CIRCADIAN AND SEASONAL RHYTHMS

The roles of vasoactive intestinal polypeptide in the mammalian circadian clock

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Abstract

Biological oscillations with an endogenous period of near 24 h (circadian rhythms) are generated by the master circadian pacemaker or clock located in the suprachiasmatic nuclei (SCN) of the hypothalamus. This clock is synchronised to recurring environmental signals conveyed by selective neural pathways. One of the main chemical constituents of SCN neurones is vasoactive intestinal polypeptide (VIP). Such neurones are retinorecipient and activated by light. Exogenous application of VIP resets the SCN circadian clock in a light-like manner, both in vivo and in vitro. These resetting actions appear to be mediated through the VPAC2 receptor (a type of receptor for VIP). Unexpectedly, genetically ablating expression of the VPAC2 receptor renders the circadian clock arrhythmic at the molecular, neurophysiological and behavioural levels. These findings indicate that this intrinsic neuropeptide acting through the VPAC2 receptor participates in both resetting to light and maintenance of ongoing rhythmicity of the SCN.

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Introduction

Over the past 30 years, a critical mass of evidence has been collected demonstrating that the suprachiasmatic nuclei (SCN) of the hypothalamus house the dominant mammalian circadian clock or pacemaker (Weaver 1998). This pacemaker and its synchronisation (entrainment) to the environmental light–dark cycle modulates the 24 h organisation of physiological and behavioural processes. Studies in which the rodent SCN is lesioned electrolytically or neurochemically have demonstrated that this leads to the abolition of wheel-running rhythms (although the rodent can still move freely) and the loss of daily variation in endocrine function such as the luteinising hormone surge in female rats (Raisman & Brown-Grant 1977) and secretion of melatonin (Klein & Moore 1979). In humans, damage to the SCN region arising through craniopharyngioma can lead to loss of sleep–wake and endocrine rhythms (Cohen & Albers 1991). Transplantation of fetal SCN material can rescue rodent behavioural rhythms (Lehman et al. 1987, Ralph et al. 1990), but not endocrine rhythms (Meyer–Bernstein et al. 1999), indicating that hard-wiring of the SCN input to neural structures of the neuroendocrine axis is required for the normal expression of overt neurohormonal rhythms. Surprisingly, neural contacts between SCN graft and host are not required for the restoration of wheel-running rhythms (Silver et al. 1996). This suggests that paracrine signals convey phase information from the graft to the neural structures regulating locomotor activity. Two substances, transforming growth factor-α (TGF-α) (Kramer et al. 2001) and prokineticin 2 (Cheng et al. 2002) have recently been implicated as key messengers in communicating phase information from the SCN to motor control sites in intact rodents.

The SCN clock is reset by light or photic stimuli (Meijer 2002) as well as arousal-inducing or non-photic stimuli (Mrosovsky 1996). Extensive studies in nocturnal rodents maintained in constant darkness and housed in cages equipped with running wheels revealed that light pulses given during their active phase (their subjective night) phase-dependently phase-reset the onset of the wheel-running activity rhythm (Daan & Pittendrigh 1976, Takahashi et al. 1984). A light pulse given during the early part of the subjective night slows or phase-delays the clock, while a similar light pulse given during the latter part of the subjective night accelerates or phase-advances the clock (Fig. 1A). Pulses of light given during the middle of the inactive phase or subjective day have no resetting effects, even though the animal may be awake at the time of the pulse (Meijer 2002). Clock processes therefore gate these actions of light on the circadian clock such that light
resets the clock predominantly during the subjective night. In contrast, non-photic stimuli, such as changing the cage or giving the animal a new running wheel, which promote arousal, lead to an acceleration or phase-advance in the clock during the subjective day (Fig. 1B) and have few prominent phase-shifting effects during the subjective night (Mrosovsky 1996). Thus, like light, the resetting actions of non-photic stimuli are phase-dependently gated by the SCN clock.

To date, investigations on the neurochemical basis of photic and non-photic resetting have focused on the influence of SCN afferents. Research with neuroanatomical tracers such as cholera toxin subunit β and fluorogold has demonstrated that photic information is relayed from the retina directly to the SCN by the monosynaptic retinohypothalamic tract (RHT) (for reviews see Colwell & Menaker 1996, Ebling 1996) (see Fig. 2). Immunohistochemical and electron microscopy studies have identified the excitatory amino acid glutamate and pituitary adenylate cyclase-activating polypeptide (PACAP) as the main neurochemicals of the RHT (Castel et al. 1993, Hannibal et al. 2000, Hannibal & Fahrenkrug 2002). Previously, substance P (SP) had been implicated as a candidate transmitter of the rat RHT (Takatsuji et al. 1991, 1995), but subsequent neuroanatomical studies (Piggins et al. 2001, Hannibal 2002) and functional studies (Piggins & Rusak 1997) have suggested that SP is unlikely to be an RHT neurochemical in many rodent species.

Some RHT afferents bifurcate and, together with other retinal fibres, innervate the intergeniculate leaflet of the visual thalamus (Pickard 1985, Smith et al. 2000). Cells here project rostrally to the SCN to form the geniculo-hypothalamic tract (GHT), thus providing another pathway (Fig. 2) by which environmental light information can be conveyed to the SCN circadian clock (Harrington 1997). In contrast to the neurochemicals of the RHT, neuropeptide Y (NPY) and GABA are most readily identified in GHT fibres that innervate the SCN (Harrington 1997). Further, this pathway appears to be more important in relaying non-photic information to the SCN since destruction of the GHT blocks the resetting effects of many arousal-inducing stimuli (Mrosovsky 1996), but only has subtle effects on photic entrainment. Microinjection of NPY into the SCN region phase-advances behavioural rhythms during the subjective day, thus emulating the temporal pattern of phase-resetting by non-photic stimuli (Huhman & Albers 1994). A third major SCN afferent arises from median Raphe cells of the brainstem (Fig. 2). Serotonin has been identified in this

Figure 1 Resetting effects of photic and non-photic stimuli on nocturnal rodent wheel-running rhythms. (A) Solid rectangles depict running-wheel activity under light–dark and constant dark (DD) conditions. The animal initially entrains to lights off (illustrated by the top bar) and then free-runs with a tau of >24 h when released into DD. Note phase-dependent phase-shifting effects of 15-min light pulses (*) delivered at late subjective night, early subjective night or middle subjective day phases respectively. In contrast, confinement to a novel running wheel during the middle of the projected day (hatched bar) phase-advances the wheel-running rhythm. (B) Phase-response curves for photic (solid line) and non-photic stimuli (broken line) with phase-advances and -delays plotted as positive and negative values respectively. The magnitude of the shift is plotted according to the time of the circadian cycle at which the stimulus was delivered.
brainstem pathway and has been implicated in both non-photic and photic entrainment (for review see Morin 1999).

While much research has been geared to resolving the information conveyed by these pathways as well as the neurochemical messengers that they utilise, there have been comparatively few investigations on the role of endogenous neurochemicals in SCN circadian function. In this paper, evidence indicating that neurochemicals intrinsic to SCN neurones can themselves regulate the phase of the SCN circadian clock will be reviewed. Moreover, we also propose that activation of a certain class of neuropeptide receptor may be important in the maintenance of ongoing circadian clock function.

Intrinsic neurochemicals

The neurochemicals synthesised within the rodent SCN have been established for a number of years (for review see Piggins et al. 2002) and, on the basis of these phenotypes as well as the neural connections formed by these neurochemically defined cells, it has been hypothesised that the SCN is subdivided into a ‘shell’ and ‘core’ (Moore & Leak 2002). Neurones contained within the shell include AVP-synthesising cells and somatostatin (SS)-synthesising cells, while gastrin-releasing peptide (GRP) and vasoactive intestinal polypeptide (VIP) immunoreactive (ir) neurones constitute the core of this structure (Fig. 3). Many neurones within the core are retinorecipient, while retinal efferents are sparse or absent in the shell. This SCN neurochemical organisation is also seen in other mammals including the human where VIP- and AVP-containing cells are key markers of the SCN (Mai et al. 1991, Moore 1992). In most mammalian species studied, GABA is expressed in many SCN neurones and frequently co-localises with a number of neuropeptides (Buijs et al. 1995, Castel & Morris 2000).

VIP is synthesised from preproVIP and cleavage of this molecule forms both VIP and peptide histidine isoleucine, a peptide found in abundance in the SCN and thought to be co-localised with VIP. VIP and peptide histidine isoleucine are structurally related to PACAP and, in rodents, these three peptides show 68% homology. Accordingly, these peptides bind to three classes of receptors present in the brain: VPAC1, VPAC2 and PAC1 (of which there are a number of isoforms). The VPAC1 and VPAC2 receptors bind VIP/peptide histidine isoleucine and PACAP with almost equal affinity, while PAC1 preferentially binds PACAP over VIP (although there is an isoform that has similar affinity for PACAP and VIP, the so-called PAC1_short, which is present in the SCN; Shinohara et al. 2002 but see also Ajpru et al. 2002).
Radioligand studies have demonstrated an abundance of $[^{125}\text{I}]$VIP-binding sites in the rodent SCN (Robinson & Fuchs 1993), while in situ hybridisation investigations have shown that mRNA for VPAC2 and, to a lesser extent, PAC1 mRNA are present in the rodent SCN (Usdin et al. 1994, Sheward et al. 1995, Cagampang et al. 1998b, c). By contrast, VPAC1 mRNA has not been detected in the rodent SCN (Usdin et al. 1994). The heavy expression of VPAC2 mRNA and intense immunostaining of VIP in SCN neurones suggests that VIP acting through the VPAC2 receptor may play an important role in circadian rhythm processes.

The VIP-ir neurones in the rat SCN have extensive intra- and inter-SCN projections. Among the intra-SCN targets of VIP cells are the AVP-ir and SS-ir neurones in the shell region, as well as VIP-ir neurones and GRP-ir neurones in the core (van den Pol & Gorcs 1986, Daikoku et al. 1992, Romijn et al. 1997). The observation that VIP-ir axons innervate VIP-ir neurones suggests that VIP may form part of an autoreceptor circuit (Fig. 3). Extra-SCN projections of VIP-ir neurones include the hypothalamic subparaventricular zone, gonadotrophin-releasing hormone neurones of the preoptic region (van der Beek et al. 1997), the dorsomedial and posterior hypothalamus, and the paraventricular nucleus of the thalamus (Watts & Swanson 1987, Kalnsbeek et al. 1993). The observations that VIP cells receive a direct retinal input (Ibata et al. 1989, Tanaka et al. 1993) and that photic stimulation activates VIP neurones in the SCN (as indicated by the presence of cFos protein in such cells; Romijn et al. 1996) implies that VIP neurones interface incoming environmental information and relay it to the circadian clock to adjust its phase accordingly.

VIP and photic entrainment

As noted above, VIP-ir neurones in the core SCN are contacted directly by retinal efferents, and photic stimulation induces cFos expression in these VIP cells. In mouse, hamster and rat SCN, some but not all VIP-ir neurones express cFos, a marker of neuronal activation, following retinal illumination during the projected nighttime (Romijn et al. 1996, Castel et al. 1997, Aïoun et al. 1998). Expression of VIP mRNA in the rat SCN varies over the light–dark cycle, but not in constant lighting conditions (Zoeller et al. 1992). Similarly, levels of VIP-ir in the adult rat SCN do not show circadian variation, but are altered by photic stimulation (Shinohara et al. 1993). This suggests that VIP synthesis and/or release in the rodent SCN is not under strong circadian control and is instead driven by variations in environmental lighting levels (although the possibility of circadian-regulated low amplitude rhythms of VIP synthesis cannot be discounted).

Functional studies of VIP in the rodent SCN have indicated a possible role for VIP and VPAC2 signalling in photic entrainment. In rat brain slices, exogenous VIP alters the extracellularly recorded firing rate of spontaneously active SCN neurones, with suppression of activity being the predominant response (Reed et al. 2002). The selective VPAC2 receptor agonist, Ro 25–1553, mimics these suppressive actions of VIP. Consistent with these agonist studies, suppressions to VIP were blocked by the VPAC2 receptor antagonist, PG 99–465. VIP activates a smaller proportion of rat SCN neurones and the selective PAC1 receptor agonist, maxadilan, mimics these excitatory actions of VIP. By contrast, equimolar application of the VPAC1 receptor agonist [K15,R16,L27]VIP(1–7)GRF(8–27) has no effect on SCN neuronal activity; a result consistent with the apparent absence of VPAC1 mRNA in the rat SCN. An important observation from these acute neurophysiological studies was that a significantly greater proportion of rat SCN neurones responded to VIP when tested during the
projected night phase as compared with the projected day phase, suggesting that the actions of VIP on the SCN circadian clock may be more prevalent during the projected night.

A very useful model for determining the phase of adult SCN brain slices in vitro is to monitor the spontaneous electrical activity of SCN neurones across the projected day and night. Early studies demonstrated that rat SCN neuronal activity peaks during the middle of the projected day phase (around Zeitgeber time (ZT) 6–7, where ZT0=lights on and ZT12=lights off) and declined to reach a nadir during the middle of the projected night phase (around ZT18–19) (Green & Gillette 1982, Groos & Hendriks 1982, Shibata et al. 1982). The timing of the peak in this firing-rate rhythm can be reset in a phase-dependent and predictable direction by exogenous application of neurochemicals associated with the RHT such as glutamate and PACAP (Ding et al. 1994, Chen et al. 1999) and the GHT such as NPY (Biello et al. 1997). Using this model, application of VIP (at a concentration shown to be effective in altering rat SCN activity – see above) phase advances the firing-rate rhythm when applied during the late projected night, but has little effect when given during the middle of the projected day phase (Reed et al. 2001). Application of VIP during the early projected night causes small phase delays. The phase-advancing actions of VIP are dose-dependent and are mimicked by equinolar doses of the VPAC₂ receptor agonist Ro 25–1553, but neither the VPAC₁ receptor agonist [K₁₅,R₁₆,L₂₇]VIP(1–7)GRF(8–27) nor PACAP alter the timing in the peak of the rat SCN neuronal firing-rate rhythm. These long-term neurophysiological investigations show that VIP acting presumably through the VPAC₂ receptor can reset the rat SCN neuronal activity rhythm during the projected night in a manner resembling the actions of light pulses on nocturnal rodent behavioural rhythms. This night-time phase of sensitivity is coincident with the phase when a greater proportion of rat SCN neurones are responsive to VIP, indicating circadian gating in the SCN clock to the actions of VIP. The precise mechanism(s) underpinning this altered sensitivity is unknown, but may be related to temporal variation in the expression of the VPAC₂ receptor (Cagampang et al. 1998a) and/or coupling of this receptor complex to second messenger systems that influence clock gene expression at this phase of the circadian cycle (Cagampang et al. 1998a).

These in vitro studies are remarkably consistent with an earlier in vivo investigation of the influence of VIP on the hamster SCN circadian clock. The hamster shows a robust circadian rhythm in this measure of locomotor activity and the circadian clock regulates the onset of this rhythm. Microinjection of VIP alone or in combination with other endogenous neuropeptides into the SCN region of hamsters free-running in constant dark or constant light was found to reset the clock during the projected nighttime (Piggins et al. 1995). This pattern of resetting resembles that of light pulses on hamster wheel running, suggesting that VIP participates in photic resetting. Moreover, application of VIP during the late projected night increases the expression of the core clock genes, pper₁ and pper₂, in the rat SCN in vitro (Nielsen et al. 2002) indicating that VIP can invoke the molecular machinery necessary to phase-reset the circadian clock.

The notion that VIP acting through the VPAC₂ receptor is important in photic entrainment is supported by a recent study in which mice were transgenically manipulated to overexpress the VPAC₂ receptor gene. In comparison with control wild-types, these mutant mice show a shortened circadian period when maintained in constant dim red light and an increased rate of re-entrainment when the light–dark cycle is reset (Shen et al. 2000). Moreover, Watanabe et al. (2000) demonstrated that exogenous VIP reset the secretion of AVP from organotypic SCN cultures with a temporal pattern of sensitivity resembling that of a photic phase-response curve. These results suggest that VIP and the activation of the VPAC₂ receptor are important in resetting to light.

VIP and VPAC₂ receptor activation in maintenance of circadian clock phase

The idea that the VIP–VPAC₂ signalling system may be necessary for maintaining the rhythmic function of the SCN is supported by studies on mice in which the expression of the VPAC₂ receptor gene has been genetically altered (Harmar et al. 2002). In these Vipr₂⁻/⁻ mice, a LacZ-Neo’ cassette was inserted in the translation start site of the VPAC₂ receptor gene (Vipr2) such that this gene is not expressed, as indicated by the absence of Ro 25–1553 binding. Under light–dark conditions, Vipr₂⁻/⁻ mice show apparent normal entrainment of locomotor rhythms, with pronounced bouts of wheel running coincident with lights off. When the light–dark cycle was shifted by 8 h, Vipr₂⁻/⁻ mice re-entrain almost immediately and more quickly than wild-type mice. Unexpectedly, these mice fail to exhibit robust behavioural rhythms when released into constant dim red light conditions. The core clock genes (mper1, mper2, mcry1 and bmal1) and the clock-controlled AVP gene are expressed at significantly lower levels in the Vipr₂⁻/⁻ mouse SCN compared with wild-type mouse SCN, and the pattern of expression of these genes does not vary significantly over the circadian cycle. This study suggests that the SCN circadian clock function is severely impaired in Vipr₂⁻/⁻ mice.

In recent in vivo and in vitro investigations, we have confirmed the findings of Harmar et al. (2002). Under light–dark conditions, Vipr₂⁻/⁻ and wild-type mice show apparent entrainment of locomotor activity to lights off. However, when maintained in constant darkness, Vipr₂⁻/⁻ mice are behaviourally arrhythmic, while
C57BL/6J and Ola/129 wild-type mice display robust wheel-running activity rhythms with endogenous periods (taus) of 23.8 h and 23.7 h respectively (Cutler et al. 2003). Verification of the absence of functional VPAC2 receptors in Vipr2−/− mice came from the observation in vitro that a significantly lower proportion of SCN neurones from Vipr2−/− mice respond to VIP, compared with wild-type SCN neurones. Moreover, 60% of wild-type SCN neurones maintained in vitro respond to the selective VPAC2 receptor agonist, Ro 25–1553, compared with only 4% of SCN neurones from Vipr2−/− mice. The proportion of SCN neurones responding to other G-protein-coupled receptor agonists (AVP and GRP) was similar in Vipr2−/− and wild-type mice, indicating that inactivation of the Vipr2 gene does not appear to impact generally on neuropeptide receptor function in the murine SCN. Remarkably, when the firing-rate rhythm of SCN slices in vitro were assessed, we found that SCN neuronal activity in Vipr2−/− mice is very low and does not show pronounced circadian variation (Fig. 4). In contrast, wild-type mice show significant circadian variation in SCN electrical activity, with a readily discernible peak in the firing-rate rhythm detected during the middle of the subjective day (ZT 6.5–7.5) (Fig. 4). The peak to peak interval between the peak on day 1 in vitro and day 2 in vitro was 23.9 h in C57BL/6J mice and 23.8 h in 129/Ola mice (Cutler et al. 2003). Thus, firing-rate rhythm, the most proximal of physiological measures of SCN clock function, strongly resembles the circadian behaviour phenotype of mutant and wild-type mice. The reduced cellular responsiveness to VIP, coupled with the paucity of responses to Ro 25–1553, indicate that residual responses to VIP are mediated through receptors other than VPAC2. The identity of such receptors is unknown but they are likely to be isoforms of the PAC1 receptor, as opposed to the VPAC1 receptor which has not been detected in the rodent SCN. It is noteworthy that while these non-VPAC2 receptors mediate some cellular actions of VIP within the SCN, their activation by VIP is insufficient to sustain overt circadian clock function. These studies reveal that the absence of behavioural and molecular rhythms also manifests at the level of neuronal activity, results that show remarkable consistency in the circadian phenotype of these mice.

**Summary and model**

Thus, unexpectedly, these studies show novel, unique functions for an intrinsic neurochemical in SCN circadian time-keeping processes. Neurophysiological studies in rat SCN brain slices in vitro established that VIP alters SCN neuronal activity via the VPAC2 receptor and that more SCN cells respond to VIP during the projected night phase. During the late night phase, VIP and the VPAC2 receptor agonist Ro 25–1553 phase-advance the SCN circadian clock. Similarly, microinjection of VIP into the SCN region during the late subjective night phase-shifts hamster wheel-running rhythms. Overexpression of the VPAC2 receptor in mice shortens tau and accelerates re-entrainment to the light–dark cycle. In the absence of functional VPAC2 receptors, the mouse circadian clock...
appears to stop such that there is no circadian rhythm in core clock gene expression, cellular activity or wheel-running behaviour.

The notion that VIP, an intrinsic SCN neurochemical, plays key roles in circadian clock processes is not without precedence. We have shown previously that GRP, an intrinsic neurochemical of the core SCN, potently alters rodent SCN electrical activity, phase-shifts rat and hamster SCN neuronal rhythms, and phase resets hamster wheel-running rhythms in a pattern like light (Piggins & Rusak 1993, Piggins et al. 1994, 1995, McArthur et al. 2000). These resetting effects of GRP appear to be mediated by the bombesin type 2 (BB2) receptor. BB2 receptor mRNA is heavily expressed in the rodent SCN (Battey & Wada 1991) and antagonists to this receptor block these actions of GRP on the SCN clock. Like VIP-ir neurones, rat GRP-containing neurones are directly contacted by retinal efferents (Tanaka et al. 1997) and some GRP-ir cells express cFos in response to a nocturnal light pulse (Aioun et al. 1998). Further, light-time GRP microinjection induces mper1 expression, particularly in the dorsal SCN. However, unlike Vip2-/- mice, genetically deleting the BB2 receptor in the SCN does not dramatically alter murine circadian activity, but does diminish the magnitude of phase-shifts to light pulses (Aida et al. 2002). These findings suggest that both VIP and GRP neurones are important in photic entrainment, but that activation of VPAC2 receptors is critical to the maintenance of circadian phase. The co-localisation of these peptides with GABA in SCN neurones indicates possible modulatory interactions between these intrinsic neurochemicals (Gillespie et al. 1996).

Conceptually, the VIP- and GRP-containing neurones appear to interface between photic inputs and central clock activity. These VIP and GRP neurones receive direct retinal innervation and levels of these peptides in the SCN appear to be dependent on the photic environment: neither VIP mRNA nor GRP mRNA in the SCN appear to cycle with a circadian rhythm in animals maintained in constant photic conditions. These neurones are activated by RHT input and release VIP/GRP into the shell and core SCN where their targets include AVP and VIP/GRP neurones respectively. VIP or a VIP-like peptide is secreted to stimulate VPAC2 receptors and this is involved in the maintenance of circadian phase.

Collectively, the above studies indicate that neurochemicals synthesised by SCN neurones may play a role in both the maintenance of circadian phase in constant conditions as well as mediating the resetting actions of some environmental Zeitgebers. Further studies are required to determine the intracellular pathways linking activation of receptors of GRP or VIP to the regulation of core clock genes, as well as determining which population of SCN neurones represents the necessary targets to mediate the phase-shifting actions of these intrinsic neuropeptides. Moreover, as both peptides are thought to be part of the SCN output signal, determining how they alter activity in brain areas that regulate the neuroendocrine axis will also be essential to elucidate the nature of SCN control of neurohormonal rhythms.

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