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Site-specific effects of gastrin-releasing peptide in the suprachiasmatic nucleus

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Abstract

The effects of gastrin-releasing peptide (GRP) on the circadian clock in the suprachiasmatic nucleus (SCN) are dependent on the activation of *N*-methyl-D-aspartate (NMDA) receptors in the SCN. In this study, the interaction between GRP, glutamate and serotonin in the regulation of circadian phase in Syrian hamsters was evaluated. Microinjection of GRP into the third ventricle induced *c-fos* and p-ERK expression throughout the SCN. Coadministration of an NMDA antagonist or 8-hydroxy-2-di-n-propylamino-tetralin [a serotonin (5-HT)_{1A,7} agonist, DPAT] with GRP limited *c-fos* expression in the SCN to a region dorsal to GRP cell bodies. Similar to the effects of NMDA antagonists, DPAT attenuated GRP-induced phase shifts in the early night, suggesting that the actions of serotonin on the photic phase shifting mechanism occur downstream from retinorecipient cells. *c-fos* and p-ERK immunoreactivity in the supraoptic (SON) and paraventricular hypothalamic nuclei also increased following ventricular microinjection of GRP. Because of this finding, a second set of experiments was designed to test a potential role for the SON in the regulation of clock function. Syrian hamsters were given microinjections of GRP into the peri-SON during the early night. GRP-induced *c-fos* activity in the SCN was similar to that following ventricular administration of GRP. GRP or bicuculline (a γ -aminobutyric acid_A antagonist) administered near the SON during the early night elicited phase delays of circadian activity rhythms. These data suggest that GRP-induced phase-resetting is dependent on levels of glutamatergic and serotonergic neurotransmission in the SCN and implicate activity in the SON as a potential regulator of photic signaling in the SCN.

Keywords

circadian; clock; *c-fos*; supraoptic; *Mesocricetus auratus*

Introduction

The mammalian suprachiasmatic nucleus (SCN) contains an endogenous clock that regulates physiological and behavioral circadian rhythms (Moore & Eichler, 1972; Stephan & Zucker, 1972; Moore & Card, 1985). In Syrian hamsters (*Mesocricetus auratus*), light is an essential entraining cue for this clock. Photic information is transmitted to the SCN by the retinohypothalamic tract (RHT), which is a monosynaptic projection from the retina to the SCN (Hendrickson et al., 1972; Pickard, 1982). The RHT is essential for photic entrainment, given that surgical destruction of this projection leads to the inability to entrain to environmental light–dark cycles (Johnson et al., 1988). Glutamate (Ding et al., 1994; Mintz & Albers, 1997; Mintz et al., 1999) and pituitary adenylate cyclase-activating

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polypeptide (Hannibal et al., 1997) are thought to be the critical neurotransmitters of this pathway.

Several reports have identified gastrin-releasing peptide (GRP) as a primary candidate for the transduction of photic information within the SCN (Piggins & Rusak, 1993; McArthur et al., 2000; Karatsoreos et al., 2004; Antle et al., 2005; Piggins et al., 2005). GRP cells receive direct input from the RHT, suggesting that GRP plays a role in mediating the effects of light on the circadian clock (Tanaka et al., 1997; Abrahamson & Moore, 2001). GRP cell bodies are located in the ventral SCN in the rat (Dardente et al., 2002) and in the central region of the hamster SCN (Karatsoreos et al., 2004, 2006) and the receptors that GRP acts on are localized dorsally. Animals in constant conditions presented with a light pulse express the immediate early gene *c-fos* in GRP cells (Earnest et al., 1993; Romijn et al., 1996). Exogenous application of GRP to the third ventricle (Antle et al., 2005), or near the SCN (Albers et al., 1991; Piggins et al., 1995), phase shifts the clock in a manner similar to light. The effects of GRP on the circadian clock are dependent on the level of excitation provided by activated *N*-methyl-D-aspartate (NMDA) receptors (Kallingal & Mintz, 2006; 2010) and can be opposed by neuropeptide Y (NPY; Kallingal & Mintz, 2007). Finally, previous reports indicate that the administration of GRP in the early subjective night increases *c-fos* (Piggins et al., 2005) and the phosphorylated form of extracellular regulated kinase (p-ERK) (Antle et al., 2005) in a region just dorsal to GRP cell bodies. These data suggest that GRP plays an active role in mediating the effects of light on the circadian clock. However, we still have a relatively poor understanding of the functional role of GRP in the photic signaling pathway and GRP's function may relate to the structural organization of the SCN.

The current study was designed to determine the immunohistological and behavioral effects of GRP administered to the third ventricle in the presence or absence of glutamate antagonists. In addition, serotonin agonists inhibit the phase shifting effects of light, but parallel effects on the phase shifting effects of GRP have not been investigated. Therefore, we also tested the effects of GRP in the presence of a 5-HT_{1A,7} agonist. Finally, in light of our initial results, we also investigated the activation of neuronal markers within the SCN and the behavioral response to GRP and a γ -aminobutyric acid (GABA) antagonist following administration of either substance to a region just dorsal to the supraoptic nucleus (SON).

Materials and methods

Subjects

In total, 115 adult male Syrian hamsters (2–4 months) were bred from hamsters 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 followed all guidelines laid down by the United States National Institutes of Health regarding the care and use of animals for experimental procedures.

Surgical procedure—Hamsters were anesthetized with an anesthesia cocktail (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 either the third ventricle or a region just dorsal to the SON. Stereotaxic coordinates for the third ventricle were 1.2 mm anterior to bregma and 7.2 mm ventral to dura along the midline. Coordinates for the region just dorsal to the SON were 1.2 mm anterior and 1.7 mm lateral to bregma and 8.1 mm ventral to dura. Prior to cannula insertion, the skull was leveled between bregma and lambda. The guide cannulas were implanted to a depth of 2.0 mm below dura (third ventricle) or 2.9 mm below dura (SON). 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. These cages were equipped with a running wheel (diameter 18 cm) that was monitored by a computer using ClockLab software (Actimetrics, Wilmette, IL, USA).

Microinjection—After 10 days of free-running activity, microinjections were given with a 32-gauge needle that extended beyond the guide cannula by 5.2 mm (to reach the final stereotaxic coordinate depth), attached by polyethylene tubing to a 1- μ L Hamilton syringe. The polyethylene tubing was filled with distilled water and an air bubble was introduced to avoid dilution of injected substances. Injections were given with the aid of night vision goggles while the hamsters were gently restrained by hand. The final volume of each microinjection was 1 μ L for ventricular injections and 250 nL for SON injections, both of which were administered over a period of 10 s. After each microinjection, the needle was left in place for 10–15 s. Microinjections were given at circadian time (CT) 13 $\pm$ 15 min, and were all unilateral. Following microinjection, hamsters were returned to their home cages.

Experiments 1 and 2—Hamsters implanted with guide cannulas aimed at the third ventricle received the following treatments in randomized order: microinjection of 0.5 nmol GRP (Phoenix Pharmaceuticals, Belmont, CA, USA), 4.94 nmol ($\pm$)-2-amino-5-phosphonopentanoic acid (AP5; Research Biochemicals International, Natic, MA, USA) and 7.6 nmol 8-hydroxy-2-di-n-propylamino-tetralin (DPAT; Sigma-Aldrich, St Louis, MO, USA), each dissolved in phosphate-buffered saline (PBS), a GRP/AP5 cocktail, a GRP/DPAT cocktail or PBS. Following injection, hamsters were returned to their home cages for either 1 h (for analysis of c-fos immunoreactivity) or 30 min (for p-ERK immunoreactivity) before being killed and processed for immunohistochemistry.

Experiment 3—Hamsters received microinjections aimed at the third ventricle of 0.5 nmol GRP, 7.6 nmol DPAT, and a cocktail of 0.5 nmol GRP and 7.6 nmol DPAT, each dissolved in PBS, or PBS. Following microinjections, hamsters were returned to their home cages and their behavioral rhythms were monitored. The order of injections was counterbalanced and a minimum of 10 days separated each injection.

Experiment 4—Hamsters implanted with guide cannulas aimed at a region just dorsal to the SON received the following treatments in randomized order: microinjection of 125 pmol GRP, 1.24 nmol AP5, 1.9 nmol DPAT, a cocktail of GRP/AP5 or GRP/DPAT or PBS. These doses represent the same concentrations as in the ventricular injections in Experiments 1–3 but in a smaller volume. Following injection, hamsters were returned to their home cages for 1 h before being killed and processed for c-fos immunohistochemistry.

Experiments 5 and 6—Hamsters prepared as in Experiment 4 received microinjections aimed at a region just dorsal to the SON of 125 pmol GRP (same concentration as in Expt 4) or 0.9 nmol (–)-bicuculline methobromide (BIC; Tocris, Ellisville, MO, USA), or PBS. Following microinjections, hamsters were returned to their home cages and their behavioral rhythms were monitored. The order of injections was counterbalanced and a minimum of 10 days separated each injection.

Perfusion—Hamsters used for immunohistochemistry were deeply anesthetized with sodium pentobarbital (200 mg/kg) in the dark and their heads were covered in aluminum foil during trans-cardial perfusion. Each hamster was perfused with 150 ml of PBS followed by 250 ml of 4% paraformaldehyde. Hamster brains were extracted and post-fixed in 4% paraformaldehyde at 4°C for 1–2 days prior to sectioning.

Immunohistochemistry—Coronal sections (40 μm thickness) were sliced on a vibratome and free floating sections were saved in three vials. c-fos immunohistochemistry (rabbit anti c-fos, 1:10,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and p-ERK immunohistochemistry (rabbit anti-phospho-42/44 MAPK, 1:1,000; Cell Signaling Technology, Danvers, MA, USA) were carried out using standard avidin–biotin labeling techniques. Ninety-three sections were washed with PBS and hydrogen peroxide. After additional rinses with PBS, sections were incubated in a solution containing 5% normal donkey serum in antisera diluent with 0.3% Triton-X for 1 h to reduce nonspecific labeling. Sections were then incubated overnight with primary antibodies in antisera diluent. Tissue was rinsed with PBS several times prior to incubation with biotinylated-SP-conjugated affipure donkey anti-rabbit IgG (1:500; Jackson ImmunoResearch, West Grove, PA, USA). Sections were subsequently washed and then incubated in avidin–biotin complex (Vector Elite Kit; Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature. Following additional rinses with PBS, reaction product was visualized using DAB (Vector Laboratories). Sections were then mounted on gel-coated slides, dehydrated through sequential series of alcohol washes and then cleared with Citrisolv and coverslipped with DPX.

Histology for behavioral experiments—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 μm thickness) were sliced on a vibratome and counterstained with hemotoxylin. Injection sites were verified using light microscopy. Only animals with visible needle tracts that penetrated the third ventricle or were within 300 μm of the SON were used in this study. Twenty-three animals (18% of total) were excluded from this study because the microinjection sites were either outside of the third ventricle or farther than 300 μm from the SON.

Data analysis of phase shifts—Phase shifts in the circadian activity rhythm were quantified using Actimetrics Clocklab software. A line was fitted to activity onsets that occurred on the 10 days preceding the microinjection. 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. Differences between groups were evaluated using a one-way analysis of variance (ANOVA; Expt 3) or two-sample *t*-tests (Expts 5 and 6).

Quantification of c-fos immunoreactivity—To assess the distribution of labeled cells in the SCN following ventricular injections, images of the anterior, medial and posterior SCN were captured using an Olympus BX-51 microscope. Additional images of extra-SCN regions, including the SON, paraventricular nucleus (PVN) and cortex, were also obtained. To assess the distribution of labeled cells following injections aimed at the SON, similar images were captured but the ipsilateral and contralateral components of the SON, SCN and PVN were analysed separately. Images were loaded into ImageJ software and the background was removed by setting a manual threshold to include labeled cells. The average area of a c-fos-positive nucleus was determined and the total stained area was calculated. The average number of c-fos-positive nuclei was estimated as the total stained area divided by the average size of positive nuclei. A set of 30 c-fos-labeled sections was also manually counted and compared with the results from the estimation method to verify the validity of the results. Results of the two methods showed a Pearson's correlation of 0.94. Differences between groups were analysed using a one-way ANOVA for third ventricle injections and a

two-way repeated-measures ANOVA for SON injections. Dunnett's test was used to assess differences between each group and GRP injections.

Quantification of p-ERK immunoreactivity—To assess the distribution of p-ERK immunoreactivity in the SCN, SON, and PVN following ventricular injections, images of the anterior, medial and posterior SCN were captured using an Olympus BX-51 microscope. Images were analysed using ImageJ software. The optical density of each region was determined by subtracting the optical density of a nearby unstained brain region (background) from that of the selected region. The region of interest was drawn freehand for each section by an individual blind to the experimental condition. Differences between groups were assessed by one-way ANOVA. Dunnett's test was used to assess differences between each group and GRP injections.

Results

Experiment 1: effects of ventricular administration of GRP on c-fos expression in the SCN, SON and PVN

This experiment was designed to test whether GRP-induced c-fos expression in the SCN is altered by co-administration of an NMDA antagonist or a 5-HT_{1A,7} agonist. Animals received a microinjection of GRP, GRP and AP5, GRP and DPAT, or vehicle ($n = 6$ per group). Significant differences between injection types were detected in the anterior (one-way ANOVA, $F_{3,16} = 4.34$, $P = 0.02$), medial (one-way ANOVA, $F_{3,18} = 31.93$, $P < 0.00001$) and posterior (one-way ANOVA, $F_{3,16} = 6.82$, $P = 0.004$) SCN (Fig. 1). At the level of the medial SCN, the number of c-fos-expressing cells was significantly larger in GRP-injected groups than GRP/AP5, GRP/DPAT or vehicle (Dunnett's test, $P < 0.05$). At the level of the anterior and posterior portions of the SCN, the number of c-fos-expressing cells was significantly larger in the GRP-injected groups than GRP/DPAT or vehicle (Dunnett's test, $P < 0.05$). When further analysis was conducted to differentiate c-fos activation in the ventral and dorsal regions of the SCN, significant differences between treatment groups were detected in the ventral third (one-way ANOVA, $F_{3,18} = 27.06$, $P = 0.00001$) and dorsal two-thirds (one-way ANOVA, $F_{3,18} = 17.82$, $P = 0.00001$) regions of the medial SCN (Fig. 2). In both the ventral and the dorsal regions, the number of c-fos-positive cells was significantly larger in the GRP-injected groups than GRP/AP5, GRP/DPAT or vehicle (Dunnett's test, $P < 0.05$). To assess whether the magnitude of drug effects differed between region, values were normalized to the mean of the saline groups for the corresponding dorsal or ventral analysis, and compared with a two-way repeated-measures ANOVA. There was a significant interaction between region and treatment ($F_{3,18} = 8.09$, $P = 0.001$). GRP increased c-fos by a factor of 10.33 ± 1.01 in the dorsal SCN but only by 5.88 ± 0.82 in the ventral SCN (Dunnett's test, $P < 0.05$). In the ventral SCN AP5 reduced GRP-induced c-fos to 0.91 ± 0.16 times that of the saline group and DPAT reduced GRP-induced c-fos to 0.93 ± 0.21 times that of the saline group. By comparison, AP5 and DPAT reduced GRP-induced c-fos to 6.29 ± 1.01 and 7.44 ± 0.55 times that of saline in the dorsal SCN, both significantly higher than expressed in the ventral SCN (Dunnett's test, $P < 0.05$). Extra-SCN regions were also analysed, including the SON, PVN and a small part of the cortex from the same sections as the SCN analysis. Significant differences between injection types were detected in the SON (one-way ANOVA, $F_{3,18} = 18.92$, $P = 0.00001$; and the PVN Fig. 2) and the PVN (one-way ANOVA, $F_{3,18} = 8.08$, $P = 0.001$; Fig. 2), but not the cortex (one-way ANOVA, $F_{3,15} = 2.79$, $P = 0.08$). In the SON and PVN, the number of c-fos-expressing cells was significantly larger in GRP-injected groups than vehicle (Dunnett's test, $P < 0.05$).

Experiment 2: effects of ventricular administration of GRP on p-ERK expression in the SCN, SON and PVN

This experiment was designed to test whether GRP-induced p-ERK expression in the SCN is altered by co-administration of an NMDA antagonist or a 5-HT_{1A,7} agonist. Animals received a microinjection of GRP ($n = 6$), GRP and AP5 ($n = 7$), GRP and DPAT ($n = 6$), or vehicle ($n = 5$). In general, the results were similar to those found for c-fos in Expt 1. Significant differences between injection types were detected in the anterior (one-way ANOVA, $F_{3,13} = 6.87$, $P = 0.005$), medial (one-way ANOVA, $F_{3,15} = 6.37$, $P = 0.005$) and posterior (one-way ANOVA, $F_{3,15} = 5.60$, $P = 0.009$) SCN (Fig. 3). At the level of the anterior and medial SCN, the amount of p-ERK immunoreactivity was significantly larger in the GRP-injected groups than GRP/AP5, GRP/DPAT or vehicle (Dunnett's test, $P < 0.05$). At the level of the posterior SCN, positive p-ERK immunoreactivity was significantly larger in the GRP-injected groups than GRP/DPAT or vehicle (Dunnett's test, $P < 0.05$). As in Expt 1, when further analysis was conducted to differentiate p-ERK activation in the ventral and dorsal regions of the SCN, significant differences between injection types were detected in the ventral third (one-way ANOVA, $F_{3,14} = 8.37$, $P = 0.002$) and dorsal two-thirds (one-way ANOVA, $F_{3,14} = 3.75$, $P = 0.04$) regions of the medial SCN (Fig. 4). In the ventral region, the amount of p-ERK immunoreactivity was significantly larger in the GRP-injected groups than GRP/AP5, GRP/DPAT or vehicle (Dunnett's test, $P < 0.05$). In the dorsal region, however, p-ERK immunoreactivity in the GRP-treated groups was significantly larger than vehicle but not the AP5- or DPAT-treated groups (Dunnett's test, $P < 0.05$). To assess whether the magnitude of drug effects differed between region, values were normalized and compared with a two-way repeated-measures ANOVA as in Expt 1. There was a significant interaction between region and treatment ($F_{3,14} = 10.06$, $P = 0.001$). GRP increased p-ERK by similar amounts in the dorsal and ventral SCN (1.73 ± 0.14 and 0.99 ± 0.39 times saline, respectively, Dunnett's test, $P > 0.05$). However, in the GRP/DPAT group pERK was significantly higher in the dorsal SCN than the ventral SCN (2.66 ± 0.36 and 0.65 ± 0.24 times saline, respectively, Dunnett's test, $P < 0.05$), suggesting a greater effect of serotonin receptor activation ventral SCN. Similar to Expt 1, extra-SCN regions of the hypothalamus were analysed, including the SON and PVN. Significant differences between injection types were not detected in the SON (one-way ANOVA, $F_{3,15} = 2.48$, $P = 0.1$) or the PVN (one-way ANOVA, $F_{3,15} = 2.31$, $P = 0.12$) (Fig. 4).

Experiment 3: effects of a 5-HT_{1A,7} agonist on GRP-induced phase delays

This experiment was designed to test whether microinjection of a 5-HT_{1A,7} agonist would alter the phase shifting effects of GRP when administered into the third ventricle in the early subjective night. To investigate the behavioral effects of increased serotonergic neurotransmission on GRP-induced shifts, the effect of DPAT on GRP-induced phase delays was evaluated. Animals received a microinjection of GRP ($n = 9$), GRP and DPAT ($n = 7$), DPAT ($n = 11$) or vehicle ($n = 12$) and significant differences were detected between the injection types (one-way ANOVA, $F_{3,35} = 9.11$, $P = 0.0001$). GRP-microinjected animals showed phase delays of the circadian activity rhythm (0.70 ± 0.08 h) that were significantly greater (Dunnett's test, $P < 0.05$) than those that received a cocktail of GRP and DPAT (0.01 ± 0.13 h), DPAT (0.16 ± 0.11 h) or vehicle (0.03 ± 0.09 h) (Fig. 5).

Experiment 4: effects of GRP administration near the SON on c-fos induction in the SCN, SON and PVN

As ventricular administration of GRP induced c-fos expression in the SON, we wanted to determine whether GRP could directly elicit this effect through action in the SON region. Animals received a microinjection of GRP ($n = 7$), GRP and AP5 ($n = 8$), GRP and DPAT ($n = 7$) or vehicle ($n = 6$) into a region immediately dorsal to the SON (i.e. near the SON but

not through it, Fig. 6). Factors in the analysis included treatment (injection type) and sidedness (ipsilateral vs. contralateral). In the anterior SCN, there were no significant factors [two-way repeated-measures ANOVA of treatment ($F_{3,12} = 2.28$, $P = 0.13$), sidedness ($F_{1,12} = 0.37$, $P = 0.56$), or interaction ($F_{3,12} = 0.76$, $P = 0.54$)]. In the medial SCN, there was a significant effect of treatment ($F_{3,12} = 4.93$, $P = 0.02$), but not sidedness ($F_{1,12} = 0.07$, $P = 0.80$) or the interaction term ($F_{3,12} = 0.10$, $P = 0.96$). In the posterior SCN, there were no significant factors: treatment ($F_{3,12} = 2.32$, $P = 0.13$), sidedness ($F_{1,12} = 0.01$, $P = 0.92$) or interaction ($F_{3,12} = 0.04$, $P = 0.99$, Fig. 6). At the level of the medial SCN, the number of c-fos-expressing cells was significantly larger in the GRP-injected groups than GRP/AP5 or vehicle (Dunnett's test, $P < 0.05$). When further analysis was conducted to differentiate c-fos activation in the ventral and dorsal regions of the SCN, significant differences in both treatment and sidedness between injection types were detected in the ventral third [two-way repeated-measures ANOVA: treatment ($P = 0.01$); sidedness ($P = 0.02$); interaction ($P = 0.13$)] and only differences in treatment were noted in the dorsal tw-thirds [two-way repeated-measures ANOVA: treatment ($P = 0.03$); sidedness ($P = 0.41$); interaction ($P = 0.65$)] regions of the medial SCN (Fig. 7). While in the ventral region, the number of c-fos-positive cells was significantly larger in the GRP-injected group than in all other groups, in the dorsal region, the number of c-fos-positive cells was greater in the GRP-injected groups than only GRP/AP5 and vehicle (Dunnett's test, $P < 0.05$). In the ventral SCN, the number of c-fos-expressing cells was larger in the ipsilateral and contralateral SCN of GRP-injected groups than all other groups examined (Dunnett's test, $P < 0.05$). After normalizing data to the mean saline value for each region, no significant interaction was detected between treatment and region ($F_{3,16} = 1.39$, $P = 0.28$). We also analysed c-fos expression in the SON, PVN and cortex. Significant differences between treatment, but not sidedness, were detected in the SON [two-way repeated-measures ANOVA: treatment ($P = 0.005$); sidedness ($P = 0.87$); interaction ($P = 0.11$); Fig. 7] and the PVN [two-way repeated-measures ANOVA: treatment ($P = 0.038$); sidedness ($P = 0.47$); interaction ($P = 0.85$); Fig. 8], but not the cortex [two-way repeated-measures ANOVA: treatment ($P = 0.95$; data not shown)]. In the SON, the number of c-fos-expressing cells in GRP-injected groups was significantly larger than in GRP/AP5-injected groups (Dunnett's test, $P < 0.05$). In the PVN, the number of c-fos-expressing cells in GRP-injected groups was not significantly larger than any of the other groups examined (Dunnett's test, $P > 0.05$).

Experiment 5: effects of GRP administration near the SON on circadian phase

This experiment tested the hypothesis that exogenous application of GRP to a region just dorsal to the SON would induce delays of the circadian activity rhythm. Microinjection of GRP near the SON at CT 13 produced delays (0.743 ± 0.159 h, $n = 10$) in circadian phase that were significantly different from those produced by vehicle (0.186 ± 0.069 h, $n = 10$) ($t_{18} = 3.20$, $P = 0.005$) (Fig. 9A and B).

Experiment 6: effects of a GABAergic antagonist administered near the SON on circadian phase

Because GRP microinjected near the SON induced phase delays in the early subjective night, we tested whether a GABA antagonist would similarly induce shifts of the circadian phase. Infusion of BIC into the SCN should excite most neurons in the area, and because BIC does not shift rhythms when injected into the SCN region (Gillespie et al., 1996), any phase shifts resulting from microinjection of BIC into the SON region would not be attributable to diffusion of the drug to the SCN. Microinjection of BIC in a region just dorsal to the SON at CT 13 elicited delays (0.598 ± 0.132 h, $n = 11$) in circadian phase that were significantly different from those produced by vehicle (0.067 ± 0.099 h, $n = 11$; $t_{20} = 3.20$, $P = 0.005$) (Fig. 9C and D).

Discussion

The results of this study add to the growing body of evidence that GRP plays an important role in the phase-resetting mechanism of the circadian clock. Our data provide new information on potential sites of action of GRP in the circadian system, and implicates the SON as having a potential role in regulating circadian phase. Recent studies have suggested that one potential role of GRP is to affect circadian phase by transmitting information from the retinorecipient region of the ventral SCN to oscillator cells of the dorsomedial SCN (Abrahamson & Moore, 2001; Karatsoreos et al., 2004, 2006). While the precise location of GRP-immunoreactive neuronal cell bodies varies between species, they are generally localized in the ventral half of the SCN, from the central to ventrolateral region of the SCN (Tanaka et al., 1997; Abrahamson & Moore, 2001; Karatsoreos et al., 2004, 2006) and GRP receptors are localized dorsal to the GRP cell bodies (Karatsoreos et al., 2006). Also, a brief light pulse presented to animals in constant conditions induces the expression of *c-fos* and *Per1* in GRP-containing cells (Karatsoreos et al., 2004) that receive direct retinal innervation through the RHT (Tanaka et al., 1997), and result in long-lasting changes in firing rate (LeSauter et al., 2011). Furthermore, it has been previously reported that microinjection of GRP into the third ventricle induces the expression of *c-fos*, *Per1*, *Per2* and the activated form of ERK in the dorsal SCN (Antle et al., 2005; Gamble et al., 2007). Finally, GRP receptor knockout mice show attenuation of both light-induced phase shifts and the induction of *Per* and *cfos* protein in the dorsal SCN (Aida et al., 2002). Together, these data strongly support the notion that GRP plays an important role in transducing photic information to affect circadian phase and cellular activity within the SCN.

The initial aim of this study was to examine the interactions of GRP with other neurotransmitter systems, specifically glutamate and serotonin. Our lab has previously shown that the effects of GRP in the SCN are mediated in part by the concurrent activation of NMDA receptors (Kallingal & Mintz, 2006, 2010), and can be opposed by carriers of non-photoc signals such as NPY (Kallingal & Mintz, 2007). Previous reports using hamsters have indicated that the ventricular administration of GRP induces *c-fos* in a subset of cells that are dorsal to GRP cell bodies (Antle et al., 2005; Piggins et al., 2005). In Expt 1, the expression of *c-fos* in the dorsal SCN following ventricular administration of GRP was similar to these earlier reports; however, in this study and contrary to earlier findings, GRP induced *c-fos* expression in the ventral SCN as well. We cannot explain why our results differ from those studies, although it is worth noting that in mice, GRP microinjections produce cellular responses in a pattern similar to our data for hamsters (Gamble et al., 2007). Our results showed that GRP-induced increases in the expression of *c-fos* were attenuated when GRP was combined with either AP5 or DPAT, suggesting that the actions of GRP in the SCN are modulated in part by glutamate and serotonin. These results are significant in that the expression of *c-fos* in the ventral SCN following the ventricular administration of GRP correlates nicely with the behavioral response of GRP administration on the circadian activity rhythm. In other words, the activation of cells in the ventral region of the rodent SCN may be necessary for phase shifts to occur. Our data suggest that serotonin can inhibit the effects of GRP on the SCN in addition to its ability to inhibit the release of GRP itself (Francel et al., 2010). The ability of AP5 to attenuate GRP-induced *c-fos* expression in the ventral SCN and the complete inhibition of GRP-induced shifts by AP5 *in vivo* (Kallingal & Mintz, 2006) further support the hypothesis that activation of the ventral SCN is a necessary component of the GRP phase-shifting pathway.

Additionally, ventricular administration of GRP increased *c-fos* expression in the SON and PVN. Unlike their effects on *c-fos* expression in the SCN, AP5 and DPAT did not suppress GRP-induced *c-fos* in the SON and PVN, indicating that the response to GRP in these regions is not mediated by the levels of glutamatergic or serotonergic neurotransmission,

and that this interaction between neurotransmission systems is specific to the SCN among the targets investigated. These findings support earlier reports which indicate that neuroendocrine structures of the hypothalamus interact with the SCN to drive circadian activities. Circadian rhythms in plasma arginine-vasopressin and oxytocin concentrations, both of which are synthesized by the PVN and SON, have been identified in a number of species (Greeley et al., 1982; Wathes et al., 1992; Saeb-Parsy & Dyball, 2004). Furthermore, stimulation of the optic nerves can cause electrical stimulation of these neuroendocrine structures (Saeb-Parsy & Dyball, 2004). Although the exact mechanisms remain unclear, it is becoming increasingly clear that the SCN, PVN and SON comprise a neuronal network that may affect photic entrainment.

To expand on our results from Expt 1, we examined another marker of neuronal activation to examine the effects of GRP on the cells of the SCN and other hypothalamic regions. By tracking the expression of p-ERK in response to GRP we could identify specific pathways involved in GRP signaling. The results generally paralleled those found for changes in c-fos expression. Ventricular administration of GRP in the early subjective night induced p-ERK activation throughout the SCN. AP5 and DPAT inhibited GRP's effects on p-ERK induction in the ventral SCN. Again, it appears that the activation of cells within the ventral region of the hamster SCN, illustrated by increases in both c-fos and p-ERK, may be necessary for phase shifts. NMDA antagonists or serotonergic agonists, both of which inhibit light (Colwell & Menaker, 1992; Weber et al., 1998), or GRP-induced phase shifts (Kallingal & Mintz, 2006) restrict GRP-induced p-ERK activation to the dorsal SCN. Similar to the results obtained for c-fos, p-ERK immunoreactivity was also examined in the PVN and SON and GRP injections increased p-ERK immunoreactivity in both regions.

Since the results of Expts 1 and 2 showed that both AP5 and DPAT restrict GRP-induced c-fos and p-ERK immunoreactivity to a subset of cells in the dorsal SCN and because our group has already shown that AP5 could attenuate the behavioral effects of GRP (Kallingal & Mintz, 2006), we examined whether DPAT would similarly be able to block GRP-induced shifts. Previous reports have indicated that non-photic stimuli presented to animals held in constant conditions could inhibit light-induced shifts (Ralph & Mrosovsky, 1992; Biello & Mrosovsky, 1995; Weber et al., 1998). In support of these reports, our lab has shown that NPY, a non-photic stimulus, can oppose the actions of GRP in the SCN (Kallingal & Mintz, 2007). Serotonergic agonists (Weber et al., 1998) and serotonergic receptor antagonists (Rea et al., 1994) can respectively attenuate and potentiate light-induced shifts of the circadian activity rhythm in hamsters. The mechanisms by which non-photic signals can attenuate the phase-shifting response to photic signals are poorly understood, although several possible explanations have been postulated. One possibility includes the effects of non-photic signals on the expression of clock genes. Administration of GRP has a stimulatory effect on the expression of *Per1* and *Per2*, both of which are involved in regulating clock phase (Dardente et al., 2002; Gamble et al., 2007). Previous reports have shown that serotonin or serotonergic agonists can oppose increases in expression of immediate-early genes and clock genes (Horikawa et al., 2000), thereby blocking the effects that photic-like stimuli have on circadian phase. Another potential mechanism is an increase in serotonin release in the SCN during non-photic stimuli, which would inhibit the release of glutamate and thereby reduce the effects of GRP on circadian phase (Kallingal & Mintz, 2006).

The GRP-induced increase in c-fos expression in the SON was not an anticipated result from our experiment. It seemed unlikely that the SON could directly respond to substances injected into the third ventricle, so a second set of experiments was designed to evaluate potential roles of the SON in the regulation of clock function. There is limited evidence indicating that reciprocal projections exist between regions of the SCN and SON (Saeb-

Parsy et al., 2000; Kriegsfeld et al., 2004). The results of Expt 4 appear to add to these findings as microinjection of GRP into a region just dorsal to the SON induced c-fos in the SCN. GRP also increased c-fos expression in the PVN, which is not surprising as the SON and PVN share neuronal projections that mediate neuroendocrine responses (Thellier et al., 1994). Such increases in the PVN were not attenuated by either AP5 or DPAT. Interestingly, substances microinjected near the SON had a slightly greater (but not statistically significant) effect on the contralateral nucleus. As previous evidence indicates, the two nuclei of the SON project to a region known as the dorsochiasmatic area to facilitate communication between these distant regions (Thellier et al., 1994). Greater or equivalent activation of the contralateral SON argues against the idea that the effects on the SCN in Expt 4 are caused solely by diffusion of GRP from the injection site to the SCN. Previous studies have shown that lateral spread of microinjected neuropeptides is minimal (Caldwell & Albers, 2003) and insufficient to explain these results.

Our final two experiments were designed to further evaluate the role of the SON in mediating clock function. To address the question of whether stimulation of the SON region can alter circadian phase, we examined the behavioral effects of substances applied directly to the SON region. Microinjection of GRP to a region just dorsal to the SON at CT 13 elicited phase delays of the circadian activity rhythm in a manner similar to either ventricular administration of GRP (Antle et al., 2005; Kallingal & Mintz, 2006) or microinjection of GRP near the SCN (Albers et al., 1991; Piggins et al., 1995; Kallingal & Mintz, 2007). In Expt 6, we injected bicuculline, a GABA_A antagonist, to the same region and found that the inhibition of GABAergic signaling in the SON served as a sufficient stimulus to induce phase delays. Microinjection of bicuculline into the SCN region does not cause shifts (Gillespie et al., 1996), so this effect is unlikely to be a result of diffusion of bicuculline into the SCN. Ultimately, any shift in the timing of the locomotor activity rhythm must result from a change in the underlying clock located in the SCN, so it is reasonable to hypothesize that a projection from the SON or peri-SON region is capable of stimulating the SCN in such a way that mimics the effects of light. One possible explanation is that bicuculline disinhibits excitatory projections to the SCN, resulting in a light-like phase delay. This could occur via a glutamatergic mechanism, but our data do not provide evidence for or against this. One way to check this possibility would require disinhibiting the peri-SON region with bicuculline while blocking glutamate in the SCN.

In summary, these experiments provide data indicating that GRP plays an important role in the signaling pathways mediating photic entrainment. By tracking c-fos and p-ERK immunoreactivity in the SCN and other hypothalamic regions following the ventricular administration of GRP, and subsequently examining the immunohistological and behavioral interactions of GRP with glutamate and serotonin, it is clear that increased glutamatergic neurotransmission and decreased serotonergic activity may potentiate the phase-resetting actions of GRP. Additionally, the SON appears to have a potential role in regulating photic entrainment pathways. Because substances delivered to a region near the SON can both activate neuronal markers in the SCN and alter the phase of circadian activity, it is likely that these two regions of the hypothalamus are connected by a neuronal network that may involve direct, reciprocal projections. These connections may be part of a system that allows the brain to regulate the photic sensitivity of the circadian clock based on physiological parameters monitored by other hypothalamic nuclei.

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Abbreviations

5-HT	serotonin
ANOVA	analysis of variance
AP5	(±)-2-amino-5-phosphonopentanoic acid
BIC	bicuculline
CT	circadian time
DD	constant darkness
DPAT	8-hydroxy-2-di- <i>n</i> -propylamino-tetralin
GABA	γ-aminobutyric acid
GRP	gastrin-releasing peptide
NMDA	<i>N</i> -methyl-D-aspartate
NPY	neuropeptide Y
p-ERK	phosphorylated extracellular-related kinase
PBS	phosphate-buffered saline
PVN	paraventricular nucleus of the hypothalamus
RHT	retinohypothalamic tract
SCN	suprachiasmatic nucleus
SON	supraoptic nucleus

Fig. 1.
 (Top) Representative photomicrographs of the hamster SCN at anterior, medial and posterior levels following microinjection of GRP, GRP/AP5, GRP/DPAT and saline.
 (Bottom) Mean + SEM c-fos counts induced by microinjection of GRP, GRP/AP5, GRP/DPAT and saline into the third ventricle at CT 13. Asterisks denote groups significantly different from corresponding GRP group (Dunnett's test, $P < 0.05$).

Fig. 2. (Top) Mean + SEM c-fos counts in the ventral third and dorsal two-thirds of the medial SCN induced by microinjection of GRP, GRP and AP5, GRP and DPAT, and saline into the third ventricle at CT 13. (Middle) Representative photomicrographs of the hamster SON and PVN following microinjections. (Bottom) Mean + SEM c-fos counts in the SON and PVN after microinjections into the third ventricle at CT 13. Asterisks denote groups significantly different from corresponding GRP group (Dunnett's test, $P < 0.05$).

Fig. 3.

(Top) Representative photomicrographs of the hamster SCN at anterior, medial, and posterior levels following microinjection of GRP, GRP and AP5, GRP and DPAT, and SAL into the third ventricle at CT13. (Bottom) Mean + SEM p-ERK optical density induced by microinjection of GRP, GRP and AP5, GRP and DPAT, and SAL into the third ventricle at CT 13. Asterisks denote groups significantly different from corresponding GRP group (Dunnett's test, $P < 0.05$).

Fig. 4. (Top) Mean + SEM p-ERK optical density in the ventral third and dorsal two-thirds of the medial SCN induced by microinjection of GRP, GRP and AP5, GRP and DPAT, and saline into the third ventricle at CT 13. (Middle) Representative photomicrographs of the hamster SON and PVN following microinjections. (Bottom) Mean + SEM p-ERK optical densities after microinjections. Asterisks denote groups significantly different from corresponding GRP group (Dunnett's test, $P < 0.05$).

Fig. 5.

Representative actograms of circadian activity rhythms before and after microinjection of (A) GRP, (B) DPAT, (C) GRP and DPAT, and (D) saline. 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). (E) Mean + SEM phase shift induced by microinjection of GRP, GRP and DPAT, DPAT, and saline into the third ventricle at CT 13. Asterisks denote groups significantly different from corresponding GRP group (Dunnett's test, $P < 0.05$).

Fig. 6. (Top) Representative photomicrographs of hamster SCN at anterior, medial and posterior levels following microinjection of GRP, GRP and AP5, GRP and DPAT, and saline into a region just dorsal to the SON at CT 13. (Middle) Mean + SEM c-fos counts induced by microinjections. Asterisks denote groups significantly different from corresponding GRP group (Dunnett's test, $P < 0.05$). (Bottom) Brain atlas image (Morin & Wood, 2001) with optimal injection site denoted by an asterisk.

Fig. 7.

(Top) Mean + SEM c-fos counts in the dorsal two-thirds and ventral third of the SCN (bilateral total) induced by microinjection of GRP, GRP and AP5, GRP and DPAT, and saline near the SON at CT 13. (Middle) Representative photomicrographs of the ipsilateral SON and contralateral SON following microinjections. (Bottom) Mean + SEM c-fos counts induced by microinjections. Asterisks denote groups significantly different from corresponding GRP group (Dunnett's test, $P < 0.05$).

Fig. 8.

Representative photomicrographs of the PVN following microinjection of (A) GRP, (B) GRP and AP5, (C) GRP and DPAT, and (D) saline into a region just dorsal to the SON at CT 13. (E) Mean + SEM c-fos counts induced by microinjection of GRP, GRP and AP5, GRP and DPAT, and saline into a region just dorsal to the SON at CT 13.

Fig. 9.

Representative actograms of circadian activity rhythms before and after microinjection of GRP and saline (A) or BIC and saline (C). 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). Mean + SEM phase shift induced by microinjection of GRP and saline (B) or BIC and saline (D) into a region just dorsal to the SON at CT 13. Asterisks denote groups significantly different from corresponding GRP group (t -test, $P < 0.05$).