

Neuropeptide Y applied *in vitro* can block the phase shifts induced by light *in vivo*

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The mammalian suprachiasmatic nuclei (SCN) can be synchronized by light, with direct glutamatergic input from the retina. Input to the SCN from the intergeniculate leaflet contains neuropeptide Y (NPY) and can modulate photic responses. NPY can reduce the phase-resetting effect of light or glutamate. We investigated the effect of NPY applied *in vitro* on light-induced phase shifts of the SCN neural activity rhythm. Light pulses delivered *in vivo* induced phase shifts in brain slice preparations similar to those as measured by behavioral activity

rhythms. NPY applied after the light pulse blocked the phase shifts during both the early and late subjective night. NPY applied 30 min after the light pulse could block the phase delay induced by light. Our results show that NPY can inhibit photic resetting of the clock during the subjective night. The time course of this inhibitory effect suggests a mechanism downstream of the glutamate receptor. *NeuroReport* 11:1–5 © 2000 Lippincott Williams & Wilkins.

Key words: Circadian rhythms; Entrainment; Light; NPY; Photic/non-photic interaction; Single unit activity; Suprachiasmatic nuclei; Syrian hamster

INTRODUCTION

The hypothalamic suprachiasmatic nuclei (SCN), the area of the brain which functions as an endogenous circadian pacemaker in mammals, can be synchronized to the 24 h light:dark cycle mainly through a direct glutamatergic projection from retinal ganglion cells. Light resets circadian rhythm phase when applied during the subjective night, and has a well characterized phase response curve (PRC) (for review see [1]). The pacemaker receives also a variety of modulatory inputs, one of which arises from the thalamic intergeniculate leaflet (IGL) and utilizes neuropeptide Y (NPY) [2].

The daily oscillation in the firing rate of SCN neurons that can be observed for 3–4 ~24 h cycles *in vitro* [3] provides a unique system in which to examine the effect of resetting stimuli. Both glutamate and NMDA have been used to reset the phase of circadian rhythms *in vitro*, and both show resetting which depends upon time of day of application in a manner roughly similar to light, although exact magnitude and dose dependence of shifts is not identical to that observed in studies using light [4–7]. Pituitary adenylate cyclase activating peptide (PACAP), a peptide co-localized with glutamate in retinal terminals in the SCN [8], shows phase resetting actions which depend dramatically upon the dose applied [9]. The work conducted to date indicates that we do not yet fully understand how to exactly mimic the neurochemical message

associated with light exposure at critical circadian times. Thus, a more physiological approach, but still with the advantages of the *in vitro* preparation, would be useful. While one study reported that light could induce phase shifts measured *in vitro* [6], another study reported that phase shifts were induced by sacrifice in the subjective night, even without light exposure [10]. The present study was designed to re-examine the usefulness of this approach while investigating the interaction between light and modulatory neuropeptides at the level of the SCN.

Although NPY has been largely studied in relation to behaviorally mediated entrainment, evidence indicates that this input exerts a modulatory influence on photic responses (for reviews see [2,11]). NPY is able to reduce the phase-resetting action of light *in vivo* [12] and local SCN application of antiserum to NPY enhances photic phase shifts [13]. At a cellular level, NPY inhibits the spontaneous discharge of SCN cells in hamsters [14,15] and rats [16,17], and also induces a long-term depression in both glutamate-elicited electrical activity and intracellular calcium levels in rat SCN slice and cell culture [16]. While the inhibitory effect of NPY can be measured *in vitro*, using glutamate to mimic light [7], NPY was seen to inhibit both advances and delays *in vitro*, whereas *in vivo* experiments showed that microinjection of NPY into the SCN region of hamsters decreased the magnitude of light-induced phase advances, without affecting phase delays [12]. When

NMDA was used in another study to mimic light, *in vitro* experiments showed that in rats co-application of NPY actually potentiated the phase shifting effect [5]. Since results are not consistent between *in vivo* and *in vitro* studies, the effect of NPY on light-induced responses, as well as the mechanism involved, remains unclear.

In this study we report that light pulses delivered to a hamster *in vivo* can induce phase shifts of the SCN firing rhythm that can be recorded *in vitro*, with a PRC similar to that of light. Preparation of brain slices in the subjective night without light exposure did not induce phase shifts in the neural firing rhythm. Moreover, we show that NPY applied *in vitro* after *in vivo* light pulses can block the phase shifts induced by light. This effect is seen even when NPY is applied 30 min after the photic stimulation. These results suggest that the *in vivo-in vitro* system is a reliable method to measure the effects of light on the circadian clock, allowing description of the extended temporal window for NPY sensitivity following light exposure.

MATERIALS AND METHODS

Animals and tissue preparation: Male golden hamsters (LVG, Charles River 40–60 days old) were individually housed in cages equipped with 17.5 cm diameter running wheels, and maintained under a light:dark schedule of 14:10 h (lights on at 17.00 h), with food and water available *ad lib*. Each turn of the wheel was detected by a magnet attached to the wheel and a magnetic switch attached to the roof of the cage, connected to a data collection and acquisition system (Dataquest III, Minimitter Co, Sunriver, OR). After a baseline recording, the animals were transferred to dim red light (DRL) conditions, provided with a safelight lamp (Coastar), and were killed as stated below between days 3 and 7 of DRL. Circadian time was defined as CT 12 being the projected time of the animal's onset of activity on the day of the sacrifice. Hamsters were administered an overdose of halothane anesthesia and decapitated during the subjective day (CT6) or night (CT14, 18 or 19). 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 provided continuously.

Electrophysiological recordings: Extracellular single unit activity of SCN cells was detected with glass micropipette electrodes filled with ACSF, advanced through the slice using a hydraulic microdrive. The signal was fed into an amplifier for further amplification and filtering, and was continuously monitored by an oscilloscope and audio monitor. Firing rate was analyzed using data acquisition software and a customized program for calculation of descriptive statistics. A number of experiments in each condition were recorded blind where the person recording data had no knowledge of the contents of the microdrop or the previous history of light exposure. In the majority of

the cases only one slice was recorded from each animal, but when a second slice containing SCN was recorded, the treatment applied was different from that applied to the first slice of the same animal. A total of 55 slices was analyzed in the present study, and a total of 59 animals were used.

Drugs and treatments: Light pulses (5 min, 150 lux, 200 $\mu\text{W}/\text{cm}^2$) were delivered individually to the animals placed under two white fluorescent tubes (Phillips, model F30T12), at CT 14, CT 18, CT 19 or CT 6. Animals were killed immediately after the light pulse (light-treated group), or at the defined CT under dim red light (control group). Animals killed under DRL were carefully handled in order to avoid stress-related alterations. Neuropeptide Y (rat, human, Bachem Bioscience, Torrance, CA) prepared in ACSF was applied as a 200 nl microdrop (234 μM) delivered to the SCN area of the slice 15, 30, 60 or 90 min after the start of light pulse administration at CT 14, and 15 min after dissection at CT 18 and CT 19. The recordings started the following day, 2–3 h before CT 30 (the projected CT 6 of the second day *in vitro*) and lasted until the firing rate returned to pre-peak levels in all cases. The number of slices per treatment (*n*) is stated in the corresponding figure or is mentioned in the text. Recordings were performed for 6–8 h.

Data analysis: 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 circadian 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 Student *t*-test, ANOVA followed by Student–Newman–Keuls' methods or Kruskal–Wallis followed by Dunn's methods (for all pairwise comparison) when multiple comparison procedures were required. Data are presented as mean \pm s.e.

RESULTS

Experiment 1: Measuring *in vitro* the effects of light applied *in vivo*: Figure 1 shows average phase shifts of the firing rhythm recorded *in vitro* from hamsters which were given light pulses *in vivo* (or killed under DRL) at CT 6, CT 14, CT 18 and CT 19. In animals killed without light exposure, the time of day of slice preparation did not affect the observed phase of the rhythm. Light pulses (5 min, 150 lux) delivered at CT 6 did not have any effect on the phase of the rhythm, but induced phase delays when applied at CT 14 (average phase shift -2.36 ± 0.26 h), and advances when applied at CT 18 and CT 19. At CT 19 the phase advances appeared more pronounced and less variable than at CT 18, however this difference was not statistically significant (average phase shift at CT 18 1.54 ± 0.5 h; average phase shift at CT 19 2.83 ± 0.07 h, $t = 1.95$, $p = 0.12$, Student *t*-test).

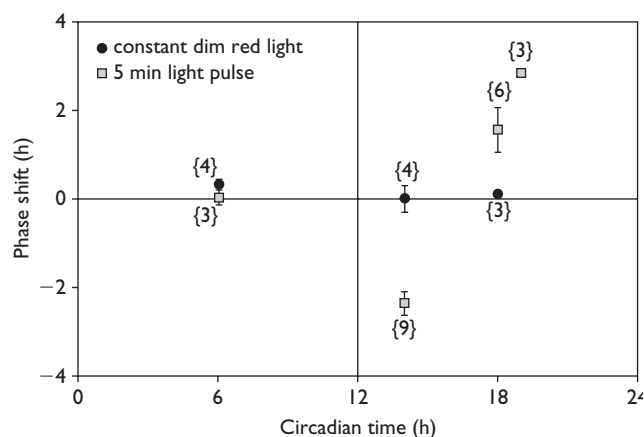


Fig. 1. Phase response curve of the SCN firing rate rhythm recorded *in vitro* to light pulses applied *in vivo*. Data are means \pm s.e., with number of treated slices denoted in brackets. Hamsters were maintained under dim red light (DRL), and wheel-running activity was recorded to determine the phase of the rhythm (CT 12 defined as time of activity onset). After 3–7 days, brief (5 min, 150 lux) light pulses were delivered at designated circadian times, and the brain slice preparation was performed under light in the light-treated group (square symbols), or under dim red light in the control (DRL) group (circles). The slices obtained were recorded on the second day *in vitro*, as stated in methods section. A two way ANOVA (time and treatment factors) revealed a significant time effect for the light-treated group ($F = 21.58$, $p < 0.0001$) and a significant time \times treatment interaction ($F = 17.30$, $p < 0.0001$).

Experiment 2: Blocking effect of NPY at CT 14 and CT 18–19: As depicted in Fig. 2, NPY applied *in vitro* 15 min after light stimulation significantly blocked the phase shifts induced by light delivered *in vivo* during the early subjective night (CT 14) as well as late subjective night (CT 18–19). We also tested the effect of NPY at CT 18 and CT 19 separately (light + NPY at CT 18: 0.5 ± 0.7 h; light + NPY at

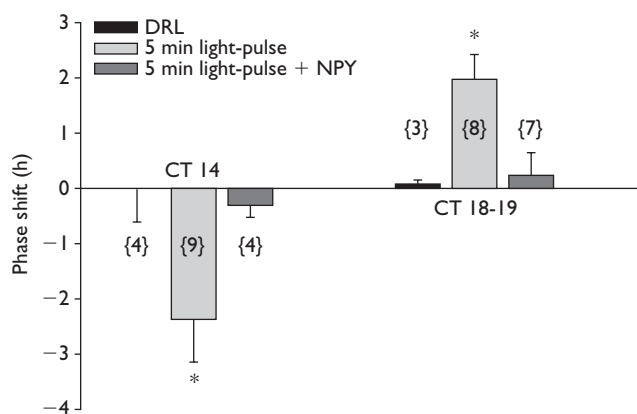


Fig. 2. Effect of NPY applied *in vitro* on phase shifts induced by light pulses applied *in vivo*. Light-treated or DRL-treated animals were killed at designated CTs, and slices obtained as stated in Methods. NPY was applied as a 0.2 μ l microdrop (200 ng) onto the SCN region, 15 min after dissection. Data are means \pm s.e., with the number of slices recorded per group denoted in brackets. NPY significantly blocked light-induced phase delays at CT 14 ($p < 0.05$, ANOVA followed by Student–Newman–Keuls' method) as well as phase advances at CT 18–19 ($p < 0.05$, Kruskal–Wallis followed by Dunn's method). No significant differences were found between NPY and control groups.

CT 19: -0.15 ± 0.07 h; $t = -0.8$, $p = 0.45$, Student *t*-test). Since no significant differences were observed in NPY blocking effect or light-induced phase shifts at both CT's, the effects of light as well as the effect of NPY during the late subjective night for graphic and statistical purposes were pooled under CT 18–19. Animals killed under DRL at various circadian phases did not show any differences in the phase of the rhythm (Fig. 1, Fig. 2). As reported previously, NPY applied during the subjective night did not induce a change in the phase of the rhythm [18]. Figure 3 shows representative recordings of control (DRL), light-treated and light + NPY-treated animals killed at CT 14 and CT 18–19.

Experiment 3: NPY effect at CT 14: time course study: Figure 4 shows the time course effect of NPY application on light-induced phase delays at CT 14. NPY blockade was statistically significant even when applied up to 30 min after the beginning of light stimulation, and lacked inhibitory effect when applied 90 min after light stimulation. NPY applied after 60 min of light stimulation had no significant effect, although the light-induced phase shift in one of the three slices was blocked by NPY.

DISCUSSION

In cultured hypothalamic slices from rat brain prepared during the donor's dark period, the SCN oscillator undergoes large shifts during the first cycle [10]. Thus, it was thought that sacrificing under dark conditions *per se* would induce phase shifts, and consequently this would eliminate the possibility of measuring *in vivo*-induced phase shifts. However, another report indicated that in rats the SCN firing rhythm could be phase shifted when animals that had been previously maintained under constant darkness were exposed to light pulses of 15 min prior to sacrifice. Moreover, the direction and magnitude of the phase shifts were similar after light exposure before sacrifice, optic chiasm electrical stimulation and glutamate administration *in vitro* [6], although results from constant darkness controls for light exposure treatment were not presented. In the present report we demonstrate that brief light pulses given before slice preparation induced phase shifts that mimic the phase response to light *in vivo*, and the phase response to NMDA and glutamate *in vivo* and *in vitro*, respectively [4,6,19]. Light pulses of 5 min did not induce significant phase shifts in the firing rate rhythm when applied during the subjective day. Slices prepared from animals under DRL did not show phase shifts at any circadian time. Therefore, we propose that this *in vivo*–*in vitro* system could represent a more physiological alternative to study the effect of light on the circadian system *in vitro*.

Our present results show that NPY applied *in vitro* significantly blocked the phase shifts induced by light *in vivo*. The mechanism(s) by which NPY blocked the effect of light even 30 min after light administration might be different to those mediating the non-photoc effects. NPY phase shifts in the subjective day are mediated via a Y2 receptor [7,17,20], while the action of NPY to block photic phase shifts depends on the Y5 receptor (unpublished results). This effect may involve the direct inhibitory effects of NPY on electrical activity and glutamatergic responses. Deple-

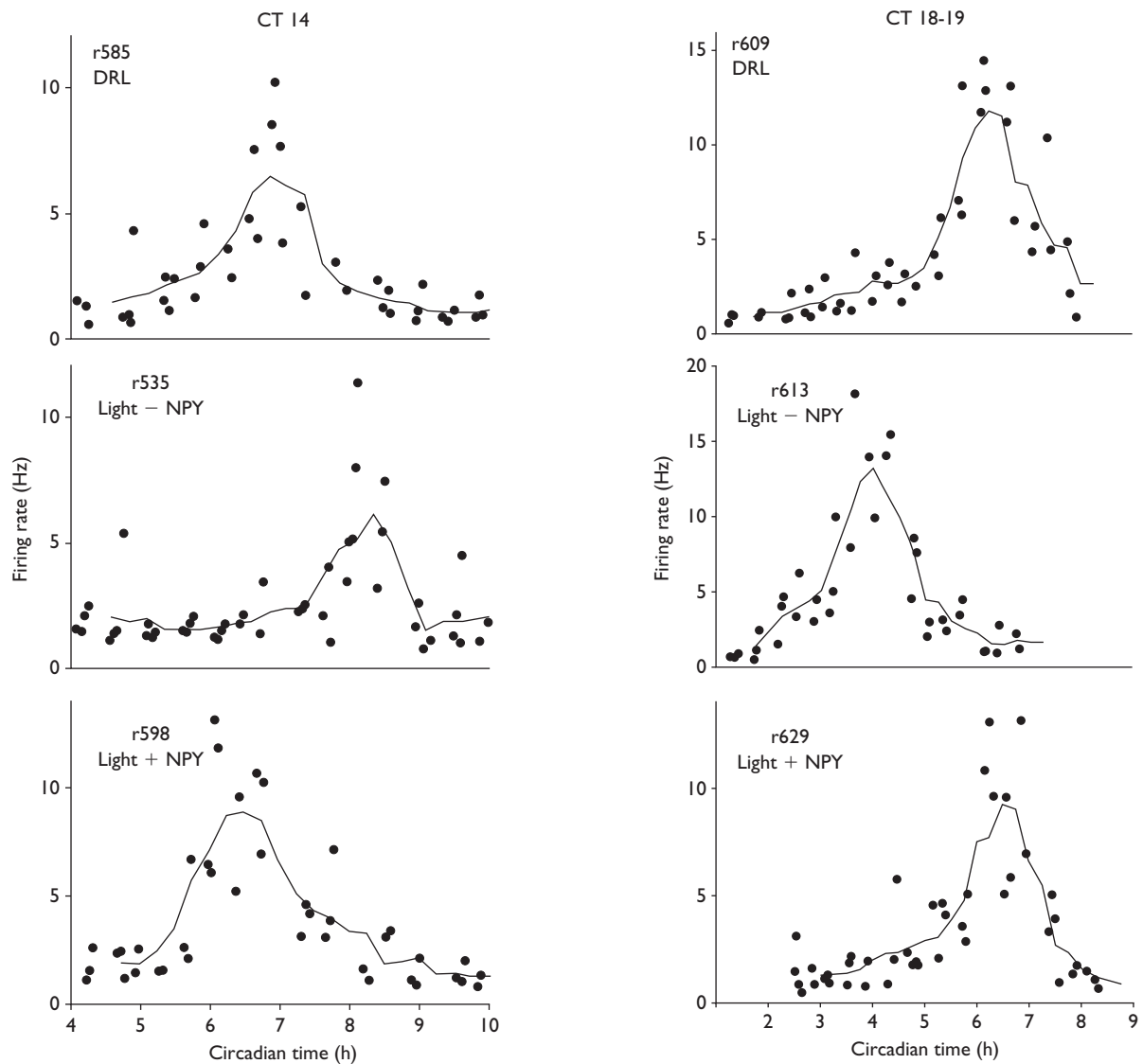


Fig. 3. Representative individual recordings. The firing rate of individual neurons (dots) was plotted against the circadian time of recording during the second day *in vitro*. Drugs were applied as stated in methods. Peak times as indicated by the running mean smoother (line) and treatments are denoted in each graph.

tion of intracellular Ca^{2+} stores during early night blocks the effects of glutamate [21]. Since NPY induced a glutamate-dependent long term depression in neuronal activity and intracellular Ca^{2+} levels in SCN cells [16], the inhibition of photic responses could be achieved by this mechanism. It should be noted that 30 min after the beginning of the light pulse the sites or pathways affected by NPY are more likely to be downstream from the glutamatergic receptors. The same time course for NPY inhibition was observed previously for glutamate-induced phase shifts in slice preparations (Biello SM, unpublished results). Moreover, since photic phase shifts were completed within 1–2 h [22], the time course of NPY inhibition of photic responses seems remarkable. In fact, given the recent report that novel wheel access, a resetting stimulus thought to be mediated by NPY [11], can decrease expression of

mper1 and *mper2* mRNA [23], it is possible that the inhibitory action of NPY on photic responses might eventually be acting at the level of gene expression.

NPY can inhibit both phase delays and advances elicited by *in vivo* administration of brief light pulses. However, *in vivo* and *in vitro* studies show that there are differences regarding the effect of NPY on light-induced phase shifts. NPY applied directly to the SCN *in vivo* inhibited light-induced phase advances but not delays [12]. On the other hand, *in vitro* studies showed that NPY could block glutamate-induced phase delays and advances [7]. One possible explanation is that *in vivo* locomotor activity or some other behavioral factor is highest during the early subjective night, saturating the system so that it cannot be further modulated by behavioral stimulation or neurochemicals such as NPY which are associated with beha-

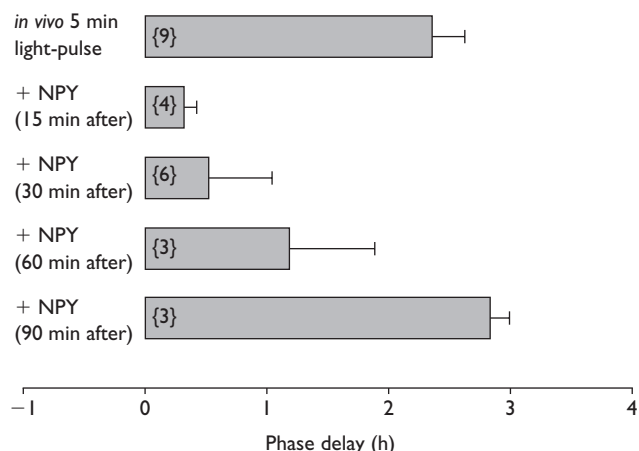


Fig. 4. Time course effect of NPY applied *in vitro* on phase delays induced by light *in vivo*. Light-treated animals were killed at CT 14, and slices obtained as stated in Materials and Methods. NPY was applied as a 0.2 μ l microdrop (200 ng) onto the SCN region, 15, 30, 60 or 90 min after the beginning of the light pulse. Data are means \pm s.e., with the number of slices recorded per group denoted in brackets. NPY significantly blocked light-induced phase delays at CT 14 when applied either 15 or 30 min after the light stimulation, but not when applied 60 or 90 min after light stimulation (* p < 0.05, ANOVA followed by Student–Newman–Keuls' method).

vioral activity. There is evidence for a direct effect of the animal's behavior on electrical activity of the SCN *in vitro*, providing further electrophysiological basis for the known effects of behavioral stimuli on the circadian pacemaker [24]. Regarding *in vivo* procedures that also inhibit photic responses, other considerations might be pertinent. In hamsters, novel wheel access can inhibit light-induced phase advances but not delays [25,26], and this effect could be blocked by metergoline, a serotonergic antagonist [26]. Administration of serotonin agonists blocked light-induced phase advances but not delays *in vivo* [27], and electrical stimulation of the raphe nuclei at CT 14 inhibited light-induced c-Fos expression in the SCN, although this effect could not be blocked by metergoline [28]. Serotonin, which also negatively modulates the effectiveness of the light entrainment pathway (for review see [1]), has been shown to decrease light-induced c-Fos expression in the SCN [29]. In rats, at a cellular level, it also inhibited the rising in Ca^{2+} levels stimulated by glutamate [30]. These results suggest the participation not only of NPY but also of serotonin for the novel wheel access inhibition of photic responses. In this sense, it is possible that circadian differences between *in vivo* and *in vitro* findings could be due to the interactions between the raphe nuclei and the IGL at a level other than the SCN. Since NPY can block

serotonergic phase advances during the subjective day in rats [31], NPY–serotonin interactions might have physiological significance.

CONCLUSION

The effect of light given *in vivo* can be measured *in vitro*, and even modulated by substances like NPY, applied *in vitro*. We also reported that NPY can block photic responses elicited during early and late subjective night, probably through a mechanism downstream of the glutamatergic receptor, with a temporal window of at least 30 min after the light stimulation.

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