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Albers, H. Elliott

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The suprachiasmatic nucleus (SCN), which appears to act as a circadian clock, contains a large subpopulation of local circuit neurons in which vasoactive intestinal peptide (VIP) and peptide histidine isoleucine (PHI) are co-localized. We are continuing to investigate the hypothesis that VIP/PHI-containing neurons are essential for the synchronization of circadian rhythms with the day-night cycle. Using in situ hybridization, we have demonstrated that some, but not all SCN neurons that contain VIP/PHI mRNA also contain the mRNA encoding a third biologically active peptide, gastrin releasing peptide (GRP). Furthermore, comparison of the cellular levels of VIP/PHI and GRP mRNA revealed that these mRNAs have distinctly different 24 hr patterns within the SCN, (Continue)

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Dr. J.A. Majde	202-696-4055	ONR

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In other experiments we examined whether VIP, PHI and GRP interact within the SCN to produce a signal important for circadian control. Co-administration of VIP, PHI and GRP within the SCN *in vivo* mimicked the phase delaying effects of light on circadian control, and activated SCN single units recorded *in vitro*. In contrast, administration of VIP, PHI or GRP alone, or co-administration of any two of these peptides did not produce the full behavioral or cellular response observed following co-administration of VIP, PHI and GRP. These data indicate that the interaction of VIP, PHI and GRP may be required for regulation of circadian rhythms by the SCN.

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INTRODUCTION

Several variations in the molecular mechanisms producing co-localized peptides have been observed. In some cases, the co-localized peptides come from the same precursor, while in other cases the co-localized peptides are derived from different precursors. One population of hypothalamic neurons in which both of these mechanisms appear to be active is in the suprachiasmatic nucleus (SCN). A large subpopulation of local circuit SCN neurons contain two peptides derived from the same precursor, VIP and peptide histidine isoleucine (PHI) and, also appear to contain a third peptide derived from a different precursor, gastrin releasing peptide (GRP) (Okamura, et. al., 1986).

A large body of evidence supports the view that the SCN acts as a circadian clock that both generates rhythms in behavior and physiology, and synchronizes those rhythms with the environmental light-dark (LD) cycle (Minors & Waterhouse, 1986). The SCN can be divided into anatomically distinct subpopulations of intrinsic neurons, however it is not known how these different neuronal subpopulations contribute to the generation and synchronization of circadian rhythms with the LD cycle (Moore & Card, 1985). Previously, we hypothesized that VIP/PHI-containing SCN neurons are involved in mediating the effects of environmental lighting important in the synchronization of circadian rhythms with the LD cycle. This hypothesis is supported by the findings that environmental lighting and enucleation selectively alter VIP and PHI immunoreactivity and mRNA within the SCN, and that the cellular levels of VIP/PHI mRNA vary over the day-night cycle (Albers, et. al., 1987; Stopa, et. al., 1988; Albers, et. al., 1990). The majority of VIP/PHI immunoreactive neurons are contained within the ventrolateral SCN, which is also the site of termination of most SCN afferent pathways, including two well defined photic projections, the retinohypothalamic tract (RHT) and the geniculohypothalamic tract (GHT). The possibility that VIP, PHI and GRP are co-localized in local circuit neurons in the afferent terminal fields of the SCN suggest that synchronization of circadian rhythms with the LD cycle may depend on the co-release of these three peptides. If so, the combined effect of VIP, PHI and GRP on circadian rhythms should be different from the effects of each peptide given alone, or in combination with one of the other two peptides. In the last year we have demonstrated that: 1) VIP/PHI and GRP mRNAs can be co-localized in SCN neurons, 2) VIP and GRP mRNAs exhibit different 24 hr patterns within the SCN, and 3) VIP, PHI and GRP interact to phase shift circadian activity rhythms and activate single unit discharge within the SCN.

METHODS

Microinjection Experiments

Hamsters were deeply anesthetized with sodium pentobarbital

and stereotaxically implanted with 26 gauge guide cannulas aimed at the suprachiasmatic region. The stereotaxic coordinates were 1.6 mm anterior and 1.7 mm lateral to bregma, and -7.7 mm below dura. The incisor bar was set at 0 and the stereotaxic arm was set 10 degrees from perpendicular. Peptides were dissolved in 0.9% NaCl (saline) and injected with a 1 ul Hamilton syringe connected to a 33-gauge needle by polyethylene tubing. Peptides were purchased from Bachem, Inc, Torrance, CA. A cocktail containing approximately equimolar concentrations of VIP, PHI and GRP was made by dissolving approximately 50 pmol VIP, 50 pmol PHI and 50 pmol GRP into 400 nl of saline. In all protocols a total of 150 pmol of peptide was injected in 400 nl of saline. Following the experiment hamsters were deeply anesthetized with sodium pentobarbital and microinjected with 400 nl of dye. Following intracardial perfusion with Perfix (Fisher Scientific), the brain was removed and examined to verify the site of injection. Phase shifts in the activity rhythm were calculated by linear regression.

Hypothalamic Slice Experiments

In brief, coronal brain slices containing the SCN (400-450 um thick) were harvested from hamsters housed in a LD 14:10 cycle. Immediately after sectioning, the slices were placed in incubation medium (i.e. modified Krebs solution) oxygenated with 95% O_2 and 5% CO_2 at 35 ^{o}C for at least one hr. Extracellular recordings were obtained using glass micropipettes with DC resistances of 25-40 M filled with 0.5 M sodium acetate containing 2% Fast green. The micropipette was introduced into the slice with the aid of a microscope. At the end of the recording session, a current of 5 uA was passed through the electrode for 3-5 min to deposit a blue spot. All recording sites used in the present study were in the ventrolateral SCN, where the VIP, PHI and GRP immunoreactive neurons are primarily located.

<u>in situ</u> hybridization

Rats maintained in a LD 14:10 cycle were sacrificed by decapitation at 4 hr intervals (N=4-7 per time point). Twelve micron thick coronal sections were cut through the anterior-posterior extent of the SCN. Hybridizations were performed using synthetic oligonucleotide probes specific to GRP and VIP mRNAs by methods described previously (Albers, et. al., 1990). The probes recognizing GRP and VIP mRNA were used for hybridizations conducted on adjacent coronal sections of the SCN. Cellular levels of the hybridization signal were determined with Bioquant Image Analysis software.

Statistical Analyses

Statistical differences among groups were assessed with the one-way classification of the analysis of variance. Subsequent to the analysis of variance, a priori comparisons were made using t-tests and post hoc comparions between individual groups were made with Duncan's Multiple Range test.

RESULTS AND DICUSSION

Co-localization of VIP/PHI and GRP mRNA within SCN Neurons

The co-localization of VIP/PHI and GRP in SCN neurons was examined by comparison of VIP/PHI and GRP mRNA within neurons of the ventrolateral SCN using in situ hybridization. As expected, the autoradiographic signal produced by probes complementary to VIP/PHI and GRP mRNAs was restricted to the ventrolateral aspect of the SCN. Analysis of adjacent sections labeled separately for VIP/PHI mRNA or GRP mRNA indicated that GRP mRNA-containing neurons also appeared to contain VIP/PHI mRNA. However, not all neurons displaying hybridization signal for VIP/PHI mRNA also contained hybridization signal for GRP. In summary, these studies suggest that some SCN neurons contain both VIP/PHI and GRP mRNA, while others contain only VIP/PHI mRNA.

Rhythms of VIP and GRP mRNA within the SCN

Comparison of the cellular levels of VIP/PHI and GRP mRNA in alternate sections of the SCN by in situ hybridization revealed that these mRNAs displayed distinctly different 24 hr patterns (Figure 1). As we had seen previously (Albers, et. al., 1990), VIP/PHI mRNA was greater at night than during the day. In contrast, GRP hybridization signal was greater during the day than at night. These data indicate that the ratio of VIP/PHI to GRP within the SCN may vary over the circadian cycle, and thereby could represent a "time of day" signal. In the upcoming year we will investigate whether the rhythmicity of afferent activity, or endogenous circadian activity induces the rhythms in VIP/PHI and GRP mRNA.

Behavioral Effects of VIP, PHI and GRP within the SCN

If VIP, PHI and GRP combine to form an important circadian signal within the SCN, co-administration of VIP, PHI and GRP within the SCN should influence the timing of circadian rhythms in vivo. To examine this possibility a cocktail containing equimolar concentrations of VIP, PHI and GRP was microinjected into the SCN region of hamsters maintained in constant illumination. As can be seen in Figure 2, VIP/PHI/GRP co-administered into the SCN region around the time of the onset of locomotor activity produced large phase delays in the free-running circadian activity rhythm. However microinjection of VIP/PHI/GRP at other times within the circadian cycle did not phase shift the activity rhythm (Figure 3). The phase shifts produced by VIP/PHI/GRP were the result of the action of these peptides in the SCN region, since microinjection of VIP/PHI/GRP into the cerebroventricular system did not phase shift the activity rhythm. Comparison of the phase response curve for VIP/PHI/GRP microinjection with the phase response curves for various lighting stimuli revealed that VIP/PHI/GRP mimicked the phase delaying, but not the phase advancing effects of light

pulses.

Although VIP/PHI/GRP mimicked the phase delaying effects of light within the SCN region, it was important to determine whether an interaction between all three peptides is required to produce these circadian effects, or whether the action of one or two of these peptides was sufficient. The phase shifting effects of administration of VIP, PHI and GRP and the co-administration of VIP/PHI, VIP/GRP and PHI/GRP were compared with the phase shifting effects of co-administration of VIP/PHI/GRP. The phase delays produced by microinjection of VIP/PHI/GRP were nearly two-fold greater than the phase delays produced by any of these peptides microinjected alone, or in combination with one of the others (Figure 4, Top).

Cellular Effects of VIP, PHI and GRP within the SCN

The hypothalamic slice preparation was used to investigate the cellular effects VIP, PHI and GRP on spontaneously active SCN neurons. Administration of a cocktail containing VIP/PHI/GRP into the perfusate was found to increase the spontaneous discharge of SCN neurons in a dose-dependent manner. At the phase of the circadian cycle in which microinjection of VIP/PHI/GRP phase delayed locomotor rhythms, the firing rate of neurons recorded in the ventrolateral SCN increased by approximately 4 impulses/s in response to VIP/PHI/GRP. The excitation produced by VIP/PHI/GRP was significantly larger than that produced by VIP, PHI, GRP, VIP/PHI, VIP/GRP or PHI/GRP (Figure 4, Bottom; Figure 5 and 6). These data indicate that VIP, PHI and GRP may function interactively within the SCN to regulate circadian rhythms.

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GRANT RELATED PUBLICATIONS

Abstracts

1. Albers, H.E., Liou, S.Y. and Zoeller, R.T. Peptide co-localization: Functional significance within the circadian timing system. Society for Neuroscience, Fall, 1989.

2. Albers, H.E., Liou, S.Y., Stopa, E.G. and Zoeller, R.T. Molecular, cellular and behavioral analysis of the circadian functions of vasoactive intestinal peptide-(VIP), peptide histidine isoleucine (PHI) and gastrin releasing peptide (GRP). Symposium entitled Suprachiasmatic Nucleus: The Mind's Clock. National Institute of Child Health and Human Development, Fall, 1989.

3. Zoeller, R.T., Licklider, N.R., Anderson, E.R. and Albers, H.E. Cellular levels of messenger RNAs encoding VIP/PHI and gastrin releasing peptide (GRP, Bombesin) exhibit different 24 hr rhythms in the rat SCN. Endocrine Society, 1990, Submitted.

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1. Albers, H.E., Stopa, E.G., Zoeller, R.T., Kauer, J.S., King, J.C., Fink, J.S., Mobtaker, H. and Wolfe, H. Day-night variation in vasoactive intestinal peptide/peptide histidine isoleucine mRNA within the rat suprachiasmatic nucleus. Molecular Brain Research, 7:85-89, 1990.

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4. Liou, S.Y., Shibata, S., Albers, H.E. and Ueki, S. Effects of GABA and Anxiolytics on the single unit discharge of suprachiasmatic neurons in rat hypothalamus slices. Submitted.

5. Liou, S.Y. and Albers, H.E. Single unit response of neurons within the suprachiasmatic nucleus to GABA and

low chloride perfusate during day and night. Submitted.

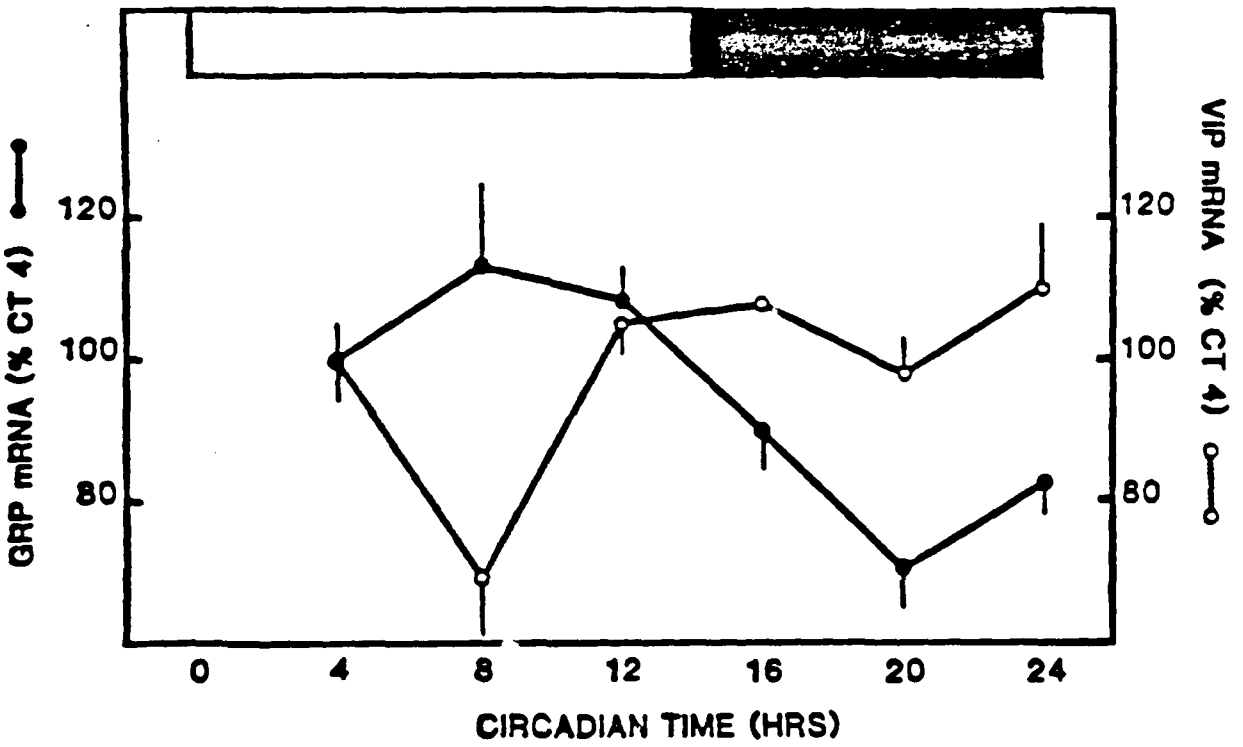

Figure 1. Patterns of the relative cellular levels of GRP and VIP mRNA within the SCN of rats were housed in a LD cycle consisting of 14 hrs of light and 10 hrs of darkness. Both VIP and GRP mRNA were determined within the SCN of each rat by in situ hybridization. For both GRP and VIP mRNA each point is plotted as a percentage of the value observed at circadian time 4. However at all times of day the cellular levels of GRP mRNA were considerably lower than those of VIP mRNA.

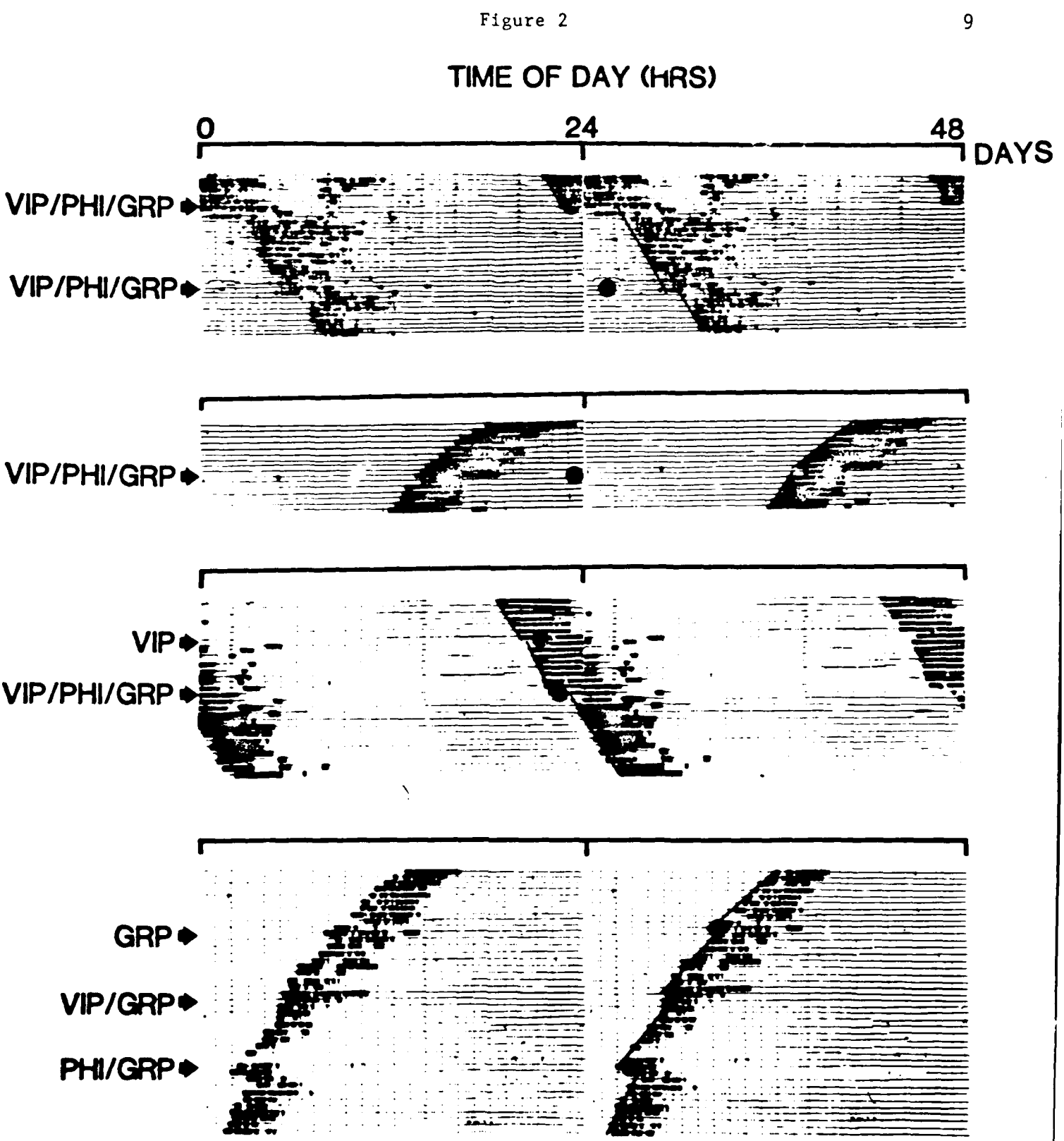

Figure 2. Effect of various combinations of VIP, PHI and GRP on the phase of free-running activity rhythms following microinjection into the suprachiasmatic region of hamsters. Co-administration of VIP (50 pmol), PHI (50 pmol) and GRP (50 pmol) produced large phase delays when given around the time of activity onset, but had little effect at other times within the circadian cycle. Microinjection of VIP (150 pmol) or GRP (150 pmol), or co-administration of VIP (75 pmol) and GRP (75 pmol) or PHI (75 pmol) and GRP (75 pmol) produced only small delays in circadian phase.

Figure 3

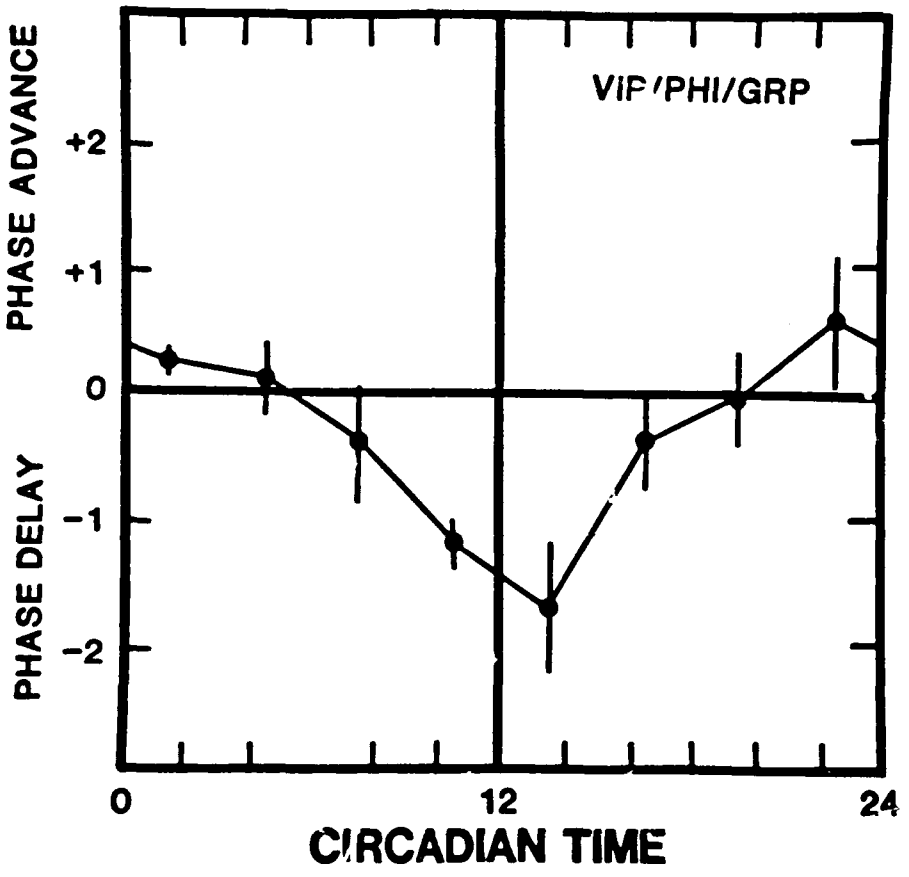

Figure 3. Summary of the effects of microinjections of VIP/PHI/GRP (N=39) into the suprachiasmatic region plotted as a phase response curve. Solid circles represent mean phase shifts (±SEM), in hrs, at 3 hr intervals throughout the circadian cycle (circadian time 12 refers to the time of activity onset). VIP/PHI/GRP produced phase delay shifts around the time of activity onset, but had no effect at other times within the circadian cycle. VIP/PHI/GRP microinjection mimics the phase delaying but not the phase advancing effects of brief light pulses.

Figure 4

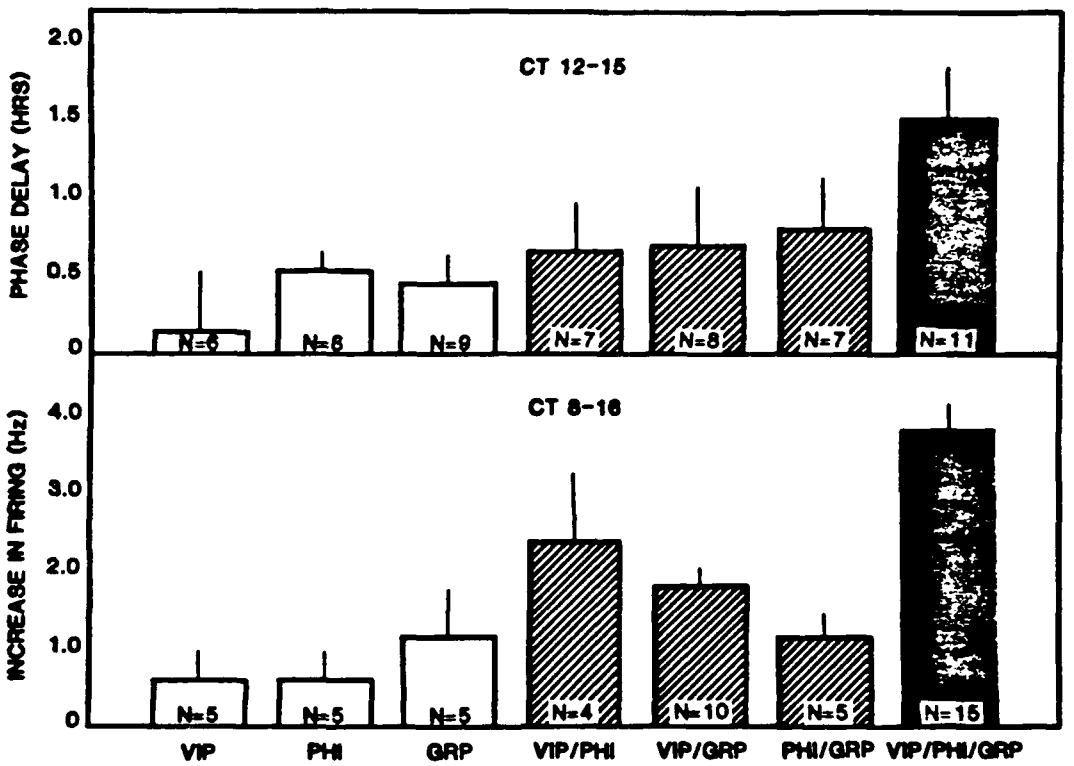

Figure 4. Summary of the behavioral and cellular effects of administration of VIP, PHI, GRP alone, co-administration of two of these peptides (i.e. VIP/PHI, VIP/GRP and PHI/GRP) and co-administration of all three peptides (VIP/PHI/GRP). TOP: Phase shifts produced in hamster activity rhythms following peptide microinjection into the suprachiasmatic region during the 3 hr interval following activity onset (i.e. circadian time 12-15). BOTTOM: Increase in spontaneous discharge of SCN single units following administration of VIP, PHI and GRP and co-administration of all possible combinations of these peptides in a concentration of 10^{-7} M. All single unit recordings were made during an 8 hr interval (circadian time 8-16) of the circadian cycle beginning 4 hrs before the time corresponding to activity onset (circadian time 12).

Figure 5

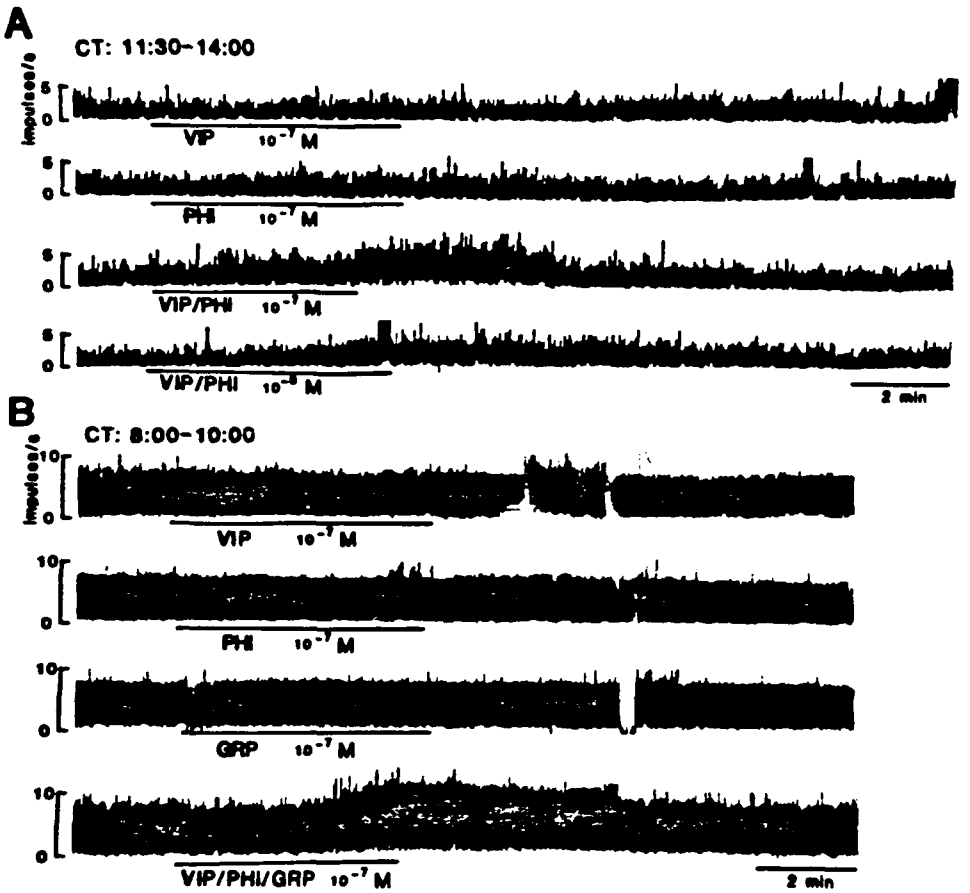

Figure 5. Integrated firing rate records from two suprachiasmatic neurons recorded extracellularly in the hypothalamic slice preparation. (A) Response of a SCN neuron to VIP, PHI, and co-administration of VIP/PHI. (B) Response of a SCN neuron to VIP, PHI, GRP and co-administration of VIP/PHI/GRP.

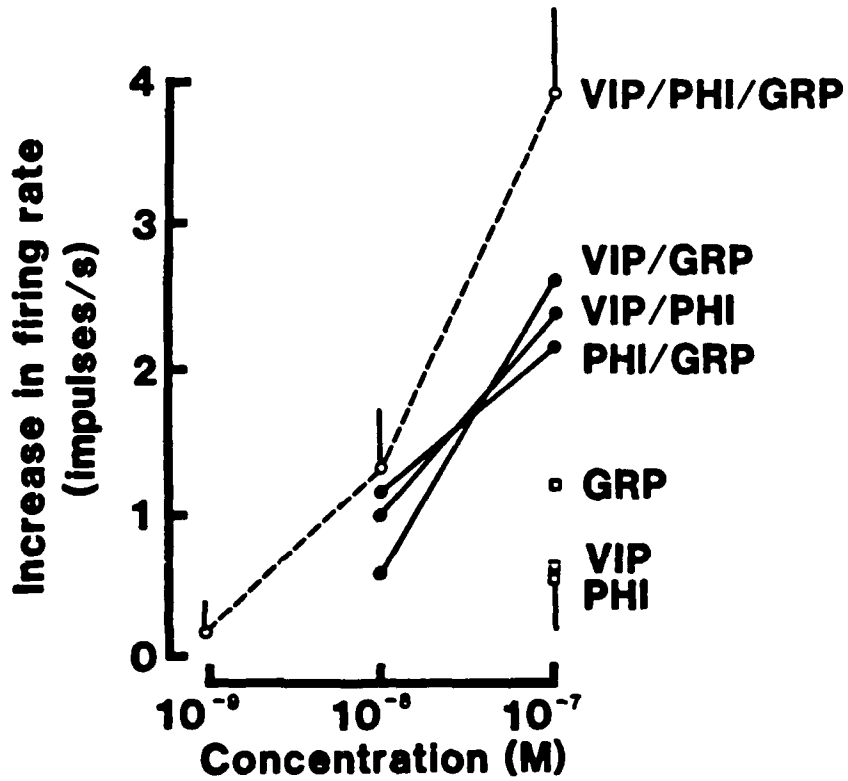

Figure 6. Dose-response of SCN single units to co-administration of VIP/PHI/GRP (N=5), VIP/PHI (N=4), VIP/GRP (N=3), PHI/GRP (N=3), VIP (N=5), PHI (N=5) and GRP (N=5).

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INVESTIGATORS

Dr. H. Elliott Albers
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Georgia State University
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Dr. Gwen V. Childs
Dept. of Anatomy & Neuroscience
Univ. of Texas Medical Branch
Galveston, TX 77550

Dr. Carl E. Creutz
Dept. of Pharmacology
University of Virginia
Charlottesville, VA 22908

Dr. Mary F. Dallman
Dept. of Physiology
University of California, Box 0444
San Francisco, CA 94143-0444

Dr. Caleb E. Finch
Dept. of Neurobiology
Univ. of Southern California
Los Angeles, CA 90089-0191

Dr. Thackery S. Gray
Department of Anatomy
Loyola University Medical Center
216 South First Avenue
Maywood, IL 60153

Dr. Richard F. Ochillo
College of Pharmacy
Xavier Univ. of Louisiana
7325 Palmetto Street
New Orleans, LA 70125

Dr. Terry Reisine
Dept. of Pharmacology
Univ. of Pennsylvania
School of Medicine
36th and Hamilton Walk
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