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Neuropeptide Y does not reset the circadian clock in NPY Y2−/− mice

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Abstract

Mammalian circadian rhythms are modulated by neuropeptide Y (NPY), a peptide contained in the projection from the intergeniculate leaflet to the suprachiasmatic nuclei of the circadian pacemaker. NPY resets the circadian clock during the subjective day, mediating non-photic inputs. Previous studies using receptor-selective agonists have indicated that this action of NPY is mediated by the Y2 receptor in hamsters. The present study determined if NPY applied to the suprachiasmatic nuclei in the mid-subjective day can phase-advance the rhythm of spontaneous firing rate of Y2−/− mice. We observed that NPY did reset the rhythm of control mice but did not significantly shift the phase of this rhythm in the Y2−/− mice. These results provide strong evidence for the role of the Y2 receptor mediating neuropeptide Y subjective day phase-advance shifts in mice.
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The hypothalamic suprachiasmatic nucleus (SCN), the master circadian clock, controls internal rhythms with a cycle length of 24 h. Photic and non-photic inputs to the clock have the ability to alter these rhythms, producing a phase shift as a function of the time of input [11]. Photic information from the retina reaches the SCN via the retinohypothalamic tract, which utilizes glutamate and pituitary adenylate cyclase-activating polypeptide as its primary neurotransmitters [4]. Non-photic input to the SCN arises from the geniculohypothalamic tract, a pathway that utilizes neuropeptide Y (NPY) as its principle neurotransmitter [5], and from the raphe nuclei in a pathway linked with serotonin [14].

Examples of non-photic stimuli include novel running wheels, sleep deprivation, or exercise. Non-photic phase response curves demonstrate phase-advances during the subjective day, and small delays or no shifts during the subjective night [16]. The key variable that results in non-photic phase shifting is unknown. Locomotor activity may play a primary role, but clock resetting can occur in hamsters by sleep deprivation alone, without locomotor activity [15].

The geniculohypothalamic tract arises from NPYergic neurons located in the intergeniculate leaflet and ventral lateral geniculate nucleus [5]. NPY has the ability to phase-advance circadian rhythms when microinjected onto the hamster SCN during the subjective day in vivo [8] and in vitro [7]. Furthermore, hamsters that phase shift to a novel running wheel are unable to demonstrate a similar shift in rhythm when pretreated with antiserum to NPY [1]. Lesions of the geniculohypothalamic tract disrupt non-photic phase shifting, further implicating that NPY plays a role in non-photic resetting of circadian rhythms [10,20].

Previous research has demonstrated that the NPY Y2 receptor plays a role in modulating the daytime effects of NPY in hamsters. The Y2 agonist, NPY (3–36), can phase shift the hamster SCN in vivo and in vitro, whereas the Y1/Y5 agonist [Leu^{31}, Pro^{34}]NPY, does not induce phase shifts [2,9]. In an as yet unexplained finding, the in vitro hamster SCN is several log units more sensitive to the phase resetting action of NPY 3–36 than to NPY itself [6]. Application of several other Y2 receptor agonists, NPY 22–36, NPY 13–36, peptide

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YY and C2-NPY, were found in subsequent in vitro studies to reset the circadian rhythm in firing rate during the subjective day [3,6], whereas avian pancreatic polypeptide (Y4 agonist) and [D-Trp^{32}]NPY (Y5 agonist) did not induce shifts [6].

The present study uses electrophysiology in order to examine the ability of NPY to induce resetting of the circadian clock during the subjective day in NPY Y2−/− mice.

Y2+/+ and Y2−/− male mice (on a background of mixed 129S and Balb/c; bred from founder mice courtesy of Patrik Ernfors, Ph.D., Karolinska Institutet [17]) were housed under a 12-h light:12-h dark cycle. Mice were relatively young at the time of death, between 2 and 6 months of age, this age range was not associated with age-related differences in electrophysiological recording of their circadian rhythms. Genotype was confirmed by PCR using primers as described in [17]. Brain slices were prepared between zeitgeber time (ZT) 6 and ZT 12, with ZT 0 being the time of lights on in the housing room. Each mouse received an overdose of halothane, and the brain was quickly dissected. Following the dissection, a hypothalamic brain slice containing the SCN was placed into a gas:fluid interface chamber. Tissue survived due to 95% O_2:5% CO_2, and artificial cerebrospinal fluid (ACSF: 125.2 mM NaCl, 3.8 mM KCl, 1.2 mM KH_2PO_4, 1.8 mM $CaCl_2$, 1.0 mM $MgSO_4$, 24.8 $NaHCO_3$, 5 mM D-glucose (pH 7.4)). Treatment was applied to the SCN at ZT 6 on the first day in vitro using a 1 μL Hamilton Syringe. The SCN from each mouse (Y2+/+ and Y2−/−) was treated with 200 ng NPY in 200 nL ACSF at ZT 6, and compared to untreated controls. Although control slices were not administered an application of ACSF, in past studies such control ACSF application were not shown to alter the time of peak firing rate on the subsequent day in vitro. Each mouse contributed one SCN slice, and treatment groups consisted of three to four slices per group. Each SCN slice was recorded for 1 day.

Electrophysiological recordings were performed on the second day in vitro. The frequencies of single cells were sampled for 6–8 h. Since the peak in the firing rate rhythm is at approximately CT6, and phase shifts to NPY are ∼2 h, the sampling period used was adequate for detection of NPY-induced shifts. Extracellular activity was recorded using an electrode filled with ACSF. The electrode was placed into regions of the SCN at random. The signal was amplified (AC differential amplifier: BAK Electronics, Germantown, MD), filtered, and discriminated (window discriminator: BAK Electronics, Germantown, MD). A rate/interval meter (FHC, Brunswick, ME) converted the signal and the spontaneous firing rate of each cell measured was recorded for 1 min using a computer program (Labtech Aquire; Laboratory Technologies Corporation, Wilmington, MA).

Analysis began with data from an individual SCN slice taken from one animal, either knockout mouse or control mouse. The average firing rate of each cell recorded from this one slice was plotted against the ZT of the recording. Data were initially grouped into 1 h bins, and an ANOVA was used to determine whether any bins differed from the others. If an ANOVA determined any 1 h bins significantly different from the others, the entire dataset was then 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 a one-way ANOVA followed by a Fisher's post hoc test. All results are reported as mean ± standard error of the mean.

The average time of peak in frequency for the untreated brain slice from a Y2+/+ mouse ($n = 4$) was ZT 6.22 ± 0.31 h (see Fig. 1A). For the Y2+/+ brain slices treated with NPY ($n = 4$), the peak time was ZT 4.37 ± 0.2 h (see Fig. 1B). The average peak firing rate for the untreated brain slices from the Y2−/− mice ($n = 4$) was ZT 6.43 ± 0.5 h (see Fig. 1C). The peak in frequency was ZT 6.56 ± 0.23 h for the Y2−/− brain slices treated with NPY ($n = 5$; see Fig. 1D).

Fig. 2 demonstrates the size of the phase shifts under each condition. A one-way ANOVA demonstrated that the Y2+/+ mice treated with NPY showed a significant advance in rhythm of approximately 1.85 h when compared to controls ($F(1,6) = 24.66$, $p = 0.003$). The Y2−/− mice treated with NPY showed a shift of approximately 0.13 h, which did not differ significantly from controls ($F(1,7) = 0.06$, $p > 0.8$).

NPY was not able to reset the circadian clock in the Y2−/− mice. This study supports the results of previous research, which demonstrate that the Y2 receptor mediates

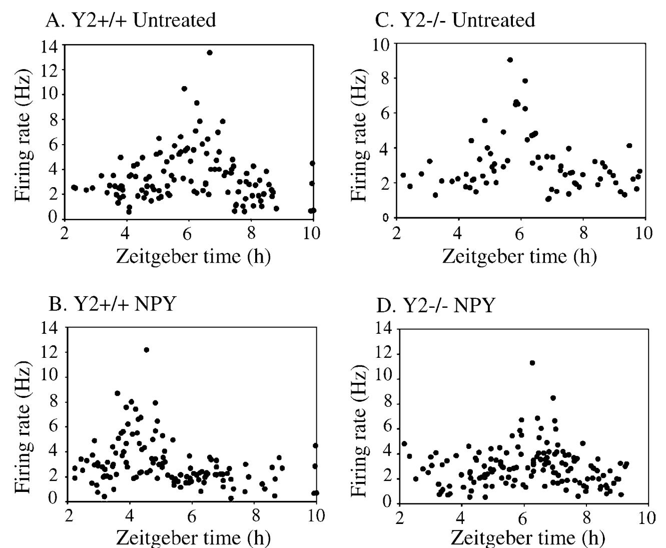

Fig. 1. Frequency of cells represented over time for each treatment group: average firing rate of the cell plotted against the zeitgeber time of the recording. Zeitgeber time 12 is defined as the time of lights off. The number of subjects in $a = 4$, $b = 4$, $c = 4$, and $d = 5$. Each point on the scatter plot represents the firing rate of a single cell. In these figures the cells from the four to five individuals in each treatment group are all plotted to allow a graphical representation of the results in each group. Note that the quantitative analysis summarized in the text was conducted by keeping each individual animal's data separate, fitting a running mean smoother to that individual's data, and then averaging the peak times.

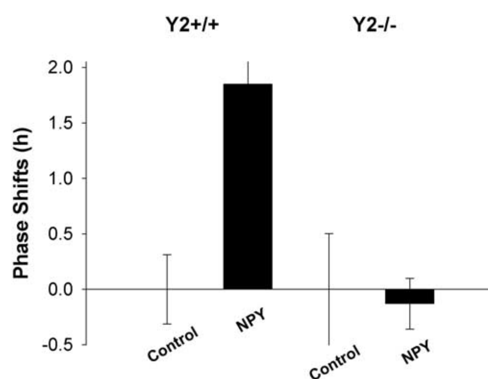

Fig. 2. Phase shifts of Y2+/+ and Y2−/− mice under both treatment conditions (applied at ZT 6). The mean phase-advance induced by NPY application was 1.85 ± 0.2 h for the Y2+/+ mice and ZT -0.13 ± 0.23 h for the Y2−/− mice. Error bars represent the standard error of the mean.

phase shifts to NPY during the subjective day in the hamster. Prior studies used Y2 agonists in hamsters and rats in order to show that the receptor mediates the effects of NPY [2,3,6]. To our knowledge, experiments using a Y2 antagonist have not been conducted in order to further verify the role of the receptor, although one study demonstrated that the Y1 receptor antagonist BIBP-3226 did not alter the ability of NPY to induce a phase shift during the subjective day in the rat SCN in vitro [3].

Further exploration of the role of NPY and the Y2 receptor could also include a full NPY phase response curve in the Y2−/− mice and control mice in order to give assurance that NPY phase shifts within a restricted period of time in the controls, and not at all in the Y2−/− mice. It would also be interesting to examine the behavioral activity patterns of the Y2−/− mice. It is difficult, however, to provide the mice with a non-photic stimulus in vivo. Non-photic stimuli such as novel wheels will phase-advance a hamster, but do not alter phase in mice, although some resetting is observed in mice following treadmill running and morphine injections [13,12]. Due to the fact that NPY cannot phase-advance the clock in vitro, either of the previously mentioned stimuli should not be able to reset the clock in vivo in the Y2−/− mice if they are mediated solely via NPY afferents. The role for serotonergic afferents in such behavioral responses is not yet clear.

There are some caveats to keep in mind when experimenting with knockout mice. There may be a loss of cells in knockout mice if they happen to depend on NPY, or the NPY Y2 receptor, for development. Furthermore, it is possible that other receptors in the brain are repressed or upregulated in certain types of knockout mice. For example, increased Y2 receptor mRNA and Y2 receptor binding was observed in brains of NPY−/− mice [19]. Y2−/−mice show reduced NPY Y1 receptor binding in the hippocampus [21], but Y1 mRNA levels were not altered, suggesting post-transcriptional regulatory mechanisms. Regulation of other neural systems are affected by the lack of NPY and the NPY Y2 receptor as well [18], providing phenotypically different animals from their wildtype littermates.

In sum, this study provided strong evidence for the critical role of the Y2 receptor in mediating the circadian clock phase shifting abilities of NPY during the subjective day.

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