Accepted Manuscript

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PII: S0306-4522(16)30065-3

DOI: <http://dx.doi.org/10.1016/j.neuroscience.2016.04.010>

Reference: NSC 17042

To appear in: *Neuroscience*

Accepted Date: 7 April 2016

Please cite this article as: R.K. Chan, R. Sterniczuk, Y. Enkhbold, R.T. Jeffers, P. Basu, B. Duong, S-L. Chow, V.M. Smith, M.C. Antle, Phase shifts to light are altered by antagonists to neuropeptide receptors, *Neuroscience* (2016), doi: <http://dx.doi.org/10.1016/j.neuroscience.2016.04.010>

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Neuroscience

Section: Behavioral and Cognitive Neuroscience

Editor: Liisa Galea

Phase shifts to light are altered by antagonists to neuropeptide receptors

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Running head: Photic shifts with GRP and VIP antagonists

Figures: 6

Pages: 31

Abstract words: 257

Introduction words: 706

Discussion + Conclusion words: 1525

Total number of words: 7049

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List of Abbreviations

CT	circadian time
DD	constant darkness
DMSO	dimethyl sulfoxide
LD	light/dark cycle
GRP	gastrin-releasing peptide
SCN	suprachiasmatic nucleus
VIP	vasoactive intestinal polypeptide

Abstract

The mammalian circadian clock in the suprachiasmatic nucleus (SCN) is a heterogeneous structure. Two key populations of cells that receive retinal input and are believed to participate in circadian responses to light are cells that contain vasoactive intestinal polypeptide (VIP) and gastrin-releasing peptide (GRP). VIP acts primarily through the VPAC2 receptor while GRP works primarily through the BB2 receptor. Both VIP and GRP phase shift the circadian clock in a manner similar to light when applied to the SCN, both *in vivo* and *in vitro*, indicating that they are sufficient to elicit photic-like phase shifts. However, it is not known if they are necessary signals for light to elicit phase shifts. Here we test the hypothesis that GRP and VIP are necessary signaling components for photic phase shifting the hamster circadian clock by testing two antagonists for each of these neuropeptides. The BB2 antagonist PD176252 had no effect on light-induced delays on its own, while the BB2 antagonist RC-3095 had the unexpected effect of significantly potentiating both phase delays and advances. Neither of the VIP antagonists ([D-p-Cl-Phe6, Leu17]-VIP, or PG99-465) altered phase shifting responses to light on their own. When the BB2 antagonist PD176252 and the VPAC2 antagonist PG99-465 were delivered together to the SCN, phase delays were significantly attenuated. These results indicate that photic phase shifting requires participation of either VIP or GRP, and only when signaling in both pathways is inhibited are phase shifts to light impaired. Additionally, the unexpected potentiation of light-induced phase shifts by RC-3095 should be investigated further for potential chronobiotic applications.

Key words: network, core, shell, bombesin, rodent

1. Introduction

The suprachiasmatic nucleus (SCN) serves as the master circadian clock in mammals and regulates daily oscillations in physiology and behavior (Antle and Silver, in press). The rhythms regulated by this clock are entrained to light-dark (LD) cycles by light signals relayed to the circadian clock from the retina. The SCN is a heterogeneous structure: the ventrolateral core plays a major role in receiving and responding to these light signals, while the dorsomedial shell is less retinoreceptive but exhibits high amplitude circadian oscillations in gene expression (Antle and Silver, 2005).

The retinoreceptive core of the hamster SCN contains at least three main overlapping cell phenotypes. A small cluster of calbindin D28K-containing neurons receive retinal input (Bryant et al., 2000) and exhibits intense light-induced expression of *cFos*, *Per1* and *Per2* (Silver et al., 1996, Hamada et al., 2003). A ventral population of cells containing vasoactive intestinal polypeptide (VIP) also receives retinal input (Reuss and Decker, 1997) and responds strongly to light (Earnest et al., 1993, Romijn et al., 1996). Finally, a third population in the lateral SCN extending dorsally from the VIP cells contains gastrin-releasing peptide (GRP). This population also receives retinal input (Tanaka et al., 1997, Abrahamson and Moore, 2001) and responds strongly to light (Earnest et al., 1993, Romijn et al., 1996). Light exposure triggers the release of both VIP (Franci et al., 2010a) and GRP (Franci et al., 2010b) in the SCN.

Both VIP and GRP are thought to participate in circadian responses to light (Antle and Silver, 2005). When injected directly to the SCN *in vivo*, both GRP and VIP can phase shift the circadian clock in a manner that mimics responses to light (Piggins et al.,

1995, Antle et al., 2005, Kallungal and Mintz, 2006, 2007, Sterniczuk et al., 2008, Kallungal and Mintz, 2010, Sterniczuk et al., 2010, Kallungal and Mintz, 2014). Similar responses are observed *in vitro* (McArthur et al., 2000, Reed et al., 2001). These responses are mediated through VPAC2 receptors for VIP (Cutler et al., 2003) and bombesin 2 (BB2) receptors for GRP (McArthur et al., 2000). Both systems appear to require participation of the protein kinase A signaling cascade (Meyer-Spasche and Piggins, 2004, Kudo et al., 2013, Sterniczuk et al., 2014). GRP-deficient mice have fairly normal circadian rhythmicity, but exhibit some diminished responses to light (Aida et al., 2002). VIP^{-/-} mice and mice lacking the VPAC2 gene (i.e., Vipr2^{-/-} mice) exhibit variable phenotypes. While a small number of mice with impaired VIP signaling are largely normal, the majority have severely impaired rhythmicity (Harmar et al., 2002, Colwell et al., 2003, Hughes et al., 2004). Normal gating of circadian responses to light is lost in Vipr2^{-/-} mice (Hughes et al., 2004). While VIP^{-/-} mice can entrain to both full photoperiods and skeleton photoperiods, such entrainment is unstable and occurs with atypical phase angles (Colwell 2003). These VIP^{-/-} mice do not appear to phase shift to nocturnal light exposure, but such assessments are complicated by the instability of their rhythms (Colwell et al., 2003). There may be some compensation by GRP in mice where VIP signaling is impaired. The severely compromised rhythmicity of Vipr2^{-/-} mice can be partially rescued by applying GRP *in vitro*, or can be further degraded by the application of BB2 receptor antagonists (Brown et al., 2005).

These data suggest that GRP and VIP are integral components of the circadian system, with GRP participating in photic responsiveness and VIP playing an integral role in both coherence of the SCN rhythm as well as photic responses. However, the severely

compromised rhythmicity of mice lacking VIP or VPAC2 receptors limits our ability to use these models to understand the role of VIP in photic responses. Furthermore, data from constitutive knockouts must be interpreted cautiously as developmental compensation may alter the underlying system, which is likely in this case because GRP appears to compensate for some of VIP's function (Brown et al., 2005). To test the hypothesis that signaling by these neuropeptides is necessary for normal circadian responses to phase shifting light, we pretreated hamsters with intraSCN injections of pharmacological antagonists to BB2 and/or VPAC2 receptors prior to exposing them to phase shifting light pulse. Our results demonstrate that light responses are only attenuated when receptors to both neuropeptides are blocked.

2. Experimental Procedures

A total of 138 male Syrian hamsters (*Mesocricetus auratus*, 80-90 g) obtained from Charles River Labs (Kingston, NY) were used. Hamsters were initially housed in groups of two or three and maintained under a 14:10 LD cycle (lights on at 4:00 h, off at 18:00 h). Cage level illuminance was approximately 300 lux. Animals had access to food and water *ad libitum*. Following cannula implantation, each animal was transferred to an individual cage and maintained in their home environment, in a 14:10 light cycle, for a minimum of one week. After this recovery period, hamsters were transferred to individual polycarbonate cages (20 x 45 x 22 cm) equipped with a running wheel (14 cm in diameter), and maintained in constant darkness (DD) for the duration of the study. All protocols were approved by the Life and Environmental Sciences Animal Care Committee at the University of Calgary and adhered to the Canadian Council on Animal Care guidelines.

2.1 Cannula Implantation

Cannula implantation was performed approximately one week after hamsters arrived into their new environment, or when the animal weighed roughly 110 g. Each animal received a subcutaneous injection of the analgesic butorphanol (2 mg/kg; Wyeth) prior to surgery. While under the anesthesia of sodium pentobarbital (~90 mg/kg; CEVA) injected intraperitoneally, the hamsters were stereotactically implanted with a 9 mm 22 gauge stainless steel cannula (Plastics One Inc., Roanoke, VA) cemented to the skull with dental acrylic and jeweler's screws. In some experiments, the cannula was aimed at the third ventricle (coordinates 1.36 mm anterior to bregma, 0.0 mm lateral to the midline, 7.0 mm ventral to the skull surface), while in other experiments the cannula was aimed at the SCN region (coordinates 0.0 mm anterior to bregma, 0.3 mm lateral to the midline, 7.0 mm ventral to the skull surface). The incisor bar was set to 2 mm below the interaural level for both surgeries. A dummy cannula was inserted to maintain patency. The dummy cannula and injection cannula extended 1 mm beyond the tip of the guide.

2.2 Drugs and Reagents

To block GRP receptors, we used the potent BB2 receptor antagonist RC-3095 (Sigma-Aldrich, 0.9 mM in sterile 0.9% saline) or the non-peptidergic BB2 antagonist PD176252 (Tocris, 0.342 mM in 50% dimethyl sulfoxide (DMSO)). To block VIP receptors, we used the VIP antagonist [D-p-Cl-Phe⁶, Leu¹⁷]-VIP (Tocris, 1 mM in sterile 0.9% saline) or the potent VPAC2 antagonists PG99-465 (Bachem, 1 mM in 0.9% sterile saline). To test the antagonistic properties of RC-3095, we also used porcine GRP (Sigma-Aldrich, 0.3 mM in sterile 0.9% saline). Cocktails of RC-3095 + PG99-465 and of PD176252 + PG99-465 were prepared so that the molarity of each substance was

identical when administered alone and in the cocktail. Molarity of antagonists were selected to be about 10x greater than the minimal molarity of GRP and VIP that yielded maximal phase shifts in previous studies (Piggins et al., 1995). Injections occurred in DD with the aid of night-vision goggles (BG15Alista, Richmond Hill, Ontario, Canada). All manipulations occurred approximately 10 days prior to and following cage changes.

2.3 Activity Rhythms

Wheel-running activity was continuously monitored using magnetic switches connected to a computer running the Clocklab data collection software package (Coulbourn Instruments, Allentown, PA). Prior to the start of manipulations, the hamsters were allowed to free run in DD for a minimum of 10 days. Graphical records of wheel-running behaviour (actograms) were generated and analyzed using Clocklab Analysis software. In order to predict the onset of wheel running activity for the manipulation day, a regression line was fit to the activity onsets for the 10 days prior to the day of injection.

Phase shifts were analyzed using Clocklab's automatic fitting function to create a line of best fit to the activity onsets for about the 10 days prior to the injection day. Another line of best fit was created for 10 days following the injection, beginning on approximately the fourth day so as not to be affected by transient activity onsets on the days immediately following treatment. The degree to which each hamster phase advanced or delayed was based on the horizontal difference between the predicted onsets from both regression lines on the day following the treatment. Subsequent treatments were given two weeks following the previous treatment. Drug treatment conditions were counterbalanced within light treatment conditions. Light treatments were delivered first, as the quality of the rhythms and patency of the cannula frequently deteriorates over the

course of the experiment, thus ensuring that the main hypotheses (i.e., if these drugs affect responses to light) could be tested first.

2.4 Experiments

2.4.1 Experiment #1 – Effects of the BB2 antagonist RC-3095 on circadian responses

The peptidergic BB2 antagonist RC-3095 was used to test the hypothesis that blocking GRP-preferring BB2 receptors in the SCN would attenuate phase shifts to light. A large 3rd ventricle injection was used in order to maximize the area affected. Animals (n=15) received an injection of RC-3095 (5 µl over 5 minutes) or vehicle (n=23) 0.5 h after activity onset (i.e., at circadian time (CT) 12.5, where activity onset is defined as CT12 by convention), followed by a 15 minute, 40 lux light pulse (LP) at CT13. Treatments were given in a counterbalanced fashion. These injections were followed-up in a subset of the animals using higher doses (2.26 mM, n=13; 4.52 mM, n=12) prior to a CT13 LP. To test effects on phase advances, another set of animals (n=8) were used to examine these same treatments at CT17.5, followed by a LP at CT18.

Because preliminary results suggested that RC-3095 might not be acting as a pure antagonist, its ability to attenuate GRP-induced phase shifts was tested with an additional set of animals (n=11). To match previous studies, animals were given a small intraSCN injection of either RC-3095 (2 mM, 0.25 µl over 30 s) or vehicle at CT12.83, followed by GRP (0.3 mM, 0.25 µl over 30 s) or vehicle at CT13.

2.4.2 Experiment #2 – Effects of the BB2 antagonist PD176252 on circadian responses

A second BB2 antagonist was used to test the hypothesis that blocking GRP-preferring BB2 receptors in the SCN would attenuate phase shifts to light. Given the unexpected findings with RC-3095, we elected to use the non-peptidergic BB2 antagonist

PD176252. As this drug is not soluble in saline, it was first dissolved in DMSO, and then diluted with sterile 0.9% saline to 1 mM in 50% DMSO. To minimize adverse effects of the vehicle, we used small (0.5 μ l / 30 seconds) intraSCN injections rather than the larger 3rd ventricle injections. Animals (n=8) were given an injection of PD176252 or vehicle at CT12.5 followed by a 15 minute 40 lux LP at CT13. Following these, animals received injection-treatments (PD176252 or vehicle) without a subsequent LP in a counterbalanced fashion.

2.4.3 Experiment #3 – Effects of the VIP antagonist [D-p-Cl-Phe6, Leu17]-VIP on circadian responses

The peptidergic antagonist [D-p-Cl-Phe6, Leu17]-VIP was used to test the hypothesis that blocking VIP receptors in the SCN would attenuate phase shifts to light. To maximize the area affected, a large 3rd ventricle injection was used. Animals (n=10) received an injection of [D-p-Cl-Phe6, Leu17]-VIP (5 μ l over 5 minutes) or vehicle at CT12.5, followed by a 15 minute, 40 lux light pulse (LP) at CT13. Treatments were given in a counterbalanced fashion. Afterwards, a subset of these animals (n=7) were used to test the no-light control treatments.

2.4.4 Experiment #4 – Effects of the potent VPAC2 receptor antagonist PG99-465 on circadian responses

Given the lack of effect of [D-p-Cl-Phe6, Leu17]-VIP on light-induced phase delays, and the unexpected observation that it induced phase delays on its own without light, we tested a second antagonist, the potent VPAC2 receptor antagonist PG99-465. Hamsters (n=7) received an intraSCN injection (0.5 μ l over 30 s) of either PG99-465 or vehicle at CT12.5, prior to a 15 minute, 40 lux LP at CT13. A subset of these animals

were then used to test either vehicle alone (n=2) or PG99-465 alone (n=4). These control manipulations were not done in a repeated measures fashion, and therefore were analyzed using an independent samples t-test.

2.4.5 Experiment #5 – Effects of VIP and GRP antagonist cocktails on circadian responses

The combined effects of GRP and VIP antagonists were tested, both with and without a subsequent light exposure. Specifically, the effects of the VPAC2 antagonist PG99-465 was tested in separate trials with either the BB2 antagonist RC-3095 (n=14) or the BB2 antagonist PD176252 (n=7).

In the first trial, animals were given an intraSCN injection of the PG99-465/RC-3095 cocktail, PG99-465 alone, RC-3095 alone, or saline alone at CT12.83 followed by a 15 minute 40 lux LP at CT13. Treatments were given in a counterbalanced fashion.

In the second trial animals were given an intraSCN injection of the PG99-465/PD176252 cocktail or vehicle control at CT12.5 and were then exposed to a 15 minute 40 lux LP at CT13. Following this, a subset of animals received the cocktail (n=5) or vehicle (n=2) at CT12.5 without subsequent light exposure. These control manipulations were not done in a repeated measures fashion, and were therefore analyzed using an independent samples t-test.

2.5 Histology

At the end of the study, animals were perfused with 4% paraformaldehyde. Brains were harvested, cryoprotected, and 35 μ m sections were collected through the anterior hypothalamus using a cryostat. Tissue was mounted on gelatin-coated slides and stained with cresyl violet to confirm cannula placements. For intraSCN injections, only animals

with injection cannula tips within 500 μm of the margin of the SCN were retained for analysis. For 3rd ventricle injections, only animals with injection cannula tip within the 3rd ventricle were retained for analysis.

2.6 Statistical Analysis

Within each particular experiment, animals were only retained for analysis if they contributed data to both the drug and vehicle pretreatment conditions. Some animals from each experiment were also used, when possible, to explore the effects of the drug or vehicle alone without a subsequent light pulse. For experiment 1, a two-way repeated measures ANOVA was run on the absolute value of the phase shifts, as the main effect of drug was the focus rather than the main effect of phase. In cases where all animals contributed to the no-light control conditions, 2 x 2 ANOVAs were used to examine the results. If only a subset of animals provided control data, then paired *t*-tests were used to compare drug versus vehicle in the light pulse conditions, while an independent samples *t*-test was used to compare drug versus vehicle in the no-light condition. In all cases the planned comparison between light with drug versus light without drug was examined to specifically address the hypotheses of interest. Additionally, the planned comparison of drug without light versus vehicle without light was examined to determine if the drug produced any effect on circadian phase on its own. Statistical tests were run with SigmaPlot 11.0 (Systat Software Inc., San Jose, CA) and alpha was set to 0.05.

3. Results

Of the 138 hamsters that were implanted with a cannula, 80 hamsters both completed their experiment and had implants sufficiently close to their targets to be retained for analysis.

3.1 The BB2 antagonist RC-3095 significantly increases phase shifts to light

Phase shifts to light were significantly potentiated following pretreatment with the BB2 receptor antagonist RC-3095 (main effect of drug, $F_{(1,21)}=7.597$, $p=0.012$, Fig 1). There was no significant interaction between phase and drug ($F_{(1,21)}=0.196$, $p=0.663$). The response to light pulses that produce phase delays was investigated at a range of doses. The maximal effect was observed with the lowest dose of RC-3095 (0.9 mM). Phase delays to light were still larger in animals pretreated with the higher doses (1.85 ± 0.27 h for 2.26 mM, 1.90 ± 0.21 h for 4.52 mM) compared to saline (1.32 ± 0.14 h), although not significantly so (2-tailed paired t-tests, 2.26 mM, $t_{(12)}=1.81$, $p=0.095$; 4.52 mM, $t_{(11)}=1.56$, $p=0.147$). While the phase delays to light following these higher doses were smaller than to the lower dose (0.9 mM, 2.42 ± 0.36 h), a pairwise analysis in animals that had both the lowest dose and one of the higher doses revealed that these differences were also not significant (0.9 mM vs 2.26 mM, $t_{(9)}=0.08$, $p=0.941$; 0.9 mM vs. 4.52 mM, $t_{(8)}=0.66$, $p=0.525$).

Given that this effect was contrary to the hypothesis, we explored the effects of RC-3095 on its own, as well as its ability to attenuate phase shifts to intraSCN GRP. GRP injections at CT13 induced large magnitude phase delays (-1.17 ± 0.25 h) that were significantly greater than those to saline (-0.47 ± 0.12 h, main effect of GRP, $F_{(1,8)}=10.097$, $p=0.009$). There was no significant interaction between GRP and RC-3095 ($F_{(1,8)}=0.474$, $p=0.51$). The following planned comparisons were examined: RC-3095 alone (-0.35 ± 0.12 h) vs. saline alone ($p=0.652$) and RC-3095+GRP (-0.86 ± 0.19 h) vs. GRP alone ($p=0.105$). These comparisons were consistent with the lack of a main effect for RC-3095 ($F_{(1,8)}=2.319$, $p=0.153$).

3.2 Phase shifts to light are unaffected by the BB2 antagonist PD176252

Light-induced phase shifts were significantly greater than those to the dark controls (main effect of light, $F_{(1,6)}=32.114$, $p<0.001$, Fig 2). PD176252 had no effect on the resulting phase shifts in either condition (main effect of drug $F_{(1,6)}=0.631$, $p=0.449$). There was no interaction between light and drug ($F_{(1,6)}=0.0009$, $p=0.977$), consistent with PD176252 neither causing phase shifts on its own nor altering light-induced phase shifts.

3.3 Phase shifts to light are unaffected by the VIP antagonist [D-p-Cl-Phe6, Leu17]-VIP

Light-induced phase shifts were significantly greater than those to the dark controls (main effect of light, $F_{(1,5)}=43.771$, $p=0.001$, Fig 3). Overall, phase shifts in animals treated with [D-p-Cl-Phe6, Leu17]-VIP were not significantly greater than those in saline-treated animals (main effect of [D-p-Cl-Phe6, Leu17]-VIP, $F_{(1,5)}=5.596$, $p=0.064$), and there was no interaction between drug and light ($F_{(1,5)}=3.106$, $p=0.138$). However, while the planned comparison exploring the effects of light with and without [D-p-Cl-Phe6, Leu17]-VIP revealed no significant effect ($p=0.771$), the planned comparison of drug versus vehicle in the no light condition did reveal that [D-p-Cl-Phe6, Leu17]-VIP caused significant phase delays on its own ($p=0.016$).

3.4 Phase shifts to light are unaffected by the VPAC2 antagonist PG99-465

PG99-465 did not attenuate the size of light-induced phase shifts. In fact, phase shifts to light and PG99-465 were marginally larger than those to saline and light, albeit not significantly so (paired $t_{(6)}=1.86$, $p=0.11$, Fig 4). Phase shifts to PG99-465 were not significantly different than those to saline alone ($t_{(4)}=0.06$, $p=0.958$).

3.5 Potentiating effects of RC-3095 are not affected by the VPAC2 antagonist PG99-465

Replicating the results of our first experiment, but with intraSCN injections rather than 3rd ventricle injections, the BB2 receptor antagonist RC-3095 significantly potentiated phase shifts to light (main effect of RC-3095, $F_{(1,13)}=71.845$, $p<0.001$, Fig 5). The VPAC2 receptor antagonist PG99-465 did not alter phase shifts responses (main effect of PG99-465, $F_{(1,13)}=0.0287$, $p=0.868$). There was no interaction between the two drugs ($F_{(1,13)}=0.002$, $p=0.964$).

3.6 Phase shifts to light are attenuated by a cocktail of the BB2 antagonist PD176252 and the VPAC2 antagonist PG99-465

Combining the BB2 antagonist PD176252 and the VPAC2 antagonist PG99-465 significantly attenuated light-induced phase shifts relative to vehicle-treated controls (paired $t_{(6)}=3.39$, $p=0.014$, Fig 6). Phase shifts to the cocktail without light were not significantly different from those to vehicle without light ($t_{(5)}=1.31$, $p=0.247$).

4. Discussion

Both GRP and VIP have been shown to be sufficient signals to induce photic-like phase shifts of locomotor rhythms in Syrian hamsters (Piggins et al., 1995, Antle et al., 2005, Sterniczuk et al., 2008). The present study was designed to test the hypothesis that these are necessary signals in the photic response. Our results suggest that neither neuropeptide on its own is necessary for normal phase shifts to light, as long as signaling by the other neuropeptide is unimpaired. Phase shifts to light are only impaired when both GRP and VIP receptors are blocked. The idea that GRP is able to compensate for a lack of VIP signaling in the circadian system has been proposed by others (Brown et al., 2005), in that brain slices from *Vipr2*^{-/-} mice with compromised rhythmicity in their firing rate rhythms could be improved when GRP was added to the perfusate.

Previous work on GRP has suggested that phase shifts to light are impaired in GRP-deficient mice (Aida et al., 2002). However, in that study, phase delays to light in GRP-deficient mice were only significantly smaller for bright light pulses (15 min, 300 lux), while phase shifts to dimmer light pulses (15 min, 30 lux) were not significantly different from wildtype controls. The light pulses in the present experiments (15 min, 40 lux) were close to the dim light used by Aida and colleagues (2002). It is possible that PD176252 may have attenuated phase shifts to brighter light. If so, this would be consistent with GRP playing a necessary role in responses to bright light, but a redundant role (together with VIP) in circadian responses to dimmer light.

Two studies have investigated the interaction between VIP and GRP (and peptide histidine isoleucine) when co-administered to the SCN *in vivo*. It was initially reported that a cocktail of these peptides produced phase shifts greater than the additive sum of their individual effects (Albers et al., 1991). A later study failed to replicate this finding, instead showing that the cocktail did not elicit greater phase shifts than were produced individually by the various peptides (Piggins et al., 1995). If these peptides participate in photic responses, then these two studies would seem to make opposite predictions about the present experiments. The study by Albers and colleagues (1991) would predict that removal of one of the peptides would diminish the resulting phase shifts to light, having lost one of the critical peptidergic components and its associated synergism, while the study by Piggins and colleagues (1995) would predict that the full magnitude response should persist, as each peptide on its own can elicit the full response, such that those peptides not blocked would completely compensate for the loss of the other. Thus the

present findings lend support to the conclusions presented by Piggins and colleagues (1995).

Previous work on the role of VIP in photic responses is somewhat limited by VIP's apparent dual role in the SCN. Mice lacking either VIP or its receptor have severely compromised locomotor rhythms, as well as impaired electrical firing rate rhythms (Harmar et al., 2002, Colwell et al., 2003, Piggins and Cutler, 2003). Given the instability of rhythmicity in these knockout models, it is difficult to assess phase shifts to light. One study to attempt this (Colwell et al., 2003) found that phase shifts to light in the heterozygous mice ($VIP^{+/-}$) were half the magnitude of those observed in wildtype mice. No significant phase shifts to light were observed in the homozygous $VIP^{-/-}$ knockout mice, but the authors cautioned that the instability of the locomotor rhythms made this assessment exceptionally challenging (Colwell et al., 2003). A similar study conducted with $Vipr2^{-/-}$ mice found that light-induced phase shifts persisted in mice that remained rhythmic in DD, albeit with an altered phase response curve (Hughes and Piggins, 2008). Given that VIP applied to the SCN *in vivo* or *in vitro* mimics the phase-shifting effects of light (Piggins et al., 1995, Reed et al., 2001), and that VIP cells in the SCN receive retinal input and express both Fos (Romijn et al., 1996) and *rPer1* (Kawamoto et al., 2003) in response to phase-shifting light, it is likely that VIP does participate in circadian responses to light. In LD cycles, both $VIP^{-/-}$ and $Vipr2^{-/-}$ mice exhibit nocturnal patterns of behavior (Harmar et al., 2002, Colwell et al., 2003, Piggins and Cutler, 2003, Hughes and Piggins, 2008). This could represent masking or entrainment. In those mice that are rhythmic upon release into DD, the phase of activity onset freeruns from an advanced phase relative to wildtype animals, suggesting that if

photic signaling persists in these animals then it is severely compromised. Based on the present results, we hypothesize that signaling through GRP is maintaining this responsiveness, and that a dual knockout of both GRP and VIP would not be able to entrain to the LD cycle.

While phase shifts to light were significantly attenuated by the cocktail of PG99-465 and PD176252, they were not completely abolished. This could be due to an incomplete receptor blockade by the antagonists, in which case higher doses of PG99-465 and PD176252 may elicit a more complete inhibition of the phase shift to light. Another option could include the possibility that the duration of action of the drugs was less than the duration of action of the light pulse, so receptor blockade might not have endured for a sufficient period of time. A third option could be that a third signaling system may be responsible for the persistent shifts. Nearly all SCN cells, including those that release VIP or GRP, also use GABA as a neurotransmitter (Moore et al., 2002). While most studies suggest that GABA signaling impairs circadian responses to light (Gillespie et al., 1996, Gillespie et al., 1997, Gillespie et al., 1999, Lall and Biello, 2003, Ehlen et al., 2008, Ehlen and Paul, 2009), a recent study has suggested that sustained activation of GABA-A receptors is necessary and sufficient to elicit photic-like phase shifts (Hummer et al., 2015). Finally, it is possible that the residual phase shift might be due to initial phase shifting of the circadian clock in only those retinal-recipient cells that exhibit oscillations, such as the VIP cells (Hermansteyne et al., 2016), and that this shifted population is then able to reset the rest of the circadian clock once peptidergic signaling in the circadian network resumes.

We tested two antagonists for each neuropeptide. While the cocktail of PD176252 and PG99-465 was able to significantly attenuate responses to light, neither antagonist on its own was able to significantly alter circadian responses. The other VIP antagonist employed ([D-p-Cl-Phe6, Leu17]-VIP) also did not alter responses to light. However, unlike PG99-465, [D-p-Cl-Phe6, Leu17]-VIP induced significant phase shifts on its own. This indicates that [D-p-Cl-Phe6, Leu17]-VIP might act as a partial VIP agonist. Alternatively, [D-p-Cl-Phe6, Leu17]-VIP may exhibit secondary binding to a different class of receptors that may mediate this effect. The fact that there was no additive effect when combined with light suggests that, on its own, [D-p-Cl-Phe6, Leu17]-VIP may be activating a pathway that is normally activated by light. These results should be interpreted with caution as only a single dose was investigated, and this was the only drug to be investigated using only 3rd ventricle injections, leading to less certainty about the site of action. Similarly, the lack of effect of PD176252, PG99-465, [D-p-Cl-Phe6, Leu17]-VIP when given alone prior to a phase-shifting light should be interpreted cautiously, as only a single dose was evaluated.

The significant potentiation observed with RC-3095 was quite interesting. It occurred with both large, 3rd ventricle injections (Figure 1) as well as with small, intraSCN injections (Figure 5). It has been suggested by others that RC-3095 may actually be a partial agonist for BB2 receptors rather than a full antagonist (Akiyama et al., 2013, Merali et al., 2013). This mirrors the effects of another putative BB2 antagonist (BIM26226), which appears to act more like a partial agonist in that it can induced light-like cFos expression in part of the SCN (Piggins et al., 2005). While RC-3095 acting as a partial agonist might explain the potentiated light responses observed, RC-3095 did not

produce significant phase shifts on its own at the doses used in the current study (on average -0.35 h phase delays, relative to -0.48 h phase delays to saline). It is unlikely that removal of GRP signaling is the mechanism by which responses to light are enhanced by RC-3095, because GRP-deficient mice do not show larger phase shifts (Aida et al., 2002) and the non-peptidergic BB2 receptor antagonist used here (PD176252) did not produce the same potentiation. It is more likely that RC-3095, at the doses used here, is activating another receptor, although it is unclear as to what this may be.

5. Conclusions

In summary, the present results demonstrate that GRP and VIP play critical roles in regulating circadian responses to light. While the system is able to function when the receptors for either neuropeptide are blocked independently, pharmacological blockade of the primary receptors for both neuropeptides together significantly attenuates phase shifting responses to light. This helps delineate the roles of the separate input populations within the SCN circadian clock.

6. Acknowledgements

This work was supported by an NSERC Discovery Grant to MCA, NSERC-USRA awards to RKC, YE and BD, NSERC-CGS awards to RS and VMS, and an Eyes-High post doctoral fellowship from the University of Calgary to PB.

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8. Figure Captions

Figure 1 Representative actograms from animals pretreated with the GRP antagonist RC-3095 (grey circle) or its vehicle (white circle) prior to a CT13 light pulse (top actogram) or a CT18 light pulse (bottom actogram). Each horizontal line represents a record from a single day, with each subsequent day plotted below the prior day. Vertical deflection from above each line denote times when the animal was engaged in wheel running, with the height of the deflection proportional to the magnitude of activity. The bottom panel depicts the mean phase shifts ($\pm$ SEM) of animals for each pretreatment condition for light pulses at each circadian phase. The absolute value of the light-induced phase shifts were significantly larger (* on the graph) following RC-3095 pretreatment than following vehicle control (saline; main effect of treatment: $F_{(1,21)}=7.597$, $p=0.012$).

Figure 2 A representative actogram from an animal exhibiting representative responses to the 4 different treatments: an intraSCN injection of the vehicle (50% DMSO, white circle) or the BB2 receptor antagonist PD176252 (grey circle) at CT12.5 prior to a CT13 light pulse or no light control. The bottom graph depicts the mean phase shift ($\pm$ SEM) for the various treatments. Light pulses produced significant phase shifts that were not affected by pretreatment with PD176252.

Figure 3 Representative actograms from an animal given a 3rd ventricle injection of either vehicle (saline, white circle) or the VIP antagonist [D-p-Cl-Phe6, Leu17]-VIP (grey circle) at CT12.5 prior to a CT13 light pulse (top actogram) or an animal given just the VIP antagonist [D-p-Cl-Phe6, Leu17]-VIP (grey circle) at CT12.5

without a subsequent light pulse (bottom actogram). The bar graph at the bottom depicts the mean phase shift ($\pm$ SEM) for the various treatments. Light pulses produced significant phase shifts that were not affected by pretreatment with [D-p-Cl-Phe6, Leu17]-VIP. In the no light control condition [D-p-Cl-Phe6, Leu17]-VIP produced significant phase shifts on its own (* $p < 0.05$).

Figure 4 A representative actogram from an animal given an intraSCN injection of the vehicle (saline, white circle) or the VPAC2 receptor antagonist PG99-465 (grey circle) at CT12.5 prior to a CT13 light pulse. Also depicted is this animal's response to the control condition of PG99-465 at CT12.5 without subsequent light exposure. The bottom graph depicts the mean phase shift ($\pm$ SEM) for the various treatments. Light pulses produced significant phase shifts relative to no-light-pulse controls that were not affected by pretreatment with PG99-465.

Figure 5 A representative actogram from an animal given an intraSCN injection of vehicle (saline, white circle), the VPAC2 receptor antagonist PG99-465 alone (circle with grey on the left), the BB2 antagonist RC-3095 alone (circle with grey on the right), or a cocktail of PG99-465 and RC-3095 (full gray circle) at CT12.5 prior to a CT13 light pulse. The bottom graph depicts the mean phase shift ($\pm$ SEM) for the various treatments. Light pulses produced significant phase shifts relative to no-light-pulse controls that were not affected by pretreatment with PG99-465. Phase shifts to light were significantly larger following RC-3095 pretreatment and PG99-465 had no effect on the resulting phase shifts.

Figure 6 A representative actogram from an animal given an intraSCN injection of vehicle (50% DMSO, white circle), or a cocktail of the VPAC2 receptor

antagonist PG99-465 and the BB2 receptor antagonist PD176252 (full gray circle) at CT12.5 prior to a CT13 light pulse. The bottom graph depicts the mean phase shift ($\pm$ SEM) for the various treatments. Light pulses produced significant phase shifts that were significantly (* $p < 0.05$) attenuated by the cocktail pretreatment.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Highlights

- Gastrin-releasing peptide antagonists do not block circadian responses to light
- Vasoactive intestinal peptide antagonists do not block photic phase shifts
- A cocktail of specific GRP and VIP antagonists attenuate photic phase shifts
- The GRP antagonists RC3095 potentiates photic phase shifts