

Blockade of the NPY Y5 receptor potentiates circadian responses to light: complementary *in vivo* and *in vitro* studies

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Abstract

Neuropeptide Y (NPY) is delivered to the suprachiasmatic nuclei (SCN) circadian pacemaker via an input from the thalamic intergeniculate leaflet. NPY can inhibit light-induced responses of the circadian system of Syrian hamsters. Here we studied whether an antagonist to NPY receptors can be used to potentiate photic phase shifts late in the subjective night. First we determined by *in situ* hybridization that both NPY Y1 and Y5 receptor mRNA are expressed in the SCN of Syrian hamsters. Second, similar to our previous findings at Zeitgeber time 14 (ZT 14, where ZT 12 was the time of lights off), we found that NPY applied at ZT 18.5 onto the SCN region of brain slices maintained *in vitro* could block NMDA-induced phase advances of the spontaneous firing rate rhythm, and this blocking effect was probably mediated by the Y5 receptor, since co-application of Y5 receptor antagonists completely reversed the effect of NPY, while application of a Y1 receptor antagonist had no effect under the same conditions. Third, we found that co-treatment with a Y5 receptor antagonist *in vivo* (s.c., 10 mg/kg) not only reversed the effect of NPY applied to the SCN *in vivo* through a cannula but also significantly potentiated the light-induced phase advance in the absence of NPY. This is the first report of a NPY receptor antagonist having such an effect, and indicates that NPY Y5 receptor antagonists could be clinically useful for potentiating circadian system responses to light.

Introduction

Circadian rhythms are daily physiological cycles that are internally generated and reset each day by external cues to maintain a 24 h rhythm synchronized to the environment. The main resetting cue is light. Photic input has access to the hypothalamic suprachiasmatic nuclei (SCN), which functions as an endogenous circadian pacemaker in mammals, through a direct projection from retinal ganglion cells (Ebling, 1996), and resets circadian rhythm phase during the subjective night mainly through glutamate release. As well, other stimuli relevant to the particular organism (including novelty and/or activity-mediated arousal and several neurochemicals such as neuropeptide Y (NPY), benzodiazepines, serotonin and opioids) can reset the clock during the subjective day when the SCN is unresponsive to light (Hastings *et al.*, 1998; Byku & Gannon, 2000; Meijer *et al.*, 2000). These stimuli are generally known as nonphotic cues, although it is not clear whether they share neurochemical mediators or even act through the same mechanism in affecting the phase of the clock.

NPY, present in an input pathway to the SCN, can block the phase-resetting effects of light on circadian rhythms. NPY inhibits light-induced phase shift responses *in vivo* (Weber & Rea, 1997; Lall & Biello, 2002) and also blocks the resetting effects of glutamate or glutamate receptor agonist application *in vitro* (Biello *et al.*, 1997; Yannielli & Harrington, 2001). NPY inhibits *in vitro* glutamate-induced shifts both in the early subjective night, when light delays the circadian cycle, and in the late night, when light advances circadian cycles into the next period (Biello *et al.*, 1997).

This negative interaction has been studied at the molecular level and has been shown to involve the genes that generate the self-sustained oscillation as well as mediate the adjustments of this clock gene machinery in response to light stimulation. NPY inhibited the rapid light-induction of both the period clock genes *per1* and *per2* (McKinley-Brewer *et al.*, 2002). However, although NPY caused a striking and prolonged suppression of *per2* mRNA, *per1* mRNA levels rebounded quickly to equal the unsuppressed *per1* induction peak at 1 h and mirrored the control light induction pattern thereafter (McKinley-Brewer *et al.*, 2002). A similar negative effect on gene expression was recently found for NPY application during the day, on diurnal *per1* and *per2* mRNA peaks (Maywood *et al.*, 2002).

Daytime phase-shifting effects of NPY are mediated via the Y2 receptor, whereas night-time effects of NPY are probably mediated by a different receptor (Golombek *et al.*, 1996; Huhman *et al.*, 1996). One recent study, conducted entirely *in vitro*, implicates the NPY Y5 receptor as critical in the action of NPY in blocking the response to NMDA early in the subjective night *in vitro* (Yannielli & Harrington, 2001). The Y1/Y5 receptor agonist ([Leu³¹, Pro³⁴]NPY) as well as Y5 receptor agonists ([hPPI-17, Ala³¹, Aib³²]NPY, and [Ala³¹, Aib³²]NPY) were equally effective in mimicking NPY blockage, while only the Y5 antagonist (RWJ-57926) could reverse this action.

Does NPY serve to inhibit photic responses *in vivo*? This has been difficult to test given the tools available. It has been shown that NPY antiserum administered directly into the SCN through cannula implants potentiated light-induced phase advances (Biello, 1995). Studies of animals with intergeniculate leaflet (IGL) lesions present mixed results (Harrington & Rusak, 1986; Pickard *et al.*, 1987; Redlin *et al.*, 1999). Our *in vitro* approach to this problem relies on the fact that SCN cells maintain a self-sustained oscillation *in vitro*, allowing

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measurement of phase-shifting behaviour from an isolated clock (Biello *et al.*, 1997). Circadian clock phase shifting is measured *in vitro* by sampling spontaneous activity from SCN neurons in the brain slice preparation during cycles following application of phase-shifting stimulation. The *in vivo* experimental design includes recording a behavioural overt-rhythm such as running-wheel activity and applying NPY through a cannula aimed directly to the SCN. Also, the development of a selective Y5 receptor antagonist with excellent brain penetration allowed us to examine *in vivo* effects using peripheral administration, and highlights a potential approach to clinical treatment of circadian disorders.

Materials and methods

Animals and tissue preparation

Male golden hamsters (LVG, Charles River 40–60 days old) were housed under a light: dark (LD) schedule of 14:10h, with food and water available *ad libitum*. Zeitgeber time was defined with ZT 12 as the projected time of lights off in the animal room. Hamsters were administered an overdose of halothane anaesthesia and decapitated during the subjective day at the stated ZT. Hypothalamic slices (500 μ m) containing the SCN were placed in a gas–fluid interface slice chamber (Medical Systems BSC with Haas top), continuously bathed (1 mL/min) in artificial cerebrospinal fluid (ACSF) containing 125.2 mM NaCl, 3.8 mM KCl, 1.2 mM KH_2PO_4 , 1.8 mM CaCl_2 , 1 mM MgSO_4 , 24.8 mM NaHCO_3 , 10 mM glucose. ACSF (pH 7.4) was supplemented with an antibiotic (gentamicin, 50 mg/L) and a fungicide (amphotericin, 2 mg/L) and maintained at 34.5 °C. Warm, humidified 95% oxygen:5% carbon dioxide was continuously provided. For *in situ* experiments the animals were processed in the same way as for electrophysiological studies except that the hypothalamus was immediately frozen on dry ice and stored at –70 °C until sectioning. Animals were maintained and utilized according to American Association for Accreditation of Laboratory Animal Care standards. All studies were reviewed and approved by the Smith College Institutional Animal Care and Use Committee.

In situ hybridization

In situ hybridization was performed to evaluate Y1 and Y5 receptor expression within the SCN slice. All *in situ* procedures were adapted, single-label versions of a dual-label protocol (Petersen & McCrone, 1994). Frozen brains were sliced into 20 μ m-thick sections on a cryostat and thaw-mounted on coated (Superfrost, Fisher) glass slides. Prehybridization included a 10-min room temperature thaw, fixation with 4% formalin in phosphate-buffered saline, and 0.25% acetic anhydride in triethanolamine hydrochloride (0.1 M TEA-HCL, 0.9% NaCl, pH 8.0), interspersed with saline sodium citrate (SSC, 1 \times , 0.15 M NaCl/0.015 M sodium citrate, pH 7.0) rinses, dehydration with an ethanol (ETOH) series, delipidation with chloroform and partial rehydration. Alternate slices from each animal were hybridized with Y1 receptor or Y5 receptor cRNA probes. Sections obtained from different animals were hybridized and processed together. [35S]-UTP-labelled cRNA 593 bp sense and antisense probes were transcribed from a rat NPY Y1 receptor cDNA fragment (GenBank No. Z11504.1, approx nt127–720). Similarly, 576 bp sense and 419 bp antisense probes were transcribed from a rat Y5 receptor fragment (GenBank No. AF044264 nt761–1180). All fragments were gifts of Dr Herbert Herzog (Garvan Institute, St Vincent's Hospital, Sydney). The Y1 receptor plasmid was linearized with EcoRI or PstI and transcribed with T3 or T7 RNA polymerase for antisense and sense probes, respectively. NcoI with T3 or T7 RNA polymerase were used to create Y5 receptor antisense and sense probes. Slices were incubated with

1.0×10^6 dpm probe per 25 μ L hybridization buffer (0.01% sodium pyrophosphate, 10% dextran sulphate, 50% formamide, SSC, 1 \times Denhardt's solution (0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.02% bovine serum albumin), 0.5 mg/mL tRNA, 0.5 mg/mL heparin and 0.4 mg/mL single-stranded sheared salmon sperm DNA) for 16–20 h at 55 °C. Post-hybridization washes included multiple SSC washes, SSC/50% formamide at 52 °C, then treatments with RNaseA (50 μ g/mL) at 37 °C and SSC/50% formamide, each preceded by a pair of SSC rinses. Finally, slices were washed in an ascending series (70–95%) of ETOH/SSC baths with a 70% ETOH last rinse. Air-dried slides were exposed to Kodak BioMax MR film for 2–6 days, and were subsequently stained with Toluidine Blue O for evaluations of the SCN region and tissue conditions.

Electrophysiological studies

Extracellular single unit activity of SCN cells was detected with glass micropipette electrodes filled with ACSF, advanced through the slice using a hydraulic micro drive. The signal was further amplified and filtered, and was continuously monitored using an oscilloscope and audio monitor. Firing rate was analysed using data acquisition software and a customized program for calculation of descriptive statistics. A number of experiments in each condition were recorded 'blind', that is, the person recording data had no knowledge of the treatment. One slice was recorded from each animal. A total number of 42 slices were recorded in the present study.

Intra-cranial implants

For *in vivo* treatment, hamsters of 80–100 g were deeply anaesthetized with nembital (80 mg/kg, i.p.), administered an analgesic (buprenorphine, 0.05 mg/kg, s.c.) and mounted in a stereotaxic instrument with the skull set level. They were surgically implanted with a stainless steel guide cannula held at a 10° angle (25 gauge, Plastics One, VA, USA) aimed at the SCN (coordinates: incisor bar, –2 mm; 0.9 mm AP, –1.55 mm L and 5.75 mm DV). After 1 week of recovery under LD 14:10, animals were individually transferred to cages (48 \times 27 \times 20 cm) equipped with wheels. Wheel running activity was recorded with ClockLab hardware and software (Actimetrics, Evanston, IL, USA).

Drugs and routes of administration

NPY (0.2 μ L, 234 μ M) was dissolved in artificial cerebrospinal fluid (ACSF, see Animals and tissue preparation section), and administered through a cannula with a 1 μ L Hamilton syringe connected with polyethylene tubing to a 13.1 mm stainless steel injector cannula (30 gauge). Y5 receptor antagonist (0.6 mL, 10 mg/kg) was dissolved in 32% 2-hydroxypropyl-B-cyclodextrin, and injected s.c. at circadian time (CT) 18.5, 30 min before NPY and/or light stimulation at CT 19. Light pulses (5 min, 200 lux) were delivered individually by placing animals under four white fluorescent tubes (Phillips, model F30T12), at a fixed distance of 45 cm above the bottom of the cages. This light intensity appears to be at the saturating level; we compared phase shifts for a separate sample of hamsters administered 5 min light pulses of 200 lux or 2000 lux (using an Apollo Slide EXR Projector Lamp) and we measured no difference in the phase shift at CT 19 (200 lux, 2.2 h \pm 0.3 ($n = 5$); 2000 lux, 2.7 \pm 0.48 ($n = 6$); $t_9 = 0.89$, $P = 0.2$).

A mixture of oxygen and isoflurane was administered by means of a gas anaesthesia machine (2.5% isoflurane to induce anaesthesia, 1.5% to maintain anaesthesia through a nose-mask) to avoid stress induced by restraint during cannula injections. NPY-treated as well as control animals were briefly anaesthetized with isoflurane before any injections, or as a control procedure when no intracranial injection was

administered. A preliminary set of experiments were conducted in order to test the effect of a brief, single exposure to isoflurane: (i) alone (~1 min) at CT 6 and CT 19; and (ii) immediately before the administration of a 5-min light pulse at CT 19. Brief exposure to isoflurane alone had no effect on the phase of the activity rhythm at CT 6 or 19 (phase shifts \pm SEM, 0.008 ± 0.011 h, $n = 5$; -0.004 ± 0.012 h, $n = 5$, respectively). Isoflurane did not alter the phase advancing effect of a 5-min light pulse as compared with previous measurements by our lab ($t_{15} = 2.13$, $P = 0.45$). These results validated the utilization of isoflurane for the purpose of intracranial cannula injections and subsequent measures of circadian rhythm phase shifts. In order to test the placement of the cannulae, we administered intracannula injections of NPY at CT 6. This is a treatment that reliably induces phase advances in hamsters with the cannula appropriately located near the SCN (Biello & Mrosovsky, 1996) and thus provides a functional check for proper cannula placement. In this experiment, 5 of 21 animals failed to show an advance of at least 30 min to CT6 NPY application and were discarded for cannula treatments.

Animals were allowed at least 10 days under LD in order to establish a stable rhythm, and then housed under constant dim red light (DRL) provided by a safelight lamp (Coastar, Inc. < 1 lux). Two sets of experiments comprising five treatments were delivered at CT 19, in a counterbalanced design: NPY alone, NPY + light; light alone; light + Y5 antagonist, NPY + Y5 antagonist + light. After two treatments (only one of them involving light stimulation), animals were resynchronized to the previous LD cycle for 7–10 days, and then exposed again to DRL for the second set of treatments. In this way, the animals did not spend more than 3 weeks under DRL, and did not receive more than one light pulse or more than four treatments overall. Controlling the number of light pulses was important since in a previous study conducted under the same conditions as the present one, we found that animals receiving two phase advancing light pulses over a period of a month under continuous dim red light showed a significantly greater phase advance to the second light pulse compared with the magnitude of the shift induced by the first light pulse. Similar results were published previously by Mistlberger & Holmes (2000).

Data analysis

For *in situ* hybridization results, film images were scanned using a high-resolution scanner (UMAX) with Photoshop 5, on a PC, adjusted to maximize the sensitivity range within that available on each image. Optical density values for sense probes did not register above threshold in any case.

For electrophysiological results, data were initially grouped into 1 h bins and an analysis of variance test was used to determine whether any bins differed from the others. If the analysis of variance test indicated significant differences, data were smoothed using 1 h running means with a 15-min lag. The Zeitgeber time of the middle of the 1 h bin with the highest mean firing rate after processing by this smoother was taken as the time of peak firing rate for that slice. Phase shifts of individual slices were measured relative to the average time of peak firing of control slices. Significant differences between groups ($P < 0.05$) were determined by ANOVA followed by the Bonferroni method. The number of slices per treatment (n) is stated in the corresponding figure. Means are reported \pm SEM.

For *in vivo* experiments, data were automatically collected and analysed with the Clocklab software bundle (ActiMetrics Software, Evanston, IL, USA). Two investigators blind to the treatment analysed phase shift magnitudes using the Clocklab software, and the average phase shift was used in further analysis. Statistical analyses were performed by means of ANOVA followed by Student–Newman–Keul's test.

Results

Y1 and Y5 mRNA expression in the SCN

In situ hybridization studies support the presence of both Y1 and Y5 receptor mRNA in SCN cells. Sections processed for Y5 receptor mRNA indicated high levels of expression in the SCN (see Fig. 1). Moderate levels of expression were observed for Y1 receptor mRNA in the hamster SCN.

Using a separate group of animals to look for diurnal changes in expression, we saw no difference in the hybridization signal for either receptor across the four times tested, ZT 1, 7, 13 and 19 ($n = 2$ per time point).

Electrophysiological recordings

Control experiments were conducted to determine the time of peak firing rate in SCN brain slices given no drug treatment (see Fig. 2, top panel). A phase advance in the time of peak firing was observed in slices given NMDA to mimic the effects of light in the late subjective night (ZT 18.5). This is the expected result for NMDA application at this circadian phase (Ding *et al.*, 1994). Slices treated with NPY 5 min after the application of NMDA demonstrated a peak in firing rate at a time similar to that observed in the control slices, indicating no phase shift (see Fig. 2, bottom panel). Thus, this work establishes that NPY blocks the phase advance elicited by NMDA at ZT 18.5. Our previous work (Yannielli & Harrington, 2001) had been conducted at another circadian phase, ZT 14, so these studies extend that previous work.

The effect of NPY Y5 and Y1 receptor antagonists on the spontaneous firing rate of the SCN was evaluated. The NPY Y5 antagonists used had nanomolar affinity for the Y5 receptor (CP-760,542, K_i , 1.7 nM; CP-781,214, K_i , 0.078 nM) with low affinity ($K_i > 1000$ nM) for NPY Y1 or Y2 receptors as well as for at least 40 other receptors tested, while the NPY Y1 antagonist CP-671,906 had a high affinity for the Y1 receptor (K_i , 0.94 nM) but low affinity for the NPY Y5 receptor ($K_i > 1000$ nM) (J.S. Sprouse, Pfizer, Inc., Groton, CT, USA, personal communication). The NPY Y5 antagonist CP-760,542 was applied at ZT 18.5 at a concentration of 10 μ M in the artificial cerebrospinal fluid (with 0.1% DMSO), bathing the slice for 60 min centred on the time of the applications for NMDA and/or NPY. Application of the antagonist alone (in ACSF with 0.1% DMSO) did not induce a shift in the phase of

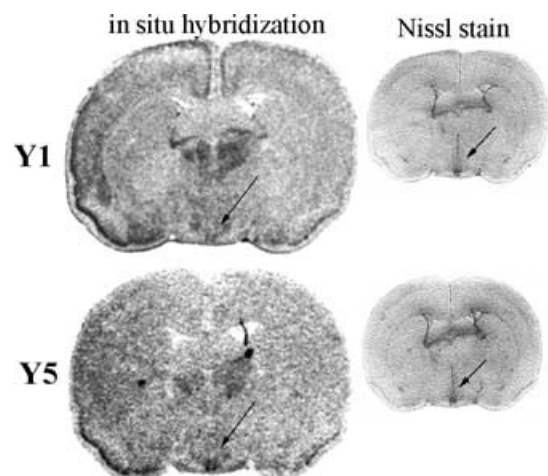


FIG. 1. Autoradiographic images of representative slices for Y1 and Y5 mRNA expression in the Syrian hamster SCN. On the right are sections with a Nissl stain, to demonstrate the SCN clearly present in the ventral hypothalamus. The same sections are shown on the left: Y1 receptor mRNA (top left) and Y5 receptor mRNA (lower left) film autoradiographs.

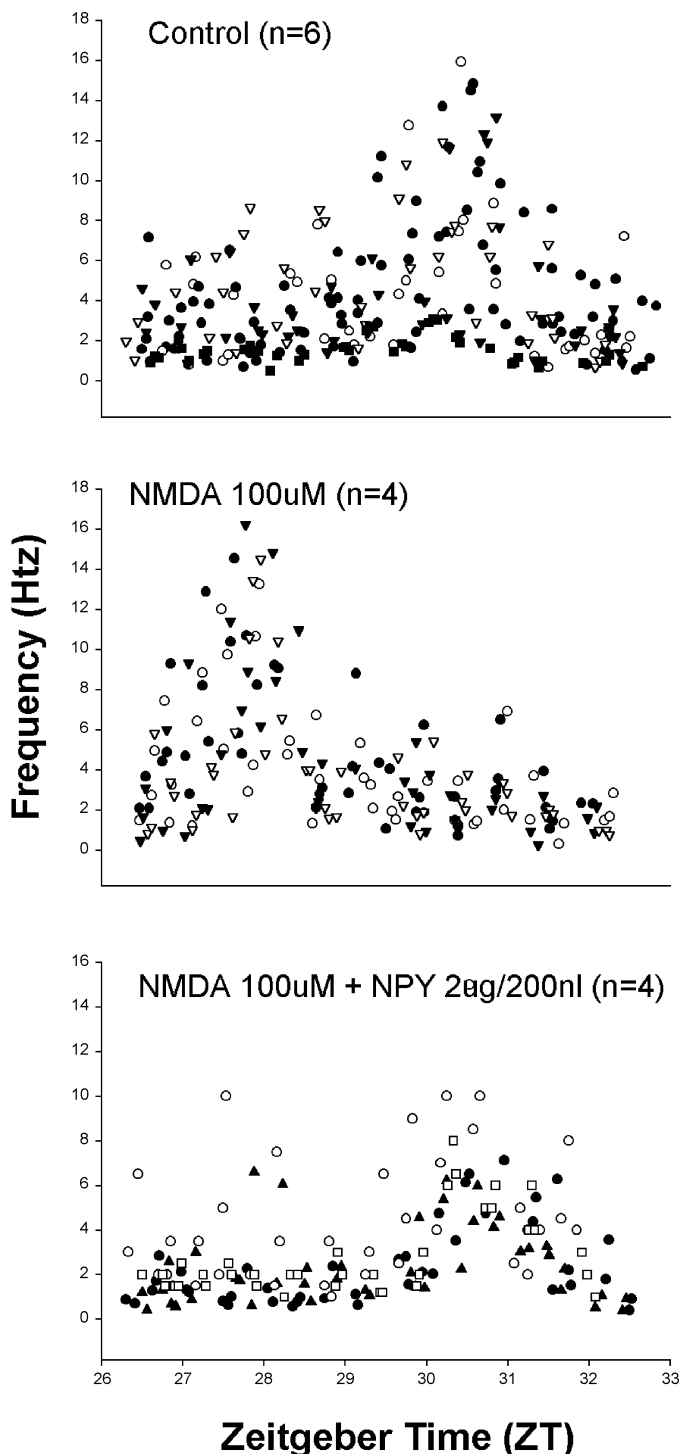


FIG. 2. Top panel, control. Spontaneous firing rate of individual cells recorded from the SCN in untreated brain slices on the second day *in vitro*. The time of peak firing for each slice is determined by the fit of a running mean smoother. The six slices varied from ZT 29.8 to ZT 31.05 in the time of peak firing. The mean time of peak firing was ZT 30.4. Middle panel, NMDA. Spontaneous firing rate of individual cells recorded from the SCN in slices treated with NMDA (100 μ M, 200 nL micro drop) applied at ZT 18 on the first day *in vitro*. The time of peak firing is advanced in these slices relative to the time of peak firing in untreated control slices. The mean time of peak firing was ZT 27.6. Bottom panel, NMDA + NPY. Spontaneous firing rate of individual cells recorded from the SCN in slices treated with NMDA (100 μ M, 200 nL micro drop) followed by NPY (2 ng/200 nL) applied at ZT 18 on the first day *in vitro*.

the spontaneous firing rate (see Fig. 3). The antagonist CP-760,542 was able to prevent NPY from blocking the NMDA-induced phase shift, showing a peak in firing rate at the advanced phase as seen in experiments with NMDA alone. This result supports our previous studies using another Y5 receptor antagonist (RWJ-57926, Yannielli & Harrington, 2001), extending those results to a new circadian phase.

We conducted similar studies using another Y5 receptor antagonist, CP-781,214. This antagonist does not shift circadian phase when applied alone at ZT 18.5 (see Fig. 3). This antagonist also prevented NPY from blocking the NMDA-induced phase shift. The phase shift in response to NMDA (2.77 ± 0.08 ; $n = 4$) was not altered by application of CP-760,542 or CP-781,214 (NMDA + CP-760,542, 3.16 ± 0.07 h ($n = 3$); NMDA + CP-781,214, 0.262 ± 0.37 h ($n = 3$); ANOVA, $F = 2.01$, $P = 0.2$).

The NPY Y1 receptor antagonist CP-671,906 was tested in similar studies. Surprisingly, we measured a phase-resetting action of this antagonist alone in control experiments (antagonist at 10 μ M, in ACSF with 0.1% DMSO; $n = 2$, phase advances of 3.04 and 02.58 h). We dropped the concentration to 1 μ M, and found that at this lower concentration there was little resetting action of the antagonist alone. The Y1 receptor antagonist did not alter the phase resetting action of NMDA, nor did it alter the effect of NPY on the NMDA-induced phase shift at the 1 μ M dose (see Fig. 3).

Intra-cranial injections

Hamsters implanted with a cannula to allow microinjection of compounds directly to the area of the suprachiasmatic nuclei (SCN) were

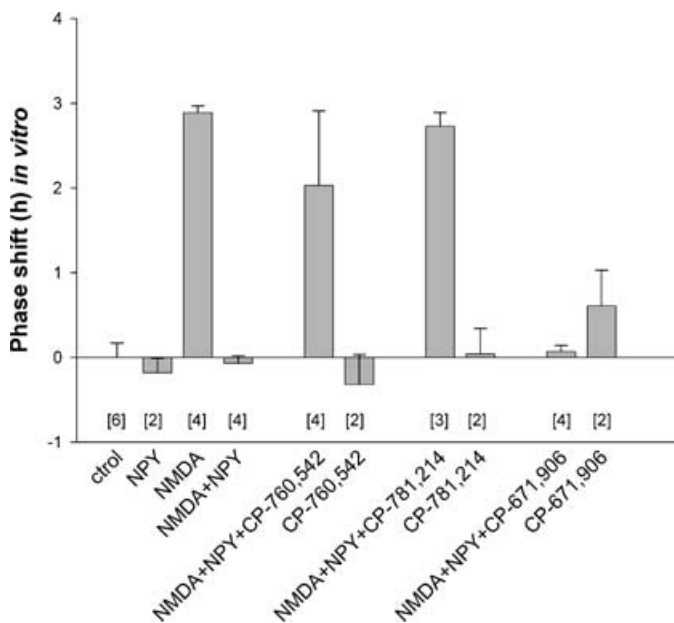


FIG. 3. Average (\pm SD) phase shifts of the peak of the rhythm of spontaneous firing rate of SCN cells in slices treated with various compounds. Shifts are measured relative to the mean of the control group (mean time of peak, ZT 30.4). The n value for each group is indicated below each histogram. Drug treatment was given at ZT 18 on the first day *in vitro*, and firing rate recorded on the subsequent day *in vitro*. NMDA (100 μ M, 200 nL micro drop) induced a phase advance shift. NPY alone (2 ng/200 nL micro drop) induced no significant phase shift relative to controls. The phase advance in firing as observed in response to NMDA alone (100 μ M, 200 nL micro drop) is blocked by application of NPY (2 ng/200 nL micro drop) 5 min later. The effect of NPY to block the NMDA-induced shift is blocked by Y5 receptor antagonists CP-760,542 (10 μ M) and CP-781,214 (10 μ M), but is not altered by the Y1 receptor antagonist CP-671,906 (1 μ M).

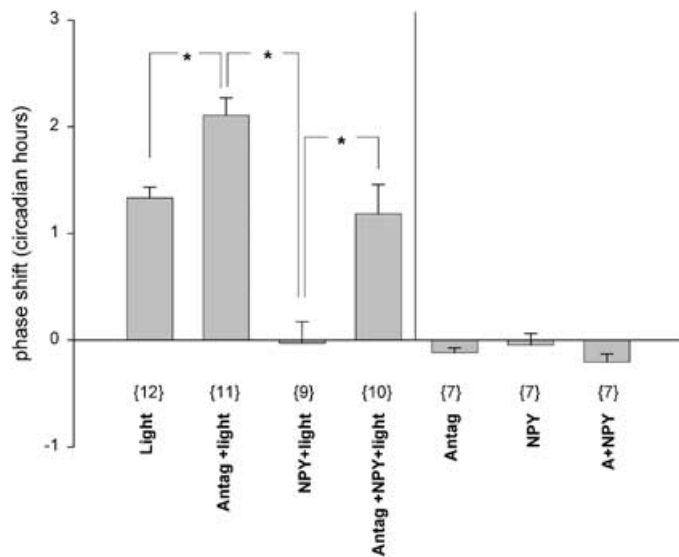


FIG. 4. Results from *in vivo* studies. Phase shifts in wheel-running rhythms are summarized (mean \pm SD) for hamsters treated with various combinations of light, the antagonist CP-781,214 (10 mg/kg), NPY (200 ng/200 nL; 234 μ M) at CT 19. The *n* value for each group is indicated below each histogram. The CP-781,214 compound was injected s.c., 30 min prior to other treatments. Light treatment was a 5 min, 200-lux light pulse at CT 19, late in the subjective night, 7 h after the onset of activity. We have tested each hamster twice, leaving at least 1 week between treatments, and ensuring each hamster received only one light pulse.

entrained to a light : dark cycle in cages equipped with running wheels. We recorded baseline activity records for 1 week under LD, and then for 1 week under constant dim red light. To check that the cannulae were located in the area of the SCN, we injected neuropeptide Y at CT 6, in the middle of the subjective day. This is a treatment that reliably gives a phase advance in activity onset, a response we were able to measure from 75% of our animals. We used isoflurane to anaesthetize the animal briefly during the handling necessary for the cannula injection. As stated in the Methods sections, isoflurane did not induce any change in the phase of the locomotor activity rhythm, alone or before a brief light pulse, at any circadian time. The animals were entrained to a LD cycle for 1 week, placed in dim red light and received the experimental series as described in the Methods section. We selected the NPY Y5 receptor antagonist CP-781,214 for all *in vivo* studies. Briefly, treatments administered were: Light, NPY, Light + NPY, Light + NPY + Y5 receptor antagonist, Light + Y5 receptor antagonist and Y5 receptor antagonist alone. As shown in Fig. 4, results show that: (i) NPY blocked light-induced phase shift at CT 19; (ii) the Y5 antagonist reversed this blockade by NPY; and (iii) most significantly, the Y5 antagonist potentiated the phase shift induced by light when applied alone, 30 min before light stimulation. Neither the Y5 antagonist applied alone, nor NPY or the combination of both induced any change in the phase of the wheel running rhythms in the absence of light stimulation at that circadian time (Fig. 4, right side). Representative actograms for the four relevant treatments are shown in Fig. 5.

Discussion

These results indicate that blocking the effects of NPY increases the effect of light stimulation on the circadian system during the dark, or 'light-sensitive' phase of the light : dark cycle. Also, the Y5 but not the Y1 receptor is responsible for the negative modulatory effect of NPY on circadian responses to light, since only antagonists of the Y5

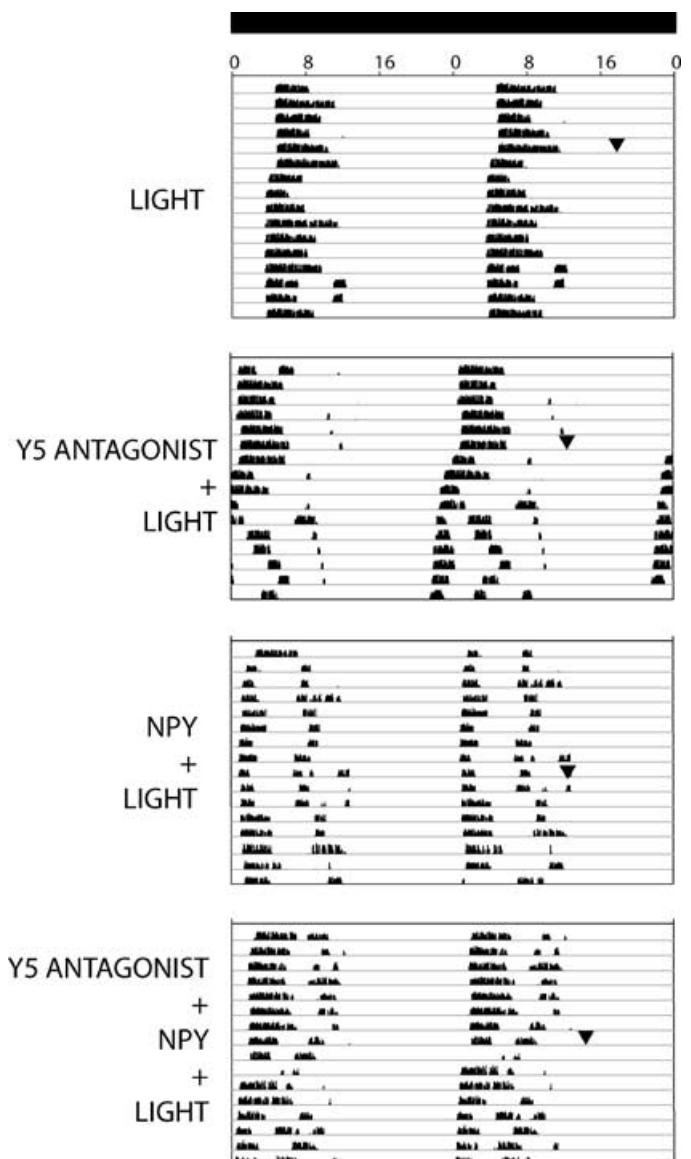


FIG. 5. Representative actograms for hamsters in the various treatment groups summarized in Fig. 4. Wheel revolutions are shown as black, with each day's activity plotted below the previous day, and the entire record duplicated on the right ('double-plotted'). Treatments are shown as an inverted triangle, with the type of treatment indicated by the label on the left.

receptor can negate the blockade induced by NPY on circadian responses to light both *in vivo* and *in vitro* experimental designs.

We previously demonstrated that NPY blocked NMDA-induced phase delays *in vitro* (Yannielli & Harrington, 2001) as well as light-induced phase delays measured in a novel *in vivo-in vitro* paradigm (Yannielli & Harrington, 2000). This effect early in the subjective night was mediated by the Y5 receptor *in vitro* (Yannielli & Harrington, 2001).

In the present study, we also demonstrated for the first time that the mRNA of both the Y5 and the Y1 receptors are expressed in the SCN of Syrian hamsters. This finding supports our previous hypothesis and results about the postsynaptic nature of these receptors, and consequently the postsynaptic nature of the inhibiting effect of NPY on circadian responses to light. High and moderate expressions were found, respectively, for the Y5 and the Y1 receptor mRNA, as revealed by a complete series where consecutive slices were alternately

hybridized with each probe. We could not detect any difference in mRNA expression at different time points throughout the cycle, that is, two 'light phase' (ZT 1 and ZT 7) and two 'dark phase' (ZT 13 and ZT 19) time points, although this conclusion must be taken with caution since only two animals were analysed at each time point. One previous study has indicated that binding of ^{125}I -PYY was higher 2 h after dark onset (ZT 14) when compared to 4 h and 1 h before dark onset (ZT 8 and ZT 11) in the Syrian hamster (Stopa *et al.*, 1995).

NPY can completely block *in vitro* NMDA-induced phase advances when both substances were co-applied at ZT 18.5. The presence of two different and equally potent Y5 receptor antagonists (CP-760,542 and CP-781,214) in the perfusion bath for 1 h around the time of NMDA and NPY micro drop application could counteract the negative effect of NPY, while a Y1 receptor antagonist (CP-671,906) showed a lack of significant effect under the same conditions. Interestingly, the Y1 antagonist at a concentration of 10 μM induced a significant phase advance when applied alone, possibly due to nonspecific actions on other receptors, or areas outside the SCN that could innervate it even in a slice preparation. When the concentration was dropped to 1 μM , no significant effect was evident, and at this concentration, there was no significant antagonism of the NPY effect on the NMDA response, even though this compound remains a selective Y1 receptor antagonist at this dose. Since the results *in vitro* were more consistent (i.e. showed smaller variance) with CP-781,214 than with CP-760 542, we decided to use CP-781,214 for the whole set of *in vivo* experiments. We refer to the latter as 'Y5 antagonist', in order to simplify the notation. We found that a brief exposure to isoflurane anaesthesia did not induce any change in the phase of the wheel running rhythm at CT 6 or 19, nor did it affect the magnitude of a light-induced phase advance at CT 19, when compared with previous results from our laboratory. These data validate the employment of brief isoflurane anaesthesia to perform intracranial injections, in order to minimize stress to the animals.

In the present series of experiments, we found that NPY applied immediately before a light pulse at CT 19 could significantly block the expected phase advancing effect of light, confirming and extending previous *in vivo* studies performed with similar concentrations of NPY (Weber & Rea, 1997; Lall & Biello, 2002). We demonstrated that this effect was significantly reversed by a single systemic injection of the Y5 antagonist, 30 min before NPY and light treatment, since there were no significant differences between the effect of a light pulse alone and the effect of a light pulse preceded by the Y5 antagonist + NPY treatment.

Lall & Biello (2003) reported that NPY could partially or completely block light-induced phase shifts, and that this effect was also achieved through microinjection of agonists expected to bind to both Y1 and Y5 receptors. With the current study we were able to discriminate the Y1 and Y5 receptors using potent and newly developed pharmacological antagonists, thus further extending those findings.

The Y5 receptor antagonist significantly potentiated phase shift responses to light *in vivo*. This is the first description of a light-response potentiation when endogenous NPY-ergic activity is antagonized at the level of specific receptors. Since the Y5 antagonist was applied systemically, we cannot rule out an action of the antagonist in areas other than the SCN; for such a conclusion a study using microinjections through an intracranial cannula would be necessary. On the other hand, our results show that systemic treatment with this antagonist can have the predicted effect. This was a major advantage of the compounds used in our study, since they have excellent brain penetration when administered systemically. Similar results were found following microinjections of NPY antiserum (thus indirectly decreasing the endogenous NPY effect on light-induced effects at the

level of the SCN; Biello, 1995) and compounds that act at postsynaptic and somatodendritic 5-HT 1 A receptors, such as NAN-190 (Rea *et al.*, 1995), WAY 100635 (Smart & Biello, 2001) and MKC-242 (Moriya *et al.*, 1998; Takahashi *et al.*, 2002). Interestingly, neither of these Y5 antagonists induced any potentiation *in vitro*, since they did not affect the magnitude of the NMDA-induced phase advance of the electrical activity rhythm in the slice preparation. This lack of effect under *in vitro* conditions is as yet unexplained. It might be that the site of action for the potentiation is not the SCN. It is also possible that these results could be explained by the lack of a necessary innervation or neurotransmitter from outside the SCN. Because the communication between the clock and the rest of the brain is lost in a slice preparation, it is plausible that the antagonist can induce a potentiation of the light response *in vivo* because it interferes with an endogenous NPYergic activity, which is normally higher during the subjective night. Our results might also be explained by the difference between inducing a phase shift using light compared with one induced by NMDA, which must only partially mimic the effects of light. Overall, more experiments are needed to elucidate the mechanism by which the NPY Y5 antagonist can induce a potentiation of the circadian photic response *in vivo*.

These findings suggest that both serotonin and NPY, two relevant circadian modulators thought to be involved mainly in nonphotic circadian responses during the day, might be in fact intimately related with an endogenous, negative modulation of light responses during the night and that these inputs can be modified to increase photic effects.

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Abbreviations

ACSF, artificial cerebrospinal fluid; CT, circadian time; DRL, dim red light; ETOH, ethanol; LD, light : dark; NMDA, *N*-methyl-D-aspartate; NPY, neuropeptide Y; SCN, suprachiasmatic nuclei; ZT, Zeitgeber time.

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