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Progress in Neurobiology 74 (2004) 59–76

progress in Neurobiology

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Let there be ‘‘more’’ light: enhancement of light actions on the circadian system through non-photic pathways

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Received 6 April 2004; accepted 18 June 2004

Abstract

Circadian rhythms are internally generated circa 24 h rhythms. The phase of the circadian pacemaker in mammals can be adjusted by external stimuli such as the daily cycle of light, as well as by internal stimuli such as information related to the physiological and behavioral status of the organism, collectively called ‘‘non-photic stimuli’’. We review a large number of studies regarding photic–non-photic interactions on the circadian system, with special focus on two widely described neurotransmitters associated with non-photic input pathways: neuropeptide Y (NPY) and serotonin 5-HT. Both neurotransmitters are capable of phase advancing the master pacemaker oscillation when applied during the subjective day, as do several behavioral manipulations. Also, both are capable of inhibiting light-induced phase shifts during the subjective night, suggesting a dynamic interaction between photic and non-photic stimuli in the fine-tuning of the pacemaker function. Suppression of the NPYergic and/or serotonergic non-photic input pathways can in turn potentiate the phase-shifting effects of light. These findings pose new questions about the possibility of a physiological role for the dynamic interaction between photic and non-photic inputs. This might be particularly important in the case of circadian system adjustments under certain conditions, such as depression, shift work or jet lag.

Contents

Abbreviations: LD, light–dark; NPY, neuropeptide Y; 5-HT, serotonin; SCN, suprachiasmatic nuclei; PACAP, pituitary adenylate cyclase activating peptide; RHT, retinohypothalamic tract; GHT, geniculohypothalamic tract; IGL, intergeniculate leaflet; NMDA, *N*-methyl-D-aspartate; 8-OH-DPAT, 8-hydroxy-2-(di-*n*-propylamino)-tetralin hydrobromide; CT, circadian time; DD, dark–dark; AURE, adenosine-uridine rich elements; PYY, peptide YY; GABA, gamma-amino butyric acid

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doi:10.1016/j.pneurobio.2004.06.001

1. Introduction

Circadian rhythms are internally generated circa 24 h rhythms. The phase of the circadian pacemaker or master clock in mammals can be adjusted by external stimuli such as the daily cycle of light–dark (LD), as well as by internal stimuli such as information related to the physiological and behavioral status of the organism. Synchronizing information is conveyed to the suprachiasmatic nuclei (SCN) of the hypothalamus, the anatomical site of the central pacemaker, by means of several different input pathways (see Fig. 1).

The response of the internal clock to external stimuli is different in the internal or subjective night versus the subjective day. Light synchronizes or entrains circadian rhythms largely by action during the subjective night. It generally does not alter the phase of the circadian rhythm when presented during the subjective day, but during the subjective night detection of light signals the clock to reset. Light early in the night produces phase delays in circadian rhythms, while light later in the subjective night produces phase advances. Through this mechanism, the endogenously generated circadian rhythm maintains entrainment with the exogenous LD cycle (Johnson et al., 2003; Pittendrigh and Daan, 1976).

Photic entraining stimuli are delivered via the retinohypothalamic tract (Johnson et al., 1988a; Morin, 1994). Briefly, a special class of retinal ganglion cells (Rollag et al., 2003) provides direct neural input to the SCN, utilizing glutamate as a neurotransmitter (Ebling, 1996), with the peptide PACAP (pituitary adenylate cyclase activating peptide) possibly serving as a neuromodulator (for review see Hannibal, 2002).

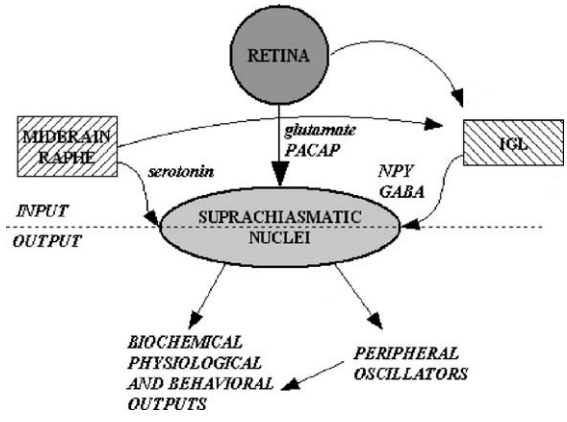

Fig. 1. Schematic representation of input pathways to the suprachiasmatic nucleus (SCN). The main synchronizing afferent comes directly from the retina (retinohypothalamic tract, or RHT) and utilizes glutamate and PACAP as the most important neurotransmitters that convey the photic information to the clock. Two main pathways transmit the non-photic information to the SCN: the serotonergic one, from the midbrain raphe, and the NPYergic and GABAergic afferents, from the intergeniculate leaflet of the thalamus, or IGL (modified from Yannielli and Harrington, 2001a, 2001b).

Recent years have seen a major advance in our understanding of the molecular mechanism of the circadian clock. The self-sustained oscillation of the clock is generated by the periodic, sequential activation of the transcription of genes whose products in turn influence their own transcriptional activation. These interacting transcriptional and translational feedback loops include a negative feedback that involves the regulation of two period genes (*per1* and *per2*) and two cryptochrome genes (*cry1* and *cry2*), whose transcription is driven by the transcription factors CLOCK and BMAL1 and whose products, CRY and PER proteins, act as negative regulators of CLOCK and BMAL1 transcription. The genetic machinery of the clock also includes a positive feedback that involves PER2 protein acting as an activator of *bmal1* transcription (Fig. 2; for review see Dunlap, 1999; Reppert and Weaver, 2002). This auto-regulatory loop of about 24 h has to be synchronized periodically to external cues such as light. Certain clock genes (mainly *per1* and *per2*) are light responsive, thus are capable of mediating the effect of light on the state of the clock (Reppert and Weaver, 2002).

Light-induced resetting is accomplished within a few hours of light onset (Best et al., 1999) and can occur even when the SCN is isolated in vitro, as when an SCN brain slice is prepared immediately following a 5 min light pulse (Yannielli and Harrington, 2000). Light modifies histones in the mouse SCN leading to chromatin remodeling suggesting transcriptional changes (Crosio et al., 2000). Transcription of the *c-fos* gene in SCN neurons appears to be increased by light, with a rapid response (mRNA levels peak about 30 min after light onset; for review see Schwartz et al., 1995). Interestingly, this response is gated; light during most of the subjective day does not increase *c-fos* just as it does not reset circadian rhythms during these times. Some of the genetic clock components that participate in the main oscillatory processes are also implicated in light synchronization. The period genes *per1* and *per2* are rhythmically expressed in the SCN with peaks around midday. Both have been shown to rapidly respond with increases in mRNA following light stimulation during the subjective night, a time when their mRNA levels are minimal (Albrecht et al., 1997; Moriya et al., 2000; Shearman et al., 1997; Shigeyoshi et al., 1997; Zylka et al., 1998). The mRNA levels start to rise about 15 min after light onset during the subjective night, peak 1–2 h later, and return to baseline after 3 h, with *per2* slightly lagging *per1*. Blocking *per1* and *per2* mRNA with antisense can block the phase resetting action of light (Akiyama et al., 1999; Wakamatsu et al., 2001), indicating these genes may be necessary for photic entrainment. On the other hand, light can synchronize circadian rhythms of mice with the *per1* or *per2* gene function blocked (Albrecht et al., 2001; Bae and Weaver, 2003; Cermakian et al., 2001). Mice with both *per1* and *per2* genes deleted do not show circadian rhythms, so the necessity of the two genes for entrainment cannot be demonstrated with this approach (Bae et al., 2001).

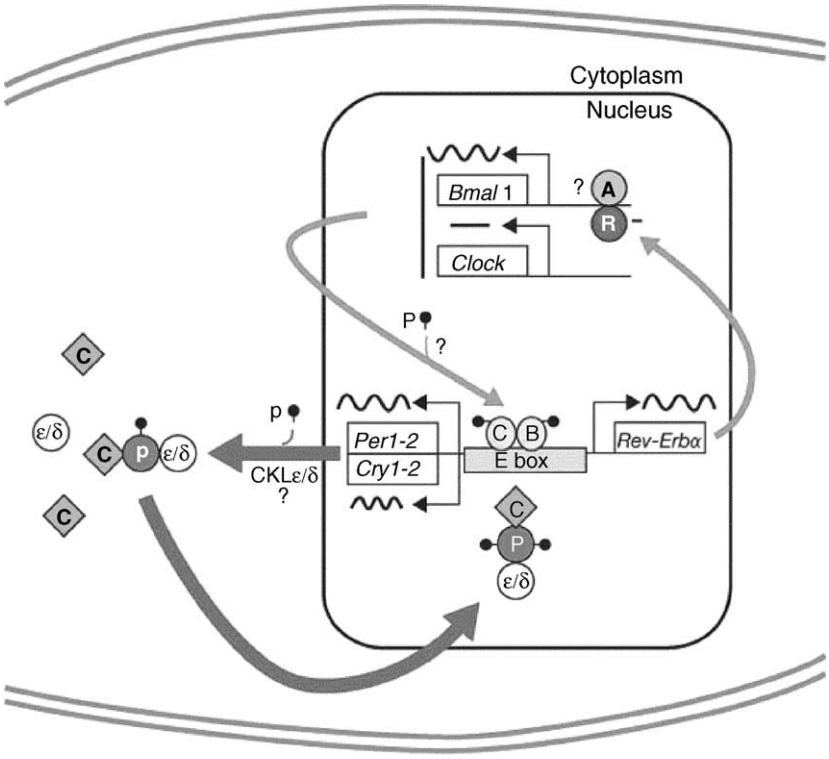

Fig. 2. Mammalian circadian clock genetic machinery. The interacting transcriptional and translational feedback loops include a negative feedback (dark grey lines) that involves the regulation of two period genes (*per1* and *per2*) and two cryptochrome genes (*cry1* and *cry2*), whose transcription is driven by the transcription factors CLOCK (C, oval) and BMAL1 (B, oval) and whose products, CRY (C, diamond) and PER (P, circle) proteins, act as negative regulators of CLOCK and BMAL1 transcription. The genetic clockwork also includes a positive feedback (light grey lines) that involves PER2 protein acting as an activator of *bmal1* transcription. These auto-regulatory loops of about 24 h have to be synchronized periodically to external cues such as light. The *per1* and *per2* genes are light responsive, thus are capable of mediating the effect of light on the state of the clock (from Reppert and Weaver, 2002).

Signal transduction pathways connecting retinohypothalamic tract (RHT) activation to circadian clock resetting are still under investigation. Is glutamate release sufficient? What is the role of the neuromodulator PACAP? Which glutamate receptors activate which signal cascades and how do these cascades link to specific gene transcription? Do pathways differ between early and late in the night? How is the resetting system gated so that light does not reset the clock during the subjective day? While we can point to studies that address these questions, such as the different initial signal transduction pathways for photic delays and advances (Gillette and Mitchell, 2002) as well as for the genetic response (Albrecht et al., 2001; Yan and Silver, 2002), much remains as future challenges.

2. Non-photic inputs are identified by their phase-shifting effects during the subjective day

Non-photic manipulations that increase arousal, such as novelty-induced activity (Reebs and Mrosovsky, 1989), social interaction (Mrosovsky, 1988), and saline injection and/or handling (Mead et al., 1992), can phase advance circadian rhythms if they occur during the subjective day. Some of them can also induce phase delays when applied during the subjective night, but these delays are smaller and less consistently observed (i.e., Marchant and Mistlberger, 1996). They can be potentiated by pre-exposure to 2 days of constant light (Knoch et al., 2004). Several pharmacological manipulations, such as short-acting benzodiazepine administration and morphine injections induce daytime phase advances which are ultimately dependent upon locomotor activation (Marchant and Mistlberger, 1995; Mrosovsky, 1996; Van Reeth and Turek, 1989). Behavioral activity has been shown to differentially and significantly decrease the spontaneous firing activity rhythm of the SCN cells of freely-moving animals (Schaap and Meijer, 2001), suggesting that there is a direct feedback of the behavioral state on the clock. Additional evidence has been recently published regarding the direct effect of the vigilance state on the electrical activity of the SCN, both recorded in vivo in freely-moving animals (Deboer et al., 2003).

Non-photic stimuli arrive into the SCN via the geniculohypothalamic tract (GHT) from the intergeniculate leaflet of the thalamus (IGL), utilizing neuropeptide Y (NPY), gamma-amino butyric acid (GABA), and endorphins (Harrington, 1997), or from the median (and/or dorsal, depending on the species) raphe nuclei, utilizing, in part,

serotonin (5-HT) (Meyer-Bernstein and Morin, 1996; Mistlberger et al., 2000). Although there are most probably more regulatory substances coming from other parts of the brain, the GHT and the raphe input mediate (alone and/or in combination) the phase resetting effects of many non-photic entraining stimuli during the subjective day. Examples of such non-photic stimuli include locomotor activation (spontaneous wheel running activity, forced treadmill running), handling and i.p. saline injections, dark pulses, short-acting benzodiazepines, opioids, and sleep deprivation (Antle and Mistlberger, 2000; Biello et al., 1994; Glass et al., 2003; Grossman et al., 2000; Gannon and Rea, 1995; Byku and Gannon, 2000; Harrington and Rusak, 1986; Hastings et al., 1992; Janik and Mrosovsky, 1994; Johnson et al., 1988b; Marchant et al., 1997; Maywood et al., 1997; Meyer-Bernstein and Morin, 1998; Sumova and Illnerova, 1996).

Neurotransmitters associated with non-photic input pathways such as NPY, 5-HT, GABA and enkephalins are capable of phase advancing the SCN master oscillation when applied during the subjective day, in vivo and in vitro (for review see Hastings et al., 1998; Harrington and Schak, 2000; see Fig. 3). Although there are some variations in the circadian time of maximal efficacy, all of these compounds appear to have a phase advancing effect on the circadian system during the subjective day. The mechanisms by which such effect can be achieved appear to be related to the basic genetic machinery of the clock.

During the subjective day, NPY, serotonin, benzodiazepine treatment and behavioral activation can decrease the normal midday rise of *per1* and/or *per2* (Maywood et al., 1999; Maywood and Hastings, 2000; Horikawa et al., 2000; Yokota et al., 2000; Fukuhara et al., 2001). Previous work published by our lab demonstrated that although wheel-running pulses can temporarily decrease *per1*, phase shift occurrence is more closely related to *per2* suppression (Yannielli et al., 2002; see Fig. 4). The signal transduction path leading to phase shifts differs for the various non-photic phase-shifting pathways. For example, NPY subjective day phase shifts depend on protein kinase C activation (Biello et al., 1997b; Harrington and Schak, 2000) whereas serotonin phase shifts depend on both protein kinase A and K^+ channel activity (Prosser et al., 1994). The NPY Y2 receptor mediates subjective day phase advances by NPY (Golombek et al., 1996; Huhman et al., 1996) while the 5-HT1A/7 receptors mediate serotonin-induced advances (Ehlen et al., 2001; Mintz et al., 1997).

An upsurge of spontaneous locomotor activity in the subjective day can increase Fos-immunoreactivity (-ir) in the hamster IGL and pretectum, and decrease Fos-ir in the SCN (Mikkelsen et al., 1998). Effects of the serotonergic agonist quipazine on Fos-ir in the SCN vary with species (reviewed in Antle et al., 2003). This may be a correlate rather than a cause of the phase shift observed following quipazine treatment in some species, because quipazine was observed to decrease Fos-ir in hamsters but not to induce a phase shift (Antle et al., 2003).

Light can interact with non-photic phase advances during the subjective day. Subjective day phase-shifting actions can be blocked by light, as has been shown for NPY microinjections (Biello and Mrosovsky, 1995; Maywood et al., 2002), novel wheel activity pulses (Yannielli et al., 2002), serotonin (Ehlen et al., 2001) and sleep deprivation (Grossman et al., 2000). This effect can be reproduced in vitro using glutamate to mimic light (Biello et al., 1997a) and has been shown to depend upon protein synthesis (Hall et al., 1999). Also, NPY-induced suppression of *per1* and *per2* genes in the SCN was attenuated when a light pulse was delivered immediately after the infusion (Maywood et al., 2002).

3. Non-photic inputs can block effects of light in the subjective night

Behavioral arousal in hamsters can alter entraining effects of light (Mrosovsky et al., 1989, 1992; Mrosovsky and Salmon, 1987; Sinclair and Mistlberger, 1997), and

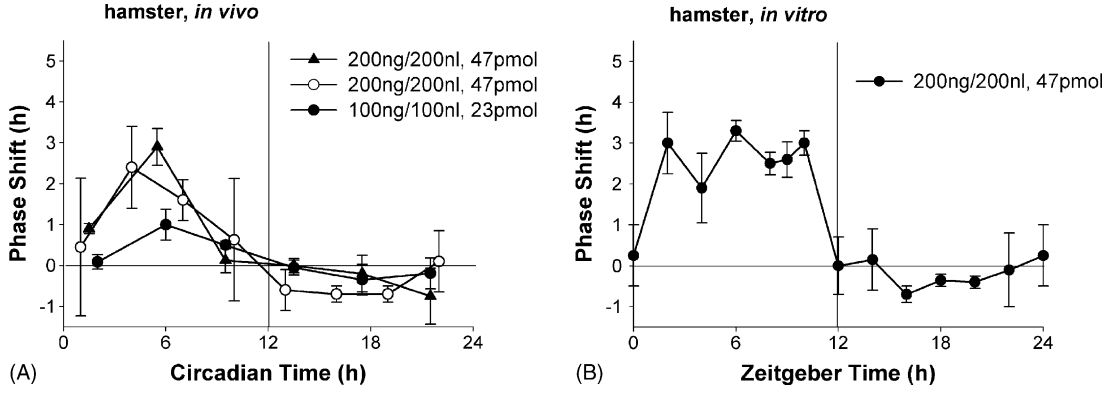

Fig. 3. Neuropeptide Y induces phase advances in circadian rhythms when applied during the subjective day. Panel A depicts the phase response curve to NPY in vivo for Syrian hamsters, according to Biello and Mrosovsky (1996) (triangles), Albers and Ferris (1984) (open circles) and Huhman and Albers (1994) (dark circles), at different doses. NPY consistently induced a phase advance around CT6, and this effect seems to be dose-dependent. Panel B depicts the phase response to NPY in the SCN of hamsters in vitro, according to Harrington and Schak (2000). Here, again, NPY induced a phase advance of the spontaneous electrical firing rhythm, with a broad range of sensitivity throughout the day (modified from Harrington and Schak, 2000).

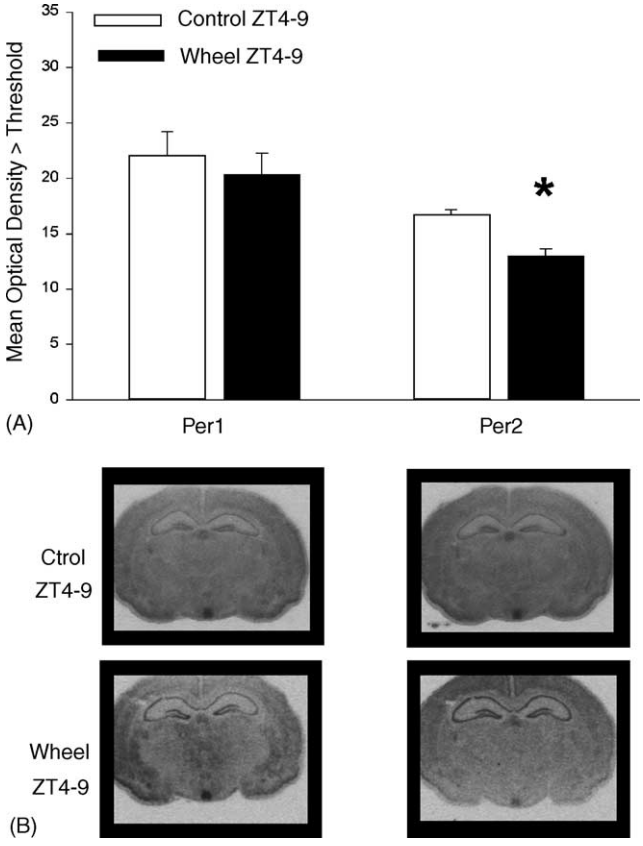

Fig. 4. Panel A shows *per1* and *per2* expression in the SCN of hamsters sacrificed at ZT9, with or without running wheel access for 5 h of dim red light conditions. This treatment had a significant phase advancing effect on the rhythm of spontaneous firing activity of SCN cells, as measured on brain slices maintained in vitro (Yannielli et al., 2002). The effect of a novel wheel pulse on diurnal basal expression of *per1* and *per2* was evaluated through in situ hybridization. After novel wheel access from ZT4 to ZT9 and/or transfer to dim red light, animals were killed, the brains quickly dissected and frozen. Optical density (^{35}S-labeled *per1* and *per2* autoradiograph) is plotted as mean ± S.E.M. Expression of *per2* was significantly decreased after 3 h of novel wheel, while expression of *per1* was unaffected at this time. Panel B shows representative images of *per1* and *per2* mRNA levels in the SCN at the time of the end of each treatment (ZT4–9, wheel access or control group) (from Yannielli et al., 2002).

decrease the phase advance shift response to light (Ralph and Mrosovsky, 1992; Mistlberger and Antle, 1998; Edelstein et al., 2003). Similarly, in mice, increased activity can alter entrainment (Mistlberger and Holmes, 2000) and can reduce phase advances to light (Mistlberger and Holmes, 1999; effects on delays were similar but not statistically significant, $P = 0.06$). Also, sleep loss or attendant low-intensity continuous activity appear to modulate the response of the hamster circadian system to light (Mistlberger et al., 1997).

These effects of behavioral arousal and/or increased activity can be mimicked by many of the same neurochemicals capable of phase advancing the circadian system during the day. Thus, NPY, serotonin, and opioids all inhibit light-induced phase shifts during the subjective night. Effects of GABA are complex, probably due to the presence of GABA in SCN neurons as well as in multiple input pathways and sites of action, including the retina. In general, it has been shown that GABAergic activation during the subjective night interferes with light or light-mimicked induced phase shifts in vivo (Gillespie et al., 1997; Mintz et al., 2002; Della Maggiore and Ralph, 1999). Recently, it has been described that selective agonists of the adenosine A1 receptors can attenuate light-induced phase advances and delays in hamsters and mice (Elliott et al., 2001; Sigworth and Rea, 2003) by interfering with the activation of phosphokinases, an event that occurs early in the photically initiated signaling cascade. Finally, delta opioids have been recently shown to inhibit light-induced phase advances in hamsters (Tierno et al., 2002). Of all these neurotransmitters, NPY and serotonin are the best characterized at the moment, due to the direct (and, in the case of serotonin, indirect as well) input pathways to the SCN, NPY from the thalamic intergeniculate nuclei, and serotonin from the raphe nuclei.

In vivo, it was previously shown that NPY could inhibit light-induced phase advances but not delays (Weber and Rea, 1997), although a recent study establishes that NPY reduces both advances and delays (Lall and Biello, 2003a, 2003b; see Fig. 5). In vitro, NPY can inhibit the phase-shifting effect of glutamate (Biello et al., 1997a, 1997b), *N*-methyl-D-aspartate (NMDA; Yannielli and Harrington, 2001a, 2001b) and in vivo delivered light pulses (Yannielli and Harrington, 2000).

This inhibition is mediated by the NPY Y5 receptor type (Yannielli and Harrington, 2001a, 2001b; Yannielli et al., 2004; Lall and Biello, 2003a). Inhibitory effects of NPY on light-like phase shifts in vitro (using NMDA to mimic the effects of light in the SCN slice preparation) were blocked by a selective Y5 antagonist, RJW-57926 (Yannielli and Harrington, 2001a, 2001b). Several Y5 selective antagonists were able to counteract the inhibitory effects of NPY on phase shifts to NMDA measured in vitro, and light-induced phase shifts in vivo (Lall and Biello, 2003a; Yannielli et al., 2004). The mechanism by which NPY exerts this blockade appears to be via a sustained inhibition of light-induced *per2*, compared with a brief transient inhibition of *per1* (McKinley Brewer et al., 2002; see Fig. 6). In agreement with our results, a recent study shows that novelty-induced activity during the subjective night decreased light-induced phase advances without modifying light-induced c-Fos protein or *per1* mRNA expression at the level of the SCN (Edelstein et al., 2003). Future experiments should focus on *per2*, a likely target for this effect of NPY.

Serotonin can inhibit phase shifts to light when 5-HT1A/7 agonists are applied onto the SCN (Weber et al., 1998). 8-Hydroxy-2-(di-n-propylamino)-tetralin hydrobromide (8-OH-DPAT; a 5-HT1A/7 agonist) reduces light-induced phase shifts in hamsters when either delivered via a cannula near the SCN or injected systemically (Ehlen et al., 2001; Rea et al., 1994; Weber et al., 1998), although one study found that 8-OH-DPAT suppressed phase advances to low

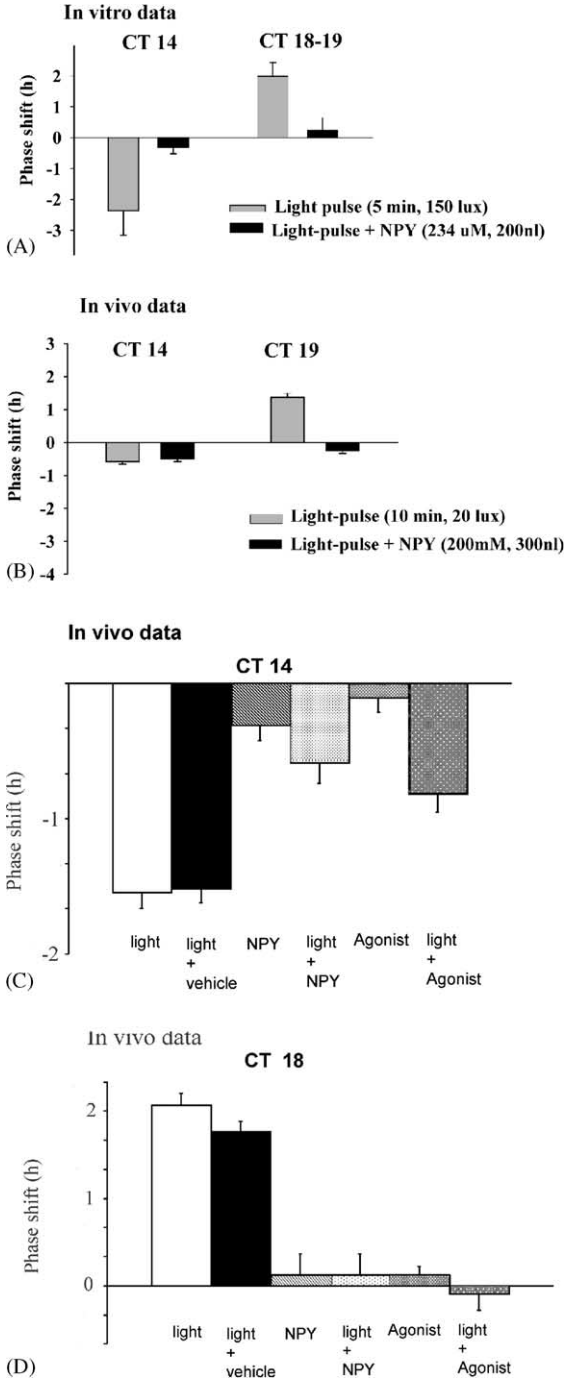

Fig. 5. Microinjection of NPY can reduce or block both phase advances as well as phase delays to light. Panel A shows the NPY blocking effect of light-induced phase shifts on the electrical activity of the SCN as recorded in vitro (Yannielli and Harrington, 2000). The effect of NY applied immediately after the light pulse and slice preparation was significant at both circadian times. Panel B shows the effect of NPY microinjected directly into the SCN of hamsters, according to Weber and Rea (1997). Here, NPY blocked the phase advancing effect of light, without affecting delays. Panel C shows in vivo data from Lall and Biello (2003), where NPY microinjected into the SCN significantly inhibited both light-induced phase advances and delays. These effects could be mimicked using an NPY Y1/Y5 agonist.

intensity (5 lx) light but not to higher intensity light (20 and 60 lx; Moriya et al., 1998). Micro-iontophoretic application of serotonin or 5-HT1A/7 agonists inhibits the spontaneous activity and photic responses of cells within the SCN (Ying and Rusak, 1994), and electrical stimulation of the median and/or dorsal raphe nuclei significantly attenuates light-induced c-Fos induction early in the subjective night (Meyer-Bernstein and Morin, 1999). Surprisingly, 8-OH-DPAT had no effect on light-induced phase shifts in mice (Antle et al., 2003), although it was shown that serotonin reduces the effect of RHT input to the SCN by acting at both 5-HT1B and 5-HT7 receptors in mice (Smith et al., 2001). Regarding the serotonergic receptors involved in this effect, pre-treatment with the 5-HT1 antagonist metergoline prevented effects of induced activity on light-pulse phase advance shifts (Mistlberger and Antle, 1998). This effect might be explained by direct action of metergoline on 5-HT receptors in the SCN, but might also be explained by effects of metergoline on raphe neurons. Metergoline might reduce auto inhibition of serotonergic raphe neurons, which would be expected to reduce serotonergic inhibition of IGL neurons (Mistlberger and Antle, 1998).

Another type of serotonin receptor is involved in the serotonergic modulation of circadian responses to light. These are the 5-HT1B receptors, located on the terminals of the RHT axons, where glutamate is released upon photic stimulation. Thus, the serotonergic effect mediated by these receptors is presynaptic. Phase shifts to light are reduced when the presynaptic 5-HT1B receptors of the retinal axon terminals are activated (Rea et al., 1994; Selim et al., 1993). Activation of the 5-HT1B receptors appears to reduce light-induced glutamate release from RHT terminals, in mice (Smith et al., 2001) and hamsters (Pickard et al., 1999), and decreases light-induced c-Fos expression at the level of the SCN (Pickard et al., 1996). Accordingly, 5-HT1B agonists applied systemically inhibit light-induced phase shifts and light-stimulated c-Fos expression in the SCN of hamsters (Pickard et al., 1996). The free-running period under constant light housing conditions lengthens significantly more in 5-HT1B knockout mice compared with controls, but the genotype fails to show any other changes in entrainment or photic synchronization (Sollars et al., 2002). Taken together, these results suggest that serotonin is able to counteract the effect of light during the subjective night, through two different receptors and mechanisms, i.e., a presynaptic one (5-HT1B) and a postsynaptic one (5-HT1/7).

In agreement with these findings, chronic treatment with the antidepressant clorgyline attenuates phase shift responses to light (Duncan et al., 1998), an effect that might be related to the predicted elevated levels of serotonin and/or serotonergic activity caused by a chronic antidepressant (Yannielli et al., 1999). As well, chronic clomipramine treatment of neonate hamsters (a procedure thought to cause a long-lasting set of symptoms that resemble endogenous depression) also induced changes in circadian photic responses, i.e., lower re-entrainment rate to a phase advance

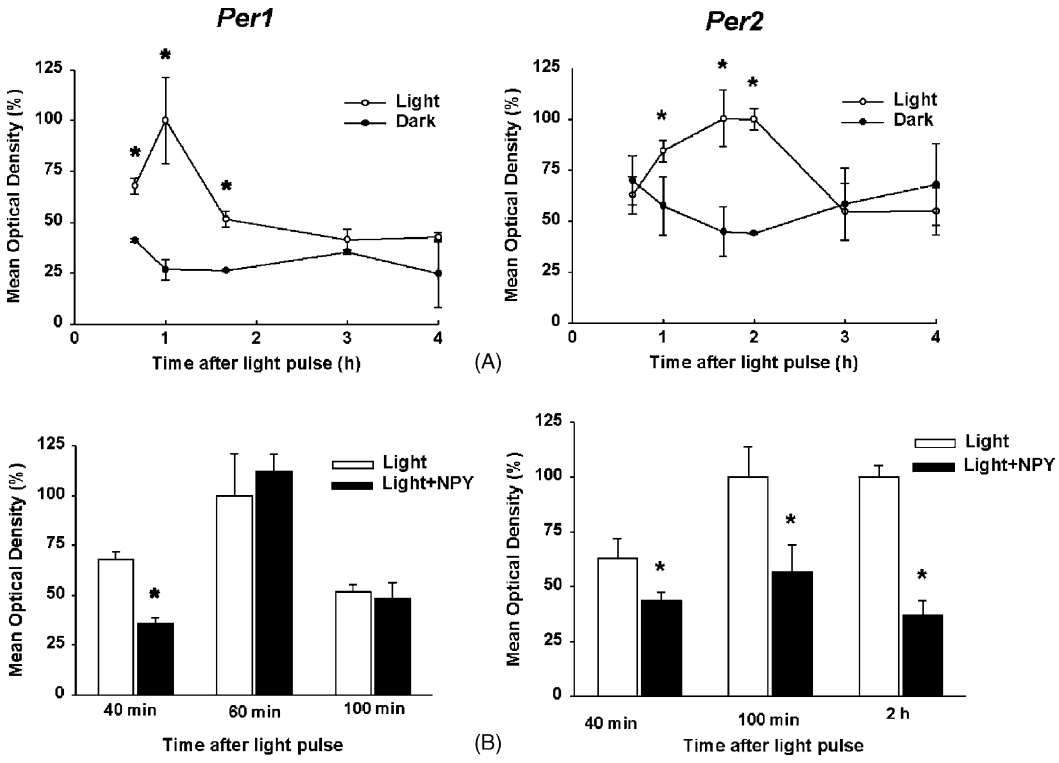

Fig. 6. Light induction of *per1* and *per2* mRNA in hamster SCN, measured after selected time periods in vitro following light stimulation at CT14. *per* expression was evaluated through in situ hybridization. *Upper panels*: Mean optical density (^{35}S-labeled *per1* and *per2* autoradiograph) is plotted as a percentage of the peak of light-induced *per1* and *per2*. *per1* is rapidly induced, with maximal expression 1 h after light stimulation. *per2* rise takes longer, with maximal expression between 1.5 and 2 h after light stimulation. *Lower panels*: NPY inhibition of light-induced *per1* expression is apparent 40 min after light stimulation, while NPY inhibition of light-induced *per2* expression was significant and sustained at least 2 h after light stimulation, and throughout the time examined (modified from McKinley Brewer et al., 2002).

of the LD cycle, and reduced response to light late in the subjective night (Yannielli et al., 1998). The reduced photic response following chronic neonatal clomipramine could be due to decreased autoinhibition of neurons in the raphe, resulting in increased serotonin in the SCN, as will be discussed later. Thus, the inhibitory effect of serotonin on circadian photic responses might be of importance in the context of certain mental disorders presumably linked to low levels of serotonin in the brain, such as endogenous depression or seasonal affective disorders.

3.1. Mechanisms

Serotonin-induced inhibition of photic responses was accompanied by decreased light-induced c-Fos expression in the SCN (Selim et al., 1993; Glass et al., 1994) and decreased light-induced expression of *per1* and *per2* clock genes (Horikawa et al., 2000; Yokota et al., 2000). In the case of NPY, preliminary results from our lab indicate that it has no effect on light-induced c-Fos protein expression (Molyneux et al., 2003), but it transiently decreases light-induced *per1* mRNA expression and completely suppresses light-induced *per2* mRNA expression (McKinley Brewer et al., 2002). Novel wheel access had no effect on light-induced Fos protein and *per1* mRNA (Edelstein et al., 2003). Also, a recent study from our lab demonstrated that three days of wheel running access under constant dark conditions decreases the magnitude of light-induced phase delays (as compared with three days of constant darkness without wheel running access), without any significant modification of light-induced *per1* expression levels in the SCN (Christian and Harrington, 2002). A general mechanism appears to be that non-photic inputs can decrease light-stimulated transcription of *per1* and *per2* genes (Maywood and Mrosovsky, 2001). Our results suggest that expression of *per2* mRNA is most relevant for non-photic effects on light-induced phase shifts (McKinley Brewer et al., 2002).

Moreover, the time course of the NPY effect on photic circadian responses is interesting. NPY is capable of blocking light-induced phase shifts even when applied 60 min after the light (Yannielli and Harrington, 2000; Lall and Biello, 2002). A similar time course is observed for novel wheel-induced activity (Lall and Biello, 2002; see Fig. 7). This suggests that the mechanism by which these modulators can decrease the response of the circadian system to light involves later events, more than early events, in the light-induced cascade of second messengers and gene activation that leads to a phase shift. One possibility is that hyperpolarization of the cell membrane, potentially by the

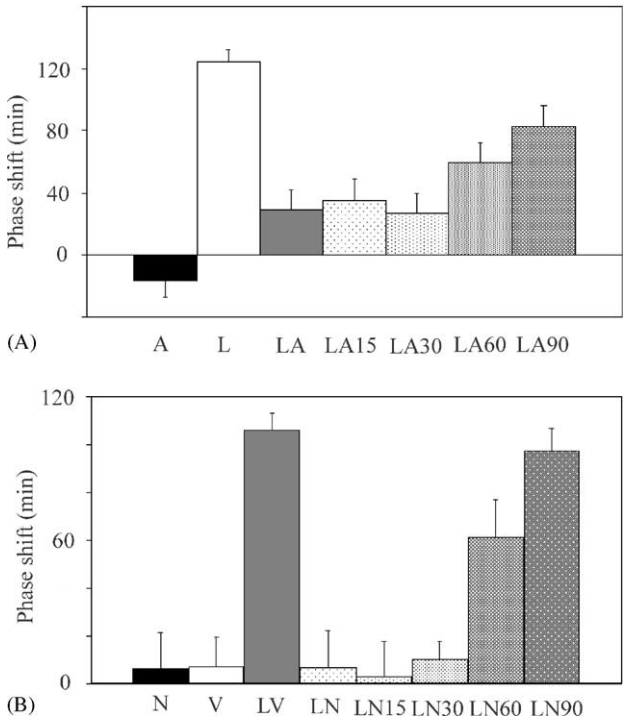

Fig. 7. Panel A shows the ability of 3 h of novel wheel access to attenuate light-induced phase advances when presented within 60 min of the photic stimulus. Panel B shows the ability of NPY to attenuate (in a comparable way as 3 h of wheel running access) light-induced phase advances, within the same time frame (from Lall and Biello, 2002).

activation of potassium channels long after the light stimulus is concluded, can bring to an end the normal sequence of events that take place within the SCN following light exposure, rendering the system unable to complete the process. Another possibility is that these inputs are affecting the output of the system, perhaps by blocking communication within the SCN (see Yan and Silver, 2002). The first hypothesis appears to be supported by the fact that NPY significantly and differentially suppresses *per2* mRNA expression, one of the later transactions in the photic synchronization cascade. As well, results from our lab demonstrated that NPY causes increased conductance through the potassium channels in SCN neurons (Hall et al., 1999). This topic has not yet been thoroughly investigated, so definitive answers are not possible.

3.2. Asymmetry of the behavioral feedback effect on photically induced circadian responses

One striking phenomena among the narrow range of ''non-photic'' modulators of circadian responses to light is that NPY and increased locomotor activity appear to be more effective later in the subjective night, and less effective earlier, in the phase-delaying portion of the photic phase response curve. One of the earliest studies demonstrated that access to a novel wheel could inhibit light-induced phase advances, while the same stimulus did not affect the light-induced phase delays (Ralph and Mrosovsky, 1992). Confinement to a novel wheel before and during the administration of a light pulse can attenuate phase advances, but not delays, and this effect has been shown to be reversed by the serotonin antagonist metergoline (Mistlberger and Antle, 1998).

The inhibitory action on light-induced phase shifts is different, in this sense, among NPY or locomotor activation and serotonin: in hamsters, serotonin appears to counteract light throughout the circadian cycle: 5-HT1A/7 agonists such as 8-OH-DPAT injected systemically inhibit light-induced phase advances and delays, although delays are affected at higher doses (Rea et al., 1994). Intra-SCN administration of 8-OH-DPAT also inhibits light-induced phase advances (Weber et al., 1998). Systemic administration of 5-HT1B agonists inhibits both light-induced phase advances and delays (Pickard et al., 1996). Median and/or dorsal raphe electrical stimulation paired with saturating light stimulation significantly attenuates light-induced c-Fos expression in the SCN of hamsters early in the subjective night (Meyer-Bernstein and Morin, 1999). Altogether, these findings suggest that the serotonergic system, and behavioral changes associated with it, can get to the clock and negatively adjust the way the circadian system responds to light stimulation. This effect is likely to be achieved through two different mechanisms, presynaptic and postsynaptic, both of them involving events in the light-elicited cascade of events that occur very early, or immediately, after light stimulation. In other words, serotonin affects light input at a stage that is common for both phase delaying and phase advancing.

Although early studies indicated that NPY applied in vivo significantly decreased light-induced phase advances but not delays (Weber and Rea, 1997), recently it has been shown that NPY applied into the SCN concurrently with a light pulse can decrease (although to a lesser extent when compared with the same effect on phase advances) the phase-delaying effect of light (Lall and Biello, 2003a, 2003b). In vitro studies, for example, reveal that NPY can decrease NMDA-induced and in vivo administrated light-induced phase advances and phase delays as well (Yannielli and Harrington, 2000, 2001a, 2001b). It is not clear why non-photic modulators would affect light only at a certain circadian phase, or if this distinctiveness has a ''within the clock'' neurochemical base, or it relies more on the behavioral state of the whole organism. These findings suggest that the lack of effect of behavioral activation early in the subjective night is more likely related to the complex series of inputs that can provide feedback to the circadian clock, and that are lost when the experiments are conducted on brain slices (Vansteensel et al., 2003).

One might wonder which is the stimulus, or the set of stimuli, that are taking part in the non-photic–photic negative interaction, particularly when a complex behavioral activation is involved. What is most significant about novel wheel access late at night — increased locomotor activity, increased

arousal, or increased anxiety? Could this activation be significantly higher at this circadian time and not at the beginning of the subjective night, when the locomotor activity rhythm goes through its acrophase? Does some other non-photic stimulation, such as sleep deprivation, have a role as well? Does light itself induce a different behavioral state early and late in the subjective night, rendering the circadian system uniquely susceptible to certain behavioral stimuli?

Although the effect of IGL lesions on photic synchronization could have resolved this divergence, the outcome of IGL lesions is complex and inconclusive, and will be discussed further.

3.3. Lesion studies

From the studies reviewed earlier, one would expect that ablation of the IGL or the serotonergic inputs to the SCN would potentiate phase shifts to light. Is this the case?

Studies of hamsters with IGL lesions have supported our prediction showing an increase in phase delays to light as compared to controls (Pickard et al., 1987). As well, the normal lengthening of free-running period under constant lighting conditions did not occur in IGL-lesion animals (Harrington and Rusak, 1988), suggesting that the lesion significantly alters both phasic and tonic effects of light on the circadian system. Other studies do not support our hypothesis. Phase advances to light are either not affected (Redlin et al., 1999) or are decreased (Harrington and Rusak, 1986; Pickard et al., 1987), and two studies reported no alteration in phase delays to light following IGL ablation (Redlin et al., 1999; Harrington and Rusak, 1986). IGL lesions do not alter entrainment to standard laboratory LD cycles (Harrington, 1997) but can alter phase of entrainment of hamsters under sinusoidal lighting cycles (Pickard, 1989) and prevents entrainment of rats to a skeleton photoperiod of two 1 h light pulses (Edelstein and Amir, 1999).

There are many caveats necessary when interpreting IGL lesion studies. For one, the IGL input to the SCN contains more than NPY. Lesions of the hamster IGL lead to loss of enkephalin-ir fibers in the SCN (Morin and Blanchard, 2001). All IGL neurons in the rat contain GABA (Moore and Speh, 1993). Secondly, damage in this area would be expected to damage axons and target cells of the projection from SCN and peri-SCN neurons to the geniculate (Watts et al., 1987) with likely degeneration of that subpopulation of SCN area neurons. Third, lesions of the IGL also damage fibers of passage; most importantly, retinal fibers providing input to most visual system terminal areas. While a few studies have employed excitotoxic substances to inflict cellular damage sparing fibers of passage, damage to cells can lead to degeneration in areas afferent or efferent to the site of damage. Most critically, retinal degeneration is a real possibility. Geniculate lesions or optic tract damage in adult animals causes progressive degeneration of the retina (Hollander et al., 1984; Pearson and Thompson, 1993). Specific retinal ganglion cell subclasses are affected more than others, leading to major alterations in retinal physiology (Hollander et al., 1984). Thus, it may be that interpretation of photic responses of animals with IGL lesions is complicated by a host of poorly defined progressive degenerative changes, as well as by the loss of more neurochemicals than simply NPY. A final consideration is that there are sparse NPY afferents to the SCN that arise from an area other than the IGL (Harrington et al., 1985); the source of these afferents is unknown, but it remains possible that some effects of NPY on the SCN are attributable to these terminals.

With regard to lesions that affect the serotonergic innervation to the SCN, treatment with 5,7-DHT (a toxin that specifically deplete the brain of serotonin) increased phase delays to light (30 min pulse, between CT13 and CT15; 450 lx) in most hamsters and was associated with strong (“Type 0”) resetting in some animals (Morin and Blanchard, 2001). Mice treated with 5,7-DHT also showed increases in phase delays to light (30 min, 30 lx, between CT12 and CT20) (Bradbury et al., 1997).

Once again, cautions in interpreting these studies are in order. Studies have shown re-growth of serotonergic fibers following damage induced by intraventricular injection of 5,7-DHT (Morin et al., 1992). It is important to note that this approach does not damage the entire raphe input to the SCN and IGL: only about 50% of the medial raphe neurons projecting to the SCN are serotonergic, and about 40% of the dorsal raphe neurons projecting to the IGL are serotonergic (Meyer-Bernstein and Morin, 1996). Because the dorsal raphe nuclei innervates the IGL, the destruction of the raphe-SCN projection alone does not rule out the co-participation of serotonin and NPY in the attenuation of light. It has been shown that specific median raphe lesions induce changes in entrainment in hamsters, while dorsal raphe (DR) lesions do not affect the circadian system (Meyer-Bernstein and Morin, 1996). Destruction of DR only slightly modulates the wheel-running circadian rhythm in mice (Barbacka-Surowiak and Gut, 2001).

4. Pharmacological compounds that target non-photic inputs can potentiate the entraining effects of light

Given the general findings that non-photic inputs can block the effects of light in phase-shifting rhythms during the subjective night, a natural question arises. Is it likely that those treatments that decrease the action of endogenous non-photic compounds potentiate the phase-shifting effects of light? There is some support for this possibility.

The earliest study (Biello, 1995) used antiserum to neuropeptide Y because selective antagonists were not yet available. Hamsters with a cannula directed to the SCN received injections of NPY antiserum at circadian time CT18 followed by a light pulse. Treatments were given to hamsters held in constant darkness for 3 weeks or longer. NPY antiserum was able to potentiate phase shifts to light. Hamsters showed larger phase shifts when pre-treated with

antiserum (mean shift: 1.92 h) than when pre-treated with normal serum (mean shift: 1.18 h). Administration of NPY antiserum alone had little effect on phase (mean shift: 0.28 h; Biello, 1995).

Because this effect of NPY is mediated by the Y5 receptor (Yannielli and Harrington, 2001a, 2001b; Lall and Biello, 2003a), an NPY Y5 antagonist should have a similar effect. Two recent studies suggest that it does. As is shown in Fig. 8, hamsters given light pulses following pre-treatment with a systemically administered NPY Y5 receptor antagonist developed by Pfizer Inc. (CP-781214) showed phase shifts significantly greater than those recorded from animals given light alone (Yannielli et al., 2004). Similar findings have been found by Lall and Biello (2003c) using a different compound developed by Johnson & Johnson Pharmaceutical Research Institute (RWJ-57926; McNally et al., 2000; Youngman et al., 2000) with administration directly to the SCN via a cannula. Interestingly, although Y5 receptor antagonists did inhibit the effect of NPY on NMDA-induced phase shifts in vitro, they did not potentiate phase shifts to NMDA in vitro (Yannielli and Harrington, 2001a, 2001b; Yannielli et al., 2004; see Fig. 8). This result may be explained by differences in endogenous NPY tone between in vivo and in vitro situations.

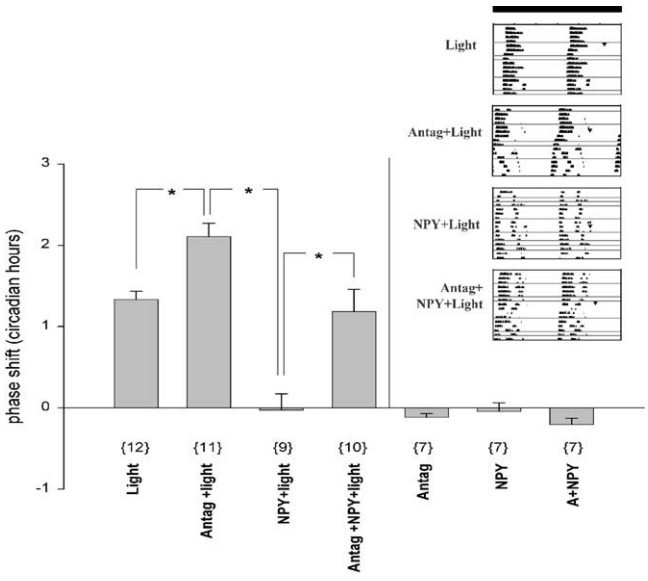

Fig. 8. Phase shifts of wheel-running rhythms are summarized for hamsters treated with various combinations of light, NPY Y5 antagonist CP-781214 (10 mg/kg) and NPY (200 ng/200 nl; 234 μM), at CT19. The CP-781214 compound was injected s.c., 30 min prior to other treatments. Light treatment was a 5 min, 200 lx light pulse at CT19. NPY Y5 antagonist could potentiate light-induced phase advance, while NPY blocked the effect of light. The antagonist could also reverse the blocking action, demonstrating that both effects of NPY on photic responses (blockade and potentiation) are mediated through the Y5 receptor. *Inset*: Representative actograms for the treatment groups summarized in the graph. Wheel revolutions are shown as black, with each day's activity plotted below the previous day, and the entire record duplicated on the right (''double-plotted''). Treatments are shown as an inverted triangle, with the type of treatment indicated by the label on the left (from Yannielli et al., 2004).

Suppression of serotonergic non-photic input pathways can similarly potentiate the phase-shifting effects of light. The only report of a pure 5-HT receptor antagonist potentiating photic responses of circadian rhythms uses WAY-100635, a compound specific for the 5-HT1A receptor (Smart and Biello, 2001). This drug potentiated phase delays to light but not phase advances. Other compounds (reviewed in detail further) are able to potentiate both light-induced advance and delay phase shifts, but possibly through their ability to act as agonists at the somatodendritic autoreceptors in the raphe and/or as antagonists of the same 5-HT1A postsynaptic receptors at the serotonergic terminals in the SCN, thus indirectly causing a strong decrease in serotonin levels in the SCN (e.g., Dudley et al., 1998). Given that presynaptic 5-HT1B receptors, located at the RHT terminal, have been shown to significantly inhibit light-induced phase shifts, it would be worthwhile to determine if antagonizing these receptors potentiates the effects of light.

NAN-190, an agonist at the raphe autoreceptors and a 5-HT1A/7 antagonist potentiates the circadian response to light in hamsters (Rea et al., 1995). Most dramatically, pre-treatment with systemic injections of NAN-190 increased phase advances to light (10 min, 20 lx) at CT19 from an average of 1.6–4.1 h (delays were increased from 0.5 to 0.9 h). Phase advances to light were potentiated at all light intensities tested (0.2, 2, 20 and 200 lx).

Several studies have focused on MKC-242, a selective 5-HT1A receptor agonist (Matsuda et al., 1995). Treatment of hamsters with MKC-242 on the day of a shift in the LD cycle (3 mg/kg i.p. at ZT20, over 2 days) accelerated re-entrainment to both advances and delays (Moriya et al., 1998). Pre-treatment with MKC-242 30 min prior to a light pulse greatly potentiated the phase advance shift (mean shift without MKC-242: 1.98 h; mean shift with MKC-42: 4.25 h; Moriya et al., 1998). This potentiating effect of MKC-242 was also observed when the drug was injected 20 or 60 min after light onset, after the light pulse was ended, a delayed time course reminiscent of effects of NPY (see Fig. 7). MKC-242 was also effective in potentiating phase delay shifts to light in mice, although the magnitude of this effect was not as large as the effect observed in hamsters (Takahashi et al., 2002; see Fig. 9). Pre-treatment with MKC-242 did not alter light-induced c-Fos in the SCN or IGL (Moriya et al., 1998). Whereas MKC-242 did not increase peak levels of light-induced *per1* or *per2* (although effects on *per2* approached significance, $P = 0.08$), the duration of time that these levels were high following a phase-shifting light pulse was increased (Takahashi et al., 2002). Levels of *per1* and *per2* were still high 3 h following a light pulse (5 min, 70 lx, CT16) in mice pre-treated with MKC-242. The potentiation of *per2* expression by MKC-242 was more dramatic in mice housed 9–10 days in constant darkness versus those housed 2 days in dark–dark (DD) prior to the light treatment.

Three compounds (BMY 7378, S 15535, and MDL 73005 EF; see Gannon, 2003 for references) are reported to

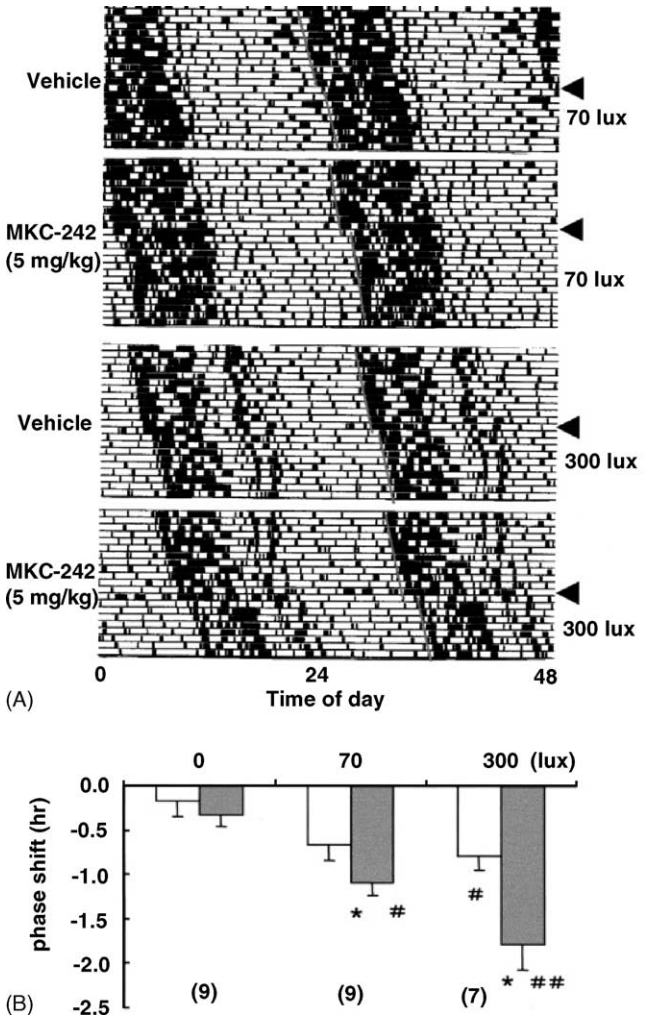

Fig. 9. Panels A and B show the effect of MKC-242 on light-induced phase delays in mouse circadian activity rhythm. *Panel A*: Double-plotted actograms. Each line in the figure shows the onset of activity. Arrows exhibit the day of drug injection and light exposure. MKC-242 or vehicle was injected into each mouse at CT15.5; 30 min later light (70 or 300 lx) was applied for 5 min at CT16. *Panel B*: Individual animals that received vehicle (open column) or MKC-242 (5 mg/kg i.p.) (grey column). Numbers in parentheses represents the number of animals. MKC-242 significantly potentiated light-induced phase delays at both light intensities tested, namely, at 70 and 300 lx light pulses (from Takahashi et al., 2002).

potentiate light-induced resetting of circadian rhythms in hamsters. All share the pharmacology of being agonists at presynaptic 5-HT1A receptors and antagonists at postsynaptic 5-HT1A receptors. Most consistent potentiating effects were observed for the drugs BMY 7378 and S 15535 (given i.p. 45 min prior to the light pulse) and for phase advancing light pulses at CT19. Phase delay shifts following light pulses at CT14 were not increased by either compound.

Because most (if not all) of these studies have used systemic administration of the serotonergic antagonists, the question of where the drug acts is yet to be conclusively answered. It might be possible that the potentiation of light-induced phase shifts is achieved only by the combination of effects at both levels, the raphe and the SCN, although indirect effects on other structures, such as the IGL, and other neurotransmitters, such as NPY, cannot be ruled out at the moment.

One mechanism that could be mediating this potentiation is an increase in stability of the transcripts signaling light to the circadian clock. Such a mechanism could explain the increased duration of light-induced *per1* or *per2* reported following MKC-242 pre-treatment (Takahashi et al., 2002). Because it contributes to the level and timing of gene expression, mRNA stability has emerged as an important regulatory factor in cellular processes. Changes in mRNA turnover could certainly contribute to adaptive changes in gene expression in response to changing environmental conditions. Stabilizing a transcript would allow a prolonged translational window for other genes that would not be engaged or sustained otherwise (for review see Guhaniyogi and Brewer, 2001). The rates at which degradation processes occur are determined by *cis*-acting elements within the transcript, and/or *trans*-acting elements that bind it. These include poly(A)-binding proteins, adenosine-uridine rich elements (AURE) or AURE-binding proteins that stabilize transcripts, estrogen, glucocorticoids, cytokines, calcium and iron (Ross, 1996). The signal transduction pathways that ultimately affect these factors are still unknown, but the possibility stands that a neurotransmitter such as NPY or serotonin could actually exert their effects by activating pathways that lead to the stabilization of the clock genes transcripts. This is a speculative hypothesis, but is supported by the observation of increased duration of light-induced *per2* expression following MKC-242 pre-treatment (Takahashi et al., 2002).

5. Endogenous levels of non-photic inputs throughout the day

As reviewed above, both serotonin and NPY have inhibitory actions on photically related SCN responses during the subjective night. As well, administration of serotonin 1A receptor antagonists or NPY Y5 receptor antagonists, directly onto the SCN or systemically, potentiates the phase-shifting effect of light, especially late in the subjective night. This finding might imply the existence of an endogenous tone of serotonin and/or NPY with some physiological role in the circadian system.

Extracellular levels of serotonin in the SCN are highest early in the night, when locomotor behavior is activated by the dark phase in rats and hamsters (Shioiri et al., 1991; Cagampang and Inouye, 1994; Dudley et al., 1998). Similar results are seen for its principal metabolite, 5-HIAA (Glass et al., 1992, 1993), reuptake binding sites (Wirz-Justice et al., 1983) and uptake of [^{3}H]5-HT (Meyer and Quay, 1976). Serotonin release in vivo at the level of the SCN is higher during the subjective night compared with subjective day, both under LD and constant dark housing conditions

(Dudley et al., 1998). Serotonin release measured at the SCN comes mostly from the median raphe innervation in hamsters (Dudley et al., 1998, 1999). Moreover, serotonin release at the level of the SCN is suppressed by novel wheel running activity late in the subjective night, systemic injection of the 5-HT1A agonist/antagonist BMY 7378, and median raphe microinjection of the 5-HT1A/7 agonist 8-OH-DPAT. These data suggest that the potentiation of light-induced phase shifts observed after in vivo administration of 5-HT1A agonist/antagonists is due to the activation of somatodendritic autoreceptors at the level of the raphe, inhibition of firing rate of serotonergic median raphe neurons, and consequent decrease of serotonin at the SCN. It is not clear if this effect can be seen also early in the subjective night, when the serotonin release is spontaneously maximal, or if other stimuli, such as a light pulse, might exert an acute change in the 5-HT release in the SCN.

NPY content and/or release at the SCN have not been studied so extensively, and results come from different species. For example, in rats, immunoassay of NPY levels shows two peaks, one around the time of each transition in a LD cycle (Shinohara et al., 1993b). Under constant dark housing conditions, peak NPY levels were seen early in the subjective night. Light stimulation during the night increased the amount of NPY measured at the SCN, with levels rising quickly (15 min after the beginning of the stimulation), and remaining high for more than an hour, even after the termination of the light pulse (Shinohara et al., 1993a). The distribution of NPY receptors in the hamster SCN overlaps the projection field of the RHT (Morin et al., 1992; Stopa et al., 1995; Yannielli et al., 2004). NPY receptor specific binding of ^{125}I-peptide YY (^{125}I-PYY) was greater 2 h after the onset of darkness than 4 and 1 h before dark onset in the ventrolateral SCN of hamsters under LD conditions (Stopa et al., 1995). Although the extensive studies of NPY content were conducted using rats and the receptor binding and mRNA studies used hamsters, preliminary results of our lab suggest that immunoassay of NPY levels in hamster SCN confirms higher levels 2 h after dark onset than at the middle of the light phase (data not published). Thus, these findings suggest that there is increased NPYergic activity (content and binding) during the early portion of the dark phase, and this activity may be increased following photic stimulation.

We can now explain the lack of a potentiating effect of the NPY Y5 antagonist when co-applied with light-mimicking compounds in vitro (Yannielli and Harrington, 2000; Yannielli et al., 2004), a perplexing result given the ability of such an antagonist to potentiate light shifts in vivo. Effects observed in vivo are likely due to suppression of the high endogenous levels of NPY, levels that are high in the early subjective night and increased further following light stimulation. In the in vitro brain slice preparation, endogenous tone is low, with circuitry unlikely to support regulated release in response to glutamatergic stimulation mimicking light. Thus, in the brain slice preparation the antagonist has no effects of NPY to oppose. We can explain another perplexing difference between in vivo and in vitro studies as well. The magnitude of phase delay shifts to light given prior to brain slice preparation or shifts to neurochemicals mimicking the effects of light are larger in vitro than the phase delays measured in response to light in vivo (see Biello et al., 1997a, 1997b; Yannielli and Harrington, 2000, 2001a, 2001b). This may indicate that endogenous tone of NPY in the early night normally acts to reduce the phase-resetting action of light, and the brain slice preparation, free of this suppressive input, expresses the true capability of light to reset the circadian clock at this phase.

The role of enkephalins and adenosine seems to be limited to a negative one. Delta opioid agonists were shown to inhibit light-induced phase advances of hamster circadian rhythm but antagonists for delta, mu, or kappa opioid did not potentiate light-induced phase shifts (Tierno et al., 2002). Similarly, adenosine A1 receptor agonist could reduce phase delays to light, but an A1 antagonist did not alter the magnitude of the light-induced phase shift (Elliott et al., 2001; Sigworth and Rea, 2003). These results imply the endogenous tone of enkephalin and adenosine levels in the SCN are not sufficient for a role in modulating light-induced shifts in phase, at least under the conditions tested.

6. Future directions

A first priority for future research on this topic is to more firmly establish the basic findings, especially where there are small effects reported in initial reports. For example, the effects of the NPY Y5 antagonist in potentiating light pulse phase shifts were relatively small in the initial report (58% potentiation of phase advances; Yannielli et al., 2004). Attention should be given to replication of this effect, using other compounds that have similar actions, as well as using other experimental approaches. For example, in an important study, Lall and Biello (2003c) delivered an NPY Y5 antagonist directly to the area of the SCN and measured a potentiation of ~50% of the light pulse phase shift. Effects of serotonergic compounds are larger (e.g., NAN-190: circa 250% increase; Rea et al., 1995) and replicated across several labs using varied compounds (see Section 4).

A second priority is to establish if such effects are general for light pulses early in the night as well as those later in the night, corresponding to light-induced phase delays or phase advances. One difficulty with NPY antagonists given early in the night is that they may not block effects of NPY from the endogenous tone, if such tone begins to increase prior to antagonist treatment. A long-lasting NPY Y5 receptor antagonist would make possible an experiment in which the receptor is blocked prior to the rise in endogenous tone, and effects on light-induced phase delays measured some hours later. Such an experiment might reveal effects of the

antagonist not apparent when the blocker is given closer to the time of the light pulse.

It will be very important to determine if the results reported here generalize to other species. Reports of non-photic phase resetting effects have been quite variable across species (for review see Mrosovsky, 1996; Hastings et al., 1998). The effects of non-photic inputs in modulating photic resetting have been observed in hamsters and mice (see Section 3). Measures of NPY endogenous tone have been published only for rats (Shinohara et al., 1993a, 1993b), while similar measures for serotonin are reported for hamsters and rats (see Section 5). The major focus of our review, the ability to potentiate phase resetting actions of light by altering non-photic inputs, has been reported in hamsters and mice. Although we would ultimately like to extend this work to humans, simply extending it to other laboratory animals, and especially to diurnal laboratory animals, is an important next step.

Future research should seek to distinguish tonic effects from phasic effects in the interaction of light and behavioral stimuli. Examination of the effects of tonic enhancement or suppression of non-photic input pathways have been stymied by the lack of appropriate research tools. Problems with the interpretation of lesions studies have been discussed above. Newer studies using transgenic mice will allow further examination of these questions, albeit with a new set of cautions regarding the interpretation of the data. Studies using tools to knockdown gene expression in a reversible way would be welcome.

We know that the non-photic input pathways to the SCN are much more complex than our efforts to model their activation in these initial studies reflects. For example, most studies manipulate only one neurotransmitter or receptor subtype, while multiple neurotransmitters act on multiple receptor subtypes in the entire system. These are appropriate first studies, but should be followed by studies considering combinations of the relevant players, serotonin and NPY predominantly, but also considering opioid, GABA, adenosine, and neurotensin inputs. Similarly, our efforts to measure endogenous tone have been collected under restricted conditions; to the lack of representation of multiple species, as mentioned earlier, we might include the lack of representation of multiple environmental conditions. An example of this direction is recent studies on serotonin (Glass et al., 2003) including multiple housing conditions. Studies on NPY content are hampered by the lack of ability to conduct microdialysis experiments; technical adaptations to make such measures possible would allow measures of greater utility than the immunoassays available. Recent studies suggest responses to serotonin may be greatly potentiated by 2 days of exposure to constant light (Knoch et al., 2004). Prior housing conditions might also alter interactions of NPY with light input pathways; one study reported that NPY potentiated NMDA resetting in rats pre-treated with 2 days of constant darkness (Shibata et al., 1994).

The cellular mechanisms by which NPY and serotonin interact with photic input pathways constitute an area for much future research. An intriguing finding is the extraordinary delay where NPY can be applied even 1 h after light and will still block the resetting action of the light at CT14 (Yannielli and Harrington, 2000; Lall and Biello, 2002). Even if the phase of the clock is already changed by light or NMDA stimulation at the moment of the delayed application of NPY, its blocking effect cannot be explained by competing phase-shifting actions (i.e., light-induced phase delay + NPY-induced phase advance), because at the estimated new phase (circa CT12.5) NPY alone induces, if anything, a small phase delay, both in vitro and in vivo (see Fig. 3).

Suggestion of a similar delay is seen in one study of serotonergic inputs (Moriya et al., 1998) but has not been widely investigated. The significance of this delay is that these compounds must not be acting on the early cellular responses to light. Less is known of paths by which the light response can be blocked 1 h into the signal cascade. We suggest that RNA stability might be altered, either for the *per2* mRNA or for other light-responsive genes. Other possibilities exist. Examination of this question might provide better understanding of the way membrane potential can interact with expression of circadian clock genes (Nitabach et al., 2002). Both NPY and serotonin alter potassium channel activity in SCN neurons in a long-lasting manner (Hall et al., 1999; Miller et al., 1996) and this might play an important role in interactions with light input. Testing if hyperpolarization of SCN neurons 1 h following a light pulse can block the phase resetting action of light would be an approach to test this hypothesized mechanism. The link with *per2* gene activation should also be better investigated. Researchers might determine if NPY can block the resetting action of light in $per2^{-/-}$ mice or in animals with prior suppression of *per2* gene. It is clear from recent research that the internal circuitry of the SCN is important in processing inputs and resetting phase. In particular, transmission of photic input from retinal-receptive neurons in the ''core'' area of the SCN to neurons in the ''shell'' may occur with a delay (Yan and Silver, 2004). Perhaps NPY, or serotonin, is able to modulate the transmission of this information within the SCN. Finally, it is possible that some of the action of these compounds occurs outside the SCN. The role of extra-SCN areas in setting circadian phase is under investigation (Vansteensel et al., 2003) and one study suggests effects of novel wheel exposure involve extra-SCN processing (Yannielli et al., 2002).

A full understanding of the role of non-photic inputs in circadian rhythm entrainment would involve incorporating these inputs into models of formal properties of entrainment (e.g., Pittendrigh and Daan, 1976; Johnson et al., 2003). Light is clearly the predominant resetting cue for circadian rhythms of most if not all species. Incorporation of non-photic inputs would require consideration of the conditions where resetting information from light is either not available

(as in early development) or should be ignored (as in cases of extreme environmental disruptions). Both circadian entrainment and responses to seasonal changes in photoperiod should be considered. Every ''photic'' stimulus may involve photic–non-photic interactions, in that light may be arousing to a diurnal animal and anxiety-provoking to a nocturnal animal. No one has extensively studied this interaction between the emotional valences of the light with the circadian system response to the light, except the modulation of light-induced phase shifts in response to certain odors (Amir and Stewart, 1998; Amir et al., 1999).

Although the entrainment process is not necessarily similar in humans as compared to other mammals, particularly laboratory rodents, the human circadian system can be entrained by photic and non-photic stimuli. Different oscillatory components of the circadian system (i.e., the rest–activity cycle and the circadian oscillation of other variables, such as core temperature or plasma melatonin levels) have different sensitivity to zeitgebers of different nature. Temperature, for example, entrains to photic cues while the rest–activity cycle seems to respond to non-photic ones (for review see Honma et al., 2003) The interaction between photic and non-photic stimuli within the human circadian system has not been clearly established, though the stable entrainment showed under ''real world'' conditions seems to be a direct result of this interaction.

In addition, there are many clinical applications for this research. In cases of entrainment disorders, such as delayed or advanced sleep phase syndrome, potentiating the effects of light at one time while blocking effects of light at another phase might be an approach to correct the phase of entrainment. Symptoms of jet lag might be shortened in duration if resetting effects of light in the new time zone are potentiated. Shift workers might benefit from blocking the effects of light at night. The progress of cancer is slower in patients with enhanced circadian rhythmicity (Sephton and Spiegel, 2003), a benefit that might accrue from better circadian entrainment. Patients with fatigue from illness or from chronic fatigue syndrome might benefit from enhanced circadian effects of light. As discussed earlier, studies on hamsters indicate that chronic antidepressant treatment may have side effects that can be treated by stimulation of non-photic inputs. Much remains to be done to determine if work with laboratory animals applies to humans. We do not yet know if altering NPY or serotonergic tone will alter circadian rhythm response to light in humans. Given that these are redundant pathways in rodents, perhaps both inputs need to be modified to produce an effect. It is not clear if behavioral manipulations can be used although some studies have reported effects of exercise or sleep deprivation. For example, exercise during the day can induce a phase advance in hormonal rhythms in humans (Miyazaki et al., 2001), and sleep deprivation has been shown to have antidepressant effects (for review see Giedke and Schwarzler, 2002).

Overall, the capability of NPY and serotonin antagonists to potentiate and agonists to attenuate the effects of light on the circadian system, could be a potentially useful tool in clinical research. Indeed, both positive and negative interactions between photic and non-photic or behavioral entrainment stimuli could be of importance for human therapeutics.

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