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The effects of electrical stimulation of the optic nerves and anterior optic chiasm on the circadian activity rhythm of the Syrian hamster: involvement of excitatory amino acids

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Abstract

The circadian pacemaker of the suprachiasmatic nuclei (SCN) is entrained to the environmental light–dark cycle via the retinohypothalamic tract (RHT). It is unknown whether light activates or suppresses firing of the retinal ganglion cells which mediate photic entrainment. We therefore electrically stimulated the optic nerves and the anterior optic chiasm of hamsters with free-running activity rhythms in continuous darkness. These electrical stimulations are thought to induce a release of neurotransmitter at the RHT terminals. Electrical stimulation mimicked the phase dependent shifts induced by light pulses. The phase shifts were significantly larger than the shifts induced by sham stimulation in the same animals or by electrical stimulation in animals with an electrode outside the optic nerves and chiasm. Our results indicate that the retinal ganglion cells which project to the SCN are activated by light. Intraperitoneal administration of MK-801, a non-competitive antagonist of the NMDA-receptor, attenuated the phase delays induced by electrical stimulation in the early subjective night. This suggests that an excitatory amino acid mediates the effects of light upon the circadian pacemaker.

Key words: Circadian rhythm; Glutamate; Electrical stimulation; MK-801; Retinohypothalamic tract; Suprachiasmatic nucleus

1. Introduction

A great variety of behavioral and physiological functions in mammals have rhythms of approximately 24 h. These circadian rhythms are driven by an internal pacemaker situated in the suprachiasmatic nuclei (SCN) of the anterior hypothalamus [26]. The retinohypothalamic tract (RHT) is a direct retinal projection to the SCN [29] which mediates photic entrainment of the circadian pacemaker to the environmental light–dark (LD) cycle [17].

The pacemaker of the SCN can be phase shifted by light pulses. The activity rhythms of hamsters free-running in continuous darkness (DD) are phase delayed when short light pulses are applied during the early part of the subjective night, and are phase advanced following light pulses during the late subjective night [9,14]. The effects of light pulses differ from those obtained by pulses of darkness. Long dark pulses applied to hamsters housed in continuous light (LL) induce phase advances during the animals' subjective day, while small phase delays are obtained during the subjective night [4,14].

Recent studies suggest that an excitatory amino acid (EAA) functions as neurotransmitter of the RHT [5,11,22]. Most findings indicate that EAAs are released from terminals of the RHT when the animal is exposed to light. A strong indication is the finding that intraventricular administration of antagonists of EAA receptors can block the phase shifting effects of light pulses [8] and the light induced immediate-early gene expression in the SCN [2].

However, micro-injection of neither glutamate nor aspartate into the SCN region mimics the effects of light pulses on the circadian activity rhythm [10,25]; instead the effects of glutamate are similar to the effects of dark pulses. Moreover, the effects of glutamate are significantly larger in animals housed in LL, compared with animals kept in DD [25]. Using in vivo microdialysis, a significant peak in glutamate and as-

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partate release in the SCN-area is not found during the light but during the dark period of the LD cycle in the Syrian and Siberian hamster [16,32]. These studies suggest that EAAs are released during darkness, and not during light (see Rusak and Bina [35]).

Thus far it has not been investigated whether light activates or suppresses firing of the ganglion cells of the retina which mediate behavioral phase shifting. To simulate activation of the ganglion cells, the optic nerves and anterior optic chiasm of Syrian hamsters free-running in DD were electrically stimulated. Electrical stimulations were performed at different times of the circadian cycle and the effects on the running wheel activity rhythm were analyzed. Moreover, the effect of the non-competitive *N*-methyl-D-aspartate (NMDA) receptor antagonist MK-801 on the phase shifting effects of electrical stimulation of the optic nerves and chiasm was investigated. Part of these results have been published as an abstract [12].

2. Material and methods

2.1. Animals

On arrival, male Syrian hamsters (*Mesocricetus auratus*, Harlan/CPB, Zeist, The Netherlands) 4–7 weeks of age, were individually housed in cages (l×w×h = 36.5×25.0×16.0 cm) with a running wheel (diameter: 26.0 cm). Groups of 12 cages were kept in light controlled boxes (L:D 14 h:10 h). The boxes were placed in a sound attenuating ventilated room at a temperature of 23°C. A computer system registered the presence or absence of running wheel activity each minute. Food (Hope Farms B.V.) and water were continuously available. Clean cages were only provided in between the experiments.

2.2. Electrode implantation

At the age of at least 10 weeks, animals were anaesthetized (75 mg sodium pentobarbital/kg b.w.) and stereotaxically implanted with a bipolar electrode with a diameter of either 0.25 or 0.15 mm (MS 303/2 or MS 303/3, Plastics One Inc., VA, USA). The twisted electrodes were cut to a length of 9.0–9.5 mm. The electrodes were insulated except for the tips of 0.2 mm (diameter: 0.20 or 0.125 mm) which were 0.2–0.6 mm apart. The electrode was aimed at the anterior optic chiasm at an angle of 5–5.5° to the vertical (coordinates: 2.3–2.8 mm anterior and 0.55–0.70 mm lateral to bregma) and lowered to the bottom of the skull, with the toothbar 2.0 mm below the interaural line. The electrode was fixed to the skull with three jewellers' screws and dental acrylic. After the implantation, the animals were placed in DD.

2.3. Experimental protocol

Experiment 1: Hamsters were electrically stimulated with a Grass S48 stimulator in series with a Grass stimulus isolation unit (SIU 4678). The electrode was connected to the stimulator via an overhead rotating electrical contact. The connection was made under red darkroom light (light intensity ≤ 2.0 lux) while the animals' eyes were covered with a black cap. Electrical and sham stimulations were performed in a circular plastic container under red darkroom light (light intensity ≤ 0.1 lux). Square wave pulses of 1 ms duration and 20 Hz were applied for 30 min. Most stimulations involved currents of 300–450 μA, while a small number of pulses involved higher currents (500–800 μA). During the stimulation the polarization direction of the current was changed after 15 min. After stimulation the animals were left undisturbed for at least 7 days (10 days when transients occurred) before receiving a next stimulation. The stimulations were clustered in three phase intervals: circadian time (CT)7–10, CT12–16 and CT18–23, with the onset of activity defined as CT12. None of the animals contributed more than two points to each phase interval of the phase-response plot.

Experiment 2: In six animals the effect of MK-801 ((+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,b]cyclohepten-5,10-imine maleate) on the phase shifts induced by electrical stimulation at CT14 was investigated. Animals were subjected to three treatments: (1) intraperitoneal (i.p.) injection of vehicle (0.15 ml saline) plus electrical stimulation; (2) i.p. injection of MK-801 (6 mg/kg b.wt. in 0.15 ml saline) plus electrical stimulation; and (3) i.p. injection of MK-801 plus sham stimulation. The injections were performed 30 min prior to stimulation. Electrical stimulations involved currents of 350–430 μA for 30 min. Between different treatments animals were left undisturbed for at least 10 days. After these experiments five of the animals were also electrically stimulated at CT14 without receiving an injection prior to the stimulus to test for a possible treatment order effect. Moreover, the animals were electrically and sham stimulated between CT7–10, CT12–16 and CT18–23. The phase shifts induced by these stimulations are included in the phase-response plots (Fig. 2A, B).

2.4. Data analysis

To determine the magnitude of a phase shift a straight line was eye fitted through the 7–10 activity onsets before the stimulation. A second line was fitted by eye through the next 7–10 activity onsets. When transients occurred, two or three activity onsets following the stimulation were excluded from the data analysis. Phase shifts were assessed by measuring the difference to the nearest 0.05 h between the pre- and post-stimulation lines on the day of the first activity onset following the stimulation. Phase advances are expressed as positive values, and delays as negative values. The magnitude of the phase shift was plotted against the midpoint of the 30 min stimulation period.

2.5. Histology

At the end of the experiments the site of electrode implantation was verified. Brains were imbedded in gelatin, sectioned (40 μm) and stained with Cresyl violet. For all animals we measured the minimal distance between the border of the electrode implantation site and the borders of the SCN.

2.6. Statistics

We tested for differences between the effects of electrical and sham stimulation in animals with the electrode in the optic nerve/chiasm with the Wilcoxon matched pairs signed-ranks test. To compare the effects of electrical stimulation in animals with the electrode inside and outside the optic nerve/chiasm we used the Mann-Whitney test. The relationship between the magnitude of a phase shift and the distance of the implanted electrode to the SCN was evaluated by linear regression. The effects of the different treatments in experiment 2 were compared with repeated measures analysis of variance, followed by a Scheffe's test. The significance level was set at 0.05.

3. Results

3.1. Histology

Forty-five animals were implanted with an electrode and stimulated in DD. In 28 animals one or both tips of the electrode were either partly or totally situated in the optic nerves or anterior in the optic chiasm. The mean distance between the most caudal sign of the electrode implantation site and the anterior SCN was 640 μm (range: 240–1080 μm). In 17 of the 28 animals the electrode was implanted anterior in the optic chiasm, and in 11 animals the most rostral sign of the electrode tips was in one ($n = 6$) or two ($n = 5$) of the optic nerves, while the most caudal sign of the implantation site was also in the optic nerve(s) or in the anterior optic chiasm.

In eight animals, the electrode was situated outside the optic nerves and optic chiasm. The mean distance between the most caudal sign of the electrode implantation site and the anterior SCN in these animals was 925 μm. In six animals, the most caudal sign of the electrode tips was between the optic nerves or just dorsal of the optic chiasm (range: 640–1280 μm rostral to the SCN). One electrode was placed 300 μm lateral of the optic chiasm (720 μm rostral of the SCN), while in another animal the electrode was situated anterior of the paraventricular nucleus, 800 μm dorsal to the SCN.

In six animals, the electrode was situated posterior in the optic chiasm and touched the anterior ($n = 5$) or medial ($n = 1$) SCN. In three other animals, the electrode had damaged the optic nerve to such an extent that results of these animals will not be presented.

3.2. Experiment 1: effects of electrical stimulation

Electrical stimulation between CT12–16 in 28 animals with an electrode implanted in the optic nerves and optic chiasm induced mainly phase delays (mean $\Delta\varphi \pm$ S.E.M.: -0.63 ± 0.11 h, $n = 33$) (Figs. 1, 2A). Phase delays were significantly different from the effects of sham stimulation between CT12–16 in these animals (-0.05 ± 0.03 h, $n = 10$, $P = 0.012$) (Figs. 1A, 2B). They were also significantly different from the phase shifts induced by electrical stimulation in eight animals with an electrode outside the optic nerves and chiasm (-0.05 ± 0.06 h, $n = 6$, $P = 0.0028$) (Fig. 2C).

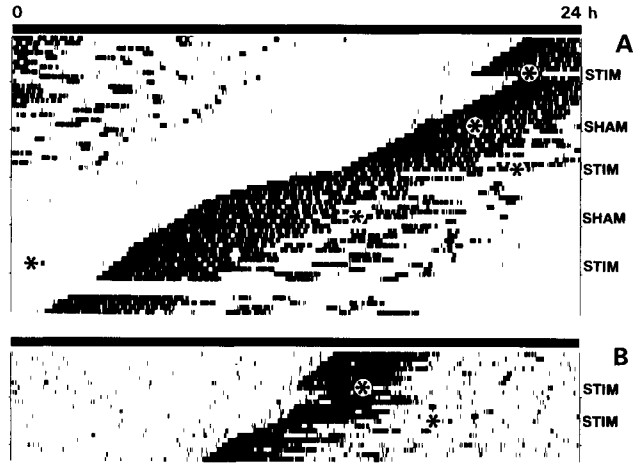

Fig. 1. A,B: activity records of hamsters with an electrode in the anterior optic chiasm. The time of day (in hours) is indicated above the actograms, the successive days are plotted beneath each other. Electrical stimulation (STIM) during the early subjective night induced phase delays, while stimulation during the late subjective night induced phase advances. Sham stimulation (SHAM) and electrical stimulation during the subjective day had no effect on the phase of the rhythm. The three days without running wheel activity in A indicate missing data caused by computer failure.

Electrical stimulation between CT18–23 in the animals with an electrode in the optic nerves and optic chiasm induced predominantly phase advances (0.67 ± 0.16 h, $n = 40$) (Figs. 1, 2A). Phase advances were significantly different from the effects of sham stimulation in the same animals (0.05 ± 0.04 h, $n = 14$, $P = 0.0058$) (Figs. 1A, 2B) and from those induced by

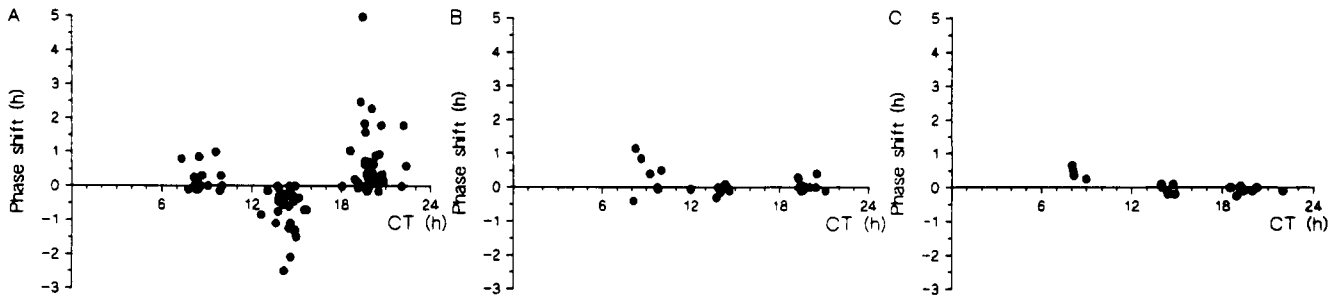

Fig. 2. Phase response plots for electrical (A) and sham (B) stimulation in animals with an electrode in the optic nerves and anterior optic chiasm and for electrical stimulation in animals with an electrode outside the optic nerves and chiasm (C). The magnitude of the phase shift was plotted against the midpoint of the 30 min stimulation period.

electrical stimulation outside the optic nerves and chiasm (-0.05 ± 0.02 h, $n = 13$, $P = 0.00004$) (Fig. 2C). Small phase advances could be induced by electrical stimulation of the optic nerves and chiasm between CT7–10 (0.17 ± 0.08 h, $n = 18$) (Figs. 1A, 2A). Small phase advances were also induced between CT7–10 by sham stimulation in the same animals (0.35 ± 0.20 h, $n = 7$) (Fig. 2B) or electrical stimulation in animals with the electrode outside the optic nerves or chiasm (0.44 ± 0.09, $n = 4$) (Fig. 2C).

In six animals the electrode was situated posterior in the optic chiasm and touched the SCN. A mean phase delay was obtained with electrical stimulation between CT12–16 (-0.47 ± 0.87 h, $n = 6$). In one animal two phase advances (2.85 and 0.70 h) were induced during these circadian hours. Between CT18–23 electrical stimulation induced a mean phase advance (0.85 ± 0.66 h, $n = 8$). During these circadian hours a large phase delay (-1.10 h) was obtained in one animal.

Linear regression over all phase shifts obtained after electrical stimulation between CT12–16 and CT18–23 did not reveal a significant relationship between the distance of the electrode from the SCN and the magnitude of the phase shift, neither for phase shifts between CT12–16 ($n = 45$, $P = 0.90$) nor for phase shifts between CT18–23 ($n = 61$, $P = 0.14$).

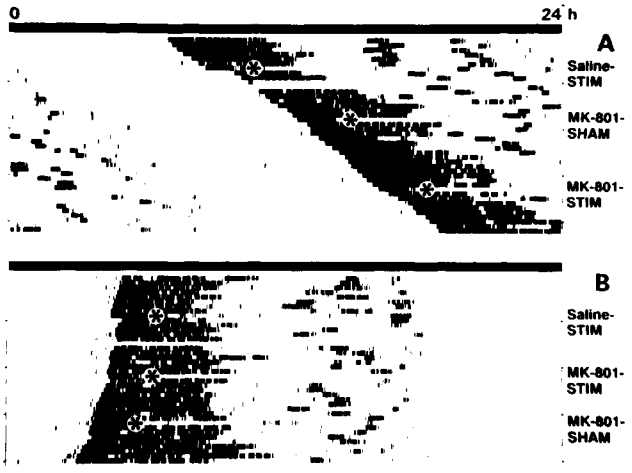

Fig. 3. A,B: activity records of two hamsters with the electrode in the optic nerves and anterior chiasm. The time of day (in hours) is indicated above the actograms, the successive days are plotted beneath each other. Electrical stimulation at CT14 in combination with saline administration 30 min prior to the stimulation induced phase delays (Saline-STIM). MK-801 administration attenuated the phase delaying effects of electrical stimulation at CT14 (MK-801-STIM). Only minor phase shifts were induced by administration of MK-801 in combination with sham stimulation (MK-801-SHAM). The absence of wheel running during the second part of day 10 and day 11 in A and during the second part of day 14 and day 15 in B are caused by a computer failure.

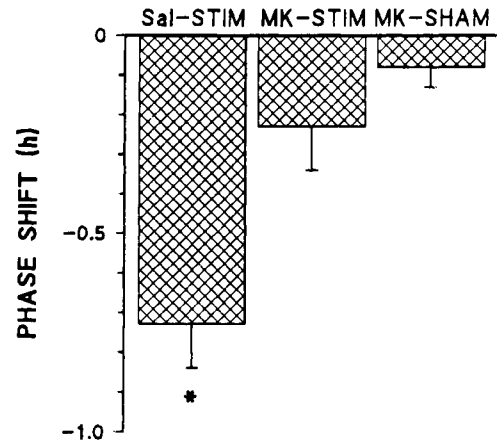

Fig. 4. Mean phase shift (±S.E.M.) in the wheel running activity rhythm induced by electrical stimulation of the optic nerves and chiasm plus i.p. administration of vehicle (Sal-STIM) or MK-801 (MK-STIM) and administration of MK-801 plus sham stimulation (MK-SHAM). Injections were performed 30 min prior to stimulation at CT14. All treatments were performed in six animals. * $P < 0.005$.

3.3. Experiment 2: effect of MK-801 on electrically induced phase shifts

Six of the 28 animals with the electrode in the optic nerves or anterior optic chiasm were used to investigate the effect of MK-801 on the effects of electrical stimulation at CT14. In these animals the mean distance between the electrode and the SCN was 580 μm (range: 240–1080 μm). After i.p. injection of saline, electrical stimulation at CT14 induced a mean phase delay of -0.73 h (± 0.11 h) (Figs. 3, 4). I.p. administration of MK-801 significantly attenuated the phase delaying effects of electrical stimulation (-0.23 ± 0.11 h, $P = 0.002$). The phase delays induced by electrical stimulation plus saline injection were also significantly larger than the effects of sham stimulation in combination with MK-801 administration (-0.08 ± 0.05 h, $P = 0.003$). The shifts induced by electrical stimulation plus MK-801 administration were not significantly different from the shifts induced by sham stimulation in combination with MK-801 injection ($P = 0.16$). After these experiments five of the animals were electrically stimulated at CT14 without receiving an injection prior to stimulation. A mean phase delay was obtained (-0.67 ± 0.21 h), which was not significantly different ($P = 0.85$, two-tailed paired Student's t-test) from the delays obtained with electrical stimulation at CT14 in combination with saline injections in the same animals. This indicates that the attenuation of the phase delays by MK-801 is not caused by the order of treatment.

4. Discussion

There is increasing evidence that EAAs function as neurotransmitter of the RHT. The release of gluta-

mate and aspartate in brain slices containing the SCN increases following optic nerve stimulation [22]. In RHT terminals of both rat and mouse, increased glutamate-immunoreactivity has been described [5,11]. Thus far, it has not been confirmed whether light or darkness triggers the release of RHT transmitter. Most studies indicate that EAAs are released from RHT terminals in the SCN when the animal is exposed to light. Intraventricular and i.p. administration of antagonists of EAA receptors can block the phase shifting effects of light pulses [6,7,8], and the light induced Fos protein expression in the SCN [1,2]. Injections with agonists of EAAs into the SCN mimic the effects of light on melatonin production [31,37]. Moreover, retinal illumination and glutamate application to the SCN have similar effects on the firing rates of the majority of SCN cells [24,27,30].

However, micro-injection of glutamate or aspartate into the SCN region fails to mimic the effects of light pulses on the circadian activity rhythm [10,25]. Instead phase advances are induced during the subjective day. The effects of glutamate are significantly larger in animals housed in LL, compared with animals kept in DD [25]. This suggests that glutamate may be released during darkness. This is consistent with the observations that the glutamate and aspartate release in the SCN-area peaks during the dark hours of the LD cycle [16,32], and that firing rates in optic chiasm recordings from just below the SCN increase during darkness and decrease during light [21].

To test whether the transmitter of the RHT is released by light we have electrically stimulated the optic nerves and anterior chiasm of hamsters free-running in DD. These electrical stimulations simulate activation of firing of the retinal ganglion cells and will cause the release of neurotransmitter from the terminals of the RHT in the SCN. Electrical stimulations induced phase dependent shifts. Phase delays were obtained between CT12–16, while phase advances were obtained between CT18–23. These phase shifts were significantly larger than the effects of sham pulses in the same animals, and than the effects of electrical stimulation outside the optic nerves and chiasm in other animals. Between CT7–10 electrical stimulation could induce small phase advances of the activity rhythm. However, small phase advances were also induced by sham stimulation. Thus, electrical stimulation in the optic nerves and anterior chiasm induced phase shifts that are very similar to the effects of light [9,14].

Rusak and Groos [33] have shown that electrical stimulation of the SCN produces phase delays at the beginning of the subjective night and phase advances at the end of the subjective night. Therefore, the question arises whether our phase shifts may have been induced by direct stimulation of the SCN, rather than by stimulation of RHT fibres. Indeed, electrical stimulation in animals with an electrode posterior in the optic chiasm which touched the SCN could delay the activity rhythm between CT12–16 and advance the rhythm between CT18–23. However, the findings that (1) electrical stimulation outside the optic nerves and chiasm did not induce phase shifts and that (2) no significant relationship was observed between the distance of the electrode from the SCN and the magnitude of the phase shift make it unlikely that the phase shifts in animals with an electrode in the optic nerves and anterior chiasm were a consequence of spread of current to the SCN. To exclude this possibility unequivocally, we investigated the effect of i.p. administration of MK-801 on the phase shifting effects of electrical stimulation at CT14. The phase delays induced by electrical stimulation were significantly attenuated by this non-competitive NMDA receptor antagonist, which indicates that the effects of electrical stimulation are not caused by direct depolarization of SCN cells.

We conclude that phase shifts obtained in animals with an electrode in the optic nerves and anterior chiasm are induced by stimulation of fibres of the RHT. This indicates that the effects of light pulses can be mimicked by electrical stimulation of RHT fibres when animals are kept in DD. This finding is consistent with the hypothesis that light activates firing of the retinal ganglion cells which mediate photic entrainment and that the RHT transmitter is released by light. It is also in agreement with the recent findings of Shibata and Moore [36]. With electrical stimulation of the optic nerve and chiasm in vitro they also obtained phase shifts that mimicked the effects of light on the circadian pacemaker.

The finding that the release of glutamate and aspartate in the SCN-area is significantly increased during the dark hours of the LD cycle [16,32] probably reflects the presence of glutamate and aspartate in the SCN area which has not been released by the RHT. This is consistent with a recent finding in the Syrian hamster that the glutamate rhythm persists in DD, and thus is of circadian origin [15]. Several studies indicate the presence of non-retinal glutamatergic input to the SCN. In hypothalamic slices which contain the SCN, the intracellular EPSPs evoked by electrical stimulation lateral or dorso-caudal of the SCN could be depressed by antagonists of EAA receptors [20]. Non-retinal glutamate-immunoreactive terminals have been described in the SCN [5,11]. Glutamate injections into the SCN [25] have a different effect on the activity rhythm than electrical stimulation of the optic nerve (this study) and light [9,14]. These injections may indirectly have activated SCN afferents such as the geniculohypothalamic tract [25]. Stimulation of this indirect photic pathway also results in phase shifts resembling those induced by dark pulses [34].

The phase delaying effects of electrical stimulation

at CT14 could be attenuated by i.p. administration of MK-801. This indicates that an EAA is the transmitter which mediates the effects of electrical stimulation. Since electrical stimulation of the RHT fibres simulates the activation of retinal ganglion cells by light this supports the hypothesis that an EAA is involved in photic entrainment of the circadian pacemaker. The effects of light on the circadian pacemaker have previously been blocked with i.p injection of MK-801 [1,6,7]. These studies suggested that EAAs are involved in the process of photic entrainment. Although intraventricular injections with EAA receptor antagonists can block the light induced phase shifts and Fos expression in the SCN [2,8], the exact site of action of NMDA receptor antagonists has not yet been localized unequivocally (see Colwell and Menaker [8]). Besides blocking NMDA receptors in the SCN, MK-801 may have had its effect in the retina. EAAs are probably involved in the transmission of photic information from the outer to the inner retina. Glutamate-immunoreactive retinal bipolar cells have been described in fish, reptile and bird [13,18,19] and electrophysiological studies in rat, rabbit and salamander indicate the presence of both NMDA and non-NMDA receptors on retinal ganglion cells [3,23,28]. We have simulated the effects of light by electrical stimulation of the RHT fibres downstream from the retina. The effects of this stimulation could be blocked with MK-801 administration. Our experiments localize the NMDA receptor involved in photic entrainment downstream from the retina, most probably in the SCN itself.

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