

Neuropeptide Y rapidly reduces *Period 1* and *Period 2* mRNA levels in the hamster suprachiasmatic nucleus

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Abstract

The mammalian suprachiasmatic nucleus (SCN) contains the main circadian clock. Neuropeptide Y (NPY) that is released from the intergeniculate leaflet of the lateral geniculate body to the SCN, acts in the SCN to advance circadian phase in the subjective day via the NPY Y2 receptor. We used semi-quantitative in situ hybridization to determine the effect of NPY on circadian clock genes, *Period 1* (*Per1*) and *Period 2* (*Per2*), expression in SCN slices. Addition of NPY to the brain slices in the subjective day resulted in reduction of *Per1* and *Per2* mRNA levels 0.5 and 2 h after treatment. NPY Y1/Y5 and Y2 agonists decreased *Per1* within 0.5 h. These results suggest that NPY may induce phase shifts by mechanisms involving or resulting in reduction of *Per1* and *Per2* mRNA levels. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

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Circadian rhythms are endogenous oscillations whose period approximates 24 h under constant environmental conditions [11]. The suprachiasmatic nucleus (SCN) of the mammalian hypothalamus contains a central circadian pacemaker. *Period 1* (*Per1*) and *Period 2* (*Per2*), putative clock components in rhythm generation, have been localized to the SCN [15–17]. The internal clock is entrained to environmental time, primarily by light. Involvement of *Per1* and *Per2* in light-induced phase shifts is indicated by previous investigations: their mRNA levels are increased upon exposure to light during the subjective night; antisense oligodeoxynucleotides to the *Per1* sequence block light- or glutamate-induced phase shifts [1,2,18,19].

In contrast to light-induced phase shifts, access to a novel running wheel, dark exposure, or administration of neuropeptide Y (NPY) can cause a different pattern of phase shifts [3,9,14]. Maywood and colleagues found that access to novel running wheels during the subjective day provoked

a reduction of *Per1* and *Per2* mRNA levels in the hamster SCN [13]. Thus, light and novel running wheels have opposite effects on regulation of mRNA levels, suggesting that these two types of phase shifts are achieved by different mechanisms.

NPY is a neurotransmitter originating in cells of the intergeniculate leaflet (IGL) of the lateral geniculate body which project to the SCN [7]. NPY is known to mediate novel running wheel-induced phase shifts [3].

In the present study, we investigated whether *Per1* and *Per2* mRNA levels in the SCN were affected by NPY and if so, which NPY receptor subtype(s) mediated these effects using our previously characterized in vitro preparation, in which NPY applied at Zeitgeber Time (ZT)6 induced a 3–4 h phase advance in the rhythm of spontaneous firing rate [6].

Male Syrian hamsters (LVG, Charles River Laboratories, 21 days old at purchase) were housed under 14:10 h light/dark cycles for at least 2 weeks. Animal care and experimental procedures were in accordance with the guidelines of Smith College and the Institutional Animal Care and Use Committee. Efforts were made to minimize animal suffer-

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ing, and to reduce the number of animals used. Animals were decapitated following an overdose of halothane anesthesia between ZT2 and ZT3. Brains were immediately removed from the animals, then hypothalamic slices (500 μm) 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. NPY (200 ng/200 nl, 234 μM) (rat, human, Bachem Bioscience, Torrance, CA, diluted in ACSF), NPY Y1/Y5 agonist [Leu^{31} , Pro^{34}]NPY (200 ng/200 nl, 200 μM) (Sigma), and the Y2 agonist NPY fragment 3–36 (NPY3–36) (200 ng/200 nl, 195 μM) (Sigma) were added to the SCN region of the slices as a microdrop at ZT6 after at least 3 h of culture, and slices for the NPY and NPY receptor agonist evaluations were collected at 0.5 h, NPY slices were also collected 1 and 2 h after treatment. Control slices with ACSF treatment were collected at the same time as drug treated slices were collected. Samples were immediately frozen on a coverslip, and kept at -70°C .

Details about in situ hybridization technique used in this study are reported in Ref. [5]. In brief, [^{35}S]-UTP (1250 Ci/mmol) (New England Nuclear) labeled probes were obtained by in vitro transcription. Hamster *Per1* cDNA fragment (GenBank accession number AF249882, 800 bp) cloned into the pBluescript II KS (–) vector (STRATAGENE) (generous gift of Dr J. Takahashi) [12] was linearized with *ApaI* or *BstXI* for antisense or sense probes, and radiolabeled using T7 or T3 RNA polymerase, respectively. Mouse *Per2* (GenBank accession number AF035830, nucleotides 9–489, cloned in the PCR-II vector (Invitrogen)) (generous gift of Dr S. Reppert) was linearized with *NotI* or *BamHI* for antisense and sense probes and radiolabeled using SP6 or T7 RNA polymerase. The cultured frozen brain sections (500 μm) were sliced into 20- μm -thick sections on a cryostat, and hybridized over night at 55°C. Sections were processed [5], and exposed to Kodak BioMax MR film with ^3H standards (Amersham Life Science) for 3–5 days at room temperature. All the sections were subsequently stained with toluidine blue for evaluations of the SCN region and tissue conditions. Paired samples (NPY experimental and vehicle control) were sectioned together, collected on the same glass slide, hybridized and processed together.

Film images were scanned using high resolution scanners (UMAX, Powerlook II and HP Scan Jet 4C, with Photoshop 5, on a Macintosh). Optical density (OD) values were determined above a selected background (adjacent hypothalamus) area using NIH image (version 1.62). The range of OD value on the film was within the linear range as determined by the ^3H standards. OD values for sense probes did

not register above threshold in any case. At least five sections were used from one animal, and the average value was represented as that animal's OD value. In each experiment, maximum OD value was represented as 100, and other values were calculated relative to the maximum value [5,17].

Comparisons among different groups/treatments were performed using parametric statistics (*t*-tests and ANOVA).

NPY significantly reduced *Per1* within 0.5 h of treatment ($P < 0.05$, *t*-test, Fig. 1A), but the effects 1 or 2 h after treatment did not reach statistical significance. In contrast with *Per1*, levels of *Per2* were not significantly reduced until 2 h after NPY treatment ($P < 0.05$, *t*-test, Fig. 1B). NPY had no significant effect on *Per2* mRNA levels 0.5 or 1 h after application.

Y2 agonist NPY3–36 (195 μM) significantly reduced *Per1* in 0.5 h ($P < 0.005$, *t*-test, Fig. 2). [Leu^{31} , Pro^{34}]NPY (200 μM , Y1/Y5 agonist) also reduced *Per1* ($P < 0.05$, *t*-test). There was no significant difference among NPY, NPY3–36 and [Leu^{31} , Pro^{34}]NPY (ANOVA).

The present study demonstrated that NPY reduced *Per1* and *Per2* mRNA levels in the SCN. Both NPY Y1/Y5 and Y2 agonists reduced *Per1* mRNA levels.

NPY reduced *Per1* and *Per2* mRNA levels in the SCN in our study (Figs. 1 and 2). The effect was rapid and transient for *Per1*; *Per1* mRNA levels were decreased 0.5 h after treatment, but no longer different from controls 2 h after treatment. *Per1* mRNA levels in the NPY treated group tended to be lower than control levels 1 h after NPY treatment, although the difference was not significant, perhaps due to variability and the small number of animals in the control group. *Per2* mRNA levels showed a slower response; it took 2 h to show a significant decrease. Preliminary in vivo results using rats also showed that *Per1* and *Per2* are reduced by exposing animals to darkness after 0.5 and 1 h, respectively (C. Fukuhara and G. Tosini, unpublished observation). Interestingly, the time-courses of *Per1* and *Per2* mRNA reduction are similar to the time-courses of *Per1* and *Per2* mRNA induction following light exposure [2,19]. These observations suggest that *Per1* and *Per2* mRNA transcription/degradation rate may have similar sensitivity to the light, dark, and NPY stimuli, and change in the concentration of *Per1* mRNA precedes that of *Per2*.

We also show here that one treatment which does not phase shift the SCN, application of the Y1/Y5 agonist [Leu^{31} , Pro^{34}]NPY [6,10], induces a similar decrease in *Per1* mRNA levels (Fig. 2). NPY-induced phase advance is mediated by the NPY Y2 receptor [6,10]. In the previous studies, application of 195 μM NPY3–36 induced phase advances in the rhythms of spontaneous firing rate [6] and running wheel activity [10], while 200 μM [Leu^{31} , Pro^{34}]NPY failed to cause significant phase shifts. In the present study, we used the same concentration of Y2 and Y1/Y5 agonists that was used in the previous studies, however not only Y2 but also Y1/Y5 agonists decreased *Per1* mRNA levels (Fig. 2). These results suggest that

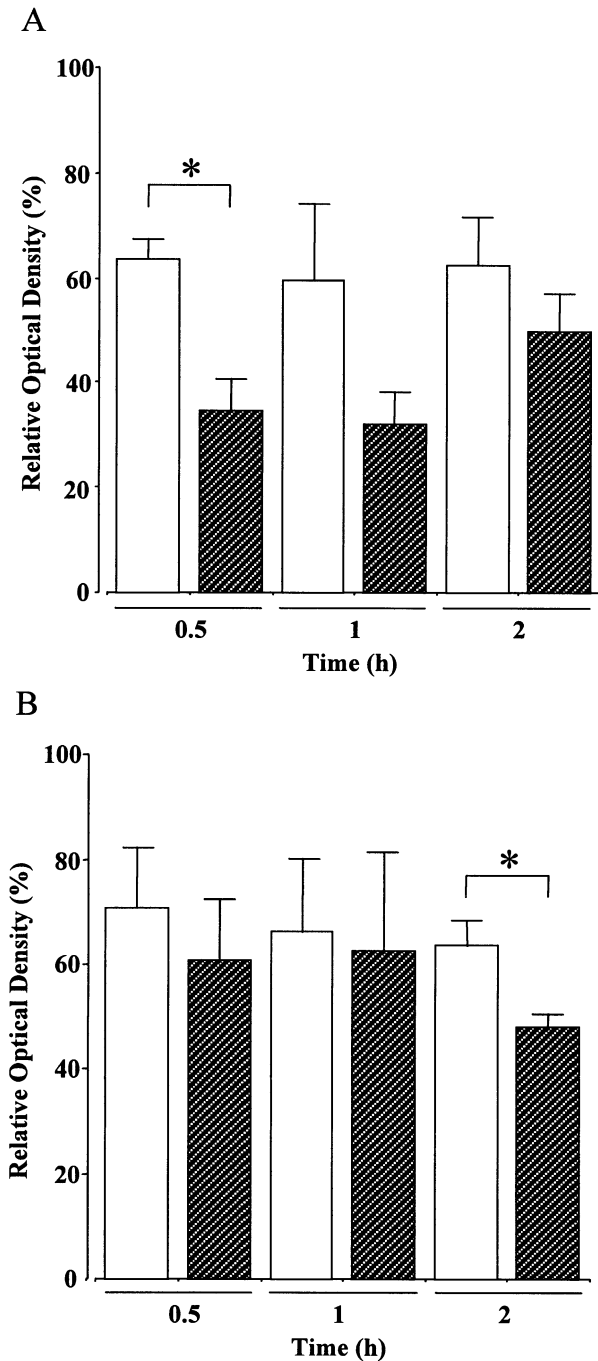


Fig. 1. Effect of NPY on *Per1* (A) and *Per2* (B) mRNA levels in the SCN. Brain slices were cultured for at least 3 h, NPY (200 ng/200 nl, 234 μ M) was added to the SCN region at ZT6 as a microdrop, and slices were collected 0.5, 1, and 2 h after treatment. Open bars represent the levels measured in control, and the hatched bars show NPY groups. (* $P < 0.05$, *t*-test). Values are means \pm SEM ($n = 3-4$).

reduction of *Per1* mRNA is not caused in a NPY receptor subtype-specific manner. The Y2 receptor, but not Y1/Y5, may stimulate certain cellular mechanisms that are responsible for phase shifts. Further studies are necessary to reveal what intracellular signal transduction pathways are acti-

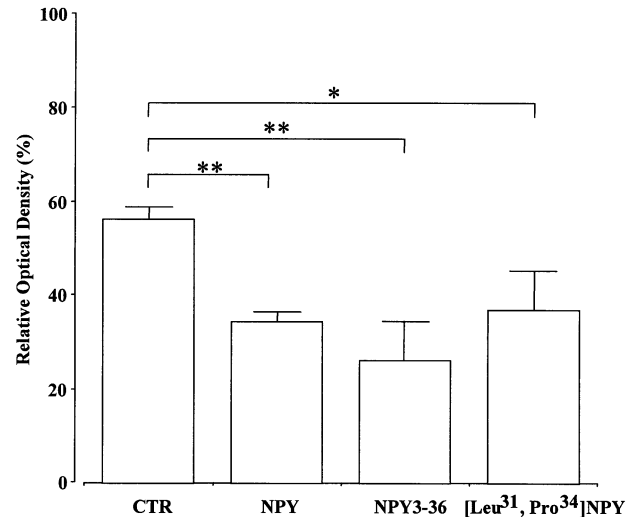


Fig. 2. Suppression of *Per1* by NPY receptor agonists. Brain slices were treated with the NPY Y2 agonist NPY3-36 (195 μ M) and Y1/Y5 agonist [Leu³¹, Pro³⁴]NPY (200 μ M) at ZT 6 for 0.5 h, then *Per1* mRNA levels were measured by in situ hybridization (* $P < 0.05$, ** $P < 0.005$, *t*-test). Values are means \pm SEM ($n = 4-6$).

vated/suppressed by stimulation of the NPY Y2 receptor in the SCN.

It is reported that NPY and light oppose each other's phase shifting effect [4]. During the day, light does not induce phase shifts but can block NPY-induced phase shifts. During the night, NPY itself has little effect on phase, but attenuates light-induced phase shifts. Our results suggest that light and NPY have opposite effects on *Per1* and *Per2* mRNA levels in the SCN; light induces *Per1* and *Per2* mRNA levels [2,15,18,19], while NPY reduces their mRNA levels.

Novel running wheel activity at CT4-7 reduced *Per1* and *Per2* mRNA levels in the SCN [13]. NPY mediates running wheel-caused phase shifts via the IGL of the lateral geniculate body, since novel running wheel stimulation can not induce phase shifts after the intergeniculate leaflet lesions [7], suggesting that NPY action is downstream or closer to the event in the SCN. Therefore NPY may cause reduction of gene expression more quickly than activity stimulation during the subjective day. To clarify this hypothesis, a more detailed time course analysis of NPY release from the intergeniculate leaflet to the SCN using the wheel pulse would be helpful.

It is striking that many different stimuli which phase advance the circadian clock in the subjective day are reported to reduce *Per1* and *Per2* mRNA levels. Such an effect has now been reported for a serotonergic agonist (8-OH-DPAT), triazolam, NPY and novel running wheel treatment [8,13]. Most reports suggest the reduction in mRNA is transient. Such a transient reduction in mRNA might not be reflected in changes in protein levels, as has been suggested in results from novel wheel treatment [13].

In conclusion, application of NPY in the subjective day reduces *Per1* and *Per2* mRNA levels. Although it is still unclear if the reduction of mRNA levels following treatments is necessary or sufficient for the phase resetting, *Per1* and *Per2* may be involved in the NPY-induced phase shifts in the SCN.

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