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13. ABSTRACT *(Maximum 200 words)*

It is now recognized that sleep results from neuronal activity which is regulated by the interactions of neurons with substances produced by neurons and other cellular components of the brain. Over the past 30 years, our technical abilities to identify and synthesize biochemicals has greatly improved; these advances have led to the identification of several endogenous sleep factors (SFs) (substances that promote sleep). Over the past five years of this contract, we have identified several new SFs and synthesized analogs that may be developed into new, more effective, and safer somnogenic agents. The broad goal of this USAMRDC-supported work has been to develop the information needed to determine if it is reasonable to propose either endogenous SFs or synthetic analogs as potential sleep-inducing agents for military use. To meet this goal, we previously reported our progress

14. SUBJECT TERMS

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ABSTRACT (#19 continued)

on 21 sets of experiments; this year, an additional 5 sets of experiments were performed. Briefly we found: 1) That several synthetic fragments of tumor necrosis factor α that contain the TNF_{31-36} sequence are somnogenic. A patent application has been filed on this discovery; 2) We also showed for the first time that the cell walls of archaebacteria (pseudomurein) have biological activity in mammals; 3) We found that prostaglandin D_2 or E_2 failed to greatly affect rabbit sleep suggesting that sleep altering abilities of these substances are species specific; 4) We showed that cholecystokinin elicits sleep in rabbits; this hormone is probably responsible for the feelings of sleepiness after a meal; 5) Finally, we began a series of studies examining on a cellular level, how various SFs alter the expression of surface antigens on brain cells. Our results are discussed within the context of a theory on sleep regulation.

This report is the fifth and last annual report under our current USAMRDC contract. The progress we have made over the past five years under the auspices of this contract will probably revolutionize the way we think about sleep. The details of this progress will be provided in the final report, but a brief list of our major findings is provided here:

1) We showed for the first time that sleep is, indeed, altered over the course of infectious disease.
2) We determined for bacteria what microbial components (and their chemical structural requirements) drive sleep responses (muramyl peptides and endotoxin).
3) We have learned much about which endogenous products induced by the microbial agents are responsible for sleep responses.
4) We have begun to describe on a cellular level how these endogenous SFs affect brain cells.
5) We have developed several completely new somnogenic agents based on the chemical structures of endogenous SFs; two patents have been applied for.
6) Finally, we have developed a theory of brain organization in regard to sleep regulation and theorized how various SFs interact with each other forming regulatory feedback loops that regulate sleep.

Some of this progress has been theoretical, but much has also been very practical. From the view point of pharmacological enhancement of performance, it seems likely to us that the new synthetic somnogenic agents we discovered can be developed into usable sleep-inducing agents.

FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council [DHEW Publication No. (NIH) 78-23, Revised 1978].

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ABBREVIATIONS

aCSF	artificial cerebrospinal fluid
BSA	bovine serum albumin
CCK	cholecystokinin
CNS	central nervous system
CSE	control standard endotoxin
DMSO	dimethyl sulfoxide
DSIP	delta-sleep inducing peptide
EEG	electroencephalographic
ELISA	enzyme linked immunosorbant assay
FBS	fetal bovine serum
FITC	fluorescien isothiocyanate
GFAP	glial fibrillary acidic protein
GH	growth hormone
GRF	growth hormone releasing hormones
GSSG	oxidized glutathione
HLA	human leukocyte antigen
ICAM	intercellular adhesion molecule
ICV	intracerebroventricular
IL1	interleukin-1
IP	intraperitoneal
IV	intravenous
LAL	limulus amoebocyte lysate
LPS	lipopolysaccharide
MDP	muramyl dipeptide
MEM	minimal essential medium
MHCII	major histocompatability complex class II
NREMS	non-rapid-eye-movement sleep
OD	optical density
PFS	pyrogen-free saline
PG	prostaglandin
PGD_2	prostaglandin D_2
PRL	prolactin
REMS	rapid-eye-movement sleep
rhIFNγ	recombinant human interferon γ
RIA	radioimmunoassay
5-HT	serotonin
SF	sleep factor
SRIF	somatostatin
SWS	slow-wave sleep
T_{br}	brain temperature
T_{co}	colonic temperature
TGF	transforming growth factor
TNF	tumor necrosis factor
VIP	vasoactive intestinal peptide
W	wakefulness

INTRODUCTION

The causes of sleep have been intensely debated for the past two or more millennia. Hippocrates concluded that sleep results from the retreat of blood and warmth into the body's inner regions. Aristotle proposed an alternative hypothesis that captured the minds of Europeans for nearly 2,000 years. He proposed that vapors arising from food digesting in the stomach were transported throughout the body via the humors, thereby inducing sleep. Aristotle's idea provides the fundamental concept of sleep factors (SFs); that a change in the amount of some substances(s), in response to a physiological process, acts to promote sleep. Of course the specifics of what is changed, what induces the change, where it happens, etc. have changed over the years and still remain in debate.

Our modern views of the causes of sleep began early in the twentieth century. Legendre and Pieron (114) experimentally addressed the hypothesis that the brain produces, during wakefulness (W), substances that induce sleep. To investigate this hypothesis, cerebrospinal fluid from sleep-deprived dogs was transfered into normal dogs. The recipient animals were then observed to sleep longer than usual. They concluded that a "hypnotoxin" (SF, sleep-inducing substance) was present in the cerebrospinal fluid. Although the chemical nature of the hypnotoxin described by Legendre and Pieron was not determined. These experiments, as well as similar experiments published by Ishimori in Japanese (1909) about the same time, established the fundamental observations to which others in the latter half of this century were to return.

By the 1920s, a new line of inquiry began to dominate the field concerned with sleep regulation. These new hypotheses emphasized the dominant role that neurons and their dynamic patterns of electrical impulses play in sleep regulation. About sixty years ago, these neurophysiological theories of sleep regulation received a major boost when von Economo (183) correlated lesions produced by a viral infection in the hypothalamus to sleep disturbances; when the lesions were in the anterior hypothalamus, patients were insomniacs. Von Economo used these data to postulate that the anterior hypothalamus is involved in sleep regulation and that sleep results from active neural processes within the brain rather than from the simple withdrawal of neural stimulation (a massive amount of subsequent data support both of these hypotheses). Ironically, although an infectious disease (von Economo's virus) played an important role in the support of neural theories of sleep, the connections between the sleepiness that is symptomatic of nearly all infectious diseases and the hypnotoxin theories of sleep were not made for another 60 years. Throughout this time, the neurophysiological theories of sleep regulation rapidly evolved in sophistication and detail in parallel with the explosive growth in knowledge and techniques available in electrophysiology.

More recently, there also has been an impressive growth in our technical ability to isolate and detect biochemicals. These advances have led to the recognition that neurons and glial cells produce, transport, package, release, and respond to a large numbers of biochemicals. Some of these substances change the patterns of electrical activity of neurons and also induce sleep. As a consequence, today these previous, seemingly dichotomous theories of sleep regulation, the hypnotoxin and

neural theories, are now viewed as complementary to each other. Furthermore, these advances in biochemical techniques have led to the intense pursuit of SFs over the past twenty years and resulted in the identification of several SFs. These results suggested to us that new, safer and effective somnogenic agents can be developed. Thus, the major objective of our USAMRDC-supported work has been to develop information needed to determine if it is reasonable to propose either endogenous SFs or synthetic analogs as potential sleep-inducing agents. As part of this goal, we have initiated the process to obtain patents on two different classes of somnogenic compounds.

With the above objective in mind, in the first year of our contract period (June 1, 1986 - May 30, 1987), five sets of experiments were performed; in the second year (June 1, 1987 - May 30, 1988) six sets of experiments were carried out; in the third year (June 1, 1988 - May 30, 1989) five sets of experiments were performed and in the fourth year (June 1, 1989 - May 30, 1990) five sets of experiments were reported. Results from those experiments are described in our annual reports dated June 1, 1987; June 1, 1988; June 1, 1989 and June 1, 1990. This year, June 1, 1990 - May 30, 1991 five sets of experiments were performed; they are numbered one through five as follows:

1) In the first experiment, we show that several synthetic fragments of tumor necrosis factor (TNF)α that contains the TNF_{31-36} sequence are somnogenic. (A patent application for this discovery has been filed).

2) Second, we showed that the cell walls (pseudomurein) of an archaebacteria (Methanobacterium thermoantotrophican) has biological activity (sleep promoting) in mammals.

3) Third, we showed that neither prostaglandin (PG)E_2 or PGD_2 were somnogenic in rabbits thus suggesting that the somnogenic actions of these agents are species specific.

4) Fourth, we showed that cholecystokinin (CCK) elicits sleep in rabbits. This hormone is released after food intake thus suggesting that it may be responsible for the feelings of sleepiness after eating.

5) Finally, we described how the expression on astrocytes of major histocompatability class II antigen is altered by a variety of somnogenic agents.

METHODS

Animals. Male New Zealand White Pasteurella-free rabbits (3-4 kg) were provided with a lateral cerebral ventricular guide cannula, a brain thermistor and stainless steel screw electroencephalographic (EEG) electrodes as previously described (102, 139, 145). A calibrated thermistor, (50 k Ω, model 4018, Omega Engineering, Stanford, CT) was implanted through a burr hole in the skull over the parietal cortex to measure brain temperature (T_{br}). EEG electrodes were placed over the frontal and parietal cortices. The leads from the EEG electrodes and the thermistor were led to an Amphenol plug; the wires, electrodes, and guide tube were held in place with dental acrylic (Duz-All, Coralite Dental Products, Skokie, IL). Animals were allowed to recover from the surgery two or more weeks.

The rabbits were kept on a 12:12 hr light:dark cycle (light on at 0600 hr) at 21° ± 1°C. Rabbits were placed in the recording chambers

(Hotpack 352600, Philadelphia, PA) for at least two 24 h habituation sessions before recordings were taken from animals for the first time. When recordings were taken from animals, they were placed in the experimental chambers the preceding evening. The light:dark schedule and ambient temperatures of the recording chambers were kept the same as that of the housing facility. Food and water were available *ad libitum*.

Recordings and sleep analyses. A flexible thether connected an electronic swivel to the Amphenol connecter on the rabbit head, this apparatus was suspended by a shock-absorbing system; this allowed the animals to have relatively complete freedom of movement within the experimental cages. To detect body movements, ultrasonic motion detectors were used. Cables from the motion detectors and the electronic swivel were connected to Grass 7D polygraphs in an adjacent room. EEG, T_{br}, and movement were recorded on the polygraphy paper. In addition, the EEG signal was led through two band pass filters, (0.5-3.5 Hz δ and 4.0-7.5 Hz θ) the signals were rectified and the ratio of θ/δ activity was continuously computed and recorded on the polygraph paper simultaneously with the EEG, T_{br} and body movements. This θ/δ ratio served as an aid in identifying periods of rapid-eye movement sleep (REMS) (139). T_{br}s were also recorded using a data logger (Acrosystem 400, Beverly, MA); values taken every 10 min were stored on computer disks.

Vigilance states were identified as previously described (139). Non REMS (NREMS) (also called slow wave sleep [SWS] in this report) was associated with decreasing T_{br}s, low EEG θ/δ ratio and a high amplitude EEG. In contrast, REMS was characterized by a rapid rise in T_{br} upon entry into REMS, high θ/δ ratios and a relative low-amplitude EEG. Polygraph records were visually scored in 12 sec epoches (102). The

duration of a particular sleep or wake episode was measured by entering episode lengths (distances on the recording paper) into a computer with a calibrated digitizer board (Jandel Scientific). The percentages (duration) of NREMS and REMS were calculated for 1 and 6 hr periods. Differences between results obtained on experimental days and control days were evaluated by use of the Wilcoxon matched-pair signed rank test. The Kruskal-Wallis one way analysis by ranks were used to determine dose dependency effects.

Test substances were dissolved in small volumes of dimethyl sulfoxide (DMSO) or ethanol; aliquots of those preparations were diluted with artificial cerebrospinal fluid (aCSF) (2 mM KCl, 1.15 mM $CaCl_2$ and 0.9 mM $MgCl_2$ in pyrogen free saline, 115 mM NaCl, Abbott, Chicago, IL) such that the doses used were in 25 µl. For control experiments and equal amounts of aCSF containing DMSO or ethanol was used as injectant. These recordings were obtained from the same animal thus a matched-pairs design was used. Most substances were injected into a lateral cerebral ventricle (intracerebroventricular, ICV). Injection volume was 25 µl; each injection took about 2 min. In the case of CCK, it was also injected IP. Finally, suspensions of pseudomurein were injected IV before recordings began. Rabbits were injected between 0845 and 0915 hr. After injections, animals were returned to the experimental cages and were recorded from for the next 6 hr.

Specific Experimental Protocols

Experiment No. 1: Somnogenic activity of TNF fragments.

Compounds tested. Human recombinant TNFα (hu r TNFα) and hu r TNFβ were obtained from R & D Systems, Inc., Minneapolis, MN, TNFα peptides 10-69 and 140-157 were gifts from Dr. Louis Chedid (University of South Florida). TNFα peptides 10-36, 31-45, 46-65, 31-68, 44-68, 31-45, 45-65, 69-100, and 101-135 were synthesized by Dr. Jerome Seyer. FMLP was purchased from Sigma Chemical Co., (St. Louis, MO). Peptides were dissolved in small volumes of DMSO before being diluted with aCSF for ICV injection.

Experiment No. 2: Somnogenic activity of pseudomurein.

Materials. Lyophilized *Methanobacterium thermoautotrophicum* was a gift from O. Kandler and W. König. Pseudomurein was isolated as described before for staphylococcal cell walls (77, 111). Briefly, whole cells were disintegrated by shaking the cell suspension with glass beads in a cell mill. Membranes and proteins were removed by successive treatment of the cell walls with EDTA, Triton X-100, and trypsin. Possible other contaminants were removed by treatment with trichloroacetic acid. After extensive washing the cell walls were lyophilized.

In preparation for IV injection into rabbits, the lyophilized pseudomurein was suspended in appropriate volumes of pyrogen free saline (PFS) (0.9% NaCl, Abbott, North Chicago, IL, USA) to provide the desired concentrations (Table). One ml of sample or vehicle alone was injected into a marginal ear vein. In some cases it was suspended in PFS containing 1 mg/ml Polymyxin B sulfate (Sigma, St. Louis, MO, USA) and pre-

incubated for one hour at 37°C prior to the injection to inactivate possible endotoxin contamination.

In a separate control experiment polybead polystyrene microspheres (0.98 µm diameter, 2.5% solids = $5x10^{10}$ spheres/ml, Polysciences, Wassington, PA, USA) were diluted to $5x10^{9}$/ml in PFS before IV injection.

Amino acid analysis of the pseudomurein sample was performed by Dr. Jerome M. Seyer (Veterans Administration Medical Center, Memphis, TN) to verify the purity of the sample. Conforming with the known structure of pseudomurein (95), only three amino acid were detected: alanine (2.08 nmol/µg pseudomurein), glutamic acid (1.84 nmol/µg), and lysine (1.79 nmol/µg). No other amino acids were found at a sensitivity that would have detected < 50 pmol/µg. Amino sugars could not be identified under the conditions used.

The Limulus Amoebocyte Lysate assay (LAL, sensitivity 0.25 EU/ml) and Control Standard Endotoxin (CSE, E. coli 0113, Associates of Cape Cod, Woods Hole, MA) were used to test samples for endotoxin contamination and to determine the amount of Polymyxin B sulfate necessary to neutralize the minimal LAL reactivity detected in the pseudomurein samples.

A within subject experimental design was used: experimental and control recordings were obtained from the same rabbits on different days. Controls for pseudomurein or microsphere samples were injected with PFS, controls for pseudomurein plus polymyxin samples received a solution of 1 mg/ml polymyxin B in PFS which was pre-incubated at 37°C for 1 h prior to injection. Average hourly values were used to evaluate

effects across the six hour recording period by means of the the Wilcoxon matched-pairs signed-ranks test.

Experiment No. 3: Back of somnogenic activity of PGE_2 and PGD_2.

Compounds tested. PGD_2 was obtained from Sigma Chemical Co. (St. Louis, MO) and from Cayman Chemical Co. (Ann Arbor, MI). Two different lots of PGD_2 were obtained from Cayman Co. Results obtained using these two lots and those obtained using the Sigma preparation did not differ from each other and thus were pooled when appropriate. The ability of the PGD_2 used to bind with antibodies used in radioimmunoassays (RIA) was determined using either the RIA method described by Leffler (Department of Physiology, University of Tennessee, Memphis) or using a commercially available RIA PGD_2 kit obtained from Advanced Magnetics, Inc. (Cambridge, MA). In both cases, the antibodies bound the PGD_2 preparation providing indirect evidence that the chemical form of the PGD_2s tested were indeed PGD_2. PGE_2 was obtained from Sigma Chemical Co.

Experimental Protocols. PGE_2 or PGD_2 were dissolved in small volumes of ethanol (10 mg/0.1 ml ethanol). Small aliquotes of these stock solutions were added to pyrogen free saline (PFS) (0.01-70.5 ml/1.0 ml PFS) in preparation for injection into animals. Twenty five µl of these solutions were injected ICV over a 1 min period beginning at 0830 hr under sterile conditions. For control studies, vehicle containing equal amounts of ethanol were used as injectants on different days. The small amount of ethanol used in this study did not affect sleep compared to using PFS alone (data not shown). After ICV injections, animals were connected to the electronic swivels then recorded from for the

next six hours. Estimated range of time between the end of injection and the beginning of recording was 5-15 min. In one case, when 0.25 nmol of PGD_2 was used, 75 µl of solution was infused slowly over a 45 min period into a lateral ventricle; this slower method of administration was used to replicate the methods used in a previous preliminary study (100). The percentages (duration) of NREMS and REMS were calculated for 12 min periods during the first postinjection hour and for 1 and 6 hr periods. Differences between results obtained on experimental days and control days were evaluated across the 6-h recording periods by the Friedman's nonparenetic two-way analysis of variance and within discrete time blocks by use of the Wilcoxon matched-pairs signed-ranks test. The Kruskal-Wallis one way analysis of variance by ranks was used to determine dose related effects. All analyses were performed by the SPSS^x international analysis system and an alpha level of $p \leq 0.05$ was accepted as indicating significant departure from control.

Experiment No. 4: Somnogenic effects of CCK in rabbits.

On the experimental days, the rabbits were injected intraperitoneal (ip) or ICV with saline (control) or CCK-octapeptide sulphate ester (Bachem Inc., Torrance, CA) dissolved in isotonic saline. The injections were done between 0845 and 0915 h. The volume of the ip injection was 2 ml/kg. Each ICV injection (25 µl/rabbit) lasted for 1 min and an additional min was allowed before removing the injection cannula. After the treatments the sleep-wake activity and T_{br} were recorded for 6 h. Three doses were tested ICV: 0.05 µg/rabbit, 0.5 µg/rabbit, 2 µg/rabbit, three different doses were injected IP: 2.5 µg/kg, 10 µg/kg, 40 µg/kg. The experimental protocol followed a self-control design,

,i.e.,each rabbit received saline treatment (control day) and 1-3 treatments of the various CCK doses. The differences between the effects of CCK and the saline injection were calculated. The experiments were separated by at least 2 days.

The durations of vigilance states were calculated hourly, and were expressed as average percent of time (± SE). These hourly values after CCK and saline injections were compared across 6 h by two-way ANOVA followed by an hour-by-hour comparison by paired Student's t-test. The maximal delta amplitudes during NREMS were compared after CCK and saline treatment in the first postinjection h by paired Student's t-test. The temperature data were averaged in 10 min intervals (± SE). Comparisons were made between the effects of saline and CCK injections for the first 3 postinjection h by two-way ANOVA. A p value < 0.05 was taken to indicate significant difference.

Experiment No. 5: Modulation of human leukocyte antigen DR expression in glioblastoma cells by putative sleep factors.

Reagents. Recombinant human IFNγ (rhIFNγ) was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN) with activity > 2×10^7 units (U)/mg in an EMC virus/WISH cell assay. Human IFNα was purchased from Chemicon (Temecula, CA) with activity of 10^6 U/mg. The following cytokines and antibodies were purchased from R&D Systems (Minneapolis, MN) and their activities were determined by R&D Systems as indicated; a) recombinant human TNFα (rhTNFα), activity > = 2×10^6 U/mg measured by its cytolytic effect on L929 cells; b) recombinant human IL-1β (rhIL-1β), activity > = 5×10^8 U/mg in a Lymphocyte Activation Factor assay; c) porcine transforming growth factor (TGF)β1 and β2, ED_{50}

for the inhibition of ^{3}H-thymidine incorporation in mink lung epithelial cells was 0.05 ng/ml; d) the neutralizing doses$_{50}$ (ND_{50}) of antibodies against porcine (TGFβ) raised in chicken were 2-3 mg/ml for blocking the effect of TGFβ1 at a dose of 0.5 ng/ml; ND_{50} of antibodies against TGFβ2 raised in rabbit was 1-2 mg/ml for blocking the effect of TGFβ2 at a dose of 0.25 ng/ml; the ND_{50} of antibodies against rhTNFα was 0.2 mg/ml for blocking the effect of rhTNFα at a dose of 0.25 ng/ml. Serotonin (5-HT), PGE_2, PGD_2, and hydrocortisone were purchased from Sigma Chemical Co. (St. Louis, MO). Vasoactive intestinal peptide (VIP) was obtained from Bachem, Inc. (Torrance, CA). Delta sleep-inducing peptide (DSIP) was purchased from Peninsula Laboratories, Inc. (Belmont, CA). Muramyl dipeptide (MDP) was a gift from Dr. L. Chedid.

Cell Culture

Cell growth. Three human glioblastoma cell lines, HTB14, HTB16 and HTB17, and a human neuroblastoma cell line HTB11 were purchased from American Type Culture Collection (Rockville, MD). Cells were grown in 100 x 15 mm tissue culture dishes in modified Eagle's minimum essential medium (MEM) (Flow Laboratories, Inc., McLean, VA) supplemented with 5% fetal bovine serum (FBS) (Biocell, Carson, CA), and 100 IU/ml penicillin G and 100 mg/ml streptomycin (Sigma Chemical Co.) at 37°C in an atmosphere with 5% CO_2 in air. Cells in confluent culture were incubated with 0.25% trypsin solution for 1-2 min, resuspended in fresh culture medium, and about 2.5 x 10^3 cells/well were transferred to 96-well plates. Cell number in each of 6 wells was counted every day for up to 11 days, and cells in parallel wells were digested with 30 ml of 0.6N

NaOH overnight and then the protein amount in each well was determined using BCA protein assay kit (Pierce, Rockford, IL).

Immunofluorescence staining for glial fibrillary acidic protein (GFAP) and neurofilament.

Glioblastoma cells were stained for GFAP, a unique intracellular interfilament for astrocytes. Confluent cell cultures in 100 x 15 mm dish were trypsinized and 4 x 10^4 cells were transferred to 12-well plates with a round glass coverslip in each well, and incubated at 37°C, with 5% CO_2 in air for 2 days. As previously described (122), cells were rinsed twice with medium and fixed with 3.8% formaldehyde at room temperature for 5 min following 6 brief (about 1/2 sec.) immersions in acetone at -20°C. Then cells were incubated 4 hours at room temperature with 500 µl of polyclonal rabbit anti-human GFAP (Sigma Chemical Co.) diluted 1:200 in culture medium as the primary antibody. After washing 5 times with PBS containing 0.1% bovine serum albumin (BSA), cells were incubated in the dark for one hour with goat anti-rabbit IgG-conjugated to fluorescien isothiocyanate (FITC) (HyClone Laboratories, Inc., Logan, UT) diluted 1:100 in 1% BSA-PBS as the secondary antibody. Finally, coverslips were mounted on slides with 1:1 PBS:glycerol before microscopic examination. The procedures for staining neurofilament was the same as to that for GFAP except that the primary antibody was monoclonal mouse anti-human neurofilament (Dakopatts, Carpinteria, CA) and the secondary antibody was goat anti-mouse IgG-conjugated to FITC (Sigma Chemical Co.).

Experimental Design

Experiment A: Expression and induction of HLA_{DR} by rhIFNγ on glioblastoma and neuroblastoma cells

HLA_{DR} expression and the effects of rhIFNγ on HLA_{DR} expression were determined in all three glioblastoma cell lines HTB14, HTB16 and HTB17, and the neuroblastoma cell line HTB11. To obtain confluent cultures within 1-2 days, cell suspensions containing 2-3 x 10^4 cells were added into 96-well culture plates. At the beginning of an experiment, the original medium was aspirated off, and either 200 µl of fresh medium or media containing various concentrations of rhIFNγ (10^{-1}, 1, 10, 10^2, 10^3, 5 x 10^3, and 10^4 U/ml) were added to individual wells. Each treatment for each dose of rhIFNγ was performed in 6 replicate wells. Culture plates were incubated at 37°C with 5% CO_2 for 4 days. HLA_{DR} expression on the cell surface was determined using an enzyme linked immunosorbant assay (ELISA) method on the whole cells as described below. To determine the effects of IFNγ on HLA_{DR} expression over time, HTB16 cells were treated with 1000 U/µl of rhIFNγ. The culture media with rhIFNγ was removed on the fifth day and replaced with 200 µl of fresh culture medium. HLA_{DR} expression was assessed every day in triplicate samples from day 0 to day 9.

Experiment B: Effects of cytokines TNFα, TGFβ1 and β2, IL-1β on HLA_{DR} expression in HTB16 cells.

Cell culture preparation was the same as that for experiment A; each treatment was performed in 6 wells.

I. rhTNFα

HTB16 cells were treated with 0.1, 1, 10, 50, and 250 ng/ml of rhTNFα diluted in fresh medium or in the media containing a fixed dose of rhIFNγ (100 U/ml or 500 U/ml) for 4 days to determine effects of TNFα doses on IFNγ-induced HLA_{DR} expression. To determine the effects of TNFα on IFNγ-induced HLA_{DR} over time, cells were incubated with 10 ng/ml of rhTNFα combined with 100 U/ml of rhIFNγ for 8 days; HLA_{DR} expression was quantitated every day. Cells were also treated with neutralizing antibody against rhTNFα at concentrations of 10, 20, and 40 µg/ml in combination with 10 ng/ml of rhTNFα and 100 U/ml of rhIFNγ for 4 days in order to verify that the effects of TNFα were specific.

II. TGFβ1 and β2

HTB16 cells were treated with 0.01, 0.1, 1, and 10 ng/ml of TGFβ1 and β2 diluted in fresh medium and in the media containing 100, 500, and 1000 U/ml of rhIFNγ respectively for 6 days to determine the effects of TGFβ1 and β2 doses on IFNγ-induced HLA_{DR} expression. The effects of TGFβ1 and β2 on IFNγ-induced HLA_{DR} over time were carried out over 8 days; cells were incubated with 10 ng/ml of either TGFβ1 or β2 in combination with 100 U/ml of rhIFNγ and HLA_{DR} expression was quantitated every day. To block the inhibitory effect of exogenous TGFβ1, cells were treated with neutralizing antibody against TGFβ at concentrations of 1, 5, 10, and 25 µg/ml in combination with 10 ng/ml of TGFβ1 and 100 U/ml of rhIFNγ for 6 days. A similar experiment was carried out for TGFβ2 using the same doses of neutralizing antibody against TGFβ2 but combined with three different treatments of TGFβ2 and rhIFNγ: a) 1

ng/ml of TGFβ2 plus 100 U/ml of rhIFN; b) 1 ng/ml of TGFβ2 plus 500 U/ml of rhIFNγ; and c) 10 ng/ml of TGFβ2 plus 500 U/ml of rhIFNγ.

III. IL-1β

To examine the effects of interleukin-1β (IL-1β) on HLA_{DR} expression and on IFNγ-induced HLA_{DR} expression, HTB16 cells were treated with rhIL-1β using concentrations of 0.5, 2, 10, 50, and 250 pg/ml in the absence or presence of 500 U/ml of rhIFNγ for 4 days before HLA_{DR} expression was assessed.

Experiment C: Effects of PGE_2, PGD_2, IFNα, VIP, DSIP, 5-HT, hydrocortisone, and MDP, on HLA_{DR} expression in HTB16 cells.

HTB16 cells were treated with the following: PGE_2, PGD_2, IFNα, VIP, DSIP, 5-HT, and hydrocortisone. All agents were incubated with cells in the absence or presence of 500 U/ml of IFNγ for 4 days before HLA_{DR} expression was assessed. The doses for these substances used were PGE_2 and PGD_2, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} M; IFNα, 1, 10, 10^2, and 10^3 U/ml; VIP, DSIP, and 5-HT, 10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} M; and hydrocortisone, 10^{-5}, 10^{-4}, and 10^{-3} M. MDP was tested using doses of 0.02, 0.2, 2, and 20 mM in the absence and presence of 500 U/ml of IFNγ; incubations were 7 days. Each treatment was performed in 6 independent wells.

Quantitative analysis of cell surface HLA_{DR} expression using ELISA

After the experimental treatments described above, media were aspirated off and cells were fixed with 100 µl of 3.8% formaldehyde at room temperature for 5-7 min. After rinsing with culture medium twice,

100 µl of monoclonal mouse antihuman HLA_{DR} antibody (Dakopatts) diluted 1:200 in culture medium was applied into designated wells and the plate was incubated at 4°C overnight. The plate was then washed gently with 300 µl of 0.1% BSA-PBS three times. The next incubation with 200 µl of secondary antibody, goat anti-mouse IgG-conjugated to horseradish peroxidase (Pierce) diluted 1:4000 in 1% BSA-PBS, was carried out at room temperature for 2 hours. Following three washes with 0.1% BSA-PBS, 100 µl of peroxidase substrate solution containing 2,2'-azino-di[3-ethylbenzthiazoline sulfonate] (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) was placed into each well. After 40 to 50 min, the optical density (OD) was read at 405 nm by a Microplate Autoreader (Bio-Tek Instruments, Inc., Winooski, VT). Finally, the cells in each well were digested with 30 µl of 0.6N NaOH overnight, and the amount of protein in each well was determined using BCA assay reagents (Pierce). The HLA_{DR} expression is presented as OD/mg protein.

RESULTS

Experiment No. 1:

I. TNFα

TNFα elicited a dose-dependent increase in NREMS and T_{br}. The threshold dose for both the hypnogenic and pyrogenic effects was 0.05 µg. This dose of TNFα induced a significant increase in NREMS across the 6 h experimental period (Table 1). Most of the TNFα-induced excess NREMS occurred during postinjection hours 2-4 (Fig. 1) although sleep tended to be elevated for the next two hours as well. Injection of 0.5

μg and 5 μg TNFα promoted NREMS for a more prolonged period extending to the sixth postinjection hour elicited an overall increase in NREMS across the 6 h recording period (Table 1). However, neither the 0.005 nor 0.05 μg dose of TNFα altered REMS. The effects of TNFα on REMS were not dose-related. REMS was significantly reduced after the injection of the two higher doses. TNFα-induced changes in T_{br} paralleled the TNFα-induced NREMS effects; there was a significant correlation between the pyrogenic effects and hypnogenic effects of TNFα. Monophasic fever induced by 0.05 μg TNFα peaked at 60 min postinjection and the T_{br} stayed elevated thereafter throughout the entire recording period. The pyrogenic effects of the two highest doses were biphasic; an initial peak occurred at about 60 min postinjection and was followed by a second rise in T_{br} that persisted until the end of the recording period.

II. TNFβ

TNFβ also elicited dose-dependent increases in T_{br} and NREMS. The three lowest doses tested induced slight increases in NREMS (Table 1) though only the increase in NREMS after the lowest dose was significant. The highest dose of TNFβ (0.2 μg) elicited a relatively robust increase in NREMS of about 15% across the 6 h experimental period. Only the highest dose affected REMS; TNFβ induced a slight but significant decrease in REMS across the 6 h. Although there were statistically significant correlations found between the hyperthermic and hypnogenic effects of TNFβ, the changes in NREMS in response to TNFβ did not parallel the febrile effects as clear as in the case of TNFα. Though the lowest dose of TNFβ (0.0005 μg) enhanced NREMS, it did not increase T_{br}.

The highest dose of TNFβ (0.2 μg) elicited a biphasic fever comparable to the effects of 0.5 μg TNFα.

III. TNFα fragments

TNFα fragments 44-68, 46-65, 69-100, 101-135 and 140-157 failed to alter NREMS. One of these ($TNF\alpha_{44-68}$) did induce a reduction in REMS and caused a slight fever (Table 1). Another fragment $TNF\alpha_{69-100}$ also induced fevers but failed to alter the sleep parameters measured; this fragments elicited a dose-dependent increases in T_{br}. It is noteworthy that unlike the whole TNFα molecule, the effect of the fragment on the T_{br} was monophasic with a latency of 100-120 min (Fig. 4).

Two fragments with overlapping sequences ($TNF\alpha_{10-36}$ and $TNF\alpha_{31-68}$) were both pyrogenic and somnogenic. These fragments were virtually equipotent; the 5 μg dose did not show any significant effect on T_{br} or sleep, but 25 μg of both fragments elicited a monophasic fever with a maximum amplitude of about 1-1.2 °C paralleled with a significantly increased NREMS across the 6 h recording period. A statistically significant correlation was found between the thermoregulatory and NREMS responses to these fragments. The pyrogenic effects of these fragments resembled the second phase of the TNFα-induced fever although the amplitudes of changes were much lower even after the injection of doses as high as 25 μg. The fragment $TNF\alpha_{31-45}$ increased NREMS but failed to significantly affect T_{br}. The fragment $TNF\alpha_{10-69}$ also increased NREMS and reduced REMS after the injection of 10 and 5 μg doses, respectively.

IV. FMLP

One facet of TNFα activity is its chemotactic activity for neutrophils and monocytes. Previously, it was suggested that this activity could be due to contaminating amounts of N-formylated methionyl peptides. Therefore, we determined whether FMLP was somnogenic. After doses of 10 pmol or 100 pmol were given ICV, sleep and T_{br} remained close to normal values.

Experiment No. 2

The injection of 1-2 mg pseudomurein per rabbit did not result in significant effects on SWS, REMS, or EEG delta wave voltages (Table 2) nor did it affect T_{br} or colonic temperature (T_{co}) (data not shown). The injection of 4 mg pseudomurein per rabbit increased SWS significantly over the six hour recording period (Table 2). The differences in SWS occurred during the first 4 hours of the experiment; no differences were observed in hours five and six (Fig. 1 A). A similar time course in enhanced EEG δ wave voltage was observed (Fig. 1) and these increases were significant across the 6 hour recording period (Table 2). REMS was not affected by this dose of pseudomurein (Table 2, Fig. 1 B). While T_{co}s measured at the end of the six hour recording period were not effected by injection of pseudomurein, T_{br}s recorded at 10 min intervals revealed that a transient increase in T_{br} occurred, peaking during the second postinjection hour (Fig. 1, D).

To evaluate the possibility that endotoxin contamination might have contributed to the observed sleep effects, the LAL test was used to estimate, and polymyxin B sulfate was used to inactivate, any possible endotoxin contamination: A suspension of 1 mg/ml pseudomurein in PFS

contained a LAL reactivity of between 1.25 ng/ml and 12.5 ng/ml CSE. A concentration of 0.01 mg/ml Polymyxin B sulfate was sufficient to inactivate 10 ng/ml of CSE in the LAL assay. Interestingly, 1 mg/ml of Polymyxin B sulfate was needed to inactivate the LAL activity of 1 mg/ml pseudomurein. Since a much higher dose of Polymyxin B was need to inactivate the pseudomurein LAL activity than needed to activate CSE activity, this suggests that the pseudomurein itself had LAL activity.

When pseudomurein was preincubated with 1 mg/ml Polymyxin B sulfate prior to injection (4 animals with 2 mg, 4 animals with 4.5 mg pseudomurein), the observed effects were similar, although less pronounced, to those obtained after injection of pseudomurein not pretreated with Polymyxin B. SWS and δ-wave voltages were significantly increased and a slight nonsignificant enhancement of REMS was observed (Table 2). T_{co}s measured at the end of the recording period were not affected (data not shown). A transient increase in T_{br} occurred during the second postinjection hour and was of equal magnitude, shape, and timing as the increase observed after injection of pseudomurein that was not pretreated with polymyxin B (data not shown).

To investigate whether the observed pseudomurein effects on sleep parameters and T_{br} were due to the particulate or to the chemical nature of the sample, polystyrene microspheres were injected via the same route and in comparable concentration. ($5x10^9$ sacculi are contained in 4.5 mg of a cell wall preparation of *Staphylococcus aureus* that was prepared in the same way as the pseudomurein [79]). The injection of $5x10^9$ microspheres in 1 ml PFS had no effects on sleep parameters (Table 2), or on T_{co} or T_{br} (data not shown).

Experiment No. 3

PGE_2 induced dose-dependent increases in T_{br} (Fig. 2) thereby confirming previous observations (44, 131). The increase in T_{br} began within minutes of the ICV injections and then peaked about one hour later. The lowest dose of PGE_2, (0.25 nmol), induced about a 0.5° rise in T_{br}. The response after this dose lasted about one hour. The highest dose of PGE_2, 10 nmol, induced a maximum increase of about a 1.5° increase in T_{br} and the duration of the hyperthermic response lasted about 2.5 hour. Within three hours of injection, T_{br}s were close to control values.

The effects of PGE_2 on sleep/wake cycles were not as obvious as its effects on T_{br}. None of the doses of PGE_2 tested altered duration of either NREMS or REMS across the six hour recording period (Table 3). However, if the analyses were confined to the first hour after injection, PGE_2 induced significant dose-dependent biphasic effects on NREMS (Fig. 3). During the first twenty four min after injection, NREMS was decreased relative to corresponding control values. In contrast, during the last 24 min of the first postinjection hour, duration of NREMS was significantly elevated above control values. PGE_2 also altered REMS. After injection of the 2.5 or 10 nmol PGE_2 dose, but not after the 0.25 or 5 nmol dose, REMS was inhibited during the last 36 min of the first postinjection hour. However, the control REMS values of the rabbits used for assaying the 5 nmol dose were very low, thus rendering it difficult to distinguish an inhibitory response to this dose of PGE_2. After the first postinjection hour, there were no significant effects of PGE_2 on sleep. Although PGE_2-induced behavioral effects were not sys-

tematically quantified, at the doses tested PGE_2 did not induce any gross behavioral abnormalities.

PGD_2 failed to induce significant changes in T_{br} after ICV injection of the six lowest doses tested. However, after the highest dose of PGD_2, 500 nmol, there was an increase in T_{br} of about 0.5° which began about forty min after injection then persisted for about two hours (data not shown). PGD_2 also failed to affect either NREMS or REMS across the six hour recording period (Table 4). In addition, if the analyses were confined to the first postinjection hour as done for PGE_2, no dose of PGD_2 tested significantly altered either NREMS or REMS (*e.g.*, Fig. 4). As described above for PGE_2, PGD_2 did not appear to induce abnormal behavior at the doses tested although this was not systematically documented.

Experiment No. 4

a) Effects of ip injections of CCK

Ten µg/kg CCK IP significantly decreased W and 40 µg/kg CCK significantly increased NREMS in the first postinjection h (Figure 5), the time period within which CCK affects rat sleep (87). The sleep-wake activity returned to the baseline level by the second postinjection h, and there were not significant changes found in the sleep-wake activity across the 6-h period (ANOVA, $p>0.05$). After the injection of 2.5 µg/kg CCK there was a nonsignificant increase in sleep in the first postinjection h. Thereafter, NREMS was suppressed in the remainder of the experimental period (Figure 5) [2.5 µg/kg CCK, ip: ANOVA for NREMS: drug effect: $F(1,6)=4.2$ $p<0.05$, time effect: $F(5,6)=1.5$, interaction: $F(5,6)=1.5$; ANOVA for REMS: drug effect: $F(1,6)=0.3$, time effect:

$F(5,6)=0.1$, interaction $F(5,6)=0.3$; ANOVA for W: drug effect: $F(1,6)=4.6$ $p<0.05$, time effect: $F(5,6)=2.2$, interaction: $F(5,6)=1.5$]. None of the tested doses of CCK affected the maximal EEG delta wave amplitudes during NREMS in the first postinjection h (Table 5).

The somnogenic effects of CCK were accompanied by dose-dependent decreases in T_{br}. While 2.5 µg/kg CCK did not affect T_{br}, 10 µg/kg CCK slightly decreased T_{br} for about two h, and 40 µg/kg CCK elicited a significant hypothermia for about three h. (Figure 5) [ANOVA for 40 µg/kg CCK: drug effect: $F(1,18)=15.8$ $p<0.05$, time effect: $F(17,18)=0.2$, interaction: $F(17,18)=0.2$].

b) Effects of ICV injections of CCK

In contrast to the effects of IP injections, ICV injections of CCK did not cause any significant increase in NREMS. Rather, 0.05 µg CCK reduced REMS across 6 h [ANOVA, drug effect: $F(1,6)=4.2$ $p<0.05$, time effect: $F(5,6)=1.6$, interaction: $F(5,6)=0.2$] and 0.5 µg CCK slightly, but statistically significantly decreased NREMS during the first postinjection h (Figure 6). The highest dose of CCK (2 µg ICV) did not cause significant changes in any of the measured sleep parameters. The maximal EEG delta wave amplitudes were not affected by any doses of CCK (Table 5).

Injections of 0.05 µg and 2 µg, but not 0.5 µg CCK, elicited statistically significant decreases in T_{br} for about one h, although the amplitudes of these changes were minor (Figure 6) [ANOVA for 0.05 µg CCK: drug effect: $F(1,18)=7.1$ $p<0.05$, time effect: $F(17,18)=0.2$, interaction: $F(17,18)=0.2$; and for 2 µg CCK: drug effect: $F(1,18)=4.7$ $p<0.05$, time effect: $F(17,18)=1.6$, interaction: $F(17,18)=0.7$)].

Experiment No. 5

Cell culture and immunofluorescence staining

All cell lines grew well after subculture; the three glioblastoma cell lines stained positive for GFAP, and neuroblastoma cells stained positive for neurofilament. Neuroblastoma cells reach confluence as determined by cell number at 7 days (data not shown). Growth curves of each glioblastoma cell line were also developed (Fig. 7); all cell cultures reached confluence in 5-7 days in the 96-well plates. There also was a correlation between the number of cells per cm^2 and mg protein in wells (Fig. 7)

IFNγ-induction of HLA_{DR} antigen

Of the three glioblastoma cell lines, HTB14 moderately expressed HLA_{DR} antigen prior to rhIFNγ treatment (Fig. 8), thus confirming a previous result (73) that some glioblastoma cell lines spontaneously express HLA_{DR}. Glioblastoma cell lines HTB16 and HTB17, and neuroblastoma cell line HTB11 were all HLA_{DR} negative before treated with rhIFNγ (Fig. 8). The effect of rhIFN was dose dependent from the dose 10 to 5 x 10^3 U/ml for all three glioblastoma cell lines, and reached a plateau at a dose of 100 U/ml for HTB14, and 1000 U/ml for HTB16 and HTB17 (Fig. 8). IFNγ failed to induce HLA_{DR} in the neuroblastoma cell line. The effect of IFNγ on HLA_{DR} over time was determined in HTB16 cells. After the treatment with 1000 U/ml of rhIFNγ for 48 hrs, HLA_{DR} antigen was detectable on HTB16 cells. This inducing effect reached a peak on the fifth day (Fig. 9). On day 5, IFNγ was removed from the culture medium and HLA_{DR} expression was still detectable two days later.

Effects of TNFα, TGFβ1 and TGFβ2 on IFNγ-induced HLA_{DR} expression

Consistent with other reports (9, 120), rhTNFα alone was unable to induce HLA_{DR} expression on HTB16 cells. However, when simultaneously added with rhIFNγ (100 U/ml or 500 U/ml), hrTNFα amplified the induction of HLA_{DR} by rhIFNγ in a dose-dependent manner. Enhanced HLA_{DR} expression was detectable at the lowest concentration of rhTNFα (0.1 ng/ml) used in the experiments; optimal effects were obtained by incubating cells with rhTNFα at 50 ng/ml and rhIFNγ at 100 U/ml for 4 days (Fig. 10A). The specificity of this synergistic effect of TNFα was confirmed using neutralizing antibodies against TNFα. The antibody at the concentration of 10 μg/ml almost completely blocked the effect of TNFα (Fig. 10B).

The potentiation of IFNγ-induced HLA_{DR} expression by TNFα in HTB16 cell line was evident only 24 hours after incubation began (Fig. 11A). Thus, rhTNFα not only amplified the effect of rhIFNγ, but also induced a more rapid HLA_{DR} expression. This synergistic effect of TNFα reached its peak on the fourth day and then persisted to the eighth day of the study (Fig. 11A).

TGFβ1 and β2 both reduced IFNγ-induced HLA_{DR} expression. The lowest concentrations of both types of TGFβ used (0.01 ng/ml) were sufficient to produce a marked inhibitory effect; TGFβ1 at 1 ng/ml and TGFβ2 at 10 ng/ml generated maximal effects (Figs. 12A & 13A). If a higher concentration of rhIFNγ (1000 U/ml) was used, the inhibitory effects of both TGFβ1 and β2 were not observed (data not shown). The inhibitory effects of TGFβ1 and β2 on IFNγ-induced HLA_{DR}-expression was reversed if neutralizing antibodies against TGFβ1 or β2 were added to the culture medium (Figs. 12B & 13B). It was important to combine rhIFNγ, TGFβ2,

and respective antibodies at appropriate concentrations to obtain ideal blocking effects of antibodies. For example, the effects of anti-TGFβ2 antibodies were dose-dependent at concentrations ranging from 1-25 μg/ml when the dose of rhIFNγ was 100 U/ml or 500 U/ml and the dose of TGFβ2 was 1 ng/ml (Fig. 13B). Using a higher dose of TGFβ2 (10 ng/ml), only the highest dose (25 μg/ml) of the anti-TGFβ2 antibody could block the effect of TGFβ2 (Fig. 13B).

The effects of TGFβ1 and β2 on IFNγ-induced HLA_{DR} expression over time were also determined. The inhibitory effects of TGFβ1 and β2 on IFNγ-induced HLA_{DR} expression were first observed on day 5 of incubation. The maximal effects were observed after 7 days of treatment (Fig. 11B).

IL-1β and PGE_2 impaired IFNγ-induced HLA_{DR} expression

In agreement with data obtained using brain endothelial cells (120), hrIL-1β induced a dose-dependent decrease of IFNγ-induced HLA_{DR} in HTB16 cells (Fig. 14A). The effect of IL-1β resembled, but was not as potent as, that of TGFβ. Since IL-1 and PGE_2 reciprocally affect each others production in some cell types (109), and PGE_2 inhibits Ia expression on macrophages (166), the effect of PGE_2 on HLA_{DR} expression was also examined. A dose-dependent suppression of IFNγ-induced HLA_{DR} expression by PGE_2 was observed after 4 days of incubation (Fig. 14B).

Experiment 3 - Failure of IFNα, 5-HT, PGD_2, VIP, DSIP, MDP and hydrocortisone to regulate HLA_{DR} expression

Several additional substances were tested alone and in combination with IFNγ to determine their effects on HLA_{DR} expression. These sub-

stances (IFNα, 5-HT, VIP, DSIP, MDP and hydrocortisone) failed to affect HLA_{DR} expression or IFNγ-induced HLA_{DR} expression in HTB16 cells (Table 6).

DISCUSSION

Experiment No. 1

During infectious disease NREMS is enhanced (174, 175). This central nervous system response to infection seems to be mediated via the ability of specific bacterial (99, 101, 105) or viral (119) products to enhance cytokine production (reviewed 105, 106). Previously, studies of the somnogenic actions of cytokines were focused on IL1; both IL1α and IL1β, as well as a fragment of IL1β, $IL1\beta_{208-240}$, enhance NREMS (106, 139) (see June 1, 1990 annual report). IL1s are part of a larger complex cytokine network which includes TNFα (also called cachectin) and TNFβ (also called lymphotoxin). There are many regulatory relationships between IL1s and TNFs, as well as other cytokines, but these relationships in brain are very poorly understood. For example, TNF induces IL1 and TNF production but whether this occurs in brain is not known. Nevertheless, TNFα mRNA is in brain, glia produce TNF (43), and there are neurons that contain TNFα-like immunoreactivity (13). Systemic production of TNF is enhanced during and after sleep deprivation whereas, other forms of stress are less effective in altering TNF production (192). Previously, the somnogenic activities of TNFα were described (164). Both TNFα and TNFβ interact with the same receptors and though they are distinct molecules, they often have similar biological activi-

ties (3). However, if TNF receptors are solubilized, TNFα but not TNFβ binds to them. Furthermore, in some instances TNFα and β have distinct biological activities. For example, TNFα and β have opposite effects on the symphatic nervous system (67). We thought it important, therefore, to investigate more thoroughly the somnogenic properties of TNF and to determine if TNF somnogenic activity could be localized to part of the TNF molecule. Both TNFα and β are somnogenic and that several TNFα fragments in the region of amino acid residues 10-69 are somnogenic. Other TNFα fragments, though possessing other TNF activities, were not somnogenic.

Experiment No. 2

The pseudomurein preparation used in this study increased the time animals spent in SWS, EEG δ-wave voltage, and caused a transient increase in T_{br} though REMS was not affected. These effects of pseudomurein on rabbit sleep and T_{br} are to our knowledge the first observations of CNS effects of this substance. Although only limited information is available on biological effects of pseudomurein, it was demonstrated earlier that in a rat arthritis model, pseudomurein-polysaccharide fragments purified from *Methanobacterium formicicum* were without effects after intraperitoneal injection; only intraarticular injection of high doses caused an acute inflammation (170). Also, pseudomurein was shown to be antigenic in animals and humans (22).

Previously, we have shown that cell wall preparations of *Staphylococcus aureus* or *Neisseria gonorrhoeae* injected IV in rabbits increased SWS, EEG δ-wave voltages, T_{co}, and T_{br}, and reduced REMS (79). About 4-5 times more pseudomurein was needed to obtain significant

effects as compared to staphylococcal cell wall preparations used earlier. In contrast to the staphylococcal and gonococcal cell wall preparations, pseudomurein did not decrease REMS and the onset of the increases in SWS and EEG δ-wave voltage were not delayed by one hour. After pseudomurein treatment, the increases in SWS and EEG δ-wave voltage were limited to the first 4 postinjection hours, this was similar to results obtained after injections the gonococcal peptidoglycan preparation. In contrast, after injection of staphylococcal cell walls, these effects persisted until the end of the 6 hour recording period. The increase in T_{br}, after pseudomurein injection, was rather transient while this effect was longer lasting after injection of eubacterial cell walls.

When staphylococci are phagocytized by murine bone marrow-derived macrophages, their cell walls can be partially digested (186), most probably by lysozyme. In the process of digestion, the macrophages produce and release into their medium low molecular weight cell wall fragments including muramyl peptides. Some of these fragments have somnogenic activity in rabbits (78). Also, the somnogenic actions of muramyl peptides are well known and several chemical structure - somnogenic activity relationships have been established (80, 99, 105). Importantly, if the muramic acid moiety of muramyl peptides contain an internal 1-6 anhydro structure, the substances are more potent than the corresponding hydrated compounds (102). When energy calculations are performed on the glycan strand of pseudomurein with talosaminuronic acid in 1C conformation, which represents the normal glucose chair conformation as it exists in muramic acid of murein, the resulting 3-dimensional structure of the sugar strand is dissimilar to that of the sugar strand

of murein (115) and, indeed, makes it very difficult to construct a net-like structure for pseudomurein. Instead, it was deducted from energy calculations that the 1C conformation of talosaminuronic acid, the reversed chair conformation, results in a 3-dimensional structure of the glycan strand which resembles that of murein (116). Interestingly, this 1C conformation is the one that must be assumed for the 1,6-anhydro-muramic acid.

It seems inadequate at this time to propose degradation pathways by which pseudomurein exerts its somnogenic and pyrogenic effects: Pseudomurein is resistant to lysozyme (84), the key enzyme for the degradation of eubacterial peptidoglycan in macrophages. Also, the accumulation of unusual ε- and γ-bonds in pseudomurein may protect it against attack by proteases (84). However, some enzymatic degradation processes for pseudomurein have been described recently: An extracellular enzyme produced by a streptomycete from cow manure has the capability to lyse a pseudomurein-containing methanogen (14). Also, a peptidase, hydrolyzing the ε-ala-lys bond of pseudomurein, was purified from *Methanobacterium wolfei* (91). Wether such processes are involved in the biological response of pseudomurein remains undetermined.

Despite the similarities in the somnogenic and pyrogenic responses of rabbits to pseudomurein or eubacterial peptidoglycan, there are also differences. Most notably the failure of pseudomurein to decrease REMS. This suggests that IL-1 is not the mediator of the pseudomurein-induced sleep and temperature responses, as has been proposed for muramyl peptides, since IL-1 decreases REMS when injected IV in rabbits (164).

Although endotoxin is not a constitutional component of archaebacteria, the pseudomurein sample used in the current experiments contained

some LAL assay reactivity. However, to inactivate this LAL reactivity a hundred-fold more Polymyxin B sulfate was needed than for the control standard endotoxin. This would point to contamination with an endotoxin that is very LAL reactive or very inaccessible to inactivation by polymyxin B or, more likely, that pseudomurein by itself has some LAL reactivity. It has been shown that the LAL assay is not specific for endotoxin and that eubacterial peptidoglycan contains LAL reactivity 3 to 5 orders of magnitude less potent than endotoxin (5, 187). Thus, the effects of pseudomurein in the LAL test might be considered a further aspect of biological activity of this substance, possibly due to the structural similarity to eubacterial peptidoglycan. Also, the sleep effects of endotoxin observed after IV administration are characterized by suppression of REMS and can be completely abolished by polymyxin B pre-treatment (104), unlike those observed here for pseudomurein.

The effects on sleep and temperature could be due to activation of macrophages or other mammalian cells which then release effector molecules like cytokines or digest pseudomurein into smaller components which might act similar to sleep inducing muramyl peptides, which can be tailored from eubacteria (78). Polystyrene microspheres of 1 mm diameter are phagocytized by macrophages *in vitro* (86). Also, microspheres of 3 mm diameter injected IV in beagle dogs are found consistently within phagocytizing cells; they eventually relocated in the liver and spleen where they remained indefinitely (85). The failure of a suspension of polystyrene microspheres of similar size and concentration to elicit any sleep and temperature effects in the assay systems used here excludes the possibility that any suspension of particles similarly

sized as bacteria is capable of eliciting the observed effects by activation of macrophages.

Experiment No. 3

The pyrogenic actions of PGE_2 reported here are consistent with those of previous studies. Thus, the time course of the fever responses and the doses of PGE_2 needed to elicit those responses are similar to those previously reported (*e.g.*, 44, 131). The effects of PGE_2 on sleep are also similar to those reported by Ueno, *et al.* (179). In rats the bolus injection of PGE_2 into the preoptic anterior hypothalamus induced a transient biphasic sleep response similar to the results reported here. These relatively short PGE_2-induced sleep responses stand in contrast to the prolonged inhibition of rat sleep if PGE_2 is continuously infused into the third ventricle (126). Such differences may be due to PGE_2 instability *in vivo*, thus with continuous infusion of PGE_2, the initial inhibitory effects observed with bolus injections of PGE_2 may persist. However, it is noted that other putative sleep factors also have short half lives, *e.g.*, IL-1 in blood has a half life of about 10 min, yet are capable of eliciting prolonged changes in sleep (98). The inhibitory actions of PGE_2 on sleep are consistent with the report that an antagonist of PGE_2 (AH6809) (126) enhances sleep. However, it is more difficult to interpret the reports suggesting that inhibitors of PG synthesis (e.g. indomethacin and diclofenac) inhibit sleep (71, 133). Finally, while PGE_2 seems to increase wakefulness in both rats and rabbits, PGE_2 decreases vigilance and motor activity in cats (72), indicating species differences in its effects.

The results reported herein using PGD_2 are also consistent with the literature to the extent that the high dose of PGD_2 induced a hyperthermia (51, 52). However, the almost complete lack of effects of PGD_2 on rabbit sleep seem to differ from the somnogenic actions of PGD_2 previously reported for cats (112), rats (178) and monkeys (140). In fact, in rabbits, the highest dose of PGD_2 used inhibited sleep for about one hour instead of promoting sleep. The reasons for these differences are unknown though there are some plausible explanations. Similarly, as for PGE_2, there are likely species differences in responses to PGD_2. Alternatively, the lack of a somnogenic action of PGD_2 may be due to the time of day it was injected. Inoué and colleagues (76) reported that in rats, PGD_2 and other putative sleep factors had little effect on sleep if given during daylight hours when rats normally sleep near maximum values. However, circadian variations in sleep duration are small in the rabbit, and a great variety of sleep promoting substances have been found effective when injected during the light cycle (163).

The current results obtained using PGD_2 fail, in part, to replicate a previous preliminary report by one of us (100). However, in that study, only one somnogenic dose was found and only four animals were tested. Further, at the time of that work, we were unaware of the sleep-promoting actions of endotoxin (101), which is a common contaminant of many reagents. The sample tested was a gift and was not shown to contain immune-reactive PGD_2 as was done for the current study nor was an assessment of endotoxin contamination made. Regardless of those preliminary studies, current results indicate that over a very wide dose range, PGD_2 lacks somnogenic activity in rabbits.

The clearest difference between the present study and those of Hayaishi and colleagues is methodological in that the latter group usually used a continuous infusion of PGs into the third cerebral ventricle, whereas in the current study, PGs were injected as a bolus into a lateral ventricle. This difference could lead to the differences in results obtained, particularly, as mentioned above, for the duration of the waking actions of PGE_2. This suggests that if PGs are involved in sleep-wake regulation then their production (and effects) are probably dependent upon the continuous stimulation of arachidonic acid metabolism by another substance (sleep factor). Indeed, several putative sleep factors have the capacity to enhance PG production (*e.g.*, IL-1 [35, 98], $IFN\alpha_2$ [103], and growth hormone releasing hormone [44, 138]). However, whether the sleep-inducing actions of these substances are dependent upon enhanced PG production is unknown, although the somnogenic actions of muramyl dipeptide persist in rabbits treated with the cyclooxygenase inhibitor acetaminophen (96). It is also noted that other substances that enhance PG production, *e.g.*, interleukin-6, and induce fever, do not affect sleep (142).

Experiment No. 4

The effects of CCK on sleep-wake activity of rats are well documented (30, 87, 121, 151). Although preliminary attempts to demonstrate somnogenic activity of CCK have been done in cats (42), our present work represents the first systematic description of the effects of exogenously administered CCK on normal sleep in a second species.

We report here that 10 µg/kg CCK decreases W, and 40 µg/kg CCK selectively increases NREMS and elicits hypothermia in rabbits. In con-

trast, ICV injections of CCK or IP injection of 2.5 µg/kg CCK did not induce NREMS in rabbits, in fact, after 0.05 µg ICV and 2.5 µg/kg IP CCK NREMS was decreased and after 0.05 µg CCK REMS was reduced, although these effects were very small. The results are similar to those obtained in rats where increases in NREMS (87) and decreases in temperature (87, 88, 193) were also found after IP administration of CCK, furthermore ICV bolus injection (155) or chronic ICV infusion of CCK (29) failed to promote sleep. The dose-range for the hypnogenic effects of CCK is similar in both species. A threshold dose of 10 µg/kg CCK in rats was reported (87), others also observed sleep increases after 10 µg/kg CCK (151), and in a third study 16, but not 8, µg/kg CCK promoted NREMS (30). IP injection of 4 µg/kg (87) or 2 µg/kg (155) CCK were ineffective on sleep of rats.

The enhanced sleep after CCK treatment was not accompanied with changes in EEG delta wave amplitudes. In contrast, recovery sleep following sleep deprivation (146), the sleep that accompanies acute infections (174, 175) and the sleep elicited by several sleep-promoting substances such as IL-1 (98), MDP (96) or growth hormone-releasing hormone (138) are characterized by increased EEG delta wave amplitudes during NREMS. This suggests that the underlying mechanisms of CCK-induced sleep and sleep induced by the above mentioned interventions may be partially independent.

The dose-dependent hypothermic effects of systemically injected CCK are similar to those reported in rats (87, 88). These thermoregulatory effects parallel the hypnogenic effects of CCK. ICV administration of 0.05 and 2 µg CCK also elicited a statistically significant decline in T_{br}. The magnitude of these changes, however, were so minor that the

physiological significance of these responses seems questionable. Similarly, Lipton and Glyn reported that ICV injection of 5 μg CCK does not alter the body temperature in rabbits (117).

The mechanisms mediating the effects of exogenous CCK on sleep-wake activity are not known. Since the peripheral-circulating and the central-neuronal CCK pools are separated by the blood-brain barrier, which is practically impermeable to CCK (147), we assume that systemic injection of CCK mainly mimics the effects of the endogenous, peripherally circulating hormone, whereas ICV administration of CCK mimics the effects of the neurotransmitter/neuromodulator CCK. Since no somnogenic effects were observed after central injection of CCK it seems that the site of hypnogenic action of CCK is peripheral. It is known that sensory inputs conveyed by the n. vagus contribute to the regulation of sleep-wake activity (153). It has also been reported that several effects of CCK are mediated by the n. vagus (23, 47, 165, 182). It is possible, therefore, that the somnogenic effects of CCK are also mediated by n. vagus. Another possibility is that CCK causes the release of other sleep-promoting substance(s) from the periphery. The role of insulin seemed likely, since insulin elicits sleep in rats (26, 158), and CCK is a potent stimulator of insulin release (*e.g.*, 149, 180). Experiments in rats indicate, however, that insulin plays little if any role in the hypnogenic effects of CCK (Kapás *et al*, submitted).

It is possible that the somnogenic doses of CCK are not physiological although it is difficult to know since the concentration of CCK at the hypnogenic site, when its secretion is physiologically stimulated (*e.g.*, after eating), is unknown. It is likely that when CCK is released within the small intestine it reaches high concentrations in circum-

scribed areas. To achieve such high concentrations experimentally, systemic injections of relatively large amounts of the peptide are needed. Regardless of the specific mechanism by which CCK affects sleep and thermoregulation, current results clearly show that the hypnogenic effects of CCK are not species specific. Furthermore, CCK enhances sleep in a species, in which it also suppresses food intake. It supports the idea that the sleep-promoting and food intake reducing effects are closely associated, presumably different manifestations of satiety. Finally, the experience of postprandial sleepiness, when the secretion of CCK is physiologically stimulated, further suggests that one of the primary actions of peripherally released CCK may be sleep promotion.

Experiment No. 5

Regulation of Class II MHC expression on astrocytes was investigated because of its association with CNS pathogenesis of autoimmune diseases, such as narcolepsy, and its importance in the initiation of immune responses during infection within the CNS. Transformed human CNS cell lines were used as a model *in vitro* system to examine the ability of some substances to alter HLA_{DR} expression (150, 195). Our results confirmed those of others in that IFNγ stimulated glial cells to express HLA_{DR} *in vitro*. The CNS was previously thought to be an immune privileged site because of the blood-brain barrier and its lack of lymphatic drainage. It is now recognized that, similar to peripheral systems, the immune response in the CNS requires the presence of several cytokines, antigen-presenting cells, and lymphocytes. Furthermore, activated T lymphocytes enter into the rat brain without major histocompatability complex (MHC) restriction and antigen specificity, supporting the con-

cept that the CNS is actively involved in immune reactions (66). These T lymphocytes could serve as a source of IFNγ. However, Traugott and Lebon (176) report HLA_{DR} antigen and IFNγ are both present on astrocytes at the edge of active multiple sclerosis lesions and in the adjacent area, suggesting astrocytes may also have the ability to synthesize IFNγ, which could be responsible for local induction of Ia antigen expression.

In our preliminary study, HLA_{DR} expression in glioblastoma cells (HTB14) was also examined using immunofluorescence staining techniques. Visibly, the intensity of the staining seemed stronger, and a greater number of cells were positively stained if cultures contained IFNγ. However, this method for determining HLA_{DR} expression was not sufficiently quantitative for our purposes. Therefore, we then established an ELISA to quantify HLA_{DR} expression on the cell surface. Furthermore, the amount of protein in each cell was quantified, thus allowing HLA_{DR} to be expressed as absorbance per mg protein. This is important because the hyperplasic and/or hypertrophic actions of cytokines *in vitro* are relatively uninvestigated. Thus, using the ELISA and protein assay, OD values are adjusted without the necessity of determining the reason for the increase or decrease of HLA_{DR} expression (due to stronger or weaker expression of HLA_{DR} per cell or changes in the number of cells expressing HLA_{DR}). Another advantage of using a protein assay is that determining protein amount is more rapid and accurate than counting cell numbers.

There are conflicting reports on the effects of TNFα on MHCII (also called Ia in mouse and rat) expression in systemic cells. TNFα was reported to induce Ia antigen and potentiate IFNγ-induced Ia antigen in

a murine macrophage cell line (17), and pancreatic cells (189), but it suppressed IFNγ-induced Ia expression on murine peritoneal macrophages (128). In human umbilical endothelial cell cultures the effects of TNFα on IFNγ-induced MHCII expression were dependent upon the order that cells were exposed to TNFα and IFNγ (113). TNFα enhanced IFNγ-induced MHCII expression when added twenty-four hours after IFNγ treatment, whereas TNFα inhibits IFNγ-induced MHCII expression when added simultaneously with, or before, IFNγ. Data presented here suggest that TNFα alone does not directly alter HLA_{DR} expression, but markedly elevates the rate and extent of IFNγ-induced HLA_{DR} expression in human glial cells. This synergistic effect of TNFα also occurs in rat astrocytes and brain endothelial cells (9, 120). TNFα is produced *in vitro* by astrocytes and microglia cells, (21, 159) and is secreted by fetal mice brain cells (191), thus suggesting that this cytokine is present in the brain. The mechanism for the TNFα-IFNγ synergistic effect on MHCII expression is not known; however, one possible mechanism may involve IFNγ up-regulation of TNFα receptor expression (9).

The effects of TGFβ1 and TGFβ2 are equivalent in most *in vitro* biological assay systems (20). Both TGFβ1 and β2 antagonize Ia expression in astrocytes (160, 195). In agreement with these results, our evidence indicates that TGFβ1 and β2 decrease IFNγ-induced HLA_{DR} in the human glioblastoma cell line HTB16. We also used neutralizing anti-TGFβ2 antibody to examine whether endogenous TGFβ2 was present in our culture system. The antibody inhibited the effect of porcine TGFβ2 but did not influence HLA_{DR} expression when cells were incubated with the antibody alone or with the antibody and IFNγ (data not shown here). We demonstrated that the antibody was effective with exogenous porcine TGFβ2 but

did not determine if it was able to cross react with human glioblastoma-derived TGFβ2 probably present in supernatants of HTB16 cell cultures. However, there is evidence that TGFβ2 is (immunologically) identical to glioblastoma-derived T cell suppressor factor. Therefore, TGFβ2 activity may not exist in our HTB16 glioblastoma cell line cultures. In contrast to the effects of TNFα, which enhanced IFNγ-induced MHCII within 24 hours, TGFβ required more than 5 days of incubation before its effect became manifest. These different time courses of effects may be important in the regulation of the immune response *in vivo*. Thus, the sequences of the actions of cytokines and/or cytokine-cytokine interactions on the expression of MHCII might be crucial in the initiation and development and termination of the immune response in brain.

Previous studies dealing with the relationships between IL-1 and Ia expression were focused on the association of positive Ia expression with the production of IL-1 (48, 56, 184), because the activation of T cells requires the following signals from APC: antigen, Ia molecules, IL-1, and recently noticed, intercellular adhesion molecules (ICAM) (54). Although the effects of IL-1 on Ia expression have not been addressed adequately, IL-1α and β inhibit IFNγ-induced Ia expression in rat synovial fibroblasts and brain endothelia (81, 120). In current studies, the inhibitory effects of IL-1β on HLA_{DR} expression in glioblastoma cells were not as potent as these of TGFβ or PGE_2, suggesting IL-1β may play a moderate, but not a key, role in regulating class II MHC expression in glial cells.

The importance of PGE_2 in mediating inflammation and immune responses has been extensively studied (108, 168). PGE_2 inhibits IFNγ-induced Ia expression in macrophages (166), and is involved in a feed-

back loop mediating the inflammatory response. PGE_2 causes a dose-dependent decrease of IL-1 production by lipopolysaccharide-stimulated macrophages, and addition of IL-1 to macrophage cultures results in an increase of PGE_2 level in supernatant (109). In the CNS, almost all cell types produce PGs, though the patterns of PG release of different cell types may vary (90) and PGs have many biological actions in the brain. Current findings suggest the possibility that locally present PGE_2 inhibits HLA_{DR}/Ia expression on glial cells and thus may play an important role in immune reaction within the CNS.

Unlike PGE_2, PGD_2 does not affect HLA_{DR} expression in glioblastoma cells indicating it is not involved in MHCII regulation. The other substances tested in this study also failed to modulate HLA_{DR} expression either alone or in combination with IFNγ. Although IFNα inhibits MHCII expression in systemic cells and in cells of the CNS, we could not replicate those results. The biological activity of our TNFα was verified in an anti-virus assay. Our data obtained with VIP, another recognized immunomodulator within the CNS (136), also are not in agreement with earlier results (53) which indicates that VIP could attenuate IFNγ-induced Ia expression on astrocytes derived from newborn rats. Besides the possible species specificity, there may be differences between cells of adults and newborns. There may also be differences in responsiveness of malignant cell lines from that of primary cells cultures to VIP. DSIP is another neuropeptide included in this study because it is found both within the brain and in immune cells (60). It also failed to affect HLA_{DR} expression in HTB16 cells.

Within the concentrations ranges used in this study (10^{-8} to 10^{-5} M), 5-HT failed to suppress IFNγ-induced HLA_{DR} expression in glioblas-

toma cells. In contrast, 5-HT was reported to alter Ia expression in murine macrophages (169). This may indicate the possibility that 5-HT is not involved in counteracting the stimulatory effect of IFNγ on MHCII expression in astrocytes, or the cell line we used lacks receptors for 5-HT.

It is well known that glucocorticoids have profound immunosuppressive effects and they are used clinically in many inflammatory and autoimmune diseases, although the precise mechanisms of their actions are still unclear. There is no consensus in the literature on the effect of glucocorticoids on MHCII expression (55, 185). It was, therefore, not surprising that hydrocortisone did not affect HLA_{DR} expression in the glial cell lines used in this study.

Massa and ter Meulen (124) suggested the bacterial cell wall component MDP as a possible candidate for MHCII induction in the CNS. However, we were unable to replicate in our human glioblastoma cells their result on MDP-induced Ia expression in rat astrocytes. It is noted here that MDP also failed to induce Ia expression in rat macrophages (124). The reasons for these failures of MDP to induce HLA_{DR} are unknown.

Regardless of whether bacterial products or certain neuropeptides alter MHCII expression, it is currently clear that many substances known to be brain products, *i.e.*, IFNα, TGFβ, IL-1β, TNFα and PGE_2, do modulate MHCII expression. It is thus likely that MHCII expression is regulated by locally present endogenous products within the CNS and that positive and negative feedback loops for this regulation exist. Our results further strengthen the concept that MHCII antigen plays a crucial role in initiating the immune response in the CNS. IFNγ and TNFα

may serve a major role in the development of immune response in the CNS, while TGFβ, PGE_2 and IL-1β may be involved in local immunosuppression.

GENERAL DISCUSSION

Criteria for Sleep Factors

Four fundamental experimental paradigms have been used to identify SFs. The first two are based on the hypothesis that some substances (SFs) are increased in concentrations during prolonged wakefulness or during infectious disease (reviewed, 12). As already mentioned, Legendre and Pieron (114) were the first to experimentally address this hypothesis using sleep deprivation. Several SFs have been identified using such paradigms; the list includes oxidized glutathione (GSSG), muramyl peptides and IL-1. A third approach was to isolate substances that are released during sleep. These substances might be responsible for the mediation of at least certain aspects of physiological sleep. This approach has resulted in the discovery of DSIP. A fourth method used to identify SFs was to assay substances for sleep-promoting activity known to be involved in the biological regulation of another substance known to be linked to sleep/wake cycles. Perhaps the best known SF identified in this manner is growth hormone releasing hormone (GRF); it was investigated because NREMS onset is associated with growth hormone (GH) release. To date, a variety of substances have been shown to alter sleep (Table 7); the list includes about 30 substances, many of which are well known for their other biological activities. Regardless of the experimental paradigm under which a SF was isolated or of its

other biological activities, it is generally agreed that in order for a substance to be classified as a SF it should induce physiological sleep. However, in practice, this criterion is not possible to test directly because animal models are used in which either excess sleep is induced or in which sleep is restored after some manipulation reduces it. Despite these experimental limitations, physiological-like sleep can often be recognized (Table 8) and several SFs appear to induce/enhance normal sleep. Several additional criteria for SFs have been proposed (reviewed, 11, 83) though often these are based on preconceived ideas of sleep rather than our knowledge of sleep regulation (106). Nevertheless, there are a few obvious criteria for SFs (Table 9). There are several putative SFs that meet these requirements; these include cytokines, the GRF/GH/somatostatin (SRIF), and the vasoactive intestinal peptide/peptide histidine isoleucine/prolactin (VIP/PHI/PRL) systems.

An Integrative Model for SFs

Historically, it has been a postulate within sleep research that the "true" SF would have biological actions specific to sleep and would act on the CNS executive sleep centers, which were also assumed to be primarily concerned with sleep regulation. However, there is much evidence strongly implicating at least three sets of compounds, cytokines, the GRF/GH/SRIF system and the VIP/PHI/PRL system, each of which have other biological actions, in physiological sleep regulation. Furthermore, a single center in brain necessary for sleep has not been demonstrated. It is possible, however, that multiple SFs, which are not specific for sleep, can elicit a specific sleep response if they operate in concert with each other (106). The SFs shown in Fig. 15 have been

shown in a variety of *in vivo* and *in vitro* systems to interact; they may enhance or inhibit the production or actions of one another. In this model, SFs are regulated through cascades of biochemical events though several of the pathways can also work in relative independence. Further, many feedback loops are involved; several of these are illustrated in Fig. 15 although additional aspects of regulation, *e.g.*, the role of endogenous receptor antagonists are not illustrated. At present, we do not know the relative contributions of the individual substances to physiological regulation. However, it is emphasized that we think that the evidence for the central involvement of some of these compounds in sleep regulation, *i.e.*, IL1, GRF, and VIP, is far greater than that for the other substances mentioned. Finally, the biochemical interactions shown were derived from experiments in which sleep was not monitored, thus the role that these interactions in sleep regulation needs to be evaluated.

The multiplicity of SFs, the numerous sleep-promoting pathways, which often act in parallel, and the many feedback loops provide a high degree of stability to sleep. Thus, changes in a single SF are not likely to alter sleep greatly, though the degree of change is likely to be dependent upon the relative importance to sleep of the specific SF. For example, blocking IL1 actions with the IL1 receptor antagonist (144, 145) or GRF actions with an anti-GRF antibody (Obál, *et. al.*, unpublished) each reduce NREMS by about 25% thus indicating their importance. In contrast, cyclooxygenase inhibitors, which inhibit prostaglandin production, have little net effect on sleep. All the experimental conditions that reliably produce increases in sleep, *i.e.*, prolonged sleep deprivation, infectious disease, excessive exercise, and

prolonged starvation, are pathologic. In these cases, multiple sites of actions and SFs are likely to be involved. For example, almost all of the substances mentioned in Fig. 15 are altered by infection. After less aggressive sleep-promoting experimental paradigms (*e.g.*, increase in ambient temperature) the occurrence of sleep is statistical rather than an obligate phenomenon.

The putative SFs mentioned in Table 7 have biological activities in addition to altering sleep; some of thes are not associated with normal sleep. For example, IL1 and certain PGs are pyrogenic, whereas entry into NREMS is usually associated with a decrease in T_{br}. The sheme in Fig. 16 illustrates how it is conceptually possible for substances that are not specific to sleep in their actions to elicit a specific sleep response. In this model, it is envisioned that a single SF will interact with more than one neuronal set. Each neuronal set will be involved in the regulation of two or more physiological parameters. Finally, some neuronal sets may not always be involved in sleep generation. By differentially driving the various neuronal sets shown in Fig. 16 by SFs, responses specific to sleep can be generated. (See ref. 106 for a numerical illustration of this concept). In contrast, most scenarios for driving the neuronal sets would involve the linkage of sleep to other physiological functions, which, in fact, is the usual case. In reference to the effects of SFs on sleep, the following points which are consisted with experimental findings can easily be envisioned from the model:

1) Some neuronal sets are more important than others for sleep propensity (have larger hypothetical output values (*e.g.*, $x>y>z$). As a

consequence, some SFs are more important than others (*e.g.*, B has a greater potential to enhance sleep than C).

2) If the output of two neuronal sets are held constant, stimulation of a third set could permit the sleep threshold to be reached; thus the exogenous administration of a SF could induce excess sleep.

3) In contrast, if neuronal sets x and y were at some minimal output level for sleep propensity, SF-D, which only interacts with neural set z, would not initiate sleep. Thus under certain experimental conditions, a SF may not initiate sleep while under others it would.

4) If one neuronal set was lesioned, total sleep propensity would be reduced. However, sleep would occur if the remaining sets were driven to a greater degree. This would also predict that after a lesion, the relative importance of various SF should shift. Further, an inherent feature of this model is that no individual neuronal set causes sleep, but all contribute to overall sleep propensity.

If the models in Figs. 15 and 16 are considered further within the context of dynamic properties of neurons (reviewed, 107), a more striking hypothesis is reached. It is: that sleep can, at different times, be caused by different neuronal sets (*i.e.*, the elusive executive sleep center may be so because it is mobile). Many physiological systems affect sleep and, in some cases, the sensory neurons involved in those systems are altered by SFs. For example, sleep is closely linked to temperature regulation. IL1 can induce changes in firing rates of hypothalamic neurons (162). More importantly, some neurons that initially are insensitive to temperature changes become sensitive to heat after exposure to IL1 (41). This suggests that the intrinsic properties of these neurons are dynamic and subject to SF influence. Thus IL1

could change the sensory response characteristics of populations of neurons (this is one reason why there is a range of hypothetical outputs of the neuronal sets in Fig. 16). Furthermore, a single neuron can be a part of more than one sensory network (69) thus indicating that extrinsic properties are also dynamic. If a SF *e.g.*, IL1, alters the responsiveness of neurons (left side of Fig. 16), such neuromodulatory actions could create dynamic neuronal networks that regulate sleep via constantly changing individual neural components (right side of Fig. 16).

Depending upon the specific physiological function driving sleep (*e.g.*, temperature, appetite, circadian rhythms, etc.) the specific neuronal sets involved would vary. Under homeostatic conditions, variations in sleep-relevant sites would be minimal, thereby, leading to apparent sleep centers. However, under pathological conditions, sleep would occur but the specific neurons responsible for it could be different (*e.g.*, sleep recovery after brain lesion). Furthermore, SFs that affect various aspects of physiology could alter sleep under some conditions (*i.e.*, when neurons sensitive to them participate in the sleep network) but not under other conditions. Dynamic changes of electrical intrinsic properties of neurons have previously been recognized as possible mechanisms leading to cyclic variations of vigilance states (118). These dynamic electrical properties, though very important, are but one of many end products of chemical influences on the neuron.

It is thus proposed that animals experience sleep as a result of the activities of different neurons under different conditions. A vast number of lesion experiments support this view to the extent that if the animal lives, a recognizable form of sleep eventually occurs regardless of which neurons were lesioned.

Regardless of the specific mechanisms by which the brain is organized to produce sleep, the relatively recent explosive growth in information about SFs has led to new concepts about sleep regulation, some of which directly challenge long-held beliefs about sleep. The work on SFs has also emphasized that sleep must be considered within the context of other physiological processes. Finally, it is currently clear that several endogenous SFs are capable of enhancing sleep, that they do so by their direct (or indirect) effects on neurons and that their interactions are biochemically linked to each other. The regulation of these substances, therefore, has become an important facet of sleep regulation. It is also important since it will likely lead to the development of new safe somnogenic agents that induce natural sleep.

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TABLE 1: Effects of TNFα, TNFβ, TNFα fragments and F-MLP peptide on rabbit sleep-wake activity and brain temperatures.

	dose (ug)	n	NREMS+		REMS		T_{br}
			Cont.	Exp.	Cont.	Exp.	
TNFα	0.005	5	41.3±1.6	41.0±1.3	5.6±1.2	5.1±0.7	0.12±0.14
	0.05	8	39.1±2.4	46.3±2.2*	4.3±0.8	4.0±0.5	0.55±0.10*
	0.5	8	44.3±1.6	57.0±3.3*	3.8±0.7	1.4±0.4*	0.82±0.17*
	5.0	4	44.3±1.1	68.7±4.2*	4.1±0.7	0.9±0.4*	2.00±0.26*
TNFβ	0.0005	7	43.2±1.6	47.5±1.4*	5.5±0.9	5.0±0.8	0.07±0.08
	0.005	6	41.5±1.6	44.9±2.8	6.3±0.7	5.1±0.5	-0.22±0.05*
	0.05	7	42.5±1.9	44.8±2.1	5.0±0.8	6.1±0.5	0.23±0.08*
	0.2	6	41.1±1.5	56.3±3.7*	6.3±0.7	3.5±0.6*	1.02±0.09*
$TNF\alpha_{10-36}$	5	5	40.1±1.7	42.4±1.7*	5.9±0.9	6.2±0.9	0.07±0.06
	25	8	46.5±2.2	55.5±1.7*	5.9±0.5	4.1±0.7*	0.88±0.26*
$TNF\alpha_{31-45}$	25	10	40.7±1.8	45.8±2.2*	6.2±0.6	4.6±0.6	0.27±0.17
$TNF\alpha_{46-65}$	25	10	44.7±1.8	47.8±2.4	5.0±0.6	4.1±0.5	0.46±0.11*
$TNF\alpha_{31-68}$	5	5	43.9±1.4	44.1±1.1	10.8±0.4	5.6±1.2*	0.29±0.10*
	25	7	45.8±1.5	54.8±2.5*	9.8±0.7	5.8±0.6*	0.96±0.14*
$TNF\alpha_{10-69}$	5	4	50.3±2.9	53.9±0.8	6.3±1.2	4.4±1.1*	n/a
	10	4	54.5±4.2	62.7±4.2*	3.6±0.8	3.0±1.0	n/a
	100	3	57.9±2.5	63.2±4.8	10.8±1.8	7.0±1.8	n/a
$TNF\alpha_{44-68}$	25	7	47.1±1.3	52.3±2.3	10.6±0.6	8.5±0.8*	0.52±0.12*
$TNF\alpha_{69-100}$	1	4	44.9±1.6	47.4±2.4	10.3±1.3	5.4±0.7	-0.19±0.18
	5	9	45.2±1.8	45.1±1.9	8.2±0.9	6.5±0.8	0.59±0.13*
	25	8	48.2±1.5	49.6±2.1	7.8±1.1	6.2±0.7	0.76±0.11*
$TNF\alpha_{101-135}$	25	7	45.6±1.7	42.9±3.5	8.2±1.1	7.1±0.8	0.06±0.10
$TNF\alpha_{140-157}$	10	4	56.1±2.4	56.7±4.6	3.5±0.3	4.2±1.1	n/a
	100	4	49.4±1.1	54.1±2.0	4.4±0.3	4.2±0.5	n/a
F-MLP	10 pmol	4	47.1±1.8	49.2±1.9	5.8±0.4	5.0±0.8	n/a
	100 pmol	8	45.7±2.4	47.3±1.0	6.4±0.5	7.9±0.9	n/a

[+]The values indicate percent time spent in NREMS and REMS during the entire 6 h experimental period ± SE. The effects on T_{br} are expressed as the maximum 1 h average temperature changes in response to peptides. Asterisks indicate significant drug effects ($p < 0.05$).

Table 2: Effects of intravenous injection of pseudomurein or microspheres on rabbit SWS, REMS, EEG δ voltage, and T_{co}

dose	n	SWS		REMS		EEG δ Voltage	
mg		Contr	Exper	Contr	Exper	Contr	Exper
1-2	4	44 ± 2	51 ± 3	3.0 ± 0.4	4.2 ± 0.8	83 ± 2	93 ± 6
4	4	47 ± 2	56 ± 2***	6.5 ± 0.8	5.8 ± 0.6	68 ± 9	81 ± 9***
Polymyxin B pre-treatment							
2-4.5	8	46 ± 1	51 ± 2*	3.7 ± 0.4	4.8 ± 0.4*	71 ± 9	76 ± 11*
injection of $5x10^9$ microspheres							
	4	47 ± 2	50 ± 1	6.5 ± 0.8	4.3 ± 0.1	68 ± 9	67 ± 9

Values are means ± SEM. Sleep values are 6-h post-injection averages; n, no. of rabbits; SWS, slow-wave sleep; REMS, rapid-eye-movement sleep; EEG δ voltage, electroencephalographic δ-wave voltage [μV]; T_{co}, colonic temperature 6-h post-injection; * $P < 0.05$, *** $P < 0.01$ Wilcoxon matched-pairs signed-ranks test.

TABLE 3: Effects of PGE_2 on Rabbit Sleep

		% NREMS+		% REMS+	
dose nmol	N	Cont	Expt	Cont	Expt
0.25	7	51.6 ± 1.9	54.0 ± 1.1	9.2 ± 0.7	7.5 ± 0.8
2.5	5	50.6 ± 3.1	50.8 ± 1.5	6.8 ± 1.0	5.6 ± 0.9
5.0	5	52.7 ± 3.8	48.6 ± 3.1	7.2 ± 0.8	6.5 ± 0.4
10.0	15	48.1 ± 1.4	48.5 ± 1.2	9.5 ± 0.7	7.3 ± 0.7

+ Average values of the 6-hr recording periods ± SE.

TABLE 4: Effects of PGD_2 on Rabbit Sleep

		% NREMS[a]		% REMS[a]	
dose nmol	N	Cont	Expt	Cont	Expt
0.25[bc]	4	44.7 ± 2.0	45.3 ± 2.9	6.2 ± 1.1	8.1 ± 0.5
0.5[c]	8	41.7 ± 2.4	43.8 ± 1.4	6.5 ± 0.5	8.0 ± 1.7
5.0[d]	7	43.5 ± 1.7	44.2 ± 1.8	7.9 ± 1.7	5.9 ± 1.7
10.0[d]	7	43.5 ± 1.7	42.2. ± 1.8	7.9 ± 1.7	6.1 ± 1.8
20.0[d]	6	43.3 ± 1.9	46.0 ± 3.7	5.1 ± 1.9	5.9 ± 2.0
50.0[c]	6	43.4 ± 2.8	48.2 ± 3.4	7.1 ± 0.7	6.1 ± 2.0
500.0[c]	7	43.4 ± 2.5	45.1 ± 1.3	7.8 ± 0.9	10.7 ± 1.8

[a] Average values of the six hour recording periods ± SE.

[b] This dose was infused over a 45 min period prior to time 0; other doses were injected ICV over a 1 min period.

[cd] PGD_2 used from Sigma[d] and two different lots [c] from Cayman.

TABLE 5. The effects of saline and CCK injections on the maximal EEG delta wave amplitudes during NREMS in the first postinjection hour

dose	route	n	saline[+]	CCK
0.05 µg	icv	7	306.9 ± 22.2	303.6 ± 27.2
0.5 µg	icv	9	306.3 ± 27.3	232.7 ± 18.3
2 µg	icv	5	259.5 ± 26.0	284.8 ± 33.1
2.5 µg/kg	ip	11	300.1 ± 13.3	287.2 ± 17.5
10 µg/kg	ip	4	316.1 ± 5.5	280.8 ± 18.6
40 µg/kg	ip	7	266.2 ± 22.4	259.9 ± 19.4

[+] The values represent average maximal delta wave amplitudes in µV ± SE

Table 6

Summary of effects of substances tested on HLA_{DR} expression

Agents		Dose range	Incubation	Cell lines used	Effect
rhIFNγ		0.1-5 10^3 U/ml	4 days	HTB14	↑
		0.1-5 10^3 U/ml	4 days	HTB16	↑
		0.1-10^4 U/ml	4 days	HTB17	↑
		0.1-10^2 U/ml	4 days	HTB11	—
rhTNFα		0.1-250 ng/ml	4 days	HTB16	—
+rhTNFα	+rhIFNγ++ (100 U/ml)	0.1-250 ng/ml	4 days	HTB16	↑
rhTNF	+rhIFNγ (500 U/ml)	0.1-250 ng/ml	4 days	HTB16	↑
TGFβ1		0.01-10 ng/ml	6 days	HTB16	—
TGFβ1	+rhIFNγ (100 U/ml)	0.01-10 ng/ml	6 days	HTB16	↓
TGFβ1	+rhIFNγ (500 U/ml)	0.01-10 ng/ml	6 days	HTB16	↓
TGFβ2		0.01-10 ng/ml	6 days	HTB16	—
TGFβ2	+rhIFNγ (100 U/ml)	0.01-10 ng/ml	6 days	HTB16	↓
TGFβ2	+rhIFNγ (500 U/ml)	0.01-10 ng/ml	6 days	HTB16	↓

Table 6 (cont.)

rhIL1β		0.5-250 pg/ml	4 days	HTB16	—
rhIL1β	+rhIFNγ (500 U/ml)	0.5-250 pg/ml	4 days	HTB16	↓
PGE_2		10^{-9}-10^{-5} M	4 days	HTB16	—
PGE_2	+rhIFNγ (500 U/ml)	10^{-9}-10^{-5} M	4 days	HTB16	↓
PGD_2		10^{-9}-10^{-5} M	4 days	HTB16	—
PGD_2	+rhIFNγ (500 U/ml)	10^{-9}-10^{-5} M	4 days	HTB16	—
IFNα		1-10^3 U/ml	4 days	HTB16	—
IFNα	+rhIFNγ (500 U/ml)	1-10^3 U/ml	4 days	HTB16	—
VIP		10^{-8}-10^{-5} M	4 days	HTB16	—
VIP	+rhIFNγ (500 U/ml)	10^{-8}-10^{-5} M	4 days	HTB16	—
DSIP		10^{-8}-10^{-5} M	4 days	HTB16	—
DSIP	+rhIFNγ (500 U/ml)	10^{-8}-10^{-5} M	4 days	HTB16	—
5-HT		10^{-8}-10^{-5} M	4 days	HTB16	—
5-HT	+rhIFNγ (500 U/ml)	10^{-8}-10^{-5} M	4 days	HTB16	—

Table 6 (cont.)

Hydrocortisone		10^{-5}-10^{-3} M	4 days	HTB16	—
Hydrocortisone +rhIFNγ	(500 U/ml)	10^{-5}-10^{-3} M	4 days	HTB16	—
MDP		0.01-10 ug/ml	7 days	HTB16	—
MDP	+rhIFNγ (500 U/ml)	0.01-10 ug/ml	7 days	HTB16	—

+ ↑ indicate increase, — no effect, and ↓ decrease in HLA_{DR} expression. ++When a substance was used in combination with IFNγ, direction of effects are relative to those observed with IFNγ alone.

TABLE 7. List of putative sleep factors (NREMS factors, REMS sleep factors, or both) and putative waking factors.

	Sleep Factors	References
I.	Identified	
	Delta sleep-inducing factor (DSIP)	Schoenenberger, 1984; Graf and Kastin, 1984
	Arginine vasotocin (AVT)	Pavel, 1979
	Vasoactive intestinal peptide (VIP)	Drucker-Colin, *et. al.*, 1984; Riou, *et. al.*, 1982
	Growth hormone-releasing factor/peptide (GRF)	Obál, 1986; Ehlers, *et. al.*, 1986
	Somatostatin (SRIF)	Danguir, 1986
	Growth hormone (GH)	Drucker-Colin, *et. al.*, 1975
	Cholecystokinin (CCK)	Mansbach and Lorenz, 1983; Kapás, *et. al.* 1988
	Insulin	Danguir and Nicolaidis, 1984
	Desacetyl-alpha-melanocyte-stimulating hormone (desacetyl-alpha-MSH)	Chastrette and Cespuglio, 1985
	Corticotropin-like intermediate lobe peptide (CLIP)	Chastrette and Cespuglio, 1985; Chastrette, *et. al.*, 1990
	Neuropeptide Y	Zini, *et. al.*, 1984
	Interleukin-1 (IL1)	Krueger, *et. al.*, 1984
	Interferon alpha-2 ($IFN_{\alpha 2}$)	Krueger, *et. al.*, 1987
	Tumor necrosis factor (TNF)	Shoham, *et. al.*, 1987
	Muramyl peptides (MPs)	Krueger, *et. al.*, 1982
	Lipid A	Cady, *et. al.*, 1989
	Prostaglandin D_2	Hayaishi, 1988
	Steroid hormones and metabolites	Heuser, *et. al.*, 1967
	Serotonin (5-HT)	Jouvet, 1984; Koella, 1984
	Melatonin	Datta and King, 1980
	Uridine (SPS-A-1)	Inoué, *et. al.*, 1984
	Adenosine	Radulovacki and Virus, 1985
	Oxidized Glutathione (GSSG)	Honda, *et. al.*, 1990
II.	Unidentified	
	Motor activity-reducing factor	Borbély and Tobler, 1980
	REM sleep factor	Sallanon, *et. al.*, 1985
	PS (paradoxical sleep)-inducing factor	Adrien and Dugovic, 1985
	Peptide-like urinary sleep factor	Ursin, 1984
	Waking Factors	
I.	Identified	
	Alpha-melanocyte-stimulating hormone (alpha-MSH)	Opp, *et. al.*, 1988
	Corticotropin-releasing factor/adrenocorticotropic hormone (CRF/ACTH)	DeWied, *et. al.*, 1976: Ehlers, *et. al.*, 1986, Opp, *et. al.*, 1989
	Thyrotropin-releasing hormone (TRH)	Takahashi, *et. al.*, 1985
	Endogenous opioids	Dzoljic *et. al.*, 1985
	Prostaglandin E_2	Hayaishi, 1988
	Interleukin-1 receptor antagonist (IL1ra)	Opp and Krueger, in press
II.	Unidentified	
	Factor E	Fencl, *et. al.*, 1971
	Motor activity-enhancing factor	Sachs, *et. al.*, 1976

*Updated reviews are cited where possible.

TABLE 8. Criteria for NREMS Factors with Regard to Physiological Sleep

1. A NREM-SF should induce increased incidence and/or duration of NREMS.
2. A NREM-SF should reduce the latency to NREMS during periods of maximum effects.
3. A SF should have different quantitative effects at different times of the circadian day.
4. A NREM-SF should enhance EEG slow wave amplitudes.
5. Sleep structure should be maintained, although altered duration of NREMS and/or REM sleep may occur; i.e., it should still cycle through stages of W, NREMS, and REM.
6. The enhanced NREMS induced by a SF should be readily reversible by appropriate stimuli.
7. Behavior following appropriate doses of SF should be normal.
8. Autonomic changes, e.g., brain temperature changes, that are tightly coupled to sleep states, should persist after SF treatment.

TABLE 9. Criteria for Sleep Factors

A. Testable criteria:

   1. The SF should induce and/or maintain physiological sleep (see Table 8).
   2. The SF should be present in the animal.
   3. The concentration and/or turnover of the SF and/or the concentration and/or turnover of its receptor and/or its receptor regulators should vary with the state of vigilance.
   4. The SF should be chemically defined.
   5. A dose-effect relationship for the SF should prevail within a certain dosage range.
   6. The range of biological activities should be similar across species.

B. Criteria that should apply as our knowledge of sleep is expanded:

   1. A SF should act directly on those brain mechanisms responsible for sleep.
   2. Inactivation of SFs (at the production or receptor level) should result in insomnia.

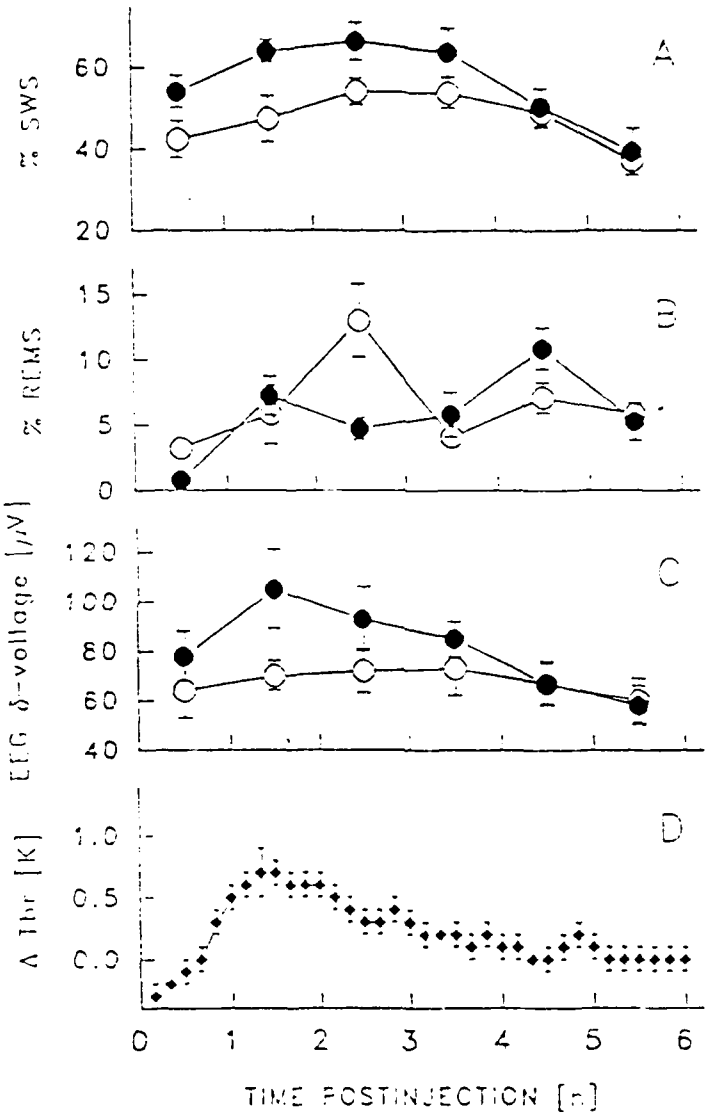

Fig. 1. Effects of intravenous injection of 4 mg pseudomurein in 1 ml PFS (filled circles) or vehicle (1 ml PFS into same rabbits on different days; open circles) on rabbit slow-wave sleep (SWS) (A), rapid-eye-movement sleep (REMS) (B), δ-wave amplitudes (C), and brain temperatures (D). A, B, C. hourly averages for indicated hour. D: differences from control recording (ΔT_{br}). n = 4. Data points are means ± SEM. SWS and EEG δ voltage were increased during post-injection hours 1 through 4. REMS was not affected. Tbr was transiently increased with a peak 1.5 hours post-injection.

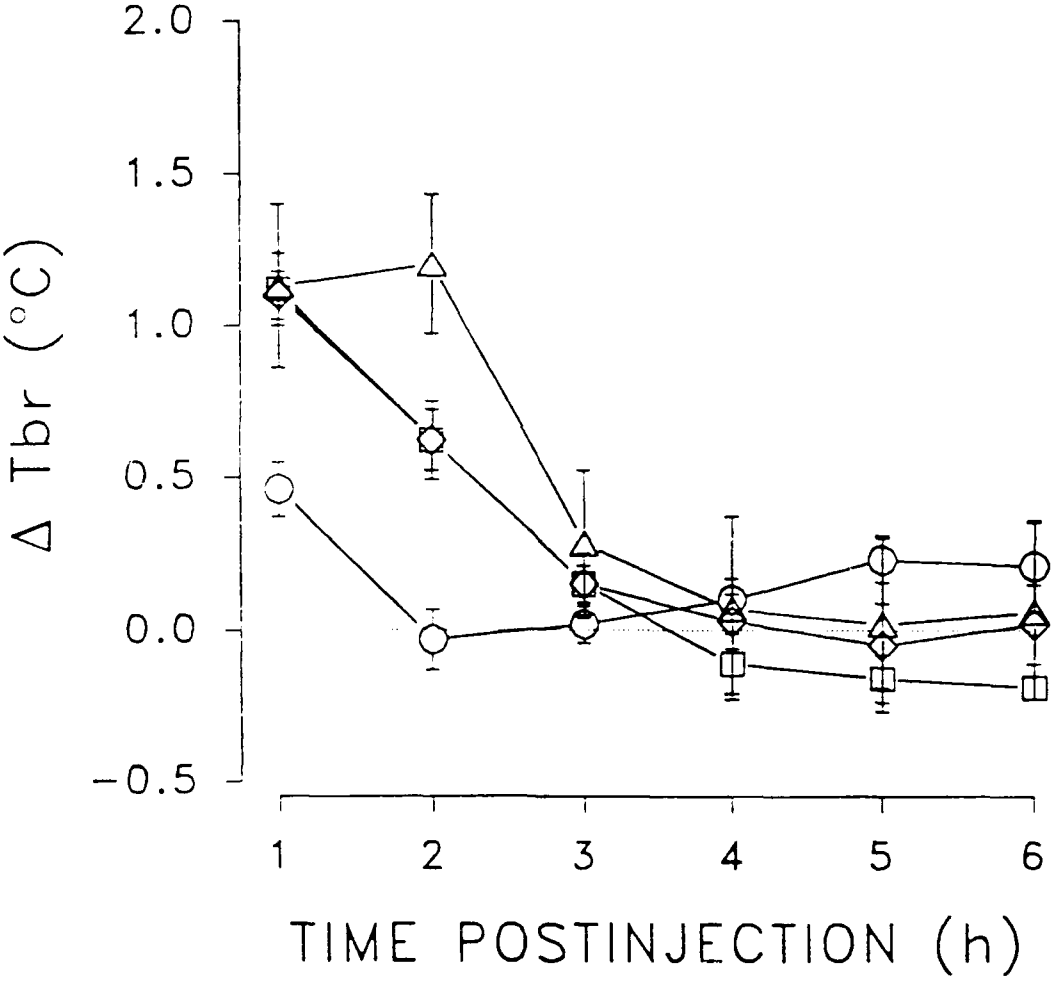

Fig. 2. Effects of intracerebroventricular injection of PGE_2 on brain temperature of rabbits. Values are the hourly means (±SEM) for the following PGE_2 doses: 0.25 nmol (circles; N = 5); 2.5 nmol (squares; N = 5); 5.0 nmol (triangles; N = 5), 10.0 nmol (diamonds; N = 7). PGE_2 induced dose-related increase in brain temperature that were confined to the first two hours postinjection.

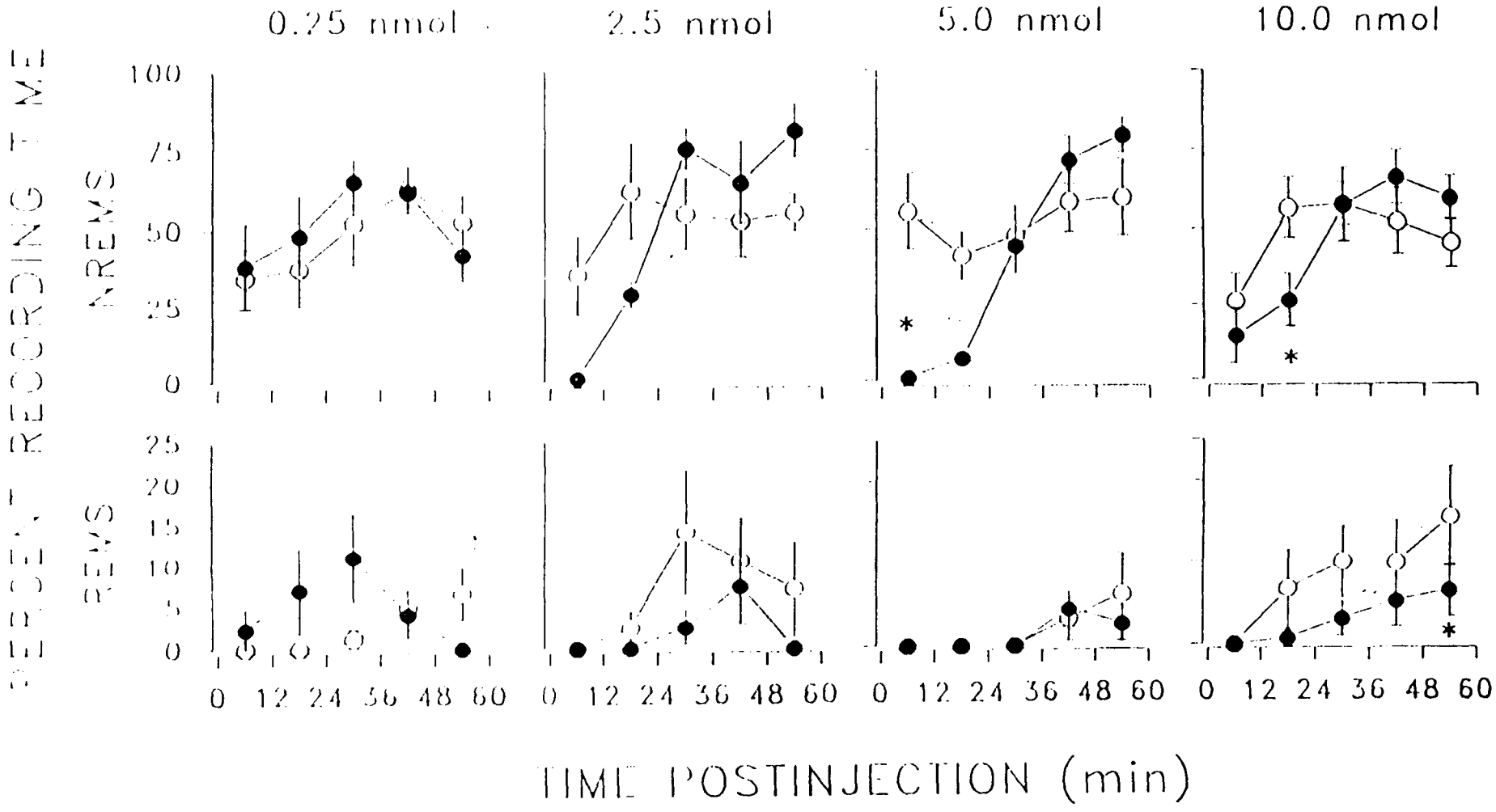

Fig. 3. Effects of intracerebroventricular injection of PGE_2 on NREMS and REMS duration in the first postinjection hour. Each data point represents the mean (±SEM) of 5-15 rabbits after control vehicle injections (open circles) or after PGE_2 (closed circles). * devote significant departure from control (Wilcoxin; $p < 0.05$).

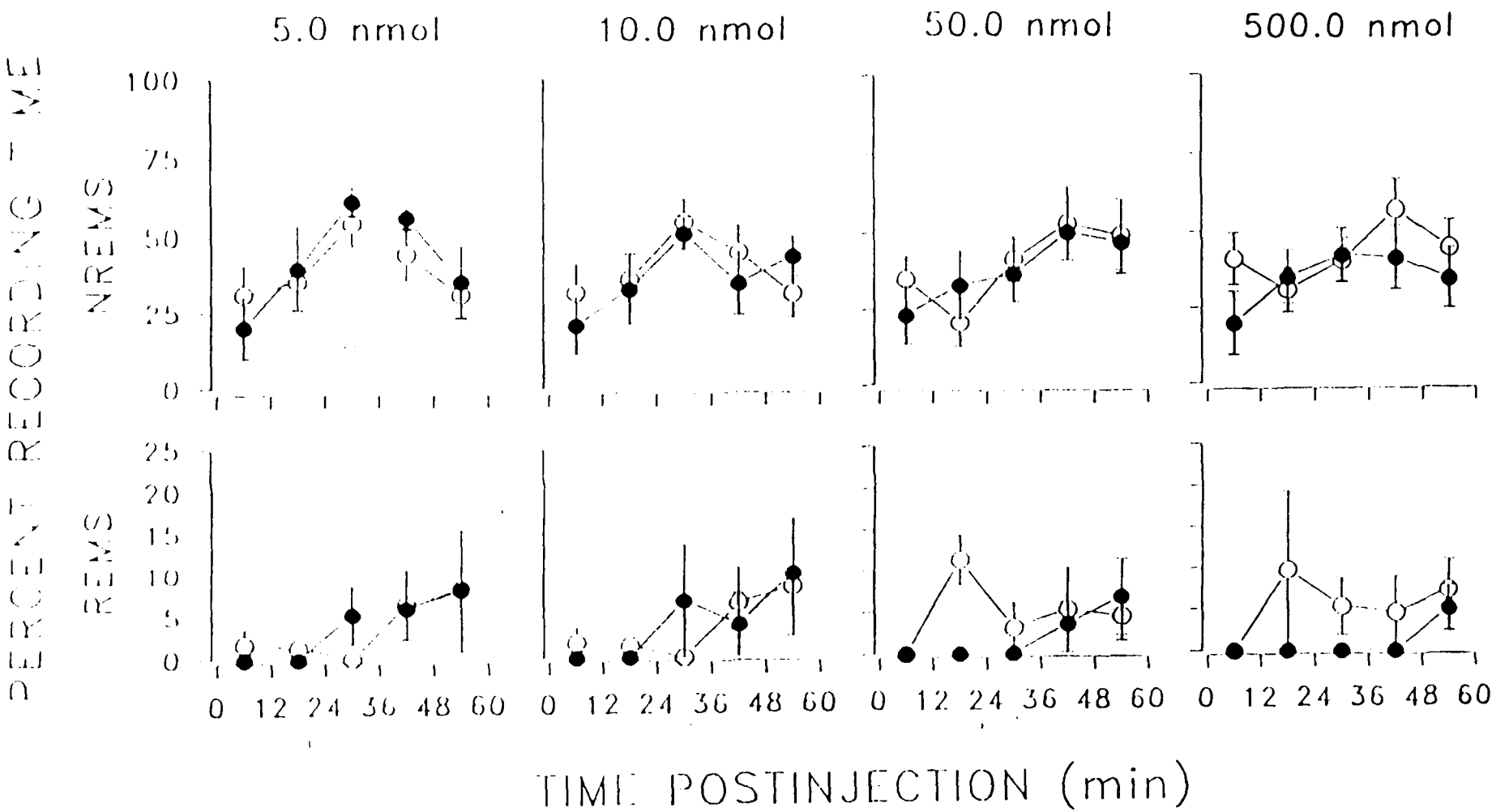

Fig. 4. Effects of intracerebroventricular injection of PGD_2 on duration of NREMS and REMS during the first postinjection hour. Each data point is the mean (±SEM) of 6-8 rabbits expressed as percent recording time after control vehicle (open circles) or after PGD_2 (closed circles). There were no significant changes in NREMS or REMS duration after injection of PGD_2.

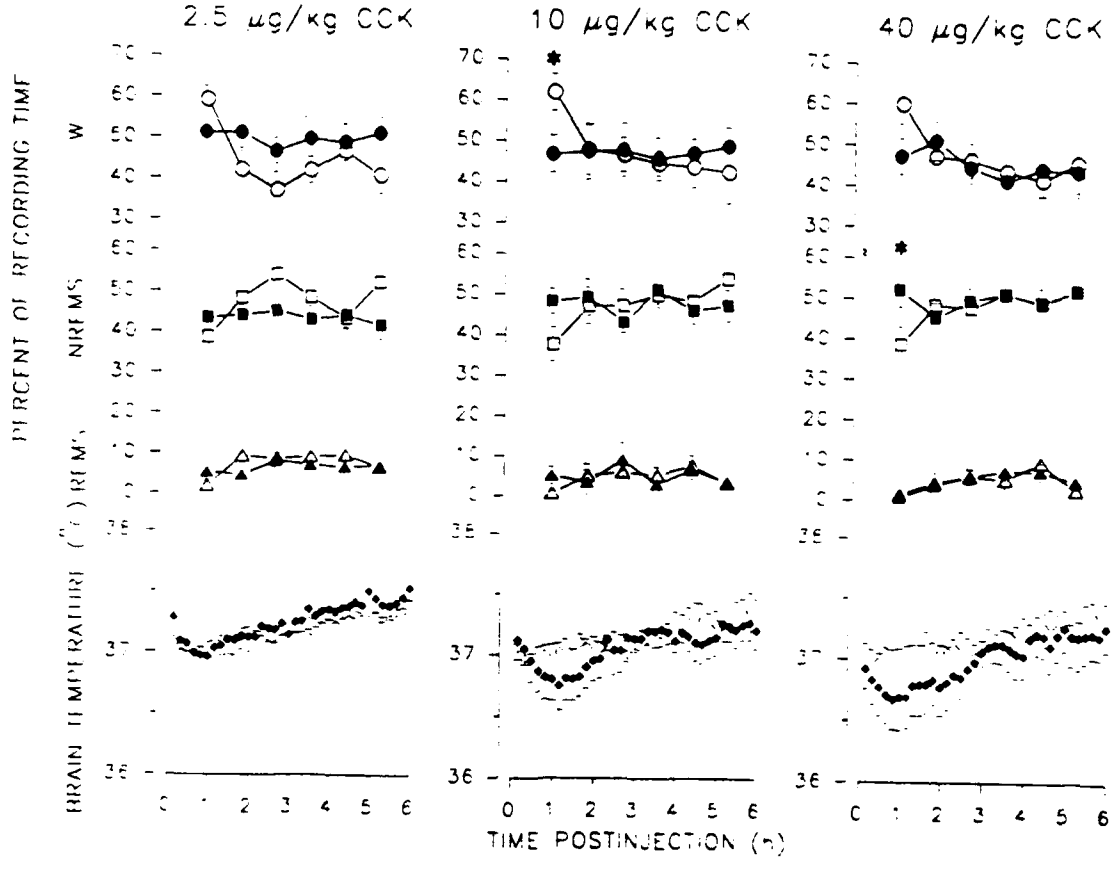

Figure 5. The effects of ip injection of various doses of cholecystokinin (CCK, filled symbols) and saline (open symbols) on sleep-wake activity and brain temperature. The injections were done at time "0". Error bars indicate SE, asterisks indicate significant differences between the effects of CCK and saline (paired Student's t-test, $p<0.05$). The highest dose of CCK induced an enhancement of NREMS for about 1 h.

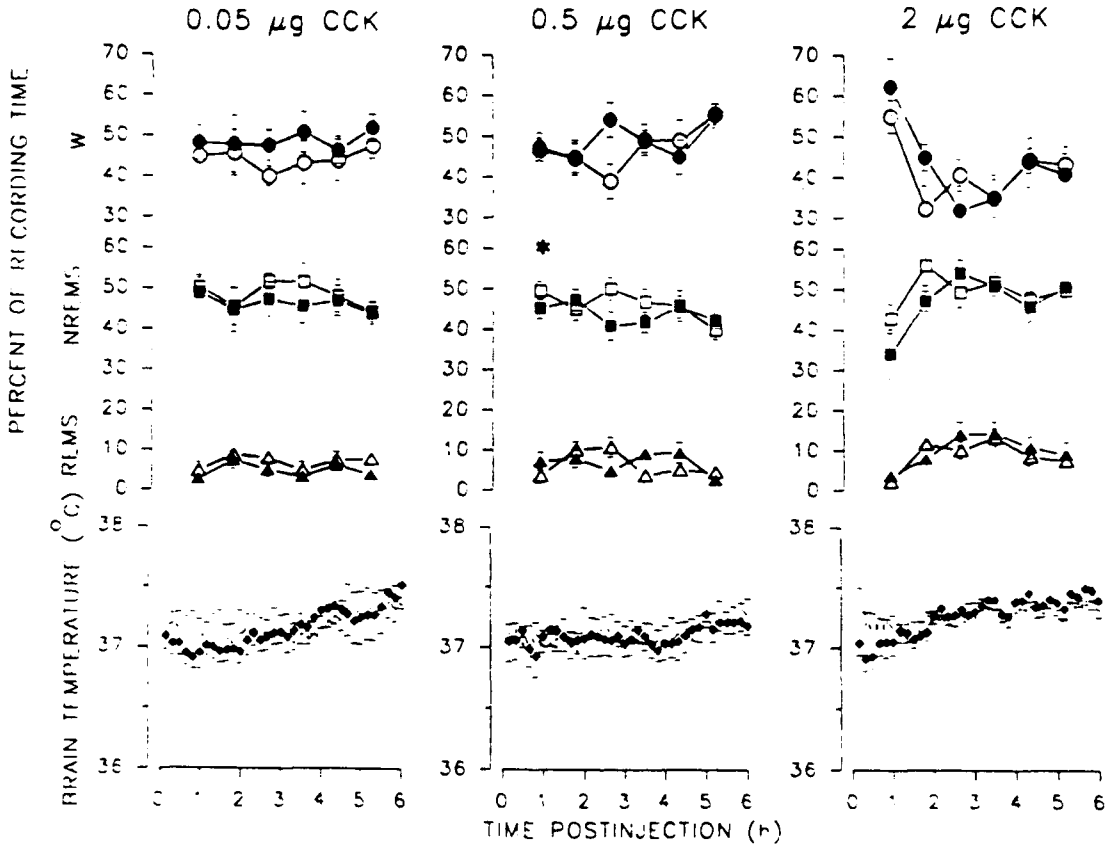

Figure 6. The effects of icv injection of various doses of CCK and saline on sleep-wake activity and T_{br}. See legend to Fig. 5 for details. Unlike high doses of CCK injected ip, icv CCK failed to enhance sleep.

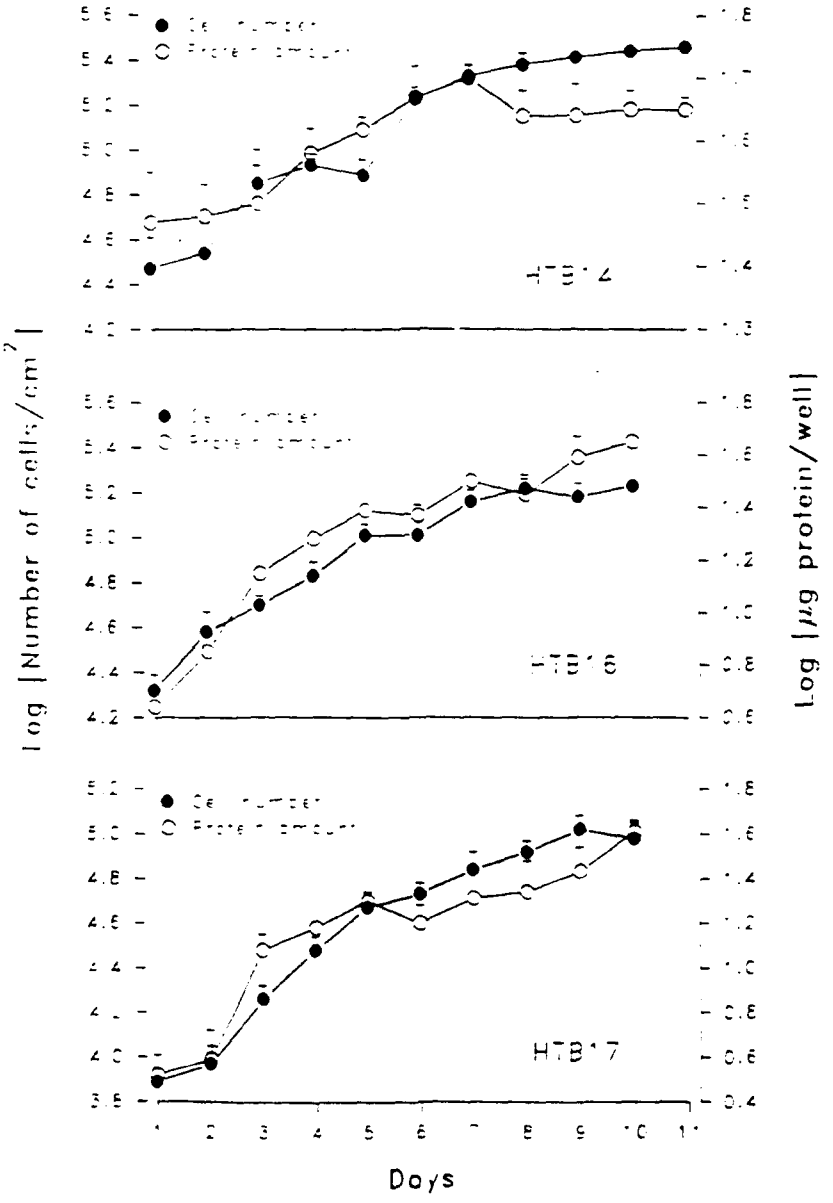

Figure 7. Growth curves of glioblastoma cell lines HTB14 (top panel), HTB16 (middle panel) and HTB17 (bottom panel), and the correlation between cell number and protein amount. Cells were seeded in 96-well plates; cell number and protein amount were determined every day for 11 days. Each point shows the mean and standard deviations from 6 replicate wells. (●) indicates cell number/cm^2; (○) indicates protein amount determined from wells parallel to the wells giving cell number.

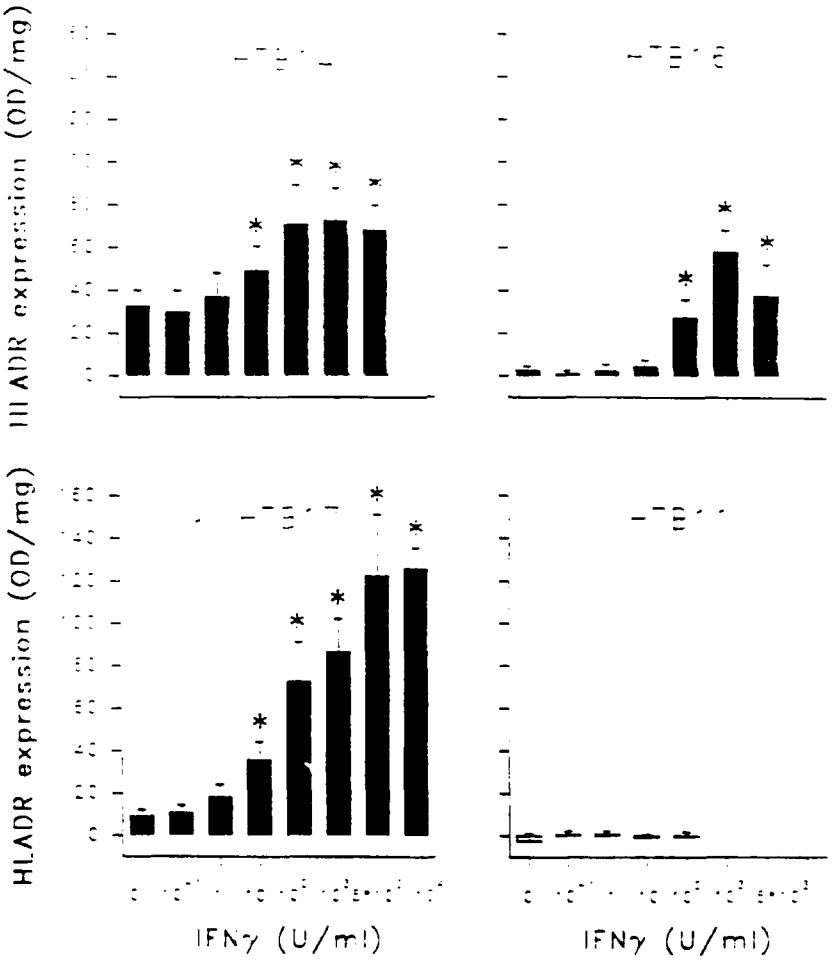

Figure 8. rhIFNγ-induced HLA_{DR} expression in glioblastoma and neuroblastoma cell lines after incubation with various concentrations of rhIFNγ for 4 days. HLA_{DR} expression is presented as OD/mg protein. Data are shown as mean + standard deviations from 6 replicate wells. An * indicates significant difference from the control value of HLA_{DR} expression obtained from the cells incubated with culture medium.

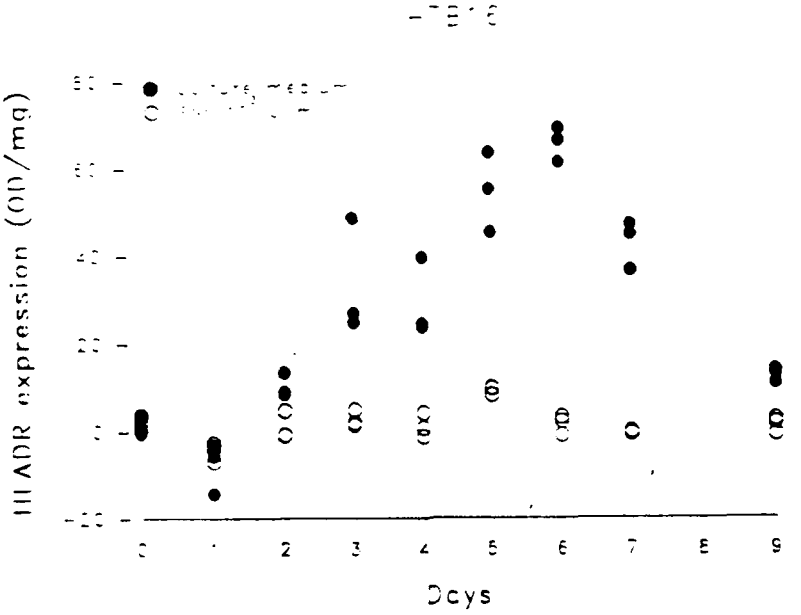

Figure 9. Time course response of rhIFNγ-induced HLA_{DR} expression in glioblastoma cell line HTB16. (●) indicates cells were incubated with culture medium. (○) indicates cells were incubated with 1000 U/ml of rhIFNγ. Media containing rhIFNγ were removed on day 5, and replaced with fresh culture medium, and HLA_{DR} expression was assessed every day. HLA_{DR} expression is presented as OD/mg protein. rhIFNγ-induced HLA_{DR} expression is noticed on day 3, reaches peak on day 5, and still detectable two days after the removal of rhIFNγ from culture media. Data are obtained from triplicate wells.

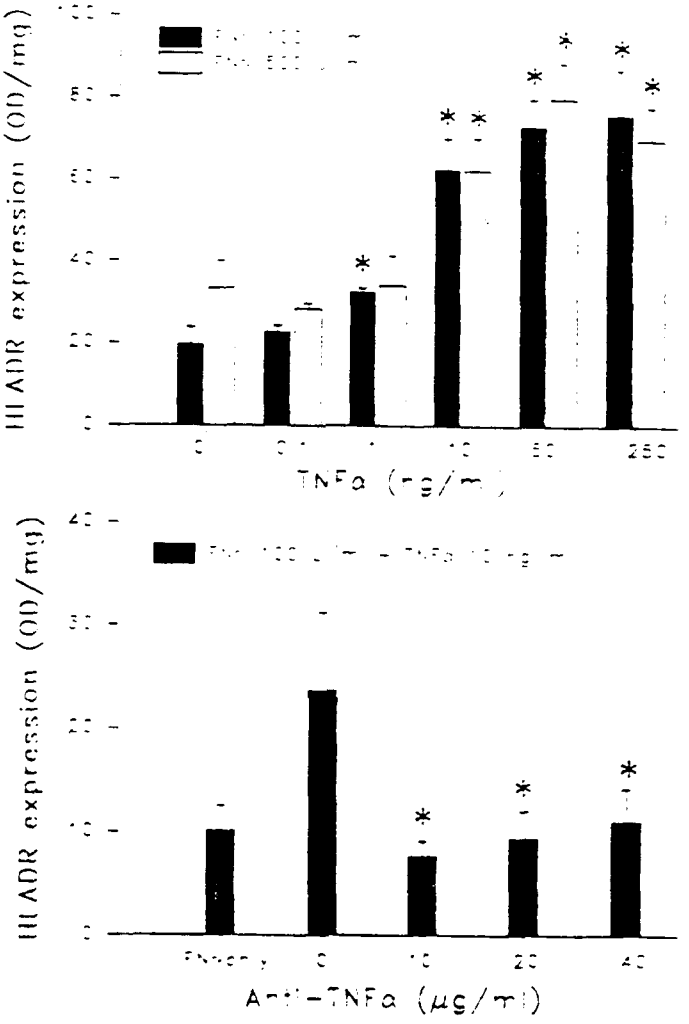

Figure 10. Effects of rhTNFα and anti-TNFα on rhIFNγ-induced HLA_{DR} expression in HTB16 cells. HLA_{DR} expression is presented as OD/mg protein; data are shown as means + SD from 6 replicate wells. Upper panels; cells were incubated with various concentrations of rhTNFα combined with 100 (solid bars) or 500 U/ml (open bars) of rhIFNγ for 4 days. An * indicates significant difference from the control values obtained in the presence of either 100 or 500 U/ml of rhIFNγ. Lower panel; anti-TNFα blocked synergistic effect of rhTNFα on rhIFNγ-induced HLA_{DR} expression. Cells were incubated with 100 U/ml of rhIFNγ only (first column), combined with 10 ng/ml of rhTNFα (second column), and 100 U/ml of rhIFNγ combined with 10 ng/ml of rhTNFα plus various concentrations of anti-TNFα for 4 days. Anti-TNFα at a dose of 10 mg/ml was sufficient to completely block the effects of rhTNFα. An * indicates significant difference from the value obtained from wells incubated with 100 U/ml of rhTFNγ + 10 ng/ml of rhTNFα.

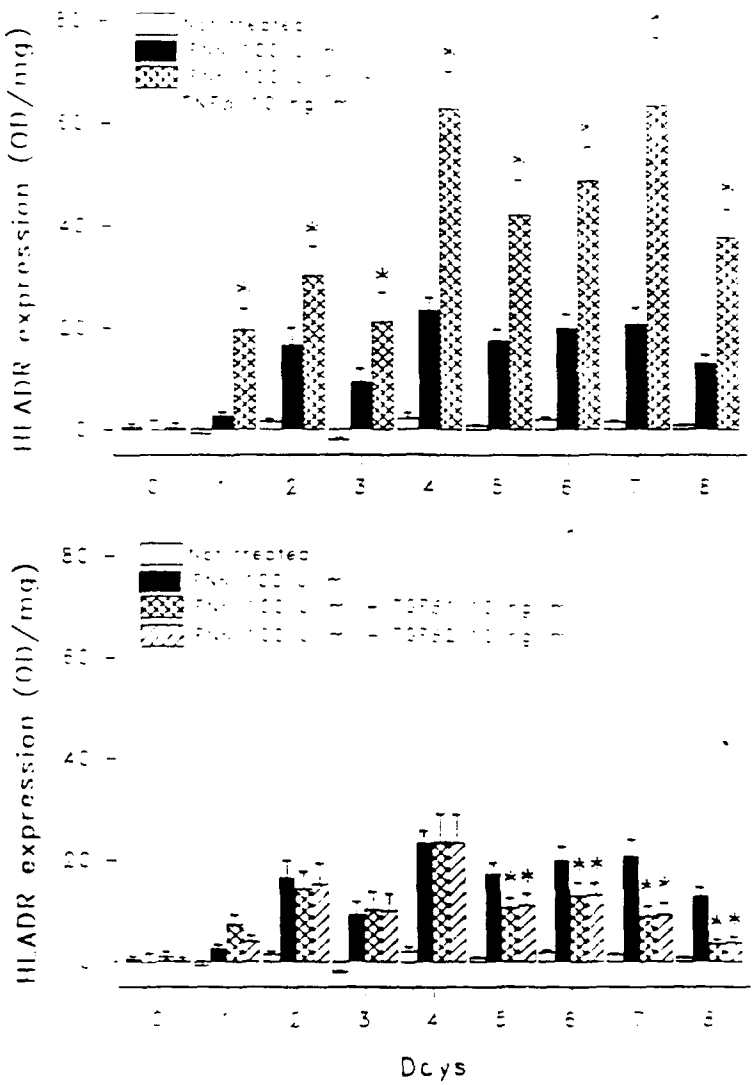

Figure 11. Time course response of the effects of rhTNFα and TGFβ1 and β2 on rhIFNγ-induced HLA_{DR} expression in HTB16 cells. HLA_{DR} expression is presented as OD/mg protein; data are shown as means + SD from 6 replicate wells. Upper panel; cells were incubated with culture medium (open bars), 100 U/ml of rhIFNγ (solid bars), and 100 U/ml of rhIFNγ combined with 10 ng/ml of rhTNFα (crosshatched bars) for 8 days. The effects of TNFα were noticed after 24 hour incubation, reached peak on day 4, and still detectable on day 8. An * indicates significant difference from the values obtained in the presence of 100 U/ml of rhIFNg on the same day. Time course response of inhibitory effect of porcine TGFβ1 and β2 on rhIFNγ-induced HLA_{DR} expression (lower panel). Lower panel; cells were incubated with culture medium (open bars), 100 U/ml of rhIFNγ (solid bars), and 100 U/ml of rhIFNγ combined with 10 ng/ml of either TGFβ1 (crosshatched bars) or TGFβ2 (hatched bars). The effects of TGFβ1 and β2 were detectable on day 5 of incubation, reached maximal level on day 7, and remained on day 8. An * indicates significant difference from the values obtained in the presence of 100 U/ml of rhIFNγ only on the same day.

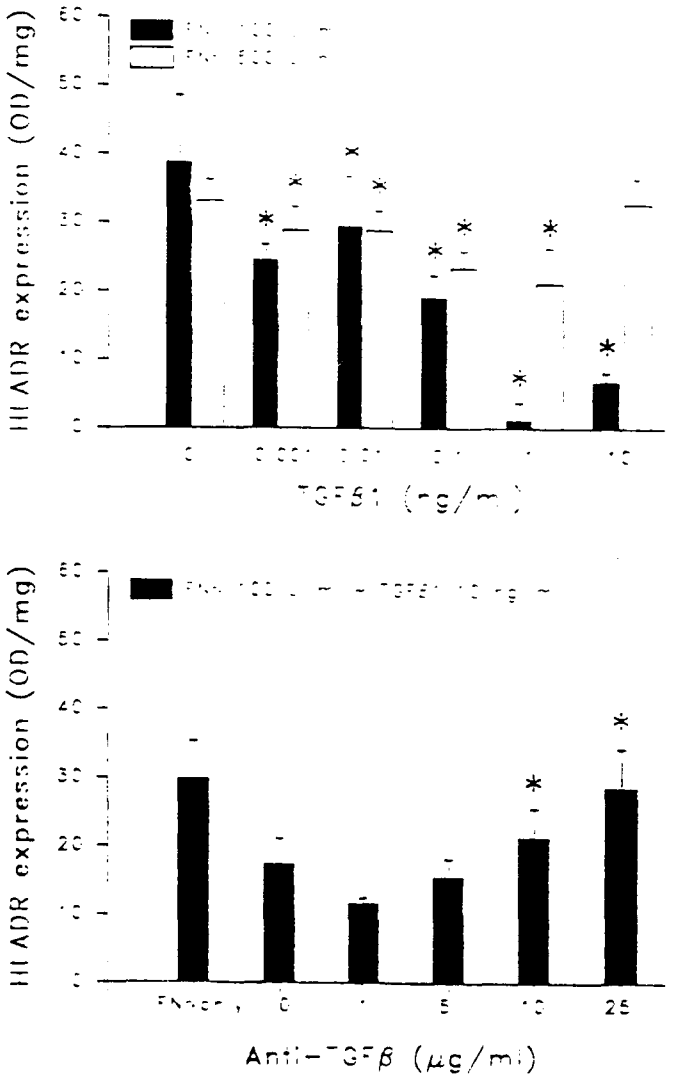

Figure 12. Effects of TGFβ1 and anti-TGFβ on rhIFNγ-induced HLA_{DR} expression in HTB16 cells. Upper panel, cells were incubated with various concentrations of TGFβ1 in the presence of 100 U/ml (solid bars) or 500 U/ml (open bars) of rhIFNγ for 6 days. An * indicates significant difference from the values obtained in the presence of either 100 or 500 U/ml of rhIFNγ. Lower panel, anti-TGFβ1 blocked the inhibitory effects of TGFβ1 on IFNγ-induced HLA_{DR} expression. Cells were incubated with 100 U/ml of rhIFNγ only (first column), 100 U/ml of rhIFNγ combined with 10 ng/ml of TGFβ1, and 100 U/ml of rhIFNγ combined with 10 ng/ml of TGFβ1 plus various concentrations of anti-TGFβ for 6 days. An * indicates significant difference from the value obtained in the presence of 100 U/ml of rhIFNγ and 10 ng/ml of TGFβ1.

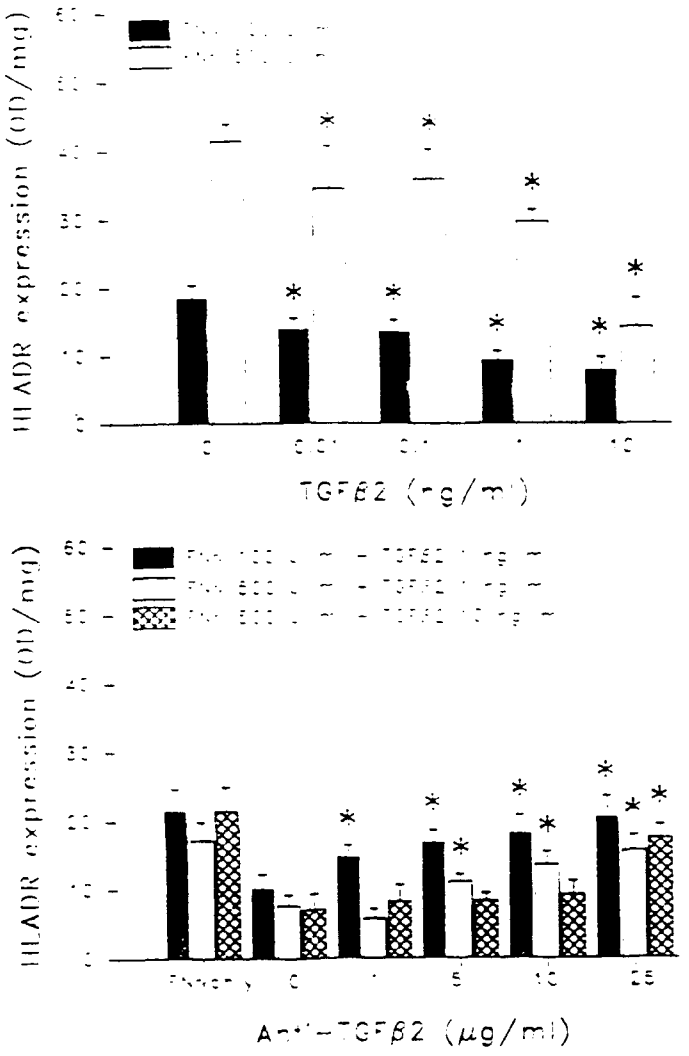

Figure 13. Effects of TGFβ2 and anti-TGFβ2 on rhIFNγ-induced HLA_{DR} expression in HTB16 cells. HLA_{DR} expression is presented as OD/mg protein; data are shown as means + SD. Upper panel, cells were incubated with various concentrations of TGFβ2 in the presence of 100 (solid bars) or 500 U/ml (open bars) of rhIFNγ for 6 days. An * indicates significant difference from the values obtained in the presence of either 100 or 500 U/ml of rhIFNγ. Lower panel, anti-TGFβ2 blocked the inhibitory effects of TGFβ2 on IFNγ-induced HLA_{DR} expression. Cells were incubated with rhIFNγ (100 U/ml, solid bar in first column; 500 U/ml, open and crosshatched bars in first column), rhIFNγ (100 U/ml) combined with 1 ng/ml of TGFβ2 and various concentrations of anti-TGFβ2 (solid bars), and rhIFNγ (500 U/ml) combined with 1 ng/ml of TGFβ2 plus various concentrations of anti-TGFβ2 (open bars) or 10 ng/ml of TGFβ2 plus various concentrations of anti-TGFβ2 (crosshatched bars) for 6 days. An * indicates significant difference from the values obtained in the presence of either 100 or 500 U/ml of rhIFNγ, combined with either 1 or 10 ng/ml of TGFβ2 (second column).

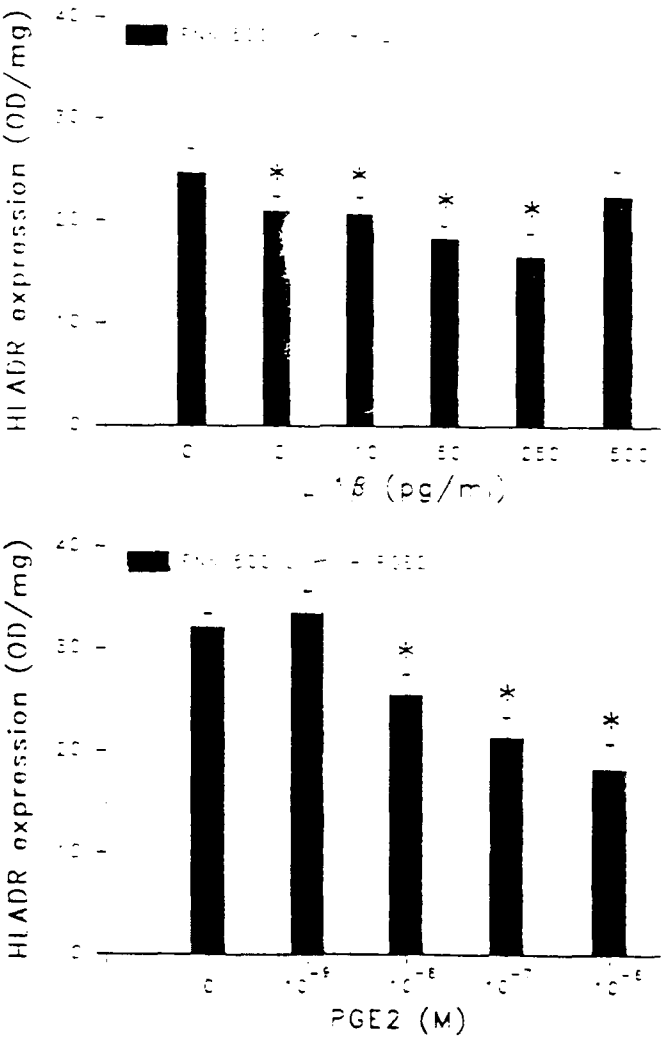

Figure 14. Effects of rhIL-1β and PGE_2 on rhIFNγ-induced HLA_{DR} expression in HTB16 cells. HLA_{DR} expression is presented as OD/mg protein; data are shown as means + SD. Upper panel, cells were incubated with various concentrations of rhIL-1β in the presence of 500 U/ml of rhIFNγ for 4 days. An * indicates significant difference from the basal value obtained in the presence of 500 U/ml of rhIFNγ. Lower panel, PGE_2 inhibited rhIFNγ-induced HLA_{DR} expression. Cells were incubated with various concentrations of PGE_2 in the presence of 500 U/ml of rhIFNγ for 4 days. An * indicates significant difference from the value obtained in the presence of 500 U/ml of rhIFNγ.

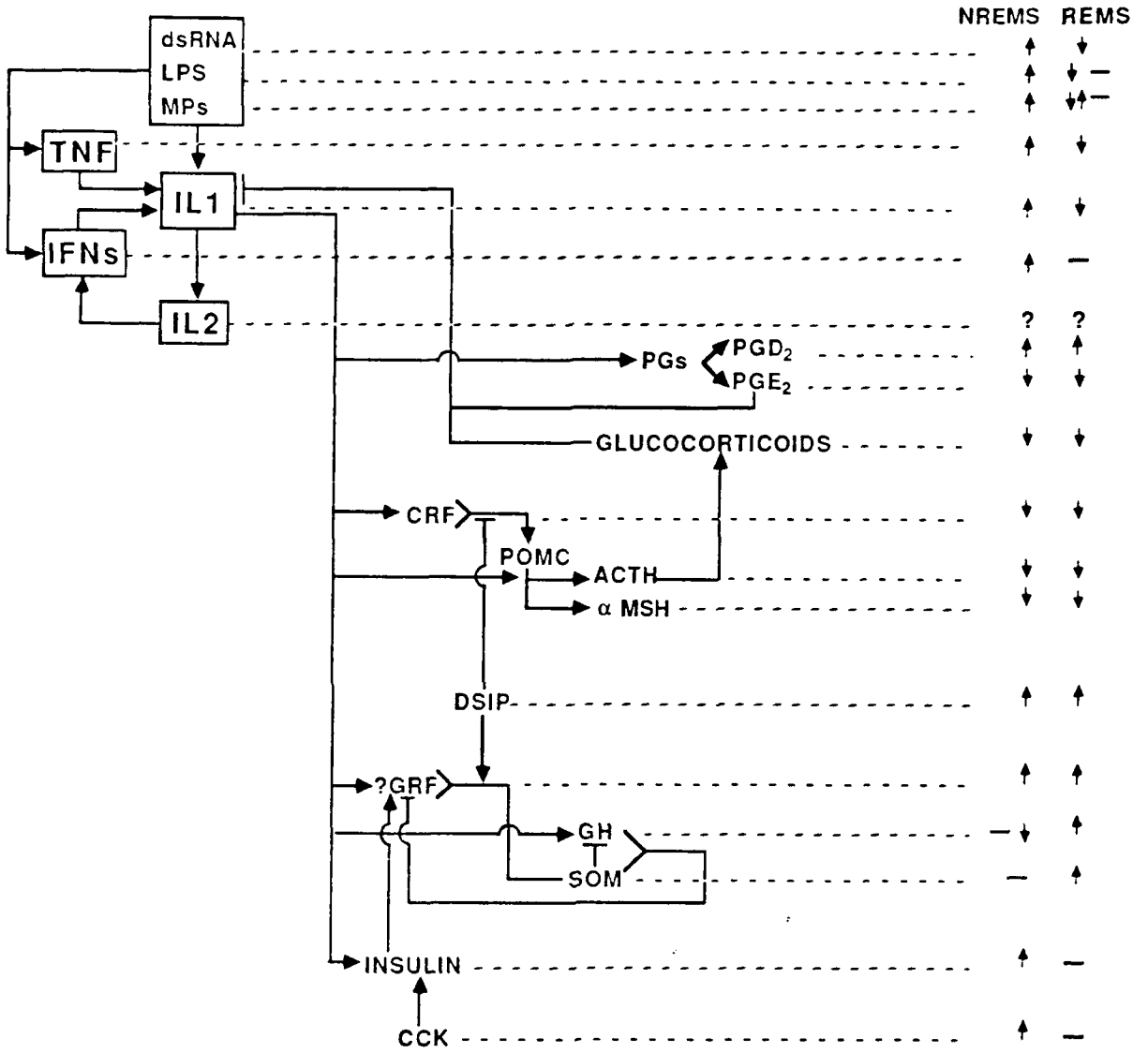

Figure 15: Activational system for sleep indicating possible interactions between putative SFs (left side) and the effects of those SFs on sleep (right side). Some of the interactions shown have been tested using sleep as the assay end point. For example, the CRF/ACTH, αMSH/glucocorticoid inhibiting actions on sleep may be related to their inhibitory effects on IL1 production or activity. Similarly, during infection when the supplies of muramyl peptides, endotoxin or viral dsRNA are enhanced, it is likely that sleep is enhanced via the ability of the microbial-derived substances to enhance cytokine production. See Table 7 for abbreviations used. Left lines; → indicates stimulation and ⊣ indicates inhibition. Arrows on right indicate sleep effects; ↑ increases, ↓ decrease and — no effect on sleep.

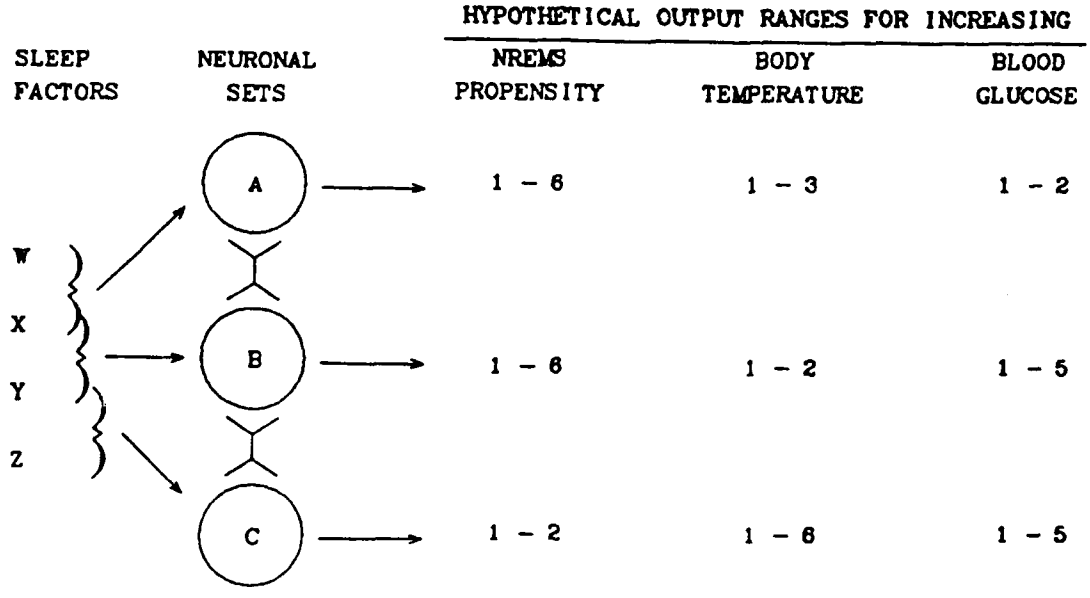

Figure 16: Sleep factors with multiple activities may selectively alter sleep by differentially interacting with various neuronal sets. The interactions may involve changes in intrinsic properties of the neurons involved. Each neuronal set has its own dynamic range of outputs affecting specific physiological systems. The involvement of any one set of neurons in sleep at any specific time is dependent upon which physiological system (SFs) are driving sleep.

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