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GASTRIN-RELEASING PEPTIDE AND NEUROPEPTIDE Y EXERT OPPOSING ACTIONS ON CIRCADIAN PHASE

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Abstract

Microinjection of gastrin releasing peptide (GRP) into the third ventricle or the suprachiasmatic nucleus (SCN) induces circadian phase shifts similar to those produced by light. Administration of GRP during the day does not alter circadian phase. In contrast, neuropeptide Y (NPY) induces phase shifts of circadian rhythms during the day but has little effect when administered at night, similar to the effects of most nonphotic stimuli. NPY inhibits the phase shifting effects of light, and GRP is thought to be part of the photic signaling system within the SCN. This experiment was designed to test whether GRP and NPY inhibit each other's effects on circadian phase. Adult male Syrian hamsters equipped with guide cannulas aimed at the SCN were housed in constant darkness until stable free-running rhythms of wheel running activity were apparent. Microinjection of GRP during the early subjective night induced phase delays that were blocked by simultaneous administration of NPY. During the middle of the subjective day, microinjection of NPY caused phase advances that were blocked by simultaneous administration of GRP. These data suggest that GRP and NPY oppose each other's effects on the circadian clock, and that the actions of NPY on the photic phase shifting mechanism in the SCN occur at least in part downstream from retinorecipient cells.

Keywords

photic; suprachiasmatic; Syrian hamster; entrainment

Introduction

The suprachiasmatic nucleus (SCN) drives physiological and behavioral circadian rhythms and responds to both photic and non-photoc cues to entrain to a 24-hour light dark cycle [8]. Photic information is conveyed to the SCN primarily by the retinohypothalamic tract (RHT), a monosynaptic projection from the retina to the SCN [30]. Non-photoc information, particularly relating to the activity level of the animal, is communicated to the SCN through a neuropeptide Y (NPY)-containing projection from the intergeniculate leaflet (IGL) [15;17] and a serotonergic projection from the median raphe nucleus [27]. These inputs are integrated by the SCN to regulate the entrainment of circadian rhythms.

The SCN is heterogeneous in structure and function [4;28]. The cells that comprise the SCN have notable variability in morphological appearance, electrophysiological firing rates, and

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patterns of gene expression. In Syrian hamsters, one neurochemical marker of the SCN is the presence of cells expressing gastrin releasing peptide (GRP) in a central region of the nucleus [25]. These cells are thought to be mediators of photic entrainment [21;35]. Animals exposed to a brief light pulse during the subjective night show increased immunoreactivity for c-fos in GRP cell bodies [12;20;33]. Microinjection of GRP into the third ventricle [3], or near the SCN [18;31], elicits phase shifts in a pattern similar to exposure of light. In mice, the loss of the GRP receptor results in a reduced phase shifting response to bright light [1]. These data suggest that GRP plays a role in the establishment of circadian phase.

NPY, synthesized in the intergeniculate leaflet of the thalamus and released in the SCN, conveys both photic and non-photoc signals [37]. Microinjection of NPY into the SCN region during the middle of the subjective day phase advances the circadian clock, while having little effect during the subjective night [2;16]. However, NPY can affect clock function during the subjective night by opposing the ability of light to induce a phase shift [23;36] and induce the expression of *Per1* mRNA [7;14]. These data suggest that NPY plays an active role in both photic and nonphotic effects on the circadian clock. Since light and NPY appear to exert opposing influences on the circadian clock, and GRP is part of the photic signal transduction pathway, we sought to determine whether NPY inhibited the effects of GRP administration on circadian clock phase. In contrast, there is little data regarding a role for GRP in nonphotic phase shifting. However, light can inhibit the phase shifting effects of NPY[6] and the phase shifting effects of behavioral activation [29]. Therefore, in order to determine whether GRP plays a role in mediating the effects of light on nonphotic phase shifting, we sought to determine whether GRP could inhibit NPY-induced phase advances.

Materials and Methods

Thirty-eight adult male Syrian hamsters (*Mesocricetus auratus*, 2-4 months) were bred at Kent State from stock derived from animals purchased from Harlan Sprague Dawley (Indianapolis, IN, USA). Animals were group-housed in a 14:10 h light:dark cycle with food and water available ad libitum. All experimental procedures were approved by the Kent State University Animal Care and Use Committee, and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Hamsters were anesthetized (110 mg/kg ketamine, 22 mg/kg xylazine and 1.83 mg/kg acepromazine) and stereotactically implanted with a 4-mm, 26-gauge guide cannula (Plastics One, Roanoke, VA, USA) aimed at SCN. Stereotaxic coordinates were 1.1 mm anterior and 1.7 mm lateral to bregma, with the depth such that a microinjection needle inserted through the guide cannula would end at 7.4 mm below dura. The cannulas were implanted at a 10° angle toward the midline. Prior to cannula insertion, the skull was leveled between bregma and lambda. After surgery, hamsters were individually housed in Plexiglas cages (24 × 45.5 × 21 cm) and given 24 h to recover in their original colony room. Hamsters were then placed in a continuously dark environment (DD), and were allowed to establish free-running activity rhythms. Cages were equipped with a running wheel (diameter 18 cm) that was monitored by a computer using ClockLab software (Actimetrics). After 10 days hamsters received the following treatments in randomized order: microinjection of 125 pmol gastrin releasing peptide (GRP; Phoenix Pharmaceuticals, Belmont, CA, USA) and 29.3 pmol Neuropeptide Y (NPY; Phoenix Pharmaceuticals), each dissolved in phosphate buffer saline (PBS), both GRP and NPY, or PBS alone. Microinjections were given with a 32-gauge needle that extended beyond the guide cannula by 5.2 mm, and was attached by polyethylene tubing to a 1 µl Hamilton syringe. The polyethylene tubing was filled with distilled water and an air bubble was introduced at the tip to avoid dilution of injected substances. Injections were given using night vision goggles while the hamsters were gently restrained by hand. The final volume of each microinjection was 250 nl, which were administered over a period of 10 seconds. After each microinjection, the needle was left in

place for 10-15 seconds. Microinjections were given at circadian time (CT) 6 ± 15 min or CT 13 ± 15 min. Following microinjection, hamsters were returned to their home cages.

After completion of each behavioral experiment, animals were deeply anesthetized with sodium pentobarbital (200 mg/kg) and killed by decapitation. Hamster brains were extracted and post-fixed in 4% paraformaldehyde. Coronal sections (100 μ m thick) were sliced on a vibratome and counterstained with hemotoxylin. Injection sites were verified using light microscopy. Only animals with visible needle tracts that were within 300 μ m of the SCN were used in this study. Six animals (16% of total) were excluded from this study because the microinjection sites were farther than 300 μ m from the SCN. Phase shifts in the circadian activity rhythm were quantified using the linear regression method [10]. A line was fitted to activity onsets that occurred on the 10 days preceding the microinjection. The onsets of activity for each day were initially determined by Clocklab data analysis software (Actimetrics), and then visually inspected for artifacts. Days when the software could not calculate an onset were omitted from further calculations. A second line was fitted to activity onsets that occurred 4-10 days after microinjection. Days 1-3 post-microinjection were not used in the data analysis to avoid including transient effects. Phase shifts were determined by the difference between the two regression lines on the day following treatment. All of the work involved in phase shift calculation was performed by individuals blind to experimental treatments. Phase shifts are shown as mean $\pm$ standard error. Differences between groups were evaluated using a 1-way ANOVA and the Tukey-Kramer test.

Results

Experiment 1: Effects of NPY on GRP-induced phase delays

This experiment tested the hypothesis that NPY would inhibit phase delays induced by microinjection of GRP into the SCN region. There was a significant difference among experimental groups injected at CT 13 (1-way ANOVA, $P = 0.000006$). Microinjection of GRP induced delays of the circadian activity rhythm (-0.67 ± 0.12 h, $n=8$) that were significantly different from animals that received NPY (0.16 ± 0.10 h, $n=9$), a cocktail of GRP and NPY (-0.05 ± 0.08 h, $n=9$), or vehicle (0.07 ± 0.07 h, $n=9$) (Tukey-Kramer test, $P < 0.05$) (Fig. 1 and 3).

Experiment 2: Effects of GRP on NPY-induced phase advances

This experiment tested the hypothesis that GRP would attenuate NPY-induced phase advances. There was a significant difference among experimental groups at CT 6 (1-way ANOVA, $P = 0.00001$). Microinjection of NPY into the SCN region induced advances of the circadian activity rhythm (0.53 ± 0.12 h, $n=11$) that were significantly different from animals that received GRP (-0.16 ± 0.03 h, $n=7$), a cocktail of NPY and GRP (-0.07 ± 0.10 h, $n=7$), or vehicle (0.05 ± 0.06 h, $n=8$) (Tukey-Kramer test, $P < 0.05$) (Fig. 2 and 3).

Discussion

The results of this study suggest that GRP and NPY have mutually inhibitory effects on the other's influence on circadian rhythmicity. During the early subjective night, microinjection of NPY into the SCN region blocked the phase shifting effects of GRP. Previous studies have shown that NPY [23] or an NPY receptor agonist [13] is able to attenuate light-induced phase advances in the late subjective night, even when NPY is administered up to 60 minutes after the light pulse [22]. However, the data on the ability of NPY to inhibit light-induced delays in the early subjective night is mixed. Some reports indicate that NPY can attenuate light-induced delays [23;24]. However, others show that NPY in the early subjective night does not attenuate light-induced delays, even when administered at high concentrations [13]. In addition to

altering behavioral phase shifts during the late subjective night, NPY has been shown to inhibit the effects of light on the induction of clock genes [7;14]. Taken together, these data support the idea that the transmission of photic information to the circadian clock mechanisms does not follow a linear pathway. The fact that NPY is capable, at least in some circumstances, of blocking both light and GRP-induced phase delays would suggest that NPY is acting downstream from GRP signal reception in the SCN to inhibit light-induced phase shifts. However, GRP-induced phase delays are also dependent on the level of glutamatergic neurotransmission in the SCN [18], and glutamatergic input from the retinas is thought to be upstream from GRP signaling. This suggests that there are parallel pathways for photic information within the SCN, including both GRP-dependent and GRP-independent routes. This idea is supported by data from GRP receptor knockout mice that show a moderately attenuated phase-shifting response to bright light [1], but not complete inhibition.

There is a continually growing body of evidence that indicates that GRP plays a major role in photic resetting of the circadian clock by acting as a signal that communicates photic conditions to the oscillator cells of the SCN. GRP immunoreactivity is localized in the retinorecipient regions of the rodent SCN [26;28], GRP receptors are localized in the dorsal SCN [19], microinjection of GRP near the SCN induces expression of c-fos in the dorsal SCN [3;32], and microinjection of GRP into the third ventricle induces *Per1*, *Per2*, and the phosphorylated form of extracellular related kinase (p-ERK) in the dorsal SCN [3]. Additionally, the presentation of a light pulse to animals kept in constant conditions induces *Per1* expression in GRP-containing cells [11]. Together, these data strongly support the idea that GRP plays a significant role in transmitting photic information within the SCN, thereby affecting the circadian phase and cellular activity of the SCN. Non-photoc input, mediated at least in part by NPY, would then inhibit these effects of GRP through the suppression of GRP-induced period gene expression.

Although NPY does not alter circadian phase when injected during the early subjective night, it does induce phase advances when given during the subjective day, both *in vivo* [2;16] and *in vitro* [5;34]. Because the results of experiment 1 indicated that NPY can inhibit GRP-induced delays in the early night, and because exposure to light during the subjective day can inhibit the phase shifting effects of NPY [6], we tested whether GRP inhibits NPY-induced phase advances. The results of experiment 2 indicate that NPY-induced phase advances are attenuated by GRP, and suggests the possibility that NPY-induced phase advances are regulated by the level of stimulation being provided by GRP release within the SCN.

The precise signaling mechanism for the interaction between GRP and NPY-induced signaling is unknown. One possibility is that receptors for both GRP and NPY are colocalized to the same cells, and that the inputs from each neuropeptide activate opposing intracellular signaling cascades. A second possibility is that each neuropeptide does not prevent SCN cells from responding to the other, but rather prevents the change in clock phase from being communicated to the rest of the SCN. GRP appears to activate a select subset of neurons in the dorsal SCN, which are then likely to transmit that information to other parts of the nucleus [3;32]. Blocking communication from these GRP-responsive neurons could inhibit the transfer of phase information to the rest of the SCN. A direct interaction between GRP and NPY is supported by neuroanatomical evidence: NPY fibers in the SCN show broad overlap with both GRP-containing cell bodies and GRP fibers [28].

It is important to note however that the effects of NPY on GRP-induced phase advances in the late subjective night were not examined in this study. There is evidence indicating that phase delays and advances are not always mediated by the same factors, or signaling events [9]. However, a consensus exists in the literature that NPY is able to block light-induced advances

in the late night. Therefore, we hypothesize that NPY would also attenuate GRP-induced advances when administered near the SCN in the late subjective night.

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Figure 1.

Representative actograms of circadian activity rhythms before and after microinjection of (A) GRP, (B) NPY, (C) GRP/NPY cocktail, and (D) SAL. Each line represents one 24-h period. Dark bars depict wheel-running activity. All injections were given with the aid of night vision goggles at CT 13 (indicated by a white circle). The regression lines used for calculating the phase shifts are shown along the onsets of activity, and the calculated phase shifts are shown.

Figure 2.

Representative actograms of circadian activity rhythms before and after microinjection of (A) NPY, (B) GRP, (C) NPY/GRP cocktail, and (D) SAL. Each line represents one 24-h period. Dark bars depict wheel-running activity. All injections were given with the aid of night vision goggles at CT 6 (indicated by a white circle). The regression lines used for calculating the phase shifts are shown along the onsets of activity, and the calculated phase shifts are shown.

Figure 3. Mean $\pm$ SEM phase shift in hours induced by microinjection of GRP, NPY, GRP/NPY, and SAL near the SCN at CT 6 and CT 13. NPY is different from all other groups at CT6, and GRP is different from all other groups at CT 13 (Tukey-Kramer, $p < 0.05$).