, Berkeley, CA.
- 242 LEPTIN EFFECTS ON IMMUNE FUNCTION AND ENERGY BALANCE ARE PHOTOPERIOD-DEPENDENT IN SIBERIAN HAMSTERS (*PHODOPUS SUNGORUS*). Deborah L. Drazen, Dept of Psychology, Johns Hopkins University, Baltimore, MD.
- 243 PHOTOPERIOD AFFECTS IMMUNE FUNCTION AND SICKNESS BEHAVIOR IN SIBERIAN HAMSTERS. Stacy Bilbo, Dept of Psychology, Johns Hopkins University, Baltimore, MD.
- 244 PHOTOPERIOD MODULATES THE INHIBITORY EFFECTS OF *IN VITRO* MELATONIN ON CELL-MEDIATED IMMUNE FUNCTION IN FEMALE SIBERIAN HAMSTERS. Brian J. Prendergast, Dept of Psychology, Johns Hopkins University, Baltimore, MD.
- 245 *IN VITRO* MELATONIN TREATMENT ENHANCES CELL-MEDIATED IMMUNE FUNCTION IN MALE PRAIRIE VOLES (*MICROTUS OCHROGASTER*). Lance Kriegsfeld, Dept of Psychology, Columbia University, New York, NY.

- 246 PHOTOPERIODIC EFFECTS ON BROMODEOXY-
URIDINE INCORPORATION IN THE BRAINS OF
ADULT SIBERIAN HAMSTERS. Victor B. Tsirlin,
Neurobiology & Physiology, Northwestern University,
Evanston, IL.
- 247 DIFFERENTIAL REGULATION OF FOLLICLE STIMU-
LATING HORMONE (FSH) AND LUTEINIZING
HORMONE (LH) BY PHOTOPERIOD AND FEMALE
EXPOSURE IN MALE SIBERIAN HAMSTERS. Sonali
Anand, Neurobiology & Physiology, Northwestern
University, Evanston, IL.
- 248 TESTOSTERONE INFLUENCES EXPLORATORY
BEHAVIOR IN SIBERIAN HAMSTERS INDEPEN-
DENTLY OF PHOTOPERIOD. Joy F. Shen,
Neurobiology & Physiology, Northwestern University,
Evanston, IL.
- 249 AN INTERVAL TIMER SYNCHRONIZES PUBERTAL
DEVELOPMENT IN SUCCESSIVE COHORTS OF
SUMMER- AND FALL-BORN SIBERIAN HAMSTERS.
Michael R. Gorman, Dept of Psychology, Univ of
California San Diego, La Jolla, CA.
- 250 AMBIENT TEMPERATURE MODIFIES PHOTO-
PERIODIC RESPONSES IN SYRIAN HAMSTERS BY
AFFECTING RATES OF CIRCADIAN ENTRAINMENT.
Jennie Larkin, Dept of Psychology, University of
California, Berkeley, CA.
- 251 REGULATION OF PITUITARY LACTOTROPHS BY THE
PARS TUBERALIS: PATTERNS OF PROLACTIN GENE
EXPRESSION. J. Anne Stirling, School of Biological
Sciences, University of Manchester, United Kingdom.
- 252 INTRASPECIFIC VARIATION IN METABOLIC AND
ENERGETIC RESPONSES TO PHOTOPERIOD IN THE
WHITE-FOOTED MOUSE, *PEROMYSCUS LEUCOPUS*.
Michelle Rightler, Dept of Biology, College of William &
Mary, Williamsburg, VA.
- 253 A CRITICAL PHOTOPERIOD OF L13.5:D10.5 AND AN
INCREASED RESPONSIVENESS TO GRADUAL
CHANGES IN PHOTOPERIOD IN F344 RATS. M. Eric
Galvez, Dept of Biology, College of William & Mary,
Williamsburg, VA.
- 254 PHOTORESPONSIVE FISCHER 344 RATS DIFFER IN
2-[¹²⁵I] IODO-MELATONIN BINDING FROM NON-
PHOTORESPONSIVE SPRAGUE DAWLEY RATS. Paul
D. Heideman, Dept of Biology, College of William &
Mary, Williamsburg, VA.
- 255 A TEST FOR A CIRCAANNAL RHYTHM OF PHOTO-
RESPONSIVENESS IN FISCHER 344 RATS. M.
Benjamin Shoemaker, Dept of Biology, College of
William & Mary, Williamsburg, VA.
- 256 PHOTOPERIODIC HISTORY AND MELATONIN
SECRETION IN REINDEER. B. E. H. van Oort, Dept of
Arctic Biology, University of Tromso, Norway.

SATURDAY, MAY 13

08:30-10:30 Cumberland Ballroom
Symposium 7
Sleepy Genes

Chair: Helena Illnerova
Czech Academy of Science

Speakers: Emmanuel Mignot
Stanford University
Hypocretin (orexins) and narcolepsy

Masashi Yanagisawa
Univ of Texas Southwestern Medical Ctr
Role of the orexin pathway in sleep regulation

Louis Ptacek
University of Utah
Familial advanced sleep-phase syndrome: a short period circadian rhythm variant in humans

Joan Hendricks
University of Pennsylvania
Drosophila rest-more than just inactivity

08:30-10:30 Talbot Conference Room
Symposium 8
Control and Regulation of Circadian Outputs

Chairs: Shin-Ichi Inouye
Yamaguchi University

Speakers: Andries Kalsbeek
Netherlands Institute for Brain Research
SCN transmitters and the circadian control of hormonal rhythms

David Klein
NICHD
Biology of the melatonin rhythm enzyme-serotonin N-acetyltransferase

Rae Silver
Columbia University
SCN signaling systems

Gianluca Tosini
Morehouse School of Medicine
Expression and regulation of clock genes in rat pineal

08:30-10:30 Ossabaw Conference Room
Symposium 9
Photoperiodism and Melatonin

Chair: Herbert Underwood
North Carolina State University

Speakers: Eberhard Gwinner
Max-Planck-Institute
Circadian patterns of melatonin secretion in birds: significance of duration and amplitude

Randy Nelson
Johns Hopkins University
Melatonin and seasonal changes in immune function

Andrew Loudon
University of Manchester
Seasonal control of prolactin secretion

Gregory Ball
Johns Hopkins University
Steroid-melatonin interactions and the seasonal regulation of the avian song control system

10:30-11:00 Coffee Break
Conference Center Patio

11:00-13:00 Cumberland Ballroom
Slide Session 7
SCN Physiology

Chairs: Rebecca Prosser
University of Tennessee

11:00
257 TIME PASSES ON: IMMORTALIZED SCN2.2 CELLS CONFER CIRCADIAN RHYTHMICITY ON NIH-3T3 FIBROBLASTS. Gregg C. Allen, Dept of Human Anatomy, Texas A&M University, College Station TX.

11:15
258 COUPLING AMONG CIRCADIAN OSCILLATORS IN THE SCN DEPENDS UPON CELL DENSITY AND ACTION POTENTIALS. Erik D. Herzog, Dept of Biology, University of Virginia, Charlottesville, VA.

11:30
259 PHASE OF ELECTRICAL ACTIVITY OF INDIVIDUAL, ADULT SUPRACHIASMATIC NEURONS *IN VITRO* DEPENDS UPON *IN VIVO* LIGHT HISTORY. Fabienne Aujard, Lab d'Ecologie Generale, CNRS UMR 8571, Brunoy, France.

11:45
260 Efferent neurons in the mouse supra-chiasmatic nucleus (SCN) express photo-inducible c-FOS. Horacio de la Iglesia, Dept of Neurology, University of Massachusetts Medical School, Worcester, MA.

12:00
261 NEUROTENSIN ACTIVATES NEURONES OF THE SUPRACHIASMATIC NUCLEUS *IN VITRO*. Andrew N. Coogan, School of Biological Sciences, University of Manchester, United Kingdom.

12:15
262 CELLULAR ORGANIZATION OF MULTI-OSCILLATORY PACEMAKING SYSTEM IN THE RAT SUPRACHIASMATIC NUCLEUS; AN ANALYSIS USING A MULTI-ELECTRODE DISH. Sato Honma, Dept of Physiology, Hokkaido University School of Medicine, Sapporo, Japan.

- 12:30
263 VASOPRESSIN IN THE CIRCADIAN CLOCK OF COMMON VOLES: A PUTATIVE CAUSAL FACTOR IN TIMING OF LOCOMOTION. Koen Jansen, Animal Behavior, University of Groningen, Haren, Netherlands.
- 12:45
264 A MATHEMATICAL MODEL OF THE SCN CLOCK INCORPORATING PHYSIOLOGICAL AND ANATOMICAL DATA. Joseph D. Miller, Dept of Pharmacology, Texas Tech University Health Sciences Center, Lubbock, TX.
- 11:00-13:00 Talbot Conference Room
Slide Session 8
Molecular Basis of Circadian Timing II
- Chair: Mary Harrington
Smith College
- 11:00
265 CIRCADIAN MODULATION OF THE GATING OF CYCLIC GMP-ACTIVATED CATIONIC CHANNELS IN VERTEBRATE RETINAL PHOTORECEPTORS. Stuart E. Dryer, Dept of Biology & Biochemistry, University of Houston, TX.
- 11:15
266 UNMASKING ARRHYTHMIA: THE FUNCTION OF ELF3 IN THE *ARABIDOPSIS* CIRCADIAN CLOCK. Harriet McWatters, Dept of Biological Sciences, University of Warwick, Coventry, United Kingdom.
- 11:30
267 CIRCADIAN AND PHYTOCHROME CONTROL ACT AT DIFFERENT PROMOTER REGIONS OF THE TOMATO *Lhca3* GENE. Birgit Piechulla, Department of Biochemistry, University of Rostock, Germany.
- 11:45
268 MELATONIN AFFECTS PHOTOPERIODIC FLOWER INDUCTION IN THE DICOT PLANT *CHENOPODIUM RUBRUM*. Jan Kolar, Institute of Experimental Botany, Czech Academy of Sciences, Prague, Czech Republic.
- 12:00
269 STRUCTURES OF COCKROACH CIRCADIAN SYSTEM VIEWED FROM CLOCK GENE PRODUCTS AND ROLES OF MELATONIN IN THE OUTPUT PATHWAY. Makio Takeda, Grad School of Science, Kobe University, Kobe, Japan.
- 12:15
270 ANALYSIS OF THE COUPLING-PATHWAY OF THE CIRCADIAN CLOCK OF THE COCKROACH *LEUCOPHAEA MADERAE*. Monika Stengl, Biology and Animal Physiology, University of Marburg, Germany.
- 12:30
271 THE *NEUROSPORA* UPPER LIP I: INTERDEPENDENCE OF *frequency* AND *white collar* GENES AND PRODUCTS. Martha Merrow, Institute of Medical Psychology, University of Munich, Germany.
- 12:45
272 THE *NEUROSPORA* UPPER LIP II: CIRCADIAN GATING OF LIGHT INPUT PATHWAYS--A ZEITNEHMER FEEDBACK? Till Roenneberg, Institute of Medical Psychology, University of Munich, Germany.
- 11:00-13:00 Ossabaw Conference Room
Slide Session 9
Physiological Aspects of Biological Timing
- Chair: Laura Smale
Michigan State University
- 11:00
273 ECOLOGICAL IMPORTANCE OF AN SCN PACE-MAKER IN WILD GOLDEN-MANTLED SQUIRRELS. Patricia J. DeCoursey, Dept of Biological Sciences, University of South Carolina, Columbia, SC.
- 11:15
274 POST-HIBERNATION ARRHYTHMICITY AND REDUCTION OF AVP IMMUNOSTAINING IN THE SCN OF THE EUROPEAN GROUND SQUIRREL. Roelof A. Hut, Zoological Laboratory, University of Groningen, Haren, The Netherlands.
- 11:30
275 THE CIRCADIAN ACTIVITY-REST RHYTHM OF AGED RHESUS MACAQUES: INFLUENCE OF MELATONIN. Henryk F. Urbanski, Division of Neuroscience, Oregon Regional Primate Center, Beaverton, OR.
- 11:45
276 PHOTOPERIOD-DEPENDENT AND -INDEPENDENT REGULATION OF MELATONIN RECEPTORS IN AREA X OF SONGBIRDS: EFFECT OF REPRODUCTIVE STATE AND INTERPRETATION OF SEX AND SPECIES DIFFERENCES. G. Bentley, Dept of Psychology, Johns Hopkins University, Baltimore, MD.
- 12:00
277 CLOCK MUTANT MICE SHOW ALTERED SLEEP AND BODY TEMPERATURE RHYTHMS UNDER BASELINE AND STRESSFUL CONDITIONS. Amy Easton, Neurobiology & Physiology, Northwestern University, Evanston, IL.
- 12:15
278 GENETIC ANALYSIS OF SLEEP-LIKE BEHAVIOR IN *DROSOPHILA MELANOGASTER*. Paul J. Shaw, The Neurosciences Institute, San Diego, CA.
- 12:30
279 MOLECULAR MECHANISMS LINKING CIRCADIAN GENES AND COCAINE RESPONSIVENESS IN *D. MELANOGASTER*. Rozi Andretic, Dept of Biology, University of Virginia, Charlottesville.
- 12:45
280 ANALYSIS OF A BEHAVIORAL FEEDBACK LOOP IN THE DESERT SCORPION. W. Otto Friesen, Dept of Biology, University of Virginia, Charlottesville, VA.
- 13:00-16:30 Break
- 16:30-18:00 Cumberland Ballroom
Pittendrigh/Aschoff Lecture
J. Woodland Hastings
Harvard University

THE HAMSTER *tau* LOCUS IS ENCODED BY CASEIN KINASE I EPSILON (CKIε) A HOMOLOG OF THE DROSOPHILA CIRCADIAN GENE, *double-time*

Phillip L. Lowrey,¹ Kazuhiro Shimomura,^{1,2} Marina P. Antoch,^{1,2} Shin Yamazaki,³ Peter D. Zemenides,¹ Martin R. Ralph,⁴ Michael Menaker,³ Joseph S. Takahashi^{1,2*}

¹Department of Neurobiology and Physiology, ²Howard Hughes Medical Institute, Northwestern University, Evanston, IL 60208, USA. ³Department of Biology, National Science Foundation Center for Biological Timing, University of Virginia, Charlottesville, VA 22903, USA. ⁴Department of Psychology, University of Toronto, Toronto, Ontario M5S 3G3, Canada.

The circadian mutation, *tau*, was the first single-gene circadian mutation to be discovered in mammals (reported by Ralph and Menaker in *Science* in 1988). The *tau* mutant, arguably, has been the one of the most significant genetic animal models for the study of circadian rhythms in mammals. We report the molecular identification of the *tau* mutation using a novel approach to locate the gene by whole-genome subtraction methods (genetically directed representational difference analysis or GDRDA). We find that the *tau* locus is encoded by the casein kinase I epsilon (CKIε) gene. This finding is remarkable because in *Drosophila*, the circadian mutation, *double-time*, is also encoded by a casein kinase that is most similar to CKIε. Our results provide definitive genetic evidence that CKIε is a component of the mammalian circadian clock. Both genetic linkage analysis and molecular analysis of the specific mutation in the gene provide strong proof for identification of the underlying gene. To determine the effects of the *tau* mutation on the biochemistry of CKIε, we have compared the wild type and mutant enzyme. We find striking changes in maximal enzyme velocity and autophosphorylation state in the mutant enzyme. To link CKIε to circadian function, we have shown that it can interact with the mammalian PERIOD proteins in vitro and that these proteins are substrates. We also show that the *tau* mutation affects the phosphorylation of PERIOD proteins. Finally, we have analyzed *Per1* gene expression in the hamster SCN and find two effects: lower peak expression and an earlier decline in *Per1* in *tau* hamsters. Taken together, these results allow us to propose a mechanism for the role of CKIε within the mammalian circadian system.

POTENTIAL ROLE OF CASEIN KINASE I ε IN THE REGULATION OF MAMMALIAN CIRCADIAN RHYTHM

D. Virshup, E. Vielhaber, E. Eide, Z. Gao, A. Rivers, M. Carlson Depts. Pediatrics and Oncological Sciences, Huntsman Cancer Institute, U. Utah, Salt Lake City, UT 84112

The molecular oscillator that keeps circadian time is generated by a negative feedback loop. Controlled nuclear entry of circadian regulatory proteins that inhibit transcription from E-box-containing promoters appears to be a critical component of this loop both in *Drosophila* and mammals. The *Drosophila double-time* gene product, a casein kinase I ε (CKIε) homolog, has been reported to interact with dPER and regulate circadian cycle length. CKIε appears to regulate mammalian circadian rhythm as well, as we find that over-expression of dominant negative CKIε in Rat1 cells alters their circadian rhythm. We have investigated the interaction of CKIε with various circadian regulatory proteins. CKIε binds to and phosphorylates the murine circadian regulators mPER1 and mPER2. A CKIε binding site on mPER1 has been defined. Unlike both dPER and mPER2, mPER1 expressed alone in human embryonic kidney 293 cells is predominantly a nuclear protein. We have identified an mPER1 nuclear localization signal (NLS). Two distinct mechanisms appear to retard mPER1 nuclear entry. First, co-expression of mPER2 leads to mPER1:mPER2 heterodimer formation and cytoplasmic co-localization. Second, co-expression of CKIε leads to phosphorylation-dependent masking of the mPER1 NLS and hence cytoplasmic retention of both proteins. Mutations in mPER1 that alter its NLS or masking domain alter its localization or the effects of CKIε. mCRY1 interacts with the mPER1:CKIε complex and alters its subcellular localization as well. The data suggest that CKIε regulates mammalian circadian rhythm in part by controlling the rate at which mPER1 enters the nucleus.

3

A TIMELESS-Independent Function for PERIOD Proteins in the *Drosophila* Clock.

Lino Saez, Adrian Rothenfluh, and Michael W. Young,

Laboratory of Genetics, and National Science Foundation Science and Technology Center for Biological Timing, The Rockefeller University, 1230 York Avenue, New York, NY 10021

We have isolated a mutant allele of *timeless*, *tim^{UL}*, that causes long 33 hour rhythm. The molecular phenotype of *tim^{UL}* is prolonged nuclear localization of the PER/TIM^{UL} complex. This extended nuclear association of PER/TIM^{UL} is linked to protracted de-repression of *per* and *tim* RNA, generating very broad peaks lasting for about 20 hrs. Generation of nuclear PER by light-induced degradation of TIM^{UL} leads to strong downregulation of *per* and *tim* RNA. Thus, in the absence of TIM, PER appears to function as a negative transcriptional regulator. Two additional studies support this TIM-independent role for PER: (i) *Drosophila* expressing a form of PER that constitutively localizes to nuclei produce dominant behavioral arrhythmicity. (ii) in cultured cells, expression of constitutively nuclear PER represses dCLOCK/CYCLE-mediated transcription of *per* in the absence of TIM. We suggest that conversion of PER/TIM heterodimers to nuclear PER proteins terminates each molecular cycle by enhancing transcriptional repression while accelerating PER turnover.

4

KNOCKOUT OF THE MURINE *TIMELESS* GENE ARRESTS EMBRYONIC DEVELOPMENT. Anthony Gotter¹, Thomas Manganaro², David Weaver¹, Bernard Possidente³, David MacLaughlin² and Steven Reppert¹. ¹Laboratory of Developmental Chronobiology, ²Pediatric Surgical Research Laboratory, Massachusetts General Hospital/Harvard Medical School, Boston, and ³Department of Biology, Skidmore College, Saratoga Springs, NY.

The *Drosophila timeless* gene is essential to molecular mechanisms that control circadian behavioral rhythms. In mice, *Timeless* (*mTim*) is expressed in the SCN, and *in vitro* modestly inhibits CLOCK/BMAL1-mediated transcription. The protein also interacts with mCRY1 and mCRY2 both *in vitro* and *in vivo*. Unlike flies, however, expression of *mTim* is not rhythmic, nor is its protein product affected by entraining light pulses. To resolve the role of *mTim* in the molecular mechanism of the mouse circadian clock, the gene was disrupted by targeted mutagenesis. Mice heterozygous for the mutant *mTim* allele (*mTim*^{+/-}) develop normally and exhibit no overt behavioral abnormalities. Despite expressing attenuated levels of mTIM protein, rhythmic behavior of *mTim*^{+/-} mutants persists for more than two weeks under constant conditions, with no significant differences in free running period relative to wildtype mice. No homozygous (*mTim*^{-/-}) offspring were observed from *mTim*^{+/-} pairings, indicating that the gene is essential for embryonic development. Of 87 weanlings genotyped by southern blot, 29 were wildtypes and 58 were *mTim*^{+/-}, values that conform to the expected 1:2:0 Mendelian ratio for an embryonic lethal phenotype. Of 121 offspring evaluated *in utero*, 35% appeared nonviable whereas only 14% of embryos from wildtype X *mTim*^{+/-} crosses were abnormal. Histological analysis demonstrates that the functional gene is required for development before embryonic day 5.5.

CLONING AND CHARACTERIZATION OF *ZEITLUPE*, A NOVEL COMPONENT OF THE PLANT CIRCADIAN CLOCK SYSTEM.

David E. Somers^{†*}, Tom Schultz[†] and Steve A. Kay[†].

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*Department of Plant Biology/ Plant Biotechnology Center, Ohio State University, Columbus, OH 43210, USA.

We have characterized and cloned a locus (*ZEITLUPE*; *ZTL*) responsible for mediating the light-dependent control of the circadian period in *Arabidopsis*. The *ZTL* locus is defined genetically by two mutations (*ztl-1*, -2), which have very similar long period phenotypes as measured by the *CAB2::luciferase* luminescence assay. Fluence-rate response tests show a heightened fluence-rate effect of red and blue light on period length in these mutants. At high light intensities ($150 \mu\text{m m}^{-2} \text{s}^{-1}$) period is 2-3 h longer than wild type (WT), whereas at low fluence rates ($1-2 \mu\text{m m}^{-2} \text{s}^{-1}$) *ztl* mutations increase period by 9-10 h. Further tests with *ztl-1* show that the rhythms of cotyledon and leaf movements are also lengthened, indicating that two very different clock-controlled processes, transcription and cell expansion, are affected by this mutation. Flowering time in long days is increased, relative to WT, also consistent with a defect in regulation by the circadian clock.

In contrast, in *ztl-1* the acute (rapid) response of *CAB2* gene expression to a red or blue light pulse is identical to WT and there is only a slight effect of light intensity on hypocotyl length, suggesting that *ZTL* may be primarily dedicated to controlling light input to the circadian clock. In both the WT and *ztl-1*, *ZTL* message levels remain constant under light/dark cycles and in continuous light, indicating that *ZTL* gene expression is neither light regulated nor under circadian clock control. *ZTL* encodes a novel plant protein with an intriguing and unique domain composition that immediately suggests that it may play a role at the interface between the light input pathway and the oscillator itself.

THE *DROSOPHILA* TAKEOUT GENE IS A NOVEL MOLECULAR LINK BETWEEN CIRCADIAN RHYTHMS AND FEEDING BEHAVIOR

Lea Sarov-Blat*, W. Venus So, Li Liu*, Daniel Lee and Michael Rosbash*

*Howard Hughes Medical Institute NSF, Center for Biological Timing Department of Biology Brandeis University Waltham, Massachusetts 02254

ABSTRACT

We report the functional characterization of a novel *Drosophila* clock-regulated output gene, *takeout* (*to*). *to* mRNA and protein levels undergo circadian cycling, and gene expression is down-regulated in all of the clock mutants tested. The *to* amino-acid sequence shows similarity to two lipophilic ligand-binding proteins, juvenile hormone binding protein and JP29. *to* mRNA is mainly expressed in the adult head, but mRNA is also localized to the cardia, crop and antennae - structures related to feeding. In addition, *to* expression is induced by starvation, especially in the gastrointestinal tract. This starvation-induced expression is blocked in the circadian mutant *per⁰¹*, suggesting a direct molecular link between the circadian clock and the feeding/starvation response. A *to* mutant with low mRNA and protein levels has an aberrant locomotor activity response to starvation, indicating a link between locomotor activity and food status. The mutant phenotype is largely rescued by expression of a wild-type *to* transgene. As a consequence, we propose that *to* participates in a novel circadian output pathway that conveys temporal and food status information to feeding-relevant metabolisms and activities.

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THE XPER2 GENE IN *XENOPUS LAEVIS* RETINA IS PRIMARILY DRIVEN BY LIGHT
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The retina of the African clawed frog, *Xenopus laevis*, contains a circadian oscillator that controls rhythmic events such as melatonin release and gene expression. In order to investigate the molecular components of the retina clock, we cloned a *Xenopus* homologue of the clock gene mPer2 and have analyzed its expression patterns in retina. The xPer2 gene exhibits a rhythm at the mRNA level in LD with a peak at ZT 8, however this rhythm is abolished in constant darkness (DD). In vivo, we show an increase in xPer2 mRNA by light pulses given to frogs maintained in constant dark. Six hours of light is able to cause a 3-5 fold increase in the amount of xPer2 mRNA regardless of circadian time (subjective day ZT 2, late subjective day ZT 10, or late subjective night ZT 18). To better characterize the effect of light, we analyzed isolated eyecups in vitro pulsed with 4×10^{-4} Watts/cm² light for 3 hours. A timecourse of light induction shows a 2 fold increase in xPer2 mRNA after 3 hours from three circadian times compared to dark controls. In addition, no induction is seen for another clock gene xPer1, or for a clock-controlled gene, tryptophan hydroxylase in these samples. Since light seems to play such an important role in the regulation of xPer2, one possibility is that xPer2 is a part of the phase shifting apparatus and must be able to respond to light signals at all times of day. Dopamine is a neuromodulator of the retina that is normally at high levels during the day. Dopamine is able to mimic the effects of light on phase shifting the melatonin rhythm in *Xenopus* retina. We found 3hr pulses with 500nM dopamine are also able to induce the xPer2 mRNA by approximately 3 fold. Similar results were found with a D2 dopamine receptor agonist, quinpirole. Further work with selective dopamine receptor agonist and antagonists will help uncover the mechanism by which dopamine signaling induces xPer2 mRNA. This work provides another example of the variation of circadian clock mechanisms among species. While post-transcriptional and translation controls may play a role in the regulation of the xPer2 protein, the lack of rhythmicity of the xPer2 mRNA in the absence of light leads us to speculate that it may play an alternative role in the basic clock mechanism in *Xenopus* retina.

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CHARACTERIZATION OF A NOVEL CIRCADIAN GENE ISOLATED FROM A CHICK PINEAL cDNA LIBRARY

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The chicken pineal is a complete circadian system having direct photosensitivity, an endogenous oscillator and rhythmic outputs. Our goal has been the identification and characterization of key molecular elements of this circadian clock. Using a PCR-based equalized subtractive hybridization strategy to screen a nocturnal pineal cDNA library, we have isolated a novel clone, which we call *Chrono*. Database searches have thus far not identified any significant vertebrate homologues to chick *Chrono*. Northern blot analysis showed that the expression of the *Chrono* transcript (4 kb) in the pineal is highly circadian *in vivo*, i.e. the rhythmicity persists under constant conditions (DD or LL) with maximal transcript levels during the subjective night and basal levels during the subjective day. This circadian rhythm persists *in vitro* and can be entrained to a reversal of the light-dark cycle. Northern blotting experiments with various chicken tissues revealed high expression of *Chrono* only in the pineal gland, although weaker signals could be detected also in chicken retina and lung. Comparative Northern blotting studies revealed *Chrono* expression in the goldfish pineal and in the rat SCN, but not in the trout or rat pineal. We interpret these data to mean that the *Chrono* gene product is involved in circadian pacemaker functions, rather than in output or input pathways. Using 5' and 3' RACE-PCR techniques the complete open reading frame for this unusual new gene is now being sequenced. Studies aimed at identifying and characterizing the mammalian *Chrono* homologue will provide further insights into the molecular evolution of vertebrate cellular clocks.
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SPECTRAL SENSITIVITY OF MELATONIN SUPPRESSION IN HUMANS

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Light exposure at night causes an acute suppression of nocturnal melatonin levels. Determination of irradiance response curves to various wavelengths of light will permit construction of an action spectrum indicating the spectral characteristics of the photoreceptor(s) involved in melatonin suppression.

Healthy subjects (18M, 4F, aged 19-45 years) were exposed to monochromatic light (λ_{max} 480, 500, 535 and 560 nm at 10 nm half-peak bandwidth) for 30 mins via a spherical dome coated with white reflectance paint. The light dome was designed to ensure uniform illumination across the entire visual field. Subjects were studied on 3 consecutive nights (N1, baseline no light treatment; N2 and N3 light treatment) on 1-16 occasions. Posture and lighting were controlled each night. The timing of light exposure was individualised to occur on the rising curve of the melatonin rhythm (estimated from prestudy urinary 6-sulphatoxymelatonin data). Each night, following pupil dilation (Tropicamide 0.5 %) at -90 mins, blood samples were taken every 15 mins from -15 mins before lights on to 90 mins after light treatment for measurement of plasma melatonin by RIA. Light-induced melatonin suppression was calculated using the average melatonin values at 30 and 45 mins on treatment night as a percentage of the same average from the baseline night (N1). Light of 480 nm was tested at 7 irradiances (n=5-7 subjects/irradiance) ranging from 1.8 to 31.0 $\mu\text{W}/\text{cm}^2$ ($\approx 7.81 \times 10^{15}$ - 1.35×10^{17} photons/ cm^2). Compared with the baseline night at 30 and 45 mins it produced significant suppression of melatonin at all irradiances. 500 nm light was tested at 6 irradiances (n=4-7, 3.0 - 30.0 $\mu\text{W}/\text{cm}^2 \approx 1.22 \times 10^{16}$ - 1.22×10^{17} photons/ cm^2) and significant suppression was observed at all intensities. Light of 535 nm produced significant suppression at irradiances ranging from 4.1 to 65.0 $\mu\text{W}/\text{cm}^2$ ($\approx 1.96 \times 10^{16}$ - 3.12×10^{17} photons/ cm^2 , n=5-7). However, no significant suppression was seen at lower 535 nm irradiances (n=3-6, 0.70 - 3.25 $\mu\text{W}/\text{cm}^2 \approx 3.35 \times 10^{15}$ - 1.56×10^{16} photons/ cm^2). Light of 560 nm was also investigated (n=3-5, 7.2 - 65.0 $\mu\text{W}/\text{cm}^2 \approx 3.65 \times 10^{15}$ - 3.30×10^{17} photons/ cm^2). Significant suppression of plasma melatonin only occurred at an irradiance of 13.6 $\mu\text{W}/\text{cm}^2$ ($\approx 6.89 \times 10^{16}$ photons/ cm^2) or greater. These results show that the suppression of plasma melatonin not only depends upon the number of photons delivered but also the wavelength of light. Preliminary estimates of the 50% maximal response (ED_{50}) show that melatonin suppression is more sensitive to light of shorter wavelengths (480-500 nm) compared to longer wavelengths (560 nm).

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CIRCADIAN PHOTORECEPTION IN HUMANS: ACTION SPECTRUM FOR MELATONIN SUPPRESSION. G. Brainard, M. Rollag, J. Hanifin, J. Greeson, B. Byrne, G. Glickman, J. Gardner, B. Sanford, E. Gerner. Department of Neurology, Thomas Jefferson University, Philadelphia, PA 19107; Department of Anatomy, Uniformed Services University of Health Sciences, Bethesda, MD 20814.

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It is not known which photopigment(s) transduce light stimuli for circadian regulation in mammals. The aim of this ongoing study is to elucidate the ocular photoreceptor system for regulating the human pineal gland by establishing an action spectrum for light-induced melatonin suppression. The 33 females and 31 males who have entered this study have a mean age of 24.5 ± 0.4 yrs. and have normal color vision. Volunteers received full-field monochromatic stimuli while their pupils were dilated with a mydriatic agent. The light exposure system was illuminated by a xenon arc lamp connected to a grating monochromator, and an integrating sphere. Monochromatic light exposures were between 2:00 and 3:30 AM. Blood samples were collected before and after these light exposures and were quantified for melatonin by RIA. Each subject was tested with at least seven different irradiances of one wavelength with a minimum of one week between each nighttime exposure. Presently, over 600 individual nighttime melatonin suppression tests have been completed with eight monochromatic wavelengths ranging from 440 nm to 600 nm. Fluence-response curves were completed with 10 irradiances at 505 and 555 nm for 8 subjects, each. The entire 505 nm curve is "shifted to the left" compared to the 555 nm curve. For example, an equal photon dose of 4.19×10^{13} photon/ cm^2/sec stimulus at 505 nm induces a mean control-adjusted melatonin suppression of 61%. In contrast, the same photon density at 555 nm induced a significantly lower ($p < 0.01$) melatonin suppression of 15%. These data demonstrate that the cone system that mediates photopic vision is not the primary photoreceptor system to transduce light stimuli for melatonin regulation in the human. Support: NIH RO1NS36590 (to GB) and NSF IBN9809916 (to MR).

11 ENTRAINMENT OF TOTALLY BLIND SUBJECTS: PHOTIC OR NON-PHOTIC?

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We have previously assessed the circadian rhythm types of 37 blind subjects with no conscious light perception (NPL)¹ using urinary 6-sulphatoxymelatonin (aMT6s) acrophase (ϕ) as a marker of circadian phase. Approximately one-third (13/37, 35%) of the NPL subjects were synchronised to 24 h; 8 were normally entrained (NE; mean aMT6s ϕ range 2.1 - 6.7 h) and 5 were abnormally entrained (AE; ϕ range 7.2 - 20.6 h). The aim of the current study was to assess whether photic input could be responsible for the entrainment of these NPL subjects. This was tested by examining the suppressive effects of ocular light exposure on the plasma melatonin rhythm. Light-induced suppression of melatonin can be used as an indirect test of the functional integrity of the retina-retinohypothalamic tract-suprachiasmatic nuclei (SCN)-pineal pathway in blind individuals².

Six NE and 3 AE subjects (21-74 yrs; 5M, 4F) were studied on one ($n = 3$) or two ($n = 6$) occasions. All but one of the NPL subjects had one or both eyes intact and the remaining subject had vestigial eyes (cryptophthalmos) as confirmed by ultrasound. Prestudy measurements reassessed circadian phase from urinary aMT6s acrophase. There was a good correlation ($r = 0.97$) with the original assessment of aMT6s acrophase (mean difference $\pm$ SD = 1.8 ± 1.0 h, $n = 9$), conducted on average 28 months (range 9-43) earlier, confirming that the subjects were not free-running at a short period.

For each study, blood samples were taken every 15-60 mins for at least 10 h for two consecutive periods of melatonin production (N1, N2). From at least 1 h prior to and 4 h after light exposure, subjects were supine, in darkness (DD) and wearing eye masks. At all other times, subjects were semi-recumbent in dim light (< 10 lux). Light was delivered via fibre optic bundles to two light rings fitted into spectacles (ETA, USA). On N2, the subjects' eyes were exposed to broad spectrum white light (UV- and IR-filtered) for 30 or 60 mins (700-11000 lux, 420-7200 μ W/cm²). N1 was identical to N2 except that the fibre optic cables were not attached to the light source. Comparison of melatonin levels on N2 with corresponding times on N1 showed no significant suppression of melatonin in any of the subjects. Sighted controls ($n = 3$) studied using the same protocol (30 mins of 2250 lux) showed statistically significant suppression of melatonin ($61 \pm 6\%$). Lack of a photic effect suggests that these entrained NPL subjects have a dysfunctional retina-SCN-pineal pathway, most likely due to dysfunctional retinal processing. The findings imply that non-photoc synchronisers may be involved in the entrainment of these NPL subjects³. *This work has been supported by The Wellcome Trust, UK (grant 048197/Z/96/Z).*¹Skene et al., 1999 *Reprod. Nutr. Develop.* 39, 295-304; ²Czeisler et al., 1995 *New Eng. J. Med.* 332, 6-11; ³Klerman et al., 1998 *Am. J. Physiol.* 43, R991-6.

12 A PHASE RESPONSE CURVE TO SINGLE BRIGHT LIGHT PULSES IN HUMANS

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The human circadian pacemaker exhibits high sensitivity to light. Low light levels typical of artificial indoor illumination are capable of generating phase shifts, and 3-cycle bright light pulses can yield a strong Type 0 phase response curve (PRC). Although PRC's to single light pulses in humans have been reported, relatively small sample sizes were used (1-3). In this study we present a more comprehensive PRC to single bright light pulses.

Data from 21 healthy, entrained male and female volunteers who completed a 9-10 day in-laboratory study were analyzed. Following 3 baseline days on the subjects' habitual sleep/wake schedule, all subjects underwent a constant routine (CR) in dim light (~ 3 -7 lux) with enforced wakefulness in a semi-recumbent posture for 27-49 hrs. The timing of the end of the CR and the subsequent 24-hr light-treatment day varied across subjects in order to systematically span the entire circadian cycle. Following an 8-hr sleep episode, subjects remained awake for 16 hrs in dim light, except for a 6.7-hr bright light treatment centered in the middle of this waking episode. The bright light treatment consisted of 6-min episodes of fixed gaze ($\sim 10,000$ lux) alternating with 6-min episodes of free gaze ($\sim 4,000$ -7,000 lux). Another 8-hr sleep episode was followed by a post-stimulus CR of 30-65 hr. Core body temperature was measured at 1-minute intervals and the estimated circadian phase of the core body temperature minimum was evaluated from data collected during the CR's by fitting a dual-harmonic-regression-with-correlated-noise model. Phase shifts were calculated as the difference in phase between the pre- and post-stimulus CR's.

The PRC revealed a characteristic shape for a Type 1 PRC with robust phase shifts of the core body temperature rhythm ranging in magnitude from -4.0 hr (delay) to +2.3 hr (advance). In general, phase delays followed light pulses applied prior to the core body temperature minimum, and phase advances followed pulses applied after this phase, with no apparent "dead zone" of photic insensitivity.

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EFFECTS OF TIMED ONE HOUR PULSES OF BRIGHT BROAD SPECTRUM WHITE LIGHT AT NIGHT ON PERFORMANCE, ALERTNESS AND MELATONIN SUPPRESSION.

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Exposure to broad spectrum white light at night can increase alertness and performance ability and decrease the concentration of circulating melatonin. A positive correlation between melatonin suppression and alertness has been described (1). However, it is not known whether melatonin suppression is strongly related to performance ability. Six normal healthy female subjects ($25.8\text{yr} \pm 3.2\text{SD}$) underwent a 27hr continuous performance sessions on four occasions. A test battery was administered every 2hrs, alertness was rated by the Samn-Perelli and Karolinska sleepiness scale and saliva samples were collected for melatonin RIA at 30-60min intervals. Ambient lighting was kept at $<50\text{lux}$. A one hour pulse of broad spectrum white light ($10,000\text{lux}$) was given at (A) 20:00h, (B) 00:00h, (C) 04:00h or (D) not at all.

Circadian phase was similar on all legs, the time of melatonin onset (mid-range crossing) being $22:54\text{h} \pm 0.3\text{SD}$ with a CV of 2.3-5.7% within subjects. Melatonin was suppressed by light at 00:00h ($57.7\% \pm 26.4\text{SD}$ at 60min) and 04:00h ($29.3\% \pm 52\text{SD}$ at 60min). Positive correlations were present between melatonin suppression and four normalised performance measures. Correlations were particularly evident for DSST (A) 21:30-22:00h $r=0.9$, $p=0.007$, (B) 01:30-02:00h $r=0.9$, $p=0.005$, and for 2-letter Sternberg accuracy (C) 06:00h $r=0.9$, $p=0.004$. Correlations for alertness measures approached significance. We conclude that enhanced performance and alertness by light at night is related to melatonin suppression.

Work supported by Priority Pathfinder contract.

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ASSOCIATION BETWEEN 24-H LIGHT EXPOSURE AND MELATONIN SECRETION IN NIGHT WORKERS

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It has been suggested that exposure to natural bright light, especially in the morning, prevents the phase shift necessary for circadian adjustment of night workers, even after many consecutive night shifts. In the present study, 30 night nurses (27 W, 3 M, aged 26-55) came to the chronobiology laboratory after a minimum of 3 consecutive days of nightwork (00:00 to 08:00). During 24 hours, subjects were kept in dim light ($< 25\text{ lux}$) and urine was collected every 2 hours to measure the excretion of 6-sulphatoxymelatonin. According to the timing of their melatonin secretion, subjects were classified in 3 sub-groups: "non-shifters" ($n=21$), "advance" ($n=3$), and "delay" ($n=6$). For about 56 hours before the admission to the laboratory, light exposure was recorded every minute by an ambulatory photometer. Results were compared between the 3 sub-groups of subjects. As expected, the highest light exposure was between 08:00 and 09:00 a.m., but there was no difference between the 3 sub-groups of subjects. However, when the entire daily pattern of light exposure was considered, significant differences were noted. Compared to the non-shifters, subjects with a delay of their melatonin secretion received more light during the night (03:00-06:00) and the evening (21:00-22:00) and less light in late morning and afternoon (10:00 to 17:00). For the subjects with an advanced secretion, the only difference was in the evening (18:00 to 21:00) when they received significantly less light than the non-shifters. The fact that very distinctive patterns of light exposure can be identified even in this small number of subjects indicates that a more systematic study of global daily patterns of light exposure may help to understand and manipulate circadian entrainment in nightworkers.

15 EFFECT OF LIGHT HISTORY ON LIGHT SENSITIVITY IN HUMANS

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We investigated if after 6 days of restriction to dim light, a nocturnal light stimulus would produce more melatonin suppression than after 6 days with enforced bright light exposure. During the DIM WEEK, subjects (6 F, 6 M; age 18-38) were instructed to minimize outdoor light exposure and wear dark welders goggles when outside. During the BRIGHT WEEK, the same subjects were instructed to get at least 4-h of bright light per day, using light boxes (about 5000 lux) and/or outdoor light. Weeks were consecutive and counterbalanced. Ambient light was monitored with a photosensor. On the 6th and 7th nights of each week (baseline and melatonin suppression night respectively), salivary samples were collected in dim light (<15 lux) every 30 min for 7 hrs at night (e.g. 0:00h to 7:00h) except on night 7 in which 500 lux was presented for 3 hrs in the middle of the night (e.g. 2:00h-5:00h). For each week, melatonin suppression was calculated using the average melatonin level in the last 2-h of light exposure during the melatonin suppression night (night 7) compared to the average level during the same 2-h period in the baseline night (night 6). In 7 subjects more suppression occurred after the DIM WEEK (68 vs 45%), in 3 suppression remained the same (46 vs 49%), in 1 suppression could not be detected in either week (-18 vs -26%), and in 1 more suppression occurred after the BRIGHT WEEK (58 vs 36%). This is the first demonstration that pineal gland sensitivity to nocturnal light can be affected by a rapid change in light history. But more study is needed to confirm this, and to determine whether light history impacts pacemaker sensitivity to phase shifting by light. Supported by NIH NS23421.

16 TIMING OF BRIGHT LIGHT TO NORMALIZE CIRCADIAN PHASE IN OLDER INDIVIDUALS WITH INSOMNIA AND DEPRESSION

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Our aim was to examine the influence of different bright light schedules on circadian phase in older individuals (n=72) with insomnia and/or depression. Volunteers wore an actillum wrist monitor for 5-7 days at home. Urine was collected over two 24-hr periods, usually days 3 and 6, and assayed for 6-sulphatoxymelatonin (6-SMT) via radioimmunoassay. The best-fitting 24-hr cosine established the acrophase of 6-SMT excretion. Following home recording, volunteers spent 5 nights and 4 days continuously in the laboratory. Sleep periods in 0 lux were fixed at 8 hrs, consistent with usual schedules. Volunteers were randomly assigned to three light treatment schedules (4 hr total per day): (A) 2 hrs at 3,000 lux beginning both 1 hr after wake time and 3 hr before bedtime; (B) 4 hours at 3,000 lux in the middle of the wake period; (C) 4 hours of placebo red light in the middle of the wake period. Lighting was at 50 lux during the remainder of the wake periods. Urine was collected on the final laboratory day. Phase shifts were calculated as the 6-SMT acrophase at home minus the acrophase on day 4. A very strong phase-response relationship to the initial phase of light treatment A was found ($r_s=0.84$; $p<0.001$). Phase advanced individuals at baseline were delayed (probably due to the evening light), whereas those who were phase delayed at baseline were advanced (probably due to the morning light). Treatments B and C elicited minimal mean phase shifts.

GLUCOCORTICOIDS CAN RESET CIRCADIAN TIME IN PERIPHERAL TISSUES BUT NOT IN THE SCN

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In mammals, circadian oscillators reside not only in the suprachiasmatic nucleus (SCN), believed to harbor the central clock, but also in most peripheral tissues. Notably, we have recently shown that a serum shock induced circadian gene expression in cultured-fibroblasts (rat-1). Here we show that the glucocorticoid hormone analogue dexamethasone can also induce circadian gene expression in rat-1 cells.

Importantly, we demonstrated that an injection of glucocorticoids changed the phase of circadian gene expression in peripheral tissues of mice (liver, kidney, and heart). However, we showed that SCN neurons do not express detectable levels of glucocorticoid receptor and that the SCN pacemaker was thus not affected by dexamethasone injections. Likewise, this hormone does not influence circadian gene expression in hepatocytes of a mouse strain with a liver-specific glucocorticoid receptor gene disruption (GR^{AlfpCre}). The uncoupling of peripheral oscillators from the central SCN pacemaker has enabled us to establish a phase-shift response curve (PRC) for peripheral oscillators in intact animals. In contrast to PRC's recorded for circadian behavior, the one determined for circadian gene expression in peripheral organs does not exhibit an extended time period refractory to phase-shifting.

Using GR^{AlfpCre} mice, we have shown that the glucocorticoid receptor is not required to maintain circadian rhythmicity in the periphery. However, we demonstrated that an injection of dexamethasone in these GR mutant mice leads to a desynchronization of circadian gene expression in the liver and in the other peripheral tissues. These data clearly demonstrate that resetting of the circadian time is cell-autonomous in the context of the intact animal.

Altogether these data (and others that will be presented) suggest that glucocorticoids can directly reset circadian time in peripheral circadian clocks without affecting the central clock.

RESTRICTED FEEDING ENTRAINS CIRCADIAN RHYTHMICITY IN THE LIVER.

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Mealfeeding or restricted feeding (RF) is a zeitgeber in many animals, and in rats causes increased wheel running 2-3 hours prior to food access. The mechanisms by which RF is perceived and conveyed as an entraining signal are still largely unknown. To study these mechanisms we have used a rat-model in which the mouse *Per1* gene promoter has been linked to a luciferase reporter gene, causing circadian oscillations of light emission to occur in many tissues *in vitro*. We have investigated how RF affects such activity in the liver, which is a tissue directly associated with feeding, and compared this with its effect on the lungs; we simultaneously recorded *mPer1-luc* activity in the SCN. Newly weaned rats, kept in LD12:12 (light-off at 5pm) with individual access to a running wheel, were given food for 4 h each day, beginning at 10am. After 7 days they showed a robust, anticipatory running activity prior to the food access interval. The daily peak of light emission from incubated slices of SCN occurred at the same circadian time in food restricted and *ad lib* fed animals, at both 7 and 19 days of RF. The peak activity of the liver was 12h advanced and that of the lungs 5h advanced in RF rats compared with controls. These data are consistent with the notion that RF does not affect the rhythmic activity of the SCN, but demonstrate that circadian oscillators in the liver were entrained by this treatment. We have previously shown that the liver reentrains very slowly following shifts in the light cycle. Our new results raise the possibility that the liver (and perhaps other peripheral oscillators) is coupled to the SCN only via behavioral changes, which in turn affect the timing of primary zeitgebers such as feeding

- 19 EFFECTS OF AN ENVIRONMENTAL TOXICANT ON THE BIOLOGICAL CLOCK. Dani Binigar, Richard Dickerson, Lynn Frame, Daam Settachan, and Joseph D. Miller. Depts. of Pharmacology and TIEHH, Texas Tech University Health Sci. Ctr. and Texas Tech University, Lubbock, TX 79430.

Halogenated aromatic hydrocarbons (HAHs) are a major class of environmental toxicants. Many adverse health effects of HAHs have been documented in humans, including cognitive deficits, developmental and endocrine abnormalities, neurological problems and insomnia. Dioxin is a prototypical HAH because of its behavioral potency and its ability to bind with picomolar affinity to a particular molecular site, the PER ARNT SIM (PAS) domain, a domain shared by all known proteins (e.g., CLOCK, PER1, PER2, PER3, and BMAL1) involved in the function of the circadian clock, localized in the suprachiasmatic nucleus (SCN) of the mammalian hypothalamus. We hypothesize that the mechanism(s) of dioxin action may involve a direct effect on the hypothalamus, specifically the SCN. We have found that dioxin administration (1 ug/kg by gavage) produces large phase advances in rats and mice at CT14. Such administration also perturbs the circadian expression of clock proteins such as mPER1 and BMAL1 in the SCN. We have also found that the dioxin receptor is expressed in the SCN. These findings will be addressed in terms of possible models of dioxin action in the SCN.

- 20 THE EFFECT OF CENTRAL INFUSIONS OF NPY ON THE EXPRESSION OF CLOCK GENES IN THE SUPRACHIASMATIC NUCLEI OF THE MOUSE

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In mammals, both light and non-photoc behavioural cues can entrain the principal oscillator of the hypothalamic suprachiasmatic nuclei (SCN). Evidence suggests that NPY mediates non-photoc entrainment. These experiments tested whether central infusion of either NPY or saline control results in phase advances of the activity rhythm in mice, and to study the impact of such infusions on various clock genes. Adult male mice were individually housed with a running wheel and infra-red detector and entrained to an 8h light:16h darkness (8L:16D) schedule, with free access to food and water. The mice were surgically fitted with a chronic indwelling cannula situated in the 3rd ventricle, adjacent to the SCN. Initially, animals were infused with either NPY (400 nmoles) or saline (200nl) at Zeitgeber time (ZT) 5 (where ZT 12 is time of lights off); lights were turned off at this time and animals were maintained in darkness for 7 days post infusion. Infusion of NPY phase advanced the activity rhythm (mean $\pm$ sem; Saline: $0.55\text{h} \pm 0.22\text{h}$; $n=8$; NPY: $2.32\text{h} \pm 0.35\text{h}$; $n=9$; $p<0.01$). Following re-entrainment to the 8L:16D schedule, the infusion was repeated, animals were killed and the brains rapidly removed and frozen on dry ice 1h (ZT6) and 3h (ZT8) post infusion. Mice treated with NPY showed a highly significant suppression of *mPer1* mRNA ($p<0.01$) 3h post infusion compared to saline controls (Saline 3h: 1351 ± 42 nCi/g; $n=5$; NPY 3h: 1023 ± 87 nCi/g; $n=5$). There was no significant difference in the levels of *clock* mRNA following either treatment. These results show that NPY is capable of mediating non-photoc entrainment in the mouse, and that non-photoc resetting of the clock results in down-regulation of *mPer1*.

Circadian rhythms generated from the mammalian suprachiasmatic nuclei (SCN) of the hypothalamus can be synchronized by light and by non-photoc stimuli. While glutamate mediates photic information, non-photoc information can be mediated by neuropeptide Y (NPY) or serotonin. NPY can reduce the phase-resetting effect of light or glutamate; however, the mechanisms and level of interaction of these two kinds of stimuli are unknown. Here we investigate the effect of NPY applied *in vitro* on *in vivo* light-induced phase shift of the SCN circadian neural activity rhythm, by means of single-unit recording techniques. Light pulses delivered *in vivo* induced phase shifts similar to those reported induced by light *in vivo*, and by

Figure 2. Time course of the inhibitory effect of NPY on light-induced phase shifts measured *in vitro*. $p < 0.05$ at 15 and 30 minutes, ANOVA.

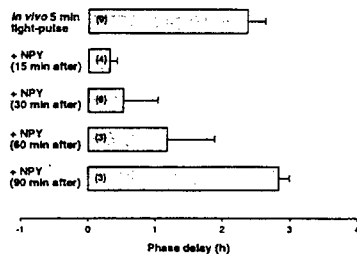
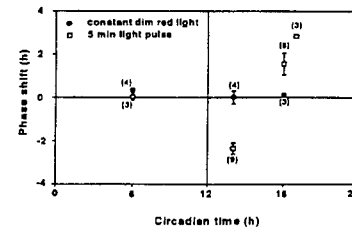


Figure 1. Phase shifting response to light-pulses administered *in vivo* (150 lux-5 min). The SCN firing activity rhythm was recorded in slices on the second day *in vitro*.



glutamate and NMDA *in vivo* and *in vitro*. NPY applied onto the SCN region of the slice 15 min after the light pulse blocked the light induced phase shifts during the early as well as late subjective night. Also, NPY applied 30 min after light pulse could block the phase delay induced by light at circadian time 14. Our results shows that a) NPY can inhibit the photic resetting of the clock during the subjective night, b) this inhibitory effect can be achieved even when NPY is applied after 30 min of light stimulation, suggesting a modulatory mechanism downstream the glutamate receptor.

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PHOTIC PHASE RESPONSE CURVE AND LIGHT-DARK MASKING OF CIRCADIAN RHYTHMS IN OCTODON DEGUS: ASSESSMENT AS A FUNCTION OF ACTIVITY PHASE PREFERENCE
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Recent studies suggest that the *Octodon degus*, a rodent species exhibiting multiple chronotypes, can invert its phase preference from diurnal to nocturnal as a function of spontaneous wheel running activity. Photic PRCs and photic masking were assessed to investigate whether phase inversion reflects a fundamental change in the core pacemaker or downstream effector mechanisms. **Photic phase response curve (PRC):** Nine animals were entrained to a 24 h light-dark cycle (LD 12:12) for at least two weeks to assess their dominant phase preference using continuous body temperature (Tb) and locomotor (LMA) recordings. The animals were then monitored in constant darkness (DD). After three weeks in DD, degus received a 1-h light pulse (30-35 lux) at different phases throughout the circadian day. A significant interaction between time of day and phase-shifts was observed ($F=15.4$; $p<0.0001$). Phase delays (-0.9 ± 0.2 h) were observed at the beginning of the subjective night (CT 14.5 ± 0.4 ; $p=0.001$). Phase advances of 1.1 ± 0.2 h and 0.6 ± 0.1 h were observed during the late subjective night (CT 22.6 ± 0.5) and very beginning of the subjective day (CT 2.5 ± 0.7), respectively; $p<0.003$. There were no significant differences in the magnitude or direction of phase shifts as a function of diurnal or nocturnal phase preference. **Light-dark masking:** Eleven *degus* were entrained to LD 12:12 prior to EEG-sleep and Tb recordings. After baseline recordings animals were subjected to a 2-h light-dark cycle (LD1:1; lights-on 108 ± 10 lux) for 24 hours. *Degus* were studied with and without access to a running wheel. Experiments were repeated four times in each condition. In LD 1:1, *degus* without access to a running wheel exhibited significantly (ANOVA) higher LMA and wakefulness levels during lights-on when compared to the diurnal baseline rhythms ($\alpha=0.05$). When averaged over the 24-hour day, *degus* without running wheels were more active and awake during lights-on than during lights-off ($p<0.0001$) akin to diurnal species. In contrast, unrestricted access to a running wheel inverted the diurnal rhythms to nocturnal under baseline conditions (LD 12:12), and during LD 1:1 light decreased wakefulness and LMA levels relative to baseline in six of the animals, akin to nocturnal species.

These studies show that the inversion of phase preference is not due to activity-dependent effects on the endogenous circadian pacemaker mechanism. Instead, diurnal and nocturnal phase preference is likely determined downstream from the fundamental entrainment mechanism in this species. Research was supported by AFOSR PRET Grant # F49620-95-1-0388 and NIH Grant # AG11084.

23 CIRCADIAN PHOTIC SENSITIVITY IS SEXUALLY DIMORPHIC IN *OCTODON DEGUS*.

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Goel & Lee (1995) noted, while studying a nonphotic odor zeitgeber, that male degus (*O. degus*) reentrained following 6 h phase advances of the light cycle significantly faster than females. This set of experiments tested the hypothesis that males are more sensitive to changes in the photic zeitgeber than are females. Ten males and females were phase advanced and delayed 9 h while housed in 580 lux and in 6000 lux. Reentrainment rate was assessed as previously described. In 580 lux, males reentrained following the phase advance significantly faster than females (19.9 ± 1.9 vs 27.0 ± 2.4 days, $p < 0.05$), but not following the phase delay (17.7 ± 2.5 vs 18.2 ± 1.1 days). In 6000 lux, males significantly decreased the rate of entrainment following the phase advance by 52% and following the phase delay by 36% ($p < 0.001$), while females had a non-significant increase in reentrainment time after each phase shift of 5-6%. We hypothesized that the difference in male sensitivity to increased light intensity might be due to a sex difference in tau or the phase response curve (PRC) under low or high light intensities. Tau was assessed for six females and seven males in DD, LL at 250 lux, LL at 580 lux and LL at 5800 lux. As previously reported the tau of males in DD was significantly shorter ($23.3 \pm .13$ h) than the tau of females ($23.7 \pm .1$ h; $p < 0.01$). However, in all LL conditions, males and females did not differ, although tau increased significantly with light intensity. Using the Aschoff II method, six animals of each sex were treated with light pulses of 250 and 6000 lux at CT4 and CT20. Males demonstrated a significantly greater phase advance at CT20 with 6000 lux than 250 lux ($1.58 \pm .21$ h [250] vs $2.50 \pm .12$ h [6000]; $p < 0.001$), while the females had a smaller phase advance with the brighter light ($2.48 \pm .29$ h [250] vs $1.77 \pm .2$ h [6000]). Similarly, females showed no effect of brighter light on the CT4 phase shift, while males had significantly larger phase delays ($-.28 \pm .07$ h [250] vs $-.44 \pm .08$ h [6000]; $p < 0.05$). We conclude that circadian photic responses of male and female degus differ significantly, and males are more sensitive to changes in light intensity.

24 A TWO-COMPONENT MODEL EXPLAINS WHY SIBERIAN HAMSTERS FAIL TO REENTRAIN TO A PHASE-SHIFT OF THE PHOTOCYCLE. N.F. RUBY AND H.C. HELLER.

DEPARTMENT OF BIOLOGICAL SCIENCES, STANFORD UNIVERSITY, STANFORD, CA 94305.

Less than 10% of Siberian hamsters (*Phodopus sungorus*) reentrain to a 5-h phase delay of a 16:8 photocycle even though they readily reentrain to a 3-h phase delay. We hypothesized that the last 2 h of light exposure on the day of the 5-h phase shift initiated loss of entrainment because it occurred in the middle of the night, a time when light exposure can severely disrupt circadian organization. The second component of our model suggests that reentrainment is prevented by continued exposure to the LD cycle. The first component of this model was tested by exposing two separate groups of hamsters to a 2-h light pulse that began 1 or 3 h before midsubjective night. All animals were then exposed to a 3-h phase delay of the LD cycle on the following day. A third group received a 2-h light pulse that began 1 h before midsubjective night but were not exposed to the phase shift. All animals exposed to the light pulse that started early in the night, but only 58% of animals that were exposed to the later light pulse, reentrained to the 3 h phase delay; the light pulse alone had no effect on entrainment. Thus, pre-exposure to light during midsubjective night, but not at an earlier time, impaired reentrainment to the 3-h phase delay. The second component of this model was tested by exposing animals to a 5-h phase delay that was followed constant darkness (DD) at the end of the 5-h light period. In those animals, activity rhythms free ran with periods of 25.0-25.5 h for the first 5-7 days of DD then shortened to < 24 h for the remainder of DD exposure. Eleven days after the phase delay, the 16:8 LD cycle was reinstated to the same phase it had prior to the phase shift and all of the animals reentrained. These data suggest that light exposure during midsubjective night, but not earlier, diminishes the pacemaker's ability to reentrain to a phase shift of the LD cycle. This diminished capacity is not permanent, however, but is restored by exposure to DD.

STRUCTURAL AND FUNCTIONAL ANALYSIS OF mPER1 : CASEIN KINASE I : mCRY1 INTERACTIONS

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Mammalian *per* proteins are essential components of the circadian oscillator, although their precise biochemical function in the clock is not yet well understood. Per proteins interact with each other, and with several other clock proteins including casein kinase I ϵ (CKI ϵ) and the cryptochrome proteins mCRY1 and mCRY2. We have begun to analyze how mPER1 interacts with these regulators. Using a series of truncations and internal deletions in mPER1, CKI ϵ and mCRY1 binding sites have been identified. Specific sequences on mPER1 responsible for regulated nuclear targeting, including a nuclear localization sequence and phosphorylation-dependent masking domain have been defined and mutated. Consequences of altering these protein-protein interactions include alteration of mPER1 intracellular localization and changes in protein stability.

ASSOCIATION AND NUCLEAR ENTRY OF mPER PROTEINS IN MAMMALIAN CELLS

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The molecular basis of circadian rhythms has been best characterized in *Drosophila*. Oscillation of circadian clock in *Drosophila* requires periodic interaction of the PERIOD (PER) and TIMELESS (TIM) proteins. Physical associations of PER and TIM allow their nuclear entry, and this is believed to be a critical step to repress *per* and *tim* transcription.

In mammals, mPER proteins are known as negative elements to suppress mPer1 transcription through several studies using *mPer1* promoter assay. In this study, we have examined nuclear import of clock proteins of the mammalian *period* gene family and the effect of serum shock, which induces circadian expression of some genes including *Per1* and *Per2* in cultured cells.

We report that nuclear translocation of mPER1 and mPER2 involves physical interactions with mPER3, which is accelerated by serum shock. Nuclear entry of mPER proteins is not prevented even in *mCry1/mCry2* double deficient cells. Truncated mutant of mPER3 which lacks nuclear localization signal (NLS) fails to allow the nuclear entry of mPER proteins both wild type and *mCry1,2* double knock-out cells. This suggests that at least in serum shock-treated cells heterodimerization among and nuclear translocation of mPER proteins can also occur independent of mCRY proteins. These data also suggest that serum shock initiates the cycle of autoregulatory feedback loop by promoting nuclear entry of induced mPER proteins in mammalian culture cells.

27 CHARACTERIZATION OF MICE WITH TARGETED DISRUPTION OF THE *mPer3* GENE.

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Neurons in the mammalian suprachiasmatic nucleus (SCN) contain a cell-autonomous circadian clock that is based on a transcriptional-translational feedback loop. The bHLH-PAS proteins, CLOCK and BMAL1, are positive regulators and drive the expression of negative regulators, CRY1, CRY2, PER1, PER2, and PER3. The importance of these genes in the core circadian feedback loop is exemplified by the loss of rhythmicity in mice with mutations in *Clock* or *mPer2*, and in *mCry1-mCry2* double knockout mice (Vitaterna et al., 1994; Zheng et al., 1999; van der Horst et al., 1999).

To assess the role of *mPer3* in circadian rhythms, we generated mice with targeted disruption of the *mPer3* gene. A 1.8 kB neomycin cassette was inserted in reverse in place of a 1.6 kB EcoR I fragment of the *mPer3* gene, deleting exon 3 and part of exon 4. Southern blot analysis with probes flanking the targeting construct confirmed homologous recombination in ES cells and founders.

Analysis of the *mPer3* knockout (KO) mice revealed only subtle effects on gene expression and circadian behavior. *mPer1*, *mPer2*, and *mCry1* rhythms in the SCN did not differ between KO and wild type (+/+) mice on the first day in constant darkness (DD). Northern blot analysis revealed rhythmic expression of *mPer1* and *mPer2* in skeletal muscle; the rhythms did not differ between +/+ and KO mice. *mPer3* transcripts in KO mice included the NEO gene due to use of cryptic splice sites. *mPer3* transcripts were rhythmically expressed in the SCN and muscle of KO mice, but at lower levels than in controls. Behavioral phenotype was assessed by monitoring locomotor activity rhythms of isogenic (129sv) male mice. Rhythms were synchronized to the 12L:12D light-dark cycle, and free ran upon release into DD. The period of rhythmicity was significantly shorter in KO mice (23.27 ± 0.18 hr, $n=12$) than in +/+ controls (23.77 ± 0.09 hr, $n=12$) ($p<0.025$, t-test).

These results demonstrate that products of the *mPer3* gene are not necessary for circadian rhythms in mice. These results contrast with the strong phenotype of *mPer2* mutant mice, and provide further evidence for specialization of function among members of the *mPer* gene family.

28 MOLECULAR INTERACTIONS BETWEEN MAMMALIAN CLOCK PROTEINS.

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Circadian rhythms observed in many organisms are driven by autonomous cellular oscillators and entrained by daily light-dark cycles. Although several homologous clock genes have been identified both in *Drosophila* and mammals, the molecular mechanisms controlling circadian rhythms differ between the two animals. In *Drosophila*, daily fluctuations of the *period* (*per*) and *timeless* (*tim*) gene products are essential for the circadian rhythm of locomotor activity. Both *per* and *tim* are induced by the dCLOCK-dBMAL1 heterodimer, and feedback-inhibited by their own products, which close the cycle of the circadian expression of both genes. In mammals, the expression of a mouse homologue of *Drosophila per*, *mPer1*, is also mediated by the concerted action of *mClock* and *mBmal1*. On the other hand, the *Per1* expression is repressed not by a mammalian homologue of *tim*, but by the products of the *Cry1* and/or *Cry2* genes. To clarify the mechanisms of the positive and negative regulation of the *Per1* expression, molecular interactions between the mammalian clock proteins (PER1, PER2, PER3, TIM, CLOCK, BMAL1, CRY1, and CRY2) were analyzed by two-hybrid assays. The interaction between CLOCK and BMAL1 was not affected by PER1, PER2, PER3, or TIM, but completely inhibited in the presence of CRY1 or CRY2. These results indicate that the transcriptional repression of PER1 is mediated by the CRY proteins which interfere with the dimerization of CLOCK and BMAL1.

Identification of Functional Domains of Human Period 1 (PER1) Protein

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Human PER1 (hPER1) encodes a nuclear protein that is essential for molecular control of the biological clock and circadian behavioral rhythms. It had been shown that PER1, as a central clock component, could functionally interact with other clock proteins in the cells. Recent studies have suggested that the regulation of nuclear translocation of PER1 may serve as a checkpoint in the negative feedback loop. However, the mechanisms by which hPER1 translocates to the nuclear compartment are unknown. By using ProfileScan, a putative nuclear localization signal can be detected in both human and mouse PER2, TIM and CRY1/2, but not in human and mouse PER1. We set out to examine the structural domains required for hPER1 protein nuclear translocation. In this report we experimentally determined that (i) singly transfected hPER1 translocates to the nucleus of cells and is present as either a patched pattern or a speckled nuclear foci; (ii) a novel Nuclear Localization Domain (NLD) is able to confer nuclear/nucleoli localization of hPER1 and a GFP reporter in transfected cells; (iii) a Cytoplasmic Localization Domain (CLD), located downstream of the PAS domain, is able to block the function of hPER1 NLD; and (iv) with co-transfection of hCRY no effect was observed on hPER1 nuclear translocation as evidenced by a co-localization assay.

FIVE CONSERVED E-BOXES ADDITIVELY CONTRIBUTE TO THE ENHANCEMENT OF *mPer1* TRANSCRIPTION. Akiko Hida, Nobuya Koike, Matsumi Hirose, Hattori Masahira, Yoshiyuki Sakaki and Hajime Tei. Laboratory of Structural Genomics, Human Genome Center, Institute of Medical Science, University of Tokyo. 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan.

Circadian rhythms are observed in many physiological and behavioral phenomena. These rhythms are generated by cellular pacemakers and are synchronized by light. The transcription of mouse *Per1* (*mPer1*) in the suprachiasmatic nucleus (SCN) oscillates in a diurnal pattern and is induced immediately after a light pulse. The results indicate that the daily oscillation of *Per1* plays an important role in the maintenance of circadian rhythms. *Per1* transcription is induced by the CLOCK-BMAL1 complex and is repressed by CRY1 and CRY2. To elucidate the molecular mechanisms of induction and repression of *Per1* transcription, the genomic structures of the human and mouse *Per1* gene were characterized. In addition, the *mPer1* promoter was analyzed by a transient transfection assay with mouse NIH3T3 cells using *mPer1::luciferase* (*luc*) reporter genes. Both of the human and mouse *Per1* genes (*hPER1* and *mPer1*) consist of 23 exons spanning approximately 16 kb. Comparisons of the entire sequences of *hPER1* and *mPer1* revealed five conserved segments in the 5' flanking region in addition to a conserved segment within the first intron. These conserved segments contained several potential regulatory elements such as five E-boxes (the binding site for the CLOCK-BMAL1 complex) and four cyclic AMP response elements (CRE). Transfection assays using several deletion mutants of the *mPer1::luc* reporter showed that the elements existing around the first exon and the first intron were responsible for the basal transcription of *mPer1*. Moreover, introduction of deletions or point mutations into the five E-boxes showed that each of the five E-boxes was functional for the *mPer1* induction by CLOCK and BMAL1, and the induction was not completely abolished unless all of the five E-boxes were mutated. These results clearly indicate that each of the identified E-boxes additively functions as an enhancer for the transactivation of *mPer1* by CLOCK and BMAL1.

31 CONSTITUTIVE EXPRESSION OF MOUSE *Per1* LENGTHENS THE CIRCADIAN PERIOD AND DISRUPTS ENTRAINMENT IN RAT.

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Circadian rhythms are observed in many biological processes and entrained by environmental light-dark cycles. A mammalian homologue of the *Drosophila period* gene, *Per1*, exhibits circadian expression in the SCN, the central pacemaker of the mammalian circadian clock, and is transiently induced by a light pulse. The function of the circadian expression of the mammalian *Per1* gene is a key question for the regulation of mammalian circadian rhythms. Transgenic rats with constitutive expression of the mouse *Per1* gene were constructed using elongation factor 1 alpha (EF1-a) or neural specific enolase (NSE) promoters. Two lines of transgenic rats (EF1-a promoter, NSE promoter) showed about 1 h longer circadian period than their sibling wild types in DD. Both of these lines of rats failed to entrain in light dark cycles (LD 12:12) although they showed relative coordination. These results indicate that the mammalian *Per1* gene is involved in rhythm generation and/or entrainment of the circadian clock. In addition, we measured the expression of the native (rat) *Per1* and *Per2* genes in the SCN and retina of the transgenic lines under DD conditions and found it to be diminished. Molecular mechanisms regulating the expression of *Per1* and *Per2* will be discussed.

Supported in part by the NSF Center for Biological Timing, NIH grant (MH56647 to M. M.) and by research grants from the Japanese Ministry of Education, Science, Sports, and Culture, and the Japanese Ministry of Health and Welfare (to H. T.).

32 SIGNAL TRANSDUCTION PATHWAYS INCLUDING RAPID RESPONSE AND CIRCADIAN EXPRESSION OF *Per1* AND *Per2* mRNA IN RAT-1 FIBROBLASTS

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Recent report revealed that a treatment of high concentration of serum (serum shock) generated circadian gene expression of *Per1,2* and some clock controlled genes in cultured rat-1 fibroblasts. This stimulation also induced immediate early response of *Per1* and *Per2* mRNA expression, which was also seen in mice suprachiasmatic nucleus (SCN) after the brief exposure of light at subjective night. Although serum shock might activate several intracellular signaling pathways, mechanisms to induce *Per1* and *Per2* mRNA and to generate the circadian gene expression have still been unknown.

We demonstrate that treatment of forskolin, which elevates intracellular cyclic AMP, generates circadian expression of *Per1,2* and albumin D-box binding protein (*dbp*) mRNAs in cultured rat-1 cells. Interestingly, although forskolin induced acute response of *Per1* expression, immediate early induction of *Per2* mRNA was not observed after the stimulation. In addition, both forskolin treatment and serum shock rapidly increased phosphorylation of CREB, which is known as a light pulse induced change in the SCN and believed to be the main pathway to reset the circadian clock. These results suggested that serum shock and forskolin is likely to reset and synchronize the clock in each cells. We also characterized other factors to generate the circadian rhythm of these gene expression in cultured rat-1 fibroblasts. We are carrying out further biochemical studies to elucidate signal transduction to generate the circadian gene expressions in rat-1 cells.

A RECESSIVE SCREEN FOR MOUSE CIRCADIAN RHYTHM MUTANTS

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Our goal is to identify mutant alleles coding for molecular components of the mammalian circadian clock. To accomplish this goal we have initiated a three-generation recessive mutation screen in the mouse. Mutant mice will be identified based on altered circadian periods as measured by wheel running activity in constant darkness. Success of this screen is dependent on several factors, the most important being a high rate of mutagenesis, a high mouse production rate and low variation of the wild type strain in the phenotypic assay. The strain that we have selected for this screen, BTBR, fits these criteria. BTBR mice have a high rate of mutagenesis and on average, produce very large litters-- close to 8 pups per litter. Most importantly, adult BTBR mice free run with a circadian period of 23.2 ± 0.13 hrs. and the circadian period for BTBR is age and sex invariant. In the early stages of this screen we have already identified several putative mutant mice. We are currently in the process of verifying and characterizing these mutant mouse lines as we continue to screen for more mouse circadian rhythm mutants.

IDENTIFICATION AND CHARACTERIZATION OF A ZEBRAFISH CLOCK MUTANT

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We have initiated a forward genetic approach to study circadian mechanisms in zebrafish. P36 is one of the mutants recovered from a pilot screen using N-ethyl-N-nitrosourea (ENU) as the mutagen and the locomotor activity rhythm of 9-17 day old larvae as the screening assay. It is a homozygous-viable and semi-dominant mutation that shortens the circadian period by about 0.5 hour in heterozygous mutants and by about 1 hour in homozygous mutants. The freerunning period of the melatonin release rhythms of cultured pineals from this mutant line is also shortened in both heterozygotes and homozygotes. This indicates that the mutant gene product affects circadian rhythmicity at the cell or tissue level as well as at the level of whole-animal behavior. P36 was mapped to LG20 near the *kit* gene by linkage analysis with simple sequence length polymorphic markers. The *kit* gene was predicted to be closely linked to the zebrafish *clock* gene from the position of its presumed ortholog on human and mouse maps. Radiation hybrid mapping using zebrafish *clock* specific primers confirmed this linkage. We then amplified the complete coding region of zebrafish *clock* gene from the mutant line by RT-PCR. Sequence analysis showed a T to A transversion in the mutant line, which predicts a substitution of Ile to Asn at amino acid position 254 (I254N). The I254N polymorphism was found to co-segregate with the P36 mutant phenotype, and was not detected in any other laboratory strains. Furthermore, the Ile at 254 position is conserved among human, mouse, *Drosophila* and zebrafish clock proteins. The functional analysis of the I254N mutation is underway. This work will contribute to our understanding of how the *clock* gene product functions.

GENETIC ANALYSIS OF CIRCADIAN RHYTHMICITY IN ZEBRAFISH.

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Widespread use of zebrafish (*Danio rerio*) in genetic analysis of embryonic development has led to rapid advancements in the technology required to generate, map, and clone mutant genes. We have initiated a screen for dominant mutations that affect the circadian periodicity of zebrafish locomotor behavior. To efficiently record behavioral rhythms from zebrafish, we designed an automated video image analysis system that can record gross locomotor activity from 150 larval (9-15 days old) zebrafish/system under constant infrared light. More than 95% of all wild type animals tested with this system expressed a circadian rhythm with a mean freerunning period of 25.5 h (± 0.2 , SD). Male zebrafish were treated multiple times with 3 mM N-ethyl-N-nitrosourea to induce point mutations in their spermatogonial stem cells, and then crossed with wild type females. The resulting G₁ progeny, each heterozygous for a unique set of mutations, were screened. Animals with atypical periods were outcrossed to wild type to confirm the mutation segregated in a Mendelian fashion. Confirmed mutant F₁'s were intercrossed to determine if the mutations were homozygous viable and whether dominance was complete. In an initial screen of 1275 animals, we have recovered two semi-dominant, homozygous viable, short period mutants with freerunning periods of 24.7-25.0 h in heterozygotes, and 24.0-24.5 h in homozygotes. Both mutations also shorten periods of melatonin rhythms measured from cultured pineals, indicating that the mutant gene products affect circadian rhythmicity at the cellular/tissue level, as well as the behavioral level. Linkage mapping illustrates that these two mutations are on different linkage groups (LGs 7 and 20, respectively), indicating they are in different genes. In order to determine whether any known clock genes are candidates for the mutations, we used Radiation Hybrid mapping to map several RT-PCR derived and Expressed Sequence Tagged clones corresponding to zebrafish clock gene homologs. To date, we have mapped seven distinct *cryptochrome* homologs (LGs 4, 8, 18, 25, two on 22, and one unlinked), two *bm11* homologs (LGs 18 and 25), and single homologs of *per2* (LG 2) and *timeless* (LG 11). The zebrafish *clock* gene maps to the same interval as the mutation on LG 20, but we have not mapped a clock gene homolog near the other mutation on LG 7. Although there are still several zebrafish clock gene homologs to map, this suggests that this mutation might affect a novel clock component. These results demonstrate the potential of zebrafish as a model system for genetic analysis of vertebrate circadian rhythmicity. This work was funded by grants from NIH, AFOSR and the Texas Advanced Research Program.

RESTRICTED DISTRIBUTION AND RHYTHMICITY OF CLOCK-IMMUNOREACTIVITY IN THE SCN: IMPLICATIONS FOR CIRCADIAN OUTPUTS CONTROLLING BEHAVIORAL RHYTHMS. Michael Lehman^{1,2}, Christopher Cutter², Jenny Nelms^{1,2}, Lique Coolen² and Rae Silver³.

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Clock was the first identified mammalian pacemaker gene, discovered on the basis of the disruptive effects of mutant CLOCK protein on circadian rhythms. The currently accepted working hypothesis of molecular events underlying pacemaker function places CLOCK as a universal component of pacemakers in mammalian cells. Recent work has shown that other critical clock genes, such as mPer1 and mPer2, are expressed in cells located throughout the SCN. In contrast, using a polyclonal antibody against mCLOCK we have found that CLOCK protein is expressed in a distinct subset of SCN cells in the Syrian hamster. Specifically, CLOCK immunoreactivity is colocalized in neurophysin (NP) and other cells localized in the rostral and dorsomedial SCN but not in vasoactive intestinal polypeptide (VIP) or calbindin-28K (CaBP) cells in the ventral and lateral portions of the SCN. Preliminary results indicate a circadian rhythm in the number of CLOCK-positive cells in the SCN but not in the piriform cortex. We are currently extending these studies to compare the distribution of CLOCK to that of PER proteins, and to examine the localization of CLOCK in the mouse SCN. Results to date suggest that the effects of the CLOCK mutation on behavioral rhythms are due to disruption of pacemaker function in a subset of SCN neurons. These cells may release output signals that either synchronize other pacemaker cells in the SCN and/or drive overt behavioral rhythms. Supported by NIH R01 NS35657 to M.N.L.

TRANSCRIPTIONAL PROFILING OF CENTRAL AND PERIPHERAL MAMMALIAN CIRCADIAN CLOCKS. Duffield, G.E., Best, J.D., Wahleithner, J.A., Schwartz, W.J.*, Loros, J.J. and Dunlap, J.C.

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The suprachiasmatic nuclei (SCN) of the hypothalamus are the site of the central circadian clock in the mammal, but recent studies have also demonstrated the existence of peripheral circadian oscillators. The aim of the current studies is to identify and characterize genes that are components of mammalian circadian clock(s).

Circadian rhythms in gene expression can be induced in Rat-1 fibroblasts *in vitro* following serum shock (Balsalobre et al., 1998). Using this system we have reproduced published data demonstrating rhythmic gene expression and further characterized other genes in this cell type. RNA samples were harvested from cells every 4 hours over a 48 hour period following treatment with 50% horse serum, and gene expression analyzed using Northern blotting. We further examined this phenomenon to see if it was conserved across species, in particular the mouse for its utility in genetic studies. We have demonstrated that NIH3T3 mouse fibroblasts exhibit the same patterns of rhythmic and non-rhythmic gene expression as that seen in the Rat-1 cells.

Studies of the SCN were carried out on C57BL/6 mice (age 8 weeks) maintained on a 12:12 L:D cycle. Animals were sacrificed in three different groups: ZT 0.2-0.5, ZT5-5.5 and ZT11-11.5 (ZT12 = lights off). RNA was extracted from SCN (0.8 mm³ punched tissue sections) and cerebella and used to construct time-of-day specific cDNA libraries. Each library contains 3.5×10^5 to 3×10^6 independent clones and we have characterized libraries by establishing the presence of specific clock and immediate early gene transcripts by PCR.

Furthermore, RNA collected at the above described time points from fibroblasts and SCN have been used to synthesize labeled cDNA probes for investigating gene expression on cDNA microarrays. We anticipate that these approaches to examine transcriptional profiling will identify novel genes whose expression change as a function of time of day, and in turn will reveal components of the central oscillator, input or output pathways to the clock. *Supported by the National Institute of Mental Health (MH44651 and MH01186 to J.C.D., W.J.S. and J.J.L) and the Wellcome Trust (058332/B/99/Z to G.E.D).*

EXPRESSION AND REGULATION OF PER1 AND PER2 IN THE MAMMALIAN PINEAL GLAND.

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The pineal gland is an important component of the vertebrate circadian system. In non-mammalian vertebrates the pineal itself contains a circadian clock, that - for unknown reason has been lost in mammals during the course of the evolution. We have recently shown that all the genes that are thought to be part of the molecular "clock" mechanism are expressed in the pineal gland of rats. Recent studies have also shown that these clock genes are expressed in several mammalian organs and tissues, but their role is unknown. Because of its unique evolutionary history, we believe that the mammalian pineal could be a useful model to understand the role and the function that these clock genes may have outside the pacemaker structures (SCN and retina).

In this work we investigated *in vivo* and *in vitro* gene expression of Per1 and Per2 in the pineal gland of rats and the mechanisms controlling such expression. Our *in vivo* results showed that both genes presented clear circadian rhythm in Light:Dark and constant dim light (with high levels of expression during the night and low during the day), but were arrhythmic in LL. Our *in vitro* data indicate that Per1 and Per2 expression in culture persisted for at least 72 hours, and Per2 expression showed a circadian oscillation that dumped out in about 3 cycles. Additional *in vitro* studies revealed that Per1 and Per2 in the pineal responded to norepinephrine stimulation.

In conclusion our results demonstrate that the expression of Per1 and Per2 in the pineal is under circadian control, and their expression is probably driven by the SCN. However, the *in vitro* rhythmic expression of Per2 also suggests the presence of a dumped circadian oscillator in this gland. The observation that norepinephrine may stimulate transcription of Per1 and Per2 suggests that the SCN may control them by pathways and mechanisms similar to those controlling pineal melatonin synthesis.

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CIRCADIAN EXPRESSION AND ADRENERGIC REGULATION OF *Per1* AND *Per2* IN THE RAT PINEAL GLAND

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Per1 and *Per2* are mammalian homologue of the *Drosophila* clock gene period, and it is known that these transcripts show the circadian rhythm in the hypothalamic suprachiasmatic nucleus (SCN). Beside SCN, both *Per1* and *Per2* mRNAs were distributed widely in many tissues regardless of nervous and non-nervous tissues. In mammals the pineal gland is responsible for the synthesis and the secretion of the hormone melatonin in response to signals originating from the endogenous clock located in the SCN. In the present study, we investigated the circadian profiles of *Per1* and *Per2* genes in the rat pineal gland. *Per1* and *Per2* genes were expressed high in the nighttime, but low in the daytime in both light-dark (LD) and constant dark (DD) conditions. These day-night variations were completely blocked by the superior cervical ganglionectomy (SCGX). The β -adrenergic receptor agonist isoproterenol (0.5 mg/kg) rapidly induced the *Per1* and *Per2* mRNAs in the pineal gland. These findings suggest that *Per1* and *Per2*, putative clock oscillating genes, were expressed high in the pineal gland under the adrenergic regulation.

PER1 IS EXPRESSED IN MOUSE SPERMATOCYTES AT ALL TIMES OF DAY. Eric L. Bittman, Liyue Huang and Thomas J. Graziano. Dept. of Biology, Neuroscience & Behavior Program, and Center for Neuroendocrine Studies, University of Massachusetts, Amherst, MA 01003.

Robust rhythms of abundance of mRNA for the clock genes *mPer1-3* have been reported not only in the central pacemaker in the SCN, but also in peripheral tissues including liver, skeletal muscle, and testis (Zylka *et al.*, Neuron 20:1103). The expression of such rhythms in primary culture and cell lines indicates that circadian clocks may exist in individual cells of differentiated organs (Balsalobre *et al.*, Cell 93:929). In order to understand the possible physiological role of PER in peripheral tissues it is necessary to identify the cell types in which it is expressed. Furthermore, it is critical to determine whether rhythms of *per* mRNA are paralleled by corresponding (and presumably time-delayed) rhythms of PER abundance, as has been reported in SCN. To this end, we have characterized immunoreactive PER1 expression in the testes of mice sacrificed at different times of day.

C57Bl6 mice housed in 12L:12D were anesthetized at 07:00, 14:00, 18:30, 22:30, or 03:00 (lights on 07:15). Testes were removed and decapsulated for immersion fixation, or animals were transcardially perfused with 4% paraformaldehyde before tissue collection. Some mice were injected with the thymidine analog bromodeoxyuridine (BrdU, 50mg/kg, ip) in order to mark cell birth at intervals ranging between 4h and 10days prior to sacrifice. Tissues were embedded in paraffin, sectioned at 6 μ m, and mounted onto subbed slides. Sections were stained using rabbit anti-PER1 antibodies obtained from either Dr. S.M. Reppert (1:4000) or Affinity Bioreagents (1:500-1:4000). Tissue from animals given BrdU was also incubated with rat anti-BrdU (Caltag, 1:500). Primary antibodies were detected using fluorescent (Cy2, Cy3 or FITC-conjugated) anti-rabbit and anti-rat secondary antibodies. Cellular colocalization of PER1 and BrdU was assessed by confocal microscopy. Absorption of the Affinity Bioreagents anti-PER1 with the peptide against which it was raised eliminated essentially all staining.

Both the Reppert and Affinity antibodies predominantly stained cells within the seminiferous tubules. Not all seminiferous tubules were labeled, indicating that PER1 expression might be confined to particular cell associations. The Affinity Bioreagents antibody generated more extensive intratubular staining; immunoreactivity was observed in several stages of spermatocytes as well as in round and elongate spermatids. Colocalization of BrdU and PER1 was not observed in mice sacrificed up to 5 days after BrdU injection, but was extensive in mice allowed to survive for 10days, indicating that PER1 is not expressed until long after DNA replication is completed. PER1 was apparent in spermatocytes of mice sacrificed at each time of day; although cell counting is not complete, there was no obvious rhythm to the pattern of PER1 expression. Our results are consistent with a role for PER1 in maturation of sperm after completion of the early stages of meiosis. Supported by NSF IBN-98-17252 to ELB.

MOLECULAR ANALYSIS OF AVIAN CIRCADIAN CLOCK

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Unlike mammals, avian circadian rhythms are regulated by a multiple oscillatory system consisting of the retina, the pineal gland and the suprachiasmatic nucleus in the hypothalamus. We have cloned *Clock* and *Period* homologs (*qClock*, *qPer2* and *qPer3*) and characterized these genes in Japanese quail to understand avian circadian system. *qCLOCK*, *qPER2* and *qPER3* showed ~79%, ~46% and ~33% amino acid identity to *mCLOCK*, *mPER2*, and *mPER3*, respectively. *Clock* was localized to quail chromosome 4 and chicken chromosome 4q1.6-q2.1. *Per2* and *Per3* genes were both localized to microchromosomes. In the eye and the pineal, *qClock* mRNA was expressed throughout the day. However, *qPer2* and *qPer3* mRNAs were high during the day and low during the night and showed robust circadian oscillation. *qPer2* mRNA was induced by light in the eye and the pineal, but neither *qClock* nor *qPer3* was induced. These results can explain the molecular basis for circadian entrainment in Japanese quail. There is a controversy about the site of the avian SCN. Two possible sites have been proposed (so-called medial SCN and visual SCN), but there are no direct evidence. The most positive proof to identify the avian SCN is the demonstration of the clock gene expression. *qPer2* was expressed in the medial SCN in the day time and its expression was induced by light. However, *qPer2* expression was not observed in the visual SCN. These results lead to the conclusion that the medial SCN is involved in the avian circadian clock.

CLONING AND INITIAL CHARACTERIZATION OF CHICKEN CLOCK GENES.

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The circadian clock system of the chicken pinealocyte has been studied extensively because of its unique equipments of both the photic-input pathway for resetting the clock and the output pathway producing melatonin in a circadian manner. To investigate the pineal circadian clock system, we isolated cDNA clones encoding chicken pineal *PER2*, *BMAL* and *CLOCK*. mRNA levels of these genes were evaluated *in vivo* by Northern blot analyses and *in vitro* by semi-quantitative RT-PCR analyses of cultured chicken pineal cells. Under both conditions, mRNA levels of the chicken clock genes exhibited rhythmic expressions not only in LD but also in DD. Interestingly, mRNA levels of *cBmal* significantly decreased upon exposure of chicks to light at early subjective night, while those of *cPer2* concurrently increased. Functionally, *cBMAL* and *cCLOCK* activated transcription from E-box elements (*mPer1*-promoter and *hAVP*-E-box-SV40 promoter), and excessive expression of *cBMAL* inhibited the *cBMAL*-*cCLOCK*-induced transcription in 293EBNA cells. These results indicate a similar but slightly different properties between the chicken pineal gland and mammalian SCN.

LEPTIN RECEPTOR IMMUNOREACTIVITY IN THE RAT SUPRACHIASMATIC NUCLEUS

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The adipocyte-derived hormone leptin has profound effects on feeding behavior and metabolism. Leptin is believed to serve an adipostat function, informing the brain of the size of adipose stores. Leptin may also affect hematopoiesis and reproduction. Recent *in vitro* studies in our laboratory suggest that leptin may also modulate the primary mammalian circadian pacemaker located in the suprachiasmatic nucleus (SCN). Specifically, leptin phase-advances the SCN pacemaker when applied during subjective day and into the early subjective night. Immunocytochemical and *in situ* hybridization studies indicate the presence of leptin receptors in several hypothalamic nuclei whose activity mediates a number of behavior patterns, including feeding. Our studies suggest that leptin receptors should also be present in the SCN. Here, we present results of immunocytochemical experiments designed to address this issue.

Two antibodies to the leptin receptor (OB-R) were used for immunocytochemistry, K-20 and M-18. The leptin receptor has five known splice variants designated OB-R_a through OB-R_e. OB-R_a and OB-R_b are both transmembrane receptors. OB-R_e is a cytoplasmic form of the receptor. The function of OB-R_c and OB-R_d is not known. Antibody K-20 is immunoreactive with the cytoplasmic portion of all leptin receptors; antibody M-18 is specific for OB-R_a and OB-R_b. Experiments were performed on brain tissue derived from adult, male Sprague-Dawley rats housed under 12:12 LD conditions. Consistent with previous studies, intense immunoreactivity was observed in the arcuate and supraoptic nuclei using either K-20 or M-18. Immunoreactivity in the SCN was also observed with K-20, though the intensity of labeling was less than that seen in other regions of the hypothalamus. Additional experiments using M-18 are in progress. These results, and those of our *in vitro* studies, suggest that leptin may act through either the cytoplasmic or transmembrane form of its receptors to modulate the circadian pacemaker in rats. Research was supported by NIH grant MH53317 (RAP).

HYPOCRETIN-LIKE IMMUNOREACTIVITY IN THE SYRIAN HAMSTER CIRCADIAN SYSTEM

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Hypocretin (orexin) is a neuropeptide which is found in the brain exclusively in neuronal cell bodies in and around the lateral hypothalamic area. It has been implicated in the regulation of numerous behavioral and physiological processes, including sleep, feeding, drinking, and luteinizing hormone concentrations. The most dramatic of these effects is its role in sleep regulation. Animals with a knockout or defect in the gene for the hypocretin-2 receptor display symptoms of narcolepsy. In the present study, we describe the hypocretin innervation of the neural circuits important in the regulation of circadian rhythms. The distribution of hypocretin in fibers was examined in both male and female hamsters, but no gender-specific patterns were observed. The suprachiasmatic nucleus is virtually devoid of hypocretin-like immunoreactivity, with just a few fibers observed along the ventral border of the nucleus. This is in obvious contrast to the surrounding areas of the hypothalamus, which contain a very high density of hypocretin-like immunoreactive fibers. The intergeniculate leaflet of the thalamus (IGL) contains a moderately dense hypocretin projection, extending along its entire rostral-caudal length. Finally, both the dorsal and median raphe nuclei contain dense hypocretin terminal fields. The heavy innervation of the IGL and the raphe nuclei suggest a possible role for hypocretin in the regulation of circadian phase.

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THE SUPRACHIASMATIC NUCLEUS DOES NOT CONTAIN THE P-75 NGF RECEPTOR BUT DOES CONTAIN BDNF IN THE DIURNAL RODENT *ARVICANTHIS NILOTICUS*. Heather Ross, Emily Lauher and Laura Smale, Departments of Zoology and Psychology, Michigan State University, East Lansing, MI, 48824.

The p-75 low affinity receptor binds to a variety of neurotrophic factors, and has been described in fibers within the suprachiasmatic nucleus (SCN) of lab rats. This receptor has been implicated in the regulation of the distribution of sleep and wheel running over the course of a 24 h day in rats and mice, respectively. For example, mice in which the p-75 receptor has been knocked-out begin to run in their wheels during the day, approximately 3-4 h before lights-out (Golombek et al., 1996, *Biol. Rhythms Res.*, 27:409-418). In the current study we used immunohistochemical techniques to determine if this receptor, or one of its ligands, brain derived neurotropic factor (BDNF) is present in the SCN of the diurnal murid rodent, *A. niloticus*. Whereas BDNF was present in a substantial number of cells evenly distributed within the SCN, the p-75 receptor was absent. This receptor was present in cells and fibers in other brain regions, suggesting that the staining methods were effective in this species, but that the receptor is not present in the SCN. These data raise the possibility that differences in the p-75 NGF receptor within the SCN of nocturnal and diurnal rodents might contribute to differences in their patterns of rhythmicity.

Distribution of substance-P and neurokinin-1 receptor immunoreactivity in the suprachiasmatic nuclei and intergeniculate leaflet of four rodent species.

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The suprachiasmatic nuclei (SCN) of the mammalian hypothalamus house the main circadian pacemaker. Entrainment to environmental stimuli, such as light, occurs via a number of pathways to the SCN including (1) the retinohypothalamic tract, (RHT) and (2) the geniculohypothalamic tract, (GHT) which arises from the intergeniculate leaflet (IGL). The peptide substance P (SP) has been postulated to function as a neurotransmitter in the mammalian circadian system. In this study, we examined the presence of SP and a SP receptor, the neurokinin-1 receptor (NK-1) in the SCN and IGL of four rodent species (Syrian hamster, Siberian hamster, Wistar rat, and C57BL/6x29SV mice).

Adult male rodents were intracardially perfused with 4% paraformaldehyde, the brains removed, post-fixed, and 30 μ m coronal sections taken on a freezing sledge microtome for immunohistochemistry. Sections were incubated with a polyclonal antibody raised in rabbit to NK-1 receptor (Chemicon), or a monoclonal antibody to the SP raised in rats (Harlan Labs), and the reaction products visualised using the ABC method with nickel-DAB as the chromagen.

SP-immunoreactivity (ir) appeared as mostly terminal staining with sparse fibres, distributed in a similar pattern in the IGL of the four species. SP-ir in the SCN was found to differ across the species. Rat SCN showed terminal staining in the ventral portion with some cell body staining in the dorsal portion. Syrian hamster, Siberian hamster and mouse showed dense terminal SP-ir staining surrounding the SCN, with little SP-ir within the SCN itself. NK-1-ir was found throughout the rostrocaudal extent of the IGL in all species examined with distinct fibre and cell body staining. NK-1-ir was mostly absent from the SCN, although NK-1-ir cell bodies and fibres were observed surrounding the SCN.

The distribution of SP-ir and NK-1-ir, seen in the SCN region of three of the four species examined, does not support the hypothesis that the SP acts as a RHT neurotransmitter in these rodents. However, the similarities in SP-ir and NK-1-ir in the IGL suggest that SP plays a role in the rodent circadian system via the GHT.

NEUROANATOMICAL STUDY OF THE RETINOHYPOTHALAMIC AFFERENTS IN MICE DURING POSTNATAL DEVELOPMENT

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The retinohypothalamic tract (RHT) forms the entrainment pathway that conveys photic information to the suprachiasmatic nucleus (SCN). Studies in rats and hamsters have shown differences in fibre innervation of retinal afferents to the SCN during development. In rats initial exuberance of retinal innervation is followed by subsequent retraction of terminal axon branches, similar to that observed in other visual structures. In hamsters, on the other hand, there appears to be no similar process of exuberance/retraction. In mice, there have been no developmental studies of the RHT afferents. The present work uses neuroanatomical tracers (choleratoxin and the carbocyanine dye DiI) to investigate the retinal projections during ontogeny in mice. Retinal axons can be detected in the SCN as early as postnatal day 0 (P0) and increase progressively up to P8-10, when the adult pattern is attained. In early stages retinal input is contralaterally predominant and the adult symmetrical bilateral distribution is reached between P6 and P8. No evidence was seen for retraction of retinal fibres from early to late postnatal stages, which may be related to basic functional differences between the visual and circadian systems. For example, in the lateral geniculate nucleus, fibre retraction during development is associated with remodelling of axon arbours from inappropriate to appropriate areas to achieve precise segregation of ocular input and establishment of a retinotopic map. In contrast, the circadian system lacks both ocular segregation and retinotopic organisation. In addition, confocal imaging shows single fibres crossing from left to right SCN which could indicate innervation of both nuclei by the same retina and consequent blurring of the visual input.

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CIRCADIAN OSCILLATIONS IN MEMORY FUNCTION: CORRESPONDENCE TO CHANGES IN MUSCARINIC CHOLINERGIC RECEPTOR DENSITY IN THE RAT SUPRACHIASMATIC NUCLEUS?

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The role of the circadian system in learning and memory is still poorly understood. Over the past decades, evidence has accumulated in favor of the view that the biological clock interacts with the process of memory consolidation and retrieval. Memory retention for a learning event oscillates on a 24-hourly basis in the rat, as studied under a 12:12 hour light dark (LD) cycle. Memory performance is highest at 24 hours, and lowest at 6, 18 and 30 hours after acquisition. Furthermore, these peaks and troughs in retention disappear when the suprachiasmatic nucleus (SCN) is lesioned, suggesting that they originate from an endogenous rhythm. We have recently shown that this fluctuation persists under free running conditions (continuous dim red light). This confirms independence of the memory fluctuations from externally imposed LD cycles.

Neurochemical alterations in the brain, and more specifically in the expression of muscarinic cholinergic receptors (mAChR) in the hippocampus and amygdala, occur within 2 and 8 hours after a learning task, respectively. Here we report similar changes in the rat SCN at specific time points after active shock avoidance training. In contrast to specific memory related areas, the SCN shows no changes at 2, 8 or 16 hours after acquisition. It does so 24 hours after such an event, the time at which retention is optimal. We are currently assessing whether the dip in memory at about 30 hours accordingly parallels a decrease in mAChR immunoreactivity, and hence whether mAChR expression in the SCN corresponds to the fluctuation in memory retention.

IGL LESIONS ABOLISH NPY FIBERS IN THE SCN OF *ARVICANTHIS*

NILOTICUS. Betty H. Gubik and Laura Smale, Department of Psychology, Michigan State University, East Lansing MI 48824

Many mammalian circadian rhythms depend on the suprachiasmatic nucleus (SCN), which is modulated by a variety of inputs. One such input comes from neuropeptide-Y (NPY) containing cells in the intergeniculate leaflet (IGL), via the geniculohypothalamic tract. This pathway plays a role in the mediation of nonphotic influences on circadian rhythms. We set out to determine if this projection exists in the diurnal murid rodent *Arvicanthis niloticus* by determining if IGL lesions would lead to a decrease in NPY fibers in the SCN. Fourteen adult male *Arvicanthis* received bilateral electrolytic lesions, with varying coordinates, aimed at the IGL. Four of these lesions appeared to be complete based on observation of cresyl violet stained tissue. Brains from these four animals, plus two control animals, were processed for immunohistochemical detection of NPY. A pronounced difference was seen in the number of NPY fibers found in the SCN of animals with complete IGL lesions compared to those of controls. Control animals had a dense plexus of NPY fibers spanning the entire SCN while the lesioned animals had very few, if any. More specifically, two of the lesioned animals had no NPY fibers in the SCN, while the other two had a small clustering of labeled fibers in the core of the caudal SCN. However, in this regard, the latter two were still very different from the control animals. NPY fibers were present in many hypothalamic regions outside of the SCN in all six animals examined. These data suggest that the primary NPY-containing cell bodies that project to the SCN are found in the IGL. This finding sets the stage for future investigation of the role of the IGL in rhythms in this diurnal species.

ANGIOTENSIN 1A RECEPTOR NULL MUTATION: EFFECTS ON CIRCADIAN RHYTHMS IN THE MOUSE. R.E. Mistlberger¹, M.C. Antle¹, M. Marcelo², M.I. Oliverio³, T.M. Coffman³, M. Morris². Dept. Psychol.¹, Simon Fraser University, Burnaby BC; Dept. Toxicol. Pharmacol.², Wright State Univ. Sch. Med, Dayton OH; Dept. Med³, Duke Univ, Durham Oh.

The suprachiasmatic nuclei in rodents contain cells and fibers immunoreactive for angiotensin II (ANG II) and a high density of AT_{1A} receptors. In the rat SCN in vitro, ANG II alters neuronal firing rate. However, the role of this neuropeptide in circadian function is unknown. Wheel running and drinking activity rhythms were monitored in 8 knockout mice (KO) lacking AT_{1A} receptors and 6 wildtype (WT) mice. All mice ran in the wheels and exhibited grossly normal running and drinking rhythms in LD 12:12 and in constant dark (DD). Daily wheel running levels were very similar between groups during the 1st week in LD, but KO mice exhibited a gradual increase and more than double the activity of WT mice by the 4th week ($p < .05$ vs WT, week 4). However, neither the L:D ratio of activity (measure of rhythm amplitude) nor the time of nocturnal activity onset relative to lights-off (phase angle of entrainment to LD) differed between groups during LD or on the first day of DD ('unmasked' phase). During each 7-14 day block within the first 60 days in DD, KO mice showed higher levels of wheel running (range of group mean KO-WT difference scores across day blocks = 2312 to 4900 wheel revs/day) and a modestly shorter free-running period (τ , range of difference scores = .04 to .27 h). This result is consistent with earlier studies showing that wheel running levels are negatively correlated with τ in rodents. However, when running wheels were locked for 20 days and then unlocked, there was no significant change in τ in either group. There was also no significant difference between groups in the size of phase shifts to light pulses (10 min, 4 lux incandescent white) either early in the subjective night (CT16, delay shifts) or late in the 'night' (CT23, advance shifts). These results demonstrate that an AT_{1A} receptor null mutation has little if any effect on general functions of the circadian system, assessed by measures of the phase angle of entrainment, free-running τ and phase resetting response to light. The modestly shorter τ in KO mice may be secondary to an effect of the mutation on locomotor activity. *Supported by grants from NSERC (Mistlberger) and AHA (Morris)*

51 VASOACTIVE INTESTINAL POLYPEPTIDE (VIP) PHASE SHIFTS THE FIRING RATE RHYTHM OF RAT SCN NEURONES *IN VITRO*.

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In mammals, the principal circadian pacemaker is localised to the hypothalamic suprachiasmatic nucleus (SCN). VIP is found in cells of the retinorecipient, ventrolateral region of the SCN. However, its role in the SCN remains unclear. Here we studied the phase-resetting effects of VIP on the firing rate rhythm of SCN neurones *in vitro*.

Adult male Wistar rats were housed under a 12:12 light:dark cycle (zeitgeber time (ZT) 0 designated as lights on). Coronal hypothalamic brain slices (500 μ m) were obtained during the light phase and maintained in a brain slice chamber by continuous perfusion with Earls balanced salt solution (EBSS). On the day following slice preparation extracellular recordings were made from individual spontaneously firing SCN neurones. All drugs were bath applied.

In untreated control slices, the electrical activity of SCN neurones exhibited a circadian variation which peaked during the middle of the day ($\sim$ ZT7). Treatment of the slice with VIP (10^{-7} M; 10min) at ZT6 (mid-subjective day) did not shift the peak of this rhythm, whereas in slices treated with VIP during the early (ZT13) or late (ZT19) subjective night, the peak of the rhythm was phase-delayed (1.40 ± 0.2 h) or phase-advanced (2.50 ± 0.6 h), respectively, in a concentration-dependent manner. To determine which receptor might mediate the effect of VIP, additional experiments examined the phase-resetting properties of agonists that selectively activate receptors currently known to bind VIP: PAC₁, VPAC₁, and VPAC₂.

Our data show that VIP phase resets the rat SCN firing rate rhythm *in vitro* in a phase-dependent manner that resembles the phase resetting effects of light on rodent behavioural rhythms. Preliminary data further suggests that this response to VIP is mediated by the VPAC₂ receptor.

52 ANISOMYCIN INHIBITS SEROTONERGIC PHASE ADVANCES OF THE SCN CIRCADIAN CLOCK IN VITRO. A. Jovanovska and R.A. Prosser, Department of Biochemistry & Cellular & Molecular Biology, Univ. of Tennessee, Knoxville TN 37996.

The suprachiasmatic nucleus (SCN) of the hypothalamus contains the primary circadian pacemaker in mammals. Mechanisms such as gene transcription and protein synthesis may be involved in circadian pacemaker functioning. In all organisms tested, protein synthesis inhibitors (PSIs) induce phase-dependent shifts in their circadian rhythms. PSIs have also been shown to block phase shifts induced by other stimuli. For example, in the *Aplysia* ocular pacemaker the PSI anisomycin blocks phase advances induced by serotonin (5-HT) (Eskin and Young, PNAS 81:7637, 1984). Here we wanted to test the requirement of protein synthesis in the 5-HT phase-shifting pathway in rats. Anisomycin, a potent, reversible PSI, has been shown to phase-shift the SCN circadian pacemaker *in vivo* (Takahashi and Turek, Brain Res. 405:199, 1987) and *in vitro* (Shibata, et al., Brain Res. 584:251, 1992) when applied during late subjective night and early subjective day.

For these experiments, SCN brain slices were obtained from adult, male Sprague-Dawley rats entrained to a 12:12 LD cycle. Slices were continuously perfused with EBSS warmed to 37°C and gassed with 95% O₂ and 5% CO₂, pH 7.4 (Prosser, Brain Res. 818: 408-413, 1999). The 5-HT agonist, (+)8-OH-DPAT, was bath-applied on day 1 for 1h at ZT 6 (ZT 0 = lights-on in the animal colony) by itself or in the presence of anisomycin (20uM). In some cases anisomycin was applied alone. Singleunit activity rhythms were monitored on day 2 *in vitro*.

8-OH-DPAT (10uM and 100uM) induced phase advances of 2.6 ± 0.2 h (n=5) and 4.17 ± 0.5 h (n=3), respectively. These phase advances were decreased to 0.9 ± 0.8 h (n=3) and 1.58 ± 0.3 h (n=3), respectively, when 8-OH-DPAT was applied in the presence of anisomycin. Anisomycin alone at ZT 6 induced phase advances of 0.7 ± 0.2 h (n= 4). Thus, anisomycin decreased the phase advances induced by 100uM 8-OH-DPAT and completely blocked the phase advances induced by 10 uM 8-OH-DPAT.

The amount of protein synthesis inhibition induced by this concentration of anisomycin was quantified by measuring [³⁵S]-methionine incorporation into newly synthesized proteins using a standard trichloroacetic acid (TCA) precipitating protocol. We found that anisomycin decreased incorporation of radioactive label by 80% within the first half-hour of its application. Complete recovery occurred within 1 h of drug removal. Together, these results suggest that protein synthesis is necessary for 5-HT to phase-shift the SCN circadian pacemaker *in vitro*. This research was supported by NIH grant MH 53317 (RAP).

ADENOSINE MODULATION OF GLUTAMATERGIC NEUROTRANSMISSION IN THE SCN

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The purine nucleoside, adenosine, is an inhibitory modulator of glutamatergic neurotransmission in the central nervous system. Adenosine production increases during periods of high neuronal activity, resulting in inhibition of glutamate release through the activation of presynaptic adenosine A1 receptors. Systemic and local administration of A1 receptor agonists attenuates light-induced phase shifts in rodents (Watanabe et al., 1996; Elliott et al., 1997). In addition, accumulation of adenosine during sustained wakefulness may promote sleep by suppressing the activity of cholinergic neurons in the basal forebrain of the cat (Portas et al., 1997; Porkka-Heiskanen et al., 1997). Thus, adenosine could serve as a behavioral state-dependent modulator of glutamate release in the SCN, thereby limiting the magnitude of light-induced circadian phase adjustments.

In order to explore a possible role for adenosine in the hamster SCN, we examined (1) the effects of local application of adenosine receptor antagonists on the extracellular concentration of glutamate in the SCN using *in vivo* microdialysis, and (2) the effect of adenosine receptor agonists on excitatory postsynaptic currents (EPSCs) evoked by optic nerve stimulation in voltage-clamped SCN neurons *in vitro*. Consistent with previous observations (Rea et al., 1993; Glass et al., 1993; Honma et al., 1996) the concentration of glutamate in SCN microdialysate was relatively low during the light phase, and rose to peak levels shortly after lights off. Infusion of the selective A1 receptor antagonist, DPCPX, into the SCN region during the subjective day raised the extracellular concentration of glutamate to near peak levels, suggesting that glutamate release may be under tonic inhibitory control by adenosine. The selective A1 receptor agonist, CHA, dose-dependently reduced the amplitude of electrically-evoked EPSCs in SCN neurons, and this effect was blocked by co-application of DPCPX. Bath application of CHA failed to alter currents evoked in retinorecipient SCN neurons by local infusion of glutamate, strongly suggesting a presynaptic site of action. Supported by AFOSR F49620-00-1-0058 (MAR).

PHASE SHIFTS OF THE CIRCADIAN BIOLOGICAL CLOCK INDUCED BY 5-HT₇ AGONISTS AND INVERSE AGONISTS: RELATIONSHIP TO cAMP PRODUCTION

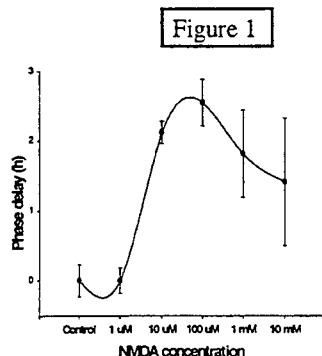
J. Sprouse, J. Braselton, L. Reynolds and A. Schmidt. Pfizer Central Research, Groton, CT 06340

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Previous studies from our laboratory (Reynolds et al., Soc. Neurosci. Abstr. 25:1888, 1999) documented the ability of 8-OH-DPAT to produce robust phase advances in rat SCN slices when superfused a day earlier at ZT6, to this extent replicating earlier work (Prosser et al., J. Biol. Rhythms 8:1-16, 1993). Selective activation of 5-HT₇ receptors was implicated with intrinsic efficacy at this subtype gauged by the ability of 8-OH-DPAT to increase cAMP levels in HEK cells expressing r5-HT₇ receptors (~120% above basal). In the present experiments, ritanserin and mesulergine, compounds employed routinely for their 5-HT antagonist properties, were observed to significantly decrease cAMP production in r5-HT₇-expressing cells in a concentration-dependent manner (K_i = 30-35 nM; maximal decrease below basal = 50-80%), thus invoking the term inverse agonist as suggested by others (Thomas et al., Br. J. Pharmacol. 124:1300-1306, 1998). In rat SCN slices, ritanserin (10 μ M) had little effect on the phase of neuronal firing following bath application alone at ZT6 but co-application with 8-OH-DPAT (10 μ M) at this pretreatment time reduced its phase advance from 2.7 ± 0.8 h to 0.4 ± 1.0 h; similar results were obtained with mesulergine. The possibility that intrinsic efficacy assignments may differ when employing expressed r5-HT₇ receptors and those existing *in situ* was evaluated by measuring cAMP production in tissue derived from rat SCN. Absolute changes from basal production in the presence of 5-HT₇ agonists and inverse agonists were smaller than those observed with expressed receptors (~10%) but consistent in terms of intrinsic efficacy. To determine whether the increases in cAMP elicited by 5-HT₇ agonists in SCN tissue were sufficient to produce the phase advances observed, pretreatment with Rp-cAMPS, a competitive antagonist of cAMP, was employed to inhibit PK-A and abort the remainder of the neurochemical cascade leading to phase changes (Prosser et al., Brain Res. 644:67-73, 1994). Co-application of Rp-cAMPS (1 μ M) and 8-OH-DPAT (10 μ M) at ZT6 reduced the phase advance to 0.5 ± 0.6 h; application with Rp-cAMPS alone had a negligible effect on phase. These data suggest that (1) 5-HT₇ inverse agonists as represented by ritanserin and mesulergine do not produce changes in the phase of SCN neuronal firing when given at the midpoint of subjective day as do 5-HT₇ agonists; (2) increases in cAMP production in the magnitude of that observed in SCN tissue lead to phase advances whereas decreases have no detectable effect; (3) 5-HT₇ agonists represent an intriguing target for drug discovery by virtue of their ability to modulate clock function and 5-HT₇ inverse agonists a valuable tool in discerning 5-HT₇ receptor function.

THE NEUROPEPTIDE Y₅ RECEPTOR MEDIATES THE BLOCKADE OF "PHOTIC-LIKE" NMDA-INDUCED PHASE SHIFTS. P. C. Yannielli and M.E. Harrington. Dept. of Psychology and Neuroscience Program, Smith College, Northampton, MA 01063

Circadian or daily rhythms generated from the mammalian suprachiasmatic nuclei (SCN) of the hypothalamus can be synchronized by light and by non-photoc stimuli. While glutamate mediates photic information, non-photoc information can in some cases be mediated by neuropeptide Y (NPY) or serotonin. NPY or serotonin can reduce the phase-resetting effect of light or glutamate; however, the mechanisms and level of interaction of these two kinds of

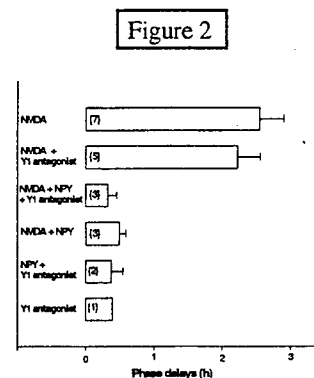


stimuli are unknown. Here we investigate the effect of NPY on the N-methyl-D aspartate (NMDA)-induced phase shift of the SCN circadian neural activity rhythm, by means of single-unit recording techniques.

NMDA (10-100 μM) applied in the early subjective night induced phase delays in the time of peak firing, while doses in the mM range disrupted firing patterns (Figure 1). The NMDA-induced phase delay was blocked by co-application of NPY (0.02-200 μM). NPY Y₁ and Y₅ receptor agonists, but not the Y₂ receptor agonist, blocked the NMDA-induced phase delay in a similar manner as NPY. The co-application of a Y₁ receptor antagonist did not alter NPY blockade of NMDA-induced

phase delays, suggesting that the Y₅ receptor is capable of mediating the inhibitory effect of NPY on photic responses (Figure 2). These results indicate that non-photoc and photic stimuli may interact at a level at or beyond NMDA receptor response, and indicate that the Y₅ receptor is involved in this interaction. Alteration of Y₅ receptor function may therefore be expected to alter synchronization of circadian rhythms to light.

Supported by NIH NS26496.



A CODE FOR LIGHT: NOCTURNAL GLUTAMATE-PACAP INTERACTIONS REGULATE SHIFT AMPLITUDE OF THE SUPRACHIASMATIC CIRCADIAN CLOCK. Martha U. Gillette, Dong Chen, Gordon Buchanan, Jian Ding, Jens Hannibal† and Shelley Tischkau. Dept. of Cell & Structural Biology, University of Illinois at Urbana-Champaign, Urbana, IL, 61801 and †Dept. of Clinical Biochemistry, Bispebjerg Hospital, Univ. of Copenhagen, Copenhagen, Denmark.

Decoding information about the external world by the brain requires integration of complex exteroceptive signals with the organism's internal state. Our objective has been to understand actions and interactions of chemical messengers that relay signals of environmental change to the suprachiasmatic nucleus (SCN), site of the circadian clock. Light information is transmitted to the SCN via direct projections from the eye via the retinohypothalamic tract (RHT). Both glutamate (Glu) and pituitary adenylyl cyclase-activating peptide (PACAP) are present within the RHT, where they colocalize in at least some terminals innervating the SCN. Whereas Glu is an established mediator of light entrainment, the role of PACAP has been unknown. We hypothesized that PACAP modulates glutamatergic effects on circadian clock resetting. To evaluate the functional significance of this colocalization, we assessed the effects of nocturnal Glu and PACAP on phasing of the circadian rhythm of neuronal firing in slices of rat SCN. When coadministered, PACAP blocked the phase advance normally induced by Glu during late night. Surprisingly, blockade of PACAP neurotransmission, either with PACAP6-38, a specific PACAP receptor antagonist, or anti-PACAP antibodies, augmented the Glu-induced phase advance. Blocking PACAP *in vivo* also potentiated the light-induced phase advance of the rhythm of hamster wheel-running activity. Conversely, PACAP enhanced the Glu-induced delay in the early night, whereas PACAP6-38 inhibited it. PACAP6-38 also decreased endogenous cAMP levels. Cellular mechanisms underlying Glu-PACAP interactions are explored in the accompanying abstract (Tischkau *et al.*, SRBR 2000). These results reveal that PACAP is a significant component of the Glu-mediated light-entrainment pathway. When Glu activates the system, PACAP receptor-mediated processes can provide gain control that generates graded phase shifts. The relative strengths of the Glu and PACAP signals together may encode the amplitude of adaptive circadian behavioral responses to the natural range of intensities of nocturnal light. (Supported by PHS grant NS22155.)

ACTIVATION OF PROTEIN KINASE A: A CELLULAR MECHANISM FOR NOCTURNAL GLUTAMATE-PACAP INTERACTIONS IN THE SCN. Shelley A. Tischkau, Eve A. Gallman, Gordon F. Buchanan and Martha U. Gillette Dept of Cell & Structural Biology. University of Illinois at Urbana-Champaign, Urbana, IL. 61801.

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Glutamate (GLU), of retinohypothalamic tract (RHT) origin, is the neurotransmitter primarily responsible for the phase resetting effects of nocturnal light on the circadian clock. Our laboratory has demonstrated that pituitary adenylate cyclase activating peptide (PACAP), a peptide also localized to the RHT, modulates glutamatergic input to the SCN, altering the magnitude of GLU phase shifts. We hypothesize that PACAP modulation of glutamatergic input to the SCN is mediated by the cAMP/PKA signaling pathway. Therefore, we evaluated 1) SCN cAMP levels after GLU treatment; 2) effects of 8-Br-cAMP, a membrane permeable cAMP analog, or KT5720, an inhibitor of PKA, applied with GLU, on the time-of-peak neuronal activity in the SCN brain slice *in vitro*; 3) effects of KT5720 on light-induced phase advances *in vivo*; 4) PCREB after KT5720 and GLU treatment; and 5) *mPer1* levels after GLU and KT5720 treatment.

cAMP was elevated after GLU treatment at CT 14 and CT 20. 8-Br-cAMP blocked the GLU-induced phase advance at CT 20, but potentiated the GLU-stimulated phase delay at CT 14. In contrast, KT5720 blocked the GLU-stimulated phase delay, but potentiated the GLU-induced phase advance. KT5720 also potentiated the light-induced phase advance *in vivo*. Thus, cAMP elevation and PKA inhibition had the same antagonistic effects on GLU-induced phase resetting as exogenous PACAP, and a PACAP antagonist, respectively.

At the molecular level, KT5720 blocked GLU induction of *mPer1* mRNA at CT 14, but did not block the *mPer1* mRNA rise at CT 20. In contrast, GLU-induced PCREB was always blocked by KT5720. Thus, PCREB may not be necessary for GLU-induced phase advances. More importantly, these data suggest a molecular mechanism for PACAP modulation of glutamatergic phase resetting. *mPer1* induction, which is required for GLU-induced phase resetting, may be mediated by PKA activation from co-release or secondary release of PACAP from the RHT in response to a light stimulus. (Supported by PHS grant NS22155 and NS10170)

HISTAMINE AND SUBSTANCE-P RESET THE CIRCADIAN CLOCK THROUGH ACTIVATION OF THE NITRIC OXIDE PATHWAY. J. M. Ding. Department of Medicine, Hennepin County Medical Center, University of Minnesota, 914 South 8th Street, D-3, Minneapolis, MN 55404.

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The circadian clock of the suprachiasmatic nucleus (SCN) is entrained by environmental light. Light information is conveyed to the SCN primarily through release of the neurotransmitter glutamate (Glu) from the retinohypothalamic tract (RHT). However, a number of other neurotransmitters also influence light entrainment through modulation of Glu neurotransmission. Recent studies have shown that both histamine and substance-p induce light-like phase shifts of circadian rhythm through modulation of Glu receptors in the SCN. Activation of the nitric oxide (NO) signaling pathway in the SCN is a key step downstream of the Glu receptors in mediating light entrainment. Here, we studied the involvement of NO in the phase resetting effects of histamine and substance-p. Using a brain slice preparation, the phase of the circadian rhythm can be determined by measuring the time-of-peak of the neuronal electrical activity of the rat SCN. Similar to previous findings, a 0.2 μ l microdrop of histamine (1 μ M) or substance-p (1 μ M) administered directly to the SCN at CT 14 for 10 min each produced 3 hour phase delays of the circadian rhythm of the SCN neuronal activity. Both histamine and substance-p induced phase delays were blocked by prior bath incubation of the NOS inhibitor L-NAME (0.1 mM) or the NO scavenger hemoglobin (0.1 mM). These findings suggest that the Glu-NO signaling mechanism may serve as a common pathway mediating the circadian rhythm phase resetting effects of histamine and substance-p.

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RHYTHMIC COUPLING AMONG CELLS IN THE SUPRACHIASMATIC NUCLEUS.

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Previous studies suggest that the basic mechanism responsible for the generation of circadian rhythms is intrinsic to individual cells. There is also evidence that the cells within the suprachiasmatic nucleus (SCN) are coupled to one another and that this coupling is important for the normal functioning of the circadian system. One mechanism that mediates coordinated electrical activity is direct electrical connections between cells formed by gap junctions. In the present study, we used a brain slice preparation to show that developing SCN cells are dye coupled. Dye coupling was observed in both the ventrolateral and dorsomedial subdivisions of the SCN and was blocked by application of a gap junction inhibitor, halothane. Dye coupling in the SCN appears to be regulated by activity-dependent mechanisms as both tetrodotoxin and the GABA_A agonist muscimol inhibited the extent of coupling. Furthermore, acute hyperpolarization of the membrane potential of the original biocytin-filled neuron decreased the extent of coupling. SCN cells were extensively dye coupled during the day when the cells exhibit synchronous neural activity but were minimally dye coupled during the night when the cells are electrically silent. Immunocytochemical analysis provides evidence that a gap-junction forming protein, connexin32, is expressed in the SCN of postnatal animals. Together the results are consistent with a model in which gap junctions provide a means to couple SCN neurons on a circadian basis.

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CONSTRUCTING SUPRACHIASMATIC NUCLEUS (SCN) CHIMERAS *IN VIVO*. Masato

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We have been exploring the possibility that transplantation of neural precursor ("stem") cells may allow for the construction of SCN chimeras, in which engrafted and endogenous cells intermingle non-disruptively and indivisibly within an intact host SCN. Donor cells were derived from E15 striatum/subventricular zone of transgenic mice engineered to express the green fluorescent protein under the control of a portion of the human cytomegalovirus major immediate early promoter. Such multipotential, self-renewing precursors propagate in culture with epidermal growth factor (EGF) and/or basic fibroblast growth factor (bFGF) as cellular clusters floating in the medium ("neurospheres"). The cells were injected into the forebrain ventricles of individual E14 embryos in order to gain access to the ventricular germinal zone and the developing brain; transplanted pups were born by normal vaginal delivery and sacrificed at P14 for analysis. Initial morphological and immunohistochemical data indicate that donor-derived cells incorporated within the SCN and appeared to differentiate into astroglial phenotypes. In addition to applications in chronobiology, SCN chimeras may be a potentially powerful model system for analyzing the structural and functional plasticity of implanted neural precursor cells.

BIOLUMINESCENCE IMAGING OF INDIVIDUAL CELLS IN TRANSGENIC MOUSE SCN

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A circadian pacemaker in the suprachiasmatic nucleus (SCN) of the mammalian hypothalamus controls rhythmic expression of immediate-early genes. To investigate these clock-controlled genes in the SCN, we imaged bioluminescence in individual SCN cells that reflects gene expression under the control of Ca^{2+} , cAMP, and other gene regulators. Brain slices from transgenic mice containing the firefly luciferase gene *luc* controlled by the immediate-early gene 1 (IE1) promoter/enhancer of the human cytomegalovirus (CMV) were placed in stationary organotypic culture. Signals in individual cells of brain slices imaged over several hours displayed ultradian rhythms with periods of about three hours and near-24 hour rhythms. Luminescence patterns were distinctly different between cultures maintained at 22° C and 30° C. Slices imaged at 22° C displayed expression in the capillaries and ependymal cells, which was confirmed with methylene blue staining and differential interference contrast (DIC). Slices maintained at 30° C expressed pronounced and persistent luminescence in the dorsal SCN, and no expression in the ependymal cells, suggesting that the signal may originate in cells expressing arginine-vasopressin or somatostatin, which have active gene regulatory elements that are also found in the CMV promoter. We predict that the 22° C expression pattern reflects a stress response mediated through the NF κ B pathway. Expression occurred in only a subset of SCN cells in the 30° C cultures. Immunolabeling for luciferase confirmed transgene expression in only a limited number of cells in brain sections. Hypothalamic primary cell cultures also showed selective expression in only a small percentage of glial cells and round, neuron-like cells. This limited expression pattern enables imaging of individual cells with minimal stray light from neighboring cells. Luminescence was highest in the cytoplasm of the dispersed cells, a pattern that was also seen with immunocytochemistry. An advantage of a strong promoter like CMV IE1 is that it can be detected with brief camera exposures, thereby enabling detection of rapid promoter activity dynamics. This transgenic mouse could be useful for distinguishing pacemaker control of gene expression and entraining signals acting through different promoter regulatory elements, such as NF κ B, Ca^{2+} /cAMP binding-protein, and basic helix-loop-helix proteins. Supported by NIH R21 RR12654-01A1 and BGSU.

LIGHT EXPOSURE PRIOR TO BRAIN SLICE PREPARATION CAN INDUCE INCREASED *PER1* AND *PER2* LEVELS MEASURED *IN VITRO*. J. McKinley Brewer, P. C. Yannielli and M.E. Harrington. Dept. of Psychology and Neuroscience Program, Smith College, Northampton, MA 01063

We have recently demonstrated that light pulses delivered *in vivo* can induce phase shifts of the *in vitro* circadian clock similar to those induced by light *in vivo* (Yannielli and Harrington, NeuroReport, in press). These results are similar to those published in 1994 by Shirakawa and Moore (Nsci Letts 178:1049). Previous studies indicate that light pulses during the subjective night can increase levels of *per1* and *per2* in the suprachiasmatic nuclei (SCN). Here we examine brain slices from hamsters exposed to light during the early subjective night to determine if levels of *per1* and *per2* can be increased by light in the SCN brain slice.

Hamsters were housed in cages equipped with computer-monitored running wheels under 14:10 h light:dark. After recording baseline data, animals were transferred to dim red light for 3 days. Hamsters were overdosed with halothane at CT14, either under the constant dim red light or after exposure to a 5 min, 150 lux, light pulse. Brains were quickly dissected and a brain slice preparation was placed in a culture chamber using techniques standard for this laboratory. At various times after the start of the light pulse (30 min, 1 h, or 2 h) the slices were removed from the chamber and stored at -70°C until cryostat sectioning. Levels of *per1* and *per2* mRNA were determined using *in situ* hybridization with ^{35}S -labeled probes.

Both *per1* and *per2* mRNA levels showed apparent increases in the SCN tissue from light-exposed hamsters vs. controls. The peak in *per1* levels was at 30 min after the light pulse, whereas the *per2* levels peaked later, 2 h after the initiation of light. These results demonstrate that light-induced increases in gene expression can be measured from SCN tissue cultured *in vitro*. In future experiments we will be interested in determining the effect of neuropeptide Y on levels of these light-induced mRNAs.

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EFFECT OF NEUROPEPTIDE Y ON *PER1* GENE EXPRESSION IN THE HAMSTER SCN *IN VITRO*.

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In our previous studies, we showed that the hamster hypothalamic slice is a suitable model to investigate the mechanism of circadian rhythm generation; we showed clear circadian rhythms of electrophysiological discharge rate, and phase advances by neuropeptide Y (NPY) in the day. In this study, we applied *in situ* hybridization technique to assess whether our model is suitable for gene expression studies to understand the molecular mechanism of circadian rhythm generation.

Male Syrian hamsters were adapted to 14h-10h light dark cycles before experiments. Halothane overdose was followed by decapitation. In most cases a brain slice preparation was cultured *in vitro* using previously published techniques. First, we determined *per1* mRNA level both immediately after decapitation or after up to 12 hours *in vitro* to see whether *per1* mRNA is rhythmically expressed in the SCN *in vitro* as seen *in vivo*. As consistent with previous studies, *per1* mRNA showed clear circadian rhythms in light dark cycles with a high level in the day. The daily change was maintained in culture conditions, high levels at CT6 and low at CT18 were observed. NPY was applied to the brain slices at CT6 to see if *per1* mRNA level is changed. NPY reduced *per1* mRNA level 1 hour ($p < 0.05$) but not 30 min or 2 hour after treatment.

These results suggest that circadian *per1* gene expression is endogenous, and clock function is intact in the SCN, although it remains necessary to establish continued cycles in gene expression *in vitro*. *Per1* may be involved in NPY-induced phase shifts, similar to results reported for novel-wheel-induced phase shifts (Maywood, et.al., PNAS, vol 96, 15211-15216, 1999).

In conclusion, our *in vitro* system is a suitable model to investigate molecular mechanism of circadian rhythm generation and that of non-photoc phase shifts.

Supported by NIH NS26496, HHMI, and the AV Davis Foundation.

GENE EXPRESSION AND NEURONAL FIRING SIMULTANEOUSLY MONITORED IN SCN

NEURONS SJ Kuhlman and DG McMahon Dept. of Physiology, University of Kentucky; Lexington, KY 40536

Endogenous cyclic activation of a specific set of genes, including *Period1* (*Per1*) appear to contribute to the generation of rhythmicity in the suprachiasmatic nucleus (SCN). Using transgenic mice in which a degradable form of recombinant jellyfish Green Fluorescent Protein (GFP) is driven by the mouse *Per1* gene promoter, a circadian rhythm in *Per1*-driven GFP fluorescence was demonstrated by imaging acute SCN slices at subjective dawn and dusk from animals housed in constant dim red light. Thus, the fluorescent signal defines the functional state of the combined action of core clock gene products, including Clock and Bmal transactivators and Period and Cryptochrome negative regulators.

Spontaneous firing of *Per1*-fluorescent neurons in SCN slices perfused with oxygenated ACSF at 33 °C was monitored using an upright Axioskop2 microscope (Zeiss) equipped with near-IR and fluorescence imaging. Electrophysiologic data was collected using an Axopatch 2000 amplifier and Clampex6 or 7 software (Axon Instruments). These data demonstrate that promoter activity and clock output can be simultaneously resolved at the level of the functional unit of rhythms generation, the individual cell. The *Per1*-driven GFP system can be employed to study coupling between individual clock neurons under stable or phase-resetting conditions. Supported by NIH AG13426 and MH60794 to DGM.

ILLUMINATION OF MOLECULAR CLOCKWORK IN THE SCN: REPORTER GENE ANALYSIS OF CLOCK AND CLOCK-CONTROLLED GENES WITHIN IMMORTALIZED CELLS. David Earnest, Rodney Walline and Gregg Allen. Dept. of Human Anatomy & Neurobiology, Texas A&M University Health Science Center, College Station, TX 77843

Recent findings indicate that immortalized cells derived from the rat suprachiasmatic nucleus (SCN) retain the capacity to generate rhythms in molecular and physiological outputs. Current analysis using luciferase reporter gene technology has allowed for non-invasive temporal analysis of clock-controlled gene (*c-fos*) expression in living cells. Using the promoter region of the *c-fos* gene linked to a luciferase reporter, we have shown a distinct circadian oscillation in luciferase-reported *c-fos* expression. When maintained in normal media (10% serum), individual cultures of SCN2.2 *fos/luc* cells derived from the same passage exhibited synchronous rhythms of luciferase activity. Administration of a 2-hour serum pulse (50%) to SCN2.2 *fos/luc* cells yielded a marked increase in the amplitude of the rhythm in luminescence compared to control cultures. Comparison of cells treated with normal media or a serum shock revealed that the phase of the rhythms in individual cultures was tightly synchronized within, but not between, groups. We have extended this non-invasive, real-time measurement of gene expression to other clock-controlled elements as well as to putative clock genes. Specifically, cells were transfected with constructs encoding either the firefly luciferase (*luc*) or green-fluorescence protein (GFP) gene fused to the 5' flanking regions of arginine vasopressin (VP) or the *period-1* gene, *Per1*. Results show that SCN2.2 *VP/luc* and SCN2.2 *Per1/luc* cells are respectively characterized by circadian variation in luciferase-reported VP and *Per1* expression. These results demonstrate that SCN2.2 cells are endogenously capable of generating luciferase reported circadian rhythms in *Per1*, vasopressin and *c-fos* expression resembling those observed within the SCN *in vivo* and *in vitro*. Together with the circadian properties of our immortalized cells, this approach should provide a unique model by which to manipulate putative core elements of the SCN clock mechanism and study resulting effects on its circadian outputs.

CA²⁺-INDUCED EXPRESSION OF CIRCADIAN CLOCK GENE, *MPER1* IN THE MOUSE CEREBELLAR GRANULE CELL CULTURE

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Various circadian clock genes such as mouse *mPer1* or *mPer2* are rhythmically expressed not only in the suprachiasmatic nucleus (SCN) of hypothalamus in which mammalian circadian clock exists, but also expressed in various brain regions, especially in the hippocampus and granule cell layer of cerebellum. However, their role outside of the SCN is barely known. Induction of circadian oscillation of these genes in the cultured Rat-1 cells by treatment of high concentration of serum suggests that oscillatory mechanism exists in the all tissues, but signaling mechanism involved in circadian oscillation is unknown. We used mouse cerebellar granule cell culture to examine signaling mechanism involved in regulation of *mPer1* gene expression.

mPer1 mRNA expression in the mouse cerebellum was dependent on K⁺ concentration in the medium and was reduced by nifedipine treatment. Medium change from low K⁺ to high K⁺ concentration induced transient increase of *mPer1* gene expression. This increased expression was suppressed by calmidazolium or KN-93, but not by PD098059. Addition of PACAP-38 into the low K⁺ medium also induced transient *mPer1* gene expression. This effect could be mimicked by dibutyl-cAMP.

These results suggest that *mPer1* mRNA expression depends on intracellular Ca²⁺ concentration through VDCC, and Ca²⁺-calmodulin and cAMP signaling pathways are concerned with the *mPer1* expression.

METABOLIC COUPLING IN CULTURED CHICK ASTROCYTES BY RHYTHMIC MELATONIN ADMINISTRATION

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Melatonin plays an important role in circadian rhythms, especially in non-mammalian vertebrates. This influence is presumably due to effects on the specific melatonin receptors, Mel_{1A}, Mel_{1B} and Mel_{1C}. Mel_{1C} receptors are expressed primarily by astrocytes and Muller cells. Recent studies have shown that astrocytes supply tricarboxylic acids, such as lactate and pyruvate to neurons as a substrate for metabolic energy. In this study, we examined the effect of rhythmic administration of melatonin on receptor expression and pyruvate release.

Polyclonal antisera raised against the Mel_{1C} receptor protein showed strong immunoreactivity with an approximately 75 kD protein, though no signal was obtained from those against the Mel_{1A} receptor. Rhythmic administration of melatonin caused the rhythmic release of pyruvate, such that levels were high in the medium without melatonin and low in medium with melatonin. Though samples collected 4-5 days later after administration showed day-night changes, the amplitude of the rhythm was much lower than that of those collected 7-8 days later. Western blot analysis demonstrated another difference between samples taken at the 4-5 and 7-8 days. The 36 kD Mel_{1C} receptor protein at days 7-8 showed not only much a stronger band than that at 4-5 days, but also showed diurnal rhythms, in which expression was high from noon (ZT6) to evening (ZT14) and low from the mid night (ZT18) to the morning (ZT2). These data suggest that rhythmic expression of Mel_{1C} receptor protein (36 kD) is necessary to regulate temporal changes of pyruvate release, and that diurnal changes of melatonin contribute the metabolic coupling between astrocytes and neurons in diencephalon.

Supported by NINDS Grant R01 NS-35822 to VMC and by Toyobo Biotechnology Foundation to AA.

INHIBITORS OF MAP AND p38 KINASES INDUCE LIGHT-LIKE AND DARK-LIKE PHASE SHIFTS IN CHICK PINEAL CELLS, BUT DO NOT POINT TO A CLEAR ROLE FOR THESE ENZYMES IN PHOTOENTRAINMENT.

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The components of the transduction pathway mediating photoentrainment in vertebrates have not yet been identified. New candidates include MAP Kinases and their relatives, JNK and p38 Kinases. The presence, temporal dynamics, and photoresponsiveness of MAP Kinase have been reported in several systems, but results in SCN, retina, and chick pineal were inconsistent, if not incompatible. As these enzymes are involved in multiple, complex and overlapping transduction pathways that mediate activation of numerous transcription factors, we tested the most simple hypothesis: Activation (or inactivation) of MAP Kinase is uniquely necessary, sufficient, and specific for photoentrainment. We used chick pineal cells in culture, which give measurable phase shifts in their circadian rhythm of melatonin release in response to light or dark pulses, and to other stimuli. If photoentrainment requires inactivation of MAP Kinase, as has been suggested, then drugs that block activation of MAPK should mimic the phase shifting effects of light and agents that promote activation of MAPK should mimic the effects of dark pulses. Drugs that are specific for other enzymes, such as p38 kinase, should not evoke phase shifts at all. We tested the effects of a putatively specific inhibitor of MAP Kinase activation (PD 98059), of a nonspecific activator of MAP Kinase (serum), and of a putatively specific inhibitor of p38 kinase (SB 203580 HCl). 4 hr pulses of PD 98059 induced phase shifts with a light-like PRC, suggesting that light might indeed promote inactivation of MAPK. However, serum failed to induce dark-like phase shifts, or any at all, and SB 203580 HCl did induce phase shifts, but with a dark-like PRC. These results argue against, but do not disprove, the simple hypothesis as stated. One is that the hypothesis tested is excessively specific and oversimplified. Effects of these inhibitors do, however, suggest that perturbations of MAP Kinase and/or its relatives can entrain the circadian clock. The exact roles of individual members of the MAP Kinase family in entrainment, however, remain to be determined.

NOCTURNIN PROTEIN EXPRESSION AND INTRACELLULAR LOCALIZATION IN *XENOPUS LAEVIS* RETINA. Julie E. Baggs, Francesca E. Anderson, Xiaorong Liu and Carla B. Green, Department of Biology, NSF Center for Biological Timing, University of Virginia, Charlottesville, VA 22903

Nocturnin was identified in a differential display screen as a circadian clock-regulated gene displaying high amplitude rhythmic mRNA expression in the retina of *Xenopus laevis*. It is expressed in the clock-containing photoreceptor layer of the retina at high levels during the early night. The nocturnin protein expression profile and intracellular localization were examined in these studies. Protein tags, including green fluorescent protein (GFP) and small peptide tags V5 and FLAG, were cloned in-frame on either the amino- or carboxy-terminus of nocturnin protein, creating several fusion proteins. When overexpressed in COS-7 cells, these fusion proteins localized primarily in the cytoplasm, although a small percentage (6-10%) of cells showed exclusively nuclear expression. Transgenic tadpoles expressing GFP-tagged nocturnin in the rod photoreceptor cells under the control of the *Xenopus* rod opsin promoter (XOP) showed similar localization: nocturnin-GFP fusion proteins localized in the cytoplasm of the rod photoreceptor cell body in most cases, though nuclear localization was observed in one animal. Endogenous expression of nocturnin protein in the retina was examined by Western blot analysis using an antibody raised against a small peptide at the carboxy-terminus of nocturnin. Nuclear and cytoplasmic retinal fractions were collected at four-hour intervals and the amount of nocturnin protein in each fraction was examined. Nocturnin protein is detected in the cytosolic fraction at ZT 16, and by ZT 20 it is detected in the nuclear fraction. Since nocturnin has no obvious nuclear localization signal, future work will focus on uncovering how its intracellular localization is regulated.

A PORTION OF THE NOCTURNIN PROMOTER CONTAINING AN E BOX-LIKE ELEMENT IS SUFFICIENT TO TARGET SIGNALS TO CLOCK-CONTAINING PHOTORECEPTOR CELLS IN *XENOPUS LAEVIS*

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Nocturnin is expressed in the photoreceptors of *Xenopus laevis* and its mRNA levels show a high amplitude circadian rhythm that is primarily regulated at the level of transcription. In this study our goal was to define the mechanism that confers this spatial and temporal pattern of expression on the nocturnin gene. Different lengths of the *Xenopus* 5'-flanking sequence were cloned upstream of the Green Fluorescence Protein (GFP) cDNA. These constructs were used to produce transgenic *Xenopus* embryos and the pattern of the GFP reporter was examined. Confocal images of a retina from a transgenic tadpole produced by using a fragment containing 2.6Kb of 5'-flanking sequence (-2.6kb/+20bp)-GFP demonstrate that this piece of promoter is sufficient to target the GFP reporter to both rod and cone cells. Deletion of the nocturnin promoter to (-108bp/+20bp) does not change the GFP expression pattern, indicating that elements necessary for spatial expression must be contained within this sequence. To identify the protein binding sites, gel shift assays were performed, using nuclear extracts isolated from *Xenopus* retina. Within the (-108bp/+20bp), a region with similarity to an E box (Noc-E-box) was identified. Using a series of mutated Noc-E-Box oligos as competitors in the gel shifts, we defined the sequence of the Noc-E-box as GTGACGTG. Gel shifts comparing the Noc-E-box with the consensus E box (CACGTG) showed that the binding proteins both are high molecular weight complexes, however, they are of slightly different size. We are currently determining the identity of the Noc-E-box-binding proteins and defining their role in the spatial and temporal pattern of the nocturnin expression.

71 OVEREXPRESSION OF DOMINANT-NEGATIVE CLOCK CAN ABOLISH MELATONIN RHYTHMICITY IN THE *XENOPUS* RETINA

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We have cloned the *Xenopus Clock* gene (*XClock*) and found that it is widely expressed in several tissues, including retina. Previous reports have indicated that *Xenopus* has an autonomous clock in the retina that regulates melatonin release in a circadian manner. We designed a dominant-negative version of XCLOCK (XCL-ΔQ) which retains the bHLH-PAS domain but lacks the 3' transactivation domain. To examine the role of the XCL-ΔQ *in vitro*, transient transfection assays were done. This truncated protein acted in a dominant-negative fashion by suppressing CLOCK and BMAL-mediated transactivation of the *Per* gene. Next, to further investigate the XCLOCK function in the retinal circadian clock *in vivo*, we performed transgenic studies using XCL-ΔQ. An IRBP (interphotoreceptor retinoid-binding protein) promoter was used to drive the transgene expression only in the photoreceptor layer. Flow-through culture was performed using eyecups of the transgenic and wild-type control tadpoles at 2-3 weeks of age. Culture medium fractions were collected over time, followed by radioimmunoassay for melatonin measurement. While wild-type eyes showed melatonin release in a circadian manner (average $\tau=23.98$) in constant darkness, rhythmic expression of melatonin was not observed in most of the transgenics (~80%). The incomplete penetrance, also observed in mouse and *Drosophila Clock* mutants, is most likely due to variability in transgene expression levels in individual tadpoles. These data indicate that XCLOCK regulates the rhythmic expression of melatonin. This is the first evidence in vertebrates directly demonstrating that CLOCK is involved in the retinal circadian clock. The transgenic approach using dominant-negative mutants can be a strong tool to selectively inactivate circadian clock function in specific cells.

72 BMAL IS EXPRESSED IN THE RETINA OF *XENOPUS LAEVIS* WITH HIGH AMPLITUDE RHYTHMS

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An autonomous clock has been localized to the photoreceptor layer in the retina of *Xenopus laevis*, and it has been shown that this tissue expresses many of the molecular clock components, such as *XClock* and *Xcrys*. To determine if the molecular mechanisms driving the clock are conserved between vertebrates, we have cloned the *Xenopus* homolog of the *bmal1* gene (*Xbmal*). By degenerate RT-PCR, we amplified a small portion of *bmal* cDNA from *Xenopus* retinal RNA. This fragment was used as a probe to screen a *Xenopus* retinal cDNA library. We obtained a 2.5 and a 3 kilobase clone. The two clones are not identical, indicating that *Xenopus* expresses two *bmal* mRNAs. The deduced amino acid sequence from the 3 kb clone is 632 amino acids long, and it is conserved between the mouse and *Xenopus*, with 86% identity and 91.5% similarity. Northern blot analysis shows that a *Xbmal* message is expressed in all tested tissues, namely retina, brain, heart, liver, spleen and testis. The message is strongly rhythmic, peaking at ZT12, and rhythmicity persists in constant darkness. This is in direct contrast to the *XClock* mRNA, which is not rhythmic. Transient transfection experiments show that XBMAL, co-transfected with XCLOCK, can activate per E-box-mediated luciferase reporter gene transcription. These results suggest that the CLOCK/BMAL1-mediated transcriptional activation mechanism is conserved between the mouse and *Xenopus laevis*, although there are clear differences in the expression patterns of these genes.

THREE CRYPTOCHROMES ARE EXPRESSED IN *XENOPUS LAEVIS* PHOTORECEPTORS

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The *Xenopus laevis* retina contains an endogenous circadian clock. The molecular aspects of this clock are mostly unknown, although a *Xenopus* homolog of *Clock* was recently cloned from retina. In order to understand the functions of the *Xenopus* clock, we decided to clone homologs of *cryptochrome* (*cry*). We designed degenerate primers based on homology between mouse *crys*. We performed RT-PCR using these primers and subsequently screened a *Xenopus* retinal cDNA library resulting in the identification of three *cry* homologs. We named them *Xcry1*, *Xcry2a*, and *Xcry2b* based on the homology to mouse *crys*. *In situ* experiments show that all the messages are expressed in the photoreceptor cell layer. We then used northern blots to study the temporal expression of these genes. At least two of the genes are rhythmically expressed (*Xcry2a* peaks at dawn and *Xcry1* peaks at middle of night). To analyze the protein function, we performed transient transfection assays by cotransfecting the three *crys* with *XClock* and *Xbmal1*. Our results show that *Xenopus* CRYs can directly suppress the activation by CLOCK and BMAL1 dimer. This suggests that *Xenopus* CRYs, like their mammalian homologs, are part of the central oscillator. However, the different expression patterns suggest some functional differences between *Xenopus* and mouse CRYs. Interestingly, the phase of the *Xcry2a* message peak is more similar to *Drosophila cry*. This leads us to believe that *Xenopus* CRYs could share functions of both mouse and *Drosophila* homologs. We hope that by studying *Xenopus crys*, we could understand how the proteins have evolved from a circadian photoreceptor in *Drosophila* to part of the central oscillator in mouse.

EVEN CHICKS CRY2

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The avian pineal gland and retina are known to contain a circadian clock capable of the generation and regulation of circadian associated genes *in vitro*. In spite of this importance, little is known about the molecular mechanisms that underlie these phenomena in the avian pineal gland or retina. Herein, we have focused on these structures to determine putative molecular clock components and their circadian expression. In order to identify the essential clock components in chicken pineal glands, cDNA libraries were generated and screened for avian orthologs of the mammalian cryptochrome genes (*Cry*). An avian ortholog of the mouse cryptochrome 2 gene (*Cry2*) was identified, and confirmed by sequence analysis. Northern blot analysis demonstrated that *cCry2* mRNA was expressed in the pineal, retina, brain, and peripheral tissues, with a transcript size of about 4.5 Kb. Daily expression levels of *cCry2* mRNA were tested in the pineal and retina, and shown to cycle under constant darkness, with peak expression levels at late subjective night. *In situ* hybridization (ISH), using digoxigenin labeled oligonucleotides, revealed *cCry2* mRNA expression in the photoreceptor layer, inner nuclear layer, and ganglion cell layer of the retina. Labeling was also observed in additional regions of the chick visual system, including the optic tectum, optic chiasm, nucleus rotundus, paraventricular nucleus, and the lateral septum. Pineal staining labeled the paraventricular and follicular pinealocytes. In summation, we have isolated an avian ortholog of mouse *Cry2* and shown its mRNA expression in clock possessing tissues (retina and pineal), and expression in regions of the chick brain associated with deep brain photoreception and visual system structures. Current studies are being performed to determine if *cCry2* may be functioning as an endogenous molecular component and as a photoreceptive element of the chick circadian system.

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75 **REGULATION OF THE CIRCADIAN OSCILLATOR BY c-JUN N-TERMINAL KINASES.**

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Transcriptional and translational feed-back loops are central mechanisms of circadian oscillators, and entrainment appears to involve transcriptional and translational regulation. Stress-activated protein kinases (SAPKs including c-Jun N-terminal kinases (JNKs) and p38 MAP kinases) are activated by several extracellular signals, some of which are known to reset circadian oscillators, and they regulate transcription. In the present study, we investigated whether SAPKs play a role in regulation of the circadian oscillator in *Xenopus* retinal photoreceptors.

Circadian rhythms of melatonin from cultured photoreceptor layers were reset by a 6-h pulse of SB 203580 (30 μ M), an inhibitor of SAPKs, in a phase-dependent manner similar to dark. In addition, light did not reset the circadian oscillator when SAPKs were inhibited by SB 203580 (100 μ M). In-gel kinase assays showed that SB 203580 inhibited a small number of protein kinases in the photoreceptor cells, and that these kinases were immunoprecipitated by a JNK antibody. The dose-dependencies of SB 203580-induced phase shifting and JNK inhibition were similar. On the other hand, we did not observe any SB 203580-sensitive p38 MAP kinase activity in the photoreceptor cells. These results suggest that SB 203580 reset the circadian oscillator and blocked responses to light by selectively inhibiting one or more members of the JNK family. We also examined the possibility that JNK is regulated by light and/or the circadian oscillator. We could detect no circadian rhythm in the phosphorylation of JNK by western blot analysis. Furthermore, no effect of light on photoreceptor JNK activity was detected in an immunoprecipitate/in-gel kinase assay. Taken together, those results suggest that constitutive JNK activity is necessary for circadian oscillator function, but that this activity is not regulated by light or the circadian oscillator. Supported by NIH grant R01-MH49757.

76 **THE CIRCADIAN SYSTEM OF AGING RODLESS + CONELESS MICE: AN ANATOMICAL AND BEHAVIOURAL ANALYSIS.**

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We have developed mouse models which lack both rod and cone photoreceptors, yet are still capable of regulating their circadian physiology and behaviour by light. Enucleation blocks these effects of light showing that mice, and presumably other mammals, have unrecognised ocular photoreceptors which regulate temporal physiology [1,2]. The most likely site for these novel photoreceptors is a sub-set of cells within the inner nuclear layer (INL) and/or ganglion cell layer (GCL) of the retina [3,4]. Studies in several other retinally degenerate mouse models have shown that photoreceptor loss can cause cell loss in the INL and GCL of the retina. Our initial studies examined rodless+coneless (*rd/cl*) mice between 80 - 100 days of age, and the present investigation was undertaken to address two questions: (1) Do we see changes in the INL and GCL of the retina in aging *rd/cl* mice? (2) Does the loss of INL and GCL cells in *rd/cl* mice parallel changes in their circadian behaviour? Our preliminary anatomical results have shown that the INL of *rd/cl* mice shows considerable cell loss after 9 months of age, and by 12 months of age the INL is completely absent in places, and the GCL is beginning to show signs of cell loss. Despite these profound insults to the retina, our initial behavioural studies have shown that *rd/cl* mice 12 months of age retain responses to light. On the basis of these preliminary data we propose that a sub-set of ganglion cells house the novel photoreceptors which mediate the effects of light on circadian behaviour. Our future studies will examine GCL cell loss and circadian behaviour in *rd/cl* mice at ages in excess of 12 months. **References:** [1] Freedman, et. al. (1999) Science 284, 502-504; [2] Lucas, et.al. (1999) Science 284, 505-507; [3] Provencio, et.al. (1998) J. Comp. Neurol. 395, 417-439; [4] Provencio, et.al. (2000) J. Neurosci. 20, 600-605.

Pupillary light reflexes in mice (C3H *rd/rd cl/+*) bearing lesions of both rod and cone photoreceptors. **Robert J Lucas¹, Ronald H Douglas² and Russell G Foster¹.** ¹Unit of Integrative and Molecular Neuroscience, Imperial College School of Medicine, Charing Cross Hospital, London W6 8RF, UK. ²Department of Optometry and Visual Science, City University, 311-321 Goswell Rd, London EC1V 7DD, UK.

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Purpose: To employ the pupillary light reflex in characterising the residual photosensitivity of C3H *rd/rd cl/+* mice, a model of retinal degeneration which retains circadian photosensitivity despite lacking both rod and cone photoreceptors. **Methods:** Standard pupillometry techniques were used to measure pupillary constriction in response to a 1 min monochromatic light exposure in unanaesthetised C3H wild type and *rd/rd cl/+* mice aged between 90 and 180 days. The wavelength and irradiance of the light pulse was varied in order to facilitate comparisons of spectral and absolute sensitivity between genotypes. **Results:** Despite the dramatic retinal degeneration of *rd/rd cl/+* mice these animals retain a robust pupillary light reflex. At 509nm the loss of classical photoreceptors was associated with a roughly 2 log unit increase in the threshold intensity required for significant constriction. However, under high irradiances, the time course and extent of constriction was the same in both genotypes. Atropine application abolished all responses, confirming that pupillary constriction was centrally regulated in both genotypes and not the result of a direct iris response. The spectral sensitivity of this response in the *rd/rd cl/+* confirms the involvement of non-rod, non-cone photoreceptors. **Conclusions:** Previous work has established that by 80 days of age, the *rd/rd cl/+* retina completely lacks rod and cone photoreceptors. Nonetheless, this animal model retains circadian photosensitivity as assessed both by phase shifts of the circadian activity rhythm and by inhibition of pineal melatonin synthesis. The demonstration of an intact pupillary light reflex in these mice indicates that the uncharacterised photoreceptors remaining in the *rd/rd cl/+* retina are also capable of regulating pupil size. Detailed stimulus response analysis of pupillary light reflexes in these mice will therefore provide useful information regarding the nature of these unidentified photoreceptors.

LOCALIZATION OF SITES OF MELATONIN SYNTHESIS IN A MAMMALIAN RETINA

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A major unresolved issue in the study of retinal melatonin has been the question of which retinal cells synthesize it. Melatonin is synthesized in a two-step process in which the enzyme serotonin N-acetyltransferase (SNAT) catalyzes the conversion of serotonin to N-acetylserotonin (NAS), and hydroxyindole-O-methyltransferase (HIOMT) converts NAS to melatonin. HIOMT has been localized to photoreceptors and bipolar cells using immunocytochemistry. However, this enzyme is present in pathways other than the melatonin biosynthetic pathway. Melatonin immunoreactivity has also been found in photoreceptors, but it is not clear whether this reflects synthesis or uptake since immunoreactivity has been shown to increase after systemic injection of melatonin. The recent cloning of SNAT has made possible the localization of SNAT mRNA in retina using *in situ* hybridization.

To determine which cells of the mammalian retina synthesize melatonin, we examined the expression pattern of SNAT in rat retina with *in situ* hybridization using a digoxigenin-labeled cRNA probe. At ZT18, which is at or near the timing of peak melatonin synthesis, SNAT mRNA was expressed in a population of photoreceptor cells in the rat retina with a distribution strongly resembling that of cones. No SNAT expression was seen outside the photoreceptor layer. We show double-labeling of SNAT mRNA with peanut agglutinin (PNA), a marker of cone cells, demonstrating that these photoreceptor cells are indeed cones. Finally, using a combined *in situ*/immunocytochemical technique, we show SNAT expression in red/green cone opsin immunoreactive (IR) cells, and lack of this co-localization in blue cone opsin-IR cells. These results indicate that the melatonin synthesizing cells of the rat retina are red/green cones.

MELATONIN RECEPTOR RNA RHYTHMICITY AND LOCALIZATION IN THE CHICK RETINA

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Melatonin is a transducer of timing information to a variety of tissues in species that make melatonin. In the chick (*Gallus gallus*), melatonin is synthesized in both the pineal gland and in photoreceptors of the retina. Within the eye, many processes are rhythmic including visual evoked potential, photoreceptor disc shedding, as well as dopamine and melatonin levels. Melatonin plays a role in at least some of these rhythms, presumably through melatonin receptors. The distribution of melatonin binding and of the mRNA of the Mel_{1A} and Mel_{1C} receptor subtypes suggests melatonin receptor expression resides in the retinae as well as in several visual system brain structures. Whereas receptor binding has been shown to be rhythmic, little is known about the rhythmicity of specific subtypes. In this study, melatonin receptor messenger RNA (mRNA) expression was examined in the chick retina at different times of day.

Mel_{1A} and Mel_{1C} receptor mRNA was examined in the chick retina of two-week old birds to determine temporal and spatial expression patterns. Rhythmicity was analyzed using RNase Protection Assay and northern blot analysis of retinal RNA, obtained every four hours, both in LD and DD. Rhythms were found in melatonin receptor RNA of both the Mel_{1A} and Mel_{1C} subtypes with levels higher during the day. Mel_{1A} and Mel_{1C} cellular localization was examined using *in situ* hybridization at the peak and trough times of the melatonin receptor rhythm. Defining melatonin receptor expression characteristics is critical to understanding the physiological effects of melatonin in the chick retina. Supported by NIH RO1 NS-35822.

EFFECTS OF TIMED MELATONIN INJECTIONS ON CIRCADIAN RHYTHMS OF CHICK ELECTRORETINOGRAM

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The indoleamine hormone melatonin is synthesized rhythmically by retinal photoreceptors, and melatonin receptors are present in the inner retina in birds. These data suggest a rhythmic element within the retina is sensitive to melatonin. Several aspects of the electroretinogram (ERG) in chicks are expressed rhythmically, which are abolished by experimental removal of melatonin rhythms. The purpose of this study was to ascertain whether the retina is differentially sensitive to melatonin depending on time of day in light: dark cycles (LD) and in constant darkness (DD). Latency, a- and b-wave implicit time, and a- and b-wave amplitude of the electroretinogram were measured at different times of day in LD and DD in the presence or absence of different doses (1 mg/kg, 100 ng/kg, or 1 ng/kg) of melatonin. An ERG rhythm of a- and b-wave amplitude was observed in both LD and DD. Melatonin (1 mg/kg and 100 ng/kg) decreased these parameters, while 1 ng/kg melatonin had no effect. B-wave amplitude was significantly decreased more during the day than during the night.

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AN OCULAR CLOCK CONTROLS ROD-CONE DOMINANCE AND SENSITIVITY IN QUAIL RETINA. Mary E. Pierce, Mary K. Manglapus and Robert B. Barlow. Department of Neurosciences and Physiology, Center for Vision Research, SUNY Upstate Medical University, 750 East Adams, Syracuse New York 13210.

A circadian clock modulates retinal sensitivity inducing cone dominance during the daytime and rod dominance at night (Manglapus et al., 1999). However, although we have hypothesized that the oscillator(s) regulating these events exist in the eye we have not demonstrated this directly. To test whether an ocular oscillator controls sensitivity, we alternatively patched the eyes of quail similar to a protocol that was used by Underwood et al. (1988). Animals were maintained on a 12L:12D cycle for 1 week, then animals were placed in constant light. A patch was placed over the right eye for 12 hrs and then it was removed. During the next 12 hrs the left eye was patched. This protocol was followed for 7 days and then ERG's and NAT mRNA levels were measured in constant darkness. Eyes exposed to opposite light:dark cycles exhibit opposite circadian rhythms in retinal sensitivity and rod-cone dominance. Thus, in constant dark one eye can be rod dominated (night state) while the other is cone dominated (day state). NAT mRNA exhibits opposite rhythmic changes in each eye. Conclusion: Ocular clocks act autonomously in the Japanese quail modulating the rod-cone dominance and NAT mRNA expression in each eye.

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SEQUENCE, GENOMIC STRUCTURE AND TISSUE EXPRESSION OF CARP VERTEBRATE ANCIENT (VA) OPSIN.

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We report the isolation and characterisation of a novel opsin cDNA from the retina and pineal of the common carp (*C. carpio* L.). When a comparison of the amino acid sequences of salmon Vertebrate Ancient opsin (sVA) and the novel carp opsin are made, and the carboxyl terminus is omitted, the level of identity between these two opsins is 81%. We have therefore termed this *C. carpio* opsin as carp VA opsin (cVA opsin). We show that members of the VA opsin family exist in two variants or isoforms based upon the length of the carboxyl terminus, and propose that the mechanism of production of the short VA opsin isoform is by premature transcription termination in intron 4 of the VA opsin gene. The VA opsin gene consists of five exons, with intron 2 significantly shifted in a 3' direction relative the corresponding intron in rod and cone opsins. The position (or lack) of intron 2 appears to be a diagnostic feature which separates the image forming rod and cone opsin families from the more recently discovered non-visual opsin families (Pineal (P), Vertebrate Ancient (VA), Parapinopsin (PP)). Finally, we suggest that lamprey P-opsin should be reassigned to the VA opsin family based upon its level of amino acid identity, genomic structure with respect to the position of intron 2, and nucleotide phylogeny.

A NOVEL ROD-LIKE OPSIN ISOLATED FROM THE EXTRA-RETINAL PHOTORECEPTORS OF TELEOST FISH.

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We have isolated a novel rod-like opsin from the pineal complex of Atlantic salmon (*Salmo salar*) and from the brain of the puffer fish (*Fugu rubripes*). These extra-retinal rod-like opsins share approximately 74% identity at the nucleotide and amino acid level with rod-opsins from the retina of these species. Using PCR on Atlantic salmon tissues, we have determined that the novel rod-like opsin is not expressed in the salmon retina, and the retinal rod-opsin is not expressed in the salmon pineal. Phylogenetic analysis suggests that the rod-like opsins arose from a gene duplication event approximately 205 million years ago, a time of considerable adaptive radiation of the bony fish. In view of the large differences in the coding sequences of the pineal/brain rod-like opsins, their extra-retinal sites of expression, and phylogenetic position we have termed these novel opsins "extra-retinal rod-like opsins" (ERrod-like opsins). We speculate that the differences between retinal rod-opsins and ERrod-like opsins have arisen from their differing photosensory roles and/or genetic drift after the gene duplication event.

CHARACTERISTICS OF THE CIRCADIAN CLOCKS LOCATED IN THE PINEAL ORGAN OF AYU (*PLECOGLOSSUS ALTIVELIS*)

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The pineal organ of teleosts is directly photoreceptive and produces melatonin in a rhythmic fashion. Although the LD cycle is the principal factor regulating melatonin production, intra-pineal circadian clocks are also involved in the regulation of melatonin synthesis in the fish pineal. Recently we have found that the pineal organ of ayu (*Plecoglossus altivelis*) exhibits robust circadian rhythms in melatonin release when maintained in a flow-through whole-organ culture (superfusion) system. In order to extend our knowledge on the circadian clock mechanism in fish, we have characterized several aspects of the circadian clocks located in the ayu pineal organ.

The melatonin rhythm was entrained to LD cycles given *in vitro*: When the pineal was entrained to reversed LD cycles, the phase of free-running rhythms under constant darkness (DD) was approximately 180 degree different from the control culture that is entrained to normal LD cycles. When a 6-h light pulse was applied to the pineal maintained in DD, circadian melatonin rhythms phase-shifted according to a typical light-type phase response curve: The light pulses during the early and late subjective-night induced phase-delay and phase-advance, respectively. While light pulses during the subjective-day were much less effective.

Next, we examined whether circadian clock located in the ayu pineal is temperature-compensated. Temperature changes (5, 10, 15, 20, 25 or 30°C) induced temperature-dependent increase in the amount of melatonin release with the Q_{10} of 1.922. However, the free-running period of the rhythm was temperature-compensated with the Q_{10} of 1.056.

Taken together, the ayu pineal provides an interesting model to analyze the circadian clock mechanism in fish.

CIRCADIAN REGULATION OF DOPAMINE AND MELATONIN CONTENT IN THE EYE OF THE GREEN IGUANA.

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We measured the circadian rhythms of melatonin, dopamine, and the dopamine metabolites DOPAC and HVA *in vivo* in the eye of the green iguana, *Iguana iguana*. We removed eyes from animals at eight different times throughout the day (n=3 for each time point) and subsequently analyzed them with radioimmunoassay (for melatonin) and HPLC. We found a robust rhythm of dopamine and its metabolites under both DD and LD conditions. The peak of retinal dopamine content occurred early in the subjective day, with peaks of metabolites lagging by several hours. Immunohistochemistry labeled dopamine-synthesizing cells predominantly in the inner-nuclear layer. The peak in melatonin content occurred during the subjective night. After the dopamine synthesizing neurons were destroyed by injection of 6-OH-Dopamine in the eye, there was an approximately ten-fold increase in the level of retinal melatonin, which also peaked earlier than in controls. The retinas of the 6-OH-Dopamine treated animals were almost completely depleted of dopamine. Our results suggest that although dopamine can inhibit the production and influence the phase of the melatonin rhythm *in vivo*, it is not necessary for maintaining that rhythm. On the other hand a robust rhythm of retinal melatonin is not sufficient to maintain the circadian ERG rhythm in the absence of dopamine (see "Circadian ERG rhythm in green iguana: effect of melatonin and dopamine" in this session.

Supported by NIMH 56647

CIRCADIAN RHYTHM OF ERG IN GREEN IGUANA: EFFECT OF MELATONIN AND DOPAMINE. Miranda-Anaya M*, Bartell PA, and Menaker M.

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The amplitude of the b-wave of the electroretinogram (ERG) of the green iguana expresses a circadian rhythm with a maximum during daytime in constant darkness (DD) or dim light-dark (LD) cycles. Pinealectomy does not abolish this rhythmicity. Since the retina produces melatonin rhythmically, we asked if retinal melatonin could influence the amplitude of the ERG rhythm and whether dopamine might participate in this regulation. ERGs were obtained in DD from urethane-anesthetized juvenile iguanas using a platinum-iridium electrode located in the vitreous and a reference electrode located subdermally in the front of the head. A 250 ms, 50 μ W cm² light pulse was given each 15 minutes over at least two days. An intraocular 10 μ l-injection of either melatonin (50 μ M) or Quinpirole (50 μ M) was given either at midday or midnight of the second subjective day. The effects of these agents were evaluated over both short and long term. Melatonin decreased the b-wave amplitude by 60% when given at midday and had no effect at midnight. Quinpirole produced a 40 % increase in ERG amplitude when given at midnight, but had no effect at midday. In a second group of animals, circadian variation in b-wave amplitude in 5 of 6 animals were abolished by treating the retina with 6-hydroxydopamine (6-OHDA). This treatment abolishes the retinal rhythm of dopamine but not the rhythm of melatonin (see "Circadian regulation of dopamine and melatonin content in the eye of the green iguana" this session). This suggests that circadian rhythmicity of the ERG depends on the rhythm of retinal dopamine. Supported by NIMH 56647

REGULATION OF THE RHYTHM OF ApC/EBP IN THE EYE OF *APLYSIA*. Samer Hattar, Carla Haramboure and Arnold Eskin. Department of Biology and Biochemistry, University of Houston, Houston, TX 77204.

Levels of the transcription factor, CCAAT enhancer-binding protein (ApC/EBP), mRNA and protein expressed circadian rhythms in *Aplysia* eyes. Therefore, ApC/EBP is controlled by a circadian oscillator in the eye. Levels of ApC/EBP were also controlled by entraining agents, light and 5-HT. Furthermore, analogs of cAMP and cGMP, which mediate effects of light and 5-HT on the rhythm, increased levels of ApC/EBP mRNA. Thus, the rhythm in ApC/EBP could be due to rhythms in levels of cAMP and/or cGMP. Indeed, preliminary results indicate that cAMP and cGMP concentrations expressed circadian rhythms in isolated eyes. The rhythm in cGMP was in phase with the optic nerve impulse rhythm and the rhythm of cAMP was in anti-phase with the optic nerve impulse rhythm. These results suggest that cAMP and cGMP rhythms could account for the rhythm in ApC/EBP as well as the rhythm in nerve impulses. The mRNA for CREB-2 (cAMP/ Ca^{+2} response element binding protein), a putative inhibitor of ApC/EBP, had a diurnal rhythm that peaked when ApC/EBP mRNA levels were low and declined when ApC/EBP mRNA were high. These results indicate that regulation of CREB and ApC/EBP by the second messengers, cAMP and cGMP, may play a central role in the ocular rhythm in *Aplysia* (Supported by NIMH MH41979).

COUPLING OF CIRCADIAN PACEMAKER CELLS IN THE MARINE SNAIL *BULLA GOULDIANA*

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The circadian rhythm of electrical activity generated by the basal retinal neurons (BRNs) in the eye of *Bulla gouldiana* is modulated by efferent inputs from the head ganglia and from the BRNs in the contralateral eye. The central neurons seem to use the peptide FMRFamide as a neurotransmitter in this efferent pathway, and a recent immunocytochemical study suggests that the BRNs may be glutamatergic.

To investigate the role of glutamate for the bilateral synaptic coupling of BRNs, the *Bulla* central nervous system with both eyes attached was placed in a Y-divided petri dish and compound action potentials (CAPs) were recorded simultaneously from both optic nerves. Synchronization of spontaneous CAP-activity was reversibly disrupted during application of the glutamate receptor blocker 6,7-dinitro-quinoxalin-2,3-dione (DNQX, 50 μM) to one eye.

The response to bath application of glutamate to the isolated eye (100 μM) and to BRNs in culture (1 μM) indicates the presence of glutamate receptors on BRNs. Agonist application led to a transient increase in CAP-frequency, a depolarisation of the BRN membrane and a rise in intracellular Ca^{2+} concentration, $[\text{Ca}^{2+}]_i$.

The BRNs can form neuronal networks in cell culture. This can be used as a model for studying coupling mechanisms of pacemaker neurons within the eye. Calcium-imaging of fura-2 loaded BRNs revealed spontaneous oscillations of $[\text{Ca}^{2+}]_i$ presumably correlated with the electrical activity of the cells. Simultaneous recordings of connected BRNs demonstrated synchronous activity, which was reversibly suspended by bath application of the gap-junction blocker carbenoxolone (50 μM). Patch clamp recordings of one BRN in a small network provided further evidence for the presence of gap junctions. Focal application of elevated K^+ to a connected but distant cell, led to immediate changes of membrane potential and induction of inward currents in the recorded BRN. We conclude that BRNs can be coupled by means of gap junctions and glutamatergic synapses.

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BRAIN OPSINS ANTISENSE CONSTRUCTS PREVENT ENTRAINMENT OF LOCOMOTOR RHYTHMS IN PINEALECTOMIZED-RETINECTOMIZED LIZARDS.

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Previous studies in Ruin lizards (*Podarcis sicula*, Lacertidae) showed that combination of pinealectomy (PIN-X) and retinallectomy (RET-X) in the same individual animal does not prevent entrainment of circadian locomotor rhythms to 24h light cycles. A new opsin, with high homology with rhodopsins was cloned from the brain of Ruin lizards. To test whether this brain opsin is involved in photic entrainment, we designed the following experiment: 1. The cDNA of the brain opsin was cloned in a plasmidic expression vector (CS2) containing the cytomegalovirus RNA-polymerase promoter and a polyadenylation site: the cDNA was oriented to produce an anti-sense polyadenilate full-length RNA; 2. Either intact or PIN-X-RET-X Ruin lizards whose locomotor rhythms entrained to a 12:12 LD cycle were subjected to an intracerebral injection of the antisense construct at the level of the third brain ventricle; 3. For control, the same PIN-X-RET-X lizards entrained to a 12:12 LD cycle were subsequently injected with a construct transcribing for the sense RNA (sense construct). A single injection of the anti-sense construct abolished entrainment of circadian locomotor rhythms to the LD cycle in 12 out of 15 PIN-X-RET-X lizards for 6-9 days. Either a single injection of anti-sense construct in intact lizards or injection of sense construct in PIN-X-RET-X lizards did not prevent entrainment to the LD cycle. Altogether, these data allow to conclude that the opsin cloned from the brain of Ruin lizards is expressed in brain photoreceptors which are certainly involved in photic entrainment of circadian rhythms.

MOLECULAR AND GENETIC ANALYSIS OF *DROSOPHILA CLOCK*. Ravi Allada, Myai Emery-Le, Leah Sarov-Blat, Patrick Emery, Michael J. McDonald and Michael Rosbash. NSF Center for Biological Timing, Howard Hughes Medical Institute, Department of Biology, Brandeis University, Waltham, MA 02454.

Clock (*Clk*) RNA cycling has been described but its role in pacemaker function remains unclear. Moreover, our anatomic analysis reveals striking tissue-specific differences: robust RNA cycling in the eyes but not in the central brain, the location of the behavioral pacemaker neurons. In addition, *Clock* protein in whole head extracts cycles very weakly (1.5x). As part of a screen for novel circadian mutants, a new recessive arrhythmic allele of *Clk* was identified, *Clk^{arr}*. *Clk^{arr}* is the first recessive allele of *Clock* to be identified in either flies or mammals. Detailed molecular and behavioral characterization of this mutant will be presented. *Clk^{arr}* can be rescued by *Clk* expression under a heterologous promoter using the GAL4/UAS system. All of these data are consistent with the idea that cycling of *Clock* activity, rather than levels, are necessary for pacemaker function.

To investigate elements important for CLOCK-mediated transcriptional activation, we transfected various deletion mutants into cultured cells. We observe that deletions in the putative activation or DNA-binding domains of CLOCK abolish activation of a *per* enhancer. However, these two deletion mutants can cooperatively activate *per* and *tim* enhancers when cotransfected into tissue culture cells. Using glucocorticoid receptor fusions, we demonstrate that this activation occurs in the presence of cycloheximide and is therefore direct. We propose that CLK can act as a transcriptional coactivator and the assembly of higher-order complexes, rather than changes in CLK level, may be important for high amplitude transcription.

91 DROSOPHILA MALPIGHIAN TUBULES: CIRCADIAN RHYTHMS OF PER AND TIM
IN *cry^b* MUTANTS

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In *Drosophila*, rhythmic expression, pairing, nuclear localization, and degradation of the core clock proteins, PER and TIM, are essential part of the circadian feed back loop. TIM's degradation by light is known to reset the clock; it was suggested that the blue-light photoreceptor, cryptochrome (CRY), is involved in mediating sensitivity of TIM to light. We studied how the CRY deficiency in *cry^b* mutants affects two independent clock systems, the lateral neurons (LNs) in larval brains and the excretory Malpighian tubules (MTs) in adults. In LD, PER and TIM proteins oscillated in the LNs and MTs of *cry^b* mutants with a phase similar to that in wild-type flies; TIM was relatively abundant during the night and significantly reduced during the day. However, light exposures applied at different times of the cycle revealed that the *cry^b* mutation dramatically reduced TIM's sensitivity to light. PER/TIM rhythms in *cry^b* flies were studied in constant darkness (DD). The rhythmic expression of the proteins persisted in LNs. In contrast, their rhythms were considerably perturbed in MTs. The level of nuclear TIM increased around CT18, as expected, but declined after CT20, four hours earlier than in wild-type flies. The pattern of PER expression also revealed more random fluctuations in DD, compared to LD.

Our data indicate that CRY is necessary for the degradation of TIM by light. In addition to its photoreceptor role, CRY appears to be essential for the free-running oscillations of the clock in MTs but not in LNs. Tissue-specific factors may account for the differential effects of *cry^b* in LNs and MTs.

92 mCRY -Inhibits CLOCK:BMAL1 -Activated Transcription Independent of the mPER and mTIM proteins.

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In vitro and *in vivo* data are consistent with a prominent role of the mCRY proteins in negatively regulating CLOCK:BMAL1-mediated transcription. The endogenous expression of clock genes in mammalian cell lines, however, has greatly obscured rigorous *in vitro* analysis of the effects of mCRY proteins on CLOCK:BMAL1-mediated transcription. We have now circumvented this problem by using an insect cell line, Schneider (S2) cells. Since S2 cells express endogenous *cyc*, transfection with *dclock* alone caused a large increase in transcriptional activity, as previously described. This activation was not inhibited by mCRY1 or mCRY2, similar to the lack of transcriptional inhibition found for *Drosophila* CRY (Ceriani *et al.*, 1999). mCLOCK:hBMAL1 and hMOP4:hBMAL1 heterodimers each induced a large increase in transcriptional activity in S2 cells that was inhibited (> 90%) by either mCRY1 or mCRY2. Immunofluorescence of epitope-tagged mCRY1 or mCRY2 expressed in S2 cells showed that each was nuclear in location, identical to their location in mammalian cells. These data indicate that mCRY1 and mCRY2 are nuclear proteins that can each inhibit mCLOCK:hBMAL1-induced transcription independent of the mPER and mTIM proteins and of each other. The results also show that the inhibitory effect is not mediated by the interaction of either mCRY1 or mCRY2 with the E-box itself. It thus appears that the mCRY proteins inhibit mCLOCK:hBMAL1-mediated transcription by interacting with either or both of the transcription factors. Yeast two-hybrid assays revealed strong interactions of each mCRY protein with mCLOCK and hBMAL1. Weaker interactions were detected between each mCRY protein and hMOP4. This is further evidence of functionally relevant associations of each mCRY protein with each of the three transcription factors.

EXAMINING THE ROLE OF *dCLK* mRNA CYCLING IN THE CIRCADIAN OSCILLATOR.

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We recently showed that the circadian oscillator in *Drosophila* is comprised of interlocked feedback loops: a *per/tim* loop that is activated by dCLK-CYC and repressed by PER-TIM, and a *dClk* loop that is repressed by dCLK-CYC and derepressed by PER-TIM. In light of this finding we are currently addressing the following questions:

1) How do dCLK-CYC repress *dClk*? There are two general possibilities. First, dCLK-CYC could repress *dClk* directly (via protein-protein or protein-DNA interactions). Second, they could repress indirectly, via activation of a *dClk* repressor/s (which itself may inhibit *dClk* transcription via protein-protein or protein-DNA interactions).

2) What does *dClk* mRNA cycling contribute to the oscillator? Since the *per/tim* and *dClk* feedback loops are both dependant on the interaction between PER-TIM and dCLK-CYC dimers, the oscillator should still function in the absence of *dClk* mRNA cycling. We believe that a potential role of *dClk* mRNA cycling is to introduce 'yet another' time delay in the oscillator.

To address these questions we are: 1) conducting a molecular analysis of the *dClk* promoter to identify regulatory elements that can drive cyclic transcription, and 2) generating transgenic flies that constitutively express *dClk* mRNA in oscillator cells. The results from these experiments will be presented.

OVEREXPRESSION OF PER DISRUPTS MOLECULAR RHYTHMS WHILE RESCUING BEHAVIORAL RHYTHMICITY IN *DROSOPHILA*

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In *Drosophila melanogaster*, transcription of *period* and *timeless* depends upon the bHLH-PAS transcription factors dCLOCK and CYCLE acting via E-boxes having the consensus sequence CACGTG. PER and TIM proteins repress their own transcription through the inhibition of dCLK/CYC binding at the E-box. In *per*, a critical E-box is located within a 69 base pair sequence known as the circadian regulatory sequence (CRS) that is capable of driving proper developmental, spatial and circadian expression and rescuing rhythmic locomotor activity.

In order to evaluate the effect of high *per* expression levels on the circadian oscillator, transgenic flies were made in which three copies of the CRS were used to drive *per* cDNA from a heatshock basal promoter (3CRS/hs/*cper*). In four independent *per*⁰¹;3CRS/hs/*cper* lines, extremely high levels of both *per* mRNA and PER protein are seen throughout the circadian cycle. Molecular rhythms are disrupted for both *per* and *dClk* genes, although *tim* mRNA and protein cycle with lower amplitude and abundance in LD cycles. In these transgenic flies, endogenous *per* transcription is constitutively repressed. Although molecular rhythms have been compromised in *per*⁰¹;3CRS/hs/*cper* flies, each independent line rescues strong behavioral rhythms having short (~22-23 hr) periods.

Overabundance of PER protein in the nucleus and lower than wild type levels of TIM throughout the circadian cycle suggests the possibility that highly abundant PER protein may enter the nucleus independent of TIM. To test this hypothesis, independent *per*⁰¹;3CRS/hs/*cper* lines were placed in a *tim*⁰ background. These flies are currently being analyzed for behavioral rhythms following either light-dark entrainment or temperature entrainment conditions. In addition, PER nuclear localization and cycling will be analyzed in behaviorally rhythmic *per*⁰¹;tim⁰;3CRS/hs/*cper* flies.

ANALYSIS OF THE *TIMELESS* PROMOTERMichael J. McDonald, Michael Rosbash, and Patrick Emery.

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At the core of the molecular oscillations of the circadian pacemaker is a negative feedback loop involving several important genes: *clock*, *cycle*, *per*, *tim*, and *doubletime*. Current data indicates that CLOCK and CYCLE bind and activate transcription from E-box elements, which control the cycling of *per* and *tim* transcripts. We have undertaken studies designed to investigate the regulation of the *tim* promoter. We have done extensive molecular and behavioral analysis of transgenic lines that harbor several different *tim* promoter constructs. We have extended the analysis to S2 cells using *tim* promoter-luciferase reporter fusions. We confirm that the E-box plays an important role in *tim* transcript cycling, but we have evidence suggesting that it is not the only region important for this cycling. Studies are underway in order to precisely determine the additional element or elements that may contribute to *tim* transcript cycling. These studies should provide important insights into the transcriptional regulation of the *tim* gene.

THE SPLICING FACTOR *DPRP43* IS REQUIRED FOR PROPER CIRCADIAN CLOCK FUNCTION IN *DROSOPHILA MELANOGASTER*Sebastian Martinek, Adrian Rothenfluh, Marla Abodeely, and Michael W. Young

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In a screen for mutations that interfere with the circadian rhythm of locomotor activity in flies, we identified a new mutation, called 2ob9, which lengthens the circadian cycle by approximately 2 hours in a dominant manner. Cloning and sequencing identified this gene as the *Drosophila* homologue of the yeast splicing factor *prp43*.

A phase response curve of the mutant shows that the 2 hour period lengthening can be entirely accounted for by a defect prior to nuclear entry of the PERIOD/TIMELESS heterodimer. Furthermore, TIMELESS protein accumulation is delayed in the circadian cycle of the mutant. These observations are in agreement with the idea, that a splicing defect in the 2ob9 mutant is responsible for the period lengthening.

Overexpression of the mutant cDNA under the control of the *period* promoter using the GAL4/UAS binary expression system resulted in decreased levels of *period* and *timeless* RNA, again in agreement with a defect in RNA processing. The specificity of the 2ob9 phenotype indicates that the circadian clock in *Drosophila* is more sensitive to defects in the function of a general splicing factor. Possible implications of this observation will be discussed.

IDENTIFICATION OF RHYTHMICALLY EXPRESSED GENES IN *DROSOPHILA* BY ENHANCER-TRAP MUTAGENESIS USING A *LUCIFERASE* REPORTER CONSTRUCT.

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*M.V. and T.S. contributed equally to the work presented.

Genetic and molecular studies of the *period* (*per*) and *timeless* (*tim*) genes have shown that both are essential components of the *Drosophila* circadian clock. PERIOD and TIMELESS exhibit phase-dependent interactions with the CLOCK/CYC heterodimer, giving rise to two interlocked feedback loops within the central oscillator.

In addition, these feedback loops are thought to control clock output processes by regulating rhythmic expression of downstream clock controlled genes (*cogs*). So far, three rhythmically expressed, putative *cogs* have been isolated in *Drosophila* (*Dreg-5*, *crg-1* and *vriille*), using approaches that depended on extracting RNA from flies. Expression of these genes continues to oscillate under constant conditions and becomes arrhythmic in the absence of *per* or *tim* gene products.

To search efficiently for rhythmically expressed genes that might function in the clock-output pathway or at the level of the central pacemaker (i.e. genes that interact with *per* and/or *tim*), we developed a genetic screen based on the mobilization of a transposable element containing the firefly *luciferase* cDNA. This P-element has been mobilized from the X-chromosome to the autosomes. The new insertion lines were screened for rhythmic bioluminescence under LD conditions, which indicates that the element is now under the control of an enhancer regulating rhythmic gene expression.

Out of 1200 lines tested ca. 30 lines show robust circadian oscillations with an amplitude comparable to that of a *per-luciferase* reporter strain (whereas the phase can differ by several hours). Expression in roughly 70% of the strains continues to be rhythmic under constant conditions. In the same lines it becomes arrhythmic or phase-altered depending on different *per* and *tim* mutant backgrounds, indicating that the transgenes' expression is clock-controlled.

22 of these lines are currently being analyzed histologically as well as molecularly. Cytological map positions have been determined via *in situ* hybridization on salivary glands. Spatial expression patterns are being examined by antibody stainings on cryosections with an antibody raised against the *luciferase* protein.

In order to obtain information about the genes flanking the P-element insertion sites, genomic DNA sequences surrounding each P-element have been isolated. 17 transcription units have been identified this way and are now being tested for cycling expression at both the RNA and the protein level. RNase protection assays showed that a subset of the candidate genes indeed cycle on the RNA level, proving the validity of our experimental approach. However, the fact that for the majority of the genes we did not detect RNA cycling suggests that rhythmic initiation of transcription does not necessarily lead to detectable fluctuations of the overall mRNA abundance. Implications of this finding will be discussed in the poster.

Future plans include the molecular, behavioral and physiological analysis of known or newly created mutations of the genes under examination. In parallel, we will study effects of artificially induced constitutive expression of the usually rhythmically expressed genes. Both approaches should lead to insights about the roles these genes play in the circadian system of *Drosophila*.

SEXUAL CLOCKS: THE INVOLVEMENT OF CLOCK GENES IN REPRODUCTION OF *DROSOPHILA MELANOGASTER*

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While most studies on clock genes have focused on their expression in the head, it has been shown that clock genes are widely expressed in peripheral tissues implying that they perform important functions in their respective organs. A peripheral organ known to exhibit robust circadian rhythms is moth testis-vas deferens complex. These complexes harbor *period*-based circadian clocks controlling rhythmic release of sperm. When male moths are exposed to constant light, they are rendered sterile due to disruption of reproductive rhythms. To further understand how specific clock genes affect reproduction we turned to *Drosophila*. We utilized flies with mutated *period* (*per*⁰) and *timeless* (*tim*⁰) genes to examine the function of each gene in reproduction of *D. melanogaster*.

Wild type (wt) and clock-mutant females were mated once with the same strain male. We observed the length of time that each pair was *in copula* and found that clock-mutants mated for a significantly longer time. The mated females were then allowed to oviposit for 4 days. The resultant progeny from these single matings were counted in three categories, larva, sterile eggs, and embryos. Both *per*⁰ and *tim*⁰ couples had significantly lower progeny count than wt flies. Lower fertility is due to two factors, slower oviposition rates, and higher sterility among laid eggs. Those trends were lost when pairs of flies remained together for the 4 days such that mating *ad libitum* could occur. In this case, the fertility of *per*⁰ and *tim*⁰ mutant pairs approached the level of reproductive success in wt flies.

Crosses between clock-mutants and wt flies demonstrate that both sexes are accountable for lower reproductive success. Clock-mutant males are responsible for the extended length of mating while the clock-mutant females show slower rate of oviposition.

EXPRESSION OF *PERIOD* GENE IN THE REPRODUCTIVE SYSTEM OF COTTON LEAFWORM, *SPODOPTERA LITTORALIS*

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The mechanisms of circadian clocks involve rhythmic expression of the *period* (*per*) gene. Recent studies demonstrated widespread expression of *per* in tissues indicating that circadian clocks resides not only in the brain but also in non-innervated peripheral tissues. The presence of autonomous and photosensitive circadian clock was demonstrated in the male reproductive tract of several moth species; the clock mechanism controls the release of sperm bundles from the testes to the vas deferens and provide a temporal framework for sperm maturation. To gain further insight into the role of circadian clocks in sperm release and maturation, we study reproductive rhythms of the cotton leafworm, *Spodoptera littoralis*. This moth has well defined rhythms of sperm release in vivo and in vitro, in isolated testis-vas deferens complexes. In order to investigate the involvement of *per* gene in the reproductive rhythms, we amplified a fragment of *per* from *S. littoralis* based on the strategy that was used for *per* amplification in other lepidopterous species. We are studying patterns of *per* mRNA expression in testis-vas deferens complexes in different light-dark conditions in relation to patterns of sperm release. To determine whether *per* is directly involved in sperm release rhythms, we are also exploring methods to interfere with *per* expression in the reproductive system of *S. littoralis*. Our research may help to understand links between the *per* gene and robust physiological rhythms associated with sperm release and maturation in moths.

ANATOMY AND PHYSIOLOGY OF NEURONS OF THE CIRCADIAN PACEMAKER OF AN INSECT.

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Lesion and transplantation experiments strongly suggest that the accessory medulla in the optic lobe of the cockroach *Leucophaea maderae* is the pacemaker for the circadian control of locomotor activity (Stengl and Homberg 1994, J Comp Physiol A 175:203-213; Reischig and Stengl 1998, in: Elsner N, Wehner R (eds) Göttingen Neurobiology Report 1998, Thieme, Stuttgart, p. 267). In this study, we have analyzed the anatomy and physiology of neurons of the accessory medulla of the cockroach with particular reference to photic entrainment pathways and outputs of the clock.

Neurons which connect the lamina and medulla to the accessory medulla showed strong responses to light stimuli, and might, therefore, act in photic entrainment of the clock. Interneurons with additional projections to the midbrain, however, hardly responded to light during the day and are, therefore, candidates for outputs of the clock. A third category of neurons interconnected both optic lobes including the accessory medullae. These neurons were sensitive to polarized light and might require a circadian input for time compensation in polarized-light orientation.

Immunocytochemical studies together with the single-cell recordings suggest multiple neuronal pathways from photoreceptors to the accessory medulla. Histamine-immunoreactive photoreceptors of the compound eye terminated in the lamina or medulla, but not in the accessory medulla. GABA-immunostaining revealed a prominent connection between the medulla and the accessory medulla. Finally, putatively extraretinal photoreceptor organs near the lamina and accessory medulla (lamina organ, lobula organ) showed immunostaining with an antiserum against *Arabidopsis* cryptochrome.

The data suggest that photoreceptors of the compound eye and of two extraretinal photoreceptor organs might cooperate in light entrainment of the cockroach circadian clock. Input from the compound eye to the accessory medulla is indirect and probably mediated through several parallel pathways. Supported by DFG grant Ho 950/9.

CRYPTOCHROME-IMMUNOREACTIVITY REVEALS HOMOLOGOUS EXTRARETINAL PHOTORECEPTOR SYSTEMS IN COCKROACHES AND BEETLES

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Two novel putative extraretinal photoreceptor organs in the optic lobes of cockroaches are described by various light- and electron microscopic methods. The lamina organ (about 400 µm long, 40 µm wide) distal to the first optic chiasm, and the lobula organ (about 150 µm long, 35 µm wide) proximal to the second optic chiasm are both composed of the same type of receptor cells. The cell bodies are tightly packed and arranged in a closed or open circle. They have long protrusions with a microvillar seam forming a rhabdom-like structure in the central lumen of the organ. Specialised cells give rise to electron-dense microvilli which partly join to a lamellated stick perpendicular to the rhabdom-like microvilli. This lamellated stick matches the structure showing strong immunoreactivity with an antibody against an *Arabidopsis* cryptochrome2. The organ cells give rise to axonal processes and receive multiple neurosecretory efferent input. Structural features, site and size of these organs suggest that they are homologous to similar organs in beetles. They may serve as photic Zeitgeber receptors in the circadian clock system.

Melatonin as a candidate messenger in the output pathway of circadian / photoperiodic clock in *Antheraea pernyi*.

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We investigated the localization of immunohistochemical reactivity to antisera against clock-associated proteins and melatonin-synthesizing enzymes in the brain of the silkworm, *Antheraea pernyi*. Antisera against *Periplaneta americana* PERIOD (PamPER), *Drosophila melanogaster* arylalkylamine-*N*-acetyltransferase (DmaANAT) and *Bombyx mori* DOUBLE-TIME (BmDBT) were raised by against GST-fusion proteins as antigens. Approx. 16 cells showed immunohistochemical reactivity against these antisera. Their colocalization was 100%. Positive cells seen to be identical to those published by Sauman and Reppert (1996) for anti-*Antheraea* and *Drosophila* PER reactivity. Moreover melatonin and hydroxyindole-*O*-methyltransferase (HIOMT) that was one of the melatonin-synthesizing enzymes, also exist in these cells. These indicate that melatonin is synthesized in the clock cells and used as a messenger for clock output pathway in *A. pernyi*.

103 A PHASE-SPECIFIC CIRCADIAN RESPONSE ELEMENT

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A critical function of the circadian clock is temporal partitioning of specific activities. Activities can be partitioned over a developmental time course, or over the daily cycle of 24 hours. It is well established that the mRNA abundance of a number of genes oscillates in a circadian fashion and that maximal mRNA abundance is regulated, or phased, to specific times of day by the circadian clock. For example, the mRNA abundance of the *CAT2* and *CAT3* catalase genes and the *RCA* (rubisco activase) gene is maximal at dawn, dusk and midday, respectively. The differential circadian regulated phasing of *CAT2*, *CAT3* and *RCA* mRNA abundance suggests that these genes contain distinct regulatory sequences that confer responsiveness to the circadian clock. Using both loss of function and gain of function assays, a 100 bp region of the *CAT3* promoter has been isolated that is necessary and sufficient to confer evening-specific circadian rhythmicity to luciferase. Within the 100 bp region is an element conserved in both *CAT2* and *RCA* promoters, and present in a 36 bp region of the chlorophyll a/b binding protein (*CAB2*) promoter that has been shown sufficient to confer mid-morning specific circadian transcription. Our efforts focus on isolating the specific cis-acting element(s) that confer evening-specific circadian phase to the *CAT3* promoter. This work has been supported by grants through the National Science Foundation (IBN-9817603) and from the United States Department of Agriculture (9602632).

104 INTERACTION OF THE PHYB AND CRY1 PHOTORECEPTORS AND DETERMINATION OF CIRCADIAN PHASE

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Multiple photoreceptors regulate *Arabidopsis* development from seedling to adult plant. Functional interactions have been shown between the blue-light photoreceptor CRY1 (CRYPTOCHROME 1) and the red/far-red photoreceptors PHYA, PHYB and PHYD (PHYTOCHROMES A, B and D). Although a direct interaction between PHYA and CRY1 has been demonstrated in vitro (Ahmad *et al.*, 1998, Mol. Cell 1, 939), our knowledge of the interplay between phytochrome and cryptochrome signaling pathways remains incomplete. A screen designed to identify mutants with altered circadian rhythmicity yielded two mutants, *oop1* and *oop2* (*out-of-phase*), in which circadian phase was altered. *oop1* and *oop2* show defects in PHYB- and CRY1-mediated hypocotyl elongation inhibition, respectively, and represent new dominant-negative alleles of *PHYB* (for *oop1*) and *CRY1* (for *oop2*). Interestingly, *oop1* affects signaling through CRY1 in a red-light dependent manner, and in some conditions behaves like a weak *phyB cry1* double mutant, while *oop2* may conditionally alter PHYA and PHYB signaling. Together, these alleles provide evidence that PHYA, PHYB and CRY1 may act in concert to gate rhythms to the proper time of day. The effect of these mutations on the phase of the circadian clock will be discussed. We also present progress toward the identification of *CTD2*, a novel locus defined by the long-period mutant *ctd2* (*circadian timing defective*), which defines a phytochrome-specific input component to the circadian clock. This work was supported by a grant from the National Science Foundation (MCB 9723482).

CALCIUM OSCILLATIONS IN PLANT CELLS AND CHLOROPLASTS

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By using tobacco (*N. tabacum*) carrying the transgene for the calcium photoprotein aequorin, the cytosolic free Ca^{2+} in plant cells was found to oscillate with a robust circadian rhythm. A model that proposed cytosolic Ca^{2+} increases as a regulator of the expression of *Lhcb* gene during light-regulated development implied that the Ca^{2+} oscillation might be the intracellular signal that drives rhythmic *Lhcb* gene expression. We tested this hypothesis by comparing the rhythm monitored by aequorin luminescence and the *LHCb1*1* (Light Harvesting Complex gene) promoter activity rhythm monitored by luciferase luminescence. We found that these rhythms free-run with different periods in tobacco seedlings in constant conditions. Moreover, robust oscillations of *Lhcb* promoter activity continued in undifferentiated tobacco calli in the absence of Ca^{2+} oscillations. Therefore, the calcium rhythm is not responsible for driving the rhythm of *Lhcb* expression.

In another strain of tobacco (*N. plumbaginifolia*) in which the aequorin transgene was targeted to the chloroplastic stroma, the free Ca^{2+} in chloroplasts did not show any circadian oscillation in constant light. However, a big sudden increase of Ca^{2+} was always seen about 20 min after each LD transition and the magnitude of this Ca^{2+} burst increased with the duration of the prior exposure of the seedlings to light. Preliminary studies indicate that blue, green, and red light can all inhibit the Ca^{2+} spike in the chloroplast.

MOLECULAR CLONING OF THE *TOC1* LOCUS IN ARABIDOPSIS

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Many physiological processes in plants show daily rhythms. Some of them are obviously driven by an internal circadian clock. In higher plants, leaf movement, stomatal rhythms, and expression of clock-controlled genes have been profoundly analyzed. Recent molecular genetic approaches using Arabidopsis have gradually revealed the molecular basis of the circadian system in plants. The Arabidopsis *toc1* mutant was isolated with a shorter period of *cab2:luciferase* expression, and further analyses indicated that all circadian-regulated phenomena tested also showed shorter periods than wild type. The phenotype of *toc1* was influenced neither by light quality nor by light intensity, indicating *TOC1* acts outside the light input. These results strongly suggested that *TOC1* is a major component of the primary oscillator of the circadian clock. In order to dissect the molecular function of *TOC1*, we have performed chromosomal walking and recently succeeded the molecular cloning of this gene. The protein structure, the expression pattern, and subcellular localization of this gene product will be discussed.

107 .INTERACTION BETWEEN THE BLUE AND RED-LIGHT SENSING SYSTEMS IN PLANTS.

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A wide variety of photomorphogenic and circadian responses in plants are mediated by two major photosensory receptors: phytochromes and cryptochromes. With these two sets of photoreceptors, plants are able to maximize their responses throughout the full spectrum of visible light. In our study, we have used cellular and molecular approaches to dissect the possible interaction between the phytochrome and cryptochrome signaling pathways in plants. The use of confocal microscopy of GFP fusion proteins allowed us to compare the subcellular localization of various photoreceptors. We have used different light treatments to analyze the changes in protein distribution and to determine the sites of action of each photoreceptor in the cell. Physiological data obtained with different *Arabidopsis* photoreceptor mutants has corroborated this intimate communication between the blue and red-light sensing pathways in plants.

108 EFFECTS OF MELATONIN AND AUXIN ADMINISTRATION ON GROWTH IN ARABIDOPSIS THALIANA AND COLEUS AND ON TESTICULAR DEVELOPMENT IN RICE RATS. Kent Edmonds, Nick Riggs, and Riki Spurrier, Department of Biology, Indiana University Southeast, New Albany, IN. 47150.

To examine whether melatonin and auxin (indoleacetic acid) administration affect growth in *Arabidopsis thaliana*, plants were exposed to 14L:10D and, from the time of germination, watered daily either 1.5 hours after lights on or 1.5 hours before lights off with 2 ml of ethanolic water or melatonin in concentrations consisting of 1×10^{-3} M, 1×10^{-4} M, 1×10^{-5} M, or 1×10^{-6} M. Growth was significantly inhibited by 1×10^{-3} M melatonin, but not by the other concentrations. There was no effect of time of administration on growth, possibly because the saturated soil caused a continuous exposure to melatonin despite daily watering at a specific time. Additional plants treated with concentrations between 1×10^{-3} M and 1×10^{-4} M melatonin had varying degrees of growth, suggesting a dose response curve for growth between 1×10^{-3} M and 1×10^{-4} M. Time of administration did affect growth in this study. Daily auxin treatment completely inhibited growth at 1×10^{-3} M, but dramatically stimulated growth at 1×10^{-5} M. Melatonin, applied in lanolin to the apex of cut *Coleus* plants, did not produce a phototropic response whereas an auxin-lanolin treatment did. This suggests that melatonin fails to act as an auxin agonist in plants. To examine the effects of melatonin and auxin implants in mammals, intact juvenile rice rats were transferred from 16L:8D to 12L:12D at 3 weeks of age and implanted with subcutaneous 20 mm implants from 3-7 weeks of age. Because melatonin implants in this paradigm block the short photoperiod-induced inhibition of reproductive development, we were interested in examining whether auxin could act like a melatonin agonist. Melatonin implants in animals housed on 12L:12D blocked the short photoperiod-induced inhibition of reproductive and Harderian gland development, but auxin did not. Auxin-treated animals housed on 12L:12D were inhibited in their development as were control animals. These results suggest that melatonin can influence both plant and animal development, but auxin cannot affect mammalian development. (Supported by IUS and NSF Grant IBN-9812824 to KE).

Distribution of Melatonin in the Plant Kingdom

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The neurohormone melatonin was first discovered in 1958. Since then scientists investigated the presence and function of this molecule in animals and man. Today we know that melatonin is present in all organs and body fluids. In some of them melatonin exhibits a circadian rhythm and represents an important role in hormonal regulation.

Between 1969 and 1973 two scientific papers were published about the action of exogenous melatonin on plant cells, but it took more than 30 years after the discovery of animal melatonin until the presence of melatonin in plant material was discovered. Today we know that melatonin is ubiquitously distributed in the plant kingdom. It is found in different parts of plants like root, stem, leaves, flowers, fruits and seed. As expected, there seems to be a great variability in melatonin concentration within and between plants. So far melatonin was estimated from not detectable to amounts of 500ng/100g fresh weight. Although further studies have to be done to determine the exact amount of this hormone in plant material, the main areas of research should be to obtain information about the role of melatonin for plants themselves. For example hormonal regulation of the day/night cycle, protection against free radicals as well as the beneficial action of nutritional plant melatonin for animals and man and especially the role of plant melatonin in preventive medicine.

Identification and characterization of *Neurospora crassa* casein kinase I ϵ , a homologue of *Drosophila melanogaster* double-time (DBT)

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Frequency (FRQ) is the central element of an auto-regulatory negative feedback loop of the circadian clock of *Neurospora crassa*. During the course of a day FRQ becomes successively phosphorylated. Phosphorylation of FRQ determines its turnover and affects the length of the circadian period of *Neurospora* (Liu et al. 2000). Similarly, phosphorylation of the *Drosophila* period protein (PER) by the casein kinase I ϵ homologue double-time (DBT) affects PER stability and the length of the circadian period (Kloss et al., 1998). The casein kinase I (CKI) family comprises two major subgroups that regulate cytoplasmic and nuclear processes, respectively. Mammalian members of the nuclear casein kinases are the closely related CK I ϵ and CK I δ . A search in the *Neurospora* EST databases revealed two sequences which encode putative homologues of CK I ϵ and CK I δ . *Neurospora* CK I ϵ shares significant sequence similarity with *Drosophila* DBT. We have cloned and expressed *Neurospora* CK I ϵ in *E. coli*. Recombinant *Neurospora* CK I ϵ is a monomeric protein with an apparent molecular mass of 39 kDa. The purified protein auto-phosphorylates and is active with β -casein as a substrate.

- 111** LIGHT RESPONSIVE ELEMENTS WITHIN THE *FREQUENCY* PROMOTER AFFECT BOTH PHASE AND RHYTHMICITY OF THE *NEUROSPORA CRASSA* CIRCADIAN CLOCK. Allan Froehlich, Jennifer Loros, and Jay Dunlap. Department of Genetics, Dartmouth Medical School, Hanover, NH 03755, USA.

The *frequency* (*frq*) locus is a key component of the *Neurospora crassa* circadian clock. Under constant environmental conditions, *frq* mRNA and protein oscillate with an approximate 22 hour period; these oscillations are part of an autoregulatory feedback loop in which *frq* gives rise to two forms of the FRQ protein which then act to repress the levels of *frq* transcript (Dunlap, J. Cell 96:271-290, 1999). Evidence gathered to date suggests that resetting of the clock by light occurs through the rapid induction of *frq* (Crosthwaite *et al.* Cell 81:1003-1012, 1995).

Resection of the *frq* promoter has uncovered two *cis*-acting light response elements (LREs), both of which are necessary for light induction of *frq* to wild type levels. Using a heterologous reporter, both LREs have been shown to be sufficient for light induction. Deletion of the proximal LRE affects phase following a light to dark transfer, but does not have an effect upon resetting following temperature treatments. Deletion of the distal LRE results in arrhythmia under both light and temperature treatments. The phenotypes of both LRE deletions can be observed at the physiological level as well as at the molecular level.

White-collar loci products (*wc-1 wc-2*) have been found to act as global regulators for light perception and as positive components of the circadian clock in *Neurospora* (Crosthwaite *et al.* Science 276:763-769, 1997). WC-1 and WC-2 proteins both contain Zn-finger domains with distinct similarity to other transcriptional activators within the GATA factor family. Using electrophoretic mobility shift assays, WC-1 and WC-2 have been shown to specifically bind to the *frq* LREs. Currently, work is under way to investigate the possible role of FRQ in WC-1/WC-2 binding of the *frq* LREs.

- 112** TEMPERATURE-DEPENDENT ALTERNATIVE SPLICING AFFECTS THE TYPE OF FRQ PROTEIN SYNTHESIZED IN *NEUROSPORA*

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FRQ is a central component of the circadian clock in *Neurospora*. Two forms of the protein (LFRQ and SFRQ) result from translational initiation at different ATGs. While each form of FRQ alone suffices for a functional clock at some temperatures, the presence of both proteins is necessary for robust rhythmicity over the physiological range of the organism. We previously observed that temperature regulates the ratio of SFRQ to LFRQ and we proposed that the findings reflect a temperature-dependent choice of the initiating ATG.

In the present work, we show that there are several forms of the *frq* transcript, arising from alternative splicing of two introns, a large one (i1) found in the 5'UTR and a small one (i2) encompassing the first ATG. Splicing of i2 removes the first ATG and leaves the second ATG intact, thereby resulting in the synthesis of only SFRQ. It is possible, therefore, that: 1) translation initiation at the downstream ATG only occurs when the first ATG is spliced out; and 2) the temperature-dependent ratio of the proteins results from temperature dependence of the splicing of i2. To test these possibilities, we mutagenized the 5' splice site of i2; this results in exclusive use of the first ATG in vivo. In addition, we undertook semi-quantitative RT-PCR studies, which demonstrate that the relative degree of splicing of i2 does indeed change with temperature in the predicted fashion. However, splicing does not appear to account completely for the temperature dependence of FRQ choice. Studies are under way to determine the extent to which translational control also plays a part in establishing the SFRQ to LFRQ ratio. Furthermore, transformation of a *frq*-null strain with cDNA constructs missing i1 from the 5'UTR does not abolish rhythms but results in aberrant periods and banding patterns on race tubes. We are attempting to understand the role that the presence or splicing of the 5'UTR intron (i1) plays in proper clock functioning.

FLO: CHARACTERIZATION OF THE *FREQUENCY*-INDEPENDENT OSCILLATOR IN *NEUROSPORA CRASSA*

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The *frq*/FRQ/WC feedback loop is an example of the transcription/translation PAS-heterodimer type feedback loop common to circadian systems in eukaryotes; it has been shown to be central to the function of the circadian clock in *Neurospora*. FRQ levels cycle with a period identical to that of the *Neurospora* circadian cycle and its expression is rapidly induced by light. Continuous light suppresses *frq* mRNA and protein cycling and dampens the circadian rhythm.

The original description of a *frq*-null strain (Loros *et al.*, 1986) included a description of oscillations in conidial banding that occasionally appeared following 3 to 7 days of arrhythmic growth. These oscillations were re-confirmed as present in *frq*-null strains when definitive *frq*-nulls were described by Aronson *et al.* (1996), and more recently by Merrow *et al.* (1999). Because no genes or gene products are yet identified that are uniquely associated with this oscillator, and because possible entraining cues such as temperature or nutritional changes will not be unique to this oscillator, we have settled on the term FLO, for FRQ-Less Oscillator, as the least ambiguous name. We have repeated and extended the previously reported observations concerning the FLO (referenced above). Unlike the intact clock, FLO is sensitive to media composition with the period length dependent on different nitrogen and carbon sources as well as concentration. FLO is insensitive to light, reiterating the role of *frq* in the interaction of light and circadian rhythmicity.

The most striking characteristic of FLO is the dependence of period length on temperature, with periods as long as 35h at 18°C and as short as 14h at 30°C. Even though period length is not temperature compensated in FLO and it is not entrainable by light pulses, this oscillator seems to retain the ability to entrain to temperature pulses. We further describe the approach taken to identify components of FLO and present possible models for how different oscillators could be coupled in *N. crassa* to assemble a functional circadian clock.

Identification of factors that regulate circadian rhythmicity of the clock-controlled *eas(ccg-2)* gene in *Neurospora crassa*. Zachary Lewis and Deborah Bell-Pedersen, Department of Biology, Texas A&M University

Viewed simply, the circadian clock is comprised of at least three components; a central oscillator, input pathways to and output pathways from the oscillator. The central oscillator regulates the expression of genes within the output pathways in a time-of-day-specific manner. However, the mechanisms and components responsible for the regulation of clock-controlled gene expression are poorly understood. In the filamentous fungus *Neurospora crassa*, several clock-controlled genes have been identified and characterized. We have identified a 68 bp promoter sequence, termed the ACE element, that is both necessary and sufficient for circadian expression of the clock-controlled *eas(ccg-2)* gene. Using gel mobility shift assays, we have identified protein factors that specifically bind to this sequence. Consistent with the factors being involved in clock-regulation of *eas(ccg-2)*, binding to ACE occurs with a circadian period. Currently, we are purifying the factors and, once isolated, we will determine their role in circadian output pathways.

Control of Conidial Development by the Circadian Clock in *Neurospora crassa*. Alejandro Correa and Deborah Bell-Pedersen Department of Biology, Texas A&M University

The *Neurospora crassa* clock regulates the timing of asexual spore development (conidiation). Several environmental signals can initiate conidiation in *Neurospora*, including light, air, carbon and nitrogen starvation; however the biological clock provides the only known endogenous signal to initiate conidiation. Three key regulators of the developmental pathway are known and include *acon-2*, *fl* and *acon-3*. Different combinations of the regulators are thought to control the expression of downstream conidiation specific genes (including *con-10*, *con-6* and *eas(ccg-2)*). A model for how these genes regulate development is currently available. To begin to understand how the clock regulates development we are investigating what genes within the developmental pathway are under circadian clock control. Our results suggest that the clock impinges early in the developmental pathway and that clock regulation of *eas(ccg-2)* is independent of the developmental regulators. In a separate study, we are using transcriptional profiling to help us understand the mechanisms by which the clock regulates development and others critical cellular events. *N. crassa* DNA microarrays are being constructed and probed with cDNA produced from mRNA obtained from cultures harvested at different times of the day. Results from these analyses will allow us to identify all the genes under circadian control at the level of transcript accumulation.

Demonstration of Circadian Rhythms of Development in *Aspergillus flavus* Andrew Greene^{1,2}, Nancy Keller^{1,3}, and Deborah Bell-Pedersen^{1,2}. ¹Program For the Biology of Filamentous Fungi, ²Department of Biology, ³Department of Plant Pathology and Microbiology, Texas A&M University, College Station, TX 77843

Endogenous circadian oscillators have been described in a variety of organisms ranging in complexity from cyanobacteria to humans. The filamentous fungi *Neurospora* and *Aspergillus* are ideal organisms in which to study the molecular basis of the circadian clock because of their well-developed genetics and ease of manipulation in the laboratory. Furthermore, in *Neurospora*, the circadian clock controls asexual spore production, providing an easy means to assay rhythmicity. Most studies of circadian rhythms in fungi have been carried out in *Neurospora*; no rhythms in *Aspergillus* have been reported. Unlike *Neurospora*, *Aspergillus* is of extreme agricultural importance because of its production of the potent carcinogen aflatoxin, which causes billions of dollars of crop losses each year. Previous studies have demonstrated that development and toxin production are closely associated. Therefore, our goals are to develop *Aspergillus* as a model system for studies of circadian rhythms and to investigate the link between the circadian clock, development and toxin production. As a first step towards these goals, we have established the presence of a circadian rhythm in overwintering body (sclerotia) production in *Aspergillus flavus*. Several approaches are currently underway to investigate the circadian clock in *Aspergillus* and these will be presented.

Circadian rhythms in abundance of Kai proteins and phosphorylation of KaiC in cyanobacteria

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A gene cluster *kaiABC* encodes central circadian clock components in the cyanobacterium *Synechococcus* sp. PCC 7942. In this study, the circadian profiles of clock proteins KaiA, KaiB and KaiC were examined. The accumulation levels of both KaiB and KaiC showed circadian cycling with peaks at CT 16. The protein rhythms lagged behind the *kaiBC* mRNA oscillation by about 8 hours.

We also found phosphorylation of KaiC *in vivo* and its circadian cycling with the same phase as KaiC accumulation. The circadian rhythms in abundance and phosphorylation of KaiC were eliminated by *kaiC* mutations that caused arrhythmia in *kaiBC* expression. These results suggest that phosphorylation of KaiC plays an important role in circadian system of cyanobacteria.

Moreover, immuno-electron microscopic analysis revealed KaiC localized both in thylakoid and karyoid regions of the cell. In contrast to many eukaryotic clock proteins, circadian change in KaiC's subcellular localization was not found in our experimental conditions.

Genetic and Biochemical Crosstalk among Clock Proteins in Cyanobacteria

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The KaiABC proteins play central roles for circadian timing in cyanobacteria. In addition, we have identified and characterized a KaiC-interacting sensory histidine kinase, SasA (in collaboration with S. Williams and S. Golden, Texas A&M Univ.; Iwasaki, Williams *et al.*, submitted).

Although KaiA and KaiC have been proposed as positive and negative elements for *kaiBC* expression, respectively, detailed molecular actions of the Kai proteins still remain obscure. We are currently analyzing levels of the *kai* gene expression and Kai protein accumulation in various *kai* and *sasA* genotypes. Biochemical properties of the Kai protein complexes are also being investigated. The results are providing novel insights on biochemical roles of the clock-related proteins in cyanobacterial circadian system.

REGULATION OF CIRCADIAN TIMEKEEPING IN *SYNECHOCOCCUS* SP. STRAIN PCC7942

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Organisms at all levels of biological complexity manifest circadian (daily) rhythms of gene expression that are controlled by an endogenous oscillator, or circadian pacemaker. Central to models for animal and fungal circadian clock timing are negative feedback loops involving clock genes that negatively regulate their own expression (for example *per*, *tim*, *frq*), and positive effector proteins that stimulate clock gene expression (*Clk*, *bmal*, *wc-2*). The single-celled cyanobacterium, *Synechococcus* sp. strain PCC7942, has a circadian pacemaker comprised of the products of at least three genes, *kaiA*, *kaiB*, and *kaiC*. The *kai* locus is expressed from two promoters, one upstream of *kaiA* (monocistronic message) and one upstream of *kaiB* (dicistronic *kaiBC* message), that are expressed in the same circadian phase in wild-type cells. Previous work has shown that KaiA is required for expression from the *kaiBC* promoter, and that overexpression of *kaiA* enhances expression from *kaiBC*, suggesting that KaiA is a positive activator of the *kaiBC* promoter. KaiC is required for normal levels of expression from its own promoter; however, overexpression of *kaiC* blocks expression from *kaiBC*, suggesting a role in negative autoregulation. These data were interpreted to be consistent with the animal and fungal circadian timing models. However, we have identified mutants of *Synechococcus* that change the phase relationship between *kaiA* and *kaiBC* expression without disrupting circadian timing, suggesting that the relative transcriptional activity of expression from the *kaiA* and *kaiBC* promoters is not important for generating circadian rhythms. To investigate the role of transcriptional regulation of the *kai* gene locus for circadian timekeeping in *Synechococcus*, we will express each individual *kai* gene from heterologous promoters. Bypassing the natural transcriptional regulation of the *kai* genes will help determine if the circadian clock is still functional. As a step towards accomplishing this goal, a series of in-frame deletions was constructed to remove each single *kai* gene to minimize the possibility of disturbing transcription or translation of downstream genes. Independent in-frame deletions in *kaiA*, *kaiB*, and *kaiC* result in arrhythmic phenotypes as measured by bioluminescence from a *kaiB::luc* reporter. Each wild-type *kai* allele with its native promoter has been cloned and inserted into a neutral site within the chromosome of each respective *kai* deletion strain for complementation. Additional constructs in which each *kai* gene is expressed from heterologous promoters that change phase information or promoter strength will indicate whether transcriptional timing and recognition of specific *cis* elements of the *kai* genes are important for circadian timekeeping in cyanobacteria.

ARS2 AS A QUANTITATIVE REPORTER AT THE ENZYME ACTIVITY LEVEL IN *CHLAMYDOMONAS* MUTANT SCREENS

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The *CABII-1* gene (also called *lhcb1*) in *Chlamydomonas reinhardtii* shows a circadian rhythm of expression at the transcriptional level, since the *Chlamydomonas* gene *ARS2* exhibits the same circadian rhythm of mRNA abundance as *CABII-1* when transcriptionally fused to *CABII-1* upstream sequences. *ARS2* encodes the enzyme arylsulfatase which is excreted into the medium. Time resolution of *ARS2* at the enzyme activity level is not high enough to report changes within a circadian cycle due to the long half-life of the protein. Instead, we tested the usefulness of *ARS2* as a quantitative reporter at the enzyme activity level when screening for mutants in accumulative expression over several circadian cycles. *ARS2* expression in our transformant Carni1 (available as CC3671 from the Chlamydomonas Genetics Center) proved stable enough for such a screen, since three single-colony isolates propagated separately for more than 4 years are statistically indistinguishable in their accumulative arylsulfatase activity pattern from the parent Carni1. After insertional mutagenesis with the *ble* marker, 1004 colonies were screened by analyzing a single sample from liquid culture under constant conditions. The optical density of this sample at 750 nm was compared to a standard curve of untransformed Carni1 to determine the amount of "wild-type" enzyme activity correlated with the culture density. Ten transformants were isolated with significantly reduced arylsulfatase activity. We are currently in the process of evaluating their mutant phenotypes.

DAILY VARIATION IN SURVIVAL FROM ULTRAVIOLET RADIATION IN *CHLAMYDOMONAS*: THE ESCAPE FROM LIGHT HYPOTHESIS. Selene S. Nikaido and Carl Hirschbie Johnson. Department of Biology, Vanderbilt University, Nashville, TN 37235.

Organisms use a variety of strategies to protect themselves from the deleterious ultraviolet (UV) wavelengths of sunlight. Among those strategies is the timing of UV-sensitive cellular processes to the night to avoid UV-induced damage. The use of this strategy was tested in *Chlamydomonas reinhardtii* by measuring cell survival following exposure to UV radiation at different phases of the day. Cells are insensitive to UV until 2 h into the night (LDT 14) on LD 12:12 when cell survival drops to 20%. At LDT 12, UV has a partial effect on cell survival which drops to 50%. Likewise, cells are most sensitive to UV at LDT 14 on LD 16:8, but at LDT 12, cell survival did not significantly drop. This phase of maximal sensitivity on LD 16:8 occurs at the end of the day. Blue-light treatment following UV reversed the effect of UV if treatments occurred at the light-to-dark transition, but not if treatments occurred only 2 h following the light-to-dark transition. On LD 12:12, UV treatment resulted in cell survival of only 23% and 12% at LDT 12 and 14, respectively. UV treatment followed by blue-light treatment resulted in cell survival of 94% and 26% at LDT 12 and 14, respectively. These results suggest that *C. reinhardtii* employs two strategies to protect itself from UV damage: programming UV-sensitive processes to the night and having blue-light activated enzymes present during the day to reverse UV damage. Supported by NIMH R01 MH43836 & K02 MH01179 to CHJ and NSF Fellowship BIR-9396285 to SSN

ROBUST OSCILLATIONS WITH TWO INTER-LOCKED FEEDBACK MODEL OF DROSOPHILA CIRCADIAN RHYTHM

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A mechanism for generating circadian rhythms has been major interest in recent years. After the discovery of *per* and *tim*, a model with a simple feedback loop involving *per* and *tim* has been proposed. However, it is recognized that the simple feedback model cannot account for phenotypes generated by various mutants. A recent report by Glossop, Lyons and Hardin (Science 286, 766 1999) on *Drosophila* suggests involvement of another feedback loop by *dClk* that is inter-locked with *tim-per* feedback loop. In order to examine whether two inter-locked feedback loops can be a basic mechanism for circadian rhythms, a mathematical model was created and examined. Through extensive simulation and mathematical analysis, it was revealed that the two inter-locked feedback model accounts for the observations that are not explained by the simple feedback model. Moreover, two inter-locked feedback model has robust properties in oscillations.

MODELING CLARIFIES THE ROLES OF DELAYS AND FEEDBACK IN CIRCADIAN OSCILLATORS. P. Smolen*, D. A. Baxter and J. H. Byrne. Dept. of Neurobiology & Anatomy, W. M. Keck Center for the Neurobiology of Learning and Memory, Univ. of Texas-Houston Medical School, Houston, TX 77303.

Mathematical modeling is useful to integrate emerging experimental data on circadian rhythms into a coherent picture. Using current data, we constructed mathematical, differential-equation based models of two circadian oscillators. In a model of the *Neurospora* oscillator, the circadian protein FREQ suppressed its own transcription independently of its phosphorylation state, although phosphorylation was required for degradation. Circadian oscillations were only obtained by assuming an explicit time delay between synthesis and degradation and that many phosphorylations were necessary for degradation. In a model of the *Drosophila* oscillator, the circadian proteins PER and TIM indirectly suppressed their own transcription by binding to CLOCK, a protein that activates their transcription. Circadian oscillations were obtained with this negative feedback. Positive feedback has been suggested to also be necessary (e.g., Crosthwaite *et al.*, 1997). However, if a recently suggested positive-feedback loop consisting of reciprocal transcriptional activation by PER and CLOCK was incorporated, circadian oscillations were suppressed. We suggest that limiting amounts of specific proteins (e.g., CYCLE) whose levels do not oscillate and which are necessary for CLOCK-mediated activation of transcription could nullify positive feedback *in vivo* and thereby restore circadian oscillations. Our analyses identify key regulatory steps in the *Drosophila* and *Neurospora* circadian mechanisms that warrant further experimental investigation. Supported by NIH grants T32 NS07373, R01 RR11626, and P01 NS38310.

Computer Simulations of "Splitting" of Activity Rhythms in Hamsters

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Long-term exposure of hamsters to DD or LL causes changes in observable properties of the circadian activity rhythm, such as τ , α (activity duration) and the PRC. Behavioral studies show that τ and α increase under DD after the animals are released from LD 12h:12h, whereas τ increases and α decreases under LL. In the latter case, "splitting" of activity into two components separated by approximately 180° arises in many hamsters. Are the periods of E (evening) and M (morning) oscillators altered by LL and DD or is coupling between them changed? We addressed this question via a series of computer simulations of two coupled Pittendrigh-Pavlidis oscillators using *CircadianDynamix*.

An exact antiphase (180°) relationship is attained only by two identical oscillators coupled by symmetric inhibitory connections, whereas an exact in-phase (0°) relationship is attained only when they are coupled by symmetric excitatory connections. Departing from these two extreme symmetric conditions, we gradually and systematically increased the asymmetry between component properties (period and amplitude) and between coupling signals and strengths. The main implications for the structure of the hamster clock derived from our simulations are: only non-identical E and M oscillators (with E presenting shorter τ and smaller amplitude) can generate covariant changes in τ and α , as seen in long-term exposure of hamsters to DD; splitting may result from a switch in the coupling signal (from excitatory to inhibitory) under long-term exposure to LL rather than from weakening of coupling; the oscillator with shorter τ and smaller amplitude phase leads under excitatory coupling and phase lags under inhibitory coupling, which can explain why the E and M oscillators cross before splitting; PRCs of each E and M oscillators during splitting do not correspond to their intrinsic PRCs, because the amplitudes of each component are greatly changed by the inhibitory coupling. Supported by FAPESP (97/13910-0) and NSF Center for Biological Timing.

A close relation between thermoregulatory and arousal related processes in basal forebrain and hypothalamic areas has been substantiated in many studies. It has been demonstrated especially - but not exclusively - in the preoptic area of the anterior hypothalamus (POAH), that a subpopulation of warm-sensitive neurons (WSNs) spontaneously increases firing rate at sleep onset, and that experimental local warming of the POAH induces a similar increase in firing rate and facilitates sleep onset. It has consequently been proposed that brain temperature may be involved in physiological regulation of sleep. However, contrary to the experimental findings of increased sleep onset probability with a locally increased hypothalamic temperature, the likeliness of sleep onset in unmanipulated conditions is actually *minimal* at the time when the circadian rhythm in temperature reaches its peak. In fact, sleep onset probability *increases* on the *falling* limb of the circadian core temperature rhythm. How can this discrepancy of increased sleep with well-controlled experimental local POAH warming be reconciled with increased sleep with local cooling under natural conditions?

A suitable explanation has been put forward⁴: in modelling based on local warming experiments, it has generally been ignored that many of the locally thermosensitive neurons also respond, in a similar way, to changes in *skin* temperature. Thus, the very changes in cell membrane properties leading to sleep-related alterations in firing rate as induced by local warming in *experimental* conditions, may be induced by warming of the skin under *natural* conditions.

Several considerations support the importance of skin temperature. First, after warming of the skin, preoptic WSNs show a markedly increased firing rate to a level that can be reached only by extreme (non-physiological) local warming. Moreover, with elevated skin temperature, WSNs have a high firing rate irrespective of the local temperature, i.e. temperature input from the skin appears to dominate when competing signals are present². Second, sleep appetitive behavior like lying down, covering etc. is associated with a redistribution of warm blood to the extremities, thus increasing their temperature. Third, and most important, the increase of sleep onset probability that occurs on the falling limb of the circadian core temperature rhythm can now be understood. Since the circadian drop in core temperature is mainly due to increased dissipation of body heat, skin temperature is actually elevated when core temperature is falling.

The preoptic area is by no means exclusive in containing thermosensitive neurons: they are present in many of the brain areas involved in arousal regulation. When sleep onset probability was modelled with human constant routine data¹ of the circadian profiles of core and peripheral temperature as inputs to thermosensitive neurons in these brain areas, we found agreement between habitual sleep onset time and peak modelled onset probability.

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TOWARDS A BIOCHEMICAL MODEL OF THE HUMAN CIRCADIAN PACEMAKER

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For almost 30 years, the van der Pol oscillator has successfully been used as a functional model of the human circadian pacemaker. However, the variables in the van der Pol oscillator assume both positive and negative values, and therefore can not directly represent chemical concentrations in a biochemical oscillator. Recently, Goldbeter has proposed several biochemically plausible models of the circadian system of *Drosophila*. Since these models are nonlinear and contain 5 or more variables, their dynamics are not easily understood. We have studied Goldbeter's (1995) [1] 5 variable model of the Per transcription/phosphorylation cycle in *Drosophila* to see if (1) Goldbeter's model is, in a sense, equivalent to the van der Pol oscillator and (2) whether such a model can be used to predict phase shifting results of human experimental protocols.

A new method has been developed to analyze quasilinear oscillations in systems with more than 2 variables (Forger and Kronauer, submitted). Using this method, we have shown that, after brief initial transients, the behavior of Goldbeter's system is analogous to a van der Pol oscillator with a stiffness of 0.22. We have previously proposed a model of the human circadian system based on a van der Pol oscillator with a stiffness of 0.23 [2]. Combining elements of these models, we construct a model of the human circadian pacemaker incorporating plausible chemical processes. This research provides (1) a simple description of the dynamics of Goldbeter's 5 variable biochemical oscillator, (2) a biochemical analog of the van der Pol equation and (3) representative human phase shift responses to conventional stimulus protocols.

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THE HUMAN CIRCADIAN SYSTEM OPERATES AS A PHASE LOCKED LOOP, Steven B. Lowen, Ph.D. and Martin H. Teicher, M.D., Ph.D., Developmental Biopsychiatry Research Program, McLean Hospital, and Consolidated Department of Psychiatry, Harvard Medical School, 115 Mill Street, Belmont, MA 02478

The human circadian system bears a remarkable similarity to a well-studied circuit from electrical engineering: the phase locked loop. These similarities include the ability to entrain to an external zeitgeber, and the generation of free-running oscillations near the desired period in the absence of zeitgebers. The entrainment strength depends on both the period and amplitude of the zeitgeber. For a given amplitude, a maximum period difference exists beyond which the system will not entrain. During entrainment, erratic shifts in the phase may occur, especially for zeitgebers with small amplitudes and periods greatly different from the free-running period. After entrainment, the phase delay between the system and the zeitgeber depends on the period difference between the zeitgeber and the free-running period. Gradually changing the period from the free-running period allows the system to remain entrained to periods that would not be possible if imposed abruptly. Upon removal of the zeitgeber, the system continues to run at the entrained period, gradually returning to the free-running period. Physiological variables which return to the free-running period more slowly than others will entrain to a narrower range of periods than others. The amount of phase shift induced by a brief zeitgeber depends on the current phase of the system, yielding a phase response curve with a characteristic "Z" shape.

A phase locked loop consists of three main components: a phase detector, a filter, and a variable-period oscillator. The phase detector compares the instantaneous phase of a zeitgeber to that of the oscillator, and generates an error signal. The filter smooths this signal, which then becomes the input to the variable-period oscillator. This loop topology ensures that the output of the oscillator is phase locked to the zeitgebers, and is distinct from the feedback loops inside the oscillator which ensure free-running oscillations in the absence of zeitgebers. Simulations of phase locked loops using van der Pol oscillators support these claims, reproducing similarities listed above including phase response curves.

This dramatic connection between human circadian systems and phase locked loops is important for two reasons. First, extensive results for phase locked loops exist in the electrical engineering literature, all directly applicable to the study of human circadian systems. Second, the structure of phase locked loops suggests avenues of research towards understanding the physiology of the human circadian system.

CHRONOBIOLOGY

A COMPREHENSIVE CD-ROM ON THE BIOLOGY OF TIMING WITH GUIDED INTERNET ACCESS TO DATABANKS AND WEB-SITES

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Chronobiology defines itself as an interdisciplinary approach to basic features of life. A traditional type of textbook cannot adequately represent deep background information, the complexity of current knowledge and its practical use at the same time. Here the almost unlimited possibilities of the modern electronic multimedial techniques can help. Therefore the authors decided to create an interactive CD-ROM "Chronobiology" with the entire scope of multimedia informations (including videos, sound tracks and animations) and organised access to internal and external databanks. The CD-ROM is organised in hierarchical complexity: 12 sections, each starting with a first level of clear statements on the main issue (similar to a textbook) comprehensible with general academic background. Via hyperlinks to further levels you reach more detailed informations on methods, data, scientific background (including papers), open problems and actual discussions, finally linked to the web-sites of the labs actually working in the respective field of research or aspects of application.

GREEN FLUORESCENT PROTEIN REPORTS REGIONAL REGULATION OF *mPER1* ACTIVITY IN THE SCN. DG McMahon, SJ Kuhlman, JE Quintero, R Silver¶. Department of Physiology, University of Kentucky, Lexington KY 40536. ¶ Department of Psychology Barnard College and Columbia University, New York, NY 10027

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The *mPeriod1* (*mPer1*) gene is a key participant of the molecular feedback loop which drives the suprachiasmatic (SCN) neuronal clock function and is critical for phase-resetting. Mice harboring degradable Green Fluorescent Protein (GFP) driven by the *mPer1* promoter were used to study *mPer1* regulation in living tissue with high reporter resolution. *mPer1*-driven fluorescence was expressed in two circadian structures, the SCN and retina. Induction of the transgene in response to a 30 minute phase-resetting night-time light pulse shown to induce *mPer1* was examined. SCN fluorescent intensity increased 2.8 fold in response to light compared to sham controls (n=3 each group, $p < 0.05$). The ventral core of SCN tended to have the strongest light-induced signal. Light-induced fluorescence in SCN sections cut in the parasagittal plane was strongest in the anterior SCN, further supporting regional regulation. In contrast, endogenous rhythmic *mPer1* activation was most apparent in the medial shell of the SCN nucleus. SCN fluorescence intensity was 2.6 fold higher at dusk than dawn (n=3, $p < 0.05$). Anti-GFP immunoreactivity showed a pattern similar to native fluorescence. Such differential regulation of promoter activity may facilitate entrainment of a multi-oscillator system to the environment.

The promoter-driven unstable GFP reporter system provides a general strategy for capturing the functional plasticity of the brain in the molecular dimension and relating changes in gene expression to the functional state of individual neurons and brain circuits in living tissue. Supported by NIH AG13426 and MH60794 to DGM.

MOUSE *PERIOD1*-DRIVEN GFP GENE EXPRESSION CYCLES IN SCN SLICES AND IN INDIVIDUAL SCN CELLS. Jorge E. Quintero, Sandra J. Kuhlman, and Douglas G. McMahon. Department of Physiology, University of Kentucky, Lexington, KY 40536.

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The suprachiasmatic nucleus (SCN) contains a heterogeneous population of cells that drive 24-hour rhythms in physiological functions in the *in vivo* animal. The advent of circadian-reporter transgenic mice has allowed the monitoring of circadian gene expression in the living SCN. Using standard methods, hypothalamic slices containing the SCN were taken from newborn, hemizygous *Per1*-driven destabilized Green Fluorescent Protein (GFP) mice housed in a 14:10 LD cycle. The *in vitro* SCN displayed two or more cycles of *Per1*-driven GFP fluorescence where the relative intensity amplitude was, on the first day, 2.1 fold, and on the second day, 1.4 fold greater than the nadir between the two cycles. The time difference between the peaks on the first and second days was 23.7 hours (± 2.3 S.D., n=8) from slices taken either early or late in the day and matches the tau (23.5 ± 0.16 hours, N=4) in free-running locomotor activity from transgenic littermates. Ninety percent of the fluorescence peak was reached at CT9.3 (± 2.9 , N=8) on the first day and CT8.7 (± 3.4 , N=8) on day 2 regardless of the time during the day when the slices were acquired.

To begin to examine the molecular dynamics of the SCN, we used confocal microscopy to image the *Per1* gene expression cycle in individual neurons. Interestingly, neuronal *Per1* gene expression cycles in the SCN slice showed a diversity of phase groupings. Many cells were in synchrony with the overall SCN fluorescence intensity rhythm (54%), another group was phase advanced by 3-4 hours (30%), and a third group phase lagged the overall SCN peak by 6-8 hours (10%) (N=78 cells in 3 slices). Six percent of cells cycled in antiphase to the main peak. Our results demonstrate distinct oscillatory phases and waveforms within the population of *Per-1* expressing neurons in the SCN and provide a basis for further assessing the relationship of these temporal clusters of gene activation to functional units within the SCN pacemaker. Supported by NIH AG13426 and MH60794 to DGM.

GENERATION AND ANALYSIS OF mPer1-LUCIFERASE TRANSGENIC MICE

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The ability to study a circadian promoter using reporter genes is well established in *Arabidopsis* and *Drosophila*, yet only recently has this method been applied to mammals. To gain a better understanding of the *in vivo* regulation at a circadian promoter, we created a reporter construct using the mouse *Period1* (*mPer1*) promoter to drive expression of luciferase. This promoter contains 6.8 kb of *mPer1* upstream sequence and includes five E box elements, exon 1, and a portion of exon 2 up to the ATG start of the mPER1 protein. We generated seven transgenic mouse lines (strain CD1) with this construct, all of which displayed a diurnal pattern of luciferase mRNA expression in the suprachiasmatic nuclei (SCN) by *in situ* hybridization. One line (P1L025) was chosen for further analysis, and a circadian rhythm of luciferase mRNA expression in the SCN of this transgenic line was confirmed. At CT17, light pulses that ranged from 30 minutes to six hours in duration resulted in the rapid induction of luciferase mRNA. This induction peaked after 90 minutes of light exposure, similar to the induction profile of endogenous *mPer1* mRNA in the CD1 mouse strain. Real-time luciferase protein activity was measured in SCN cultures from mPer1-luciferase transgenic animals; six out of six transgenic lines tested displayed a circadian rhythm of reporter activity in culture for the extent of the experiment (two to five days). These results indicate that the 6.8 kb *mPer1* upstream sequence contains elements necessary for circadian regulation and light induction *in vivo* as well as maintenance of rhythmicity in culture.

132 TRANSGENIC ANALYSIS OF A MAMMALIAN CLOCK GENE, *Per1*.

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A mammalian homologue of the *Drosophila period* gene, *Per1*, exhibits circadian expression in the SCN, the central pacemaker of the mammalian circadian clock, and is transiently induced by a light pulse. Function of the circadian expression of mammalian *Period* genes is a key question for the regulation of circadian rhythms. Transgenic rats with constitutive expression of the mouse *Per1* gene were constructed using elongation factor 1 alpha (EF1-a) or neural specific enolase (NSE) promoters. Both the circadian period of locomotor activity and entrainment to light dark cycles were severely affected in several transgenic lines. The results clearly indicate that the mammalian *Per1* gene is involved in rhythm generation and/or entrainment of the circadian clock. In addition, we measured the expression of the native (rat) *Per1* and *Per2* genes in the SCN and retina of the transgenic lines under DD conditions. The circadian expression of endogenous *Per1* and *Per2* genes was diminished in the transgenic lines. Molecular mechanisms regulating the expression of *Per1* and *Per2* will be discussed.

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RESETTING CENTRAL AND PERIPHERAL CIRCADIAN OSCILLATORS IN TRANSGENIC RATS

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We constructed a transgenic rat line in which luciferase is rhythmically expressed under the control of the mouse *Per1* promoter and have used it to study mammalian circadian organization. Light emission from cultured suprachiasmatic nuclei (SCN) of these rats was invariably and robustly rhythmic and persisted for up to 32 days in vitro. Liver, lung and skeletal muscle expressed circadian rhythms which damped after 2-6 cycles in vitro. The circadian rhythm of light emission from the SCN followed light cycle shifts more rapidly than did the rhythm of locomotor behavior or the rhythms in peripheral tissues. We hypothesize that self-sustained circadian oscillators in the SCN entrain damped circadian oscillators in the periphery to maintain adaptive phase control which is temporarily lost following large, abrupt shifts in the environmental light cycle.

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DAMPED OSCILLATION OF *PER1-LUC* IN ISOLATED BRAIN REGIONS

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The suprachiasmatic nucleus (SCN) has been identified as a central pacemaker which regulates circadian rhythms in mammals. Neural activity in the SCN is higher during the day than at night in both nocturnal and diurnal animals. In nocturnal animals, extra-SCN brain areas show electrical activity in anti-phase to the SCN. The nature of the rhythmicity in extra-SCN brain areas is relatively unexplored. We tested whether the isolated brain regions can oscillate by themselves using *mPer1-luc* transgenic rats.

Explants of specific brain nuclei were isolated from the transgenic rats and cultured on Millicell membranes in medium containing luciferin. Under these conditions, the SCN showed sustained circadian oscillations of bioluminescence, as recorded by either a photomultiplier tube or an intensified CCD camera. The peak of bioluminescence was always during the subjective day. We also recorded from isolated brain regions that highly express *Per1* including the supraoptic nucleus, hippocampus, piriform cortex, cerebellum, paraventricular nucleus and arcuate nucleus. Although they did not show sustained rhythms, they expressed damped circadian oscillations with 1-3 peaks. The peak phase of bioluminescence was during night in almost all brain regions. The caudate-putamen expressed low level of luciferase activity with no detectable peaks.

The present result indicates that brain regions other than SCN can oscillate by themselves. However, communication with SCN seems to be necessary to maintain circadian rhythmicity in these regions.

Identification of Down-Stream Genes of mPer2 *in vivo* using cDNA Microarrays

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Three putative murine clock genes (mPer1, mPer2 and mPer3) have been identified. The mPer genes share a conserved PAS domain, show a diurnal expression pattern in the SCN, and play a role in regulating transcription factors belonging to E-box binding proteins, which activate mPer gene transcription. Mutant mice with a deletion in the PAS domain of the mPer2 gene display a number of circadian abnormalities including a shorter circadian period and a loss of circadian rhythmicity in DD. The mutation also affects the expression of both mPer1 and mPer2 in the SCN, indicating that mPer2 may regulate mPer1 *in vivo*. However, nothing is known about other genes that are affected by mPer2. One approach to identify genes regulated by mPer2 is to determine genes differently regulated in mPer2 mutant tissues. Adult male mPer2 mutant and wild-type mice were sacrificed and liver and brains removed. Extracted total RNA was labeled with Cy5 (green control) and Cy3 (red experimental) and hybridised together to cDNA microarrays (discoveryARRAY™, Display Systems Biotech). The used microarrays consisted of 2688 different cDNA obtained from a mouse cDNA library spotted onto a glass surface. The hybridised array slides were scanned for the two dyes using a laser scanner and the two image files were overlaid electronically and analyzed using ImaGene software. In the liver, 2 genes were found to be up-regulated and 2 genes down-regulated more than 1.8-fold. In the brain, 3 genes were identified to be either up-regulated and two genes down-regulated using the same threshold. The regulated genes in brain and liver were in some cases the same, in others not, indicating that mPer2-dependent gene-regulation is both tissue- and promoter specific. Future analysis will determine if the genes regulated by mPer2 are expressed in the SCN and play a functional role in the output of the clock.

BEHAVIORAL REVERSION IS ASSOCIATED WITH LOSS OF DAILY BEHAVIORAL RHYTHM AND CHANGES IN *PERIOD* GENE EXPRESSION IN THE BRAIN OF THE HONEY BEE

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As part of the system of age-related division of labor in honey bee colonies, young bees care for brood ("nurse") around the clock, while older foragers have strong circadian rhythms that are involved in navigation and timing visits to flowers. Previous research has shown that this division of labor is associated with the development of endogenous activity rhythms and an increase in *period* (*per*) gene mRNA levels in the brain. We determined whether the development of rhythm is reversible at the behavioral and molecular levels. We used colonies composed only of foragers, with all bees assumed to have highly developed circadian rhythms. In the absence of nurses some individuals reverted from foraging to nursing while others continued to forage, as in previous studies of reversion. Brood was cared for around the clock in these colonies; detailed observations of individually identified bees revealed that around-the-clock nursing was accomplished not by rhythmic individuals working in shifts, but by bees that attended brood around the clock with no diurnal rhythms. Measurement of *per* mRNA in individual bees revealed that *per* levels showed circadian oscillations in all bees, but the data suggest that reverted nurses had lower amplitudes than foragers. Reverted nurses from one colony also had significantly lower *per* mRNA brain levels than foragers. These results demonstrate striking plasticity in a circadian system and suggest that socially-mediated changes in behavioral rhythms are associated with changes in molecular components of the clock.

EFFECTS OF AGING ON THE TIMING OF SPONTANEOUS DAYTIME SLEEP

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We have shown in young adults that naps occur at a preferred circadian phase during the daytime hours, just prior to the circadian temperature maximum. If napping is indeed an inherent part of the circadian sleep-wake rhythm, one might hypothesize that napping would occur earlier in old relative to young individuals, corresponding to the age-related advance in timing of nocturnal sleep and the temperature nadir.

Fourteen old (68 ± 6 y) and 13 young (24 ± 4 y) subjects were studied for 72h while in social and temporal isolation. They were instructed to sleep whenever inclined to do so, and behavioral options were limited. Body core temperature and EEG were recorded continuously. Criteria for major nocturnal sleep episodes were: initiated 2200-0400h, >4h duration, with <1h continuous wakefulness. Naps were defined as: initiated 1000-1800h, >15m duration, with <30m continuous wakefulness. Each subject's temperature minimum (Tmin) and maximum (Tmax) were determined using a 9th order polynomial curve fit.

Both Tmin and Tmax were significantly earlier in old subjects. Both nighttime sleep episodes and naps were advanced in old relative to young subjects, as well. Nighttime sleep episodes were significantly longer in young subjects (Y: 9h41m vs. O: 6h15m, $p < .01$).

The phase angle between nighttime sleep onset and Tmin was 4h40m in young and 4h08m in old groups, respectively (n.s.). The interval from nap onset and Tmax was 2h29m for young and 2h13m for old groups, respectively (n.s.).

These results support the concept that napping is an inherent part of the endogenous pattern of sleep and wakefulness in humans. Further, they provide insight into the status of sleep regulating processes in aging. Because the phase angle between sleep and Tmin is maintained in old subjects, it appears that Process C is relatively intact. In contrast, nighttime sleep episode durations were significantly shorter in the old group. Whether this reflects an alteration in Process S, phase tolerance in young subjects, or perhaps a change in sleep need in aging will be further investigated by examining the composition of these sleep episodes.

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	NIGHT SLEEP			NAP SLEEP		
	onset	offset	Tmin	onset	offset	Tmax
Young:	24:54	10:35	05:34	15:54	17:16	18:23
Old:	23:02	05:17	03:11	13:51	15:08	16:04

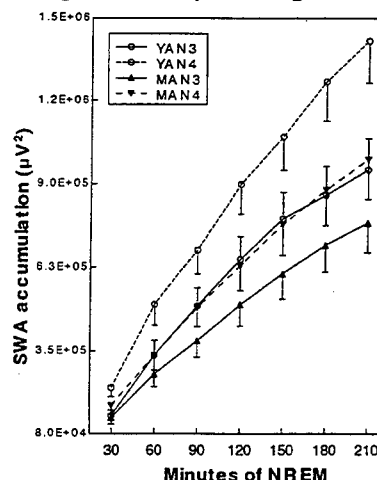
ATTENUATION OF HOMEOSTATIC PROCESS DURING DAYTIME

RECOVERY SLEEP IN THE MIDDLE YEARS OF LIFE

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It has been proposed that aging is associated with an attenuation of process S efficiency. Recently, we have reported that despite 25 hours of sleep deprivation, sleep consolidation is dramatically perturbed in middle-aged compared to young adults when recovery sleep is initiated in the morning. This study investigated the effect of a 25-hours sleep deprivation on the dynamics of sleep EEG slow-wave activity (SWA) during daytime recovery sleep in young and middle aged subjects. Twenty-two subjects (Young: 25-39 y, 5W, 5M and Middle-aged: 40-59 y, 7W, 5M) came to the sleep laboratory for 4 consecutive nights and 2 days. The morning following the third night (N3: baseline), subjects entered a mini-constant routine during which they were kept awake in bed for the next 25 hours. Recovery sleep episode (N4) started in the morning. Compared to the young, the middle-aged showed a steeper increase in the number of minutes of wakefulness during daytime recovery sleep ($p < 0.001$). Analyses per third of night revealed that this steeper increase of wakefulness in the middle-aged was more prominent in the last two thirds of night. SWA (spectral power; 0.5-4.5 Hz) was potentiated in both groups following sleep deprivation. However, SWA rebound was significantly reduced in middle-aged compared to the young (Interaction Group X Night: $p = 0.02$; Two-way ANOVA on mean SWA for each half hour of NREM sleep). This reduction in homeostatic recuperative drive following sleep deprivation in the middle-aged subjects might account for their reduced ability to maintain sleep when they have to recuperate at an abnormal circadian phase. These results may help to understand the increase in subjective sleep complaints related to shift-work and jet-lag in the middle years of life.



SLEEP DURING FORCED DESYNCRHONY IN ADOLESCENTS

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Previous studies of sleep during forced desynchrony (FD) of 28- or 20-hr with adult humans show a strong circadian influence on sleep efficiency and REM sleep, strong sleep-dependent influence on slow wave sleep (SWS), and an interaction of the two influences on sleep efficiency (wake within sleep episodes) and REM sleep (1,2). Sleep patterns are influenced by age; for example, SWS declines markedly across adolescence. We examine here whether maturation may influence the regulation of sleep as assessed in FD.

Methods. Data were analyzed from 20 adolescents (10 f) ages 9.6 to 15.1 yr. Participants lived at home for 10 to 12 days on a 14:10 LD (lights on = 0800) before coming to the lab, where a 36-h constant routine was followed by 12, 28-h cycles, with 16h20m:11h40m LD ($L \leq 15$ lux; $D < 1$ lux). Circadian phase was determined for individuals from period estimates based on salivary melatonin onset times. Sleep from scheduled sleep episodes was scored in 30-sec epochs using standard criteria (3) and aggregated for individuals in 120-min bins for 600 min after lights out and in 30° phase bins to assess sleep-dependent and circadian influences, respectively. Four variables—NREM sleep, SWS, REM sleep, and waking—were derived as a percent of recording time and assessed with repeated-measures MANOVA. Maturation influences were examined by grouping participants as pre/early pubertal (Tanner stages 1 or 2; 1 f, 5 m) or mid/late pubertal (Tanner stages 3-5; 9 f, 5 m).

Results. Significant main effects of circadian phase were found for NREM sleep, REM sleep, and Wake; significant main effects of time since lights out were found for NREM sleep, SWS, REM sleep and Wake; significant interactions were found for all 4 variables. A Tanner group by wake-dependent interaction was found for SWS only.

Discussion. Most of these findings are consistent with the adult data. Of interest were the sleep-dependent effect on REM sleep and the interaction of circadian and wake-dependent factors for SWS, neither of which was a consistent finding in adults. Furthermore, the Tanner group interaction for the wake-dependent influence on SWS indicates a maturational process.

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SLEEP ALTERS HUMAN PHASE RESPONSE TO EXTRAOCULAR LIGHT

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We have reported that light to the popliteal fossa of humans resets the circadian clock in a manner similar to that reported for ocular light exposure. Here we report that the human phase response to extraocular light is altered when the stimulus is presented during sleep.

Thirteen subjects were studied in active and control conditions. In each condition, subjects spent 5 days and nights in the lab. During the first 24 hrs, baseline circadian phase was assessed. Subjects were exposed twice, during the next two 24-hr periods, to a 3-hr pulse of extraocular light (or to a sham condition) while sleeping. Clock time of exposure was the same for each subject in control and active conditions. Light was presented using fiber-optic pads placed on the popliteal fossae. In the control condition, pads were covered with an opaque sheath. Circadian phase was assessed during the final 48 hrs in the lab, and degree of shift was determined by comparing baseline with post light phases. Temperature curves were fit by 4 individuals blind to subject and condition, and phase was determined by calculating the average time of the temperature minimum (t_{min}) of the blind fits.

As would be expected, there was a systematic variation in the degree to which extraocular light reset the biological clock, depending on the phase at which light was presented. The largest shifts occurred when light was presented close to t_{min} . The average absolute shift in response to active light presented within 5 hours of t_{min} was 100.7 minutes, compared to 30.6 minutes in the control condition ($p = .011$). In contrast to light PRCs obtained from waking subjects, this "sleep PRC" was characterized by a predominance of phase delays in response to light presented both *before and after* t_{min} . Sleep architecture was also influenced by light exposure. Specifically, the proportion of sleep spent in REM during the 3-hour light interval showed an average 47% increase in the active compared to the control condition. The increase in REM% was the result of a significant shortening in the average duration of REM/NREM cycles during the 3-hour light presentation interval (A: 85 min vs C: 105min; $p = .02$).

These findings demonstrate that the human circadian clock can be reset by extraocular light exposure, even when presented to sleeping subjects. The additional finding that extraocular light acutely, and significantly, increases REM sleep amounts indicates that non-ocular sensory stimuli are detected and responded to by multiple neuronal sites.

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WHAT IS THE RELATIONSHIP BETWEEN ASSESSMENTS OF JET-LAG AND SOME OF ITS COMPONENTS?

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Jet-lag arises after travelling across several time zones. It is generally described as consisting of several symptoms, including subjective ones linked with mood, sleep, appetite and bowel function. What has been studied less is how closely these symptoms are related to "jet-lag", and to its gradual disappearance with continuing time spent in the new time zone.

Thirty-nine subjects - varying in their age, athletic ability, objectives when in Australia, and whether or not they were taking melatonin - were studied after a flight from the UK to either Sydney or Brisbane (10 time zones to the east). For the first 6 days after arrival, they scored their "jet-lag" five times per day (at about 08:00, 12:00, 16:00, 20:00 and 24:00 h), using a 10 cm scale labelled "0 - no jet-lag" to "10 - very bad jet-lag" at its extremes. Other subjective variables were measured using visual analogue scales, the extremes being labelled "-5" and "+5" (indicating marked changes compared with normal), and the centre, "0" (indicating "normal"). These variables were fatigue (measured at the same time as jet-lag), and aspects of sleep (08:00 h), mood and appetite (12:00, 16:00 and 20:00 h), and bowel function (24:00 h). Since there were no significant differences between the melatonin and placebo groups, the results were pooled in the following analyses.

Mean daily values ($\pm$ SE) for jet-lag ($+ 3.65 \pm 0.35$) and fatigue ($+ 1.55 \pm 0.22$) were raised on day 1, and fell progressively with subsequent days; they were still raised ($P < 0.05$) on day 5 (fatigue) or day 6 (jet-lag). Times of waking were earlier on all days. By contrast, falls in mean daily concentration and motivation, and rises in mean daily irritability and the number of waking episodes during sleep, had recovered by day 4 or earlier. Bowel activity was less frequent and with harder stools, on days 1 and 2 only, and there was a decrease in the ease of getting to sleep ($- 1.33 \pm 0.55$) on day 1, but this changed to an increase from day 2 onwards. That is, not all the symptoms attributed to "jet-lag" adjusted at the same rate as did "jet-lag" itself.

Stepwise regression analysis was used to investigate the significant predictors of jet-lag at each of the five times of measurement. The severity of jet-lag at all times was strongly predicted by fatigue ratings made at the same time. The severity of jet-lag at 08:00 h was predicted by an earlier time of waking, by feeling less alert 30 min after waking, and, marginally, by the number of waking episodes during sleep. Jet-lag at 12:00 and 16:00 h was strongly predicted by a fall in the ability to concentrate; and jet-lag at 12:00, 16:00 and 20:00 h was predicted by the amount of "feeling bloated" after a meal.

These results indicate that the exact interpretation of "jet-lag" is complex, since it would seem to be affected partly by an individual's understanding of this term, and when jet-lag is measured.

ORDINARY INDOOR ROOM LIGHT CAN AFFECT THE ADAPTATION TO SIMULATED JET-LAG

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Introduction: Recent evidence has demonstrated that humans are much more sensitive to light than initially held, such that even light of ordinary indoor intensity can significantly reset the phase of the human circadian pacemaker^{1,2}. This experiment examines the efficacy of light of ordinary intensity in the readaptation to a simulated Montreal-London voyage.

Methods: 12 healthy young men, aged 18-30 years, participated in this study. After 3 weeks of maintenance of regular sleep-wake habits, subjects were admitted to the laboratory for 15 consecutive days of study in temporal isolation. Following 3 baseline days, initial circadian phase was determined using a 35-hour constant routine (CR). Subjects were then set on a sleep-wake schedule to simulate a voyage 5 time-zones eastward. Experimental subjects were assigned to one of two groups. They were exposed to a light stimulus consisting of 6 hours of ~380 lux daily, either on an adaptive ('morning' group), or inappropriate ('evening' group) schedule. They remained in very dim levels of light (<10 lux) during the remainder of their waking episodes. Following one week of phototherapy, circadian phase was reassessed by a 45-hour CR. Core body temperature (CBT) was continuously recorded throughout the experiment. Blood samples were collected every 60 minutes during CR procedures in order to assay plasma melatonin concentration. Circadian phase was determined by a dual-harmonic regression model of CBT values collected during CR periods³ without serial correlated noise.

Results: At the start of the experiment, the endogenous circadian phase ($\pm$ SEM) was comparable between both groups of subjects (5.87 ± 0.44 and 5.75 ± 0.62 , $p = 0.83$ two tailed). After one week on the London schedule, mean phase shifts were significantly different between both groups (p [two-tailed] = 0.006). Phase advances of $+6.06 \text{ h} \pm 0.31$ and $+1.32 \text{ h} \pm 1.05$, for morning and evening groups respectively, were observed. The morning group was adjusted to the "destination" sleep-wake schedule, whereas the evening group remained closer to the "departure" sleep-wake schedule.

Conclusions: These findings demonstrate that the pattern of exposure to ordinary indoor room light can significantly affect circadian adjustment to jet-lag. The results carry important implications for the alleviation of symptoms related to jet-lag and shiftwork. They further demonstrate the sensitivity of the human circadian pacemaker to room light and imply that the exposure to room light should be documented in field studies.

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DOES MORNING MELATONIN ADMINISTRATION PHASE DELAY HUMAN CIRCADIAN RHYTHMS? A. Wirz-Justice, K. Kräuchi, E. Werth, C. Renz, S. Müller, P. Graw & J. Weber *Chronobiology and Sleep Laboratory, Psychiatric University Clinic, CH-4025 Basel, Switzerland*

Although there is good consensus that melatonin administration (MEL) in the early evening can phase advance human circadian rhythms, the evidence for a phase delay portion of the PRC is sparse. We therefore carried out a double-blind randomized-order placebo-controlled study under modified constant routine conditions (58h bedrest under <8 lux, sleep 23-7h) in 9 men (24 ± 1 y). Melatonin (5mg p.o.) or placebo was administered at 07h on the first morning, phase shifts were measured 24 hours later. Thermometry (rectal and skin temperatures) and heart rate were continuously recorded, saliva was collected half-hourly for MEL assay (Dim Light Melatonin Onset or Offset threshold 3 pg/ml). Neither the timing of DLMOFF or DLMO, nor heart rate and skin temperature rhythms were phase shifted after MEL compared with placebo. After MEL administration, the mid-range crossing time of the core body temperature rise occurred earlier ($8:54 \pm 33'$ vs. $09:28 \pm 36'$), the decline later ($01:02 \pm 15'$ vs. $00:36 \pm 10'$), leading to a longer duration (16.13 ± 0.44 h vs. 15.14 ± 0.53 h). This alteration in wave form could not be interpreted as a straightforward phase delay. Under the same experimental constant routine conditions, a single administration of MEL in the evening (18h or 20:40h) was able to induce a phase advance. Thus, further studies are required to establish whether there is a significant phase delay portion of the human PRC to melatonin.

THE EFFECT OF DAY-TIME EXOGENOUS MELATONIN ADMINISTRATION ON CARDIAC AUTONOMIC ACTIVITY

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Exogenous melatonin, particularly if administered during the day, increases sleepiness, decreases core body temperature and increases peripheral body temperature¹. Melatonin receptors have been identified in the periphery, indicating that melatonin produces these effects via its action on the periphery². Melatonin may also produce its effects by altering autonomic activity.

We investigated this hypothesis by conducting Multiple Sleep Latency Tests (MSLTs) on two separate days, from 11:00 to 20:20 hrs, in a double blind, counterbalanced design. A 5mg oral dose of melatonin or placebo was administered to 12 healthy young subjects at 14:00 hrs.

Melatonin decreased mean sleep onset latency to stage 1 NREM sleep by 4.31 ± 0.76 mins ($p < 0.05$) and mean core temperature by $0.09 \pm 0.02^\circ\text{C}$ ($p < 0.05$). Mean peripheral body temperature increased by $0.43 \pm 0.07^\circ\text{C}$ ($p < 0.05$). In addition, melatonin produced a mean decrease of 1.59 ± 0.53 beats/min in heart rate ($p < 0.05$) and 8.11 ± 2.09 msec in pre-ejection period (measure of sympathetic activity) ($p < 0.05$). No change in respiratory sinus arrhythmia (measure of parasympathetic activity) was observed ($p > 0.05$). The decrease in heart rate and pre-ejection period indicates that melatonin administration decreases blood pressure. These results suggest that the daytime administration of melatonin does not influence cardiac autonomic activity, but instead alters blood pressure via peripheral melatonin receptors. Thus the previously observed presleep increase in cardiac parasympathetic activity³ is unlikely to be due to an increase in endogenous melatonin.

¹ Van Den Heuvel CJ, et al. Effects of daytime melatonin infusion in young adults. *Am J Physiol.* 1998, 275(1 Pt 1):E19-26.

² Viswanathan M, et al. Expression of melatonin receptors in arteries involved in thermoregulation. *Proc Natl Acad Sci U S A.* 1990, 87(16):6200-3.

³ Burgess, HJ, et al. Sleep and circadian influences on cardiac autonomic nervous system activity. *Am J Physiol.* 1997, 273(4 Pt 2):H1761-8.

Phosphorylation of the *Neurospora* clock protein FREQUENCY determines its degradation rate and strongly influences the period length of the circadian clock.

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Under free running conditions, FREQUENCY (FRQ) protein, a central component of the *Neurospora* circadian clock, is progressively phosphorylated, becoming highly phosphorylated prior to its degradation late in the circadian day. To understand the biological function of FRQ phosphorylation, kinase inhibitors were used to block FRQ phosphorylation *in vivo* and the effects on FRQ and the clock observed. 6-DMAP (a general kinase inhibitor) is able to block FRQ phosphorylation *in vivo*, reducing the rate of phosphorylation and the degradation of FRQ and lengthening the period of the clock in a dose dependent manner. To confirm the role of FRQ phosphorylation in this clock effect, phosphorylation sites in FRQ were identified by systematic mutagenesis of the FRQ open reading frame. The mutation of one phosphorylation site at Ser-513 leads to a dramatic reduction of the rate of FRQ degradation and a very long period (>30 hrs) of the clock. Taken together, these data strongly suggest that FRQ phosphorylation triggers its degradation and the degradation rate of FRQ is a major determining factor for the period length of the *Neurospora* circadian clock.

THE INTERPLAY BETWEEN WC-1 AND FRQ IN THE *NEUROSPORA CRASSA* CIRCADIAN CLOCK. Kwangwon Lee, Jennifer J. Loros, and Jay C. Dunlap, Department of Biochemistry and Department of Genetics, Dartmouth Medical School. Hanover NH. 03755.

The current molecular paradigm for the cellular circadian oscillator is a transcriptional/translational feedback loop which in eukaryotes is composed of negative (FRQ in *Neurospora*, PER and TIM in *Drosophila*, and mPER1, mPER2 & mPER3 in mammals) and PAS domain containing positive elements (WC-1 and WC-2 in *Neurospora*, dCLK and CYC in *Drosophila*, and CLK and BMAL1 in mammals). Additionally, the WC proteins act as positive regulators in the blue-light signal transduction pathway into the *Neurospora crassa* clock as activators of light induced gene expression of *frq*. Analyses of different alleles of *wc-1* suggested that WC-1 is necessary for the expression and the robust oscillation of FRQ in constant darkness. We find the level of the WC-1 protein is rhythmic in constant darkness, whereas the steady state transcript levels are constant, suggesting rhythmicity of WC-1 is conferred by a post-transcriptional mechanism. The peak of WC-1 protein oscillation follows that of FRQ with a delay of 4 to 8 h. Mutations at the *frq* locus control the period of the WC-1 oscillation, indicating that FRQ has a regulatory role in WC-1 regulation in constant conditions.

Experiments placing FRQ under regulatable conditions indicate that FRQ is not only a negative regulator of its own expression, but also a positive regulator of WC-1 metabolism in constant darkness by a post-transcriptional mechanism, thereby forming a second interlocked feedback loop in the *Neurospora* system. Interlocked feedback loops was also recently proposed in the *Drosophila* system where the PER-TIM complex not only down-regulates its activators, but also up-regulates *dClk* message (Glossop et al. 1999. Science 286:766-768), suggesting similar functional mechanisms of regulating circadian components in fungi and flies.

PERIOD LENGTH, TEMPERATURE COMPENSATION AND LIGHT ENTRAINMENT DEFECTS IN THE PAS PROTEIN, WHITE COLLAR-2, A POSITIVE ELEMENT IN A CIRCADIAN CLOCK. Michael A. Collett, Jay C. Dunlap and Jennifer J. Loros. Department of Genetics, Dartmouth Medical School, Hanover, NH 03755

To understand the role of *white collar-2* in the *Neurospora* circadian clock, and in photic entrainment of the clock, we examined the effect of a *wc-2* allelic series on these processes. One allele of *wc-2*, ER24, resulted in a lengthened period of the circadian clock, reduced expression of *frq* in darkness and altered temperature compensation. The temperature compensation defect correlated with an increase in overall FRQ protein levels with temperature, with the relative increase being greater in *wc-2* (ER24) than in wild type. We suggest that as temperature is elevated the increase in FRQ levels partially rescues the lowered levels of FRQ resulting from the defect in *wc-2* function, resulting in a shorter period at higher temperatures. We then examined light driven expression of *frq* in the *wc-2* allelic series. This revealed differential regulation of *frq* expression between light and dark. Additionally, we found that *frq* light signal transduction appears to be unique to *frq* expression but shares common elements of other *Neurospora* light signal transduction pathways, e.g., WC-2. Thus, both clock driven and light driven *frq* expression are differentially regulated by, but dependent on, WC-2. Normal activity of this essential clock component, a positive regulator of *frq*, is critical for photic entrainment and establishing both period length and temperature compensation in this circadian system.

To further understand regulation of *frq* transcript we have performed a selection for mutants with elevated levels of *frq* transcript (*elf* mutants). We have begun characterization of 18 arrhythmic *elf*'s isolated in this screen. It appears that at least eight of these are new alleles of *frq*. Details of the *elf* selection and characterization will be presented.

DROSOPHILA CRY IS A DEEP-BRAIN CIRCADIAN PHOTORECEPTOR

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cry (cryptochrome) is an important clock gene, and recent data reinforce the notion that it encodes a critical circadian photoreceptor in *Drosophila* (CRY). A mutant allele, *cry^b*, was previously isolated and shown to inhibit circadian photoresponses. To determine the contribution of different tissues to circadian photoreception, we restricted CRY expression to various tissues of wild-type and *cry^b* flies and tested different behavioral and biochemical photoresponses. Biochemical data suggest that CRY expression is needed in a cell-autonomous fashion for oscillators present in many different fly tissues. Consistent with this view, CRY overexpression only in the ventral lateral neurons - brain pacemaker cells- increases behavioral photosensitivity. This restricted CRY expression also rescues all circadian photoresponse and entrainment defects of *cry^b* behavior, and we show that wild-type ventral lateral neurons express CRY. The results indicate that these cells make a striking contribution to all aspects of behavioral circadian rhythms and that they are directly light-responsive. These brain neurons therefore contain an identified deep brain photoreceptor as well as the other two critical circadian elements: a central pacemaker and a behavioral output system.

THE ROLE OF CRY IN LIGHT-INDUCED DEGRADATION OF TIMELESS

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We recently showed that the *timeless*(TIM) clock protein undergoes degradation by the proteasome in response to light. Prior to its degradation TIM is phosphorylated on tyrosine residues and ubiquitinated. However, the mechanisms that transduce light to TIM are still unclear. In *Drosophila*, circadian photo-reception is mediated at least in part by the cryptochrome(*cry*) photoreceptor. In *cry^b* mutant flies, a point mutation in the flavin-binding site of CRY causes a defect in entrainment (Stanewsky et al., 1998). In addition, TIM oscillations are abolished in these flies in light-dark cycles, such that TIM levels remain high during the day. This indicates that light-induced TIM degradation is affected. In this study, we used an *in vitro* cell system to examine the role of CRY in light-induced TIM degradation. We found that *cry*, but not *cry^b*, increases TIM ubiquitination in a dose dependent manner. Overexpression of *cry*, however, downregulates ubiquitination perhaps because CRY acts as a dominant negative under these condition. After transducing the photic signal which leads to TIM ubiquitination, CRY itself is degraded by the proteasome.

EFFECTS OF A CRYPTOCHROME MUTANT ON OLFACTORY RESPONSES IN *DROSOPHILA MELANOGASTER*

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Circadian rhythms in *Drosophila melanogaster* are controlled by a system of feedback loops in expression of core clock genes *period* (*per*), *timeless*(*tim*), and *Clock*(*dClk*). *Drosophila* cryptochrome (*cry*) is involved in the synchronization of this feedback loop to the environmental light and dark cycle. *cry^b*, a mutant allele of the gene, disrupts rhythmic expression of *per* and *tim* in whole flies. However, there is no effect of *cry^b* on the locomotor activity rhythms. Behavioral rhythmicity in *cry^b* flies is maintained by molecular rhythms of clock genes in lateral neurons (LNs), which are the central oscillators governing locomotor activity rhythms. This suggests that the role of CRY may be more important in peripheral oscillators and rhythms that they govern.

We were interested in defining rhythms that emanate from peripheral tissues. We found one such rhythm in the electroantennogram (EAG) responses to odor cues. The olfactory rhythm shows a peak during subjective night and is abolished in *per⁰¹* and *tim⁰¹* mutant flies. To determine whether this peripheral rhythm is under the control of a central oscillator, we studied *per 7.2:2* transgenic flies which expresses *per* solely in the LNs and displays rhythmic locomotor activity. We found that olfactory rhythms were abolished in the *per 7.2:2* flies, suggesting that these neurons are not sufficient to drive olfactory rhythms. This indicates that olfactory rhythms require peripheral oscillators.

Having identified an output mediated by a peripheral oscillator, we can now address the question of whether CRY is required for the entrainment of EAG rhythms. We looked at the effects of the *cry^b* mutation under light-dark (LD) environment and constant darkness (DD). In LD, we observed the presence of a circadian rhythm in the olfactory responses. This weak rhythm is abolished when the flies are tested on the second day of DD. The amplitude of the EAG rhythm is intermediate between peak and trough in DD, indicating a lack of synchrony between oscillator cells. We are now characterizing molecular rhythms of clock gene expression in antennae to determine if there is an underlying lack of synchrony that is responsible for the EAG rhythms affected in the *cry^b* mutant.

PER-CRY INTERACTIONS INTEGRATE LIGHT INFORMATION TO THE CIRCADIAN PACEMAKER IN *DROSOPHILA*

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The *Drosophila* blue light receptor CRYPTOCHROME (CRY), is reported to interact with TIMELESS to derepress the CLOCK-CYCLE heterodimer which activates *period* and *timeless* circadian gene transcription. We show a strong physical interaction between CRY and PER, which is significantly weakened by the *per*^S mutation. Further reducing the strength of the PER-CRY association reveals a remarkable effect on the fly's circadian locomotor behaviour, suggesting that photoreceptor input (ocular and extra-ocular) converges on the PER-CRY dimer, which transmits the light information to the clock mechanism. Our findings suggest that CRY functions not only as a photoreceptor, but may also play a role in circadian timekeeping, as recently reported for the mammalian clock.

ANALYSIS OF CIRCADIAN BEHAVIOR AND SCN CELLULAR COMPOSITION OF *CLOCK* CHIMERIC MICE

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We produced chimeric mice containing both *Clock* homozygous mutant and wild-type (WT) cells to study how cells of different genotypes function within the suprachiasmatic nucleus (SCN) to produce behavioral rhythmicity in an intact animal. Tissue transplantation experiments, where SCN tissue from both WT and *tau* mutant hamsters were combined in single animals, do not find evidence for interaction of the two contrasting circadian periodicities associated with these genotypes (Vogelbaum & Menaker, 1992, *J. Neurosci.* 12:3619; Brokars et al., 1998, *Soc. Res. Biol. Rhythms Abstr.* 6:85).

We generated chimeras by aggregating *Clock* mutant and WT 8-cell embryos (Low-Zeddies & Takahashi, 1998, *Soc. Res. Biol. Rhythms Abstr.* 6:27). All tissues in each resultant chimeric individual, including the SCN, are populated by both *Clock* mutant and WT cellular genotypes in unique proportions and patterns, in a finely-interspersed array (Low-Zeddies & Takahashi, 1999, *Int. Cong. Chronobiol. Abstr.* 24).

Across a panel of *Clock* chimeras, in addition to WT and *Clock* mutant-like behavioral phenotypes, we see a variety of novel combinations of period, amplitude and phase-shift responses, not seen in either entirely mutant or entirely WT mice. Furthermore, given that these three circadian behavioral parameters are properties of single SCN cells (Welsh et al., 1995, *Neuron* 4:697; Liu et al., 1997, *Cell* 6:855; Herzog et al., 1998, *Nat. Neurosci.* 8:708; Liu & Reppert, 2000, *Neuron* 1:123), intermediate phenotypes that we observe suggest functional interaction between the *Clock* mutant and WT populations of cells within the SCN.

We present quantitative analyses of the relationship between period, amplitude and phase shift measurements across a spectrum of chimeric individuals. We also analyze the correlation between these behavioral measures and the proportion of the two cellular genotypes composing the SCN. By describing how components of circadian behavior vary relative to one another and relative to the cellular composition of the SCN we help to elucidate the functional organization of this pacemaker tissue.

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ACTIVATION OF ADENOSINE RECEPTORS IN THE SCN REGION BLOCKS THE PHASE SHIFTING EFFECTS OF LIGHT AND INHIBITS LOCOMOTOR ACTIVITY IN SYRIAN HAMSTERS

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Adenosine is an inhibitory neuromodulator found throughout the brain. It is known to interact with glutamate neurotransmission by strongly inhibiting the release of glutamate. Since glutamate is the neurotransmitter which conveys photic information from the retina to the SCN, we sought to examine whether adenosine would inhibit the phase shifting effects of light through direct action on the SCN. Adult male Syrian hamsters were implanted with guide cannula aimed at the SCN, placed in constant dark, and allowed to establish a stable free-running activity rhythm. Hamsters then received a microinjection at circadian time 20 directly into the SCN region, followed immediately by a 15 min, 50 lux light pulse. The injection consisted of 200nl of the following solutions in counterbalanced order: 0.9% saline (vehicle), N6-cyclopentyladenosine (N6CPA, A1 receptor agonist), or 5'-(N-Cyclopropyl)-carboxyamidoadenosine (5CCAA, A2 receptor agonist). A saline injection followed by a light pulse produced a large phase advance (approx 1 hr) which was completely eliminated by injection of N6CPA or 5CCAA. These data suggest that both A1 and A2 adenosine receptors are involved in modulating the phase shifting effects of light. In addition, these injections resulted in an acute suppression of locomotor activity and a "sleep-like" behavioral state. We propose adenosine as a candidate for mediating the inhibitory effects of sleep deprivation on light-induced phase shifts.

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CHARACTERIZATION OF SEROTONERGIC PATHWAYS AND RECEPTOR SUBTYPES IN THE CIRCADIAN SYSTEM OF LABORATORY RATS

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The mammalian circadian pacemaker in the suprachiasmatic nucleus (SCN) receives dense serotonergic projections both directly from the median raphe nucleus and indirectly from the dorsal raphe nucleus via the intergeniculate leaflet. In hamsters, serotonergic input to the SCN seems to be involved in the phase-resetting effects of non-photoc stimuli. However, recent studies performed in our lab suggest that in rats, serotonin (5-HT) mediates the effects of photic stimuli to the SCN. For example, microinjections of various 5-HT agonists, such as quipazine or 5-CT, close to the SCN induced photic-like phase shifts of locomotor activity as well as c-Fos expression in the ventrolateral area of the SCN during subjective night, but not during subjective day.

Recently, we investigated the effect of specific 5-HT agonists and antagonists in order to further characterize the pharmacological profile of the receptor subtypes mediating the effects of serotonin on the rat circadian system. These studies provided evidence for the involvement of a 5-HT_{2C} receptor subtype, because the phase-shifting effect of 5-HT was mimicked by specific 5-HT_{2C} agonists and was blocked by specific 5-HT_{2C} antagonists. In addition, our studies revealed that the photic-like effects of 5-HT depend on an intact optic nerve, because quipazine did not induce any phase shifts or c-Fos expression in blinded rats. These findings suggest that the photic-like effect of 5-HT may be mediated by presynaptic 5-HT receptors modulating transmitter release from other input pathways. However, more experiments with specific agonists and antagonists are required to further characterize the precise nature and location of the receptors involved.

Sponsored by Deutsche Forschungsgemeinschaft

- 155** **INTRA-RAPHE INJECTION OF METERGOLINE BLOCKS DRN ELECTRICALLY-STIMULATED 5-HT RELEASE IN THE SCN.** Lisa A. DiNardo and J. David Glass. *Department of Biological Sciences, Kent State University, Kent, OH 44242.*

Although there is a lack of direct serotonergic innervation from the dorsal raphe nucleus (DRN) to the suprachiasmatic nucleus (SCN) in hamsters, recent studies have demonstrated serotonin (5-HT)-like effects in the SCN following electrical stimulation of the DRN. In addition, our observation that systemic pretreatment with the general 5-HT_{1,2,7} antagonist metergoline blocks DRN-stimulated 5-HT release in the SCN suggests that the DRN contributes to 5-HT release in the SCN through a 5-HT responsive intermediate target possibly located within the midbrain raphe nuclear complex itself. The present study was undertaken to test this hypothesis by examining the effects of direct metergoline microinjections into the DRN or median raphe nucleus (MRN) on DRN-induced 5-HT release in the SCN using *in vivo* microdialysis. On the first day of testing, male Syrian hamsters received vehicle infusions (2 μ L DMSO) followed, on subsequent days, by metergoline infusions (2 μ L; 4 μ g) into the DRN or MRN 20 minutes preceding electrical stimulation of the DRN (20 min @ 150 μ A, 10 Hz, 2 msec pulse duration) at ZT 6. Infusion of metergoline into the DRN significantly blocked maximal DRN-stimulated 5-HT release (106.25 $\pm$ 2.57% of baseline) compared to vehicle controls (174.59 $\pm$ 21.52% of baseline; p <0.0001; n =5). Similarly, metergoline microinjection into the MRN suppressed maximal DRN-stimulated SCN 5-HT release (106.92 $\pm$ 6% of baseline) compared to vehicle controls (175.53 $\pm$ 20.8% of baseline; p <0.05; n =3). These results support the notion that a serotonergic projection from the DRN to intermediate target(s) localized within the midbrain raphe nuclear complex mediates DRN communication to the SCN. Moreover, our previous finding that systemic pretreatment with the 5-HT_{2,7} antagonist ritanserin, but not the 5-HT₂ antagonists ketanserin or cinanserin, blocks DRN-stimulated SCN 5-HT release implicates the involvement of a 5-HT₇-like receptor in this pathway. NIH GM19984 (L.A.D.) and NS35229 (J.D.G.)

- 156** **THE 5-HT_{1A,7} AGONIST 8-OH-DPAT DOES NOT INHIBIT NMDA-INDUCED PHASE ADVANCES IN SYRIAN HAMSTERS.** K. L. Gamble, E. M. Mintz, and H. E. Albers. *Lab of Neuroendocrinology and Behavior. Departments of Biology and Psychology. Georgia State University, Atlanta, GA 30303.*

The suprachiasmatic nucleus (SCN) of the hypothalamus is a key circadian pacemaker in the circadian time-keeping system in mammals. The SCN receives information regarding environmental light directly from the retina through the retinohypothalamic tract. Glutamate, the primary neurotransmitter of this pathway, activates NMDA receptors of the SCN. NMDA injected directly into the SCN induces phase shifts of the circadian activity rhythm that mimic the effects of light pulses (Mintz, et. al., 1999, *J Neurosci*). Furthermore, when the 5-HT_{1A,7} agonist 8-OH-DPAT is injected into the SCN just before a light pulse, light-induced phase advances are inhibited (Weber, et. al., 1998, *J Biol Rhythms*). The purpose of this study was to determine whether 8-OH-DPAT or 5-HT inhibits NMDA-induced phase advances. Male Syrian hamsters were housed in constant dark. Experimental groups received 0.2 μ l microinjections (directly into the SCN region) of either 100 μ M 5-HT or 100 μ M 8-OH-DPAT, each followed by a 0.2 μ l microinjection of 10 mM NMDA. The control group received a 0.2 μ l microinjection of vehicle followed by a 0.2 μ l microinjection of 10 mM NMDA. Circadian wheel running rhythms were measured before and after treatment. Neither 8-OH-DPAT nor 5-HT significantly altered NMDA-induced phase advances. These results suggest that serotonin inhibits the phase advancing effects of light by a pre-synaptic mechanism.

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TIMED *IN VIVO* MICRODIALYSIS PERFUSION OF THE SCN REGION WITH 5-HT AGONISTS PHASE-SHIFTS THE HAMSTER CIRCADIAN ACTIVITY RHYTHM J. Christopher Ehlen, Gregory H. Grossman and J. David Glass. *Department of Biological Sciences, Kent State University, Kent, OH, USA.*

Serotonin (5-HT) is strongly implicated in the non-photic entrainment of mammalian circadian rhythms, however the site(s) of this action are speculative. Although evidence exists for a phase-resetting action of 5-HT in the SCN, other sites have been implicated as targets for this action. Also, the concept of serotonergic phase-resetting as a whole has been challenged by findings that 5-HT antagonists do not suppress activity-induced phase-resetting. In the present study, reverse microdialysis was used as a new approach to evaluate SCN serotonergic phase-shifting response. This method offers the advantage of perfusing the SCN with 5-HT agonists for precise durations with minimal disturbance to the animal during drug delivery. This effect was evaluated in animals pretreated with the 5-HT synthesis blocker, p-CPA, to enhance 5-HT receptor sensitivity. Perfusion was performed in male hamsters outfitted with a reentry cannula aimed at the SCN. The probe was inserted 24 hrs prior to a 3 hr perfusion starting at ZT 6. Animals were released into constant

PRETREATMENT	SCN 8-OH-DPAT	CAUDAL TO SCN 8-OH-DPAT	I.P. 8-OH DPAT	SCN 8-OH-DPAT + I.P. RITANSERIN	SCN 8-OH-DPAT + LIGHT	SCN 5-HT	SCN VEHICLE
p-CPA	100±22 ^a (6)	46±8 ^{bcd} (6)	71±10 ^{abc} (6)	40±26 ^{bce} (6)	15±13 ^{def} (5)	60±12 ^{bc} (10)	-5±11 ^f (7)
VEHICLE	30±10 ^{bc} (7)	—	32±6 ^{bc} (6)	—	—	—	—

Values are phase-advance (mean±S.E.M. [mins]). Values with different letters are significantly different, $p < 0.05$. Numbers in brackets are n/group.

darkness at the start of perfusion, and phase-shifts were measured using an Aschoff type II procedure. The data show that p-CPA significantly increased the effects of i.p. and intra-SCN administration of the 5-HT_{1,7} agonist, 8-OH-DPAT. Response to intra-SCN 8-OH-DPAT was anatomically specific, as phase-shifting was significantly reduced when drug was perfused 2 mm caudal to the SCN. Suppression of 8-OH-DPAT shifting by the 5-HT_{2,7} antagonist, ritanserin, implicates involvement of the 5-HT₇ in the phase-resetting response. Finally, photic suppression of serotonergic phase-resetting appears to occur in the SCN region. Collectively, these data suggest that under appropriate conditions the SCN can respond to a direct phase-shifting action of 5-HT. NIH NS35229 (J.D.G.)

PHOTIC STIMULATION OF POLYSIALIC ACID AND ITS NCAM-180 CARRIER IN THE HAMSTER SUPRACHIASMATIC NUCLEI. Lenka Fedorkova, Lei Chen and J. David Glass. *Department of Biological Sciences, Kent State University, Kent, OH, USA.*

The adult SCN express a highly polysialylated form of neural cell adhesion molecule (PSA-NCAM) that promotes plasticity and regulates synaptic transmission in select regions of the adult brain. Enzymatic removal of cell surface polysialic acid (PSA) inhibits stimulus-induced morphological changes in glial-neuronal organization of the supraoptic nuclei, abolishes long-term potentiation in the hippocampal slice and significantly attenuates photic signaling in the SCN (Glass et al., 2000, *Neurosci. Lett.* 280:207-210). It is hypothesized that PSA-NCAM expression in the SCN serves to promote photic signaling and/or facilitate possible changes in cell-cell communication associated with plastic circadian phase-resetting responses. In the present study, western immunoblot and immunohistochemical procedures were used to examine the effects of a 30 min phase-advancing light flash (300 lux) from ZT 18.5-19.0 on the SCN contents of PSA, its NCAM-180 carrier and the other major NCAM isoforms (NCAM-140 and NCAM-120) that do not carry PSA in the adult SCN. Effects were assessed 1 hr after the end of the light pulse. Quantitative immunoblot analyses of SCN tissue punches revealed a large (2.8-fold) increase in PSA content relative to non-light pulsed controls ($p < 0.02$; $n = 12$). This was associated with a 1.9-fold increase in NCAM-180 ($p < 0.03$); however, there were no significant changes in NCAM-140 or NCAM-120 (both $p > 0.20$). Also, there were no significant light-induced alterations in expression of PSA or all NCAM isoforms assessed in hippocampal punches from the same animals (all $p > 0.37$). In a separate experiment, dual immunofluorescence labeling for PSA and Fos protein revealed a pronounced light-induced increase in extracellular PSA expression in SCN cells that was co-localized with a large proportion of cells expressing light-induced Fos immunoreactivity, as well as numerous cells not stained for Fos. Collectively, these results are strong evidence for an anatomically-specific stimulatory effect of light on SCN PSA-NCAM expression. Experiments are currently underway to determine whether this is a genomic or post-transcriptional response. Supported by NIH grant MH57034 to J.D.G.

DISTINGUISHING THE ROLES OF POLYSIALIC ACID AND NEURAL CELL ADHESION MOLECULE IN CIRCADIAN TIME-KEEPING FUNCTION IN THE MOUSE.

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The hypothalamic suprachiasmatic nuclei (SCN) are the primary circadian clock in mammals. The adult SCN express the neural cell adhesion molecule (NCAM) that carries polysialic acid (PSA), a carbohydrate polymer capable of modulating cell-cell interactions in neural tissues. The NCAM in the SCN has three main isoforms (180, 140, and 120 kD). Expression of these isoforms varies with developmental stages and physiological status in the adult. The present study was undertaken to assess the roles of the NCAM isoforms and PSA in SCN clock function by analyzing the circadian activity rhythms in three NCAM transgenic mouse lines with differing patterns of PSA and NCAM expression: (1) NCAM^{nos} (n=8) lacking NCAM and PSA; (2) NCAM^{tm1Cwr} (n=15) lacking NCAM 180, 140 and PSA in the adult; and (3) The NCAM^{tm2Cwr} (n=4) lacking NCAM 180. Locomotor activity was well entrained to a 12 hr light/dark cycle in NCAM^{tm1Cwr} and NCAM^{tm2Cwr}, but not in NCAM^{nos} mutants, where daily onset and offset of activity were irregular or even lost due to continuous bursts of activity. Under constant darkness, the free-running period was stable compared to wildtypes in the NCAM^{tm2Cwr} (24.05±0.13 vs 23.85±0.06 hrs in wildtype; p>0.05) but significantly shortened in the majority of the NCAM^{tm1Cwr} (23.50±0.09 vs 23.90±0.05 hrs in the wildtype; p<0.05) and 50% of the NCAM^{nos} mutants (23.7±0.03 vs 23.98±0.01 hrs in the wildtype; p<0.05). The remaining half of NCAM^{nos} mutants had aberrant rhythmicity throughout the circadian day. In the NCAM^{tm1Cwr} mice, duration of the active phase under DD was progressively increased until the third week rhythmicity was abolished. This analysis suggests that PSA and NCAM 140 are important in circadian time-keeping function, and that NCAM 180 is not essential in mutant mice where NCAM 140 assumes the role of primary PSA carrier. It is not possible at this time to rule out the possibility of developmental deficits contributing to the circadian abnormalities in the mutant mice. Supported by NIH Grant MH57034 to JDG.

THE ROLE OF CALBINDIN-D28K ON THE PHOTOENTRAINMENT MECHANISM OF HAMSTER SUPRACHIASMATIC NUCLEUS.

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In mammals, the suprachiasmatic nucleus (SCN) of hypothalamus has been shown to be a primary circadian pacemaker of locomotor activity and various physiological phenomena. We previously described a discrete subnucleus in the core of the hamster SCN containing Calbindin-D28K (CalB), positive cells. Cells in this subnucleus are Fos-positive in response to a light pulse (Silver et al., Neuroreport, 1996). Lesions of the CalB subnucleus result in loss of locomotor activity rhythms, even when other SCN components, marked by VIP- and VP- positive cells, are spared. Transplants of small SCN punches that contain CalB cells restore locomotor rhythms to SCN-lesioned host animals (LeSauter and Silver, J. Neurosci, 1999). Taken together, these studies suggest that cells in the region of the CalB subnucleus are necessary pacemakers for locomotor rhythmicity and these cells receive photic information.

Recent studies suggest that the period, cryptochrome, and clock genes have an important role in photoentrainment and circadian rhythmicity of locomotor activity in mammals. In order to explore the relationship between the CalB and period (Per) genes, we compared the expression pattern of CalB protein with Per1, and Per 2 mRNA in the SCN after a light pulse at CT 19. The expression of CalB protein in the SCN was not affected by light pulse, while light-induced Per 1 and Per 2 mRNA were strongly expressed in the CalB-positive region of the SCN.

These results suggest that light information may be transmitted via a CalB-Per signal transduction pathway.

PARAVENTRICULAR THALAMIC NUCLEUS ENTRAINS CIRCADIAN RHYTHMS AND MODULATES THE EFFECT OF LIGHT PULSES, IN RATS. Alberto Salazar-Juárez and Raúl Aguilar-Roblero. Departamento de Neurociencias, Instituto de Fisiología Celular, UNAM. MÉXICO

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A neural pathway from the paraventricular thalamic nucleus (PVT) to the SCN has been previously described, however its role in the circadian system remains to be established. The present work was aimed at studying the role of PVT in the synchronization of rhythmicity. In particular, we addressed whether stimulation of this nucleus is able to phase shift circadian rhythmicity, and whether PVT modulates phase shifts induced by light pulses.

In a first experiment two groups of 9 male Wistar rats were cannulated in the PVT in order to administrate 1.5 µg of glutamate in 1 µl of Phosphate Buffer Saline (PBS) or only PBS. Animals injected at CT 12 showed phase delays of -67.5 ± 8.01 min, while at CT23 showed advances of $+84.27 \pm 20.14$ min. No effects were found after vehicle administration. In a second experiment, animals with lesion of PVT or sham-lesioned (9 for each group), received 60 min light pulses (400 lux) at different circadian times (CT6, CT12 and CT23). No difference between sham- and PVT-lesioned animals was found after light stimulation at CT6 (25 ± 7.5 , 10 ± 13 min, respectively), while PVT-lesioned animals showed significantly shorter delays at CT12 (-135 ± 21 min, -85 ± 22.5 min; respectively) and longer advances at CT23 (103 ± 29 min, 143.33 ± 60 min, respectively; $F_{(5, 48)}=128$, post-hoc LSD $p < 0.05$).

Altogether present results suggest that PVT activity may phase shift the circadian oscillator independently from the RHT, and is able to modulate the response of the pacemaker to light pulses.

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PARTICIPATION OF A Rel/NF-κB TRANSCRIPTION FACTOR IN THE HAMSTER CIRCADIAN SYSTEM

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Light reaches the suprachiasmatic nuclei (SCN) through a glutamatergic pathway; several signal transduction mechanisms have been proposed as mediators of circadian entrainment, including activation of calcineurin, NOS and many others. On the other hand, immune factors (which could also be related to glutamate-calcium mechanisms) affect the sleep-wake cycle through specific cytokine-mediated pathways. NF-κB is a transcription factor related to the immune system, neuronal plasticity, apoptosis and many other responses. It is composed of two subunits, Rel A (p65) and p50; in the inactive form they are bound to an inhibitory subunit (IκB) which upon activation is phosphorylated and degraded. This transcription factor is activated by the rise of intracellular calcium, by the activity of protein kinases, by glutamate and by cytokines (such as IL-1 and TNFα), and can induce the expression of NOS, neurotransmitters, cytokines and several other genes. We have studied the presence and activity of Rel/NF-κB related proteins (p65, p50, IκB and Iκk) in the hamster circadian system analyzing running wheel activity, SCN immunohistochemistry, western blot, and electrophoresis mobility shift assay (EMSA). A small number of SCN neurons (42.6 ± 5.4 neurons per 20 µm slice) were immunoreactive for p65. The staining was cytoplasmic in all cells. The administration of pirrolidine-di-thio-charbamate (PDTC, 7 µM icv), an NF-κB activation inhibitor, significantly blocked the phase advance induced by a light pulse at CT 18 (vehicle+light pulse: 2.08 ± 0.46 hours, PDTC+light: 0.3 ± 0.35 hours). We also found specific immunoreactive bands corresponding to p65, p50 and IκB in SCN homogenates. The NF-κB activity showed by EMSA was clear and specific (i.e., was competed by κB sequences but not by SP-1 or CRE and a p65 antibody lowered activity in a super shift assay) in SCN nuclear extracts. This gel shift activity was higher during the night. These results demonstrate the presence of Rel/NF-κB family proteins in the hamster SCN and suggest that these proteins may be related to the entrainment and regulation of circadian rhythms.

RHYTHMICITY OF THE cGMP- RELATED SIGNAL TRANSDUCTION PATHWAY IN THE MAMMALIAN CIRCADIAN SYSTEM.

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Entrainment of mammalian circadian rhythms requires the activation of specific signal transduction pathways in the suprachiasmatic nuclei (SCN). The pharmacological inhibition of kinases such as cGMP-dependent kinase (PKG) or Ca^{2+} /calmodulin-dependent (CaM) kinase, or nitric oxide synthase (NOS), but not cAMP-dependent kinase (PKA) blocks the circadian responses to light *in vivo*.

In this study we have analyzed temporal variations in cGMP and PKG levels and PKG activity in the SCN *ex vivo*. We have also studied effects of PKG and guanylyl cyclase (CG) inhibition *in vivo*. cGMP exhibited diurnal (LD) and circadian (DD) rhythms with maximal values during the day or subjective day. This rhythm was dependent on phosphodiesterase but not on guanylyl cyclase activity. 5-min light pulses increased cGMP levels at CT18 but not at CT14. PKG activity and levels presented the same temporal profile as cGMP. Western blot analysis indicated that the PKG II isoform is the one present in the SCN. Inhibition of PKG or guanylate cyclase *in vivo* significantly attenuated light-induced phase shifts at CT18 (after 5-min light pulses) but did not affect c-Fos expression in the SCN. These results indicate that cGMP and PKG undergo diurnal and circadian changes and suggest that they may be involved in the signal transduction pathway for photic entrainment.

DIFFERENTIAL EXPRESSION OF PROTEIN KINASE C β I (PKC β I) BUT NOT PKC α AND PKC β II IN THE SUPRACHIASMATIC NUCLEUS OF SELECTED HOUSE MOUSE LINES.

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The functional significance of the suprachiasmatic nucleus (SCN) in circadian rhythm control of mammals has been well documented. The role of protein phosphorylation mediated by protein kinase C (PKC), however, is not well known. We report the immunocytochemical localization of three Ca^{2+} -dependent PKC isoforms (α , β I, β II) within the SCN of selected house mouse lines that differ in behavioral circadian rhythm parameters. The low-selected mice had more than three-fold higher PKC β I immunostaining in the SCN than the high-selected lines. The non-selected control lines were intermediate. PKC α and PKC β II immunostaining was not different among the lines.

The significance of these findings will be discussed in the context of differences among the lines in AVP and Fos expression in the SCN and behavioral phase-delay responses to 15-min light pulses in constant darkness. We conclude that the mice bidirectionally selected for nest-building behavior are a prominent model system for the investigation of the role of PKC β I, and the possible interactions of AVP and PKC α /PKC β I, in the regulation of circadian time keeping.

RAPID RESETTING OF THE FETAL CIRCADIAN PACEMAKER BY MELATONIN Xiaodong Li
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In rodents, the fetal circadian pacemaker is entrained by maternal rhythms. Prenatal entrainment is also likely to occur in humans beginning in the first trimester of pregnancy. Although the specific maternal rhythms that mediate prenatal entrainment have yet to be established, the pineal melatonin rhythm is a strong candidate. In hamsters, melatonin injections to pregnant hamsters predictably sets the phase of the pups' activity rhythms measured at weaning, and the fetal SCN expresses melatonin receptors. Previous studies in hamsters (Viswanathan and Davis, 1997) indicate that melatonin has strong resetting effects on the fetal pacemaker; single injections of melatonin given at opposite times of day to pregnant hamsters sets the phases of the pups' activity rhythms to opposite times of day. In addition, the average phase of the the pups' activity rhythms indicate that melatonin resets the fetal pacemaker to a nighttime phase. These results predict that melatonin rapidly resets a rhythm in *per1* expression in the fetal SCN and that low *per* expression (nighttime levels) will be set to the time of day when melatonin was given. To test this, single melatonin injections were given to two groups of SCN-lesioned pregnant hamsters at opposite times of day on the last day of gestation. Neonatal brains were collected at approx. 12, 24, and 36 hours after birth and processed for in situ hybridization to *per1* mRNA using a probe made from a PCR template amplified from a hamster cDNA library (courtesy of N. Gekakis and C. Weitz). Although the pups in the two treatment groups were of identical developmental ages, variation in *per1* mRNA abundance showed different temporal patterns. The differences indicate that *per1* rhythms had been set to opposite phases with low mRNA coinciding with the time of the melatonin injection in each group. Thus melatonin appears to strongly reset a rhythm in *per1* mRNA. The results suggest that melatonin rapidly drives the molecular rhythm to its nighttime phase and that the resulting shift of up to 12 hours is permanent. The fetal pacemaker appears to be highly susceptible to strong resetting by exogenous signals. (supported by NIH/NICHD grant HD18686 to FCD).

DISSOCIATION OF *mCry* AND *mPer* RHYTHMS IN THE SUPRACHIASMATIC
NUCLEI DURING TRANSIENT RESETTING TO LIGHT.

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The mammalian circadian clock housed within the suprachiasmatic nuclei of the hypothalamus is thought to consist, at the simplest level, of a number of autoregulatory negative feedback loops involving the *mCry* and *mPer* genes. The clock is reset by nocturnal light pulses. Phase delays occur rapidly (within 1 cycle) whereas the full expression of advances can take several cycles to be completed.

Experimental data, both *in vivo* and *in vitro*, suggest that *mCry* genes are central oscillator components insensitive to light, whereas *mPer1* and *mPer2* are up-regulated by resetting light pulses. Our own data indicate that mPER proteins are up-regulated by both delay and advancing pulses. In contrast, mCRY1 protein is not affected by advancing pulses, but its circadian decline is prevented by delaying pulses. In the absence of mRNA induction, we suggest that this represents stabilisation of existing mCRY, possibly by mPER (Field *et al.* (2000) *Neuron*, in press).

To examine the relationship between *mPer* and *mCry* cycles more closely, we have mapped the response of these genes to strongly advancing light pulses, which lead to transient resetting of the activity-rest cycle. The aim of this study is to test the hypothesis that transient resetting is a consequence of dissociation of the light-sensitive and light-insensitive coupled elements of the core oscillator.

167 NMDA RECEPTOR-MEDIATED PHOTIC INDUCTION OF PERIOD GENES IN THE HAMSTER SCN

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To clarify whether NMDA receptors mediate both photic induction of Period genes and behavioral phase shifts, we examined the effects of NMDA receptor antagonist, +MK-801 on Period transcript in the hamster SCN, utilizing in situ hybridization method, and on light pulse-induced phase shift of wheel-running activity rhythm.

First, we observed that +MK-801 attenuated light pulse (60 lux, 15 min) at CT13.5 or CT20 -induced phase delay or phase advance of activity rhythm, respectively, in a dose-dependent manner (0.1 – 5.0 mg/kg). Light at CT13.5 or CT20 elicited a rise in the level of Per1 and Per2 mRNAs in the SCN, and +MK801 suppressed the photic induction of Per1 and Per2 dose-dependently. On the basis of dose-dependent curve for inhibitory action of +MK801, there are positive correlation between amplitude of phase-shifts and Per gene mRNAs. Interestingly, an injection of +MK801 at CT1 suppressed circadian rise of Per1 and Per2 transcript at CT2 and CT4, especially in the ventrolateral portion of the SCN, while this compound failed to affect the phase of wheel-running rhythm.

These results suggest that the photic resetting of circadian clock is strongly associated with induction of Period gene via NMDA receptor activation in the SCN. We also demonstrated that NMDA receptors were involved in the circadian rises of Period genes transcript in the ventrolateral SCN although its functional role is unclear.

168 NON-PHOTIC ENTRAINMENT BY 5-HT_{1A/7} RECEPTOR AGONISTS ACCOMPANIED BY REDUCED *PER1* AND *PER2* MRNA LEVELS IN THE SUPRACHIASMATIC NUCLEI

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In mammals, the environmental light-dark cycle strongly synchronizes the circadian clock within the suprachiasmatic nuclei (SCN) to 24 hours. It is well known that not only photic but also non-photic stimuli can entrain the SCN clock. Actually, many studies have shown that a daytime injection of 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH DPAT), a serotonin 1A/7 receptor agonist, as a non-photic stimulus induces phase advances in hamster behavioral circadian rhythms *in vivo*, as well as the neuron activity rhythm of the SCN *in vitro*. Recent reports suggest that mammalian homologs of the *Drosophila* clock gene, *Period* (*Per*), are involved in photic entrainment. Therefore, we examined whether phase advances elicited by 8-OH DPAT were associated with a change of *Period* mRNA levels in the SCN. In this experiment, we cloned partial cDNAs encoding hamster *Per1*, *Per2*, and *Per3* and observed both circadian oscillation and the light responsiveness of *Period*. Furthermore, we found the inhibitory effect of 8-OH DPAT on hamster *Per1* and *Per2* mRNA levels in the SCN occurred only during the hamster's mid-subjective day, but not during the early subjective day or subjective night. The present findings demonstrate that the acute and circadian time-dependent reduction of *Per1* and/or *Per2* mRNA in the hamster SCN by 8-OH DPAT is strongly correlated with the phase resetting in response to 8-OH DPAT.

MELATONIN ADMINISTRATION ENTRAINS CIRCADIAN RHYTHMS OF TEMPERATURE AND ACTIVITY IN B6D2F₁ MICE EXPOSED TO CONSTANT LIGHT 169

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Endogenous circadian rhythms in body temperature and activity are suppressed in Sprague Dawley rats exposed to continuous light (LL), possibly as a result of an alteration of the melatonin (MLT) secretion rhythm. In this model, exogenous MLT restored temperature rhythm but had little effect on the rest-activity cycle (Deprés-Brummer *et al.*, *Am J Physiol* 1997; *Eur J Pharmacol* 1998). The B6D2F₁ mice have predominant secretion of MLT near Circadian Time (CT) 10 when they are resting (Li *et al.*, *Am J Physiol* 2000, *in press*). The ability to entrain circadian system function with exogenous melatonin was investigated in these hybrid mice exposed to LL. Sixteen male B6D2F₁ mice, aged 7 weeks, were first synchronized with LD 12:12 for 2 weeks, then exposed to LL until the end of the experiment. All the mice had a temperature and activity sensor implanted into the peritoneal cavity upon arrival (data every 10 min). After 2 weeks of LL exposure, mice received daily subcutaneous MLT (0.1, 1 or 10 mg/kg/day) or vehicle (4 mice per dose) at subjective CT 10 for 3 weeks. Spectral analysis (Fourier transform) was performed with Mathcad. LL lengthened the period of both rhythms as compared to LD 12:12 (25.6 ± 0.2 h vs 24 h). Despite daily vehicle treatment, no entrainment of either rhythm was found in controls ($\tau = 25.7 \pm 0.4$ h). Conversely, MLT entrained the temperature rhythm to 24.0 ± 0.1 h and produced a significant increase in circadian amplitude without any significant dose-related effect. The rest-activity cycle was synchronized with MLT in 10 of 12 mice, thus it was less susceptible to MLT entrainment than body temperature rhythm. Entrainment occurred when MLT injection coincided with the onsets of activity and temperature rise and was maintained until MLT withdrawal. A dominant period of 26.2 h reoccurred when MLT was withdrawn in all the mice kept in LL. Thus daily MLT injections entrained circadian rhythms of temperature and activity in B6D2F₁ mice exposed to LL. MLT entrainment was stronger for temperature rhythm than for rest-activity, an effect earlier reported in rats, which suggests a differential effect of MLT on the oscillators which generate these rhythms.

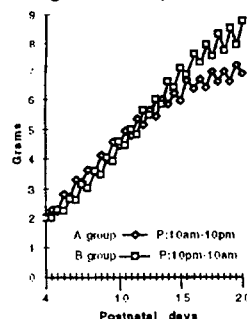
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DAILY RHYTHM IN THE BODY WEIGHT OF C57BL/6J PUPS DURING PRESENCE AND ABSENCE CYCLES OF THE MOTHER MOUSE 170

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Presence and absence (PA) cycles of mother mouse (C57BL/6J) entrain the circadian activity rhythm of pups. The mechanism causing the PA cycles entrainment is not known. It is possible that the mother's milk may contain the entraining (ingestive) cues and the cyclic availability and non-availability of milk during mother's PA cycles may entrain the pup's rhythm. The present study was conducted to examine whether there is any body weight gain at the end of the 12-hr mother's presence period i.e., after milk consumption. The mother and the litters were maintained in LD 14:10 (Light on at 6AM and off at 8PM). The size of the litters was 6-8. The litters were divided into two groups (A and B) of 3-4 pups in each group on postnatal day 4. Each group experienced 12:12 hr of PA cycles (A group 10AM-10PM; B group 10PM-10AM) starting from postnatal day 4 through postnatal day 17-20. The body weight of each group was measured at 10 AM and 10 PM. It was found that average body weight showed a daily rhythm with high body weight at the end of presence period (high body weight for A group was at 10 PM and for B group was at 10AM- See figure below).

The results suggest that the pups growth/body weight gain occur during the period of mother's presence when milk is available to the pups and milk may contain the cues causing the PA cycles entrainment. Moreover, the body weight gain of the group that experienced the mother during 10PM-10 AM was relatively greater than the group that experienced the mother during 10AM-10 PM. This result suggests that delivery of milk by the mother is rhythmic and more milk is delivered to the pups during early morning/rest hours of the mother than other timings since these mothers were entrained to 14:10 LD cycles. Supported by NICHD award HD 33805 to NV.



171 USE OF FOS INDUCTION TO ASSESS SPECTRAL SENSITIVITY OF THE MOUSE CIRCADIAN SYSTEM

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In mammals, the 24 hour period of the endogenous pacemaker in the suprachiasmatic nucleus (SCN) is synchronized to the external light-dark cycle through photic information conveyed from the retina. Previous behavioral studies in the mouse and hamster have shown an action spectrum with a peak of around 500 nm, implying the involvement of a rod or an M (green) cone opsin. However, photic entrainment of activity rhythms in transgenic rodless and/or coneless mice has suggested (1) neither rods or cones are required for the response and (2) possible input from other non-classical photopigments (melanopsin, cryptochromes). We examined the spectral sensitivity of the circadian system in adult wild-type mice (C57BL) using the amplitude of Fos expression in the SCN as an assay. The mouse retina contains a rod-opsin (498 nm), a short (UV, 370 nm) and amid (M, 509 nm) wavelength cone opsin. Mice were exposed to monochromatic light at wavelengths ranging from 365-620 nm. Total optical density of Fos immunoreactivity in the SCN was quantified using an image analysis system. The results show a peak of sensitivity in the region of 500 nm. The shape of the action spectrum from 420-620 nm corresponds to that of an 11-cis-retinaldehyde based photopigment (rod or M cone). However, the results also show an increase of sensitivity in the UV region of the spectrum, which could suggest participation of a short wavelength photopigment (UV cone or cryptochrome). The presence of two separate peaks in the short- and long-wavelength regions of the spectrum favors the hypothesis that more than one photopigment is involved in circadian photoreception.

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172 RETINAL INPUT TO THE VENTROLATERAL PREOPTIC AREA (VLPO) AND THE MODULATION BY LIGHT OF FOS EXPRESSION IN THE VLPO OF NOCTURNAL AND DIURNAL RODENTS.

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There is not much known about how neural correlates of circadian rhythms differ between nocturnal and diurnal mammals. In the nocturnal rat, *Rattus norvegicus*, Fos expression in the sleep-active ventrolateral preoptic area (VLPO) shows a daily rhythm over a 12:12 light-dark (LD) cycle with highest levels of Fos expression occurring during the light period. Under the same lighting conditions, in the diurnal murid rodent, *Arvicanthis niloticus*, Fos expression in the VLPO is highest at night. The VLPO may have a similar role in sleep in nocturnal and diurnal animals, although the periods of activity are out of phase. The VLPO receives direct retinal projections in both *A. niloticus* and *R. norvegicus*. The first goal of this study was to quantify the density of retinal projections to the VLPO in these two species using eye injections of cholera toxin. *R. norvegicus* had a significantly higher amount of retinal inputs to the VLPO when compared to *A. niloticus*, with no species differences in other areas (paraventricular nucleus of the hypothalamus, upper and lower subparaventricular zones, supraoptic nucleus, centromedial thalamus, and paraventricular nucleus of the thalamus). The second goal of this work was to assess the effects of light on rhythms of Fos expression in the VLPO of the two species. *R. norvegicus* and *A. niloticus* were kept in a 12:12 LD cycle and perfused at 6 time points over a 24 hour period. Another group of *R. norvegicus* and *A. niloticus* were kept first in the same LD cycle and then released into constant dim red light (DD) and perfused at the corresponding circadian time points. In *A. niloticus*, Fos expression was elevated during the night (in LD) and subjective night (DD) with no reduction in the amplitude of the rhythm in the absence of a LD cycle. However, in rats, the rhythm of Fos expression showed a reduced amplitude in animals kept in DD. These results raise the possibility that light differentially affects the activity of the VLPO in rats and *A. niloticus*. This difference may be related to the amount of retinal input to the VLPO typical of each species. The VLPO may integrate circadian, homeostatic and photic signals that affect the sleep-wake cycle and its relation to the illumination cycle. Supported by: MH 11232, MH 53433 and IBN 9514374.

WHEEL RUNNING INDUCES ELEVATED FOS EXPRESSION IN NEUROPEPTIDE-Y CELLS OF THE INTERGENICULATE LEAFLET OF *ARVICANTHIS NILOTICUS*. Joshua P. Nixon and Laura Smale, Department of Zoology, Michigan State University, East Lansing, MI 48824.

Arvicanthis niloticus is a diurnal murid rodent species in which a subset of individuals displays a nocturnal activity pattern when given access to a running wheel. A previous experiment demonstrated a difference in Fos labeling of neuropeptide-Y (NPY) cells in the intergeniculate leaflet (IGL) of diurnal and nocturnal *Arvicanthis*. Specifically, the proportion of NPY cells that expressed Fos was higher during periods of wheel running than when animals were inactive. This was the first salient difference in neural function shown between the nocturnal and diurnal subsets of *Arvicanthis*. The current study focused on diurnal animals in an attempt to determine whether access to a wheel induces Fos expression within NPY cells in the IGL. Adult male *Arvicanthis* that had been classified as diurnal with respect to wheel running (n=12) were housed with running wheels in a 12:12 light-dark (LD) cycle. After three weeks, six animals had wheels removed; cage lids were changed on the other six. All animals were perfused at CT 4, five days after wheels were removed. IGL sections were stained for Fos and NPY. The percent of NPY cells that expressed Fos was significantly higher in animals housed with wheels (mean=22.3±12.3%) than in animals whose wheels were removed (mean=0.6±0.9%; t=4.396, df=5, p=0.007). This indicates that the high levels of Fos expression within NPY cells in the IGL are caused by wheel running. This also suggests that the differences previously seen between nocturnal and diurnal animals with respect to Fos expression in NPY neurons may be caused by differences in the times at which they run in the wheels.

LIGHT-INDUCED FOS EXPRESSION IN THE SUPRACHIASMATIC NUCLEUS OF *ARVICANTHIS NILOTICUS*. Megan M. Mahoney¹ and Laura Smale^{1,2,3}

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The mammalian suprachiasmatic nucleus (SCN) is the neural site for the generation and synchronization of circadian rhythms. It is not yet known if the SCN is responsible for differences in rhythms of nocturnal and diurnal species. In this study we characterized the light-induced Fos response of SCN cells in the diurnal rodent *Arvicanthis niloticus*. Adult males (n=48) were transferred to constant darkness for varying intervals. Experimental animals were pulsed for 1 h with a white fluorescent light (550-650 lux) and then perfused. Pulses were given at circadian times (CT) when light either has no effect on circadian rhythms (CT 4), or induces phase delays (CT 13), or phase advances (CT 21) in this species. Control animals remained in constant darkness and were perfused at corresponding times (n=7-8 /group). Tissue was labeled for Fos-immunoreactivity (IR) and the numbers of Fos positive cells were counted in the SCN. There was a significant effect of light (P<0.001), time of sacrifice (P<0.001), and an interaction between these two variables (P<0.001) on the number of Fos-IR cells in the SCN. Specifically, light pulses induced an overall increase in Fos-IR during the subjective night but not subjective day. In conclusion, the SCN of *Arvicanthis* is similar to that of nocturnal rodents in that light increases Fos-IR during phases when it also shifts activity rhythms.

PHASE RESPONSE TO LIGHT PULSES OF RATS BEARING SCN GRAFTS. Ivette Caldelas and Raúl Aguilar-Roblero. Departamento de Neurociencias, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, MÉXICO.

The mammalian circadian system is constituted by several circadian oscillators. However, only the Suprachiasmatic Nucleus (SCN) has been yet identified, which has restrained the study of coupling among circadian oscillators. We have grafted fetal SCN tissue into intact animals as a model to learn about the coupling interactions between the host and the grafted SCN.

In order to determine the effect of the graft in the phase responses to light of the host, 24 male intact Wistar rats with SCN grafts in the third ventricle, received brief light pulses (1 h, 400 lux) during the subjective night at CT13 and at CT23, and the behavioral phase shifts were estimated. Also, we assessed the photic responses at the cellular level by immunohistochemical detection of *c-Fos* and *Jun-B* proteins. Our data suggest that SCN grafts affect the phase response to light of the host SCN mainly during the phase advance zone (CT 23), by inducing phase shifts with opposite direction and increasing the number of transitory cycles. We also observed *c-Fos* and *jun-B* expression in the grafted SCN, but only in the region where VIP immunoreactive cells and fibers were detected. These findings indicate that the SCN grafts affect the phase responses to light of the host SCN at behavioral level, while the host seems to influence the grafted SCN at the cellular level. Present results suggest that both circadian pacemakers were coupled.

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RAPID CIRCADIAN CLOCK RESETTING BY SLEEP DEPRIVATION AND ITS INHIBITION BY CAFFEINE. M. C. Antle, N.M. Steen, R. E. Mistlberger, Dept. Psychology, Simon Fraser University, Burnaby BC V5A 1S6, Canada.

Circadian rhythms in Syrian hamsters can be phase shifted by continuous locomotor activity ('exercise') during the usual sleep period. We have recently shown (Mistlberger et al, Soc. Neurosci. Abstr., 1999) that comparably large phase shifts (up to 240 min advances) of the rest-activity cycle can be induced using the sleep deprivation (SD) procedure of gentle handling, with minimal locomotion, from ZT6-9 (Aschoff Type II method of assessing phase shifts). We have now used the circadian rhythm of light-induced *c-fos* expression in the suprachiasmatic nucleus (SCN) as a phase marker to further demonstrate that shifting is accomplished within 1-h following a 3-h SD. In the absence of light, SD reduced Fos expression in the SCN, and increased Fos expression in the intrageniculate leaflet, thus mimicking the effects of exercise on Fos in two components of the circadian system (Mikkelsen et al, 1998). Finally, caffeine (75 mg/kg, i.p.) at ZT6 induced behavioral arousal without phase shifts, and dose-dependently blocked phase shifts to 3-h of exercise, by a mechanism independent of adenosine A1 receptors. Behavioral arousal with or without exercise can significantly alter circadian phase, and a widely used drug that promotes arousal can block this effect. *Supported by NSERC, Canada.*

14 HOUR PHASE SHIFTS IN *TAU* MUTANT HAMSTER IN RESPONSE TO A DOUBLE LIGHT PULSE.

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The suprachiasmatic nucleus (SCN) of wild type hamsters in constant darkness (DD) phase shifts by about 1.5 h in response to a light pulse at CT 14. Double pulse experiments with light pulses 2 h apart result in a phase delay of 2 h that can be measured the next day (Best et al. J Neurosci. 1999). The *tau* mutant hamster housed long term in DD phase delays ~ 6 h in response to a light pulse at CT13, and phase advances ~ 5 h to a light pulse at CT18 (Shimomura and Menaker, 1994). We asked whether the *tau* mutant hamster can rapidly achieve large phase delays in response to suitably timed double light pulses (CT13 & CT18). If resetting to an initial delay pulse occurs rapidly, then the second pulse given 5 h later should produce additive delay responses. If resetting to the first pulse is not rapid, a light pulse given 5 h later should produce a phase advance, and the overall response should be a short delay. *Tau* mutant hamsters (N=7) kept in DD for 7 weeks received a 1 h light pulse at either CT 13, or at CT 18, or a double 1 h light pulse at CT 13 and 18 in random order. The light pulse given at CT13 produced a 7.8 ± 1.1 h delay; at CT 18, a 2.4 ± 0.6 h phase advance. The double light pulse at CT13 and 18 produced a 14.2 ± 1.2 h delay.

We next explored whether the foregoing phase delays can be measured directly in the SCN, using c-fos immunoreactivity. Control animals received a single light pulse at CT21 or CT05. Experimental animals received a 1 h light pulse at CT14 to phase delay the animal by ~8 h. A second light pulse was given at a time when c-fos would be high if the animal had phase shifted (~CT21), and low if the animal had not phase shifted (CT05). The latter produced substantial c-fos immunoreactivity equivalent to that of control animals at CT 21, indicating that a large delay shift had occurred.

In summary, the behavioral results show that in response to a suitably positioned double light pulse, the mutant hamster displays a dramatic phase delay of 14 circadian hours, measurable the next day. The high levels of fos-ir in the SCN of the mutant hamster indicate that the delay has occurred in the SCN.

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ANTICIPATION OF LIVER METABOLISM IN RATS ENTRAINED BY RESTRICTED FEEDING SCHEDULES.

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Restriction of daily food intake entrains several behavioral and physiological circadian rhythms. This process depends from a time keeping system independent of the SCN, however the identification of the food entrained oscillator (FEO) has been elusive. The liver plays an important role in metabolic balance since the main nutrients are metabolized by the hepatic tissue depending on the anabolic or catabolic status of the organism. The hepatic function is mainly influenced by pancreatic hormones and autonomic signals. The present study was aimed to characterize regulatory parameters of liver metabolism in rats maintained under restricted feeding schedules (RFS). In addition, the temporal kinetics of insulin, glucagon and corticosterone was determined under the same conditions. Wistar rats (225-250g) maintained under RFS for three weeks (food access 12-14 h) and their ad libitum controls were sacrificed at random at 9, 10, 11, 12, 14 and 18 h by a blow on the back and liver was removed immediately and frozen. Other rats were sacrificed by decapitation, trunk blood was collected and serum was stored for hormonal determinations with standard ¹²⁵I Radioimmunoassay Kits. Liver lactate, pyruvate, β -hydroxybutyrate and acetoacetate indicated an oxidized cytoplasmic and mitochondrial redox state potential, which was normalized in both compartments after feeding. An important increase of ATP and ADP was observed before feeding, energy charge showed no significant modification, but it showed an important decrease after food intake. Rhythmicity of the three hormones was entrained by RFS. These observations suggest an "anticipatory activity" of the liver metabolism, possibly in order to optimize the processing of nutrients, they also suggest an important involvement of the liver as constituent of the FEO.

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- 179** **PHASE SHIFT OF SERUM CATECHOLAMINES RHYTHM BY RESTRICTED FOOD ACCESS IN RATS.** JL Chávez-Juarez¹, MT Martínez-Merlos², R Aguilar-Roblero¹ and C Escobar².
¹Depto. Neurociencias, IFC and ²Depto. Anatomía, Fac. Medicina, UNAM. MEXICO.

Restricted feeding schedules produce behavioral activation in anticipation to food access, as well as entrainment of metabolic parameters related with energy balance. Storage and mobilization of energy metabolites depend on endocrine and autonomic signals. Previous reports have described entrainment of pancreatic hormones under RFS, however the involvement of autonomic signals is unknown. The present study was designed to describe the rhythmicity of serum catecholamines in rats maintained under RFS and their ad libitum controls.

Adult Wistar rats were maintained under restricted food access from 12 to 14 h (RFS) or fed ad libitum (CON) during 3 weeks. Rats were beheaded at 3 h intervals to complete a 24 h cycle. Trunk blood was collected, serum was obtained and the acid extract added with glutathione-SH was frozen until processed. Serum catecholamines were separated by HPLC by means of a μ Bondapak C-18 column and quantified with a fluorescence detector (285-325 nm). The corresponding chromatograms were analyzed with the Millenium software (Waters).

Serum adrenaline showed a clear rhythm in CON rats with lowest values from 21 to 24 h. RFS rats showed a rhythm associated to food access, with a trough before and a peak after feeding. The rhythm in serum noradrenaline was evident in both groups, but the peak in RFS rats was advanced with respect to CON animals occurring just after food access. These data indicate that restricted food access entrains the rhythm of serum catecholamines in intact rats.

This study was supported by DGAPA IN-206697 and CONACyT LN0024-N9607

- 180** **FEEDING-ENTRAINED CIRCADIAN RHYTHMS ARE ATTENUATED BY LESIONS OF THE PARABRACHIAL REGION IN RATS.** A. J. Davidson, S. L. T. Cappendijk, and F. K. Stephan. Department of Psychology, Florida State University, Tallahassee, FL, 32303-1270.

Rats anticipate daily restricted meals with increased approaches to a feeder and an increase in core body temperature. Food-anticipatory activity (FAA) is thought to be under the control of a feeding-entrained circadian oscillator (FEO). Although numerous forebrain lesions have failed to permanently abolish FAA, the hindbrain has not yet been investigated. The parabrachial nuclei (PBN) integrate information from visceral and gustatory afferents. This region is also innervated by neurons in the area postrema that have access to the peripheral circulation. Therefore it is possible that this region plays a role in triggering FAA. In two experiments, a total of 19 rats were given ibotenic acid or electrolytic lesions targeted at the PBN. The PBNx subjects showed a marked attenuation in anticipatory approaches to the food bin relative to sham-operated controls. Some subjects did not anticipate the meal at all. In addition, it was shown that the expected increase in core body temperature was severely attenuated in the PBNx subjects when compared with controls. The most likely interpretation of these data is that the PBN serve as a relay for information about the zeitgeber (food in the gut) or as a clock output pathway, but not as the site of the FEO.

IMMUNOREACTIVITY FOR IMMEDIATE-EARLY GENE PROTEINS AND OREXIN IN MOUSE BRAINS AFTER FOOD RESTRICTION AND REFEEDING. Marleen H.M. de Groot¹, Elliott G. Marchant¹ and Benjamin Rusak^{1,2}. Departments of Psychology¹ and Psychiatry², Dalhousie University, Halifax, Nova Scotia, Canada B3H 4J1.

Restricted daily access to food can entrain a circadian pacemaker that is separate from the light-entrainable pacemaker in the rodent suprachiasmatic nucleus (SCN). The identity of this food-entrainable pacemaker and the nature of the afferent signals that affect it are not known. There has been recent interest in the role of orexins in the regulation of food intake and in the use of immediate-early gene (IEG) proteins as markers of neural activation. We used immunocytochemical methods to compare the patterns of immunoreactivity for the IEGs c-Fos and JunB and for Orexin A protein in the brains of male C57Bl/6j mice that were maintained on a restricted feeding schedule for 17 days. We observed strong increases in IEG expression in a number of brain nuclei in mice that were refed during their scheduled feeding time as compared to those that were killed just before an anticipated feeding opportunity. The results will also be compared to those obtained from groups of mice that were allowed to feed ad lib, were acutely food deprived for 20 h, or were refed after 20 h of deprivation. We will also report on behavioral differences among these groups and discuss the relevance of these results to identifying inputs to the food-entrainment system.

RESTRICTED FEEDING INDUCES THE ANTICIPATORY EXPRESSIONS OF MPER GENES MRNA IN THE CEREBRAL CORTEX AND HIPPOCAMPUS IN MICE

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There are two entrainment stimuli of circadian rhythm, restricted feeding and light exposure. Light-induced expression of *mPer1* in the suprachiasmatic nucleus (SCN) closely correlated with the light-induced entrainment of locomotor activity rhythm. Although *mPer1*, *mPer2* and *mPer3* express not only in the SCN but also other brain tissues, the function of these genes outside of the SCN has been poorly understood.

To better understand the role of these genes in food-entrainment rhythm, we examined the effect of restricted feeding (RF) on the expression of mouse *mPer1* and *mPer2* mRNA in the various brain area of mice. The expression of the *mPer* genes in the cerebral cortex and hippocampus showed a clear nocturnal rhythm in contrast to that in the SCN. After 6 days of scheduled-RF, mice were allowed access to food for 4 hours in the daytime, *mPer1* and *mPer2* mRNA levels were increased in the cerebral cortex and hippocampus but not in the SCN, striatum and piriform cortex in the daytime without feeding. This anticipatory increase of *mPer* expression was still observed in SCN-lesioned mice, although nocturnal rhythm of *mPer* expression in cerebral cortex was disappeared in these lesioned animals.

The present results support that food-entrainment circadian rhythm is independent of the SCN, and demonstrated that expression of *mPer1* and *mPer2* in the cerebral cortex and hippocampus are well correlated with the anticipatory activity in mice. Therefore, these brain areas may be involved in food-entrainable.

IS DAILY ADRENALINE ADMINISTRATION AN ENTRAINMENT SIGNAL FOR BEHAVIORAL CIRCADIAN RHYTHMICITY IN RATS? Jorge Mendoza¹, José Ávila¹, Raúl Aguilar-Roblero² and Carolina Escobar¹. ¹Depto. de Anatomía, Fac. de Medicina, ²Depto. de Neurociencias, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México.

The anatomical and functional properties of the food entrained oscillator (FEO) are not well understood. The identification of its entraining pathway may lead to further understand its functional characteristics. Since time of food access is the main entraining signal for FEO, metabolic parameters elicited by food intake may be its internal entraining signals. Food ingestion elicits an immediate increase of sympathetic activity among diverse metabolic changes. The present study explored the possible role of adrenaline as an internal time signal for the organism. Adult Wistar rats weighing 250-300g at the beginning of the experiment were maintained in a soundproof chamber under constant dim red light and controlled temperature (20°C). Drinking behavior was individually monitored with an automatic drinking device, and data were collected and analyzed with the program Dispac. Rats were left in free running conditions for two weeks and were randomly subjected either to vehicle (saline, 1ml/kg) or to adrenaline (12.5 µg/kg; in 1ml/kg) daily injection for 12 days. Subsequently they were left unperturbed for 10 days and were subjected to other 12 days injection in a counter balanced design. A third control manipulation was performed by handling the animals daily for the same 12 days. None of the manipulations modified the free running component. Adrenaline produced increased drinking behavior in anticipation to daily injections, while vehicle produced increased drinking behavior in response to the injection. Both responses varied according to the phase relation with the main free running component and persisted for 2-3 cycles after concluding the treatment. Anticipation observed by daily adrenaline injections suggest its possible function as time signal. Further manipulations are needed to evidence its relation to FEO.

This study was supported by DGAPA IN-206697.

GASTROINTESTINAL TRACT MELATONIN RELEASE IN FASTED INTACT AND PINEALECTOMIZED ZEBRA FINCHES (*TAENIOPYGIA GUTTATA*). Thomas J. Van't Hof, Research Center for Ornithology of the Max-Planck Society, 82346 Andechs, Germany; (Fax: 049-(0)8152-373133) Email: vanthof@erl.ornithol.mpg.de

The phenomenon of food-entrainable rhythms and the role of gastrointestinal tract (GIT) melatonin remains unsolved. I examined the relationship between the presence of food in six anatomical sections of the GIT and melatonin levels in the respective tissues overnight and during fasting in intact and pinealectomized birds. Melatonin levels in tissues were measured by RIA using an assay system validated for use with tissue samples. A subset of samples was also validated with HPLC. Birds were kept on a 12:12 LD regime. At mid-point in the dark phase the majority of the melatonin found in the GIT is of pineal origin and pinealectomy abolishes the diurnal rhythm (Van't Hof and Gwinner 1999. J. Biol. Rhythm 14: 185-189). Overnight, melatonin levels increased significantly in GIT tissues from 10-100 pg/gm at the mid-point in the dark phase to more than 1000 pg/gm just before lights on in pinealectomized birds. Levels of melatonin in the plasma, heart, liver and muscle remained unchanged and were either at or below the detection limit of the assay. In birds that were fasted an additional five hours after lights on, melatonin levels increased even further to >10 ng/gm in GIT tissues and declined precipitously and significantly after food was provided. Levels of melatonin remained unchanged in the plasma, heart, liver and muscle tissues. The response to fasting was attenuated in fasted intact birds as compared with pinealectomized birds. Melatonin levels were significantly lower in fasted intact birds than in pinealectomized birds in the first 2 hours of fasting after lights on than after 5 hours of fasting. These results indicate that melatonin levels increase throughout the night and decrease when food enters the GIT suggesting that the duration of bouts between feeding regulates melatonin release. They also indicate that the pineal gland influences the release of melatonin in the gut and confirms suspicions that the GIT has the metabolic capacity to synthesize melatonin like that of the pineal gland.

EVIDENCE OF ARTIFICIALLY ACCELERATED AGING IN THE SUPRACHIASMATIC NUCLEUS OF THE GRAY MOUSE LEMUR (*MICROCEBUS MURINUS*).

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In mammals, the suprachiasmatic nucleus (SCN) contains a circadian pacemaker that regulates a variety of physiological and behavioral rhythms. This circadian organization, including the response to environmental stimuli, is dramatically altered in aged rodents, but the underlying causes of these changes remain unknown. In the gray mouse lemur (*Microcebus murinus*), a prosimian primate, survival and longevity can be affected by long-term acceleration of seasonal rhythms (up to 2.5 times the natural photoperiodic regimen), providing a model for assessing various aspects of aging. As a first step towards understanding the effect of aging on the circadian pacemaker of this primate, we measured the light-induced expression of the immediate early gene *c-Fos* in the SCN of young and aged mouse lemurs. Aged animals had been exposed to different photocycles, and their age category was defined according to either their biological (in seasonal cycles) or their chronological age (in years). Light-induced *Fos* expression in the SCN was studied by exposing the animals to a 15-min monochromatic pulse of light (500 nm, irradiance of 10^{11} or 10^{13} photons/cm²/s) at CT 14. Control animals remained in darkness. The amplitude of *Fos* expression was determined by measuring the total optical density of *Fos* immunoreactivity in the SCN using an image analysis system. *Fos* expression following exposure to low levels of irradiance was reduced by 88% in the SCN of aged mouse lemurs compared to young controls. Animals submitted to an artificially accelerated aging demonstrated the same pattern, with a reduction of 72% in *Fos* expression compared to young animals. Exposure to higher irradiance levels showed similar results, with a reduction in *Fos* expression in the SCN of 66% in case of normal aging and 57% in case of accelerated aging. These results suggest an age-related reduction in responsiveness to light by the circadian pacemaker in mouse lemurs and provide new insights into the mechanisms underlying artificial accelerated aging at the level of the molecular machinery of the biological clock.

CIRCADIAN RHYTHMS OF PLASMA DHEAS AND CORTISOL IN YOUNG AND OLD FEMALE RHESUS MACAQUES. Garyfallou, V.T. and Urbanski, H.F. Division of Neuroscience, Oregon Regional Primate Research Center, 505 N.W. 185th Avenue, Beaverton, OR 97006.

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The adrenal steroid, dehydroepiandrosterone sulfate (DHEAS), is recognized as being an important biomarker of aging in humans and nonhuman primates. However, it is unclear whether DHEAS has a robust circadian pattern of release like cortisol, another adrenal steroid. To address this issue, 24-hour plasma profiles of DHEAS and cortisol were examined in young (4-7 years) and old (21-23 years) female rhesus macaques (*Macaca mulatta*), maintained under a 12L:12D light-dark cycle. Each animal was fitted with a subclavian vascular catheter which was connected via a swivel-tether system to a remote blood sampling port. This set-up enabled serial blood samples to be collected at hourly intervals across the day and night without perturbing the animals' sleep-wake cycle; the plasma samples were subsequently assayed for DHEAS and cortisol using radioimmunoassay. In the young animals, the plasma levels of both cortisol and DHEAS rose gradually during the night and peaked around the time of activity onset in the morning, just before lights *on* (cortisol peak: ~230 ng/ml; DHEAS peak: ~70 ng/ml). Both hormone levels then gradually fell during the daytime and reached a nadir approximately 12 hours later (at lights *off*). The old animals showed a circadian pattern of plasma cortisol that was qualitatively similar to that shown by the young animals, with little evidence for an aging-related attenuation (cortisol peak: ~200 ng/ml). In contrast, plasma DHEAS levels were lower in the old animals and in most of them a circadian pattern was not obvious (DHEAS range: 10-30 ng/ml). Because this dampening of the circadian DHEAS rhythm was evident even in premenopausal animals, it is likely to have been initiated by a sex-steroid-independent aging process. In summary, these data show that DHEAS like cortisol has a circadian pattern of release during early adulthood, but in contrast to cortisol it shows a marked attenuation during aging. One clinical implication of these data is that plasma DHEAS is likely to be a more accurate biomarker of human aging if it is measured in samples collected at dawn, rather than in the afternoon.

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AGING ALTERS CIRCADIAN GENE EXPRESSION IN THE SUPRACHIASMATIC NUCLEI OF MALE SYRIAN HAMSTERS. D.E. Kolker, D.S. Huang, H. Fukuyama, T.H. Horton, and F.W. Turek. Dept. of Neurobiology and Physiology, Northwestern University, Evanston, IL.

Aging results in marked changes in rodents' circadian rhythmicity. In Syrian hamsters, these changes include changes in τ , rhythm amplitude, and the phase-shifting responses to photic and non-photoc stimuli. As the circadian clock is comprised of an intracellular feedback loop of gene transcription, protein translation, and repression of transcription, we hypothesized that some of the effects of age may be correlated with alterations in the temporal expression patterns of recently discovered circadian clock genes. In situ hybridization from young (2 mos.) and old (18 mos.) animals sacrificed at four circadian times ($n=5-6$ per age group per time point) revealed that *BMAL1* mRNA expression is significantly lower in old hamsters than in young hamsters (ANOVA: age effect $p < 0.05$). While rhythmic expression is present in SCN from all animals (ANOVA: time of day effect $p < 0.05$), there was a trend towards a decrease in the amplitude of *BMAL1* mRNA expression although it did not reach the level of statistical significance (ANOVA: age by time interaction $p < 0.10$). Experiments are currently underway to examine the effects of age on *Per1* and *Per2* expression in this species. These results suggest that alterations in the expression levels of specific genes may underlie the age-related changes in circadian rhythmicity. Sponsored by NIH grants P01 AG 11412 and P30 HD 28048 to F.W.T. and a Glenn/AFAR scholarship to D.E.K.

AGE-RELATED ALTERATION OF CLOCK-RELATED GENES EXPRESSION IN RAT.

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It is known that circadian rhythm disorder appears in elderly person. In fact, it is reported that aging alter the free-running period and the amplitude of the circadian rhythm. Since circadian clock genes, such as *period* and *clock* were cloned in mammals, molecular study of circadian rhythm is accelerated. In order to reveal the mechanisms of the age-related circadian rhythm disorder, we analyzed the age-related alteration of *period* and *cry* mRNA levels in young (2-3 months) and old (24-26 months) male rats using *in situ* hybridization and RT-PCR methods.

In the suprachiasmatic nucleus (SCN), there were no differences of daily expression pattern of *rPer1*, 2, and *rcry1* between in young and old rat. This result suggests that the ability of the rhythm generation in the SCN is not affected by aging at least in mRNA levels. Therefore, to test whether output from the SCN alters with aging, we analyzed mRNA levels in the cerebral cortex, cerebellum and pineal body, which are regulated by the SCN. In the cortex and cerebellum, peak levels of *rPer1* mRNA were lower in old rat than those in young rat, whereas no changes were observed in the pineal. These results suggest that output signals from the SCN impaired with aging. Finally, to test whether input of light to the SCN alters with aging, we analyzed light-induced expression of *rPer1*, 2 in the SCN. Light-induced expression of *rPer1* in old rat decreased compared with that in young rat. This data suggests that input to the SCN altered with aging. From this study, we hypothesize that age-related circadian rhythm disorder is caused by age-related decline of input and / or output signals, but not by rhythm generation ability in the SCN.

Although wheel running has for many years been widely used to measure the phase and period of circadian rhythms in rodents, the functional significance of this behavior remains unclear. Running has been variously characterized as a lab analogue of foraging and migration, an animal model of obsessive/compulsive disorder and a lab artifact with no natural behavioral analogue. Osiel et al (1994, 1998) have argued that daily levels of wheel running in hamsters are conserved, suggesting homeostatic regulation. The regulatory properties of running should inform models of function. Moreover, evidence for homeostasis suggests that wheel running could serve as a model for study of 2-process regulation of behavior. To evaluate this proposal, we quantified levels of daily wheel running activity from archival data sets drawn from studies of young male Syrian hamsters and C57BL/6j mice in our lab. In a sample of 59 hamsters, daily wheel revolutions declined gradually and significantly (~50%) over the first 2 months in LD, and were relatively stable over the second 2 months. In SCN ablated hamsters (N=9) activity levels declined significantly (~48%) from the 1st to the 4th week of recording in DD. When these hamsters were run in a novel wheel for 2-h/day, there was no consistent effect on total daily running; daily wheel counts doubled in some cases, and halved in others. During 10 days of LD, activity was greatly suppressed in the 12h L period, but did not differ from DD in the 12 h of D, resulting in a ~51% reduction in total daily running. When the hamsters were placed on restricted feeding, running levels markedly increased. In mice (N=20) recorded in DD, activity levels declined significantly (~50%) from the 1st to the 3rd month. Mice in LD (N=39) did not show conservation of daily activity during 3 weeks when wheel access was restricted to either the 1st or the 2nd 6-h block of the 12 h D period. In animals with wheel access only during the 2nd half of the night, activity gradually increased over the 3 weeks, but total activity remained ~36% below baseline. In aggregate, these results provide little evidence of significant homeostatic regulation of wheel running in hamsters and mice. While rodents may find wheel running rewarding, it is doubtful that activity per se is homeostatically protected. Regulation of activity levels is more likely secondary to regulation of body weight and the acquisition of key resources (e.g., food and mates). *Supported by NSERC, Canada.*

SIMILAR GENETIC CONTROL OF NEONATAL AND ADULT SLEEP IN MICE

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While the emergence of behavioral sleep states is one of the most fundamental aspects of development in mammals, the relationship between these states in the neonatal and adult animal, if any, is not known. Early studies indicated the the behavioral sleep states in the neonate, active sleep (AS) and quiet sleep (QS), are immature forms or precursors to the EEG-defined sleep states of rapid eye movement (REM) and non-REM (NREM) sleep, respectively, in adult animals (1). In contrast, the results of more recent studies have been interpreted to indicate that AS is an undifferentiated behavioral state from which both REM and NREM sleep develop (2). Clear genetic differences in the characteristics of the behavioral sleep states in adult animals of different strains of rodents offer a potential window for examining, at least at the genetic level, how the neonatal and adult behavioral states are related to one another.

We have examined vigilance states in neonatal (8-day old) mice of four different strains (C57BL/6ByJ, BALB/cByJ, DBA/2J and C57BL/6J) which show different amounts of wake and sleep states as adults (3). Many of these differences were already evident when wake, QS and AS were monitored only via EMG recordings in 8-day old mice. These results indicate that behavioral sleep states in the neonate are under similar genetic control as the REMS and NREMS states in adult animals.

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SLEEP DEPRIVATION STIMULATES SEROTONIN RELEASE IN THE HAMSTER SUPRACHIASMATIC NUCLEUS. Gregory H. Grossman¹, Ralph E. Mistlberger², Michael C. Antle², J. Christopher Ehlen¹ and J. David Glass¹. ¹*Department of Biological Sciences, Kent State University, Kent, OH, USA.* ²*Department of Psychology, Simon Fraser University, Burnaby, British Columbia, Canada.*

Changes in behavior, associated with feeding, locomotor activity, sleep/arousal and the light-dark transition are associated with adjustments in central serotonergic activity. Experimental manipulations of behavior, including wheel-running and sleep deprivation (SD), markedly affect central serotonergic activity, as well as have pronounced phase-shifting effects on the circadian activity rhythm. Despite these findings, the effect of SD on the synaptic release of serotonin (5-HT) has never been directly measured. In view of the circadian clock resetting effects of SD (Mistlberger et al., 1999, Soc. Neurosci. Abst. 646.9), the present study was conducted to assess the impact of this manipulation on *in vivo* 5-HT release in the suprachiasmatic nucleus (SCN) using microdialysis. SD was induced over a 3 hr period from ZT6-9 by gentle handling and air puffs under dim red light (<0.5 lux). A striking effect of this procedure was the induction of a large ($170 \pm 10\%$ of baseline; $p < 0.05$; $n = 12$), reversible increase in SCN 5-HT release lasting 3 hrs. This procedure also caused a 109 ± 27 min phase-advance in the circadian activity rhythm ($p < 0.05$ vs. non-SD controls; $n = 5$). Exposure to 3 hr of dim red light alone had no effect on 5-HT release ($p < 0.05$ vs. SD groups; $n = 6$). When SD was conducted under bright incandescent lighting, SCN 5-HT release was also stimulated ($201 \pm 60\%$ of baseline; $p < 0.05$; $n = 6$). However, phase-shifting was greatly diminished (47 ± 18 min; $p < 0.05$ vs. dim red light; $n = 8$). Our results are consistent with past studies suggesting a stimulatory effect of SD on serotonergic activity. In addition, we have shown that SD stimulates SCN 5-HT release to a level similar to that induced by continuous wheel-running at the same ZT, indicating that this alteration in SCN neurochemistry could be functionally related to the phase-resetting effects of either treatment. Finally, if the phase-resetting response to SD is mediated by increased 5-HT release, light must inhibit this phase-shifting by blocking the post-synaptic or downstream actions of 5-HT. NIH NS35229 (J.D.G.) and NSERC (R.E.M.) and NSERC Fellowship (M.C.A.)

METHAMPHETAMINE SCHEDULES REINITIALIZE CIRCADIAN MECHANISMS IN RATS IN THE ABSENCE OF OTHER TIME CUES, LOCOMOTION, OR SCN TISSUE. Pecoraro, N., Kosobud, A.E.K., Rebec, G.V., Timberlake, W. Department of Psychology, Program in Neural Science, & Center for the Integrative Study of Animal Behavior, Indiana University, Bloomington, IN.

Four experiments examined the effects of scheduled methamphetamine (MA) injections (2 mg/kg) on wheel running activity rats. All experiments were conducted under conditions of constant, moderately dim light (50 lux) and rate-limited feeding (two 97 mg Noyes pellets/6min) to prevent large meals. In Experiment 1, injections were given once daily every 24 hr. The rats anticipated the injections by 1-2 hr and showed an increase in activity at the former injection time on the first no-intervention day. Experiment 2 utilized a long T injection schedule ($T = 31$ hr) on the presumption that the long T schedule would be outside the range of entrainment of animals' intrinsic periods. Rats did not anticipate the 31-hr schedule, but rather showed increased activity ensuing from the injections by 24-28 hr. Experiment 3 examined the necessity of post-injection hyperactivity on anticipatory activity by preventing wheel running and ambulation for the first 5 hr following injections by locking animals in small cages. In both restrained and unrestrained conditions rats showed increases in activity prior to injections. In addition, marked hypoactivity was observed between injections in both MA conditions. Experiment 4 used a long T MA schedule ($T = 31$ hr) in rats rendered arrhythmic by lesions of the suprachiasmatic nuclei (SCN). In spite of the lesions, animals showed increased wheel running activity ensuing from the injections by 24-28 hr, suggesting that the circadian re-initializing effects of MA injections can be SCN-independent.

Little is known about the rhythmic effects of morphine on beta-endorphin (BE). Reports of morphine's effects on BE concentration vary depending on the species or strain of animal being studied and tissue studied. These differences in results may also be due to the time of day when morphine was given and the animals were sampled. Whether the effects of morphine vary with time of day, needs to be studied because it is a widely used analgesic. Clinically, the administration of morphine is often ineffective in relieving pain and is also associated with side effects. These problems may be due to the timing of morphine administration. This study was designed to examine the effects of morphine on BE at different times of day. The first two studies described the secretion of BE following acute pain. Dilute Brown Non-Agouti (DBA) mice ($N = 244$) were exposed to a 55 °C (treatment) or room-temperature (control) hot-plate and trunk blood was collected. Beta-endorphin was analyzed using radioimmunoassay (RIA). Data were analyzed with ANOVA, harmonic analysis, and cosine regression analysis. Both groups increased their BE secretion immediately. The response of treatment mice lasted 20 min longer than the controls. A marked circadian rhythm in BE secretion was observed in both groups. In an additional study, treatment mice were injected with 2.3 mg/kg of morphine and control mice were injected with saline. Treatment ($n = 6-8$ /time point) and control ($n = 7-8$ /time point) mice were exposed to a hot-plate set at 55 °C at one of 6 different time points at 4-hr intervals for 24 hr. Control mice showed a robust circadian BE rhythm with a secretion peak at 0000 and a nadir at 1200. Morphine-injected mice showed no circadian BE rhythm. These studies demonstrated that both pain and handling initiate an increase in BE, however, BE levels remain elevated 20 minutes longer following a painful stimulus than from handling stress alone. Morphine decreases plasma BE and abolishes the circadian BE response rhythm. Supported in part by NASA grant NGT 40051 and Sigma Theta Tau International Grant (1997).

CIRCADIAN ORGANIZATION BEFORE, DURING, AND AFTER ALCOHOL ADMINISTRATION IN RAT MODELS OF ALCOHOLISM. Timberlake, W., Leffel, J.,

Kosobud, A.E.K., Pecoraro, N Department of Psychology, Program in Neural Science, & Center for the Integrative Study of Animal Behavior, Indiana University, Bloomington, IN.

Two experiments used either alcohol preferring/non-preferring (P/NP) rats or high/low alcohol drinking (HAD/LAD) rats to determine baseline circadian organization, and the effects of daily alcohol injections on activity. Animals were entrained to a 12:12 light:dark cycle for at least 60 days to establish baseline activity patterns, then given one injection daily of EtOH (1 g/kg i.p.) at circadian time 8 h (CT 08) for 10 successive days, then observed for at least 14 days without alcohol injections. Both P and NP rats generally were most active in the first half of the night before, during and after alcohol treatment. Alcohol suppressed overall activity in both Ps and NPs similarly. Neither anticipation of alcohol injections nor striking changes in circadian patterning were apparent. After alcohol injections ceased, NPs showed greater than baseline activity levels, whereas the activity levels of Ps remained below baseline. In contrast to Ps and NPs, HADs and LADs distributed their average activity evenly throughout the dark phase during baseline, and LADs were far more active than HADs in all phases. Alcohol suppressed activity in both HADs and LADs, primarily in the early part of their active phase (possibly due to the proximity of the alcohol injections). Relative to their baselines, suppression was not significantly different between HADs and LADs. After alcohol injections, daily activity patterns returned to baseline levels in LADs, while HADs, like Ps, remained below baseline. In pooled analyses, alcohol-preferring strains (HAD and P) showed less overall activity than non-preferring strains (LAD and NP), similar reductions in activity relative to baseline during alcohol treatment, and significantly less recovery of activity levels following the end of alcohol treatment. This work was supported by PHS AA07611.

- 195 **DIFFERENTIAL EFFECTS OF VASOPRESSIN ON ACTIVITY AND TEMPERATURE OSCILLATORS.** Helen M. Murphy, Cyrilla H. Wideman, and George R. Nadzam. Departments of Psychology and Biology, John Carroll University and The Cleveland Clinic Foundation, Cleveland, OH.

Telemetered body temperature (BT) and activity (AC) data were collected in vasopressin (VP)-containing, LE and VP-deficient, DI rats. The animals were initially exposed to a 12h/12h light/dark cycle (photic cue) and allowed ad-lib access to food and water. Care and handling (nonphotic cues) occurred during the dark cycle. After an habituation period, rats were subjected to continuous light (LL) or dark (DD) and had ad-lib access to food and water. Rats were fed, watered, and cages were cleaned at randomly selected times every 3 days. During the habituation period, both strains exhibited clear 24h circadian rhythms of AC and BT. LE animals shifted to a 26h cycle for both AC and BT in LL and 24.6h in DD. Care and handling were seen as minor artifact. In DI rats, although there were robust 26h and 24.6h circadian cycles of AC in the LL and DD, respectively, BT data were inconsistent and showed sporadic fluctuations. In the BT rhythm of DI animals, strong peaks were associated with feeding, care, and handling times and trough periods were characterized by a dramatic drop in temperature. In conclusion, this experiment suggests that AC and BT are controlled by separate oscillators. In addition, the importance of vasopressinergic fibers in the control of circadian rhythms of BT is evidenced by the loss of circadian rhythms in animals lacking these functional fibers when exposed to free-running paradigms where there is no entrainment of photic or nonphotic oscillators.

- 196 **REACTIVENESS TO STRESS PREDICTS THE RATE OF RESYNCHRONIZATION OF THE CIRCADIAN CLOCK IN RATS.**
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Two physiological systems are involved in the adaptation of the organism to environmental challenges : the circadian system and the stress reaction system. These two systems interact closely : exposure to stressors can alter circadian clock function, while circadian factors are able to modulate stress responses. Light is the major synchronizer of the circadian clock, and after a shift in the light : dark cycle it takes several days to readjust to the new schedule. A major physiological response elicited by stress is activation of the hypothalamo-pituitary-adrenal axis. The amount of corticosterone secreted in response to acute stress varies as a function of the type of stressor and of time of day for stressor presentation. In addition, there is a large interindividual variability in stress sensitivity, with great differences in the amount of corticosterone secreted. In this study we addressed the possibility that differences in stress sensitivity and differences in the functioning of the circadian system could be related. At 2 months of age, corticosterone secretion in response to a 20-min restraint stress was assessed in 9 animals. Two weeks later the response of the circadian system to its major synchronizing agent, i.e. light, was evaluated using a "jet lag" paradigm involving an abrupt 8-hour phase advance in the 12 : 12 light : dark cycle (light intensity : 200 lux). The rate of resynchronization of the locomotor activity rhythm was calculated. Our results showed that the number of days to resynchronization were positively correlated to plasma corticosterone levels in response to the restraint stress procedure. These data show for the first time that corticosterone secretion in response to acute stress can predict the rate of resynchronization of the circadian clock to a circadian stressor, i.e. an abrupt shift in the light : dark cycle. Thus the « stress-sensitivity » of an animal may influence the resetting of the circadian clock by light. Those results could explain interindividual differences in the susceptibility to shift work and to rhythm disturbance-related pathologies.

REST-ACTIVITY AND BODY TEMPERATURE RHYTHMS AFTER SUPRACHIASMATIC NUCLEUS DESTRUCTION IN B6D2F₁ MICE. 197

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Suprachiasmatic nucleus (SCN) destruction results in the loss of several circadian rhythms in rats and hamsters. However some controversial studies into core body temperature reported suppression and sometimes persistence of circadian rhythmicity. The aim of this experiment was to study the effect of SCN lesions on rest-activity and body temperature rhythms in B6D2F₁ mice as a potential model for investigation of coupling between circadian outputs and cellular rhythms responsible for chronopharmacology.

Methods. Sixteen male B6D2F₁ mice 6-week old upon arrival were synchronized in LD 12:12 (L: 6⁰⁰-18⁰⁰, D: 18⁰⁰-6⁰⁰) for 7 days and then had a temperature and activity sensor (Physio Tel, TA 10 TA-F20) implanted into the peritoneal cavity. Both variables were monitored every 10 min for 12 days to validate the rest-activity and body temperature rhythms. The SCN were destroyed by electrocoagulation in 14 mice and 2 mice were sham-operated. The rest-activity and temperature rhythms were monitored for 8 weeks under LD 12:12. The brains of all the mice were sampled and SCN lesions were verified with both Nissl and immunostaining for Peptide Histidine Isoleucine (PHI). Time series were analyzed by inspection and by spectral analysis (Fourier transform) using Mathcad, complemented with Cosinor.

Results. Rest-activity and body temperature rhythms persisted in both sham-operated mice. The rest-activity cycle was abolished in all the lesioned mice. Five of them retained an apparent body temperature rhythm despite the absence of remaining SCN tissue revealed in histological studies. Fourier analysis documented a dominant 24-h periodicity in body temperature, which was confirmed by cosinor analysis ($p < 0.001$) in the 2 sham and in the 5 SCN lesioned mice. The circadian amplitude was decreased in the 5 SCN lesioned mice (range 0.11 to 0.28 °C) as compared to sham (0.48 and 0.97 °C). The acrophase was spread between 12¹⁰ and 19²⁰ (mid light to early dark) in the 5 lesioned mice while it remained near mid dark in both sham-operated animals (00¹⁰ and 00³⁰). These results suggest that SCN destruction does not always induce temperature arrhythmia in mice kept in a light/dark cycle. Two hypotheses could account for these data. Firstly, the presence of the light/dark cycle could be driving the temperature rhythm even though it had no overt effect on activity, or secondly, there could be a circadian oscillator other than the SCN driving the rhythm. The equivalent experiment will be carried out in constant darkness to distinguish between these two hypotheses.

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DORSAL LATERAL GENICULATE LESIONS PREVENT THE ENHANCEMENT BUT NOT THE SUPPRESSION OF LOCOMOTOR ACTIVITY BY LIGHT IN THE MOUSE. K. Edelstein and N. Mrosovsky. Department of Zoology, University of Toronto, Toronto, Canada M5S 3G5. 198

Light exposure alters locomotor activity in nocturnal rodents, masking the overt expression of the circadian activity rhythm. Bright light pulses suppress locomotor activity, whereas dim light pulses increase locomotor activity. Such effects are known as negative masking and positive masking, respectively. Although the neural mechanisms underlying the masking responses to light are unknown, the finding that retinally degenerate (*rd/rd*) animals continue to exhibit negative masking responses to light, despite virtually total loss of rod photoreceptors, is consistent with the idea that the system mediating the attenuation of activity by light is the same as that regulating circadian rhythmicity. Furthermore, the absence of positive masking in *rd/rd* mutants raises the possibility that the increased activity in response to dim light pulses is mediated by the classical image-forming visual system. To explore this hypothesis, we compared the masking responses to light pulses given at ZT14-15 in mice with bilateral electrolytic lesions aimed at the dorsal lateral geniculate nucleus (DLG) and sham-operated control animals. Neutral density filters were used to alter light levels. The results showed that DLG-lesioned animals exhibited greater suppression of wheel running in response to light than did the controls. Moreover, lesioned mice failed to exhibit the increased activity in response to dim light pulses observed in intact controls. Histological examination confirmed that lesioned animals sustained damage to the dorsal lateral geniculate, sparing the intergeniculate leaflet (IGL). Functional integrity of the IGL was also confirmed using neuropeptide Y immunohistochemical staining in the SCN. Most lesioned animals also sustained partial damage to the hippocampus and cortex. The results support the view that the masking effects of light on behavior comprise two opposing processes, one which increases activity and is mediated by the classical visual system, and another which suppresses activity and is mediated by the non image-forming irradiance detection system.

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EFFECT OF PINEALECTOMY ON MELATONIN RECEPTOR PROTEIN EXPRESSION IN CHICK DIENCEPHALON

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The hormone melatonin has been shown to be an important component of circadian organization among many taxa, especially in birds. Presumably, the physiological action of melatonin as a timekeeping cue is mediated through binding of the ligand to one or more receptor subtypes, thus initiating a signaling cascade within target tissues. Investigators have characterized two specific receptor proteins, Mel_{1A} and Mel_{1C}, which are expressed at high levels in the chicken. A third, the Mel_{1B} receptor, is expressed in birds but at lower levels. Previously, it has been shown that 2-[¹²⁵I]-iodomelatonin binding is rhythmic in various brain structures of several avian species. Hence, emerging evidence suggests that rhythmic changes in abundance of the melatonin receptor, as well as of the ligand, are an important regulatory feature of circadian systems. Here, we use western blot analysis to investigate diencephalic expression of Mel_{1C} receptor protein in the model organism, *Gallus domesticus*. Polyclonal antibodies raised against the Mel_{1C} receptor subtype showed immunoreactivity with an approximately 36 kD protein. In addition, consistent immunoreactivity of the antisera with two larger proteins (~70kD and >121 kD) occurred. Corroborating evidence from this lab suggests these higher weight molecular species represent oligomerization of the receptor protein. In this study, the temporal distribution of the Mel_{1C} protein was assayed under LD and DD conditions. The effects of pinealectomy were also assessed. Expression of the 36 kD protein (presumed monomer) was higher during the day than in the night, and this rhythm persisted for at least two days in DD. Pinealectomy resulted in an overall increase in receptor expression and attenuation of the rhythm in both LD and DD. Furthermore, the relative levels of expression of the different molecular weight proteins were differentially altered by both a change in lighting conditions and by pinealectomy.

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DAILY RHYTHM OF PINEAL MELATONIN CONTENT IN THE DIURNAL MURID RODENT, *ARVICANTHIS NILOTICUS*. Colleen M. Novak¹, Abel Bult⁴, Ketema N. Paul¹, Laura Smale³, Gianluca Tosini², and H. Elliott Albers¹. ¹Department of Biology and Neuroscience Program, Georgia State University; ²Neuroscience Institute, Morehouse School of Medicine; ³Department of Psychology and Neuroscience Program, Michigan State University; ⁴Institute of Arctic Biology, University of Alaska Fairbanks.

The hormone melatonin has effects on sleep and circadian rhythms, as well as reproduction in photoperiodic species. In the vast majority of species, melatonin secretion from the pineal gland is rhythmic and peaks during the nighttime hours. The aim of the following study was to determine if the diurnal rodent, *Arvicanthis niloticus*, shows a daily rhythm of pineal melatonin content. Forty-eight male *A. niloticus* were housed on a 12:12 light:dark cycle. Animals were decapitated at 6 times over the light dark cycle, at zeitgeber time (ZT, hours after lights-on) 3.5, 7.5, 11.5, 14.5, 19.5, and 23.5. Pineals were suspended in medium, which was subjected to a radioimmunoassay for melatonin. Pineal melatonin content ranged from undetectable to 226 pg/pineal. A significant rhythm of melatonin was detected, with the peak at ZT 19.5. These data demonstrate that *A. niloticus* show a rhythm in pineal melatonin that peaks during the middle of the night. Supported by MH 58789 to H.E.A.; NS38483 to G.T.; NS07279 to A.B.; NIMH R01 MH 53433 to L.S.

Adult Gonadal Hormones Determine Social Cue Responsiveness in Circadian Rhythms of the Diurnal Rodent, *Octodon degus*. Tammy J. Jechura, Kush Goyal & Theresa M. Lee. University of Michigan, Dept. of Psychology, Ann Arbor, MI 48109-1109

Female *Octodon degus*, a diurnal hystricomorph rodent, respond to social cues with an increased rate of reentrainment following phase shifts of the light:dark cycle (Goel and Lee, 1995). However, male *degus* appeared unresponsive to the same cues. The purpose of this study was to determine the role of adult gonadal hormones in both male and female *degus* in their responsiveness to social cues to accelerate reentrainment. Responsiveness is operationally defined as an increase in the rate of reentrainment when exposed to social cues over reentrainment when housed alone with only photic cues for environmental cues. In the first two experiments, adult female and male *degus* were subjected to 6-hour phase advances in the presence and in the absence of a social cue donor both before and after gonadectomy. In the first experiment, OVX females were unresponsive to social cues. Their reentrainment rates did not differ when housed alone or with a social cue donor. Males, on the other hand, reentrained significantly faster with a donor when CAST than when intact ($p=.006$) and were once again unresponsive when given testosterone replacement capsules. The third and fourth experiments involved testing intact males for social cue responsiveness with increased levels of social cue stimuli to determine whether they were completely inhibited in responsiveness. Males' reentrainment after a 9-hour phase advance did not differ when housed alone or with a single sister, but when housed with two unfamiliar females, they reentrained significantly faster than when alone ($13\text{days} \pm 1.472$ vs $26.8\text{days} \pm 1.424$, $p=.012$). These results indicate that ovarian hormones are necessary to enhance sensitivity to donor cues in adult females, while testosterone in adult males suppresses sensitivity to social cue enhanced reentrainment.

LIGHT INTENSITY IN THE AFTERNOON AND DURING A SOLAR ECLIPSE: EFFECTS ON RETREAT UNDERGROUND IN A DIURNAL MAMMAL.

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European ground squirrels (*Spermophilus citellus*) in their natural habitat emerge from their burrows ~4 hours after twilight at dawn and disappear ~3 hours before twilight at dusk. Activity patterns recorded in an enclosure (the Netherlands) with light-sensitive radio collar transmitters reveal that no animals were above ground during the rapid light transitions of civil twilight at dusk and at dawn (Hut et al., 1999, JBR 14: 409-419). The classical phase response model for entrainment can not explain how the circadian system of the species remains entrained to the external, natural LD cycle while the major LD transitions are created by its own behaviour. The same data show that afternoon retreat underground is positively correlated with afternoon light intensity. A single decrease around noon of two log units in light intensity due to a 99% solar eclipse (august, 11, 1999 in Vienna, Austria) did not result in retreats underground in a natural population. Thus, if the behavioral response to decreasing afternoon light intensity is instrumental in circadian entrainment, it must depend on a phase-dependent response.

PHOTIC AND NON-PHOTIC CIRCADIAN PHASE-SHIFTING RESPONSES IN A DIURNAL MONKEY, THE COMMON MARMOSET. J. David Glass¹, Suzette D. Tardif¹, Robert Clemens¹ and N. Mrosovsky². ¹Department of Biological Sciences, Kent State University, Kent, OH, USA. ²Departments of Zoology, Psychology and Physiology, University of Toronto, Toronto, Canada.

Despite extensive literature on mammalian circadian entrainment, there is little information on this subject in diurnal mammals. Contributing to the lack of understanding in this area is the problem of separating photic from non-photoc effects in diurnal species. If non-photoc phase-response curves (PRC's) for arousal-related stimuli are linked to the active phase of the sleep-wake cycle, the advance region would be 180° out of phase with that of nocturnal species. However, this corresponds to the phase-advancing region of their photic PRC. To avoid potential confounds between different zeitgebers when generating PRC's, the present study was undertaken to produce events that were primarily photic (a 20 sec bright light pulse [450 lux]) to exclude behavioral input, and non-photoc (a 1 hr activity-inducing pulse under total darkness) to exclude light input. To study photic entrainment, marmosets with a free-running activity rhythm under constant dim light (DD; <0.5 lux; n=4) received a consecutive 12-day light pulse regimen, with the pulse occurring at the same external time. The animals quickly entrained to this stimulus with a phase angle of 10.9±0.3 hrs (using activity onset as CT 0). With a τ of 23.3 hrs, this represented a phase-delay of 0.7±0.1 hrs. A PRC produced from single light pulses had a phase-advance region at CT18-22 and phase delay region at CT 9-12. To study non-photoc entrainment, animals under DD were induced to become active for 1 hr in complete darkness at the same external time for 76 days (n=6). Most of the marmosets exhibited transient entrainment to this stimulus (mean=3 wks) with a phase angle of entrainment of 0-1 hrs, representing a phase-delay of 0.7 hrs. The phase-delaying region of the non-photoc PRC was also evident in the lengthening of τ as the rhythm neared the activity pulse during the late subjective night. Our data indicate that the non-photoc diurnal PRC depends on the phase of the pacemaker and is not tied to the circadian activity-rest cycle. These results agree with recent data from the European ground squirrel, and suggest that diurnal non-photoc PRC's may have phase similarities with those of nocturnal hamsters and mice.

DOES THE TAU MUTATION IN THE SYRIAN HAMSTER AFFECT THE TIMING OF OTHER PROCESSES, FROM MILLISECONDS TO YEARS?

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The circadian period mutation *tau* in the Syrian hamster accelerates the period of locomotor activity rhythm from 24 h in wild-type (++) to 20 h in homozygous mutants (ss) i.e. 17%. We examine the impact of circadian *tau* mutation on time constants of processes in a wide range of frequencies, from milliseconds to life span. Heart rate (circa 150 ms per beat) is not affected by the *tau* mutation. The ultradian feeding rhythm was accelerated in *tau* mutant hamsters by -10%. However, the ultradian endocrine rhythm in LH and cortisol has been shown to be longer in mutants than in wild-type hamsters (Loudon et al. 1994). The rate of metabolism in mutants has been shown to be inversely proportional to the circadian frequency (Oklejewicz et al, 1997). In the infradian range, we measured the duration of torpor episodes during hibernation (about 90 h) and the time between two consecutive euthermic stages (about 115 h) and did not find effects of the *tau* mutation. Similarly, the estrous cycle (96 h) is unaffected by circadian period mutation (Refinetti et al. 1992). In life span (time on average 2 years) *tau* mutants exceed wild-types hamsters by 14% in females and 12% in males.

In summary, the *tau* mutation has multiply pleiotropic effects besides the effect on circadian period especially on metabolism related processes (BMR, ultradian feeding rhythm, and growth), but not on all aspects of temporal organization. The positive effect on life span in spite of the enhanced metabolic rate remains an enigma.

THE CRAYFISH *PROCAMBARUS CLARKII* SHOWS CIRCADIAN VARIATIONS IN 205
DIFFERENT PARAMETERS OF THE GSH CYCLE.

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The objective of this work was to investigate possible circadian variations in the hemolymph and mid-gut gland glutathione status in the crayfish *Procambarus clarkii*. To test the above sixty adult crayfish were divided into two groups 1) 30 animals subjected to 12:12 light-dark cycles for 15 days and 2) 30 animals treated as described above, then exposed to 72 h. of complete darkness. Five crayfish from both groups were killed at six different times of day and the hemolymph and mid-gut gland were assayed for reduced (GSH) and oxidized (GSSG) glutathione by fluorometric methods. In addition mid-gut gland glutathione reductase was determined. The data were analyzed by means of single cosinor analysis. Hemolymph GSH and GSSG showed a bimodal circadian rhythm under LD that continued to free-run with an unimodal oscillation under DD. Mid-gut gland GSH and GSSG concentration as well as glutathione reductase activity showed a bimodal oscillation under both conditions. The amplitude of the rhythms that best fitted a circadian period showed maximal statistically significant difference from zero ($P < 0.05$) in DD. These results indicate that crayfish possess cyclic antioxidant mechanisms to deal with the oxidative stress of the light cycles. These antioxidant rhythms seem to be related with the activity rhythm described elsewhere (1).

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APPEARANCE AND ENTRAINMENT OF SPLIT ACTIVITY RHYTHMS IN 4-PHASE LDLD AND 206
SKELETON LIGHT CYCLES. J. A. Elliott¹ and M. R. Gorman² ¹Department of Psychiatry 92093-0667, and ²Department of Psychology 92093-0934, University of California, San Diego CA 92093.

Splitting of activity rhythms into two roughly equal-size bouts of activity, which thereafter freerun or phase shift independently, is often interpreted as evidence that the circadian pacemaker is comprised of two or more functionally distinct oscillators. The cellular basis of splitting is not known but the observation that individual SCN neurons behave as independent circadian oscillators in dispersed cell culture suggests the hypothesis that splitting may involve a temporal reorganization of SCN neurons into two populations with distinctly different phase distributions and/or coupling interactions. This study is part of an ongoing program to develop new approaches to test this hypothesis and to discover underlying principles of oscillator-oscillator interaction. Traditionally, in hamsters, split rhythms can be studied only in LL. However, we have overcome this limitation with a paradigm in which split rhythms arise in a majority of hamsters during entrainment to a 4-phase LDLD 24 h cycle. Previously, this paradigm included several days of novelty wheel running (NWR) experienced during the afternoon dark phase. However, we have found, and the current study further demonstrates, that splitting occurs readily in LDLD without NWR. The primary objectives of the present study are: 1) to characterize the incidence of splitting in hamsters exposed to a symmetric 7L5D7L5D light cycle, and likewise, in non-split hamsters subsequently exposed to an asymmetric 9L5D5L5D cycle, 2) to determine parameters of entrainment of split rhythms to these two different LDLD cycles, 3) to determine whether and how these split rhythms entrain to 24 h skeleton light cycles consisting of 4 brief light pulses (3 h, 2 h, or 1 h) per day, and 4) to begin to characterize the phase responses (PRC) of each bout to 1 h light pulses. The most striking result to date is that most split rhythms remain entrained to skeletons while most previously non-split rhythms do not. Instead, the later animals respond with bizarre sequences of phase shifts, phase jumps and/or splitting which in most cases may be characterized as relative coordination rather than stable entrainment.

THE TIME IS ZT 13: DO YOU KNOW WHERE YOUR HAMSTERS ARE?

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Over the past few years, an increasing number of male golden hamsters [LAK:LVG(SYR)] purchased from Charles River Laboratories (Kingston, NY) have either taken an extended period of time to entrain to our laboratory light:dark cycle upon arrival or have failed to shift their activity onsets to the dark portion of the LD cycle. A single hamster received in the Spring of 1996 was observed to take over 30 days to entrain to the LD cycle. In a recent shipment of 18 hamsters, only 6 animals demonstrated entrainment of the circadian rhythm of wheel-running activity to our standard laboratory LD cycle (lights on 1200:lights off 0200) within two weeks as expected from past experience. After 4 weeks in our laboratory LD cycle, 6 animals were still not entrained and their activity was almost completely confined to the light portion of the LD cycle. One animal continued to express wheel-running activity onsets 2-3 hours after lights-on for 60 days. We compared the behavior of these LAK:LVG(SYR) hamsters to a group of SASCO derived male golden hamsters from Charles River Laboratories [SAS(E)/GSH]. All 18 SASCO hamsters entrained to our laboratory LD cycle within two weeks after arrival with a mean of approximately 7 days.

LAK:LVG(SYR) hamsters were released into DD; all animals expressed free-running activity rhythms with no indication of masking during the prior LD cycle. Ten animals (including the animal with activity onsets during the light for 60 days) received a 10 min light pulse (150 lux) at CT 19. All 10 hamsters expressed light-induced phase shifts (mean = 1.88 ± 0.12 hr). We suggest that LAK:LVG(SYR) hamsters have experienced genetic drift in their breeding population resulting in an alteration of entrainment to LD cycles in some animals. Supported by NIH grant NS 35615.

EFFECTS OF LIGHT INTENSITY AND RESTRAINT ON DARK PULSE-INDUCED PHASE SHIFTING DURING EARLY SUBJECTIVE NIGHT IN HAMSTERS. Suzanne M. Dwyer and Alan M. Rosenwasser, University of Maine, Orono, ME, 04469.

Dark pulses presented on a background of constant light (LL) result in phase advances during the mid-subjective day and early subjective night, and phase delays during the late subjective night. The phase-shifting effects of dark pulses in hamsters are believed to be mediated by increased activity, as previous studies have shown that restraining animals during dark pulses blocks the phase shifts observed in mid-subjective day and late subjective night. In the present study, we focused on dark pulse-induced phase shifting during early subjective night, examining both the influence of LL intensity and the effects of restraint (via confinement to a 7.5 x 9.5 cm box) on the magnitude of these phase shifts. Hamsters were maintained in LL of differing intensity (1, 10, 100, or 600 lux) and periodically presented with a six hour pulse (dark pulse alone, restraint alone, or dark pulse + restraint), beginning at CT11. As expected, hamsters showed phase advances in response to the dark pulses. The magnitude of these phase advances was significantly greater under the higher LL intensities, but did not depend on the amount of activity (i.e. wheel turns) displayed during the dark pulse. Six-hour periods of restraint resulted in phase delays, and the magnitude of these shifts also increased with increasing LL intensity. However, the magnitude of restraint-induced phase shifts also appeared to be related to the amount of activity displayed following release from restraint (in the late subjective night), consistent with previous studies showing phase delays following behavioral activation during late subjective night. When hamsters were restrained during dark pulses, phase advances were reduced – but not blocked — and the extent of the reduction could be predicted from summation of the effects of the dark pulse and restraint conditions alone. Taken together, these results suggest that the phase-shifting effects of dark pulses during early subjective night may be mediated by photic input pathways to the circadian system, and represent a mirror image of the phase-delaying effects of light pulses presented at this phase. In agreement with this hypothesis, preliminary results show that significant phase advances are observed following 15-minute dark pulses presented between CT 11 and 13. Thus, although long duration dark pulses are assumed to be required for behaviorally mediated phase shifts, they are not required for dark pulse-induced phase shifting during the early subjective night, further supporting the independence of photic and behavioral activation at this phase.

NON-PHOTIC AND ANTI-PHOTIC MECHANISMS UNDERLYING THE DARK-PULSE PHASE-RESPONSE CURVE (PRC). Alan M. Rosenwasser and Suzanne M. Dwyer, Department of Psychology, University of Maine, Orono, ME 04469, USA

In Syrian hamsters, dark pulses presented on a constant-light (LL) background induce circadian phase advances from mid-subjective day through early subjective night, and phase delays during late subjective night. While dark pulse-induced phase shifting was originally interpreted as a negative or "mirror image" photic effect, recent observations suggest that the dark-pulse PRC may instead belong to a family of non-photoc, behavioral activity-dependent PRCs. Thus, the dark-pulse PRC is at least superficially similar to PRCs for novelty-induced locomotor activity and for triazolam administration, and phase shifts induced by both dark pulses and triazolam can be blocked by physical restraint that prevents the increased locomotor activity normally evoked by these stimuli. The novelty-, triazolam-, and dark-pulse PRCs are not identical, however: of these three stimuli, only dark pulses induce phase-advances during early subjective night. Indeed, we show in a companion poster that such phase shifts are *not* dependent on evoked activity, but *are* dependent on dark-pulse "intensity" (i.e., background LL intensity). Since light pulses induce phase delays during early subjective night, these results suggest that the phase-advancing effect of dark pulses at this phase may indeed reflect a photic mirror image or "anti-photoc" mechanism. In order to test this hypothesis, we have used polynomial functions fit to published data to produce a model dark-pulse PRC based on the summation of non-photoc (novelty and triazolam) and anti-photoc PRCs. This two-process model predicts accurately the shape of the dark-pulse PRC, particularly the extension of the phase-advance region into the subjective night.

DECREASED SAMPLING RATES MAY PROVIDE ACCEPTABLE MELATONIN PHASE, DURATION AND AMPLITUDE ESTIMATES, DEPENDING ON THE PRECISION REQUIRED

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Reducing the sampling rate for plasma melatonin while maintaining acceptable estimations of melatonin phase, duration and amplitude would be less costly and less demanding on subjects and technicians. To investigate this possibility, we created 120-Minute (Q120) and 90-Minute (Q90) data sets from original data sets collected via an indwelling catheter every 30 minutes during hours 5 to 30 of a constant routine (CR) procedure. Thirty-five young healthy subjects participated in the CR. We present here data from twenty-six of these subjects (25.3 ± 6.6 y) whose original Q30 data sets were complete. We calculated melatonin phase, duration and amplitude estimates from the Q120 and Q90 and the original Q30 data sets using previously described methods¹. The differences between the Q90 and Q30 estimates as well as between the Q120 and Q30 estimates were calculated for melatonin for the first night in the CR. Paired t-Tests were used to determine whether: 1) decreasing the sampling rate resulted in significantly different estimates on average; and 2) the absolute value of the differences in estimates were significantly different from zero.

There were no significant differences in: 1) the average phase estimates using either the midpoint method for Q90 or Q120 or the 15 pg/mL dim light melatonin offset (DLMOFF) method for Q90; and 2) the average amplitude estimates using area under the curve (AUC) for Q90 and Q120. Phase estimates using the 15 pg/mL DLMO method were 7.4 ± 12.1 min earlier on average for Q90 ($p < 0.05$) and 14.3 ± 19.5 min earlier on average for Q120 ($p < 0.002$), and estimates using the 15 pg/mL DLMOFF method were 11.1 ± 14.8 minutes later on average for Q120 ($p < 0.002$). Duration estimates (time between DLMO and DLMOFF) were 9.7 ± 19.7 min longer for Q90 ($p < 0.05$) and 25.2 ± 29.7 min longer for Q120 ($p < 0.0004$).

The absolute values of the differences between the Q90 and Q30 estimates, as well as between the Q120 and Q30 estimates were significantly different from zero ($p < 0.0001$) for all methods tested. The absolute value of phase estimates using the midpoint were 5.6 ± 4.5 min different for Q90 and 7.8 ± 5.9 min different for Q120, using DLMO were 10.1 ± 9.8 min different for Q90 and 18.2 ± 15.8 min different for Q120, and using DLMOFF were 13.3 ± 19.5 min different for Q90 and 14.6 ± 11.3 min different for Q120. The absolute value of the duration estimates were 16.8 ± 13.8 min different for Q90 and 30.2 ± 24.4 min different for Q120. The absolute value of the AUC estimates were 18.8 ± 16.1 h*pg/mL different for Q90 and 26.2 ± 35.5 h*pg/mL different for Q120.

These results suggest that a decrease in melatonin sampling rate will result in an increase in variability and a decrease in precision for estimates of circadian phase, amplitude and duration. However, while the differences between Q30 and Q90 or Q120 estimates are statistically significant, they are mainly of practical concern when using melatonin data to detect small changes in circadian phase and amplitude. Choice of sampling rates must therefore depend on the level of precision required in a given protocol.

References: ¹Benke, KB, Jewett, ME, Khalsa, SS, Czeisler, CA: Hourly and 30-Minute Sampling: Similar Phase, Duration, and Amplitude Estimates for Plasma Melatonin. Sleep, (2000) in press. Supported by: NIMH RO1-MH45130, NIA PO1-AG09975, AFOSR F49620-95-1-0388, NSBRI NCC9-58, ARO DAAD19-99-1-0241 (MEJ), NRSA F33-HL09588 (SBSK), NCRN MO1-RR02635.

A COMPARISON OF TWO CIRCADIAN PHASE MARKERS IN HUMANS

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Both dim light melatonin onset (DLMO) and the body temperature minimum (Tmin) have been proposed as reliable markers of circadian phase position. The aim of this investigation was to determine a) the group variability of temperature- and melatonin-derived phase estimates and b) the relationship between DLMO and Tmin under baseline conditions and after a phase shifting stimulus in healthy subjects whose prior sleep history had not been circumscribed.

Fifty baseline data sets from 41 subjects (11f, 30m; aged 21-68 years, mean 35.2) participating in various protocols were analyzed. Subjects entered the lab around 16:00. Between 17:00 and 24:00, saliva samples were collected in dim light (<20 lux) at 30- or 60-min intervals while the subjects were sitting in a reclined position. Simultaneously, core body temperature (CBT) was measured at 2-min intervals over 24 hours. Thirteen out of these 41 subjects also participated in a phase shift protocol. Following baseline, a 3-hr light pulse was administered starting 3 to 4 hours before the subjects' estimated Tmin in order to achieve a phase delay. Post-treatment circadian phase was assessed on the second night after light exposure. The DLMO was defined at a 3.33 pg/ml threshold and the phase of CBT was determined using a complex (24+12h) cosinor model.

The mean DLMO at baseline (n=50) occurred at 21:51 (± 79 min) and Tmin occurred at 4:52 (± 78 min). Variability between subjects was the same for both phase markers. The phase angle between DLMO and Tmin ranged from 3h 36min to 10h 24min, although 74% of all cases occurred within a window of 6-8 hrs (mean: 6h 40min ± 85 min). A significant correlation was found between DLMO and Tmin ($r=0.42$, $p<0.002$). Comparing the two phase markers before and after a 3-hr light pulse, a considerable difference in the phase shift of DLMO and Tmin was observed. Results showed a phase delay of 40 min ± 44 using DLMO and 67 min ± 86 using Tmin. The shift was significant for both phase markers ($p<0.01$). The phase angle between DLMO and Tmin was 6h 31min before and 6h 58min after the light exposure (n.s.). Looking at individual phase shifts, results showed that in 8 subjects Tmin was delayed more than DLMO (74 min. ± 62) while in 5 subjects the opposite was observed: DLMO was delayed more than Tmin (46 min ± 31). The correlation between DLMO and Tmin increased from $r=0.46$ (n.s.) at baseline to $r=0.61$ ($p<0.03$) after light administration.

While variability at baseline and phase shifts are similar for both phase markers when determined for a group of subjects, DLMO and Tmin may respond differently to a phase shifting stimulus for any given individual.

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SALIVARY MELATONIN: SAMPLING PROCEDURES AND STABILITY

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Introduction and Objective. At the 6th SRBR meeting, we reported test methods to measure low levels of melatonin in human saliva. There is a debate about the optimum saliva collection method and the stability of melatonin in saliva. We report here the recovery of melatonin from saliva samples collected in four different devices when compared to spitting. The stability of salivary melatonin when stored at room temp (RT), -20°C and -70°C is also reported.

Methods. 8 donors collected 5 samples each at early morning, late afternoon and midnight. The first sample was collected by spitting in a tube followed by samples taken in (A) polyester-type Salivette®, (B) cotton-type Salivette®, (C) cotton-type Salivette® with citric acid, and (D) cotton-type Sali-Saver™. The saliva samples were analyzed using a sensitive, direct RIA (BL, Allschwil, Switzerland). Short-term stability was determined by collecting saliva from 6 subjects at 7:00am. Aliquots were stored at -20°C, 4°C and 28°C for 24 hours. Mid-term stability was determined by incubating aliquots of 2 saliva samples each from 8 donors for 24, 48, 72 hours and 7 days at 4°C and 28°C, and comparing them with aliquots stored at -20°C. Long-term stability to 12 months at -20°C and -70°C is reported here.

Results. Compared to spitting, recovery of melatonin from the devices was as follows: (A) 88.2%, (B) 83%, (C) 19.4% and (D) 96.6%. Salivary melatonin was stable when stored at 4°C (mean: 14.4 $\pm$ 11.7 pg/ml) as well as at 28°C (mean: 15.7 $\pm$ 12.9 pg/ml) compared to a corresponding sample kept at -20°C (14.9 $\pm$ 11.3 pg/ml). No decrease of melatonin levels was observed after 12 months long-term storage at -20°C or -70°C. We report 2 samples each from 6 subjects measured with the Bühlmann direct ELISA.

Conclusion. All devices tested with the exception of the citric acid Salivette® showed satisfactory recovery. Salivary melatonin is stable for at least 3 days even at 28°C and for at least 1 year at -20°C.

CIRCADIAN RHYTHM AND SLEEP DIFFERENCES BETWEEN COMMUNITY DWELLING AND INSTITUTIONALIZED OLDER ADULTS. Chaperon, C. M. and Farr, L.A., College of Nursing, University of Nebraska Medical Center, Omaha, NE

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Sleep complaints in older adults are commonly attributed to age-related deterioration in the endogenous circadian pacemaker and the sleep homeostat. Recent laboratory research indicates that environmentally disrupted sleep can contribute to dyschronized sleep/wake cycles, poor sleep consolidation, and can impair health and daytime function, and increase sleepiness.

The purpose of this study was to investigate environmental cues such as light exposure and lifestyle as influences of differences between melatonin rhythms, core body temperature, and sleep/wake patterns in community-dwelling (CD) and in institutionalized (ID) older adults. This study used a convenience sampling of 20 older adults (CD=10, ID=10). The mean age of the subjects ranged from 72 to 102 yrs with the mean CD age of 79 and the mean ID age of 85. Activity/rest cycles were measured by actilume/actigraphy, temperature cycles by axillary temperature probe, and salivary melatonin (MLT) by RIA. Chronotype was measured with the Morningness/Eveningness Questionnaire (MEQ), fatigue with the Multidimensional Assessment Fatigue (MAF) Scale, cognitive function with the Folstein Mini Mental State Exam (MMSE), depression with the Center for Epidemiological Studies-Depression (CES-D) Scale, and sleepiness with the Stanford Sleepiness Scale (SSS).

CD subjects had greater sleep efficiency and lower sleep percentages/24 hrs than ID subjects. ID subjects took more daytime naps but still scored more sleepiness on the SSS. ID subjects were exposed to more light at night than CD subjects. Both CD and ID subjects had similar daily mean MLT levels, however, the ID subjects' MLT rhythms were advanced, had lower amplitude, and most were not rhythmic. ID subjects had higher mean body temperatures, were less active during the day, more restless at night, and had less robust circadian rhythms in both temperature and activity than did CD subjects. Supported in part by NASA grant NGT 40051

SLEEP DISTURBANCE IN PATIENTS AND THEIR CARE GIVERS FOLLOWING ARTHROPLASTIC SURGERY. L. Farr, J. Grandgenett, C. Chaperon, F. Nelson, S. Yannone, and R. Bakewell College of Nursing, University of Nebraska Medical Center, Omaha, NE, 68198-5330.

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Following surgery, sleep is disrupted, night time awakenings increase and sleep efficiency decreases (Farr et al, 1995) Circadian timing of temperature, blood pressure, and urinary metabolites are often delayed (Farr et al., 1994). This study was conducted to determine if sleep patterns and circadian melatonin rhythms were altered in arthroplastic surgery patients and to examine the sleep of their care givers. Fifteen patients/care giver pairs were studied before and after total hip or knee replacement. Patients spent one to two nights in the hospital following their surgery and then returned home. Data were collected for two days before and five days after surgery by patients and their care givers. Care giver subjects wore wrist actigraphs and patient subjects wore wrist actilumes in order to measure light exposure. Data were measured continuously and stored in 60 second bins. Patient subjects collected saliva samples every 4 hours. Saliva melatonin was measured by RIA. Care giver subjects rated their fatigue morning and evening using a visual analog scale. Data were analyzed using RM-ANOVA and cosine regression analysis. Patients experienced disrupted sleep cycles similar to those previously reported in abdominal and oral surgery patients (Farr et al., 1989, 1994). They woke more at night and slept more during the day. Care givers were awake more at night, went to bed later, and reported more fatigue. When patient and care givers were compared, care givers had more night time awakenings than did their patients and less total sleep. Patients' salivary melatonin rhythms were delayed following surgery, showed higher levels in the mornings and delayed nighttime increase compared to pre-operative data. These findings suggest that alterations in sleep/wake patterns during recovery from surgery may be related, in part, to changes in melatonin secretion. In addition the sleep of care givers is decreased following surgery, but their loss of sleep is greater than the loss of sleep of their patients. (Supported by NASA grant, NGT 40051)

TIME COURSE OF SLOW WAVE ACTIVITY DURING EXTENDED SLEEP IN ADOLESCENTS

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Introduction: Slow wave sleep (SWS) is largely dependent upon the amount of prior wakefulness. The usual time course of SWS in humans has maximal values in the initial hour of sleep and a progressive decline to minimal values about 3 to 5 hours later. Studies in adults have reported a resurgence of SWS in sleep episodes extended to 15h (1). It is not known whether a return of slow wave activity (SWA) during sleep occurs in adolescents who exhibit more abundant and higher amplitude SWS than adults. Our goal was to examine the time course of SWA in adolescents during 11h40m sleep opportunities occurring at various circadian phases during a forced desynchrony (FD) study. **Methods:** Sleep EEGs were analyzed from eight healthy adolescents (ages 13-15 years; Tanner stage 3-5; 4F). After 10 cycles of a 24h sleep-wake schedule at home, participants came to the laboratory, where lighting was held at <20 lux. After an adaptation night, participants underwent a 36h constant routine followed by a recovery night and 12 cycles of 28h FD consisting of an 11h40m sleep opportunity and 16h20m wake episode each cycle. C3-A2 EEG was recorded onto Nicolet Ultrasom workstations for all sleep episodes. Average amplitude of EEG activity in the frequency range of 0.75-4.5 Hz was determined for each 30-sec epoch scored as sleep stage 2, 3, or 4. Activity in this frequency range was estimated by squaring the amplitude of each epoch. Data were averaged in 2h bins from lights out irrespective of circadian phase and in 60° bins using estimates of circadian phase at time of lights out, based on intrinsic period obtained from dim-light salivary melatonin (0°=melatonin onset phase). Phase-dependent patterns of SWA were examined for each participant. **Results:** SWA exhibited maximal values at the beginning of the night and declined as the night progressed for all participants and regardless of the phase at the time of lights out. Returns of SWA (% deviation from the mean > 0) at the end of the sleep episode were observed when the sleep episode was initiated at 60° or 120° degrees (n=3). The magnitude of SWA recurrences was much smaller than values observed in the initial part of the sleep episodes. **Conclusions:** The exponentially declining time course of SWA in adolescents is similar to that of adults. When the sleep opportunity was initiated at 60° or 120° degrees, SWA showed a recurrent pattern in three out of eight subjects. The return of SWA when the sleep episode starts at certain circadian phases, points to the potential involvement of the circadian system in regulating slow wave activity during sleep in adolescents. (1)De Koninck GC, Hebert M, Carrier J, Lamarche C, and Dufour S. Body temperature and the return of slow wave activity in extended sleep. *Electroencephalogr Clin Neurophysiol.* 1996 Jan;98(1):42-50. Research supported by MH52415 and MH 01358.

ARE THERE HANGOVER-EFFECTS ON PHYSICAL PERFORMANCE WHEN MELATONIN IS INGESTED BY ATHLETES BEFORE NOCTURNAL SLEEP?

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Athletes ingest melatonin in the evening in an attempt to improve sleep quality or alleviate symptoms of jet lag after transmeridian travel. Past research work has been concentrated on the effects of melatonin on performances in simple psychological tests after daytime administration. Even if it is effective as a nocturnal hypnotic, it is not known whether there are residual effects of this hormone on physical performance after sleep is taken by fit subjects. These issues are relevant to athletes travelling from the American and European continents to compete in the Sydney Olympic Games.

After a sample size estimation (statistical power = 90%) involving a meaningful effect on performance of 5%, 5 mg melatonin or placebo was ingested by 12 physically-active subjects before sleep in a double-blind experiment. The following morning, subjective sleep quality (latency and maintenance) was measured together with intra-aural temperature, grip strength of the left and right hands and time to complete a time trial on a cycle ergometer over the Olympic distance of 4 km. The subjects also rated perceived exertion during the cycle ergometer test.

The null hypothesis of no effect of melatonin on either subjective sleep quality or physical performance measured the morning after administration could not be rejected on the basis of our observations ($P > 0.30$). The mean differences between treatments were less than 1% for the strength tests and time trial performance. The confidence intervals for these differences for left and right grip strength and the cycling test were -21 to 28 N, -30 to 27 N and -3 to 4.5 s, respectively.

In conclusion, we have not found that 5 mg melatonin has any meaningful effects on physical performance in the morning after fit subjects ingest the hormone. There was also little evidence that it improves subjective quality of nocturnal sleep in this population. Further research is needed concerning the residual effects of melatonin on mental performance, after nighttime administration.

Tasks relying on (pre)frontal functioning are performed disproportionately worse with aging¹. There is also an intriguing relation of frontal functioning to sleep. EEG delta activity predominates in the frontal derivations during the first two sleep cycles⁹, and is enhanced especially in these derivations after sleep deprivation¹⁰. Sleep deprivation also disproportionately attenuates frontal functioning, as shown e.g. on a verbal fluency task^{5,4}. In animals, disruption of the circadian rhythm furthermore affects memory retrieval processes². Since disturbances in the sleep-wake cycle frequently occur in healthy elderly, and even more so in demented elderly, we hypothesized that these disturbances might contribute to the worsening of frontal function at high age.

We investigated the relation between frontal functioning and the circadian sleep-wake cycle in 66 demented elderly by assessing their verbal fluency (animals) and an actigraphic recording for two weeks continuously (Actiwatch-L, Cambridge Neurotechnology). Sleep duration was calculated using the SleepWatch software (Cambridge Neurotechnology) and the stability, fragmentation and amplitude of the rest-activity rhythm was quantified nonparametrically⁸. Stepwise regression analysis was used to assess the association of the sleep-wake variables with fluency. Two likely contributors to the fluency performance were included in the analysis as covariates: the general cognitive functional level (MMSE)³ and the use of psychotropic drugs.

In the present sample of demented elderly (MMSE mean $\pm$ st.dev. 16 $\pm$ 7), fluency was positively associated only with the MMSE (beta=0.68, p<0.0001) and the amplitude of the circadian rest-activity cycle (beta=0.20, p<0.03).

The results support the involvement of sleep-wake rhythms in frontal functioning. In healthy elderly, exercise has been shown to selectively improve performance on frontal tasks⁶, as well as the circadian activity rhythm⁷. It remains to be investigated whether enhancement of the daytime activity level - and thus the circadian amplitude in the rest-activity cycle - in demented elderly could attenuate the progressive decline in frontal functioning.

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MELATONIN AND SLEEP IN DEMENTIA EN Kennedy¹, Z Grujic¹, S Benloucif², M Dubocovich², and PC Zee¹ ¹Dept. of Neurology, and ²Dept. of Molecular Pharmacology and Biological Chemistry, Northwestern University Medical School, Chicago, IL 60611

It has been reported that sleep and melatonin change as a part of normal aging and more so in dementia, but it is unclear how these changes relate to each other and to subjective sleep ratings and agitation. The purpose of this study was to systematically describe the 24 hour urinary melatonin metabolite (6-sulphatoxymelatonin) rhythm in dementia subjects and attempt to correlate it to sleep and behavioral parameters. We studied 7 dementia subjects (mean age=83.7) and 13 controls (74.7). All subjects wore Actiwatch activity monitors for 7-10 days. For dementia subjects, we collected 24 hour urine samples and caregivers' evaluations on the neuropsychiatric inventory (NPI) and Cohen-Mansfield Agitation Inventory (CMAI). Actiwatch data were analyzed for sleep parameters. Extraction of urine from diapers was followed by ELISA to measure 6-sulphatoxymelatonin (6-SMT) in each collection time. 6-SMT analysis revealed that dementia subjects have depressed levels of melatonin compared to healthy young controls and similar levels to those reported elsewhere in the literature for healthy elderly controls. However, no fluctuations in concentration were seen in 6 of 7 dementia subjects in contrast to the changes usually reported for healthy elderly. Sleep parameters generally supported the findings of other studies, but intersubject variability was high. Generally, we found that dementia subjects, compared to controls, spent more time in bed and slept more over 24 hours but had similar amounts of sleep concentrated at night. Dementia subjects had an increased fragmentation index and more wakes after sleep onset. However, no correlation was found between sleep parameters, 6-SMT levels, agitation, or subjective sleep rating from the neuropsychiatric exams. *Supported in part by USPHS grants AG11412 and AG00810.*

BRIGHT LIGHT AND FIXED SLEEP SCHEDULES COMBINE TO SPEED CIRCADIAN ADAPTATION TO NIGHT WORK. T. S. Horowitz, B. E. Cade, J. M. Wolfe, C. A. Czeisler. Circadian, Neuroendocrine and Sleep Disorders Section, Division of Endocrinology-Hypertension, Department of Medicine, Brigham and Women's Hospital, Boston, MA, 02115.

Night shift workers suffer from impaired alertness and performance on the job and degraded sleep during the daytime, among other problems, because they are operating at an adverse phase relationship between their schedules and their endogenous circadian rhythms. A number of controlled simulations of shift work have convincingly demonstrated that precisely timed bright light can overcome this circadian misalignment. However, such studies typically confound bright light treatments with scheduled sleep in darkness. It is important to know how bright light treatments are affected by the timing of sleep when designing a treatment regiment to alleviate circadian maladaptation to night work. Accordingly, we designed an experiment to manipulate independently these two factors.

27 men and 27 women participated in a 10 day high-fidelity shift work simulation consisting of 4 day shifts followed by 3 night shifts. Initial circadian phase was measured during a 6-h constant posture (CP) episode prior to the first night shift. Final circadian phase was measured during a 38-h constant routine (CR) following the final night shift. Subjects left the laboratory and slept at home. Subjects received 2,500 lux (Bright Light) or 150 lux (Room Light) during night shifts, and either were scheduled to sleep from 0800 – 1600 (Fixed Sleep) h in a darkened bedroom or were permitted to sleep *ad lib* (Free Sleep). Sleep schedules were monitored via actigraphy.

Both Fixed Sleep [$F(1,39) = 6.60, p < .05$] and Bright Light [$F(1,39) = 19.64, p < .0001$] led to significant phase delays, indicating adaptation to the night work schedule. The two factors combined additively, with bright light exposure leading to larger phase shifts. Free Sleep subjects who spontaneously adopted a consistent sleep schedule adapted better than those who did not. The same pattern of results was observed in subjective alertness.

We cannot, from these experiments, determine what aspect of scheduling sleep in darkness promotes circadian adaptation. Whatever the underlying mechanism (and more than one mechanism may be acting in concert), a consistent sleep/wake schedule may minimize exposure to competing synchronizers. Properly timed bright light exposure during scheduled work times alone was insufficient to induce complete circadian adaptation to a night work schedule. Therefore, scheduling of sleep/darkness should play a major role in prescriptions for overcoming shiftwork-related phase misalignment. Shift workers should be strongly encouraged to adopt a consistent sleep schedule that provides a stable and adequate time for sleep.

THE PHASE ANGLE OF ENTRAINMENT TO 10 MG MELATONIN IN BLIND PEOPLE DEPENDS ON PRIOR FREE-RUNNING PERIOD

A.J. Lewy, A.R. Kendall and R.L. Sack

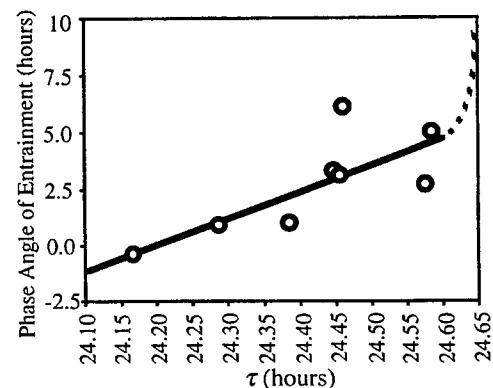
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We have recently shown that a bedtime dose of 10 mg of melatonin entrained seven out of eight formerly free-running totally blind individuals. The following analysis was undertaken to determine if there was a relationship between the pre-treatment intrinsic period (τ) and the phase angle of entrainment. The phase angle of entrainment was defined as the interval between the time of melatonin administration and the endogenous melatonin onset (MO).

There was a linear relationship between the phase angle of entrainment and the pre-treatment free-running τ , in that the longer the τ , the greater the phase angle of entrainment. When the phase angle of entrainment was corrected for variation in peak endogenous melatonin levels (range: 13-153 pg/ml), the regression line (see figure) became statistically significant ($p < .03$). According to the melatonin PRC, melatonin causes phase advances when administered between about CT (circadian time) 6 and CT 18. (We use the 10 pg/ml MO as CT 14.)

The melatonin PRC assumed a steady-state phase position such that exogenous melatonin was given within the phase-advance zone. Also according to the melatonin PRC, as administration time is moved earlier from CT 18 to CT 6, the magnitude of the daily phase-advance response increases. Therefore, these data are consistent with what we know about the phase-advance zone of the melatonin PRC.

These results provide further support for the common shape of the melatonin PRC in blind and sighted people and also indicate that melatonin's effects in blind people are directly related to entraining the endogenous circadian pacemaker. Testing different doses of melatonin in a group of blind people with a range of τ s should produce a set of curves that, with increasing τ , eventually turn sharply upwards and reach an asymptote, with higher doses shifting these curves downwards and to the right.



AGING CAUSES A SLIGHT LENGTHENING OF THE INTRINSIC PERIOD IN THE BLIND

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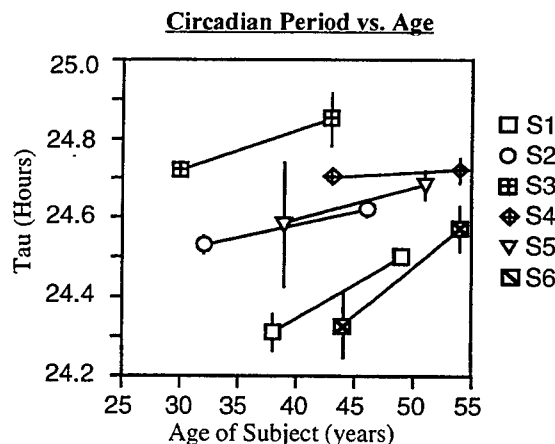
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Age-related changes in the intrinsic circadian period (τ) have been hypothesized to account for sleep symptoms in the elderly such as early morning awakening. Animal studies have suggested that τ decreases over time. Temporal isolation studies of sighted humans have shown that τ decreases or is relatively stable during aging. We sought to determine whether aging in blind individuals produces quantifiable differences in τ .

Six healthy blind men were first studied when they were 38 ± 6 (S.D.) years old. Their free-running rhythms were assessed a second time after an interval of 10 to 14 years. τ assessments consisted of three to six melatonin onset measurements, each involving hourly blood sampling for 24 hours. The melatonin onset was defined as the rise of plasma melatonin concentration above 3pg/ml. τ calculations were obtained by fitting the melatonin onsets to a linear regression.

All six subjects exhibited a longer τ in the second assessment with a mean increase of 0.128 ± 0.078 hours from the original assessment. A two-tailed t-test indicated that the new τ s were significantly different ($p < 0.01$) from the initial τ s. Post-hoc analysis yielded a moderate power ($1-\beta = .73$). The figure illustrates the initial and final τ s for each subject with 95% confidence intervals plotted against age during the assessments. Four of the six subjects exhibited non-overlapping 95% confidence intervals.

This study does not support current thinking that τ shortens or is stable during human aging. On the contrary, it appears to slightly, but statistically significantly, lengthen during at least part of the aging process. Alternatively, it is possible that the effects of age on τ are different in blind and sighted people.



PHASE SHIFTING RESPONSE TO LIGHT IN YOUNG AND OLDER ADULTS Susan Benloucif¹, Mireille L'Hermite-Balériaux⁴, Sandra Weintraub², and Phyllis C. Zee³ Depts. of ¹Molecular Pharmacology and Biological Chemistry, ²Psychiatry and ³Neurology, Northwestern University Medical School, Chicago, IL 60611, ⁴Dept of Gynecology and Obstetrics, Université Libre de Bruxelles, B-1050 Brussels, Belgium

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A decrease in the phase shifting response to light has been observed in old rodents. In the present report we assessed the effect of age on light-induced phase delays in young and older adults. Healthy young (5 M, 3 F, 30.8 ± 1.7 years) and elderly (5 M, 6 F, 67.3 ± 1.8 years) subjects with normal neuropsychological and ophthalmologic exams were admitted for a 4 night/ 3 day stay in the Clinical Research Center. Subjects remained under constant conditions (light < 50 lux and semi-recumbent in bed) during wake periods and slept for 8 h at their usual bedtime. Following habituation and baseline nights, subjects were exposed to light (4,000 lux for 3 h, bracketed by $\frac{1}{2}$ h of intermediate illuminance) centered 2.5 h before the baseline core body temperature minimum (T_{min} young: 4.76 ± 0.78 h; T_{min} elderly: 3.69 ± 0.81 h). Blood samples were collected from 19:30 to 9:30 on the nights before and after light exposure. Shifts in the timing of the melatonin rhythm are reported for 7 young and 6 elderly subjects. Results are not presented for six subjects due to baseline melatonin levels that were below detectable limits for this radioimmunoassay (2.5 pg, $n=3$) or impaired blood draws. Exposure to light delayed the dim light melatonin onset (DLMO) in both young (1.25 ± 0.35 h, $p < 0.01$) and elderly subjects (1.03 ± 0.30 h, $p < 0.05$), however, these results may be influenced by the low basal levels of melatonin observed in the elderly subjects. In contrast, the magnitude of light-induced delays in the declining phase of the melatonin profile (75% of max) was reduced in elderly subjects (0.42 ± 0.24 h) relative to young controls (1.64 ± 0.30 h, $p < 0.01$). The reduction in light-induced phase delays of the melatonin offset indicates that age alters the responsiveness of the human circadian timing system to light. This age-related change in the phase shifting response to light may contribute to the early morning awakenings commonly observed in older adults. Supported in part by USPHS grants NCRR-00048, AG11412 and AG00810.

3-CYCLE BRIGHT LIGHT STIMULUS INDUCES TYPE 0 RESETTING IN HUMAN MELATONIN RHYTHM

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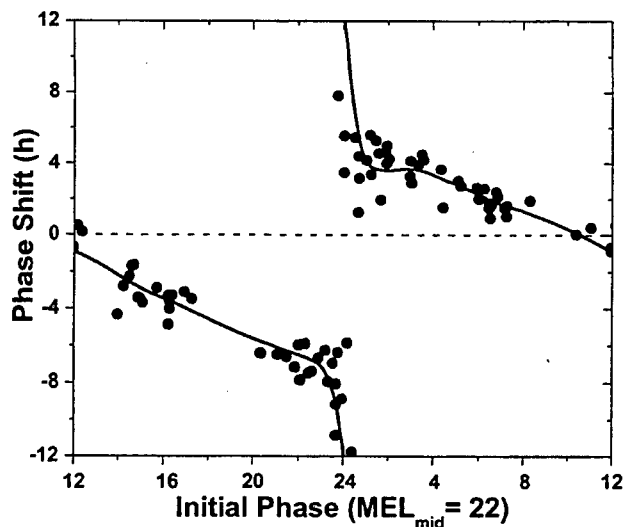
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A 3-cycle bright light stimulus (10,000 lux) presented against a background of dim light (10-15 lux) induced type 0 resetting, in which phase shifts as large as 12 h were observed, in the human endogenous core body temperature rhythm¹. We analyzed the plasma melatonin data from that experiment to determine if type 0 resetting was also induced in this hormonal rhythm.

Ninety-four phase resetting trials were conducted in 71 healthy young male subjects (23.3 ± 3.6 yrs) using a previously described protocol². Melatonin data were available for 83 trials in 64 subjects. Each trial consisted of a pre-stimulus constant routine (CR), three 24-h cycles each containing a 5 h bright light pulse, and a post-stimulus CR. Melatonin phase was defined to be the midpoint of the upward and downward crossings of the mean level of melatonin values between the first 5 and 29 h of each CR. Phase shifts (filled circles) were calculated as the difference between melatonin phases in the pre- and post-stimulus CRs. The melatonin resetting data were smoothed (solid line) with a 13-point followed by a 7-point running average, using an iterative procedure³.

The 3-cycle stimulus induced type 0 resetting in the plasma melatonin rhythm, with the largest phase shifts observed in the critical region, which occurred 2 h after the melatonin midpoint. The smoothed fit to the data is quite similar to that reported previously for core body temperature^{1,2}, and data does not show a dead zone. This supports the hypothesis that the circadian pacemaker driving both the plasma melatonin and endogenous core body temperature rhythms is sensitive to bright light throughout the subjective day, and that bright light induces both phase and amplitude resetting.

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PHASE-SHIFTING EFFECTIVENESS OF INTERMITTENT LIGHT PULSES: RELATIONSHIP TO MELATONIN SUPPRESSION

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Animal studies demonstrated that pulses of bright light of a duration of as short as 3 seconds produced small phase shifts of the circadian pacemaker.¹ The resetting action of light has been shown to be most efficient at the beginning of the light exposure, with minimal additional phase shift produced by further extension of the light stimulus.² In humans, it has been demonstrated that three days of exposure to short, intermittent pulses of bright light are sufficient to induce significant phase advances of the circadian pacemaker, but also that they are more effective on a per minute basis than is continuous bright light exposure.³ We conducted a study to determine if brief pulses of bright light administered during the early subjective night will induce a significant phase delay of the endogenous circadian timing system and whether this effect is linked to light-induced melatonin suppression. We have thus far exposed six healthy subjects (mean $\pm$ SE; 26.8 ± 3.2 yr.) to one of the three following conditions for 6.5 h: (1) continuous bright light exposure ($\sim 9,500$ lux); (2) continuous very dim light exposure (< 1 lux); (3) intermittent bright light exposure: six 15-minutes light pulses ($\sim 9,500$ lux) separated by one hour of very dim light (< 1 lux). The "light" exposure period was centered 5.8 h before their habitual waketime (corresponding to ~ 3.5 h before their core body temperature minimum). The initial melatonin phase was assessed by the midpoint between the DLMO_n and the DLMO_{ff}⁴ during a constant routine (CR) following three habituation days. The area under the curve (AUC) of the melatonin profile during the 6.5-h exposure was compared to the AUC of the same period during the CR, as an estimation of the melatonin suppression. The phase-shifting effect of the intervention regimen was assessed using the same procedure during a second CR following the light stimulus. Subjects remained in dim light (< 3 lux) during the CRs. The phase-delays measured were 0.80 h and 2.67 h after continuous bright light exposure; 0.14 h and 0.45 h after continuous very dim light exposure; 2.43 h and 2.67 h after intermittent light. Thus, the phase delays observed under the intermittent light condition were comparable to those measured after continuous bright light exposure, though the bright light represents only 23% of the 6.5 h exposure. The melatonin release was suppressed by 89% and 90% during the continuous light exposure, suppressed by 40% in one subject and increased by 7% in another one during the intermittent light exposure. These preliminary suggest that intermittent exposure to bright light is effective in inducing circadian phase shifts and that the magnitude of those phase shifts is not a function of the degree of melatonin suppression. We anticipate presenting results from additional subjects at the meeting.

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A SUBSTANCE P ANTAGONIST REDUCES THE LIGHT-INDUCED SUPPRESSION OF MELATONIN IN HUMANS
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The role of substance P (SP) and its primary receptor neurokinin-1 (NK-1) in the human circadian system is not known. The present study was designed as a pilot to assess the effect of CP-122,721 (a selective NK-1 receptor antagonist) on night-time melatonin in the presence and absence of retinal illumination.

Healthy drug free male subjects (n=6, aged 21-34 yrs) received placebo, 20 or 200 mg CP-122,721 (double blind, randomized, crossover design) 3 hrs before white light treatment (30 min, 120 lux, 91 $\mu\text{W}/\text{cm}^2$, full field, uniform retinal illumination with pupil dilation) timed to occur on the rising phase of the subjects' endogenous melatonin rhythm. According to our standard protocol¹ blood samples were taken -15, 0, 15, 30, 45, 60, 75, 90 and 120 mins after lights on and subjects' melatonin concentrations measured on night 2 (treatment night) were compared with a baseline night 24 hrs earlier (N1, no treatment). Posture and environmental lighting (<10 lux from 21.00-23.00 h, darkness and wearing eye-masks from 23.00-07.00 h) were identical on both nights except for the light treatment period. At least a week separated the treatment legs. Light-induced melatonin suppression was calculated using the average melatonin values at 15, 30 and 45 mins on treatment night (N2) as a percentage of the same average from the baseline night (N1). The F-test in an ANOVA crossover model indicated a significance difference among the 3 treatments ($P=0.048$). Post hoc pairwise t-tests demonstrated borderline significance ($P = 0.06$) between placebo (% suppression $\pm$ SEM; $60 \pm 14\%$) and 20 mg CP-122,721 ($40 \pm 11\%$). Melatonin suppression was significantly different ($P = 0.02$) between placebo and the high 200 mg dose of CP-122,721 ($34 \pm 7\%$). However, there was no statistically significant difference ($P = 0.50$) between the two doses of the drug (20 and 200 mg). In two earlier legs of the study, there was no significant effect of 200 mg CP-122,721 on plasma melatonin in the absence of retinal illumination.

In summary the findings show that, as predicted, light exposure at night produced a significant suppression of plasma melatonin. Compared with placebo, this light-induced suppression was significantly reduced following CP-122,721 administration. These results suggest that SP may have an inhibitory effect on the retina-SCN-pineal axis in humans. The exact mechanism of action remains to be elucidated.

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DIURNAL VARIATION OF RETINAL SENSITIVITY IN MORNING AND EVENING TYPES

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The purpose of this study was to evaluate the diurnal variation of retinal sensitivity, as measured with the electroretinogram (ERG), in morning and evening type subjects. Nine evening types and nine morning types (11W: 7M; aged 20-25) were recruited according to their scores on a French version of the Horne & Ostberg Morningness-Eveningness Questionnaire. ERG testing was performed twice on each subjects, at 10:30 p.m. and at 8:00 a.m., the following morning. After a 30-minute dark adaptation period, scotopic ERGs were recorded with a LKC UTAS-E-3000 system using 11 blue (400nm) flashes with intensity varying from -5.4 to -1.35 log cd.m⁻².sec. Subjects were kept in dim light (<10 lux) during all of the experiment, i.e. from 7:30 p.m. to 9:30 a.m. Salivary melatonin samples were taken every half-hour from 8:30 p.m. to 12:00 a.m. and from 6:30 a.m. to 9:30 a.m.; urine was also collected at 12:00 a.m., 6:30 a.m. and 9:30 a.m. Two variables were measured on electroretinographic data: maximal rod response (V_{max}) and retinal sensitivity (K) which is defined as the flash intensity needed to obtain an ERG response of half of the V_{max} . Statistical analysis were conducted by a 2X2 ANOVA for repeated measures. Preliminary results show that retinal sensitivity was significantly lower (higher K) in the morning than in the evening session for both evening and morning types ($p<0.05$). No significant diurnal variation was observed in the V_{max} measurements. The decreased sensitivity in the morning could be related to the burst of rod disk shedding which takes place between 0.5 and 2.5 hours after light onset in the rat. However, since this process has an endogenous circadian rhythm, we would have expected it to happen later in the evening types (mean waking time: 9h40) than in the morning types (mean waking time: 7h39). The phase difference between the two groups will be more precisely defined with the melatonin RIA results.

EVENING LIGHT: ASPECTS OF HUMAN ENTRAINMENT

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Americans over 60 years of age spend the last 4 hours of the day before bedtime in rather dim illumination. Because such dim illumination may provide only a weak stimulus to the phase-delay portion of the phase-response curve, a more advanced circadian entrainment than is desired might sometimes result. This may provoke evening sleepiness and early awakening, i.e., the advanced sleep phase syndrome. Possibly the problem becomes more troublesome with age because the eyes become less responsive to dim lighting. Postmenopausal women (N=154, age 67 years, SD 8 years) were recorded for 7 nights in their homes with Actillum instruments, so that the intensity of evening lighting could be examined. The median evening illumination was 24 lux (mean 48 lux, range 2-620 lux). Surprisingly, no significant correlations were noted between evening illumination and quality or timing of actigraph-measured sleep, symptoms of sleep disturbance, or mood. On the other hand, a pilot experimental study which provided enhanced illumination near the subject's television chair, did produce a slightly delayed sleep pattern and relief of symptoms.

WEEK-LONG BLOOD PRESSURE MONITORING FOR REFERENCE VALUES AND DIAGNOSIS OF DISEASE RISK SYNDROMES. Katarina Borer, Germaine Cornelissen, Franz Halberg

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Aim. Reference values to study effects of walking on blood pressure (BP) and heart rate (HR) of 21 postmenopausal women 49-69 years of age screened for both high BP, abnormally swinging BP (circadian hyperamplitude-tension, CHAT) or both. *Background.* CHAT, defined by a circadian amplitude exceeding the upper 95% prediction limit of gender- and age-matched peers, is associated with a 720% increase in the risk of ischemic cerebral events in a prospective 6-year study on 297 patients and studies on hypertensive rats and 221 pregnant women. *Method.* BP and HR were measured at 30-min intervals around the clock for about 7 days, using a TM2421 ambulatory monitor (A&D Co, Tokyo, Japan). Data were fitted by 24- and 12-hour periodic cosine curves to estimate parameters compared with 90% prediction limits derived on the basis of data from presumably clinically healthy women in the same age group. *Results.* Circadians are documented for systolic (S) and diastolic (D) BP and HR. *Discussion.* Of the 21 women monitored, a 53-year-old African-American (N21) had both systolic (S) and diastolic (D) CHAT, with circadian amplitudes 14.2% and 5.2% above the acceptable limits of SBP and DBP, respectively. Two other women had slight elevation of their SBP circadian amplitude only. Fatal heart attacks had occurred in N21's family at 39 (mother), 62 (father) and 54 (sister) years of age; a brother died from an aneurysm at 43 years of age, and another brother (age not specified), also hypertensive, died of lymphoma. One living sister (age not specified) is treated for hypertension and diabetes. In N21, CHAT may reflect a positive family history of cardiovascular disease, but population studies involving another 400 subjects have confirmed that CHAT represents a risk syndrome not only independent from the average BP value but also of the presence of other risk factors such as smoking and age. *Conclusion.* As suggested by Janeway in 1904, it is now cost-effectively possible to assess, in addition to a mean value of BP, indices of BP variability such as the circadian amplitude before a patient is seen in a physician's office.

CIRCADIAN RHYTHM DISTURBANCES IN ALZHEIMER'S DISEASE. David G. Harper, Andrew Satlin, Patricia C. Harlan, Rachel L. Goldstein, Barbara Manning and Ladislav Volicer. Harvard Medical School and McLean Hospital, Belmont, MA

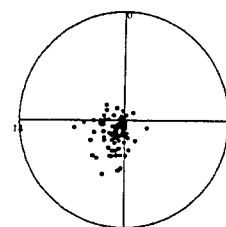
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Degenerative dementia is a devastating age-associated infirmity described as a progressive syndrome of global loss of cognitive ability sufficient to impair social or occupational functioning. The most common and well known of the degenerative dementias is Alzheimer's disease (AD). In addition to the well known cognitive symptoms of AD including deterioration of executive function and memory, there are many noncognitive symptoms associated with it. Sleep disturbance is one common accompanying symptom of AD, and caregivers report that their decision to institutionalize is often due to exhaustion as a consequence of managing sleep disturbance. This symptom could be caused or exacerbated by a reduction in amplitude and phase delay of the rhythms of core-body temperature and activity. We have studied the rhythms of core-body temperature in a group of 19 male patients with NINCDS-ADRDA probable Alzheimer's disease (pAD) and 8 male controls using a modified constant routine protocol. Nutrition was given in equicaloric, hourly snacks, temporal cues were removed from the environment and light was maintained at a constant, low level (70 lux). All subjects were allowed to sleep between the hours of 22:00 and 06:00. We have found that the circadian rhythm of core-body temperature as measured by cosinor analysis was delayed in this cohort (controls = $15:18 \pm 00:42$; pAD = $19:01 \pm 00:24$) and the amplitude was reduced (controls = $0.47 \pm 0.05^\circ\text{C}$; pAD = $0.35 \pm 0.03^\circ\text{C}$). These results are consistent with the presence of a disturbance in the endogenous circadian rhythm of patients with Alzheimer's disease.

RHYTHMS OF AGITATION: DOES SUNDOWNING EXIST? Ancoli-Israel, S. Shochat, T. Martin, J. Gehrman, P. Corey-Bloom, J. Connor, D. UCSD/VASDHS, Department of Psychiatry, 116A, 3350 La Jolla Village Drive, San Diego, CA 92161

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A number of studies have described the phenomenon of "sundowning" (i.e., agitation which is acutely exacerbated in the evening hours) in 10-25% of institutionalized dementia patients. As part of a larger study on sleep and agitation in nursing home patients, 85 patients (28 men, 57 women, mean age=83 yrs, sd=7.6, range=61-99) had minute-by-minute activity recorded for 72 consecutive hours with Actillum recorders (Ambulatory Monitoring, Inc. Ardsley, NY). Patient's agitation was rated by trained observers every 15 minutes for 61 of these 72 hours. All patients had a diagnosis of Alzheimer's disease and were rated as agitated by nursing home caregivers. The mean acrophase for agitation based on behavior ratings was 1438h, though there was considerable variability in the agitation rhythms displayed (see figure). The figure shows agitation acrophase time (circumference) and goodness of fit (R^2 ; radius) of an extended cosine model. Patients with strong agitation rhythms (high R^2) generally had an agitation acrophase between 1200-1800, not during the traditional "sundowning" period. Patients with weaker agitation rhythms (i.e. lower R^2 ; data points closer to the center of the plot) had acrophase times scattered throughout the 24-hour period. Surprisingly, only 2 patients (2.4%) were true "sundowners". Results suggest that, although sundowning is uncommon, agitation appears to have a strong circadian component in most patients. As a result, interventions targeting circadian rhythm disturbances such as light therapy may be helpful for many patients in alleviating agitation.



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CORTISOL CIRCADIAN RHYTHMS DURING THE MENSTRUAL CYCLE AND WITH SLEEP DEPRIVATION IN PREMENSTRUAL DYSPHORIC DISORDER AND NORMAL CONTROL SUBJECTS. Barbara L. Parry, Suryabanu Javeed, Gail A. Laughlin, Richard Hauger, Paul Clopton. Department of Psychiatry, University of California, San Diego, La Jolla, CA 92093-0804

Background: In this study we extended previous work by examining whether disturbances in the circadian rhythms of cortisol during the menstrual cycle distinguish patients with premenstrual dysphoric disorder (PMDD) from normal control (NC) subjects. In addition, we tested the differential response between the 2 groups to the effects of early and late partial sleep deprivation on cortisol rhythms. **Methods:** In 15 PMDD and 15 NC subjects, we measured cortisol levels every 30 minutes from 18:00 to 09:00 hours (h) during mid follicular (MF) and late luteal (LL) menstrual cycle phases and also during a randomized crossover trial of early (sleep 03:00–07:00 h) (ESD) versus (vs.) late (sleep 21:00–01:00 h) (LSD) partial sleep deprivation administered in two subsequent and separate luteal phases. **Results:** In follicular vs. luteal menstrual cycle phases, we observed altered timing, but not quantitative measures of cortisol secretion in PMDD compared with NC subjects: In the LL vs. MF phase, the cortisol acrophase was a mean of 1 h earlier in NC, but not in PMDD, subjects. The effect of sleep deprivation on cortisol timing measures also differed for PMDD vs. NC subjects: During LSD (when subjects' sleep was earlier), the cortisol acrophase was almost 2 h earlier in PMDD, but not in NC, subjects. **Conclusions:** Timing rather than quantitative measures of cortisol secretion differentiated PMDD from NC subjects during both the menstrual cycle and in response to early versus late sleep deprivation interventions.

DUAL EPILEPTIC FOCI IN A SINGLE PATIENT EXPRESS DISTINCT TEMPORAL PATTERNS DEPENDENT ON LIMBIC VERSUS NONLIMBIC BRAIN LOCATION

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How timing information is transferred from the suprachiasmatic nucleus to other regions of the brain to mediate neuronal activity, whether physiologic or pathologic, is largely unclear. Epileptic seizures are episodes of paroxysmal discharges that occur in some patients in daily patterns. A 57 y.o woman with intractable epilepsy and a well-documented, long term seizure diary provided a unique means to demonstrate how susceptibility to chronobiological modulation varies with brain region.

Evaluation for epilepsy surgery required intracranial EEG with bilateral intrahippocampal and subdural electrodes. Two independent epileptic foci were revealed, one right hippocampal causing complex, partial seizures, and the other right parietal causing ictal pain of the left hand.

Five years of an hour-by-hour seizure diary maintained by the patient and her husband yielded 1009 seizures identified by major symptoms corresponding to those confirmed by EEG. Seizure rate was stationary as determined by simple regression of seizure number and interseizure interval. Approximate entropy analysis determined data were highly ordered by time of day. Seizures from both foci occurred periodically with a dominant period of 24 hours but were out of phase with each other (clock time of phase peak by FFT– nonlinear least squares with 95%CI: temporal 12:10 (10:59 – 14:19), parietal 02:50 (00:28 – 05:12) . 573/694 (83%) of temporal lobe seizures compared to 133/315 (36%) parietal seizures occurred between 0700–1900. Beyond ultradian periods most likely from harmonic artifacts of FFT analysis, an infradian rhythm of 18 days was resolved in temporal seizures.

These data suggest that neuronal excitation and inhibition, depending on the anatomical system involved in epilepsy, may be differently affected by circadian modulation and confirms daily distributions of seizures demonstrated in groups of patients with limbic and nonlimbic partial epilepsy.

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The seasonality of symptoms and the efficacy of light therapy suggest involvement of the circadian pacemaker in Seasonal Affective Disorder (SAD). The variations of body temperature originating from the circadian pacemaker and those related to behaviour were studied in SAD patients and matched controls.

Seven (1 male and 6 female) SAD patients (mean age $\pm$ sem 36.3 $\pm$ 5.3, range 22-54) and 7 controls matched for age, sex and menstrual cycle phase (mean age $\pm$ sem 38.1 $\pm$ 4.5, range 25-56) were subjected to a 120-hour forced desynchronization protocol. SAD patients participated once during a depressive episode, once after recovery upon light therapy and once in summer. Controls were studied once in winter and once in summer. All subjects were drug-free in the months before the experiments. After 4 baseline days at home, in which sleep was scheduled between midnight and 8.00 am (verified by wrist actometry) and 1 habituation night in the time isolation unit, participants (blind to the study design) were subjected to the 120-hour forced desynchronization protocol and were forced to live on 20-hour days (13.5 hours of wakefulness in dim light (<10 lux) and 6.5 h of darkness spent in bed). Core body temperature data were stored at 1-minute intervals and analysed by an iterative method (1) to distinguish between circadian pacemaker related variation and variation due to 'masking'.

During the winter experiments, subjects entered the isolation unit in October-March. Summer experiments were performed in May-August. Before and after each experiment, subjects completed the Structured Interview Guide for the Hamilton Rating Scale of Depression (HRSD), Seasonal Affective Disorder self-rating version (SIGHSDSR), consisting of the 21-item HRSD and 8-item atypical symptom scale (ATYP) and the Beck Depression Inventory (BDI). At baseline SAD patients scored 32 $\pm$ 4 (mean $\pm$ sem) on the SIGHSDSR (HRSD 21 $\pm$ 3; ATYP 11 $\pm$ 2) and 20 $\pm$ 2 on the BDI during the depressive episode. In all other conditions, subjects had a baseline SIGHSDSR score $\leq$ 10 and BDI score $\leq$ 5. In all conditions, sleep-wake related and circadian variations of body temperature were observed. Analysis of the temperature data of patients and controls did not show significant differences in the mean level and amplitude of the sleep-wake related variation of body temperature. Between the SAD patients and controls no significant differences were found in endogenous circadian rhythm period (τ) and the time in which the circadian temperature minimum was reached. However, compared with controls the amplitude of the circadian temperature variation in winter was significantly smaller in SAD patients during a depressive episode and after recovery upon light therapy (paired-samples t-test, $p=.041$ and $p=.037$ respectively).

The obtained τ -values and the timing of the circadian temperature minimum in SAD patients during a depressive episode, after recovery on light therapy and in summer do not differ from those found in matched controls. There is support for a smaller circadian temperature amplitude during an SAD episode as well as in the remitted state in patients compared to controls in winter.

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SCN SIGNAL OF CHANGE OF SEASON IN SEASONAL AFFECTIVE DISORDER

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Most mammals use changes in day-length to detect change of season and regulate the timing of seasonal rhythms. The suprachiasmatic nucleus (SCN) plays a critical role as a mediator of these responses. The SCN generates a signal that encodes day-length, and it adjusts the duration of this signal in response to seasonal changes in day-length, which it detects via input from the retina. We hypothesized that seasonal changes in photoperiod induce winter depression in patients with seasonal affective disorder (SAD) and that the SCN mediates this response. A basic assumption of this hypothesis is that the SCN in patients with SAD detects and responds to seasonal changes in day-length. We tested this assumption, using winter and summer plasma melatonin profiles as surrogate measures of the SCN's response.

Patients (N = 55) met criteria of Rosenthal et al. for SAD. Healthy volunteers (N = 55) were matched with patients for age, sex and menstrual cycle phase. Subjects were free of medications, and patients refrained from using light treatment in the winter. In the winter and in the summer, plasma samples were obtained every 30 minutes for 24 hours while subjects remained in constant dim (< 1 lux) light. Melatonin levels were measured by RIA. The intrinsic (dim-light) duration of active melatonin secretion was assessed with the method of Lewy et al. and was used as a surrogate measure of the SCN's response to changes in night-length.

In patients, the intrinsic duration of melatonin secretion was longer in winter than in summer ($p < 0.001$). In healthy volunteers, there was no change. The difference between the groups' responses was statistically significant ($p < 0.05$). The seasonal change was more robust in male than in female patients.

The results show that the SCN in patients with SAD differs from that in healthy individuals in being able to detect seasonal changes in day-length and to transmit a signal of these changes to the pineal gland and, potentially, other sites in the organism. They confirm a basic assumption of the hypothesis that SCN-mediated responses to seasonal changes in day-length induce winter depression in SAD.

RHYTHMICITY OF LYMPHOCYTES AND MELATONIN IN HUMANS

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In a pilot study of 2 subjects, we recently documented a circadian rhythm of lymphocyte counts with increased levels during the habitual time of sleep episode (1). The aim of the present study is to clarify the relationship between the circadian oscillation of the cells and that of two reliable circadian markers: core body temperature (CBT) and plasma melatonin (MLT). Five healthy male volunteers (mean age: 23.2 ± 2.77 yrs) were recruited to undergo a 35-h constant routine procedure (CR). CBT was recorded every minute to assess endogenous circadian phase by a dual-harmonic regression model (2). Blood samples were drawn every 20 and 60 min during the entire duration of CR to assess the endogenous circadian rhythms of lymphocytes and MLT, respectively. Plasmatic melatonin concentration was determined by radioimmunoassay (Stockgrand, Guilford, UK). CBC analysis was performed on a Coulter JTTM CBC & Differential machine (Coulter Elect., Inc., FL, USA). Results were first averaged per subject using 20 and 60min bins, and were subsequently averaged per bin across subjects. Results were aligned relative to the endogenous circadian phase (attributed a relative clock time of 6h00). A significant circadian rhythm of lymphocytes was observed using a single-harmonic regression model ($p < 0.05$). Highest values of lymphocyte counts were observed during the descending limb of the endogenous circadian rhythm of CBT and during the rising limb of the circadian curve of MLT. Observations based on the lymphocyte counts median line (drawn at $\sim 1.54 \times 10^3$ cells/ul) confirm that lymphocyte counts during the habitual time of sleep period are greater than during (subjective) daytime. We have also observed that the changes in lymphocyte counts tend to occur at specific times of the endogenous circadian rhythm of melatonin secretion. Although limited to relatively few subjects, the present study confirms the presence of a clear and robust circadian rhythm in lymphocyte counts (1). These data are not explained by postural changes and are consistent with those of prior studies that were conducted without the use of the CR procedure and frequent blood-sampling (3). Possible explanation for the circadian variation of lymphocytes could be that higher plasmatic melatonin concentration induces lymphocyte recruitment to the circulation, thereby contributing to the process of "policing for foreign antigen" (4). 1) Cho PF *et al.* *SRO*, 1999;2 (sup. 1), 600. 2) Brown E and Czeisler CA. *JBR*, 1994;7:315. 3) Born J *et al.* *J Immun.*, 1997;158, 4454. 4) Zinkernagel RM and Doherty PC *Nature*, 1974;248: 701. Supported by the Medical Research Council of Canada.

GENDER DIFFERENCES IN TIME-DEPENDENT POSTPRANDIAL TRIACYLGLYCEROL VARIATIONS

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We have previously reported that triacylglycerol (TAG) is elevated during circadian night in constant routine conditions (1), that it is raised more after a standard meal during a simulated night shift compared to a day shift (2) and that in the latter case, preceding diet (low fat or high fat) strongly influences the variations observed (3). We have now compared the response to an identical standard meal (3330kJ, 37% fat, 52% carbohydrate and 11% protein) after a low fat pre meal (5.5h before the standard meal) at 1330h (simulated day shift) and 0130h (simulated night shift) in normal healthy women ($n=17$) and men ($n=18$).

Men and women aged 21-33 years (mean $\pm$ S.E.M. 25.5 ± 0.55 years) were recruited from postgraduate students and staff of the University of Surrey. Subjects maintained a controlled sleep wake cycle (2300-0700h dark/sleep) for a 3 day baseline. During the simulated day shift, they ate a pre-meal (2008kJ, 3% fat, 90% carbohydrate and 7% protein) at 0800h and the test meal at 1330h. On the simulated night shift, they ate the pre-meal at 2000h and a test meal at 0130h, remaining awake for the rest of the night. Baseline blood samples were taken at -15 and 0 min (before the test meal) and at 20, 40, 60, 90, 120, 180, 240, 300, 360, 420, 480 and 540 min after the test meal in each case. Subjects remained seated except for visits to the toilet, and in low level light (100-300 lux) throughout the blood sampling period.

We found that despite males and females having similar basal TAG levels before the day time meal and the night time meal, men showed higher TAG levels than women after the day time meal, 1.77 ± 0.14 (peak TAG mean $\pm$ S.E.M.) versus 1.24 ± 0.10 mmol/L respectively ($P < 0.01$), and after the night time meal, 2.02 ± 0.18 versus 1.24 ± 0.10 mmol/L respectively ($P < 0.001$). Moreover, the increase in TAG after the night time meal was substantially greater in men than in women, 280.1 ± 41.7 (incremental area under curve (IAUC) mean $\pm$ S.E.M.) versus 54.9 ± 16.32 mmol/L.min respectively ($P < 0.0004$). The night-day difference did not reach significance in women.

We conclude that elevated TAG response to a standard meal is found consistently in men, but not in women. These findings have important implications for heart disease risk in shift workers.

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PAP SMEAR DETECTION OF UTERINE CERVICAL EPITHELIAL CARCINOGENESIS AND PROGRESSION IS CIRCAANNUALLY RHYTHMIC AND APPARENTLY TIED TO RHYTHMS IN HUMAN SEXUALITY

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Cervical cancer screening programs uncover vastly more "pre-malignant" epithelial lesions than cancers. Most of these lesions resolve spontaneously. Mammalian reproductive biology, as well as, the biology of cancers arising in reproductive tissues are modulated seasonally. We inspected the monthly frequencies, (per thousand screening examinations) of HPV infection, mild, moderate and severe dysplasias and atypias, as well as carcinoma *in situ*, micro-invasive carcinoma and frankly invasive cervical cancer, among 920,359 consecutive interpretable pap smears obtained by a single screening laboratory in Leiden, Holland during a continuous 16-year span from 1983 through 1998. Profound and similar circannual rhythmicity characterizes each of these cervical epithelial abnormalities. Circannual rhythms in sex hormone concentration, human sexual activity and conception frequency in Northern Europe covary with these summer peaking annual rhythms in HPV, precancer and cervical cancer detection among Dutch women. Peri-equatorial conception frequency and cervical cancer detection frequency each peak in winter rather than summer. These data imply that all of the biological steps in uterine cervical epithelial carcinogenesis and progression wax and wane during the year with a pattern that is characteristic for the latitude and weather of a population's residence and that population's circannual pattern of sexual behavior.

MEANINGFUL AND REPRODUCIBLE REPRODUCTIVE CYCLE MODULATION OF CANCER BIOLOGY

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The growth characteristics of a subcutaneous, transplantable estrone binding breast cancer, a chemically induced sarcoma and a clonally manipulated murine breast cancer cell line were studied in young, cycling C₃HeB/FeJ, CD₂F₁ and BALB/c female mice, respectively, as a function of vaginal cytology determined estrous cycle stage {proestrus (P), estrus (E), metestrus (M), or diestrus (D)}. Several independent studies of each of these three *in vivo* model systems demonstrate prominent waxing and waning of tumor size reproducibly phase locked to the fertility cycle. Average tumor size, irrespective of fertility stage of measurement, shows the typical sigmoidal cancer growth. These same tumor sizes, organized according to the fertility cycling stage of measurement, unmask this rhythmic tumor biology. Tumor size changes very little between P and E, increases slightly between E and M, grows most markedly between M and D and shrinks sharply as the next proestrus is approached. Up to a six fold difference in tumor size occurs during each fertility cycle. Within a 3-5 day normal range, some mice cycle faster than others. On average, cancer grows slower in the faster cyclers. This effect of cycling frequency upon tumor growth is not dependent upon the estrous cycle locked tumor growth/shrinkage pattern. The mammalian fertility cycle rhythmically modulates the host-cancer balance and cancer growth. These results may help explain why the fertility cycle timing of breast cancer resection determines breast cancer outcome in both mice and pre-menopausal women.

THE NOCTURNAL INCREASE IN ALDOSTERONE IS BLUNTED DURING SLEEP DEPRIVATION

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The aim of this study was to determine the effect of sleep deprivation on the 24-h profile of aldosterone and its consequences on renal function. Aldosterone and its main regulatory factors, ACTH (evaluated by cortisol measurement) and the renin-angiotensin system (evaluated by plasma renin activity (PRA measurement) were determined every 10 min over 24 h in 8 healthy subjects in supine position, once with nocturnal sleep and once during total sleep deprivation. Diuresis, natriuresis and kaliuresis, plasma sodium (Na^+) and potassium (K^+) were measured in both conditions.

During sleep deprivation, aldosterone displayed lower plasma levels and pulse amplitude in the 2300-700h period than during sleep. Similarly, PRA showed lower levels and lower pulse amplitude and frequency. Plasma cortisol levels were slightly enhanced. A dual influence, by the renin-angiotensin system during sleep and by the adrenocorticotrophic system during wakefulness is exerted on aldosterone pulses throughout the 24 hours. Overnight variations in plasma K^+ and Na^+ were not significant in both conditions. Diuresis and kaliuresis were not influenced by sleep deprivation. In contrast, natriuresis significantly increased during sleep deprivation.

This study demonstrates that acute sleep deprivation modifies the 24-h aldosterone profile by preventing the nocturnal increase in aldosterone release and leads to altered overnight electrolyte and water balance.

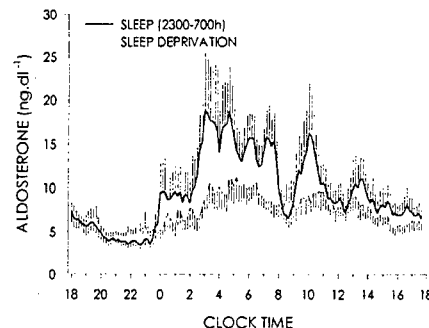


Figure : Effect of sleep deprivation on the 24-h profiles of aldosterone in 8 subjects (mean $\pm$ SE)

EFFECT OF REPEATED CAFFEINE ADMINISTRATION ON CORE BODY TEMPERATURE DURING 88 HOURS OF SUSTAINED WAKEFULNESS.

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Several studies have examined the effectiveness of caffeine administration as a countermeasure for the increased fatigue and decreased performance associated with extended periods of sustained wakefulness [1-3]. Acute administration of caffeine during these protocols has been reported to increase performance and alertness levels. In addition, significant hyperthermic effects associated with acute caffeine administration have also been reported [1, 2]. This analysis, which is part of a larger study, investigates the effects of repeated caffeine administrations during 88 hours of sustained wakefulness.

Twenty-one male subjects (aged 21-47) lived in the sleep laboratory, with light levels <50 lux, for 10 days (9 nights). Following 3 baseline days, subjects were required to remain awake for 88 hours, followed by 3 recovery nights. During the final 66 hours of sustained wakefulness subjects were assigned to receive either caffeine (0.3mg/kg/hr; N=12) or placebo (N=9) at hourly intervals, in a randomized, double-blind fashion. The initial administration occurred 22 hours after the commencement of the sustained wakefulness period. During the wake period core (rectal) body temperature was measured continuously, and blood samples were collected at 90 minute intervals via an indwelling canula.

In the caffeine group a steady increase in plasma caffeine levels was evident from within 3.25 hours of the first administration, and continued increasing until reaching a plateau after approximately 29 hours. In addition, a coincident elevation in core body temperature was observed in the caffeine group, relative to the placebo group ($p < 0.001$). The magnitude of the temperature elevation was greatest during the first few hours of caffeine administration (mean = $0.51 \pm 0.02^\circ\text{C}$). This hyperthermic effect endured for approximately 18 hours following the first caffeine administration.

The activating effects of caffeine, including increased alertness and physical activity, may underlie this elevation in core temperature. Alternatively, this hyperthermic effect may represent a possible mechanism mediating the increased alertness and performance levels associated with the ingestion of caffeine.

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Exposure to short day lengths or long duration melatonin (MEL) signals induces testicular regression in Siberian hamsters. After approximately 16-24 weeks the gonads undergo spontaneous recrudescence. Thereafter, hamsters remain unresponsive to inhibitory day lengths or MEL signals (refractoriness). Only after experiencing ≥ 12 weeks of long day lengths do individuals regain responsiveness to short day lengths and undergo a second gonadal regression.

MEL administered to the suprachiasmatic nucleus (SCN), the nucleus reunions (Nre) or the paraventricular nucleus of the thalamus (PVt) prevents gonadal growth in juvenile hamsters kept in constant light (Badura and Goldman, 1992). The neural sites involved in the development of refractoriness are unknown. MEL has not been administered to a particular neural site for an interval long enough to establish whether or not refractoriness occurs. MEL may act on one target tissue to induce gonadal regression and then trigger refractoriness. Alternatively, each MEL-binding site may become refractory, without affecting responsiveness at other target tissues. To discriminate between these hypotheses Siberian hamsters were housed in 16 hours light/day, pinealectomized and implanted with a cannula containing MEL or vehicle aimed at the SCN, Nre or PVt (week 0). A control group of hamsters was implanted at week 0 with subcutaneous (s.c.), constant release Silastic implants containing MEL or left empty. At week 40 all MEL implants were removed and hamsters infused s.c. with MEL for 12h/day for an interval of 6 weeks, at the end of which, paired testis weights were recorded.

Control animals did not undergo regression when treated with empty cannulae and 90% exhibited regression in response to the second, s.c. infusion of MEL. 50% of the hamsters with MEL-implants aimed at the SCN underwent initial gonadal regression ($n=14$) and 71% of these individuals responded to the subsequent s.c. infusion with a **second** gonadal regression. A similar result was observed in hamsters with melatonin-implants aimed at the Nre; 63% initially responded with gonadal regression, of these 71% regressed in response to the second, s.c. infusion. PVt-implants resulted in 50% of the hamsters exhibiting the initial gonadal regression and all of these individuals exhibited regression in response to the second, s.c. infusion. These results suggest that restricted long-term exposure of individual target tissues to MEL induces refractoriness limited to the sites so stimulated without compromising responsiveness at other target tissues.

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LEPTIN EFFECTS ON IMMUNE FUNCTION AND ENERGY BALANCE ARE PHOTOPERIOD-DEPENDENT IN SIBERIAN HAMSTERS (*PHODOPUS SUNGORUS*)

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Many adaptations have evolved in small mammals to maximize survival during winter. One such adaptation involves adjusting immune function in advance of the stressful conditions of winter. Leptin is a hormone produced by adipose tissue, and in addition to its central role in energy metabolism, leptin mediates the interactions among energy allocation, immune function, and reproduction. To examine this interaction further, exogenous leptin was administered to Siberian hamsters (*Phodopus sungorus*) for 2 weeks; hamsters were housed in either long or short days for a total of 12 weeks. Short-day hamsters displayed the expected reductions in immune function, body mass, fat mass, and food intake. Exogenous leptin treatment counteracted the short-day suppression of immune function and the reduction in food intake in short-, but not in long-day hamsters. In most of the measured fat depots and body mass, leptin had no effect in long days. In sum, leptin administered to short-day animals evoked long-day responses. Taken together, these data suggest that leptin may be acting as a signal to the immune system indicating energy availability. Leptin appears to act differentially, according to photoperiod, to regulate both immune and energetic parameters.

PHOTOPERIOD AFFECTS IMMUNE FUNCTION AND SICKNESS BEHAVIOR IN SIBERIAN HAMSTERS. Staci D. Bilbo and Randy J. Nelson, Department of Psychology, Behavioral Neuroendocrinology Group, Departments of Neuroscience, Biochemistry and Molecular Biology, Reproductive Biology Division, The Johns Hopkins University, Baltimore, MD, 21218-2686 USA.

Winter is often energetically more demanding than summer, and the risk of infection may be greatest at this time. Many rodent species enhance immune function in the laboratory under winter-simulated, short day lengths, and this increased energy allocation towards immune function is primarily accomplished through the curtailment of reproductive activity and growth during the winter. Infected animals generally exhibit 'sickness behaviors' that aid in the recovery against pathogens. One component of this suite of adaptive behaviors is anorexia; infected individuals that reduce food intake decrease recovery time and increase survival from infections. Because anorexia is adaptive during infections, but potentially problematic during energy shortages (e.g., during the winter), we hypothesized that anorexia would not occur in infected Siberian hamsters (*Phodopus sungorus*) housed under winter photoperiods because these animals dramatically lose body mass during short daylengths. Male and female hamsters were housed in either short (LD 10:14) or long (LD 14:10) day lengths for 8 weeks. Short-day males and females exhibited significantly lower lymphocyte proliferation 24 h following an acute mitogen challenge than animals in long days. A separate cohort of animals was acclimated to a sweetened condensed milk solution; and was assessed for anorexia following injections with lipopolysaccharide (LPS). As expected, short-day males exhibited significantly decreased body masses compared to males in long days. Short-day females exhibited winter-typical changes in pelage color, but did not lose body mass relative to long-day females. Concomitantly, males in long day exhibited significantly greater anorexia than males in short days following injections of LPS, whereas females in both photoperiods exhibited comparable levels of anorexia. These data suggest that anorexia may play a significant role in immune function, and that seasonal alterations in body mass and energy availability may mediate the expression of sickness behaviors in Siberian hamsters.

PHOTOPERIOD MODULATES THE INHIBITORY EFFECTS OF *IN VITRO* MELATONIN ON CELL-MEDIATED IMMUNE FUNCTION IN FEMALE SIBERIAN HAMSTERS

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Many non-equatorial organisms exhibit seasonal changes in physiology and behavior that are cued by changes in day length (photoperiod). Day length information is translated into a neuroendocrine signal via nocturnal secretion of the pineal hormone, melatonin. For example, in some seasonally-breeding rodents, long and short days (and their corresponding melatonin sequelae) inhibit reproductive function- regression of reproductive physiology under short days figures prominently among energy-saving adaptations that increase the likelihood of successful overwintering. Photoperiod and melatonin also affect immune function, although data regarding photoperiodic influences on immune cell function are based almost exclusively on studies of male rodents. In some long-day breeding rodents (e.g., voles, deer mice) short days, or *in vitro* melatonin, enhance cell-mediated immune function. In contrast, short days suppress cell-mediated immunity in another long-day breeding rodent, the Siberian hamster. It is unknown whether photoperiod exerts its apparent immunosuppressive effects in this species through a direct action of melatonin on lymphocytes, or if photoperiod alters immune function via indirect mechanisms. The present experiment sought to establish (1) whether photoperiod affects immune function in female Siberian hamsters, and (2) whether melatonin influences cell-mediated immunity through a direct action on lymphocyte proliferation. Adult female Siberian hamsters were housed in either long (14 h light/day, 14L) or short (10L) photoperiods for 13 consecutive weeks. Two populations of immune cells (circulating lymphocytes and splenic lymphocytes) were extracted and assayed for *in vitro* responsiveness to mitogens (phytohemagglutinin and concanavalin A) and melatonin. Short days predictably provoked reproductive regression and decreases in body weight. Short day treatment likewise inhibited basal proliferation of both circulating and splenic lymphocytes. Physiological concentrations of melatonin (50 pg/ml) inhibited *in vitro* proliferation of circulating lymphocytes, whereas higher concentrations (500-1000 pg/ml) were required to inhibit proliferation of splenic lymphocytes. In addition, the immunomodulatory effects of melatonin were restricted to lymphocytes extracted from long-day hamsters-- *in vitro* melatonin had no effect on circulating or splenic lymphocytes derived from short-day females. Threshold-effective melatonin treatments suppressed proliferation of long-day lymphocytes down to values that did not differ from those of untreated short-day lymphocytes, suggesting melatonin alone may be sufficient to mediate the effects of photoperiod on cell-mediated immunity. These data indicate that melatonin can act directly on Siberian hamster lymphocytes to suppress growth and proliferation. Furthermore, photoperiod (decoded by the CNS) modulates this cellular responsiveness to melatonin. Responsiveness to melatonin in the short-day lymphocyte may be saturated by the already expanded nightly pattern of melatonin secretion under short days.

IN VITRO MELATONIN TREATMENT ENHANCES CELL-MEDIATED IMMUNE FUNCTION IN MALE PRAIRIE VOLES (*MICROTUS OCHROGASTER*). Lance J. Kriegsfeld, Deborah L. Drazen, & Randy J. Nelson. Behavioral Neuroendocrinology Group, Departments of Psychology, Neuroscience, and Division of Reproductive Biology, The Johns Hopkins University, Baltimore, MD 21218-2686, USA.

Numerous studies have reported seasonal or photoperiod-mediated alterations in immune function. In the laboratory, exposure to short day lengths leads to enhanced immune function in most species studied to date. The present study was designed (1) to determine the extent to which male prairie voles (*Microtus ochrogaster*) alter immune status in response to short day lengths, (2) to evaluate the role of melatonin in coordinating these alterations in immune function, and (3) to assess the association between alterations in immune function and reproductive responsiveness to photoperiod. Male voles were housed in either long or short day lengths for 10 wk; voles in short days were subdivided into reproductive "responders" or "non-responders" based on testicular mass at autopsy. After 10 wk of exposure to photoperiodic conditions, cell-mediated immune function was evaluated using an *in vitro* splenocyte proliferation assay. The direct effects of melatonin on basal and mitogen-stimulated immune function were evaluated by adding melatonin (50 pg for 48 hr) to one-half of the cultures in each experimental condition. Melatonin treatment led to enhanced splenocyte proliferation for all experimental groups. Neither photoperiodic condition nor reproductive status was associated with alterations in immune function or the degree of immuno-enhancing effects of melatonin. Taken together, the results of the present study suggest that melatonin is capable of enhancing immune function in male voles potentially by acting directly on immune cells. Supported by USPHS grant MH57535 (RJN), NSF grant IBN 97-23420 (RJN), and Sigma Xi Grants in Aid of Research Award (LJK).

PHOTOPERIODIC EFFECTS ON BROMODEOXYURIDINE INCORPORATION IN THE BRAINS OF ADULT SIBERIAN HAMSTERS. V.B. Tsirlin, M.B. Sierszulski, F.W. Turek, and T.H. Horton. Department of Neurobiology and Physiology, Northwestern University, Evanston, IL 60208

The effect of photoperiod on the incorporation of the thymidine analog bromodeoxyuridine (BrDU) into the brains of adult Siberian hamsters on long and short photoperiods was examined. BrDU substitutes for thymidine during DNA synthesis; thus it can be used to label dividing cells. Neurogenesis was once believed to occur in mammals only during the embryonic or neonatal stage. Recent studies, however, have shown that new neurons are frequently seen to arise from stem cells in the subependymal zone and in the dentate gyrus of the hippocampus of adult mammals. Changes in the rates of cell birth and death have been associated with differences in circulating levels of gonadal and adrenal steroids. Short photoperiod inhibits reproductive function in Siberian hamsters (*Phodopus sungorus*). The current study examined whether exposure to short photoperiod altered the number of cells labeled with BrDU in Siberian hamsters.

Adult male hamsters were born and reared in long photoperiod (LD 16:8). Ten weeks prior to the start of the experiment, thirty-two males were transferred to short photoperiod (6L:18D); thirty-three males were retained in long days. On day 0 of the experiment, long day and short day males received 2 injections of BrDU (50 mg/kg). Injections were given 12 hrs. apart, the first injection coincided with the onset of darkness in each photoperiod. Hamsters were sacrificed 1, 8 and 15 days after the first injection of BrDU. Hamsters were deeply anesthetized with sodium pentobarbital and perfused through the heart with paraformaldehyde. Cryoprotected brains were frozen and sectioned in preparation for immunocytochemistry. Sagittal frozen sections of the brains were cut at a thickness of 40 μ m; every fifth section was collected. Sections were labeled for BrDU and counterstained using cresyl violet.

BrDU labeled cells were observed along the lateral ventricle, following the subependymal zone, and into the olfactory bulb. Considerable quantities of stained cells were also found in the dentate gyrus around the hilus and the granule cell layer of the hippocampus. To date full analysis has been completed only for the rostral regions of the brain. There is an increased number of BrDU containing cells in the rostral portion of the brain in long day Siberian hamsters as compared to short day hamsters; especially in the olfactory bulb. There are also regional differences in the distribution of cells. Finally, the number of labeled cells declines over time following injection in both photoperiods. Supported by HD 09885 to FWT.

DIFFERENTIAL REGULATION OF FOLLICLE STIMULATING HORMONE (FSH) AND LUTEINIZING HORMONE (LH) BY PHOTOPERIOD AND FEMALE EXPOSURE IN MALE SIBERIAN HAMSTERS.

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Siberian hamsters undergo decreases in gonadotropin levels and testis size following housing in short photoperiod (short-days 6L:18D). On photostimulation, (transfer to long-days 16L:8D), a selective increase in FSH and testis weight is seen. LH and testosterone remain low. Our first experiment demonstrates that long-day males, but not short-day males, show an increase in serum LH shortly after female exposure. This suggests that photoperiod modulates the response of a male to a female.

Our second experiment defines the time course following transfer from short to long photoperiod over which males regain the ability to increase LH secretion in response to a female. Four groups of males were examined: i) long-day controls, ii) short-day controls, iii) males transferred from short to long photoperiod for 4 days or iv) for 11 days. After 4 days of transfer to long-days, males show a modest increase in serum LH in response to a female, which is greatly augmented following 11 days of transfer to long-days. In contrast, FSH levels are not influenced by female exposure.

To conclude, FSH and LH are differentially regulated by photoperiod and female exposure. Moreover, photoperiod modulates the responsiveness of the male to other environmental stimuli. On transfer to long-days, FSH, but not LH, increases to promote testicular development and spermatogenesis. Subsequently, in response to a female, LH increases to cause release of testosterone, which facilitates sexual behavior. The dissection of FSH and LH secretion by different environmental stimuli within defined temporal windows makes the Siberian hamster an excellent model to examine molecular and cellular mechanisms of neuroendocrine regulation of the hypothalamic-pituitary-gonadal axis.

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TESTOSTERONE INFLUENCES EXPLORATORY BEHAVIOR IN SIBERIAN HAMSTERS INDEPENDENTLY OF PHOTOPERIOD. J.F. Shen, F.W. Farkas, S. Losee-Olson, J. Arbuzova, F.W. Turek, and T.H. Horton. Department of Neurobiology and Physiology, Northwestern University, Evanston, IL 60209-3520.

Long photoperiods (long day, LD) stimulate gonadal activity whereas short photoperiods (short day, SD) inhibit reproductive function in the Siberian hamster (*Phodopus sungorus*). Reproductive steroids influence many aspects of the brain and behavior. Experiments with the Syrian hamster (*Mesocricetus auratus*) have shown that the effects of SD on the neuroendocrine and behavioral responses to testosterone are specific for different functions. For example, while the hypothalamic-pituitary-testicular axis becomes hypersensitive to testosterone on SD, the neural substrates regulating copulatory behavior become hyposensitive to the effects of testosterone (Campbell et al. 1978). In the current study we investigate whether photoperiod alters a behavioral response of Siberian hamsters to testosterone. We have recently developed a test of exploratory behavior in Siberian hamsters. In the test each hamster is given 3 training trials during which it habituates to the testing box and the position of 4 objects within the box. Prior to a fourth trial the objects are rearranged. The number of times a hamster touches each object with its nose is recorded during all four sessions. Many males respond to the rearrangement of objects with an increase in exploratory behavior. We suspect that this response is steroid dependent and may reflect some aspect of learning or memory in this species. Males with intact testes show increased exploratory behavior following rearrangement of a group of four objects as compared to castrate males. LD males also show increased exploratory behavior as compared to SD males.

The present experiment tested the hypothesis that exposure to short photoperiod alters the effect of testosterone on exploratory behavior. Three-month old hamsters were castrated on day 1 of the experiment. Half the animals were retained in LD (LD 16:8), the other half were moved to SD (LD 6:18). Twenty-eight days after castration, animals were implanted with Silastic capsules filled to a length of 0, 2, 4, or 8 mm with crystalline testosterone mixed with cholesterol (1:2 T:C). All males were tested in the exploratory behavior test 4 wks after implantation of the capsules. The desired sample size is 8 to 10 animals per group. Because of the large number of animals required, the experiment is being conducted in replicate series. Three of four replicates have been completed. To date, the results indicate that seminal vesicle weight, an indicator of circulating testosterone levels, is significantly influenced by capsule size ($p < 0.01$), indicating the capsules resulted in elevated testosterone levels in a dose dependent manner. Increasing testosterone dose, but not photoperiod, significantly increased learning behavior (testosterone $p = 0.04$; photoperiod $p = 0.13$). To date, it appears that steroids influence exploratory behavior in Siberian hamsters independent of any additional modifying effects of short photoperiod on the brain. Supported by HD 09885 and HD 21921.

AN INTERVAL TIMER SYNCHRONIZES PUBERTAL DEVELOPMENT IN SUCCESSIVE COHORTS OF SUMMER- AND FALL-BORN SIBERIAN HAMSTERS. Michael R Gorman, Department of Psychology, University of California, San Diego, La Jolla, CA 92093-0109.

In many long-day breeding species, late summer decreases in day length (DL) induce reproductive regression in adults and retard gonadal and somatic growth among young born near the end of the breeding season. The converse transition into reproductive condition in the following late winter/early spring begins in advance of long DLs under the influence of an interval timer (IT) mechanism. The IT renders animals refractory to inhibitory short DLs after approximately 20 weeks of short DL exposure. By initiating reproductive activity in advance of favorable conditions, the IT mechanism may allow animals to deliver their first spring litters at the earliest, reliably permissive time. This proposition has not been rigorously tested. In hamsters, some reproductive adult individuals, moreover, are unresponsive to decreasing summer/fall DLs and may continue to wean litters into mild autumns, thereby generating successive cohorts of young photo-inhibited from birth. If the IT is to optimally time the spring breeding effort of these different cohorts, its duration must be plastic to compensate for the lag in birth dates. Thus, I predicted that hamsters born into August DLs would undergo gonadal maturation at a later age than hamsters born into September conditions. I examined timing of gonadal and somatic development among offspring born to Siberian hamster females maintained in a simulated natural photoperiod (SNP) at 50° latitude. Dams on this yearly cycle were paired with males to yield cohorts born approximately 7 weeks apart (early August or late September) when DLs were 14.3L and 11.8L, respectively. Pups remained in gradually decreasing photoperiods until the winter solstice (9L) when DL was either fixed at 9L or increased naturally. Body weights and testis size were measured every one or two weeks, respectively. Postnatal photoperiods were inhibitory for both groups, and rapid somatic and gonadal growth was delayed until late winter. Pubertal increases in male and female body weight and male gonad size in late winter were initiated under the influence of the IT rather than by increasing winter DLs, per se. Gonadal growth was initiated at a significantly later age among hamsters born into August compared to September DLs (23.7 ± 0.6 vs 19.7 ± 0.4 weeks, respectively, $p < 0.001$). Similarly, spontaneous increases in body weight in late winter were initiated at markedly later ages in August- versus September-born male and female hamsters. When developmental patterns of groups were assessed in relation to time of year (rather than age), differences between groups were largely eliminated. Because the IT triggers these developmental events, its duration must be plastic. This plasticity appears to facilitate a relative synchronization or entrainment of developmental milestones in hamsters born into different late summer/early fall photoperiods.

AMBIENT TEMPERATURE MODIFIES PHOTOPERIODIC RESPONSES IN SYRIAN HAMSTERS BY AFFECTING RATES OF CIRCADIAN ENTRAINMENT.

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Day length, by altering the duration of the nocturnal melatonin signal, is the primary cue photoperiodic animals use to regulate seasonal responses. Ambient temperature (T_a) modifies seasonal photoperiodic responses. In Syrian hamsters (*Mesocricetus auratus*), Siberian hamsters, and deermice, exposure to low T_a (5°C) accelerates the appearance of short-day traits relative to conspecifics maintained at 22°C. In contrast, the manifestation of short-day responses is delayed in Syrian hamsters maintained at high T_a (30-32°C). Earlier studies did not specify whether T_a acts at a pre-pineal (affecting melatonin production) or post-pineal (affecting responsiveness of target tissues to melatonin) site.

To investigate the interaction of T_a and photoperiod, male Syrian hamsters were transferred from long-days (14L:10D) at 22°C to short-days (10L:14D) and held at one of three T_a s (5°C, 22°C, or 28°C). Activity was recorded continuously using passive infra-red activity monitors; the duration of nocturnal activity (α) served as an indicator of the duration of the nocturnal melatonin signal. Body mass and estimated testis volume (ETV) were measured weekly.

Hamsters held in short-days at 22°C showed a small but significant reduction in ETV on week 5 and substantial reduction in ETV on week 6; α significantly expanded on week 2 and reached maximal values on week 4. In contrast, among hamsters held at 5°C, α had expanded significantly during the first week in short-days and reached an asymptotic value by week 2. Concomitant with accelerated expansion of α , ETV was reduced significantly after one week and substantially after two weeks in short-days at 5°C. α expanded at the same rate in hamsters at 22°C and 28°C, but the latter animals failed to undergo gonadal regression after 6 weeks of short-days. This study demonstrates that exposure to low T_a accelerates α expansion and initiation of short-day responses; future studies will investigate the role of T_a in modulating post-pineal responses to melatonin.

251 REGULATION OF PITUITARY LACTOTROPHS BY THE PARS TUBERALIS: PATTERNS OF PROLACTIN GENE EXPRESSION

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Photoperiod drive induces pronounced changes in prolactin (PRL) secretion and gene expression in seasonal mammals. Here, we show that the Syrian hamster *pars tuberalis* (PT) secretes a factor(s) which acts on pituitary lactotroph cells to regulate PRL gene expression.

In vitro co-culture of PT fragments induced a dose- and duration-dependent increase in PRL gene promoter activity in GH₃ cells stably transfected with a plasmid comprising the 5kb hPRL promoter linked to the luciferase reporter gene (PRL-LUC+ GH₃ cells). Conditioned medium from Syrian hamster and ovine PT (tuberalin) also stimulated PRL promoter activity in the PRL-LUC+ GH₃ cells after an 8-h incubation period.

Photoperiodic control of PRL gene expression was investigated in hamsters housed in long (LD;16hL:8hD) or short (SD;8hL:16hD) days for 12 weeks. *In situ* hybridization with a rPRL riboprobe showed that expression of PRL mRNA, restricted to the *pars distalis* (PD) region of the pituitary, was significantly increased on LD. There was no significant seasonal change in the proportion of PD cells expressing PRL mRNA, but on LD a greater number of cells were recruited to the high-expressing population of lactotrophs. Hamster lactotroph cells also exhibited marked heterogeneity in expression of PRL mRNA at the single cell level. Real-time imaging of PRL promoter activation in unstimulated individual living PRL-LUC+ GH₃ cells revealed profound heterogeneous fluctuations in gene expression. The frequency distribution of pituitary PRL mRNA observed in hamster PD cells may therefore represent dynamic heterogeneity in PRL promoter activity. Further studies demonstrate that PRL promoter gene expression can be synchronised to produce a homogeneous oscillatory response.

In conclusion, our results show marked heterogeneity in PRL gene expression and further support the hypothesis that seasonal modulation of the PRL axis depends upon secretion of a PT factor. (*Supported by the BBSRC*).

252 INTRASPECIFIC VARIATION IN METABOLIC AND ENERGETIC RESPONSES TO PHOTOPERIOD IN THE WHITE-FOOTED MOUSE, *PEROMYSCUS LEUCOPUS*

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There is a large amount of individual variation in the responses of small mammals to seasonal changes. Some of these changes include behavioral, reproductive, and metabolic responses to proximate seasonal cues. Day length is most often the proximate cue by which temperate-zone mammals predict the onset of winter. Short day lengths have been shown to cause many temperate-zone rodent species to inhibit reproductive activity. This response to short days may also affect food consumption and metabolic rate. In the present study, male white-footed mice (*Peromyscus leucopus*) were raised in short photoperiods (8L:16D) for 80 days and then placed in long photoperiods (16L:8D) for 70 days. Food intake and oxygen consumption were measured for a period of 48 hours for each mouse in each photoperiod treatment. In short days, mice that are not reproductively inhibited by short photoperiods had a 47% higher food intake than mice that are reproductively inhibited by the short photoperiod treatment. The two phenotypes did not differ in food intake in long photoperiods. Body weight did not differ between the two groups in either photoperiod treatment. There were no differences in metabolic rate for either phenotype in either photoperiod treatment. This suggests that the cause for the increase in food intake in reproductively nonphotoresponsive mice in short photoperiods may be due to differences in digestive efficiency or activity patterns.

A CRITICAL PHOTOPERIOD OF L13.5:D10.5 AND AN INCREASED RESPONSIVENESS TO GRADUAL CHANGES IN PHOTOPERIOD IN F344 RATS.

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Photoperiod is the major regulator of reproduction in temperate-zone mammals. Laboratory rats are generally considered nonphotoresponsive, but young male F344 rats have a uniquely robust response to short photoperiods of 8 hours of light. Rats transferred at weaning from a photoperiod of 16 hours to photoperiods of < 14 hours of light slowed both reproductive development and somatic growth rate. Those in photoperiods < 13 hours of light underwent the strongest responses. The critical photoperiod of F344 rats can be defined as L13.5:D10.5, but it requires photoperiods of ≤ 12.5 hours to fully suppress reproduction and somatic growth. This demonstrates that the 12-hour photoperiod that is standard in some laboratory colonies would have significant effects on reproductive maturation and growth rate of this common rat strain.

Young F344 rats in decreasing photoperiods that mimic natural change delayed reproductive development and slowed growth rate to a greater extent and for a longer duration than those transferred at birth to short photoperiod. The effects of gradual changes in photoperiod persisted for at least 12 weeks following weaning. In combination, these results indicate that young male F344 rats possess responses to photoperiod that would result in functional photoperiodism in a wild mammal.

PHOTORESPONSIVE FISCHER 344 RATS DIFFER IN 2-[¹²⁵I] IODOMELATONIN BINDING FROM NONPHOTORESPONSIVE SPRAGUE DAWLEY RATS.

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Many temperate-zone species use photoperiod as an environmental cue to regulate reproductive timing. Strains of laboratory rats differ in their responsiveness to photoperiod, with the Fischer 344 (F344) strain being the most responsive known. F344 rats and closely related strains that differ in photoresponsiveness may be useful as models to study the mechanisms and genetic basis for photoresponsiveness. We tested the hypothesis that the location or abundance of melatonin receptors, as estimated by the amount and location of binding of the radioligand 2-[¹²⁵I]-iodomelatonin (IMEL) in the brain, might cause variation in photoresponsiveness among rat strains. The brains and pars tuberalis of the pituitary from photoresponsive F344 rats and nonphotoresponsive Harlan Sprague-Dawley (HSD) rats were processed for autoradiography using IMEL. We found significantly higher specific IMEL binding in the anterior paraventricular nucleus of the thalamus (PVNt) and reuniens nucleus of the thalamus of F344 rats than in the same areas in HSD rats. There were no differences between strains in specific IMEL binding in the medial or posterior PVNt, reticular nucleus of the thalamus, suprachiasmatic nucleus, or the pars tuberalis. These results provide support for the hypothesis that F344 rats may be photoresponsive due to differences from other strains in the location, density, or affinity of melatonin receptors.

A TEST FOR A CIRCANNUAL RHYTHM OF PHOTORESPONSIVENESS IN FISCHER 344 RATS

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Many mammals use circannual rhythms to control a cycle of fertility and body mass. Laboratory rats are not thought to have a circannual rhythm. It has recently been found that young Fischer 344 (F344) rats respond to photoperiods below 14L:10D by a decrease in reproductive capacity, body weight, and food intake, but the responses of adults are not known. This study tested the hypotheses that F344 rats are photoreponsive as adults, and that they had a circannual rhythm of photoreponsiveness and photorefractoriness. Adult F344 rats (6 months old) were found to be photoreponsive for a period greater than 8 weeks with photoreponsiveness first exhibited after 8 weeks of a short day treatment (8L:16D). Young F344 rats were found to be photoreponsive to two photoperiods (8L:16D, 12L:12D) for 16 weeks of treatment, after which they became photorefractive. These results show that F344 rats may be a valuable model for study of circannual rhythms, and suggests that constant photoperiods of 12L:12D should not be used for F344 rats.

PHOTOPERIODIC HISTORY AND MELATONIN SECRETION IN REINDEER

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Photoperiodic conditions in the high-Arctic oscillate between continuous daylight in mid-summer and continuous darkness mid-winter, while in spring and autumn daylength changes rapidly between these extremes. Arctic reindeer are capable of extracting sufficient photoperiodic information to time seasonal events such as reproduction and autumnal fattening despite the absence of any clear daily light/dark cycle for much of the year. Presumably, this timing is influenced by the rhythmic secretion of melatonin from the pineal gland. Photoperiodic history appears to influence the amplitude of secretion of the hormone: reindeer appear to secrete more melatonin in spring than under corresponding photoperiods in autumn. To test this, we measured the plasma concentration of melatonin at 30 to 120 minute intervals over approximately 48 h in five male reindeer which were shifted from either continuous light (LL) or darkness (DD) to a daily light/dark cycle (LD12:12). Neither the amplitude nor the duration of the daily rhythmic secretion of melatonin differed during the first 48 h of exposure to LD following transfer from either LL or DD. However, six weeks later the post-DD group tended to display higher melatonin secretion compared to the post-LL group. Large individual differences in plasma concentrations of melatonin were observed under both DD and LD. There was little consistency: some individuals displayed high amplitude rhythms under one experimental treatment and low amplitude rhythms during others while other individuals showed the reverse. No animal displayed persistent circadian rhythmicity in melatonin secretion when exposed to LL or DD. We conclude (i) photoperiodic history may influence the vernal and autumnal secretion of melatonin, (ii) the putative pacemaker regulating melatonin secretion in reindeer is easily perturbed by large, short-term changes in photoperiod.

TIME PASSES ON: IMMORTALIZED SCN2.2 CELLS CONFER CIRCADIAN RHYTHMICITY ON NIH-3T3 FIBROBLASTS. Gregg Allen, Jodie Rappe, Vincent Cassone and David Earnest. Depts. of Human Anatomy and Biology, Texas A&M University Health Science Center, College Station, TX 77843

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In mammals, the biological clock responsible for circadian rhythms in molecular, physiological and behavioral processes is located within the hypothalamic suprachiasmatic nucleus (SCN). Recent analysis has revealed that molecular oscillations in gene expression are not strictly limited to the SCN, but instead are broadly expressed in many peripheral tissues. Although the capacity to oscillate appears to represent a widely distributed property, our previous studies using immortalized SCN cells suggest that only oscillators derived from the SCN are distinguished by pacemaker function and the requisite cellular milieu to confer circadian rhythmicity to the organism. Fundamental distinctions between the SCN and peripheral oscillators were explored further in the present study using co-culture techniques to determine whether SCN2.2 cells are capable of driving circadian rhythms and their phase in NIH-3T3 fibroblasts *in vitro*. Because the SCN *in vivo* and our SCN2.2 cells are characterized by circadian rhythmicity in their uptake of 2-deoxyglucose (2-DG), co-cultures were separately assessed for oscillatory behavior in this index of cellular metabolism. Colonies of NIH-3T3 fibroblasts on cell-impermeable inserts (pore=1 μ m) were co-cultured in wells containing either the same cell type or SCN2.2 cells and uptake of ^{14} C-labeled 2-DG in these co-cultures was measured at 4-hr intervals for 52 hours. Similar to our earlier findings, SCN2.2 cells showed a distinct circadian rhythm in 2-DG uptake for 2 cycles *in vitro*. When co-cultured with these cells, NIH-3T3 fibroblasts were marked by comparable rhythmicity in their 2-DG uptake. It is noteworthy that the phase of the conferred-rhythmicity in NIH-3T3 cells was delayed by 4 hours relative to the endogenous rhythm of 2-DG uptake in co-cultured SCN2.2 cells. In contrast, co-cultures containing only NIH-3T3 cells showed no evidence of rhythmic fluctuation in their uptake of 2-DG. The results reiterate that pacemaker function is a distinctive property of the SCN. Further application of this approach will provide an opportunity to explore what signal(s) are involved in pacemaker cell coupling and passing time onto other oscillators.

COUPLING AMONG CIRCADIAN OSCILLATORS IN THE SCN DEPENDS UPON CELL DENSITY AND ACTION POTENTIALS. E.D. Herzog¹, S. Yamazaki¹, M. Abe¹, M. Straume¹, H. Tei², M. Menaker¹, and G.D. Block¹ ¹NSF Center for Biological Timing and Dept. of Biology, University of Virginia, Charlottesville, VA 22903, USA ²Human Genome Center Institute of Medical Science, University of Tokyo, Tokyo, 108-8639, Japan

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The mammalian suprachiasmatic nucleus (SCN) is comprised of a population of circadian oscillators that act together to drive rhythms in behavior and physiology. The mechanism by which these multiple oscillators coordinate their activities is unknown. Using a combination of electrophysiological and reporter gene assays, we have explored the hypotheses that synchrony among the SCN oscillators depends on their proximity to each other and on action potentials. Electrical activity of individual SCN neurons was recorded from dispersed cell cultures on planar electrode arrays. Approximately 24,000 cells harvested from neonatal mice were plated over a defined area delimited by a Sylgard gasket to produce plating densities of 2000, 4000 or 6000 cells/mm². Extracellular spikes were recorded beginning on ~day 21 *in vitro*. In accordance with previous observations, low density cultures (≤ 2000 cells/mm²) expressed circadian rhythms in firing rate with differing periods in the same culture. In contrast, cells expressed apparently synchronized, in phase, circadian rhythms in higher density cultures (≥ 3000 cells/mm²). *mPer1* transcription was measured as bioluminescence from SCN explanted from adult rats carrying the *mPer1-luc* transgene. SCN were isolated from coronal sections and cultured either as explants on Millicell membranes or as dispersals on glass coverslips in medium containing luciferin. Both explants and high density dispersals expressed circadian rhythms in bioluminescence. Some cultures were subsequently treated for 10-12 days with 300 nM TTX, a blocker of sodium-dependent action potentials. Treated explants showed a circadian rhythm that diminished in amplitude while cultures treated with vehicle remained robustly rhythmic. TTX effects were reversed following washout. One interpretation is that TTX uncouples SCN cells so that they drift out of phase from one another. The results indicate that SCN cells communicate over short distances and suggest that action potentials may play a role in synchronizing rhythmicity among the multiple circadian oscillators.

PHASE OF ELECTRICAL ACTIVITY OF INDIVIDUAL, ADULT SUPRACHIASMATIC NEURONS *IN VITRO* DEPENDS UPON *IN VIVO* LIGHT HISTORY

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The suprachiasmatic nucleus (SCN) contains a clock that drives circadian rhythms *in vivo* and *in vitro*. Recently, recordings have correlated the periods of locomotor rhythms in *adult* rodents and of firing rate rhythms in *neonatal* SCN neurons. We now report a new methodology that allows us to study the effects of *in vivo* treatments on behavior and subsequently on *in vitro* firing rate patterns of SCN neurons of adult mice. Following behavioral assessment in 12:12 LD or DL, C3H mice (2- to 4-months old) were sacrificed and their SCN were dissociated by enzymatic digestion and dispersed on multimicroelectrode arrays coated with laminin and poly-D-lysine. After a 4-day incubation at 36.5°C in 5%CO₂/95%air, the cells were transferred to a recording set up, continuously perfused with culture medium (13 µl/min) and their action potentials were monitored.

SCN neurons from adult mice attached to the electrode array within 20 minutes, with approximately 30 neurons near the electrodes. Each culture produced long-term recordings from 1 or 2 neurons. Each neuron that was recorded for at least 2 days (longest recording was 5 days) expressed a robust circadian rhythm in spontaneous firing rate, with an average dominant period of 23.8 ± 0.3 h (N = 12). Their first recorded peak occurred at the expected phase (ZT 4.0 ± 0.3 h) relative to the animal's previous light-dark cycle.

Individual SCN neurons cultured from adult mice remain viable for at least one week *in vitro* and exhibit a circadian rhythm in firing rate. Direct comparisons of behavioral and neuronal rhythms of adult animals should give new insights into the mechanisms of phase adjustment and waveform regulation of circadian rhythmicity within the SCN.

Supported by grants from the AFRT and the NSF Center for Biological Timing.

EFFERENT NEURONS IN THE MOUSE SUPRACHIASMATIC NUCLEUS (SCN) EXPRESS PHOTO-INDUCIBLE c-FOS. Horacio O. de la Iglesia, William J. Schwartz. Department of Neurology, University of Massachusetts Medical School, Worcester, MA 01655.

To investigate the neuroanatomical organization of light-induced SCN gene expression at the cellular level, we have combined a stereotaxic tract-tracing technique with c-Fos immunocytochemistry. The retrograde tracer cholera toxin subunit B (CtB, 5mg/ml in 0.1 M phosphate buffer) was iontophoresed (pipette tip diameter 25-30 µm, positive current of 2 µAmp, on and off for 8 s over 10 min) into the mouse subparaventricular zone. After a postsurgical entrainment to a 12:12 light:dark cycle, animals were placed in constant darkness. One group of animals was exposed to a 1.5 h light pulse (CT 18-CT 19.5) while a second group remained in constant darkness. All animals were sacrificed by perfusion between CT 19.5 and CT 20. Forty-µm vibrotome coronal sections including the SCN through the subparaventricular zone were cut, and rabbit anti-c-Fos and goat anti-CtB were used for double-label immunocytochemistry. Analysis of individual tissue sections was performed by light or confocal microscopy. The percentage of retrogradely-labeled SCN neurons that also express c-Fos was 15 ± 2.7 % (mean $\pm$ SEM, 8 animals, 415 CtB-labeled cells total) in the light-treated animals and 7 ± 1.9 % (mean $\pm$ SEM, 7 animals, 418 CtB-labeled cells total) in the dark-treated animals (P=0.03, two-tail t-test).

c-Fos has been used conventionally as a marker for cells on the input pathway for photic entrainment of the circadian clock. Our data in the mouse show that some of these cells also innervate one of the SCN's primary target regions. Thus, we have identified a subset of SCN efferent neurons that are also light-responsive. These cells appear to represent a convergence site for input and output elements of the circadian system.

NEUROTENSIN ACTIVATES NEURONES OF THE SUPRACHIASMATIC NUCLEUS *IN VITRO*.

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Neurotensin (NT) is a tridecapeptide found throughout the CNS of many species. NT- and NT receptor-immunoreactivity is found in the suprachiasmatic nucleus (SCN) of the mammalian hypothalamus. The SCN has long been known to house the central circadian pacemaker in mammals. In this study we examined the effects of NT on the spontaneous firing rates of neurones in the rat SCN *in vitro*.

Slices of rat hypothalamus containing the SCN were prepared from adult male Wistar rats during the subjective day. Slices were then transferred to and maintained in a PDMI microincubator perfused with aCSF of standard composition. Extracellular recordings of cell firing frequencies were made. All drugs were bath applied.

324 cells were tested in this study. Of 163 cells treated solely with NT (10^{-8} M to 10^{-5} M), 87 showed increased firing rate, 15 showed decreased firing rate and 61 showed no effect. There was no regional (ventral SCN/dorsal SCN) variability in responsivity to NT. The effects of NT were not attenuated by pre-treatment with the glutamate receptor antagonists D-AP5 and CNQX or the GABA_A receptor antagonist bicuculline. The NT receptor-1 antagonist SR48692 (10^{-5} M) inhibited the effects of NT in only 3 of 14 NT responsive cells tested. Specific agonists of the NT-receptor 1 and 2 (JMV 510 and JMV 431 respectively) elicited NT-like responses in NT-responsive cells.

This study describes for the first time the neurophysiological actions of NT in the SCN and suggests that NT may play a role in regulating SCN function.

Cellular organization of multi-oscillatory pacemaking system in the rat suprachiasmatic nucleus; an analysis using a multi-electrode dish. 262

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The suprachiasmatic nucleus (SCN) is composed of multiple oscillatory neurons. Arginine vasopressin (AVP) and vasoactive intestinal peptide (VIP) in the SCN show robust circadian rhythms in their content and mRNA levels. We have shown that the two rhythms were phased in cultured rat SCN but desynchronized by antimetabolic treatment, suggesting the two subdivisional oscillators in the SCN. In order to examine the role of peptidergic neurons in the multioscillatory pacemaker in the SCN, we analyzed the circadian rhythms of peptide releases and of electrical activity from individual neurons in stationary organotypic culture as well as in dispersed cell culture of rat SCN.

The SCN of new born rats was cultured on a multi-electrode dish (MED) either in slice of 350 μ m or in dispersed cells. Extracellular single neuronal activity was continuously monitored over 2 weeks and circadian rhythm in firing rate was analyzed. Peptides in culture media were measured by EIA at 4 hr intervals for 64 hr. AVP and VIP neurons were immunochemically identified.

At the 5th day of culture, all slices examined showed significant circadian rhythms in AVP releases, the peak of which was located in the subjective day. Significant circadian rhythms in VIP release were detected in a half of slices and the peak phases were varied. Single SCN neurons with significant circadian rhythms were detected more frequently in the dorsal part than in the ventral part of the SCN. Synchronized rhythms were detected in AVP and VIP releases in dispersed cell culture where most neurons showed synchronized firing rhythms, but peptide release was arrhythmic where individual neuronal rhythms were desynchronized. These results suggest that, 1) AVP and VIP rhythms were driven by separate oscillators, 2) the oscillator which drives AVP rhythm is synchronized by external cues in neonates, 3) the oscillator which drives VIP rhythm is not entrained by the same time cue as for AVP rhythm or has a different period, 4) a loss of VIP rhythm is not due to the disturbed cytoarchitecture, 5) the neurons with robust circadian rhythms were more frequent in the dorsal SCN, 6) peptide rhythm reflects the synchronization of peptidergic neurons, which does not depend on the cytoarchitecture in the SCN, 7) cellular oscillation mechanism is steady but the synchronization is easily lost in cultured SCN, especially in ventral area.

Vasopressin in the circadian clock of common voles: a putative causal factor in timing of locomotion

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The necessary presence of the circadian pacemaker in the mammalian suprachiasmatic nucleus for the expression of behavioral rhythms is well established. Natural variation in rhythmic locomotor behavior, as for instance observed in common voles, can be exploited to study the mechanism of pacemaker control over the circadian timing of behavior. We determined daily patterns in numbers of vasopressin- and protein kinase C α -immunoreactive suprachiasmatic nucleus neurons in relation to variable expression of circadian locomotor behavior in the common vole. In addition, vasopressin release patterns from organotypic suprachiasmatic nucleus cultures in adult voles with known behavioral expression of rhythmicity was examined. Voles with pronounced circadian wheel running behavior show circadian variation in the number of vasopressin immunoreactive suprachiasmatic nucleus neurons, while voles with weak or no circadian rhythmicity did not reveal such circadian profile. The intracellular messenger protein kinase C α , part of the signal transduction system of vasopressin, changed concurrently with the vasopressin system. Organotypic suprachiasmatic nucleus cultures made from non-rhythmic vole brains did not produce any circadian pattern in vasopressin release, while five out of six suprachiasmatic nucleus cultures of rhythmic voles produced significant peak values in the release of this neuropeptide. We discuss the vasopressinergic system of the vole suprachiasmatic nucleus as a functional correlate with expression of circadian locomotor behavior, and conclude that vasopressin in the common vole may play a key role in mediating output of its circadian clock.

A MATHEMATICAL MODEL OF THE SCN CLOCK INCORPORATING PHYSIOLOGICAL AND ANATOMICAL DATA. Joseph D. Miller & Wijesuriya P. Dayawansa. Depts. of Pharmacology and Mathematics, Texas Tech University Health Sci. Ctr. and Texas Tech University, Lubbock, TX 79430.

Mathematical models of the circadian oscillator have rarely incorporated known physiological and anatomical data accumulated on the SCN. Such data include: 1) total neuron number in the unilateral SCN (8500) 2) the core/shell organization of the SCN 3) the observation that the SCN is a GABAergic neuropil and that available estimates suggest that every core neuron may receive synaptic input from up to 100% of the other core neurons (6000), while shell neurons (3500) may receive input both from the core and from all other shell neurons 3) the developmental time course of circadian rhythmicity as a function of increasing neuron number in the fetus 4) the electrophysiological observation that neuronal discharge in the SCN is only roughly synchronized over the circadian cycle, as opposed to perfect interneuronal cross-correlation 5) the standard deviation of circadian period length in individual SCN neurons is approximately one hr. We have incorporated the latest Kronauer oscillator model at the single neuron level into our model, as well as the preceding observations, implementing the activity of the SCN neuronal population in a supercomputer simulation. We have simulated the response of the population to zeitgebers, simulated lesion, variation in number of "driver" neurons, and variation in local and global coupling. Responses of the model to such perturbations will be compared with "real world" data.

CIRCADIAN MODULATION OF THE GATING OF CYCLIC GMP-ACTIVATED CATIONIC CHANNELS IN VERTEBRATE RETINAL PHOTORECEPTORS. Stuart E. Dryer and Gladys Y.-P. Ko, *Department of Biology and Biochemistry and Biological Clocks Program, University of Houston, Houston, TX 77204-5513*

Visual phototransduction in the vertebrate retina is mediated by a G protein-coupled cascade that ultimately causes changes in the gating of cGMP-activated cationic channels (CNGCs). Light causes CNGCs to close, resulting in membrane hyperpolarization and a decrease in neurotransmitter secretion onto second-order retinal neurons. Because closure of these channels is the last step in normal visual phototransduction, modulation of CNGC gating could produce significant effects on visual system function. We now report that the affinity of CNGCs for cGMP in cultured chick retinal photoreceptors (cones) is under circadian control. In initial experiments, retinal photoreceptors were dissociated at E6 and maintained on LD 12:12 for five days *in vitro*. Inside-out patch recordings from cells containing prominent oil droplets were made on the fifth day in culture, at ZT4-7 and at ZT16-19. Electrode and bath solutions were free of divalent cations. Apparent affinity (ED50) and Hill slope were calculated from cGMP dose-response curves. Mean ED50 was significantly ($p < 0.05$) greater at ZT16-19 than ZT4-7 but there was no change in Hill slope or maximum response amplitude. Identical results were obtained from retinal photoreceptors examined on the second day of DD after four days entrainment to LD 12:12. The persistence of this pattern in DD strongly suggests circadian control by a retinal oscillator. Moreover, identical changes in CNGC gating behavior were obtained when entraining LD cycles were applied to intact chicken eggs (as opposed to dissociated retinal cells). In these experiments, entrainment was started at E6, cells were dissociated at E11-12, and recordings were made on the next day. Thus, functional light-sensitive circadian oscillators are expressed early in the normal embryonic development of the chick. Analyses of channel non-stationarity with time after patch excision, biochemical assays, and the effects of inhibitors, suggest a role for protein kinases in mediating these changes. The lower affinity for cGMP is consistent with a greater likelihood of CNGC closure, and thus greater responses to light, during the subjective day in avian cones. **NIH RO1-EY11973**

UNMASKING ARHYTHMIA: THE FUNCTION OF ELF3 IN THE ARABIDOPSIS CIRCADIAN CLOCK.

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An old question in circadian studies is whether arrhythmia represents the absence of a functional clock or the masking of its output. In constant light (LL), unlike rhythmic wild type plants, *early flowering 3* (*elf3*) mutants of *Arabidopsis thaliana* show arrhythmic activity of a reporter construct, the chlorophyll *a/b* binding protein 2 promoter fused to the luciferase gene (*CAB2::LUC*). We tested the dependence of phase upon photoperiod in *elf3-1*, a null mutant, and wild type plants as well as the timing of a *CAB2::LUC* peak in darkness following varying periods of time in LL after LD cycles. Our data suggest that the oscillator had very low amplitude in the absence of ELF3 and was not sustained past the first 12 hours of LL. When low levels of ELF3 were present the oscillator was more robust. We measured the acute response to a light pulse of *elf3-1* and *elf3-7*, a partial mutant. In wild type plants the acute response shows a pattern of circadian gating. However, in *elf3-1* the acute response was high at all times, indicating abnormal gating by the circadian clock; *elf3-7* plants showed an intermediate gating phenotype for the first 12 hours of darkness. Levels of ELF3 must be high to gate the acute response, and, in the absence of gating, the circadian rhythm of *CAB2* expression in *elf3-7* is masked in LL, leading to its arrhythmic phenotype. We propose that the role of ELF3 is to act on the light input pathway, controlling input from the photoreceptors to the oscillator via gating.

CIRCADIAN AND PHYTOCHROME CONTROL ACT AT DIFFERENT PROMOTER REGIONS OF THE TOMATO *Lhca3* GENE

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Light harvesting complex protein genes are controlled by light and the circadian clock. Based on experimental hints and theoretical considerations it is possible that parts of both signal transduction chains merge or cis- and/or trans-regulatory elements are simultaneously used in both regulatory circuits. To test this hypothesis promoter deletions of tomato Lhc genes were constructed, transferred into tobacco and young plants were tested for their responsibility to red and far red light (= phytochrome mediated light control) as well as circadian expression. The deletion construct $\Delta 278$ of the *Lhca3* (*cab 8*, PSI) promoter revealed circadian mRNA oscillations in LD and DD conditions while the $\Delta 231$ resulted in constant steady-state mRNA levels indicating that the presence of approximately 50 nucleotides are important for circadian expression. Illumination of the tobacco plants with 1 or 2 minutes of red light resulted in an increase of mRNA levels in both deletion mutants. Furthermore, red light illumination followed by 10 minutes of far red light revealed a significant reduction of the transcript levels indicating that both promoter regions are sufficient to mediate phytochrome control. These results suggest that in the tomato *Lhca3* promoter the light and the circadian regulatory circuits apparently do not use the same cis-regulatory element(s).

268 MELATONIN AFFECTS PHOTOPERIODIC FLOWER INDUCTION IN THE DICOT PLANT *CHENOPodium RUBRUM*

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We investigated melatonin occurrence and function in the dicot short-day plant *Chenopodium rubrum*, a model for photoperiodic flower induction. Using an improved method employing solid phase extraction, purification on immunoaffinity columns and quantification with liquid chromatography/tandem mass spectrometry, we are able to determine melatonin in plant tissues. We had previously identified melatonin by mass spectrometry in *C. rubrum* shoots. Melatonin levels exhibited a daily rhythm in 12 h light/12 h darkness regime with a peak 4-6 h after lights off. Preliminary data also suggest persistence of melatonin rhythm in continuous darkness and its changes in different photoperiods.

These results led us to investigate possible melatonin involvement in *C. rubrum* photoperiodism. The highest flower induction in 4.5-day-old *C. rubrum* plants grown in constant light is achieved after a single 12-h darkness. 10^{-4} M or 5×10^{-4} M melatonin applied onto the cotyledons and apical parts 1 h before the beginning of 12-h darkness significantly lowered the percentage of flowering plants from 81% to 62% or 47%, respectively. Similar reduction was achieved with melatonin agonists, 6-Cl-melatonin and 2-I-melatonin. The putative nuclear melatonin receptor agonist, CGP 52608, was even more efficient, while 5-methoxytryptamine had no effect on flowering in this application schedule. None of the compounds affected the growth or appearance of treated plants.

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Structures of cockroach circadian system viewed from clock gene products and roles of melatonin in the output pathway

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The brain-subesophageal ganglion (BR-SG) of *Periplaneta americana* was immunohistochemically (IHC) investigated for *P. americana* (*Pam*) PER, *Bombyx mori* (*Bm*) DBT and *Drosophila melanogaster* (*Dm*) CRY. *Bm*DBT-like and *Pam*PER-like antigens occurred in the same neurons: (1) in the PIC, (2) the PL and (3) two clusters of small sized cells, dorsal and ventral to the outer optic chiasma. Stained granules were found in the CC / NCC I and SG. The ventral side to the inner chiasma, conventionally-thought circadian pacemaker (CPM) locus, was negative. IHC reactivity to anti-*Dm* CRY was found in (1) dorsal glia cells and (2) in the nucleus of the cortical cells of the optic lobe.

As output pathway, we investigated the temporal patterns in the BR-SG melatonin content and NAT activity. Both rhythms were bimodal and free-ran. The reactivity to anti-*Dm* NAT-GST was partially overlapped with PER/DBT-like reactivity but the locus (3) above was negative. NAT activity of tripartite BR samples showed (1) a part containing the locus (1) above did not free-run, (2) a part containing the locus (2) showed a bimodal rhythm and free-ran, and (3) a part containing the locus (3) free-ran but showed a unimodal.

Critical reevaluation of our present understanding is required on the structure of cockroach CPM. Maybe there is no CPM but the rhythm is derived from interactions of dispersed units.

ANALYSIS OF THE COUPLING-PATHWAY OF THE CIRCADIAN CLOCK OF THE COCKROACH *LEUCOPHAEA MADERAE* 270

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In the cockroach *L. maderae* lesion studies located the bilaterally paired circadian clock to the optic lobes, ventrally between medulla and lobula (Nishiitsutsuji-Uwo and Pittendrigh 1968, Z Vgl Physiol 58:1). In this general pacemaker location lies the accessory medulla (AMe) which is densely innervated by pigment-dispersing hormone-immunoreactive (PDH-ir) neurons, which are pacemaker candidates (Homberg et al. 1991, Cell Tissue Res 266:343; Stengl and Homberg 1994, J Comp Physiol A, 175:203). Lesion and transplantation studies showed that the AMe is necessary and sufficient for the generation of circadian locomotor activity in the cockroach *L. maderae* and, therefore, appears to be the circadian clock (Reischig and Stengl, Göttingen, Neurobiology Report 1998, p 267). With different experimental approaches we tested whether a subgroup of PDH-ir neurons forms the coupling pathway of the bilaterally paired circadian clock. After lesions of one optic stalk degeneration studies on the EM-level identified contralateral projections in the loose neuropil of the AMe, matching the projection area of PDH-ir neurons. HRP-backfills from the contralateral optic stalk stained a cell group matching PDH-ir neurons next to the AMe as well as contralateral projections in the loose neuropil of the AMe. Dextran-backfills into the AMe together with PDH-immunocytochemistry revealed 3 PDH-immunoreactive neurons with contralateral projections. In addition, PDH-immunocytochemistry on the EM-level found input as well as output-synapses in one AMe. Thus, morphological criteria were met for PDH-ir neurons to form a circadian coupling pathway between both clocks. Because injections of PDH into the vicinity of the AMe caused phase-dependent phase delays (Petri and Stengl 1997, J Neurosci 17:4097) it was tested whether delays at the late subjective day are sufficient to couple two model oscillators. We used a computer model based on the molecular feedback loop of the clock genes in *Drosophila*, to test whether release of PDH is involved in the coupling between bilaterally paired oscillators. The simulations predicted that a combination of all-delay and all-advance type interaction between two model oscillators matches best experimental findings in the cockroach. Also, the simulations suggested that PDH targets a phosphatase or a kinase in the molecular feedback loop decreasing the phosphorylation state of PER. We are currently testing in Western blots whether casein-kinase or a phosphatase 2 C are present in optic lobes of the cockroach and in injection studies whether PDH exerts its phase delays via inhibition of a kinase or via activation of a phosphatase. Supported by DFG grants STE 531/7-1,2

THE *NEUROSPORA* UPPER LIP I: INTERDEPENDENCE OF *frequency* AND *white collar* GENES AND PRODUCTS

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Clock genes, in general, are highly responsive to light signals. Modeling circadian rhythmicity indicates that functional assignment of clock gene location to either central rhythm generator or rhythmically expressed input pathway is problematic. In *Neurospora*, the strains that are deficient in the clock gene *frequency* (*frq*) fail to respond to synchronizing light cycles. An additional physiological measure of light response is carotenoid induction. *frq*⁻ strains are able to synthesize normal amounts of carotenoids upon light induction, however, they have a reduced light sensitivity relative to *frq*⁺ strains. Thus, there is an indication of a bifurcated Light Input Pathway (LIP) in *Neurospora*.

Light input in *Neurospora* is mediated by the *white collar* proteins. In the absence of WC, levels of FRQ are extremely low, meaning that these LIP proteins are responsible for basal expression levels of FRQ (Crosthwaite et al, 1997). When the WC levels are determined in *frq*⁻ strains, it is clear that FRQ also regulates WC-1 and WC-2 expression levels. Several predictions follow, and these have been confirmed: there is a defect in light induction in *frq*⁻ strains, and, as FRQ regulates WC-1 expression, WC-1 is rhythmic. Finally, FRQ co-immunoprecipitates with WC-2, as does WC-1, indicating a mechanism for participation in light signal transduction. These results combine to place the clock gene *frq*, functionally, in the earliest events of light reception in *Neurospora*.

THE *NEUROSPORA* UPPER LIP II: CIRCADIAN GATING OF LIGHT INPUT PATHWAYS - A *ZEITNEHMER* FEEDBACK?

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Physiological experimentation with *Gonyaulax* demonstrates circadian regulation of a blue light input pathway to the circadian clock. Indeed, distinction of circadian regulation of light input versus oscillator state is problematic in interpretation of what contributes to light PRCs. While it was previously determined that *frequency* (*frq*) of *N. crassa* is regulated by light input pathway proteins, we have now shown that FRQ, in turn, regulates its own activators. Along with demonstration of circadian rhythmicity of core light input pathway components comes the prediction that functional light inputs are rhythmic. We have evaluated this possibility in several ways. First, one of the most rapid light induced events in *Neurospora* is induction of *white collar-1* RNA. In the subjective day, its' light dependent accumulation is lower than in the subjective night, despite starting from equal basal levels. This circadian gating is found both in the upper Light Input Pathway (LIP) as well as downstream, as manifested by other light induced RNAs. On the physiological level we find that carotenoid induction is also circadian, being more efficient at subjective dusk than dawn. This timing is shifted approximately 6h compared to the *Neurospora* light PRC, which gives largest responses in the middle of the night.

ECOLOGICAL IMPORTANCE OF AN SCN PACEMAKER IN WILD GOLDEN-MANTLED SQUIRRELS. P.J. DeCoursey. Department of Biological Sciences, University of South Carolina, Columbia, SC 29208.

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Evidence has recently accumulated for the survival value of the SCN in both domesticated lab and free-living mammal species. The field data for wild species in natural habitat, however, have mainly evaluated mortality but have not identified the actual system(s) compromised by SCN loss. The present study specifically tested the role of the SCN in deep hibernation of golden-mantled ground squirrels (*S. lateralis*). The research was carried out in Ponderosa pine forest and meadows in the Cascade Mountains near Bend, Oregon, and in a Hibernaculum enclosure on the study site. Seventy golden-mantled squirrels were radio collared, using simple location-style collars, activity collars, photo collars, or body temperature collars. Computerized data logging of body temperature was employed to document winter hibernation bouts. Radio tracking and behavioral observations of the intact population for one year provided base line data on predation rate, circadian and annual activity patterns, reproductive timetable, territory size, reproductive sequence, and den site stability.

In summer 1999, 12 animals were designated as field project animals: 5 SCN-lesioned squirrels, 4 intact controls, and 3 surgical (sham) controls; Hibernaculum animals included 2 SCN-lesioned squirrels, 1 intact control, and 1 sham. Activity patterns of free-living intact and lesioned individuals differed; predation on SCN-lesioned squirrels was 80% and for combined control groups was 42.9%. Differences were seen in body temperatures patterns of the control group and the SCN-lesioned group in the Hibernaculum. Future work will further document mortality and hibernation patterns after SCN-lesioning for free-living individuals in wilderness habitat.

POST-HIBERNATION ARRHYTHMICITY AND REDUCTION OF AVP IMMUNOSTAINING IN THE SCN OF THE EUROPEAN GROUND SQUIRREL

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Circadian body temperature rhythms are distorted during the first days after natural hibernation in the European ground squirrel (*Spermophilus citellus*). This may be the consequence of low body temperature effects on the organisation of the circadian system. Alternatively, it may reflect transient cycles during post-hibernation re-entrainment to the day-night cycle. Under continuous light conditions, however, ground squirrels were found to be arrhythmic after hibernation, suggesting that hibernation affects either the internal organisation of the circadian system, or the output signal of the circadian system driving the overt rhythm. In all ground squirrels circadian rhythmicity was spontaneously restored after 1-2 weeks. The number of AVP positive neurons in the SCN correlated with the degree of post-hibernation rhythmicity ($p=0.02$) and arrhythmic animals seem to lack AVP immunostaining in the SCN. AVP is considered to function as an output signal of the SCN. AVP innervation between SCN neurons indicates that it may also serve as an internal signal, possibly synchronizing individual pacemaking neurons within the SCN. The lack of AVP immunostaining in the SCN and circadian arrhythmicity after hibernation may indicate internal desynchronisation of the circadian system. Post-hibernation arrhythmicity may be a direct consequence of low body temperatures during hibernation. It is potentially beneficial, since low amplitude oscillators are considered to be more sensitive to an entraining stimulus, and low amplitude after hibernation may thus accelerate re-entrainment to the zeitgeber which has been unavailable for many months when the animals were hibernating underground.

THE CIRCADIAN ACTIVITY-REST RHYTHM OF AGED RHESUS MACAQUES: INFLUENCE OF MELATONIN. Urbanski, H.F., Garyfallou, V.T., Kohama, S.G., and Latimer, V.S. Division of Neuroscience, Oregon Regional Primate Research Center, 505 N.W. 185th Avenue, Beaverton, OR 97006.

Similar to women, female rhesus macaques (*Macaca mulatta*) have 28-day menstrual cycles and eventually undergo menopause. However, it is unclear whether they show similar aging-associated perturbations in their circadian biology. In the present study, eight 21-23-year-old female macaques were fitted with Actiwatch recorders (Minimitter) to enable continuous monitoring of their activity-rest rhythms, and also were implanted with vascular catheters to enable remote blood sampling. Based on menstruation records and reproductive hormone profiles, five of these animals were still premenopausal. As expected, the animals showed a consolidated diurnal pattern of activity when housed together under a 12L:12D lighting regimen; daily activity began ~30 min before lights *on* and continued throughout the day until ~10 min after lights *off*. In addition, a few of the animals showed persistent low-level activity throughout the night but this was not related to whether or not they were menopausal. After exposure to continuous dim illumination (100 lux) all of the animals showed increased activity during their subjective night. Nevertheless, the main activity phase of the circadian rhythm remained synchronous between the animals, with a slight phase advancement (~1 hour) relative to the rhythm observed under 12L:12D; a corresponding phase advancement was seen for the circadian rhythm of plasma cortisol, which also remained synchronous between the animals. After 21 days of exposure to continuous dim illumination, the animals were treated for 8 consecutive days with a bolus injection of melatonin (3 mg, *iv*) during their subjective evening, but this had no effect on any parameter of the activity-rest rhythm. To address this lack of responsiveness to melatonin, an additional experiment was performed in which melatonin receptor (Mel_{1A}) mRNA expression was examined in the suprachiasmatic nuclei (SCN) of young (<1 year) and middle-aged macaques (10-15 years), using *in situ* hybridization. A marked aging-related decrease was detected ($P < 0.001$). Taken together, these data suggest that aged primates may be able to synchronize their biological rhythms without the aid of photoperiodic cues or the involvement of melatonin.

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PHOTOPERIOD-DEPENDENT AND -INDEPENDENT REGULATION OF MELATONIN RECEPTORS IN AREA X OF SONGBIRDS: EFFECT OF REPRODUCTIVE STATE AND INTERPRETATION OF SEX AND SPECIES DIFFERENCES. G.E. Bentley, P. Deviche*, J.J. Sartor, B.D. Spar and G.F. Ball. Dept. of Psychology, Johns Hopkins University, Baltimore, MD 21218; *Dept. of Biology, Arizona State University, Tempe AZ 85287-1501.

The functional significance of seasonal changes in the pineal melatonin signal in birds has remained elusive. Recently, melatonin was identified as playing a key role in fine-tuning the regulation of seasonal neuroplasticity within the telencephalic song control system of European starlings, a highly photoperiodic songbird species. Data from four studies are presented here: Experiments 1 and 2 were designed to investigate any possible seasonal regulation of melatonin receptors (MelR) within the song control system of male and female starlings. Brains were sampled from photosensitive (Phsens) starlings exposed to short days, photostimulated (Phstim) starlings exposed to long days and photorefractory (Phrefr) starlings also exposed to long days. MelR distribution was assessed *in vitro* by ¹²⁵I-melatonin (IMEL) receptor autoradiography. In general, MelR were distributed within the song control system in a similar pattern to that described in other songbird species. However, there was a striking down-regulation of MelR in Area X of Phstim birds on long days, as compared to their photorefractory counterparts also on long days, and to the short-day birds. These studies have identified markedly different patterns of melatonin receptors within birds of a single sex, but in different reproductive conditions. Changes in the distribution of melatonin receptors in Area X were associated with changes in the reproductive state of the birds, rather than with the sex of the bird, changes in photoperiod *per se* or whether the brains were collected in the light phase or dark phase of the light/dark cycle. Thus, changes in the endogenous melatonin signal from the pineal gland or other sources did not affect melatonin receptor distribution. These data on starlings raised questions about previously hypothesized sex- and species differences in MelR distribution, so we performed similar experiments on another photoperiodic songbird species, dark-eyed juncos (Expt 3), and also on a "non-photoperiodic" songbird species, zebra finches (Expt 4).

Experiment 3 essentially replicated the data from Experiment 1 in gonad-intact photosensitive and photostimulated dark-eyed juncos, indicating that distantly-related photoperiodic songbird species regulate melatonin receptor activity in Area X in a similar fashion, having implications for IMEL binding investigations performed on other photoperiodic songbird species, such as House sparrows. Experiment 4 investigated melatonin receptor regulation in Area X of male zebra finches, a "non-photoperiodic" songbird species which has been assigned as having "male" and "female" patterns of IMEL binding in Area X, with no binding in males and high binding in females. We report that male zebra finches can exhibit both high binding and low binding of IMEL in Area X. Neither pattern was consistent with photoperiod, but we hypothesize that the different binding patterns are a result of differences in centrally-mediated reproductive state.

We suggest that because other studies of songbirds have not fully investigated the possible roles of photoperiod and reproductive state upon the distribution of melatonin receptors within the song control system, hypothesized sex and species differences must be interpreted with caution until the effects of reproductive state are fully characterized.

CLOCK MUTANT MICE SHOW ALTERED SLEEP AND BODY TEMPERATURE RHYTHMS UNDER 277
BASELINE AND STRESSFUL CONDITIONS Amy Easton, Bernard Bergmann, and Fred W. Turek
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The circadian pacemaker is known to regulate the timing and distribution of sleep; however, the role of specific circadian genes in the regulation of sleep has just begun to be explored. The first mammalian circadian clock gene to be discovered, *Clock*, is highly expressed in the eye and suprachiasmatic nuclei and is an integral part of the pacemaker's molecular machinery¹. Mice bred with a mutation of the *Clock* gene (*Clock/Clock*) exhibit significantly altered free-running rhythm in running wheel activity¹. In this study, we were specifically interested in determining whether recovery sleep following an acute stress was affected by the *Clock* gene mutation. Six wild-type and 8 *Clock/Clock* male mice bred on a C57Bl/6J background and maintained on a L:D 12:12 cycle. Mice were implanted with EEG and EMG electrodes and a Minimitter transmitter to collect data on body temperature and total locomotor activity. Baseline sleep was collected for 24 hours at least 1 week after recovery from surgery. On day 2, animals were restrained in a plastic tube for 1 hour between ZT3-5. Recovery sleep was recorded for the following 24 hour period immediately after the stress ended. *Clock/Clock* mice showed a decrease in NREM sleep ($p < .001, t = -4.03$) during the light period and twice as much REM sleep during the dark period ($p < .001, t = 3.6$) during baseline recording. In addition, *Clock/Clock* mice had less delta power during the dark period compared to wild-types and the amplitude of the body temperature rhythm was decreased in *Clock/Clock* mice, which corresponded with the changes in NREM and REM sleep across the light/dark cycle ($p < .05, f = 7.8$). Following an acute stressor, *Clock/Clock* mice showed less recovery sleep than wild-type mice ($p < .05$). Correspondingly, body temperature decreased during the recovery dark period in wild-types but did not change in *Clock/Clock* mice. Finally, a negative rebound in delta power was observed in wild-type mice but not in *Clock/Clock* mice ($p < .01$). These results indicate that the *Clock* gene disrupts the pattern and amount of sleep as well as the amplitude of the body temperature rhythm under baseline and stressful conditions.

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¹ Vitaterna, M. et al. Science. 1994.

GENETIC ANALYSIS OF SLEEP-LIKE BEHAVIOR IN *DROSOPHILA MELANOGASTER*. 278

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Although the circadian organization of the rest-activity cycle in *Drosophila* is well characterized, it was not known until recently whether sustained periods of rest constitute a sleep-like state or mere inactivity. Using behavioral, pharmacological, molecular, and genetic investigations we have shown that *Drosophila* rest shares many critical features with mammalian sleep¹. Thus, like mammalian sleep, rest in *Drosophila* is characterized by increased arousal threshold and is homeostatically regulated^{1,2}. As in mammals, rest is abundant in young flies, is reduced in older flies, and is modulated by stimulants and hypnotics. Several molecular markers modulated by sleep and waking in mammals, such as mitochondrial enzymes and chaperone proteins, are also modulated by rest and activity in *Drosophila*.

To evaluate the feasibility of using *Drosophila* as a model system for the genetic dissection of sleep, we examined rest patterns and the response to rest deprivation in 74 fly lines characterized by mutations of genes expressed in the CNS. The genes included, but were not limited to, synthetic and catabolic enzymes, ion channels, protein kinases, receptors, and transcription factors. In addition, we evaluated several fly lines with mutations in clock genes. Rest/activity patterns were monitored using standard infrared monitors as well as ultrasound technology. The ultrasound system permitted the continuous measurement of fly activity by detecting movement as a change in phase and amplitude of a standing sound wave. Flies were deprived of rest by gentle handling or by an automated system. At least 16 flies (females, 3-5d old) for each line were individually studied and compared with wild type CS flies. Results indicated that the amount of rest over the 24/h period and the homeostatic response to 12h of rest deprivation were comparable to wild type flies in the vast majority of mutant lines. The stability of the phenotypes used to evaluate behavioral sleep in a variety of CNS genotypes suggests that a systematic screening for sleep-related mutants in *Drosophila* is feasible.

¹Shaw, et al. *Science* (287) 1834-1837, 2000 ; ²Hendricks et al., *Neuron* (25) 129-138, 2000.

(Supported by Neurosciences Research Foundation)

- 279 · MOLECULAR MECHANISMS LINKING CIRCADIAN GENES AND COCAINE RESPONSIVENESS IN *D.melanogaster*. Rozi Andretic and Jay Hirsh. Department of Biology, Gilmer Hall, University of Virginia, Charlottesville, VA 22903.

The development of sensitization to psychostimulant drugs of abuse is a relatively long-lasting neural adaptation that in human cocaine abusers can lead to the onset of paranoid schizophrenia, and might be important for understanding craving and tolerance.

There are striking similarities between cocaine-induced behaviors in *Drosophila* and higher vertebrates. Exposure of flies to volatilized cocaine induces dose dependent motor behaviors similar to ones seen in rodents and similar to higher vertebrates repeated cocaine exposure results in behavioral sensitization. Mechanistic studies show that the similarities extend to the level of brain chemistry where modulation of the amine system plays an essential role in the sensitization process.

We have shown that a subset of circadian genes has an unexpected but essential role in modulating cocaine responsiveness in *Drosophila*. PER (period) and several other circadian gene products are essential for the development of sensitization to repeated cocaine exposures. However, only a subset of circadian genes which affect PER expression are involved in cocaine responses. We are investigating molecular mechanisms underlying the action of circadian genes on sensitization. Evidence will be presented to show that changes in gene expression in the fly brain induced by cocaine exposure underly the behavioral responses to cocaine.

280 ANALYSIS OF A BEHAVIORAL FEEDBACK LOOP IN THE DESERT SCORPION

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Scorpions are nocturnal animals whose circadian activity rhythm is entrained via several median and lateral eyes. Photoreceptors in lateral eyes convey light signals to the lamina with little modification, whereas the circadian pacemaker modifies median eye output via at least two feedback loops. Scorpions also control their exposure to daylight by a behavioral rhythm—they emerge from their deep burrows at dusk and return before sunrise. We sought to understand how these multiple feedback loops function to regulate the circadian behavior of freely behaving scorpions in their natural environment with a computer model *CircadianDynamix* that is based on a set of differential equations developed by T. Pavlidis and C. Pittendrigh.

We examined entrainment of the scorpion circadian system by natural daylight when the simulated 'scorpion' was exposed to light only during its active phase, which is controlled by the circadian pacemaker. Under these conditions, the pacemaker exhibited stable entrainment only when the visual input to the pacemaker was at an intermediate value. With low values, the system failed to entrain, whereas with high visual gain the phase angle of entrainment became increasingly unstable until entrainment failed. Entrainment was stable over a much larger range of pacemaker tau's when the period of the pacemaker was less than 24 hours than for larger tau values. We obtained similar results for entrainment experiments simulating median eyes, which have a pacemaker-driven rhythm of sensitivity—the 'sunglasses' rhythm, and the lateral eyes, which exhibit only minor circadian alterations in sensitivity. The lateral eyes, however, did entrain the pacemaker over a greater range of visual gains. We conclude that gain control in visual pathway that controls the pacemaker phase is likely to be especially important in animals that control their own exposure to daylight. *Supported by the NSF Center for Biological Timing and the Deutsche Forschungsgemeinschaft.*

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