Seven young scientists benefited from research training in our lab due to this AFOSR-sponsored AASERT. Each made significant contribution to our overall research aim to elucidate the cellular organization and regulation of the brain’s biological clock. This structure controls daily (circadian) rhythms of behavior (e.g., performance), physiology and metabolism in mammals. The clock, located in the suprachiasmatic nucleus (SCN), survived for up to 4 days in a hypothalamic brain slice where its properties were directly probed. By applying this technique to SCN from inbred rats, trainees made progress in demonstrating that: 1) glutamate and nitric oxide mediate light-induced phase-resetting of the clock at night, but not day; 2) nocturnal activation of this pathway leads to phosphorylation of the transactivation site on nuclear Ca^{2+}/CaM response element binding protein (CREB); 3) glutamic acid decarboxylase (GAD), the biosynthetic enzyme for GABA, oscillates; 4) NPY can modulate serotonergic phase-shifts in daytime; 5) SCN neuronal activity rhythms can be monitored continuously by a carbon fiber bundle electrode; 6) neuronal nitric oxide synthase (nNOS) is the dominant SCN isoform. This project involved both individual and interactive research projects at the University of Illinois and the USAF School for Aerospace Medicine.
AASERT Evaluation Report - Final

(FY92 AASERT) AUGMENTATION OF RESEARCH TRAINING IN CHRONOBIOLOGY: REGULATION OF THE MAMMALIAN CIRCADIAN CLOCK BY NEUROTRANSMITTERS
Grant No. F49620-93-1-0413

Martha U. Gillette, P.I.
Departments of Cell & Structural Biology and of Molecular & Integrative Physiology, The Neuroscience Program and the College of Medicine, University of Illinois at Urbana-Champaign

a. Parent Award:
"The Organization and Regulation of the Rat Suprachiasmatic Circadian Pacemaker", AFOSR-90-0205 (original).

b. Funding of parent award in 12 months prior to AASERT: $209,900; supporting 2.00 FTE graduate students.

c. Funding of parent award in 12 months during/after AASERT: $216,332; supporting 1.67 FTE graduate students.

d. Funding of AASERT award: $131,439; supporting 6.08 FTE graduate students and one hourly undergraduate student.

e. The undergraduate and graduate students supported by the AASERT award were citizens of the United States of America, and are natives of the states of Pennsylvania or Illinois:

Amy Melissa Fox  (B.S. awarded in Biology)
Steven J. DeMarco  (M.S. awarded in Biology)
William J. Hurst  (Ph.D. student in Cell & Structural Biology)
Marija Medanic Madden  (Ph.D. awarded in Physiology)
Steven M. Shinall  (M.S. awarded in Biology)
Thomas K. Tcheng  (Ph.D. student in Neuroscience)
E. Todd Weber  (Ph.D. awarded in Physiology)
AASERT Final Technical Report  
(FY92 AASERT) AUGMENTATION OF RESEARCH TRAINING IN  
CHRONOBIOLOGY:  
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Our research program aims to understand the mechanisms by which the mammalian circadian clock in the suprachiasmatic nucleus (SCN) adjusts its own sensitivity to resetting by afferent neurotransmitter pathways. Our model species is the rat. The specific progress made by each of the seven students supported by this AASERT award over the three year period of its tenure is summarized below. Each of these students has maintained satisfactory grades and progress toward their degree requirements during the funding period. Five have completed the degree upon which they were working during their appointment on the AASERT award, and all of these have continued either their postgraduate education or career development in science. This speaks well for the confidence that the AFOSR placed in our research program by its support of research training through the granting of this award.

AMY MELISSA FOX worked as an hourly undergraduate biology major during the second summer (1994) of this award. She submitted this research project in support of award of her B.S. degree in Biology, "with highest distinction", for which she was recognized at Commencement in May, 1995. She was awarded a position on the Cell & Molecular Biology Training Grant (NIH) and entered the graduate program in Molecular & Integrative Physiology at the University of Illinois that fall. She is currently prospering in that program where she is studying the molecular mechanisms underlying hypertrophy of skeletal muscle.

During her appointment as an AASERT trainee, Amy studied regulators of transcriptional activation during light stimulation of the SCN at night. Specifically, we have found that the Ca\(^{2+}/cAMP\) Response Element Binding protein (CREB) is phosphorylated on the transcriptional regulatory site, \(^{133}\)serine, forming P-CREB in rat SCN treated with agonists of the putative signaling pathway mediating photic stimulation. The effect is specific to the night phase of the clock. Induction of P-CREB was studied in situ, by immunocytochemistry. Amy's work utilized a state-specific antibody, that selectively recognizes the phosphorylated form of P-CREB. She found that it was colocalized with vasoactive intestinal peptide (VIP) in SCN neurons after a brief exposure of rats to a light flash during the night. Dual labeling of neurochemical constituents within cells is a rather sophisticated technique, and we were extremely pleased that Amy was able to master it. Her distinction research project was lauded at the reception for students receiving degrees conferred with honors at commencement.

STEVEN M. DEMARCO was supported by this award during the second year of research for his M.S. degree. After completing the requirements for that degree in May, 1994, he entered the Ph.D. program in neuroimmunology at the Mayo School for Graduate Research, Rochester, MN.
The project upon which Steve DeMarco worked under this award was centered around glutamic acid decarboxylase (GAD), the key biosynthetic enzyme for GABA. GABA is the major inhibitory neurotransmitter in the SCN. Steve examined the hypothesis that the concentration and specific activity of GAD in the SCN are under circadian clock regulation. Steve evaluated GAD in SCN whose circadian clocks are spontaneously generating a 24-h timekeeping signal \textit{in vitro}. He probed Western blots of proteins that had been separated by polyacrylamide gel electrophoresis with antibodies specific to the two major isoforms. Steve demonstrated that both GAD$_{65}$ and GAD$_{57}$ are expressed in the SCN. They are present throughout the diurnal cycle, at circadian times (CTs) 4, 10, 16 and 22 in nearly equal concentrations. These isoforms paralleled each other in spontaneous circadian changes in abundance: They were high daily at CTs 10 and 22, and low at CT 16 (Tukey 2-way ANOVA, $p \leq .005$). Specific activity was evaluated at eight points in the 24-cycle. Pyrodoxyl phosphate (PLP)-stimulated activity exhibited significant highs at CT 10 and 19 ($p \leq 0.04$), whereas this cofactor-stimulated activity expressed a significant low at CT 4. These results suggest that there is circadian modulation of GAD activity, which is partially under control of the cofactor. Interestingly, enzyme levels and activity both peaked in late day and late night. These two times may represent different inhibitory states for the SCN. One may act within neurons and circuits within this structure (CT 10) as the SCN moves into its nocturnal phase of low neuronal activity. Another may act at projections sites of the SCN, to inhibit efferent targets as the circadian system moves into the daytime inactive period for the rat, a nocturnal rodent (CT 19/22).

MARIJA MEDANIC MADDEN was supported by this AASERT award during the final stages of her doctoral research. She was awarded the Ph.D. in Physiology in December, 1994. She is presently teaching Physiology at Parkland Community College here in Champaign while her husband is finishing his degree in aeronautical engineering at the University. He will be joining a USAF research facility in Albuquerque, NM in the spring, 1997. Marija intends to seek employment there in biology teaching or research.

Marija’s research under this award focused upon the potential interaction of serotonin (5HT) and neuropeptide (NPY) in regulating the phasing of the SCN rhythm of neuronal activity. Information from other brain regions to the SCN carried by these two neurotransmitter systems is thought to convey information about dark-transitional photic changes and behavioral arousal states during the daytime portion of the circadian cycle. Afferents from the raphe (5HT) and intergeniculate leaflet (NPY) terminate in the ventrolateral SCN, often upon the same neurons. Both 5HT and NPY induce phase advance of SCN rhythms in daytime when applied alone, both \textit{in vitro} and \textit{in vivo}. Marija investigated the hypothesis that these two neurotransmitter interact during the daytime and together modulate the effect that each has alone upon phasing of the SCN biological clock. Potential interactions were evaluated at CT 7 and CT 23. The SCN rhythm can be reset at these two times by NPY alone.

SCN brain slices were treated with microdrops (10$^{-11}$ ml) containing 5HT and NPY. The concentration of 5HT was held constant at 10$^{-6}$ M, while varying the concentration of NPY. The effects of these treatment on the phase of the rhythm of neuronal electrical activity were assessed on the second day \textit{in vitro}. While equimolar concentrations of NPY and 5HT caused phase advances of 3.5 $\pm$0.2 h, the same magnitude phase shift induced by NPY alone, decreasing the NPY concentration resulted in the larger phase shifts that characterize the effect of 5HT alone at CT 7. At CT 23, the shift was characteristic of NPY alone, with no effect of 5HT. These results
demonstrate that putative neurotransmitters for nonphotic zeitgebers can interact directly at the level of the SCN to integrate signals from these brain regions. This work is being prepared for publication.

WILLIAM J. HURST was supported for the first two years of his graduate training in Cell & Structural Biology as an AASERT trainee. During that period he successfully passed his Qualifying Examination for the Ph.D. He was recently awarded a position on the Systems & Integrative Biology Training Grant (NIH).

Bill Hurst in an energetic student who very successfully collaborates with other scientists in the lab. He has contributed to two studies that have recently been very favorably reviewed for publication. The first of these studies was centered in the hypothesis that light-induced phase shifts are initiated by stimulus-transcriptional coupling via the Ca\(^{2+}\)/cAMP Response Element Binding protein (CREB). Specifically, CREB is phosphorylated on the transcriptional regulatory site, \(^{32}\)P-CREB in rat SCN treated with agonists of the putative signaling pathway mediating photic stimulation. The induction of this phosphorylation event by light is limited to the right phase of the clock. Bill subjected brain slices to sonication, then a nuclear purification step followed by gel separation and Western blotting of SCN proteins with anti-PCREB. He found that application of either glutamate and nitric oxide (NO) donors to the SCN brain slice specifically induced formation of an immunoreactive band at 43 kDa; this is strong evidence for P-CREB formation. This study was recently reviewed at *Journal of Neuroscience*, and will require changing only a couple of sentences for approval for publication.

Secondly, Bill is an author on a paper that has just been revised for *Journal of Neurochemistry*. That paper characterized nitric oxide synthase (NOS) in the SCN of rat. Bill used Western blot analysis of SCN NOS as part of this characterization. He found that neither endothelial nor inducible NOS were detectable at significant levels. However, the SCN contains substantial amounts of a 155 kDa protein recognized specifically by an anti-nNOS polyclonal antibody; binding is completely blocked by preincubation of the antibody with baculovirus-expressed nNOS. Material immunoreactive for nNOS is distributed equally between the membrane and cytosolic fractions.

STEVEN M. SHINALL was supported in-part during the last year of his M.S. research. Steve is presently in a Ph.D. program in immunology at the University of Iowa.

Steve's time was largely spent acquiring the methodologies he needed to conduct research. His efforts were directed toward identifying the kinase systems that are present in the SCN during the nighttime. Steve subjected the SCN tissue from brain slices to Western blot analysis. He found that MAP kinase is present in SCN tissue. He was not successful in determining whether it is tyrosine phosphorylated by light signals.

THOMAS K. TCHENG has been supported by the AASERT award over the whole period of the award. Tom is a senior student in the Neuroscience Program; we anticipate that he will complete his Ph.D. in the summer of 1997.

Tom has succeeded in developing a multiunit electrode that can simultaneously monitor the activity of small to large populations of SCN neurons in the brain slice. He has perfected the parameters of the electrode and the recording system so that he can start the recording on one day, and then observe the SCN circadian rhythms of
neuronal activity 'on line' for 3-5 days without repositioning the electrode. A report of this method using a carbon fiber bundle electrode is In press at the Journal of Neuroscience Methods. Development of this type of recording capability has long been sought in the SCN field, and Tom's success here will be greeted with great enthusiasm by the field. In addition to his work on electrode fabrication and design of a biocompatible brain slice chamber, Tom has contributed to developing the interface necessary to analyze the complex multiunit electrical signal.

E. TODD WEBER was appointed on this award for one semester in Fall, 1995, after he completed his tenure on the Air Force Laboratory Fellowship. He held that fellowship in conjunction with Dr. Michael Rea's lab at the BRAIN Institute, Brooks AFB, TX. He was awarded his Ph.D. in December, 1995 and directly joined Dr. Rea's laboratory as an Air Force Postdoctoral Fellow. He has been invited to stay a third year as a semi-independent investigator at the BRAIN Institute.

While under his AASERT appointment here, he conducted experiments as to the nature of the kinase enzyme that mediated P-CREB formation in response to glutamate stimulation. In particular, he asked whether cGMP-dependent protein kinase could mediate phosphorylation of CREB either early or late at night. His experiments involved comparing the efficacy of cGMP- and cAMP-dependent kinases against this peptide substrate. He found that, while cGMP-stimulation was less effective that cAMP-stimulation in leading to $^{32}$P incorporation into CREB, cGMP-dependent protein kinase phosphorylated CREB as well as its best known substrate, histone. We are evaluating the $^{32}$P incorporation pattern into the CREB amino acid sequence by various elements in the signaling pathways we have identified as acting at night. This work will contribute to a paper in preparation for the American Journal of Physiology, in which the properties of the cGMP/PKG system are thoroughly analyzed.

Additionally, we undertook a number of collaborative experiments with Dr. Mike Rea (USAF-SAM, Brooks AFB) that were based upon Todd Weber's work during this award period. The most productive interactions were oriented around the hypothesis that light-induced phase-shifts might be mediated through NMDA-receptor/Ca$^{2+}$ influx leading to stimulation of nitric oxide synthase. The NO produced by this process has been demonstrated in many tissues to directly activate guanylyl cyclase, producing cGMP. This might lead to induction of phosphorylation of Ca$^{2+}$/cyclic AMP Response Element Binding protein (CREB) and Fos expression in the SCN. Todd spent 3 months in Dr. Mike Rea's lab at USAF-SAM where he conducted experiments that elegantly demonstrated that NO is a necessary element in the signal transduction pathway by which light resets behavioral rhythms in hamsters. This work was fully complementary to and supported by the studies of the SCN in the brain slice by Dr. Ding in my lab. Todd Weber, Mike Rea and I published two manuscripts reporting these findings (Science, 1994 and Brain Research, 1996).
SUMMARY OF PROGRESS

1) Glutamate applied directly to the SCN produces a light-like phase response curve, and activates a nitric oxide generating pathway. This supports the evidence that the effects of light on circadian rhythms of animals is mediated by glutamate at the retinohypothalamic tract afferents to the SCN.

2) Phosphorylation of a 43 kD nuclear protein was detected by Western blot with state-specific antibodies to 133-serine phosphorylated Ca2+/cyclic AMP Response Element Binding protein (P-CREB). This form of stimulus-transcription coupling was evident during subjective night, at times when phasing of the SCN clock can be reset by light pulses. Application of microdrops of glutamate or nitric oxide donors (SNP, SNAP) specifically induce P-CREB formation. Colocalization of P-CREB and VIP within single SCN neurons was observed. This effect is specifically block by L-NAME, a potent inhibitor of NOS, but not by the stereoisomer, D-NAME.

3) Glutamic acid decarboxylase (GAD) levels undergo significant diurnal variation over the circadian cycle.

4) Serotonin and NPY are both potent regulators of clock phasing when applied briefly and focally to the SCN in the brain slice. They both induce significant phase-advances during the daytime portion of the circadian cycle when applied focally to the site that raphe afferents terminate; at nighttime serotonin is without effect. At CT 7, when the two neuromodulators are co-applied at 10^-6 M, NPY blocks the large amplitude phase-shifts induced by serotonin; at CT 23 there is no interaction between the two neurotransmitter systems on SCN phasing.

5) The circadian rhythm of SCN neuronal activity can be measured with a carbon fiber bundle electrode. Robust circadian oscillations in the summed firing rates of a small neuronal population can be measured at one site for up to 3 days. Activities of neurons in subregions of the SCN can be measured for up to 3 days and exhibit a stable, regenerating sinusoidal rhythms of firing rate.

6) The SCN contains substantial amounts of a 155 kD protein recognized specifically by a polyclonal anti-nNOS antibody. This is the only isoform of NOS discernible in the SCN of rat by Western blot analysis. The nNOS immunoreactivity is distributed equally between membrane and cytoplasmic fractions.

Publications resulting in part from funding from this AASERT award:


* Manucripts previously submitted to the AFOSR.

**Information about the P. L. M. U. Gillette:**

**Honors and Awards:**

**Federal Government Public Advisory, National Service:**