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Avian Pancreatic Polypeptide Phase Shifts Hamster Circadian Rhythms When Microinjected into the Suprachiasmatic Region

Abstract. *The suprachiasmatic nucleus has been identified tentatively as a circadian pacemaker. To examine the functional role of peptides found within suprachiasmatic neurons, avian pancreatic polypeptide and vasopressin were microinjected into the suprachiasmatic region. Avian pancreatic polypeptide, but not vasopressin, shifted the phase of the wheelrunning rhythm as a function of the time of its injection within the circadian cycle. Avian pancreatic polypeptide or a similar peptide may be one component of the neurochemical processes underlying entrainment to the light-dark cycle.*

Many of the complex daily patterns in mammalian physiology and behavior are controlled by the circadian timing system. Circadian pacemakers generate precise rhythms in physiological systems that are close to, but rarely, 24 hours (*1*). The synchronization of these endogenous rhythms with the 24-hour light-dark (LD) cycle is achieved by daily resetting of the circadian pacemaker's phase (*2, 3*). Whether light advances or delays the phase depends on the time of the circadian cycle when light exposure occurs.

Neurons of the suprachiasmatic nuclei (SCN) of the hypothalamus have been identified as a putative circadian pacemaker. Destruction of the SCN eliminates the circadian rhythmicity of a large number of behavioral and physiological variables (*4, 5*). The SCN exhibits circadian variations in metabolic activity (*6*) and rhythms in multiple-unit electrical activity, despite surgical isolation from other hypothalamic areas (*7*). The phase resetting of circadian timing that occurs after exposure to brief light pulses can be mimicked by electrical stimulation of the SCN (*8*) or by intraventricular injections of carbachol, a cholinergic agonist (*9*).

Little is known about the physiological mechanisms that mediate the entrainment of SCN neurons to the LD cycle, generate circadian variations in SCN activity, or communicate circadian information to other physiological systems. Anatomical studies, however, have provided much information on the morphology, neurochemistry, and afferent projections of the SCN (*5*). A large population of neurons within the nucleus contain vasopressin (VP), vasoactive intestinal peptide, or somatostatin (*10*). The ventrolateral area of the SCN seems to be the primary terminus of SCN afferents, including a monosynaptic pathway from the retina, the retinohypothalamic tract (*11*). Other well-defined fiber systems that terminate in the ventrolateral SCN include a serotoninergic pathway from the midbrain raphe (*12*) and a secondary visual projection from the ventral region of the lateral geniculate nucleus (vLGN) of the thalamus (*13*) that contains avian pancreatic polypeptide (APP) immunoreactivity (*14*).

We examined the effect of microinjections of APP and VP into the suprachiasmatic region on the circadian rhythm of locomotor activity in the hamster. Adult male hamsters housed in cages containing activity wheels were implanted stereotaxically with guide cannulas aimed at the suprachiasmatic region (*15*). After surgery, each hamster was housed under constant illumination and allowed to establish a stable free-running circadian activity rhythm. At 10- to 14-day intervals the unanesthetized hamsters were removed from their cages and injected with VP (200 ng) or APP (200 ng) in 200 nl of 0.9 percent NaCl (*16*). All injection sites were verified histologically (*17*).

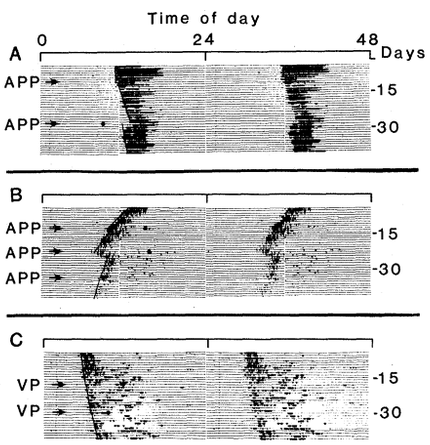

Fig. 1. Effects of avian pancreatic polypeptide (*APP*) and vasopressin (*VP*) microinjected into the suprachiasmatic region on the phase of hamster activity rhythms free-running in constant light. (A and B) Phase-shifts in the activity rhythm produced by microinjection of APP at various phases of the circadian cycle (circles indicate time of injection). (C) Absence of phase shifts in the activity rhythm after microinjection of VP.

Whether microinjection of APP into the suprachiasmatic region advanced or delayed the phase of the activity rhythm depended on the time within the circadian cycle at which it was administered. The shift in the phase of the activity rhythm was fully expressed 1 to 4 days after the injection of peptide (Fig. 1). The data obtained from all APP injections were then plotted in a phase response curve (Fig. 2) that illustrates the magnitude and direction of the phase shifts (*18*) produced by APP injected at various times throughout the circadian cycle. Injection of APP within a 10-hour interval prior to the onset of the wheelrunning [circadian time (CT) 2 through 12] consistently advanced the phase of the activity rhythm. The largest phase advances occurred when APP was administered between CT 6 and CT 10. When injected between 6 and 14 hours after the onset of wheelrunning, APP produced phase delays in the activity rhythm. The delays in the circadian phase, however, were more variable than the phase advances. The transitions between APP-produced phase advances or delays were abrupt, with little indication of a time when animals did not respond to the peptide. Despite the induction of phase shifts in the activity rhythm, the free-running circadian period was not altered after APP injection (-0.05 ± 0.03 hour).

The proximity of the injection site to the third ventricle suggested that APP could have penetrated into the ventricular system and thereby acted on more remote structures. To explore this possibility, we examined the effects of APP injected into the lateral ventricle. Intraventricular administration at times within the circadian cycle that produced maximal phase advances and delays when injected within the suprachiasmatic region had no effect on circadian phase. Injections between CT 6 through 10 and CT 20 through 24 resulted in phase shifts of -0.31 ± 0.22 hour (mean ± standard error, $N = 2$) and $+0.08 \pm 0.19$ hour ($N = 3$), respectively.

Microinjection of VP into the suprachiasmatic region at various times throughout the circadian cycle produced no consistent pattern of phase shifts in the activity rhythm (Fig. 1). The magni-

tude and direction of the small phase shifts that did result from VP injection seemed independent of the circadian phase of administration (Fig. 2). The VP administration seemed not to change any feature of the circadian activity rhythm, including its free-running period (+0.01 ± 0.02 hour).

Our data demonstrate that APP, but not VP, affects the functioning of the circadian timing system when locally administered to neurons within the suprachiasmatic region. Although immunohistochemical studies have indicated the existence of both peptides within neurons of this region, the anatomical characteristics of their distribution differ (*5, 10*). Vasopressin has been described in cell bodies of the dorsomedial SCN that project extensively within the SCN. In contrast, APP immunoreactivity is limited to a population of SCN afferents coming from the vLGN. Other studies that have used VP-deficient Brattleboro rats to examine the role of VP in the circadian timing system have found little to suggest such a role (*19*). SCN neurons containing VP could be an important component in the communication of circadian control to other systems since they project to other neural structures (*20*) and since SCN lesions eliminate the prominent circadian rhythm of immunoreactive VP in cerebrospinal fluid (*21*).

Our data suggest that APP or an APP-like peptide may play a role in the entrainment of circadian rhythmicity to the LD cycle. The phase resetting effects of APP resemble the phase shifts observed after the presentation of 2-hour dark pulses to hamsters housed in constant light (*22*). Both treatments produce maximal phase advances approximately 4 hours before activity onset and phase delays 6 to 14 hours after activity onset, and both require several days of transient cycles before the phase shift is fully expressed.

The entrainment of circadian rhythmicity may be the result of a complex interaction among multiple input pathways. The retinohypothalamic tract seems to be of major importance since ablation of all other visual tracts by postchiasmatic cuts or lesions alters, but does not eliminate, LD cycle entrainment of activity rhythms (*23*). Lesions of the LGN do not alter the pattern with which the circadian activity rhythm entrains to the LD cycle but do retard the rate at which rodents reentrain to phase shifts in the LD cycle (*24*). Visual units with functional properties consistent with the requirements necessary for LD cycle entrainment of a circadian pacemaker have been defined in both the SCN and the vLGN (*25*).

Whether APP acts as a neurotransmitter or neuromodulator within the SCN or other structures within the central nervous system remains to be determined. APP is a 36-amino-acid polypeptide that was originally isolated from chicken pancreas (*26*). More recently, a 36-amino-acid peptide with a structural sequence similar to APP and several other gut peptides has been isolated from porcine brain. This peptide, called neuropeptide Y (NPY), shares 20 amino-acid sequences with APP and cross-reacts with antibodies raised to APP (*27, 28*). Allen *et al.* (*28*) used antibodies raised to NPY that do not cross-react with APP, to describe an NPY immunoreactive system that parallels the reported distribution of APP immunoreactivity. This distribution includes a dense innervation of the ventral lateral SCN. These and other data suggest that previous studies reporting APP-like immunoreactivity may instead have identified NPY neuronal systems. Nevertheless, studies in the peripheral nervous system suggest that APP and NPY, as well as other members of this peptide family, exhibit similar biological activity (*27, 29*). Whether the native peptide found within the vLGN-SCN projection is APP or NPY, our data suggest that this pathway could play an important role in the entrainment of circadian rhythms.

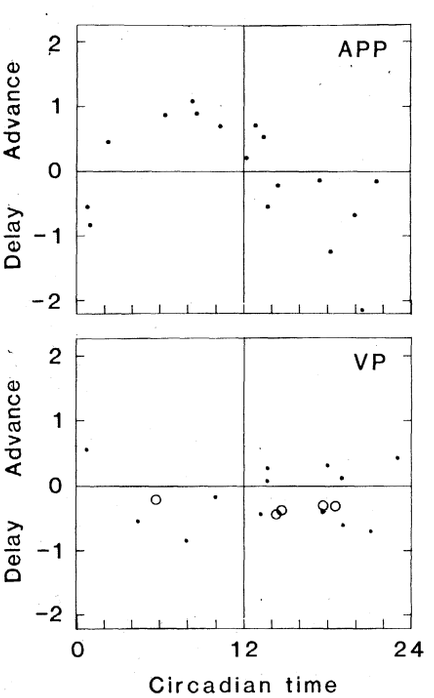

Fig. 2. Phase response curves for APP and VP microinjected into the suprachiasmatic region. Each point represents the phase shift, in hours, induced in the free-running activity rhythm by a single injection of peptide at the circadian phase indicated on the horizontal axis. Circadian time 12 is the time of the onset of activity. Symbols: ●, intact hamsters; ○, blinded and castrated hamsters.

Note added in proof: We have found that microinjection of NPY into the suprachiasmatic region phase shifts hamster circadian rhythms in a manner similar to that described for APP.

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16. Peptides were injected with a 1-μl Hamilton syringe connected to a 33-gauge needle by polyethylene tubing.
17. At the end of the experiment each hamster was anesthetized with sodium pentobarbital and then perfused intracardially with Perfix (Fisher Scientific). The brain was removed and subsequently embedded, cut, and stained with cresyl violet. Each slide was then evaluated under light microscopy. Only data from injection sites that surrounded but did not damage the SCN were included. Microinjections into the lateral ventricle were verified by inspection of the brains after Evans's blue dye had been injected.
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Lead Retention in Zircons

The report by Gentry *et al.* (*1*) is incorrect on three important points. First, they are not the first workers to determine Pb isotope compositions of single zircon grains by mounting the grains directly on a mass spectrometer filament. This technique was successfully used more than 15 years ago (*2*), but has not been extensively pursued because of the limited usefulness of Pb isotope ratios alone for a mineral that almost always yields discordant U-Pb and Pb-Pb apparent ages (*3*). Furthermore, complete U-Pb isotope analyses are readily obtained for such samples by directly loading the residue from a high-pressure HF attack in the presence of a U-Pb spike (*4, 5*).

Second, as is well known by isotope geologists, the radiogenic $^{207}Pb/^{206}Pb$ of a mineral is very insensitive to Pb loss within the past 200 million years or so, due to the fact that ^{235}U, the parent of ^{207}Pb, has largely decayed by then. Thus, a 1500-million-year-old zircon could have lost 50 percent of its Pb 65 million years ago (a well-documented time of Pb loss in the southwestern United States) without lowering the $^{207}Pb/^{206}Pb$ by even 2 percent. In other words, a rough constancy of $^{207}Pb/^{206}Pb$ for zircons from the granite studied by Gentry *et al.* is not useful evidence that these zircons have lost little or no Pb. In fact, the data of Zartman (*6*) show that zircons from this rock at 2900 m depth have lost about 25 percent of their Pb. Moreover, the observation by Gentry *et al.* "that the total number of Pb counts per zircon . . . shows no systematic decrease with depth" in fact lends no additional support to their arguments. A simple linear regression of the data shows that they are much too scattered to provide any useful resolution of a trend.

Third, and perhaps most important, because natural zircons contain only trace amounts of radioactive elements, their Pb retentivity is not germane to the question of closed-system behavior of synthetic zircons that contain large amounts of highly radioactive nuclides. Such zircons would accumulate many orders of magnitude more radiation damage than natural zircons, with the potential for much greater elemental leachability. This point is of crucial importance to any element immobilization strategy (*7*).

Abundant data from geochronologic studies already exist to show that natural zircons can quantitatively retain Pb for up to billions of years (until a time of episodic Pb loss); otherwise zircon suites with concordant Pb/U ages or isotopic systematics indicating simple episodic Pb loss would not be observed.

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Ludwig *et al.* find no fault with our basic data (*1*) but claim to find other errors, to which I must take exception.

I acknowledge that Roubault *et al.* (*2*) previously analyzed single zircon grains, and we should have cited this in our report (*1*). However, in that instance the zircon was attacked by orthophosphoric and hydrofluoric acids after being placed on the filament. This procedure could have affected the lead isotopic measurements they reported, either directly from whatever lead that was in the acids or indirectly through molecular ion interferences generated by the acid treatment of the zircons.

The important point here is that their technique, which we learned of after our experiments were concluded, introduces the possibility of contaminant mass peaks in the lead region from two sources. In contrast, our report (*1*) describes an innovative mass spectrometric method which excludes any acid treatment of the zircon, and is thus a contamination-free technique.

As to the second point of Ludwig *et al.*, I note that their arguments about the lead ratios are in reality only problematical statements. Also, by agreeing that the total lead counts per zircon show no trend with depth, they fail to realize this is in fact strong evidence in favor of high lead retention. Significant lead loss would have been accompanied by a definite trend toward smaller total lead counts per zircon for the greater depths. But this was not observed. They also apparently fail to realize that their claim of a 25 percent lead loss from zircons taken from a depth of 9527 feet, which is based on a single zircon analysis cited by Zartman (*3*), is contradicted by their own admission that the total number of lead counts per zircon we obtained reveals no variation with depth. That is, a 25 percent lead loss at 9527 feet would imply an almost total lead loss (or equivalently few if any lead counts per zircon) at greater depths due to higher temperatures. As noted above, the data show no such effect.

In their third point, Ludwig *et al.* overlook the fallacy in their comparison of radionuclide retention in natural and synthetic zircons, namely, that the concentration of nuclear waste–type radionuclides in synthetic zircons can easily be adjusted so as not to exceed the naturally accumulated radiation dose during the required waste storage period. Thus, our results remain highly relevant to the solution of the long-term waste storage problem, a fact that continues to escape the Department of Energy but not the Congress (*4*).

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