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PHASE SHIFTS TO LIGHT ARE ALTERED BY ANTAGONISTS TO NEUROPEPTIDE RECEPTORS

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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. © 2016 Published by Elsevier Ltd. on behalf of IBRO.

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

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 retinoresponsive but exhibits high amplitude circadian oscillations in gene expression (Antle and Silver, 2005).

The retinorecipient 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 (Francl et al., 2010a) and GRP (Francl 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; Kallingal and Mintz, 2006, 2007, 2010, 2014; Sterniczuk et al., 2008; Sterniczuk et al., 2010). 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

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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.

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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 et al., 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.

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 × 45 × 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.

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 stereotaxically implanted with a 9-mm 22-gauge stainless steel cannula (Plastics One Inc., Roanoke, VA, USA) 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 the 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 the 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.

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 10× 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.

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, USA). Prior to the start of manipulations, the hamsters were allowed to free run in DD for a minimum of 10 days. Graphical records of

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