

Research report

# Neuropeptide Y attenuates NMDA-induced phase shifts in the SCN of NPY Y1 receptor knockout mice in vitro

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## Abstract

Neuropeptide Y (NPY) blocks the effect of light on the mammalian circadian clock during the subjective night. The present study explores the role of the NPY Y1 receptor in this interaction. The effect of NPY when co-applied with NMDA, a glutamate agonist that can mimic the effect of light, was examined in NPY Y1<sup>-/-</sup> mice (background strain 129SVXBalb/c) using electrophysiology. Cells in the suprachiasmatic nucleus (SCN), the master circadian pacemaker, show a circadian rhythm in spontaneous firing rate that can be recorded in vitro. The results demonstrated that NPY attenuated the phase shifts to NMDA in both the Y1<sup>-/-</sup> mice and control mice, indicating that the Y1 receptor does not mediate the NPY blockade of photic-like phase shifts. The peak in frequency in the untreated control brain slices from Y1<sup>-/-</sup> mice was advanced by approximately 1 h as compared to the Y1<sup>+/+</sup> mice. The Y1 receptor may contribute to a functional model of circadian rhythms, but apparently is not essential for the effects of NPY on photic phase shifts.

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## 1. Introduction

The hypothalamic suprachiasmatic nucleus (SCN), the mammalian circadian pacemaker, displays intrinsic rhythms with a period circa 24 h. Photic and nonphotic inputs to the clock have the ability to alter these rhythms, producing a phase shift as a function of the time of input [10]. Photic information from the retina reaches the clock via two distinct pathways: the retinohypothalamic tract (RHT), a direct photic pathway which utilizes glutamate and pituitary adenylate cyclase-activating polypeptide as its primary neurotransmitters [4,8], and the geniculohypothalamic tract, an indirect photic pathway that utilizes neuropeptide Y (NPY) as its principal neurotransmitter [9]. Behavioral

actions, such as locomotor activity, have the ability to attenuate phase shifts to light when both stimuli are presented simultaneously [17]. Similarly, NPY also interacts with photic input from the RHT at the level of the SCN. NPY is increased in the SCN in the presence of light [19]. NPY has the ability to block light-induced phase advances and attenuate light-induced delays in vivo [12,23], and can also block glutamate or NMDA-induced phase shifts in vitro [2,26].

Anatomical studies have localized Y1 and Y5 receptors in the SCN in rats [24]. The distribution of NPY receptors, as visualized by <sup>125</sup>I-peptide YY binding, in the hamster SCN overlaps the projection field of the RHT [14,20]. In hamsters, the Y2 receptor is responsible for mediating the daytime effects of NPY [5], and the Y5 receptor plays a role in NPY inhibition of photic input during the night [26]. The Y5 receptor mediates inhibition of spontaneous firing rate by NPY in the rat [6]. The Y1 receptor may also contribute

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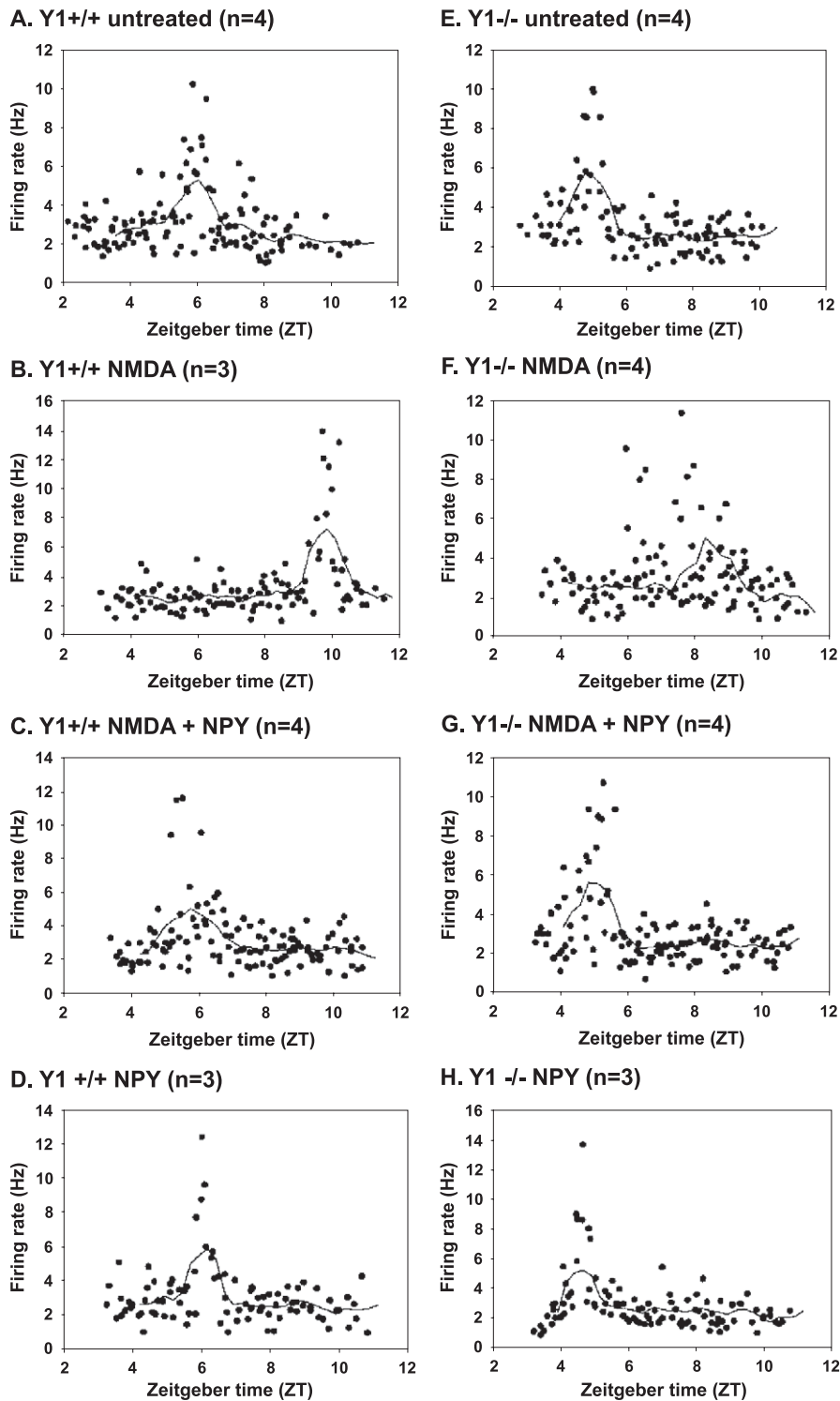


Fig. 1. Firing frequency of individual cells represented over time for each treatment group: average firing rate of the cell plotted against the Zeitgeber time (h) of the recording. Zeitgeber time 12 is set to the time of lights off in the animals housing room. ‘*n*’ signifies the number of subjects. Each point on the scatter plot represents the firing rate of a single cell, and the line depicts the running average firing rates of cells over the course of several hours of all subjects tested. In these figures, the cells from the three to four individuals in each treatment group are all plotted, with a running mean smoother fit to the grouped data, to allow a graphical representation of the variability in the results in each group. Note that the quantitative analysis summarized in further figures 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. Thus, the average of the peak times of the individuals may not exactly match the time of peak of the running mean smoother fit to the grouped data set as shown in this figure.

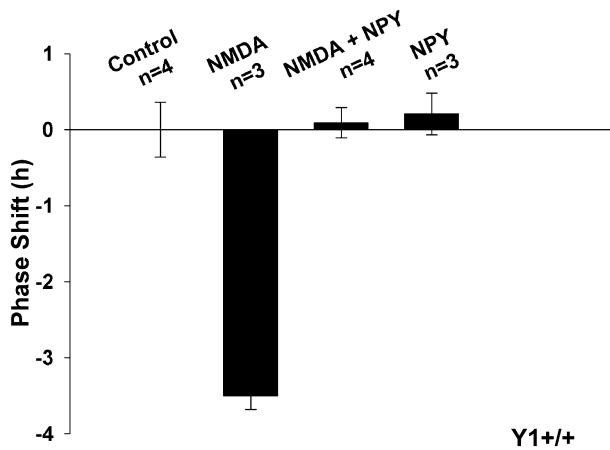


Fig. 2. Phase shifts of  $Y1^{+/+}$  under each treatment condition (applied at Zeitgeber time 15). Mean NMDA phase shift was  $-3.51$  h ( $\pm 0.17$  h), mean NMDA+NPY phase shift was  $0.09$  h ( $\pm 0.19$  h), and mean NPY phase shift was  $0.21$  h ( $\pm 0.27$  h). Error bars represent the S.E.M.

to nighttime effects of NPY in hamsters [12,26]. The role of the Y1 receptor in the circadian system of mice is not known. Mice show dense Y1 receptor mRNA in the SCN [15]. Some studies indicate the Y1 receptor may heterodimerize with the Y5 receptor, producing a receptor with pharmacological properties different from the Y1 or the Y5 [18]. Thus, it is possible that the two receptors have not yet been clearly distinguished by pharmacological tools, and therefore it is prudent to provide further evidence for the role of these receptors in function of circadian rhythmicity using transgenic mice.

The present study examines the role of the Y1 receptor on the NPY blockade of photic phase shifts using  $Y1^{-/-}$  mice. Because the Y1 and the Y5 receptors can form a heterodimer [18], we hypothesized that the presence of the Y1 receptor may be necessary for the Y5 receptor to mediate the blocking effect of NPY on phase shifts induced by light.

## 2. Materials and methods

129SV $\times$ Balb/c  $Y1^{+/+}$  and  $Y1^{-/-}$  male mice between 2 and 6 months of age (courtesy of Patrik Ernfors, PhD, Karolinska Institutet) [16] were bred in house. Mice were housed under a light/dark cycle of 12:12 h. Genotype was confirmed by PCR using primers as described in Ref. [16]. 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 maintained at  $34.5$  °C. 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)). Treat-

ment was applied to the SCN at ZT 15 on the first day in vitro using a 1- $\mu$ l Hamilton syringe. Each genotype ( $Y1^{+/+}$  and  $Y1^{-/-}$ ) had four different treatment groups: untreated, 10  $\mu$ M *N*-methyl-D-aspartate (NMDA), 10  $\mu$ M NMDA followed by 200 ng NPY, and 200 ng NPY. Each treatment application was 200 nl. When two drops were applied, there was a time of 5 min between drops. 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 SCN cells in each brain slice were sampled for 6–8 h. Extracellular activity was recorded using an electrode filled with ACSF. The electrode was placed into regions of the SCN at random, alternating between the left and right SCN. The signal was amplified (AC differential amplifier: BAK Electronics, Germantown, MD). It was then filtered, discriminated (window discriminator: BAK Electronics, Germantown, MD), and the spontaneous firing rate of each cell measured was recorded for 1 min.

Analysis began with data from an individual SCN slice taken from one animal. 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. If an ANOVA determined any 1-h bins significantly different from the others, the entire data set was then smoothed using 1-h running means with a 15-min lag. The ZT 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 correcting for multiple comparisons, or by a *t* test. Differences between treatment conditions were eval-

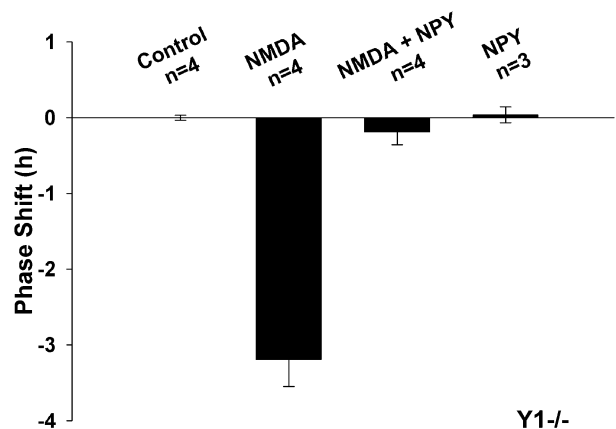


Fig. 3. Phase shifts of  $Y1^{-/-}$  under each treatment condition (applied at ZT 15). Mean NMDA phase shift was  $-3.19$  h ( $\pm 0.35$  h), mean NMDA+NPY phase shift was  $-0.19$  h ( $\pm 0.17$  h), and mean NPY phase shift was  $0.04$  h ( $\pm 0.10$  h). Error bars represent the S.E.M.

uated in each genotype, and then the two genotypes were analyzed comparatively using data from the untreated condition. All results are reported as mean  $\pm$  standard error of the mean.

### 3. Results

The average time of peak in frequency for the untreated Y1<sup>+/+</sup> mouse brain slices was ZT 6.3  $\pm$  0.36 h (see Fig. 1A). Out of four subjects, one Y1<sup>+/+</sup> mouse SCN treated with NMDA did not demonstrate an appreciable phase shift (peak time 5.58 h). For the brain slices from Y1<sup>+/+</sup> mice treated with NMDA that did produce a shift in the rhythm ( $n=3$ ), the peak time was ZT 9.81  $\pm$  0.17 h (see Fig. 1B). The peak in frequency was ZT 6.21  $\pm$  0.19 h for brain slices from Y1<sup>+/+</sup> mice treated with NMDA and NPY (see Fig. 1C); and ZT 6.09  $\pm$  0.27 h when treated with NPY alone (see Fig. 1D). The average peak firing rate for the untreated brain slices from Y1<sup>-/-</sup> mice was ZT 4.8  $\pm$  0.04 h (see Fig. 1E). The peak in firing rate frequency from SCN slices of Y1<sup>-/-</sup> mice was ZT 7.9  $\pm$  0.35 h when treated with NMDA (see Fig. 1F); ZT 4.99  $\pm$  0.17 h when treated with NMDA and NPY (see Fig. 1G); and ZT 4.76  $\pm$  0.10 h when treated with NPY alone (see Fig. 1H).

Fig. 2 demonstrates the size of the phase shifts under each treatment group for Y1<sup>+/+</sup> mice, while Fig. 3 exhibits the same for Y1<sup>-/-</sup> mice. One-way ANOVA tests showed that the Y1<sup>+/+</sup> and the Y1<sup>-/-</sup> mice had significant phase delays to NMDA; Y1<sup>+/+</sup>:  $F(3,10)=38.13$ ,  $p<0.001$ ; Y1<sup>-/-</sup>:  $F(3,11)=55.36$ ,  $p<0.001$ . These were of similar magnitude ( $\sim -3.35 \pm 0.21$  h). When treated with both NMDA and NPY, the phase delays did not differ significantly from the untreated groups for each genotype, although the Y1<sup>-/-</sup> mice had a slightly (yet insignificantly) larger phase delay ( $-0.19 \pm 0.17$  h) when treated with NMDA and NPY compared to the Y1<sup>+/+</sup> subjects ( $0.09 \pm 0.19$  h). Phase shifts to NPY were not statistically different from the untreated control group for each genotype.

The time in peak firing rate differs significantly between Y1<sup>+/+</sup> and Y1<sup>-/-</sup> untreated mice ( $t(6)=4.11$ ,  $p=0.006$ ). The Y1<sup>-/-</sup> mice demonstrate a slight advance in the rhythm.

### 4. Discussion

The results of this study demonstrate that circadian clock phase shifts induced by NMDA may be attenuated by application of NPY to the SCN in the mouse, confirming previous research [1]. This study also shows that the NPY Y1 receptor is not vital for the generation of an intrinsic electrical rhythm. This study further demonstrates that the Y1 receptor is not involved in the interaction between NMDA and NPY during the early subjective night. Mice deficient in Y1 receptors were still able to exhibit a NPY

blockade of NMDA-induced phase shifts. A recent preliminary report indicates that mice deficient in the NPY Y5 receptor did not show this effect of NPY [1]. Taken together, these studies support a role for the NPY Y5 receptor, and not the NPY Y1 receptor, in the interaction between NPY and light in the subjective night. Studies using Y5 antagonists in hamsters supported a similar conclusion: NPY is unable to attenuate light-induced phase advances in the presence of the Y5 antagonist, *in vivo* [13,27].

Although this study found that NPY attenuates NMDA-induced phase delays in Y1<sup>-/-</sup> mice, phase advances were not examined. Weber and Rea [23] found that NPY blocks light-induced phase advances, but not delays, in hamsters *in vivo*. Lall and Biello [12] found an inhibition of both advances and delays. Because differences between delays and advances concerning this effect have been noted, it would be interesting to test if the Y1 receptor plays a role in the interaction between NPY and NMDA in mice during the late subjective night. Unfortunately, advances to light are not as robust in mice as they are in hamsters, making this experiment more difficult.

Further research should also consist of a dose response curve of NPY. In this study NPY was applied in a concentration of 234  $\mu$ M, but even 0.2  $\mu$ M NPY can block the NMDA-induced phase shift in hamster brain slices [25] and preliminary results indicate that 2  $\mu$ M NPY can block phase shifts to NMDA in brain slices from mice [3]. It may be possible that a more dilute application of NPY can block phase shifts to NMDA in the Y1<sup>+/+</sup> mice, but not in the Y1<sup>-/-</sup> mice, similar to differences observed between dexas1<sup>-/-</sup> and <sup>+/+</sup> mice [3]. This would indicate a relatively subtle role for the Y1 receptor in mediating the sensitivity to NPY at low concentrations, with the Y5 receptor able to mediate effects at higher concentrations.

The Y1<sup>-/-</sup> mice demonstrate a peak in the rhythm approximately 1 h before the control mice. This difference in peak time was also apparent under every treatment condition. Previous studies have used Y1 antagonists to examine the receptor's effect concerning circadian rhythmicity. The specific Y1 antagonist BIBP-3226 when delivered to the SCN of the rat *in vitro* in 10 nM concentrations produced no difference in peak time relative to controls [6]. Similarly, Yannielli and Harrington [26] found that the same Y1 antagonist applied in 1  $\mu$ M concentrations in the hamster *in vitro* also did not affect the time of peak frequency. However, when the Y1 antagonist CP-671,906 was applied in 10  $\mu$ M concentrations, the time of peak firing rate was advanced by approximately 1 h [27]. This, in accordance with the present study, implies that the Y1 receptor may play a role in setting phase of circadian rhythms.

When glucose concentration in the bathing medium for a SCN brain slice is increased, there is a similar advance in the time of peak firing rate, indicating that peak time of the SCN is sensitive to glucose. This is a change in the phase angle of the firing rate rhythm to the underlying oscillator

because the effect is not permanent [7]. Taking this into account, it can be speculated that this difference in peak-firing rate may be linked to the Y1 receptor's role in metabolism. Y1<sup>-/-</sup> mice have been shown to have an elevated basal level of plasma insulin as well as impaired insulin secretion after glucose administration [11].

Aside from compensatory mechanisms, transgenic mouse studies have complications not found in other types of studies. There may be a loss of cells if they happen to depend on NPY, particularly the Y1 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, a 6-fold increase in Y2 receptor mRNA was observed in the CA1 region of the hippocampus in NPY KO mice [21]. In general, because knockout mice are created on mixed-strain backgrounds, details of individual experiments and reproducibility are crucial to refining system models [22].

In conclusion, the Y1 receptor does not appear to be involved in the interaction between NPY and NMDA during the early subjective night in mice. We report that SCN brain slices from Y1<sup>-/-</sup> mice display a ~1 h advance in peak frequency when compared to Y1<sup>+/+</sup> mice. This implies that the Y1 receptor may have some influence on the circadian system, but this receptor is clearly not mediating the effect of NPY to block the phase-resetting action of light.

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## References

- [1] S.M. Biello, Neuropeptide Y (NPY) does not attenuate phase shifts to NMDA in NPY Y5 receptor knockout mice, Program No. 285.13. 2003 Abstract Viewer/Itinerary Planner, Society for Neuroscience, Washington, DC, 2003, Online.
- [2] S.M. Biello, D.A. Golombek, M.E. Harrington, Neuropeptide Y and glutamate block each other's phase shifts in the suprachiasmatic nucleus in vitro, *Neuroscience* 77 (4) (1997) 1049–1057.
- [3] H.M. Cheng, K. Obrietan, B. Lee, S.W. Cain, P.V. Agostino, N.A. Joza, M.E. Harrington, M.R. Ralph, J.M. Penninger, Dexas1 potentiates photic and suppresses non-photic responses of the circadian clock, *Neuron*, in press.
- [4] F.J. Ebling, The role of glutamate in the photic regulation of the suprachiasmatic nucleus, *Progress in Neurobiology* 50 (1996) 109–132.
- [5] D.A. Golombek, S.M. Biello, R.A. Rendon, M.E. Harrington, Neuropeptide Y phase shifts the circadian clock in vitro via a Y2 receptor, *NeuroReport* 7 (7) (1996) 1315–1319.
- [6] V.K. Gribkoff, R.L. Pieschl, T.A. Wisialowski, A.N. van den Pol, F.D. Yocca, Phase shifting of circadian rhythms and depression of neuronal activity in the rat suprachiasmatic nucleus by neuropeptide Y: mediation by different receptor subtypes, *Journal of Neuroscience* 18 (8) (1998) 3014–3022.
- [7] A.C. Hall, R.M. Hoffmaster, E.L. Stern, M.E. Harrington, D. Bickar, Suprachiasmatic nucleus neurons are glucose sensitive, *Journal of Biological Rhythms* 12 (5) (1997) 388–400.
- [8] J. Hannibal, Neurotransmitters of the retino-hypothalamic tract, *Cell and Tissue Research* 309 (1) (2002) 73–88.
- [9] M.E. Harrington, The ventral lateral geniculate nucleus and the intergeniculate leaflet: interrelated structures in the visual and circadian systems, *Neuroscience and Biobehavioral Reviews* 21 (5) (1997) 705–727.
- [10] C.H. Johnson, J.A. Elliott, R. Foster, Entrainment of circadian programs, *Chronobiology International* 20 (5) (2003) 741–774.
- [11] A. Kushi, H. Sasai, H. Koizumi, N. Takeda, M. Yokoyama, Obesity and mild hyperinsulinemia found in neuropeptide Y-Y1 receptor-deficient mice, *Proceedings of the National Academy of Sciences of the United States of America* 95 (1998) 15659–15664.
- [12] G.S. Lall, S.M. Biello, Attenuation of circadian light induced phase advances and delays by neuropeptide y and a neuropeptide y1/y5 receptor agonist, *Neuroscience* 119 (2) (2003) 611–618.
- [13] G.S. Lall, S.M. Biello, Neuropeptide Y (NPY) attenuates photic phase shifts via the NPY Y5 receptor subtype, Program No. 510.14. 2003 Abstract Viewer/Itinerary Planner, Society for Neuroscience, Washington, DC, 2003, Online.
- [14] L.P. Morin, J. Blanchard, R.Y. Moore, Intergeniculate leaflet and suprachiasmatic nucleus organization and connections in the golden hamster, *Visual Neuroscience* 8 (1992) 219–230.
- [15] P. Naveilhan, I. Neveu, E. Arenas, P. Ernfors, Complementary and overlapping expression of Y1, Y2 and Y5 receptors in the developing and adult mouse nervous system, *Neuroscience* 87 (1) (1998) 289–302.
- [16] P. Naveilhan, H. Hassani, G. Lucas, K.H. Blakeman, J.X. Hao, X.J. Xu, Z. Wiesenfeld-Hallin, P. Thoren, P. Ernfors, Reduced antinociception and plasma extravasation in mice lacking a neuropeptide Y receptor, *Nature* 409 (6819) (2001) 513–517.
- [17] M.R. Ralph, N. Mrosovsky, Behavioral inhibition of circadian responses to light, *Journal of Biological Rhythms* 7 (4) (1992) 353–359.
- [18] D.A. Schober, M.M. Berglund, D.R. Gehlert, Neuropeptide Y (NPY) Y1 and Y5 receptors form constitutive dimers that elicit an enhanced response to Y5 antagonists, Program No. 615.4. 2003 Abstract Viewer/Itinerary Planner, Society for Neuroscience, Washington, DC, 2003, Online.
- [19] K. Shinohara, K. Tominaga, C. Fukuhara, Y. Otori, S.I. Inouye, Processing of photic information within the intergeniculate leaflet of the lateral geniculate body: assessed by neuropeptide Y immunoreactivity in the suprachiasmatic nucleus of rats, *Neuroscience* 56 (4) (1993) 813–822.
- [20] E.G. Stopa, J.K. Johnson, D.I. Friedman, H.I. Ryer, J. Reidy, V. Kuo-LeBlanc, H.E. Albers, Neuropeptide Y receptor distribution and regulation in the suprachiasmatic nucleus of the Syrian hamster (*Mesocricetus auratus*), *Peptide Research* 8 (2) (1995) 95–100.
- [21] P.G. Trivedi, H. Yu, M. Trumbauer, H. Chen, L.H. Van der Ploeg, X. Guan, Differential regulation of neuropeptide Y receptors in the brains of NPY knock-out mice, *Peptides* 22 (3) (2001) 395–403.
- [22] R.N. Van Gelder, J.B. Hogenesch, Clean thoughts about dirty genes, *Journal of Biological Rhythms* 19 (1) (2004) 3–9.
- [23] E.T. Weber, M.A. Rea, Neuropeptide Y blocks light-induced phase advances but not delays of the circadian activity rhythm in hamsters, *Neuroscience Letters* 231 (1997) 159–162.

- [24] M.L. Wolak, M.R. deJoseph, A.D. Cator, A.S. Mokashi, M.S. Brownfield, J.H. Urban, Comparative distribution of neuropeptide Y Y1 and Y5 receptors in the rat brain by using immunohistochemistry, *The Journal of Comparative Neurology* 464 (2003) 285–311.
- [25] P.C. Yannielli, M.E. Harrington, Neuropeptide Y in the mammalian system: effects on light-induced circadian responses, *Peptides* 22 (2001) 547–556.
- [26] P.C. Yannielli, M.E. Harrington, The neuropeptide Y Y5 receptor mediates the blockade of “photic-like” NMDA-induced phase shifts in the golden hamster, *Journal of Neuroscience* 14 (2001) 5367–5373.
- [27] P.C. Yannielli, J. McKinley Brewer, M.E. Harrington, Blockade of the NPY Y5 receptor potentiates circadian responses to light: complementary in vivo and in vitro studies, *European Journal of Neuroscience* 19 (2004) 891–897.