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Short Communications

Suprachiasmatic circadian pacemaker of rat shows two windows of sensitivity to neuropeptide Y in vitro

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The geniculohypothalamic tract carries visual information from the intergeniculate leaflet to the suprachiasmatic circadian pacemaker. NPY, found in this projection, has been shown to affect the phase of behavioral rhythms and influence photic entrainment. We now demonstrate that NPY, when briefly applied to the geniculate projection sites of rat SCN in vitro, induces permanent phase-shifts in the rhythm of neuronal electrical activity at two separate phases of the circadian cycle.

The suprachiasmatic nuclei (SCN) of the hypothalamus contain a circadian pacemaker, which acts to synchronize rhythmic processes of the organism with the daily rhythms in the environment[40,56]. The SCN clock has the ability to sustain near 24-h oscillations in isolation from external time signals[14], but it can be reset by environmental changes transmitted to the nuclei by neuronal and endocrine inputs. The SCN receive photic information via the retinohypothalamic tract (RHT) and the geniculohypothalamic tract (GHT). The RHT projects bilaterally from the retina to the ventrolateral regions of the SCN[21,42,48]. It carries primary visual information, about changes in environmental lighting, and it is essential for entrainment to light-dark cycles[19,26,27,50]. Ablation of the RHT results in the loss of photic entrainment even if other inputs to the SCN are intact[26].

RHT afferents, the axons of a specific subset of retinal ganglion cells, bifurcate at the SCN[39,49]. Some of these projections continue on to the lateral geniculate nucleus (LGN) of the thalamus. Among their postsynaptic sites are neurons of the intergeniculate leaflet (iGL). The rat iGL is a uniform lamina of neurons extending between the dorsal and ventral LGN[8,13,22,44,63]. In addition to bilateral retinal efferents[22], it receives serotonergic innervation from the dorsal raphe nuclei[10,11,41]. Neurons of the iGL in turn project back to the SCN, forming the GHT[6,8,62], as well as to the pineal gland[37], the source of the neuroendocrine signal of darkness, melatonin.

The retinorecipient cells of the iGL are immunoreactive for neuropeptide Y (NPY)[6,17,18], and in the rat they predominantly project to the ventrolateral region of the SCN via the GHT, to overlap with retinal terminals[7,8,38]. The GHT is not essential for entrainment to light–dark cycles but has been found to affect the size of the phase-shifts induced by light pulses as well as to influence photic entrainment. Lesions of the iGL in hamsters induce phase-shifts in activity rhythms[50] and alter the rate of reentrainment to varied lighting schedules[27,51]. Similarly, electrical stimulation of the GHT results in phase-shifts of hamster wheel running rhythms[36]. In addition, hamster responses to constant light are affected by GHT/iGL lesions. GHT lesioned hamsters are found to be less susceptible to splitting of activity rhythms[19], while a significant number of hamsters with split rhythms induced under constant light show fusion of activity rhythms following such lesions[19].

NPY is a 36 amino acid, C-terminally amidated peptide of the family of pancreatic polypeptides[64,65] that is thought to play a modulatory role in the hy-

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pothalamus, hippocampus and the cerebral cortex[4,12]. In the CNS, it has been implicated in the regulation of appetite[9,28,61], sexual behaviors[28] and energy and fluid balance[30]. Axons immunoreactive for NPY constitute about 60% of the GHT processes in the rat[8,44]. Moore and Speh[43] have recently found that GABA is colocalized with NPY in the hamster GHT. These NPY containing neurons of the GHT are positioned to provide modulatory inputs to the circadian pacemaker, signalling changes in the levels of luminescence[52], possibly linked to changes in the activity state of the animal[45–47,67], as well as transitions between light and dark[1].

Albers and Ferris[2] have demonstrated that NPY will phase-shift hamster behavioral rhythms in a manner similar to pulses of darkness[5]: it induces phase-advances in the middle of the day and phase-delays in the night, when it is microinjected into the SCN region. In vitro studies concentrating on the acute effects of NPY on SCN neuronal firing have shown variable responses that appeared to depend on the technique used, such that bath application of NPY to SCN slices resulted primarily in inhibition of neuronal firing rates[3,58], while more localized treatment of SCN using pressure ejection induced excitatory responses[16,31]. A recent report suggests that these results may be due to species differences rather than technique[60].

In the present study we investigated the role of NPY in the mammalian circadian system by examining the effects of NPY on the phase of the circadian rhythm of rat SCN neuronal firing rates in vitro. NPY was briefly and focally applied to GHT projection sites of the SCN in slice at different times across the circadian cycle and the effects of these treatments were assessed on the following circadian days.

Coronal hypothalamic slices (500 μm) were made in the daytime from male Long–Evans rats, 7–8 weeks of age from our inbred colony. The slices were housed in a Hatton-style brain slice chamber[20] and continuously perfused with Earle's balanced salts solution (EBSS), supplemented to 24.6 mM glucose, 26.2 mM $NaHCO_3$ and 0.005% gentamicin, at pH 7.2 and warmed to 37°C. The experimental environment was under constant illumination and saturated with a 95% O_2, 5% CO_2 gas mixture.

Microdrops of 10^{-6} M solutions of NPY were delivered to the NPY input sites of the SCN at 11 time points across the circadian cycle. Initial studies were performed using 10^{-6}M NPY/dH_2O at CT 5, 7, 9, 15 and 18 (CT 0 = time of 'lights on' in the colony; CT 12 = time of 'lights off'); subsequently, all additional experiments and time points (CT 0, 3, 5, 7, 8, 9, 12, 15, 18, 21, and 22) were performed using 10^{-6} M NPY/EBSS. Perfusion of the slices was halted at the time of treatment and a single droplet (3×10^{-11} ml) of experimental or control solution was placed on the ventrolateral region of one of the SCN in the slice for 5 min (detailed microdrop methods have been described elsewhere[34]). The treatment was rinsed off by a stream of medium directed away from the SCN, down toward the optic chiasm. The slices were otherwise undisturbed until the onset of the next circadian cycle.

The effect of NPY on the phase of the pacemaker was assessed by recording the rhythm of neuronal activity of the ensemble of SCN neurons on the second and third days in vitro. This is a robust and readily reproducible rhythm which persists in vitro for up to three circadian cycles[53], showing the same phase and period as in vivo[25].

Firing rates of single neurons, from the microdrop-treated SCN, were recorded extracellularly, sampling cells throughout the nucleus. We recorded during the projected daytime, sampling 4–6 cells each hour, with an average 40–70 units per experiment from which we determined the phase of the electrical activity rhythm. Each cell was monitored for two 120 s trials during which firing rates in 10 s bins were recorded and a mean firing rate of the unit was obtained. These firing rates were averaged and 2 h means, with 15 min lags, were calculated and plotted. The time at which these 2 h means reached a symmetrical maximum was used to define the time-of-peak. In the figures shown we have plotted the firing rates determined for hourly intervals only. Recordings of electrical activity rhythms after microdrop applications of dH_2O (at CT 7) or EBSS (at CT 9) alone show peaks at times overlapping with untreated slices[53] and were used as control experiments to which the results for NPY-treated slices were compared. The size of phase-shifts was determined by comparing time-of-peak of vehicle-treated SCN vs. NPY-treated SCN.

Microdrop application of NPY to the SCN in vitro resulted in phase shifts of the electrical activity rhythm. Extracellular recording following treatment with NPY at eleven different time points indicated that there were two distinct periods of sensitivity to NPY (Fig. 1). Phase-shifts of more than 1 h were induced during the mid-subjective day from CT 5–9. The greatest effect was produced by NPY at CT 8, a 4.8 ± 0.47 h ($n = 4$) phase-advance. Similarly, treatment of slices with NPY between CT 21–24 resulted in large phase-advances (3.5 ± 0.24 h at CT 22) in the rhythm of neuronal activity. Other time points in the subjective night were not sensitive to phase-shifting by NPY.

Preliminary studies with NPY were performed using 10^{-6} M NPY/dH_2O at CT 5, 7, 9, 15 and 18[35].

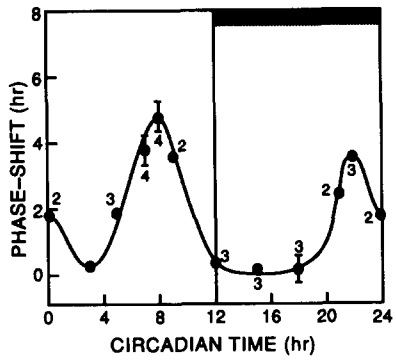

Fig. 1. Phase–response curve for NPY. The x-axis denotes the circadian time of NPY treatment (h) and the y-axis indicates the average magnitude and direction of the induced phase-shift (h). The magnitude of the shift in the time-of-peak in the electrical activity rhythm was determined in relation to time-of-peak in slices treated with microdrops of medium alone. Filled circles denote the mean ± S.E.M. phase-shift. The subscript number indicates the number of experiments performed at a particular circadian time. Points at CT 0 are replotted at CT 24. The vertical bar marks the time of 'lights-off' and the horizontal bar represents the subjective night in the colony.

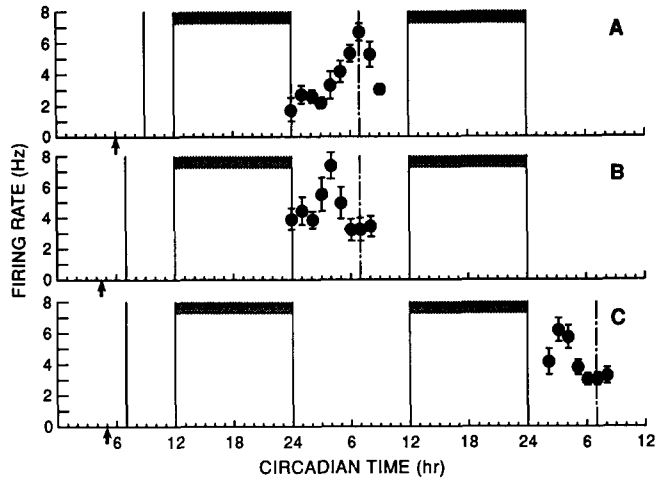

Fig. 2. NPY induces permanent phase-shifts in vitro. A: rhythm of endogenous neuronal activity recorded on day 2 after treatment with microdrops of EBSS on day 1 at CT 9. B: single SCN treated with a microdrop of 10^{-6} M NPY on day 1 at CT 7. The time-of-peak on day 2 occurred 3.25 h earlier than in control slices. C: recording on day 3, in a separate experiment, following treatment with NPY at CT 7 on day 1, resulted in a 3.5 h phase-advance. This is nearly 24 h later than the average time-of-peak seen on day 2 (3.75 ± 0.46 h). Filled circles denote the hourly means ± S.E.M. of the neuronal activity rhythm recorded on the second and third day. The vertical bar indicates the time of treatment. The interrupted line shows the time-of-peak observed in untreated slices and the horizontal stippled bar represents the time of the donor's night in the colony. The arrow points out the time of slice preparation.

Subsequently these were repeated with 10^{-6} M NPY/EBSS. There was no observable difference in the phase-shifts obtained. The average phase advance induced by NPY/dH_2O at CT 7 was 3.7 ± 0.52 h ($n = 3$), while NPY/EBSS resulted in a 4.0 h advance in the time-of-peak. Neither of the solutions induced a phase-shift in the electrical activity rhythm at CT 15 or CT 18. All following time points were tested using NPY/EBSS; the results from both procedures were found overlapping, and were grouped and averaged for analysis.

We also addressed the issue of whether NPY resets the pacemaker permanently. This was demonstrated by recording the rhythm of electrical activity on the second and third days in vitro, following treatment at CT 7 on day 1 (Fig. 2). The time-of-peak recorded on day 3 was at CT 3.5 ($\Phi_A = 3.5$ h), within the range of peak times recorded on day 2 in separate experiments (average $\Phi_A = 3.75 \pm 0.46$ h).

A dose–response curve was generated for NPY administered at CT 7 in microdrops, at 4 different concentrations, 10^{-5} M, 10^{-6} M, 10^{-8} M and 10^{-10} M (Fig. 3). Concentrations of 10^{-6} M (which were used in experiments addressing temporal sensitivities) elicited a maximal phase-shift of 3.75 ± 0.46 h, while the half maximal phase change was seen near 5×10^{-9} M. These results demonstrated that the phase-shifting effect of NPY on the circadian pacemaker is dose dependent.

We have shown that NPY can play a regulatory role in the mammalian circadian system by affecting the phase of the pacemaker directly. The dose–response relationship demonstrates that concentrations effective at inducing phase-shifts are in a physiological range. The actual effective dose is likely somewhat lower than that of the solution applied due to diffusion away from the site of microdrop application. Administration of microdrops of NPY at the GHT projection sites resulted in permanent advancing of the circadian clock during the mid to late subjective day and at the end of the subjective night.

There is notable similarity between the daytime sensitivity of the SCN to direct application of NPY and the daytime responses of animals to dark pulses[5] or to

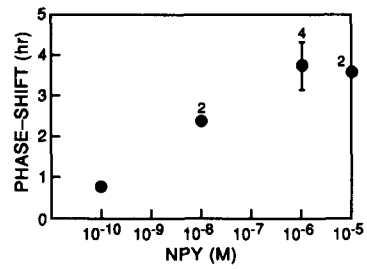

Fig. 3. NPY dose–response curve. Slices were treated with different concentrations of NPY ranging from 10^{-5} M to 10^{-10} M at CT 7. The x-axis denotes the NPY concentration (M) administered while the y-axis indicates the phase-shift response (h). The magnitude of the phase-shift in the time-of-peak was determined in relation to the peak time in vehicle microdrop-treated control slices. Filled circles represent the mean ± S.E.M. phase-shift. The subscript number indicates the number of experiments performed at a particular concentration.

injection of NPY into the suprachiasmatic regions[2]. Boulos and Rusak[5] obtained a phase–response curve for pulses of darkness (2–6 h) in hamsters, where animals housed in constant light were exposed to darkness at different phases of the circadian cycle. A 2-h pulse in the mid to late day (CT 4–12) resulted in phase advances of up to 4-h, while a 6-h pulse induced phase advances as large as 7 h. Albers and Ferris[2] found that injection of NPY near the hamster SCN in the daytime caused robust phase advances in the hamster activity rhythms. Advances as large as 4 h were observed at CT 4 with means of 2–2.5 h. The sensitive period ranged from CT 4–10. Daytime effects of dark pulses in vivo, NPY injection in vivo and NPY applied in vitro are similar both in the sign of the phase change and the sensitive periods. This suggests that NPY from the GHT may contribute to phase-shifting by pulses of darkness, or that the stimuli may act via the same transduction pathway at SCN neuronal integration sites.

In contrast, the nocturnal effects elicited by this range of treatments differ both in sign and time of sensitivity. We found that nighttime application of NPY in vitro at GHT projection sites only affected the SCN between CT 20–24, when it induced phase advances of up to 3.5 h. Albers and Ferris[2] reported phase delays (up to 1 h) after injection of NPY into the suprachiasmatic region during the subjective night of hamsters (CT 14–22) in constant light. This is comparable to the effect of dark pulses during subjective night on hamsters in constant light[5]: phase delays of up to 3 h were observed between CT 20 and 24. These differences may be attributable to anatomical species differences, as well as to differences between the in vitro and in vivo conditions. Our study used SCN from rats entrained to 12L:12D; the SCN neural activities were recorded under conditions analogous to constant dark, while the hamster in vivo studies were carried out in constant light. The fundamental difference, in the sign of the response, observed in the subjective night between in vivo and in vitro studies suggests that the nocturnal effect of NPY is different than that of the day, possibly being more complex, with multiple regulators. The integration of these regulators may be altered in the hamster in constant light, a condition that is known to cause significant alteration in SCN neuronal electrical characteristics[57]. Whether SCN remodelling due to constant light contributes to the nighttime phase-delays by NPY treatment in vivo is testable by injecting NPY, in constant darkness, during the late night and determining its effect on the phase.

The GHT is believed to provide signals of change in levels of environmental illumination[52], as in the transitions between light and dark followed by change in the activity state of the animal. A recent report by Shinohara et al.[59] has demonstrated that NPY levels in the SCN of rats oscillate with a bimodal rhythm; peaks occur 2 h after transitions in the light–dark conditions in the environment. In the same study they demonstrated that endogenous NPY levels change in rats in constant darkness, with a single daily high occurring at CT 12, about 4 h after the peak daytime sensitivity found in our study. Perhaps NPY application at CT 8 resets the clock to CT 12, mimicking the NPY peak. In light–dark conditions, a peak in NPY faithfully follows each transition, whether L/D or D/L, possibly as a neurochemical affirmation of the occurrence of a stepwise transition in environmental illumination.

The iGL receives both photic inputs from the retina and nonphotic, activity-stimulated serotonergic inputs from the raphe nuclei. This information may be integrated at the level of the iGL, and the integrated output sent to the SCN by activation of the NPY projection of the GHT. There is extensive overlap of NPY and retinal inputs as well as NPY and serotonergic terminals at the VIP neurons of the ventrolateral SCN[15,23,29,66]. The serotonergic input to the SCN is believed to carry information about the arousal state of the animal, possibly mediating behavioral and social entrainment. Honrado and Mrosovsky[24] demonstrated that hamsters forced to be inactive became entrained to novel light–dark schedules at a slower rate than unrestrained animals. Furthermore, Van Reeth and Turek[67] showed that phase-shifts by dark pulses are blocked by immobilization, implying that activity, itself, may be a necessary component in activating these phase changes. This lends further support to the notion that there is communication between pathways activated by these different sources of time cues to the circadian system.

We have found that serotonin regulates the circadian pacemaker directly at ventrolateral SCN projection sites from the raphe[34]. Phase-shifting of the neuronal activity rhythm occurred only during the daytime and was mediated by the 5-HT_{1A} receptors. The greatest sensitivity to serotonin was at CT 7, a time at which NPY also induces significant phase-advances in the electrical activity rhythm. Unlike our finding with NPY, which phase-shifts the SCN during the night, serotonergic modulation at ventrolateral SCN sites is restricted to the daytime. Intriguingly, a general serotonergic, quipazine, when bath applied, caused phase-delays in SCN slices at night[54], whereas bath applied[55] or microdropped[34] 5-HT_{1A} agonists did not.

Finally, it should be noted that the endogenous sensitive periods of the rat SCN in vitro to NPY occur at periods preceding the light transition points in the

entrained 12L:12D cycle, by 7–3 h at dusk and 4–0 h at dawn. Interestingly, these NPY sensitive periods occur just before the rat SCN shows sensitivity to the pineal hormone, melatonin[32,33], the neuroendocrine signal of darkness. Because these sensitive periods are no doubt driven by the SCN clock, this observation raises the possibility that the substrates underlying these sensitivities are sequentially linked in the clock's mechanism.

This study has confirmed that NPY can play a regulatory role in the mammalian circadian system. Further, we have established the circadian timing of sensitivities to NPY, which anticipate the transitions in the entraining light–dark cycle. While NPY is not essential for generating circadian rhythmicity, there is increasing evidence suggesting that it is an integral element of the circadian system, providing behavior-coupled photic signals necessary for everyday entrainment to the environment.

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