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We studied neurochemical mechanisms related to control of the activity of these cells. Modulation of the neuronal activity here plays animportant role in determining the phase of the circadian cycle. We examined neuromodulation of GABA and glutamate actions in cells of the SCN. Neuromodulation is based on the concept that neuromodulators can alter the release or response of SCN cells to the fast-acting neurotransmitters GABA and glutamate that regulated inhibition and excitation in the SCN, respectively. In general, this is important because we can understand how the activity of these neurons is modulated by neurotransmitters, we may be able to understand how to alter the temporal regulation of circadian cycles, important for improving human performance during what is commonly known as "jet lag".

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Overview. Circadian rhythms greatly influence performance levels in both humans and non-human mammals. Circadian rhythms are controlled by a part of the brain in the hypothalamus called the suprachiasmatic nuclei (SCN). Here a few thousand nerve cells regulate circadian rhythms. We studied neurochemical mechanisms related to control of the activity of these cells. Modulation of the neuronal activity here plays an important role in determining the phase of the circadian cycle. We examined neuromodulation of GABA and glutamate actions in cells of the SCN. Neuromodulation is based on the concept that neuromodulators can alter the release or response of SCN cells to the fast-acting neurotransmitters GABA and glutamate that regulated inhibition and excitation in the SCN, respectively. In general, this is important because if we can understand how the activity of these neurons is modulated by neurotransmitters, we may be able to understand how to alter the temporal regulation of circadian cycles, important for improving human performance during what is commonly known as "jet lag". We published a number of papers describing our work, described and listed below.

Specific findings.

A brief exposure to light can shift the phase of mammalian circadian rhythms by an hour or more. Neuropeptide Y (NPY) administration to the hypothalamic suprachiasmatic nucleus (SCN), the circadian clock in the brain, also causes a phase-shift in circadian rhythms. After a phase shift, the neural clock responds differently to light, suggesting learning has occurred in neural circuits related to clock function. Thus certain stimuli can produce effects that last for an extended period, but possible mechanisms of this long term effect have not previously been examined at the cellular level. NPY caused a long term depression in both electrical activity and intracellular calcium levels of neurons, as studied with whole cell patch clamp recording and fura-2 digital imaging. In contrast to the immediate (1 sec) recovery after relief from glutamate receptor blockade, a brief single application of NPY (100 nM) depressed cytosolic Ca^{2+} for over an hour. The mechanism of this long term calcium depression, a form of cellular learning, is dependent on the simultaneous release of glutamate and activation of NPY receptors, as both the extended response to NPY and any after-effect were blocked by co-application of glutamate receptor antagonists. Postsynaptic actions of NPY, mediated by both Y1 and Y2 receptors, were short term and recovered rapidly. The primary site of long term NPY actions may be on presynaptic glutamatergic axons, as the frequency of miniature excitatory postsynaptic currents in the presence of tetrodotoxin was reduced by transient exposure to NPY in both cultures and slices (van den Pol et al,1996).

Although NPY has been shown to influence the action of many transmitters in the brain, modulation of GABA, the primary inhibitory transmitter, has not been detected with electrophysiology. Using whole-cell patch clamp recording, we found that NPY has a large

modulatory effect on GABAergic neurons of the SCN that act as the circadian clock in the mammalian brain. NPY, acting at both Y1- and Y2-like receptors, reduced the frequency of spontaneous miniature inhibitory postsynaptic currents while having little effect on the postsynaptic GABA receptors, suggesting a presynaptic mechanism of NPY action. In single self-innervating neurons, application of either Y1 or Y2 agonists to the same neuron significantly inhibited the evoked autaptic GABA release. The use of single-neuron microcultures has allowed the demonstration that a single peptide, NPY, has two different receptors coded for by different genes in the same axon terminal. The Y1 and Y2 agonists also inhibited whole-cell calcium currents when applied to the same neuron, indicating a coexistence of Y1- and Y2-like receptors in the postsynaptic cell body. The self-innervating cell model we used may be generally applicable for discriminating presynaptic versus postsynaptic actions of other neurotransmitters and neuromodulators and locating their subtype receptors (Chen and van den Pol,1996).

GABA is the primary transmitter released by neurons of the SCN, the circadian clock in the brain. Although $GABA_B$ receptor agonists can exert a significant effect on circadian rhythms, the underlying mechanism by which $GABA_B$ receptors act in the SCN has remained a mystery. Potassium and calcium channels are two major effectors of $GABA_B$ receptor actions in the CNS. We found no $GABA_B$ receptor-mediated slow potassium conductance change, no effect on membrane potential, and no change in input resistance in SCN neurons in vitro using whole-cell patch clamp recording. However, the $GABA_B$ receptor agonist baclofen (1-100 μM) did exert a large and dose-dependent inhibition (up to 100%) of evoked inhibitory postsynaptic currents (IPSCs), and the inhibition was blocked by the $GABA_B$ receptor antagonist 2-hydroxysaclofen. Baclofen reduced the frequency of spontaneous IPSCs, but showed little effect on the frequency or amplitude of miniature IPSCs in the presence of tetrodotoxin. The activation of $GABA_B$ receptors did not modulate postsynaptic $GABA_A$ receptor responses, suggesting a presynaptic action on GABA neurotransmission. In contrast to the apparent absence of effect on potassium channels, the depression of GABA release by $GABA_B$ autoreceptors appeared to be mediated through a modulation of presynaptic calcium channels. The baclofen inhibition of both calcium currents and evoked IPSCs was greatly reduced (up to 100%) by the P/Q-type calcium channel blocker agatoxin IVB, suggesting that P/Q-type calcium channels are the major targets involved in the $GABA_B$ receptor modulation of GABA release. N-type calcium channels were also involved in the $GABA_B$ receptor modulation, but to a smaller degree. The inhibition of GABA release by baclofen was totally abolished by a pretreatment with pertussis toxin (PTX), whereas the inhibition of whole-cell calcium currents by baclofen was only partially abolished by PTX, suggesting different G proteins may be involved in $GABA_B$ receptor modulation. We conclude that $GABA_B$ receptor activation exerts a strong presynaptic inhibition of GABA release in SCN neurons primarily by modulating P/Q-type calcium channels at axon terminals (Chen and van den Pol, 1998).

Glutamate is the primary excitatory transmitter innervating the hypothalamic SCN and is responsible for light-induced phase shifts of circadian rhythms generated by the SCN. Using self-innervating single neuron cultures and patch clamp electrophysiology, we studied metabotropic glutamate receptors (mGluRs) expressed by SCN neurons. The selective agonists for group I (3,5-dihydroxy-phenylglycine (DHPG)), group II ((S)-4-carboxy-3-hydroxyphenylglycine (4C3HPG)), and group III (L(+)-2-amino-4-phosphonobutyric acid (L-AP4)) mGluRs all depressed the evoked IPSC in a subset (33%) of single autaptic neurons, suggesting a coexpression of all three groups of mGluRs in the same axon terminals of a single neuron. Other neurons showed a variety of combination of

mGluRs, including an expression of only one group of mGluRs (18%) or coexpression of two groups of mGluRs (27%). Some neurons had no response to any of the three agonists (22%). The three mGluR agonists had no effect on postsynaptic GABA receptor responses, indicating a presynaptic modulation of GABA release by mGluRs. We conclude that multiple mGluRs that act through different second messenger pathways are coexpressed in single axon terminals of SCN neurons where they modulate the release of GABA presynaptically, usually inhibiting release (Chen and van den Pol,1998).

Although metabotropic glutamate receptor (mGluR) modulation has been extensively studied in neurons, it has not been investigated in astrocytes. We studied modulation of glutamate-evoked calcium rises in primary astrocyte cultures using fura-2 ratiometric digital calcium imaging. Calcium plays a key role as a second messenger system in astrocytes, both in regulation of many subcellular processes and in long distance intercellular signaling. Suprachiasmatic nucleus (SCN) and cortical astrocytes showed striking differences in sensitivity to glutamate and to mGluR agonists, even after several weeks in culture. Kainate-evoked intracellular calcium rises were inhibited by concurrent application of the type I and II mGluR agonists quisqualate (10μM), t-ACPD (100-500μM), and L-CCG-I (10μM). Inhibition mediated by L-CCG-I had long-lasting effects (>45 min) in about 30% of the SCN astrocytes tested. The inhibition could be mimicked by the L-type calcium channel blocker nimodipine (1μM) as well as protein kinase C (PKC) activators PDBu (10μM) and PMA (500nM), and blocked by the PKC inactivator H-7 (200μM), suggesting a mechanism involving PKC modulation of L-type calcium channels. In contrast, mGluRs modulated serotonin (5HT)-evoked calcium rises through a different mechanism. The type III mGluR agonist L-AP4 consistently inhibited 5HT-evoked calcium rises, while in a smaller number of cells quisqualate and L-CCG-I showed both inhibitory and additive effects. Unlike the mGluR—kainate interaction, which required a pretreatment with an mGluR agonist and was insensitive to pertussis toxin (PTx), the mGluR modulation of serotonin actions was rapid and was blocked by PTx. These data suggest the glutamate, acting at several metabotropic receptors expressed by astrocytes, could modulate glial activity evoked by neurotransmitters and thereby influence the ongoing modulation of neurons by astrocytes (Haak et al,1997).

Light is the primary sensory stimulus that synchronizes or entrains the internal circadian rhythms of animals to the solar day. In mammals photic entrainment of the circadian pacemaker residing in the suprachiasmatic.nuclei is due to the fact that light at certain times of day can phase shift the pacemaker. In this study we show that the circadian system of mice can integrate extremely brief, repeated photic stimuli to produce large phase shifts. A train of two msec light pulses delivered as 1 pulse every 5 or 60 seconds, with a total light duration of 120 msec, can cause phase shifts of several hours that endure for weeks. Single 2 msec pulses of light were ineffective. Thus, these data reveal a property of the mammalian circadian clock, that it can integrate and store latent sensory information in such a way that a series of extremely brief photic stimuli, each too small to cause a phase-shift individually, together can cause a large and long-lasting change in behavior (van den Pol et al,1998).

Publications.

A number of publications cited support from AFOSR. Those in bold are the ones that directly address the aims listed in the application, and are focused on the circadian clock cells from the SCN. The other papers (not bold) benefited from the AFOSR funding, but are more distantly related to the aims and focus on the surrounding hypothalamus in which the SCN is located.

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Final Invention Report
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In the course of this grant, we devised one invention for which a patent is pending:
A device for phase shifting the circadian clock. The devise is the focus of a joint patent application by Stanford and Yale Universities, with A.N. van den Pol and H.C. Heller listed as inventors. The Air Force Office of Scientific Research was listed as a primary supporter of the research leading to the invention.

Background. When humans fly to a distant time zone, their circadian rhythms are out of synchrony with the new solar light cycle. This leads to a substantial decrease in both intellectual and physical performance. It generally takes several days for the endogenous cycle to synchronize with the new light cycle, a process called "phase-shifting". If the process of phase shifting could be enhanced, this would result in a more rapid resetting of the circadian rhythm, and restore human performance to an optimal level. A substantial amount of work has been done in this area, and it has been found that continuous exposure to bright light, often for several hours, at the correct time of day, can facilitate phase shifting.

In experiments with lab rodents, we found that several brief flashes of light, each less than a second in duration could enhance phase shifting. In other words, an extended exposure to constant light was not required. Rather a few 2 msec (1/500 second) flashes of bright light was sufficient to generate a robust phase shift (van den Pol et al,1998).

Device. The pending patent describes a device, something like a pair of sunglasses, that has a small light bulb that is controlled by a time piece. The time piece controls a few brief flashes of light, which then would enhance the phase shift. Alternately, a large device could be used for groups in which an entire room could be illuminated by brief flashes of light to evoke the phase shift of the circadian rhythm. The advantage of this device over other existing devices is that only a few brief flashes of light are required to obtain maximal phase shifting. The time piece could be programmed ahead of time to deliver the flashes of light at the optimal part of the circadian cycle to generate the required phase shift. The concept has been tested on lab animals, but has not yet been tested on humans. In most respects the circadian rhythms of mammals are similar, suggesting that humans should benefit from use of this device.