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Membrane Properties and Neural Circuits of the Suprachiasmatic Nucleus
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The goal of these studies was to examine local synaptic circuits in the suprachiasmatic nucleus and the effect of GABA on suprachiasmatic nucleus neurons. Electrophysiological evidence was provided to support the hypothesis that suprachiasmatic nucleus neurons are interconnected through a system of GABA-mediated local circuits. Previous studies had suggested that GABA was excitatory during subjective day, but our lab and two others corroborated and extended previous findings indicating that GABA is inhibitory during both subjective day and night. Other experiments showed that zinc and benzodiazepines modulate GABA-mediated inhibition of suprachiasmatic nucleus neurons. In particular, benzodiazepines augment the effect of GABA on these inhibitory circuits. These experiments have provided fundamental information on GABA-mediated local circuits in the suprachiasmatic nucleus, which would be expected to be a fundamental part of the circadian timing system in mammals.

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1. Local GABA-mediated circuits in the suprachiasmatic nucleus

Although the suprachiasmatic nucleus (SCN) in mammals is known to function as a biological clock that regulates and coordinates a variety of circadian rhythms, the intrinsic synaptic circuitry of the SCN has been largely unexplored. Presumably the SCN neurons in different parts of the nuclei contribute differently to the various components of the overall circadian rhythm, yet they must also be synchronized to generate the circadian rhythm of electrical and metabolic activity. Most if not all SCN neurons contain the neurotransmitter GABA, and anatomic studies have demonstrated the presence of GABAergic synapses and neurotransmitter GABA, and anatomic studies have demonstrated the presence of GABAergic synapses and local axon collaterals in the SCN. Numerous neuroactive substances have been detected in the SCN, but only GABA- and glutamate-receptor-mediated postsynaptic events have been reported. It has been unknown, however, whether these events originate from local neurons or from neurons located outside the SCN, and the physiological evidence for synaptic communication among SCN neurons has been indirect. We used three approaches to investigate local circuitry in acute hypothalamic slices of the rat SCN (Strecker et al., 1997).

Tetrodotoxin (TTX) was used to block action-potential-dependent synaptic release. This resulted in a decrease in the frequency of spontaneous inhibitory postsynaptic currents (sIPSCs) in SCN neurons, suggesting that spontaneously active cells within the slice (probably relatively close to the recording neuron) provide GABAergic synaptic input. Since TTX was applied to the entire slice, however, we could not exclude the possibility that the reduction of IPSCs was due to a suppression of firing of neurons outside the SCN.

Our second approach involved selectively stimulating small populations of SCN neurons with brief, localized, pressure-applications of glutamate onto the SCN while analyzing changes in the synaptic activity to single SCN neurons. Electrical stimulation causes release of neurotransmitter from cut axons that originate outside of the SCN, whereas glutamate does not stimulate axons directly, and selectively activates cell bodies and dendrites. By observing synaptic activity in SCN neurons during glutamate microapplication, we could test the hypothesis that SCN neurons receive local synaptic input. We found that 25% of SCN neurons showed significant increases in the frequency of GABAergic PSCs when other SCN neurons were stimulated in this way. In contrast, no evidence for local glutamatergic input was observed. These data from glutamate microapplication through micropipettes further suggest that neurons in or adjacent to the SCN provide $GABA_A$-receptor-mediated input to the SCN.

Our third approach to investigating local synaptic interactions in the SCN involved a major modification of the glutamate microstimulation method that significantly increased the temporal and spatial resolution of our stimulation (and is the basis for part of this proposal). Slices were submerged in ACSF containing inactive, caged glutamate. Flashes of UV light were focused into the SCN from beneath the slice, which uncaged glutamate and stimulated nearby SCN neurons. This approach produced mush faster and more focal glutamate stimulation within the slice. As with glutamate microapplication through micropipettes, uncaging experiments in the SCN resulted in significant increases in IPSC frequency in SCN

neurons, and no evoked EPSCs were observed. The GABAergic synapses that comprise the network shown here conceivably could be a substrate for the synchronization and amplification of the circadian rhythm of SCN firing. Alternatively, this circuitry might mediate other aspects of clock function, such as the integration of environmental and physiological information.

2. Effects of GABA as a function of circadian time

Yarom and colleagues (Wagner et al., 1997) reported that although GABA had its expected inhibitory effect during subjective night, it remarkably became excitatory during subjective day. Given our evidence for functional GABAergic interconnectivity in the SCN, this suggested that GABA may augment the day/night difference in SCN neuronal firing rate by using the synaptic interconnections to further suppress an intrinsically low neuronal firing rate at night (when GABA in inhibitory), and to further increase an intrinsically higher firing rate during the day (when GABA is proposed to be excitatory).

As an initial test of this hypothesis, we examined whether GABA excited SCN neurons during subjective day. We used extracellular cell-attached patch recording in order to maintain the normal internal chloride concentration [$Cl^-$]. In this configuration, the firing rate of the cell can be monitored by observing capacitive transients created by spontaneous action potentials within the cell. Sprague-Dawley rats (Harlan) were housed under a 12-h light, 12-h dark cycle, and cell-attached recordings were made during the light phase between CT 5:45 and 11:45. In approximately half of our experimental animals, the integrity of the light cycle prior to experimentation was confirmed independently using photosensitive recording device. Coronal slices (175 – 225 μm) of hypothalamus were cut on a Vibratome and maintained in artificial cerebrospinal fluid (ACSF) containing (in mM) NaCl 125, KCl 2.5, $CaCl_2$ 1, $MgCl_2$ 1, $NaHCO_3$ 24, and glucose 10, bubbled with 5 % $CO_2$ and 95% $O_2$ (pH 7.4). Recordings were made either at room temperature (20-22° C) or 30-32° C on rats aged 21-47 days. Patch pipettes (3-5 MΩ) contained (in mM) K-gluconate or KCl 140, $CaCl_2$ 1, $MgCl_2$ 1, NaCl 1, EGTA 5, HEPES 10, Mg-ATP 2. Experiments were also conducted with pipettes containing the extracellular solution (i.e., ACSF) or with NaCl 140, KCl 4, $CaCl_2$ 1, $MgCl_2$, and glucose 10. GABA (100 μm dissolved in ACSF) was applied by picospritzer onto the recorded cell. In some cases GABA was also bath-applied.

We found that 44 of 45 spontaneously firing SCN cells responded to the brief application of 100 μM GABA with a transient suppression of firing, whereas only 2 of 46 cells showed either transient excitation or excitation followed by inhibition. Of 8 silent neurons (i.e., not firing spontaneously), none responded at all to GABA. Two other laboratories, using different methods, have also found that GABA in inhibitory during the subjective day. There other studies used long-term multiple-unit extracellular recordings and gramicidin perforated-patch techniques, and they yielded data similar to ours. The results from our three groups argue that GABA is predominantly an inhibitory neurotransmitter in the SCN during the subjective day (Gribkoff et al., 1999), and thus they are at variance with the results of the Yarom group. We are unable to explain this disparity, but the well-known lability of intracellular chloride concentration (i.e., $[Cl^-]_i$) in neurons is likely to be important. For example, GABA is excitatory in immature neurons and excessive activation of GABA

receptors in the hippocampus has been shown to invert the polarity of the GABA response from inhibitory to excitatory. These results suggest that the efficacy and even the polarity of GABA responses in the SCN are particularly labile. Even subtle changes in GABAergic efficacy, not necessarily involving complete polarity shifts, could have profound effect on the activity of SCN neurons, particularly given the synaptic interconnectivity of the SCN (Strecker et al., 1997).

3. Modulation of $GABA_A$ receptors by zinc and benzodiazepine

A variety of pharmacological agents modulate $GABA_A$-receptor function. Zinc has been reported to be present in the SCN, and has been shown to inhibit GABA-mediated currents in some preparations but not in others, depending on the composition of $GABA_A$-receptor subunits. Benzodiazepines are commonly prescribed medications that are known to potentiate GABA-receptor-activated currents, and can produce phase shifts in circadian time in vivo. As with zinc, not all subtypes of $GABA_A$ receptor are modulated by benzodiazepines. The specific pharmacology of $GABA_A$ receptors is not well understood in the SCN, and contradictory reports regarding zinc and benzodiazepine sensitivity reveal a lack of consensus on which modulators may be effective on SCN neuronal GABA receptors. Therefore, knowledge concerning the actions of these substances on SCN neurons is important for understanding modulation of synaptic transmission in the SCN. Furthermore, given the clear importance of GABA as a transmitter within the SCN, fundamental information about its pharmacology may lead to a better understanding of its role in circadian rhythms and provide better ways to manipulate them.

The pattern of sensitivity to zinc and benzodiazepines is also relevant to understanding the specific subunit composition of $GABA_A$ receptors in the SCN. Neuroanatomical methods have identified several GABAA receptor subunits that may be present in the SCN, but which combinations of subunits actually form functional receptors in the SCN in unknown. The presence of benzodiazepine sensitivity in GABAA receptors, conferred by the $\gamma$ subunit, has often been associated with a lack of zinc sensitivity, and vice versa. More recent findings indicate that it is possible to retain both benzodiazepine sensitivity and a weaker zinc sensitivity in certain subunit combinations. Therefore, an examination of GABA responses may yield some clues regarding the structure of GABA receptors in the SCN, which in turn may be useful in predicting the specific electrophysiological behavior of the receptors in this region.

Properties of $GABA_A$ receptors in SCN neurons were examined in acutely-prepared slices of SCN from 3- 8-week-old-rats, using whole-cell voltage-clamp techniques (Strecker et al., 1999). $Zn^{2+}$ (200 μM) significantly reduced the mean amplitude of sIPSCs (i.e., without TTX) in 4 of 5 SCN neurons (Kolmogorov-Smirnov test). In the 4 cells showing significant reductions, mean sIPSCs amplitudes were attenuated to $56 \pm 11\%$ of control, while the one cell without a significant reduction showed a mean sIPSC amplitude in $Zn^{2+}$ that was 96% of control, possibly indicating some heterogeneity of receptor composition. In the presence of TTX, $Zn^{2+}$ (200 μM) also significantly reduced the amplitude of mIPSCs in 7 of 8 cells to $54\ \% \pm 5\%$ of control mean amplitude, resulting in a similar degree of attenuation as without TTX, and indicating a primarily postsynaptic effect of $Zn^{2+}$ on IPSC

amplitude. To confirm further the postsynaptic nature of $Zn^{2+}$ block, currents evoked by pressure-applied GABA (100 μM) were reduced significantly by $Zn^{2+}$ (200 μM) in 8 of 8 cells, to an average of 35 ± 10% of control amplitude. Thus, zinc proved an effective blocker of both endogenous (synaptic) and exogenous (pressure-applied) GABA responses.

The effects of the benzodiazepine flunitrazepam were also examined on currents resulting from endogenous GABA and exogenous application (pressure-applied). For sIPSCs, both amplitude and kinetics were analyzed. Flunitrazepam (100 nM) usually failed to potentiate the amplitude of spontaneous IPSCs (sIPSCs), producing significantly larger events in only 4 of 11 neurons at 30-33° C (Kolmogorov-Smirnov test). The average increase in IPSC amplitude for all 11 neurons examined was only 8 ± 5% above control. The mean amplitudes of sIPSCs in the 11 neurons in control and in 100 nM flunitrazepam were –44.1 ± 6.3 pA and –47.2 ± 6.2 pA, respectively. Thus, IIPSC amplitude was generally not affected by flunitrazepam. In contrast, IPSC kinetics, which were examined in detail in 10 of these 11 neurons, did show changes with flunitrazepam (100 nM). Two exponential components were readily resolved in the sIPSC decay constants, with time constants $\tau_{fast}$ = 7.5 ± 0.7 ms and $\tau_{slow}$ = 63.3 ± 5.0 ms, and a fractional amplitude ($fract_{slow}$) = 0.52 ± 0.02 for the slow component. Flunitrazepam increased all three parameters significantly, yielding $\tau_{fast}$ = 11.2 ± 0.6 ms, $\tau_{slow}$ = 113.9 ± 6.2 ms, and $fract_{slow}$ = 0.58 ± 0.02 (paired t test, 1-tail, n = 10 neurons). Thus, the general effect of flunitrazepam on these parameters was uniformly to increase the duration of IPSCs. Flunitrazepam produced significant increases in $\tau_{slow}$ for all 10 neurons, whereas 7 of 10 cells showed significant increases in $\tau_{fast}$, and 5 of 10 cells showed significant increases in $fract_{slow}$. Thus, benzodiazepine appears to influence all three of these IPSC decay parameters, but its greatest effect appears on $\tau_{slow}$, which is the characteristic most likely to influence total IPSC decay time.

The fact that flunitrazepam failed to strongly potentiate IPSC amplitudes while strongly potentiating the decay kinetics suggests that regardless of whether flunitrazepam is present, similar numbers of GABA channels are activated initially during the brief period of synaptic release of GABA that generates the IPSC. Once activated, however, the channels remain activated for a longer period of time when flunitrazepam is present. Consistent with this, flunitrazepam (100 nM) potentiated the amplitude of current evoked by pressure application of GABA by an average of 242 ± 22% in 13 of 13 SCN neurons held at 30-33° C, and by an average of 193 ± 10% in 6 of 8 neurons at room temperature (2 neurons failed to show any potentiation by flunitrazepam, suggesting possible heterogeneity in responsiveness to flunitrazepam). Because GABA remains present for several seconds after the pressure application, the prolongation of decay constants is presumably manifest as an increase in temporal summation and, hence, in amplitude under these conditions.

Our results demonstrate at a cellular level that both zinc and benzodiazepine are effective modulators of adult SCN neurons. It is unclear whether zinc normally plays a modulatory role in the SCN in vivo, but benzodiazepines are known to have effects on circadian rhythmicity, and our study demonstrates a direct action of this drug on GABAA-receptor-mediated synaptic mechanisms in the SCN. Therefore, these data support the hypothesis that benzodiazepine treatment enhances summation of IPSPs, thus suggesting that benzodiazepines augment the effects of the local inhibitory circuits during the subjective day

when the frequency of PSPs is expected to be greatest. The fact that benzodiazepine was generally effective on SCN neurons suggests that $GABA_A$ receptors in the SCN have a $\gamma$ subunit. Furthermore, the moderate sensitivity of SCN neurons to zinc is also consistent with the presence of $\gamma$ subunits, because receptors without $\gamma$ subunits often show much stronger block from $Zn^{2+}$. Further research with more pharmacologically specific ligands should reveal precisely which receptor combinations produce these physiological responses.

Publications

a. Refereed Publications:

Strecker, G.J., Wuarin, J.-P., and Dudek, F.E. (1997) $GABA_A$-mediated local synaptic pathways in the rat suprachiasmatic nucleus. J. Neurophysiol., 78:2217-2220.

Gribkoff, V.K., Pieschl, R.L., Wisialowski, T.A., Park, W.K., Strecker, G.J., de Jeu, M.T.G., Pennartz, C.M.A., and Dudek, F.E. (1999) A re-examination of the role of GABA in the mammalian suprachiasmatic nucleus. J.Biol. Rhyth. 14:126-130.

Strecker, G.J., Park, W.K., and Dudek, F.E. (1999) Zinc and flunitrazepam modulation of GABA-mediated currents in rat suprachiasmatic neurons. J. Neurophysiol. 81:184-191.

b. Reviews and Book Chapters

van den Pol, A.N., Strecker, G.J. and Dudek, F.E. (1996) Excitatory and inhibitory amino acids and synaptic transmission in the suprachiasmatic nucleus. Prog. Brain Res. 111:41-56.

Bouskila, Y., Strecker, G.J., and Dudek, F.E. Cellular mechanisms of circadian function in the SCN. Handbook of Behavioral Neurobiology - - Circadian Clocks, Ed. By J.S. Takahashi, F.W. Turek and R.Y. Moore (in press).