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Loss of melatonin secretion in hamsters can alter the rhythm of melatonin sensitivity in the suprachiasmatic nuclei (SCN) as tested in an in vitro slice preparation. The effect on melatonin sensitivity depended on whether pinealectomy or brief constant light exposure was used to reduce melatonin levels, with constant light increasing sensitivity and pinealectomy decreasing it. The same treatments also eliminated or reduced the amplitude of the firing-rate rhythms monitored in the SCN slice preparation. These results imply a role for pineal melatonin in the maintenance of the normal amplitude of the SCN pacemaker's output rhythms. Serotonin and melatonin were determined to suppress photic responses of SCN cells and intergeniculate leaflet cells studied in vivo. Serotonin appears to act at both targets via a receptor that is similar to the serotonin-1A receptor type, while melatonin acts via a non-serotonergic receptor. Gastrin-releasing peptide (GRP) causes increased firing of about 50% of SCN cells tested in a slice preparation; the proportion of responsive cells depends on the circadian phase tested. GRP injected into the SCN in vivo causes phase-dependent phase shifts that resemble those caused by light pulses.

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NEUROPHYSIOLOGICAL ANALYSIS OF CIRCADIAN RHYTHM ENTRAINMENT

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Benjamin Rusak

This research program has obtained the following results in the 1993 period of support.

1. Loss of melatonin secretion in hamsters can alter the rhythm of melatonin sensitivity in the suprachiasmatic nuclei (SCN) as tested in an in vitro slice preparation. The effect on melatonin sensitivity depended on whether pinealectomy or brief constant light exposure was used to reduce melatonin levels, with constant light increasing sensitivity and pinealectomy decreasing it.

2. The same treatments also eliminated or reduced the amplitude of the firing-rate rhythms monitored in the SCN slice preparation. These results imply a role for pineal melatonin in the maintenance of the normal amplitude of the SCN pacemaker's output rhythms.

3. Serotonin and melatonin were determined to suppress photic responses of SCN cells and intergeniculate leaflet cells studied in vivo. Serotonin appears to act at both targets via a receptor that is similar to the serotonin-1A receptor type, while melatonin acts via a non-serotonergic receptor.

4. Gastrin-releasing peptide (GRP) causes increased firing of about 50% of SCN cells tested in a slice preparation; the proportion of responsive cells depends on the circadian phase tested. GRP injected into the SCN in vivo causes phase-dependent phase shifts that resemble those caused by light pulses.

5. SCN cells in a slice preparation are sensitive to the drugs which are selective agonists of the metabotropic type of glutamate receptor. Selective metabotropic antagonists can block these effects but do not alter responsiveness to the ionotropic glutamate agonist, NMDA.

Annual Technical Report, 1993
U.S. Air Force Office of Scientific Research
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Benjamin Rusak

This contract has provided support for a program of neurophysiological studies of the mammalian circadian system in vivo and in brain slice preparations. The publications arising directly from this support during 1993 (and early 1994) are listed below in section E, marked with an asterisk. Other publications listed came out of this laboratory in relation to other research support. These behavioral studies and studies of gene expression in the mammalian circadian system are often closely linked to and informed by findings we make in the neurophysiological laboratory with AFOSR support. Although these studies could not be conducted without other sources of financial support, they also depend heavily for their logic and design on neurophysiological findings made under the AFOSR contract, and are, therefore, a leveraged product of that support.

A. Role of Pineal Melatonin in SCN Rhythmicity

During the first year of our renewed support under an AFOSR contract (calendar year 1993), we have pursued a number of neurophysiological studies related to the regulation of mammalian circadian rhythms. In one set of studies we examined the circadian firing-rate rhythm and melatonin sensitivity rhythm in hamster suprachiasmatic nucleus cells studied in a brain slice preparation (1). We sampled baseline firing rates of SCN cells at all circadian phases with a glass micropipette and then pressure-ejected melatonin from the recording pipette and noted whether firing rates changed significantly. We found that constant light exposure for a 48-hr period was sufficient to damp the rhythm of melatonin sensitivity, so that the usual daytime peak of sensitivity was attenuated. In fact, sensitivity to melatonin appeared to increase, rather than decrease as in controls, during the projected night phase. Overall, the proportion of melatonin-responsive cells increased as a result of constant light exposure for 48 hr. In addition, we observed that the firing-rate rhythm which was clear in control (light-dark cycle housed) animals was also damped after exposure to bright constant light, so that there was no change in mean firing rates across time of day.

In a second study (2) we examined the effects of pinealectomy a week earlier on these two rhythms in hamsters studied under light-dark cycles. Pinealectomy also eliminated the daytime peak and nighttime trough in melatonin sensitivity, but, unlike constant light exposure, the overall effect of pinealectomy was to reduce the proportion of melatonin-sensitive cells. Pinealectomy also damped but did not entirely eliminate the firing-rate rhythm of SCN cells; rather, the daytime peak was shortened and the nocturnal trough was shallow compared to control animals. These results suggest that pineal melatonin plays a role in modulating the intrinsic circadian rhythm of SCN cells, perhaps by increasing synchrony among a population of SCN cells which are each competent circadian oscillators. In addition, the results suggested one of two alternatives: that suppressing melatonin by light exposure has different effects from those following loss of melatonin from pinealectomy, or that there are transient and even opposite effects of melatonin suppression on melatonin receptor availability after 2 days and after 7 days. The latter hypothesis has support from recent receptor binding studies from Paul Pevet's laboratory. These hypotheses will be tested in studies to be conducted in the next year.

B. Serotonin and Melatonin Effects on IGL and SCN Neuron Photic Responses

A number of studies have examined sensitivity of SCN cells to serotonin (5-HT) and the effects of serotonin on rhythm entrainment. The evidence to date has suggested, however, that SCN cells that are photically responsive are not sensitive to serotonin. We re-examined this issue and also the issue of the role of melatonin in affecting SCN function. It has generally been assumed that behavioral effects of these drugs are mediated by their effects on the SCN. While this is certainly plausible, it is equally plausible that some of these effects are mediated by their influence on the activity of cells in the intergeniculate leaflet (IGL), which is also a part of the light entrainment pathway. We therefore examined the roles of melatonin and serotonin in affecting IGL and SCN cells' responses to photic stimuli, and attempted to determine the types of receptors involved in these effects.

We showed that both melatonin and serotonin when applied iontophoretically via a multibarrel micropipette suppressed baseline (dark) firing in most IGL and SCN cells, and suppressed the increased firing rate normally seen in response to retinal illumination **(6,10)**. The pharmacological specificity of these effects was demonstrated because they were dose-dependent, and reversible with appropriate antagonists, at least for serotonin. The serotonin antagonists tested were ineffective in reversing the effects of melatonin, demonstrating that melatonin was acting via a non-serotonergic receptor. No effective melatonin antagonist has yet been described, but we are currently exploring a candidate drug which has shown some promise in preliminary studies.

The results of combining light pulses, a serotonin agonist and a serotonin antagonist have yielded some evidence as to the nature of the receptor involved in mediating serotonergic effects on IGL and SCN cells. The effects of serotonin are mimicked by those of the 5-HT_{1A} selective agonist, 8-OH-DPAT, and both serotonin and DPAT effects are reversible by co-application of the nonspecific serotonin antagonist metergoline. Application of the specific serotonin 5-HT_{1A} antagonist pindobind-5-HT_{1A} also reversed the effects of both agonists on IGL cells, while an antagonist specific to 5-HT_2 receptors was ineffective (ketanserin), and one with mixed 5-$HT_{2/1C}$ affinities had both weak agonist and antagonist properties (ritanserin). Other drugs with putative $5HT_{1A}$ antagonist properties, such as spiperone and propranolol, had no antagonist activity when co-applied with serotonin or DPAT. SCN cells were tested with both serotonin and the putative 1A agonist 5-CT, both of which had similar effects in suppressing photic responses. Serotonin effects on SCN cells also responded to agonists and antagonists with a profile that resembled that of a 5-HT_{1A} receptor, although appropriate pharmacological tests to discriminate it from the newly discovered 5-HT_7 receptor have yet to be performed.

These results demonstrate that serotonin acting through a 5-HT_{1A}-like receptor can modulate photic responses at two levels of the mammalian photic entrainment mechanism, and that light-sensitive SCN cells are responsive to serotonin. They also demonstrate parallel effects of melatonin, which are not antagonized by any serotonin receptor antagonist so far tested. Presumably these effects are mediated by a specific melatonin receptor, the character of which is very likely to be revealed in the near future. Our preliminary studies with a putative melatonin antagonist supplied by Servier (S20928) has revealed some suggestive evidence of successful antagonism, although it seems to have some agonist properties as well.

C. Effects of Bombesin-like Peptides (BNLPs) on SCN Cells and Circadian Rhythms

A report by Albers et al. had suggested a novel form of interaction among three peptides found in the SCN, and this proposed mechanism was premised on the idea that one BNLP, gastrin-releasing peptide (GRP) had few neurophysiological effects on SCN cells in vitro on its own, and caused few phase shifts when applied alone to the SCN in vivo. We examined these claims and found contradictory evidence. Our results indicate that BNLPs, in particular GRP, have very potent excitatory effects on up to 50% of SCN cells tested in vitro,

that these effects vary with circadian phase, and that GRP has very potent circadian rhythm phase-shifting effects in vivo that closely resemble those induced by light. These results have been published in full (3) or in abstract form (19, 21, 22), or are about to be submitted for publication in June, 1994 (11, 12).

Our major results are that GRP_{1-27} can be applied by pressure ejection to SCN cells and cause increased firing rates in about 50% of cells tested. GRP_{1-27} is less effectively applied via iontophoresis, presumably because of the large size and therefore limited electrophoretic mobility of the molecule. Other peptide fragments which affect the same GRP-preferring receptor; namely, bombesin and GRP_{18-27}, have similar effects. The previously reported failure to observe significant effects of GRP is probably attributable to a combination of sampling error because of the very small sample size studied and the use of bath application of GRP_{1-27}, which would promote both direct and trans-synaptic effects on target cells being recorded. Some activations as a result of GRP application in the bathing medium would undoubtedly be masked by secondary suppressions arising from the GRP-induced release of the inhibitory transmitter GABA, which is found in most or all SCN cells.

The behavioral effects of GRP_{1-27} alone when applied to the SCN in vivo are equally potent and quite different from what had been reported previously. The one previous report by Albers et al. indicated that GRP caused less than 30 min delay shifts and no advance shifts. In contrast to these results, we have found very robust phase delays and advances to injections of much smaller doses of GRP as well as to doses comparable to those used previously. These results indicate that GRP has potent phase-shifting effects that are phase-dependent in a way that closely resembles both the phase sensitivity and amplitude of shifts produced by light pulses. Our results suggest that GRP release from SCN cells in response to light input to the SCN may contribute to the phase-shifting effects of light. This hypothesis is currently being tested in behavioral studies with co-application of GRP and putative GRP antagonists that we have previously identified as effective in electrophysiological studies in slice preparations.

D. Other Neurophysiological Studies

Because it has been identified as a possible transmitter in the retinohypothalamic tract, we examined the neurophysiological effects of Substance P (SP) application to SCN cells and the interaction of SP and glutamate effects on SCN cells (24). Our results indicate that SP affects a high proportion of SCN cells. There was some evidence in our results of interactions among SP and glutamatergic effects on SCN cells, but these were not very potent or consistent. A good many more data will have to be collected using these agonists in order to determine whether they do have significant additive or interactive effects on SCN firing rates.

In a newly developed line of research, we have begun to investigate the role of metabotropic glutamate receptors on SCN cell activity. In slice preparations, our initial findings are that the selective metabotropic agonist 1S,3R-ACPD has very potent, sustained and long-lasting activational effects on SCN cells. Unlike NMDA which causes a rapid increase in firing, which returns to baseline immediately after cessation of drug ejection, ACPD activation is sustained for the duration of the ejection and lasts for many seconds after cessation of drug ejection. The slow time course of recovery is characteristic of metabotropic effects on other cells. Some cells responsive to ACPD were also responsive to the NMDA. The effects of ACPD were antagonized by co-application of the putative metabotropic antagonist MCPG, which, by contrast, had no effect on activations in response to the ionotropic agonist NMDA (25).

These results point to a previously uninvestigated role for metabotropic receptors in mediating the effects of excitatory amino acids on SCN cells. These results open a new and potentially important line of investigation into the mechanisms by which light information can affect the SCN-based circadian pacemaker. We are currently pursuing these findings by evaluating the effects in vivo of application of the metabotropic agonists on circadian phase and the effects of antagonists on agonist-induced and light-induced phase shifts.

E. Refereed Journal Publications

*1. Yu, G. D., Rusak, B. and Piggins, H.D. Regulation of melatonin sensitivity and firing-rate rhythms in hamster suprachiasmatic nucleus neurons: Effects of constant light. *Brain Research* 602:191-199, 1993.

*2. Rusak, B. and Yu, G.D. Regulation of melatonin sensitivity and firing-rate rhythms in hamster suprachiasmatic nucleus neurons: Effects of pinealectomy. *Brain Research* 602:200-204, 1993.

*3. Piggins, H.D. and Rusak, B. Bombesin-like peptide effects on suprachiasmatic nucleus neurons in vitro. *Journal of Neuroendocrinology* 5:575-581,1993.

4. Bina, K.G., Semba, K. and Rusak, B. Localization of cholinergic neurons projecting to the suprachiasmatic nucleus of the rat. *Journal of Comparative Neurology* 334:1-13, 1993.

5. Harley, C.A. and Rusak, B. Diurnal variation in active glycogen phosphorylase distribution in the molecular layer of rat dendate gyrus. *Brain Research* 626:310-317, 1993.

*6. Ying, S.W., Zhang, D.X. and Rusak, B. Effects of serotonin agonists and melatonin on photic responses of hamster intergeniculate leaflet neurons. *Brain Research* 628:8-16, 1993.

*7. Rusak, B., Abe, H., Mason, R., Piggins, H.D. and Ying, S.W. Neurophysiological analysis of circadian rhythm entrainment. *Journal of Biological Rhythms* Suppl. 1: S39-S46, 1993.

8. Abe, H. and Rusak, B. Physiological mechanisms regulating photic induction of Fos-like protein in hamster suprachiasmatic nucleus. In press, *Biosystems*, 1994.

9. Fleming, A.S., Suh, E.J, Korsmit, M. and Rusak, B. Distribution of Fos-like immunoreactivity in brain after the display of maternal behavior in primiparous female rats. In press, *Behavioral Neuroscience*, 1994.

*10. Ying, S.-W. and Rusak, B. Effects of serotonergic agonists on firing rates of photically responsive cells in the hamster suprachiasmatic nucleus. In press, *Brain Research*, 1994.

*11. Piggins, H.D., Cutler, D.J. and Rusak, B. Bombesin-like peptides activate hamster SCN neurons: an *in vitro* ionophoretic investigation. In preparation.

*12. Piggins, H.D., Cutler, D.J. and Rusak, B. Injection of gastrin releasing peptide into the suprachiasmatic nucleus causes phase-dependent shifts of hamster circadian rhythms. In preparation.

Book Chapters and other Publications

*13. Mistlberger, R.E. and Rusak, B. Circadian rhythms in mammals: Formal properties and environmental influences. In: *Principles and Practice of Sleep Medicine, Second Edition*. Ed. by M.H. Kryger, T. Roth and W.C. Dement, Saunders:Philadephia, 1993, pp. 277-285.

*14. Harrington, M.E., Rusak, B. and Mistlberger, R.E. Anatomy and physiology of the mammalian circadian system. In: *Principles and Practice of Sleep Medicine, Second Edition*. Ed. by M.H. Kryger, T. Roth and W.C. Dement, Saunders:Philadephia, 1993, pp.286-300.

*15. Rusak, B. Afferent systems affecting neurons in the suprachiasmatic nucleus. In: *Sapporo Symposium on Biological Rhythm*, Ed. T. Hiroshige & K.I. Honma, Hokkaido University Press, in press, 1994.

*16. Rusak, B. and G. Haddad. Introduction: Neural mechanisms of the mammalian circadian system, Special Supplement, *Journal of Biological Rhythms* 8:S1, 1993.

Published Abstracts

17. Edelstein, K., Pfaus, J., Rusak, B. and Amir, S. Neonatal MSG treatment alters the circadian response to constant light and prevents induction of Fos protein in the intergeniculate leaflet in adult rats. *Society for Neuroscience Abstracts* 19:571, 1993.

*18. Ying, S.-W. and Rusak, B. Serotonin agonist and melatonin effects on photic responses of hamster suprachiasmatic nucleus (SCN) and intergeniculate leaflet (IGL) cells. *Society for Neuroscience Abstracts* 19:1816, 1993.

*19. Piggins, H.D. and Rusak, B. Gastrin-releasing peptide (GRP_{1-27}) phase shifts the mammalian circadian pacemaker. *Society for Neuroscience Abstracts* 19:1488, 1993.

*20. Mason, R., Piggins, H.D. and Rusak, B. Effects of the putative retinohypothalamic neurotransmitter NAAG (N-acetylaspartylglutamate) on rat and hamster suprachiasmatic neurones in vitro. *Journal of Physiology* 459:482P, 1993.

*21. Piggins, H.D., Cutler, D.J., and Rusak, B. Effects of ionophoretically applied bombesin-like peptides on Syrian hamster suprachiasmatic neurons in vitro. *Journal of Physiology* 475: 133P, 1994.

*22. Piggins, H.D., Antle, M., and Rusak, B.. Gastrin-releasing peptide phase-shifts hamster locomotor rhythms regardless of environmental lighting conditions. *Society for Research in Biological Rhythms Abstracts* 4:90, 1994.

23. Semba, K., Piggins, H.D., and Rusak, B. Distribution of neuopeptides in the hamster suprachiasmatic nucleus. *Society for Neuroscience Abstracts* 20, in press, 1994.

*24. Piggins, H.D., Cutler, D.J., and Rusak, B. Electrophysiological effects of ionophoretically applied Substance P on hamster suprachiasmatic nucleus neurons *in vitro*. *Society for Neuroscience Abstracts* 20, in press, 1994.

*25. Scott, G. and Rusak, B. Metabotropic glutamate receptor agonists modulate neuronal activity in the hamster suprachiasmatic nucleus *in vitro*. *Society for Neuroscience Abstracts* 20, in press, 1994.