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Glutamate phase shifts circadian activity rhythms in hamsters

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The suprachiasmatic nuclei (SCN) have been identified as a pacemaker for many circadian rhythms in mammals. Photic entrainment of this pacemaker can be accomplished via the direct retino-hypothalamic tract (RHT). Glutamate is a putative transmitter of the RHT. In the present study it is demonstrated that glutamate injections in the SCN cause phase shifts of the circadian activity rhythm of the hamster. In contrast, glutamate injections outside the SCN or vehicle injections inside the SCN did not affect the circadian phase. These data suggest that glutamate could be involved in photic entrainment of the circadian pacemaker.

The suprachiasmatic nuclei (SCN) function as a primary pacemaker for circadian rhythms in mammals [15]. Entrainment of circadian rhythms to the environmental light–dark cycle is mediated by the direct retino-hypothalamic tract (RHT) [6]. An indirect photic projection reaches the pacemaker via the ventral lateral geniculate nucleus and the intergeniculate leaflet (the geniculate-hypothalamic tract or GHT) [3, 17]. This pathway is characterized by neuropeptide Y immunoreactivity [7]. Although it is clear that the GHT plays a role in photic responsiveness of circadian rhythms [1, 2], lateral geniculate ablation studies indicate that the direct retino-hypothalamic projection is sufficient for entrainment of the circadian clock [4].

The neurotransmitter(s) of the RHT relevant to the processing of photic information to the circadian clock is (are) not yet known. Acetylcholine (ACh) may be involved in mediating the phase-shifting effects of light although it is unlikely to be the primary transmitter of the RHT since choline acetyltransferase is not found in the optic nerve [19]. Evidence for a role of ACh in mediating light effects on rhythms is inconsistent. Intraventricular injection of a cholinergic agonist, carbachol, mimics the phase shifting effects of light [5], while intraventricular injection with the cholinergic antagonist mecamylamine blocks these effects [8]. In addition, iontophoretic ap-

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plication of cholinergic agonists alters the spontaneous discharge rate of most SCN neurons when recorded in vivo [13]. On the other hand, light-induced phase shifts cannot be blocked by hemicholinium, a drug which depletes the store of ACh [14]. α -Bungarotoxin binding, which probably reflects cholinergic binding sites, is found in the SCN of the rat. Although ovariectomy eliminates such binding, ovariectomized rats are as responsive to the phase-shifting effects of carbachol injections as are intact females [12].

Recently, it was demonstrated in a rat brain slice preparation containing the SCN that stimulation of the optic nerve induces the release of [3 H]glutamate and [3 H]aspartate [9]. Moreover, glutamate and aspartate aminotransferase are present in the optic nerve [19] and SCN neurons are responsive to glutamate [13, 16]. These findings suggest that excitatory amino acids could be involved in the transmission of photic information to the SCN via the RHT. Light and dark pulses can phase shift the circadian pacemaker. If glutamate does mediate photic information to the SCN, one would expect that glutamate can also phase shift the circadian pacemaker. In this series of experiments we therefore examined whether glutamate micro-injections in the suprachiasmatic area can induce phase shifts of the circadian activity rhythm of the hamster.

Thirty-three male golden hamsters (*Mesocricetus auratus*) obtained from TNO, Zeist, The Netherlands at the age of 10 weeks (80–100 g) were initially housed in groups under a light–dark schedule (L:D=14:10) for 1–3 weeks. They were later transferred to individual cages (1 × w × h = 48 × 27 × 22.5 cm) with free access to food and water, and were placed in either a continuously illuminated (LL, 30–100 lux) or a dark (DD) sound attenuating room. Running wheel activity (diameter running wheel: 14 cm) was recorded both by an Esterline Angus event recorder and by a computer system in which the state of activity was accumulated over 2-min epochs.

The animals were anesthetized with sodium pentobarbital, 90 mg/kg initial dose, and implanted with a stainless-steel cannula (o.d., 0.3 mm; i.d., 0.15 mm) aimed stereotaxically at the SCN at a 5° angle to vertical (coordinates: 0.7 ± 0.2 mm anterior to bregma, 7.6 ± 0.1 mm ventral to dura and starting 0.7 mm lateral to the midline at dura), with the toothbar 2 mm below the interaural line. The cannula was fixed to the skull with dental acrylic, a wire was inserted in the cannula in order to keep it patent, and the assembly was protected by a removable cap.

After a stable freerunning rhythm was established for at least 7 days under either LL or DD conditions, the animals were injected with $0.5 \mu\text{l}$ 1 mM L-glutamate (sodium-L-glutamate-monohydrate, Merck) dissolved in artificial CSF near circadian times (CT) 0, 3, 6, 9, 12, 15, 18 and 21 h with CT 12 defined as the onset of activity. Before each injection, the hamster was lightly anesthetized with ether to enable the attachment of a plastic tube to the cannula which was also attached to a Hamilton syringe. During the injection (lasting 2–4 min) the animals could move freely in a small cage (1 × w × h = 33.5 × 19 × 13 cm). Those animals housed in DD were moved to another room immediately prior to the injection procedure to diminish possible disturbance of other animals. The attachment of the Hamilton syringe was performed under dim red light ($0.02 \mu\text{W}/\text{cm}^2$) while the animals' eyes were occluded by a black cap.

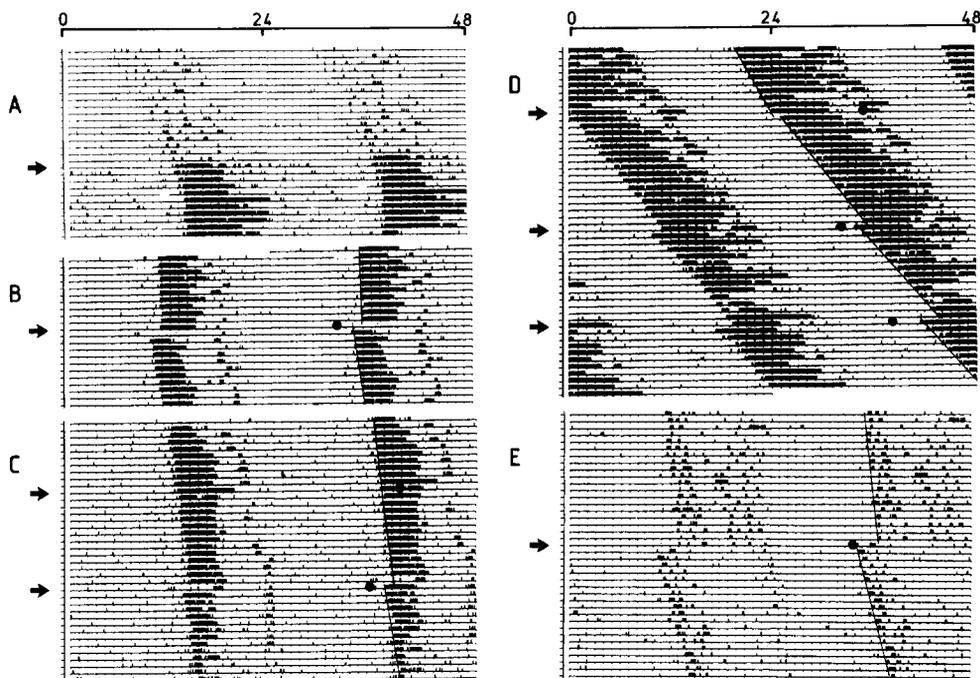


Fig. 1. The activity records are double-plotted to enable visualization of the activity rhythms. The time of day (in hours) is indicated horizontally and the consecutive days vertically. In A it is illustrated that the amount of activity increases when the hamster is placed in a somewhat smaller cage with a larger running wheel. The day of the transfer is indicated by an arrow. B–E: representative examples of glutamate-induced phase shifts of the circadian activity rhythm in DD (B,C) and LL (D,E). The day and the time of the injections are marked by arrows and dots, respectively.

Straight lines were fitted by linear regression through the activity onsets prior to the injection, and through the activity onsets following glutamate injection once a stable freerunning rhythm was re-established. The phase shifts were estimated by measuring the difference between the extrapolated pre- and post-injection regression lines at the first activity onset following an injection. At the end of the experiment the location of the cannulas was verified histologically by examining Cresyl violet-stained brain sections ($40\ \mu\text{m}$).

Hamsters placed in LL generally showed reduced levels of running wheel activity which sometimes interfered with determination of activity onsets and, therefore, with the calculation of phase shifts. In the course of the experiment, the hamsters in the illuminated room received new cages ($1 \times w \times h = 30 \times 22.5 \times 29.5\ \text{cm}$) that were equipped with a larger running wheel (diameter 25 cm), which was found to be effective in enhancing their amount of running wheel activity.

Cannulas in 8 hamsters were located in the SCN. In these animals, glutamate micro-injections caused consistent phase shifts of the circadian activity rhythm both in LL (number of injections, $n = 19$) and DD ($n = 18$) while vehicle injections ($n = 8$) did not produce phase shifts (Fig. 1B–E). When the cannulas were placed outside

the SCN ($n=19$) glutamate applications ($n=28$ in LL, $n=15$ in DD) did not produce phase dependent shifts. Phase shifts are plotted in Fig. 2 as a function of the injection time. The mean phase advances (\pm S.E.M.) in LL at CT 6 (1.7 ± 0.4 h) and in DD and CT 9 (1.3 ± 0.2 h) differed significantly from the phase advances obtained by control injections in the SCN (0.3 ± 0.6 h in LL, and 0.1 ± 0.1 h in DD) as was indicated by a Student's *t*-test ($P<0.001$). When phase shifts are grouped per animal, the experimental injections differed from the control injections with $P<0.03$ for LL housed animals and with $P<0.001$ for DD. Moreover, the pooled phase advances at CT 6 and CT 9 (1.8 ± 0.3 h in LL and 1.0 ± 0.2 h in DD) differed from pooled phase shifts obtained by glutamate injections outside the SCN (0.2 ± 0.1 h in LL, $P<0.001$ and 0.3 ± 0.8 h in DD, $P<0.005$). The complete phase-response curve (PRC) for glutamate injections in one animal indicates that phase advances and delays can be obtained by the same injection site in the suprachiasmatic area (see Fig. 3). In two animals in which the tips of the cannulas appeared $200\ \mu\text{m}$ dorsal to the SCN, glutamate injections resulted in phase delays ($\Delta\phi$) at CT 3 (n , number of injections = 1 in DD, $\Delta\phi = -0.63$ h), CT 6 ($n=2$ in LL and DD, $\Delta\phi = -0.88$ h) and at CT 18 ($n=2$ in DD, $\Delta\phi = -0.55$ h). At CT 0, 9, 14 and 21 no considerable shifts

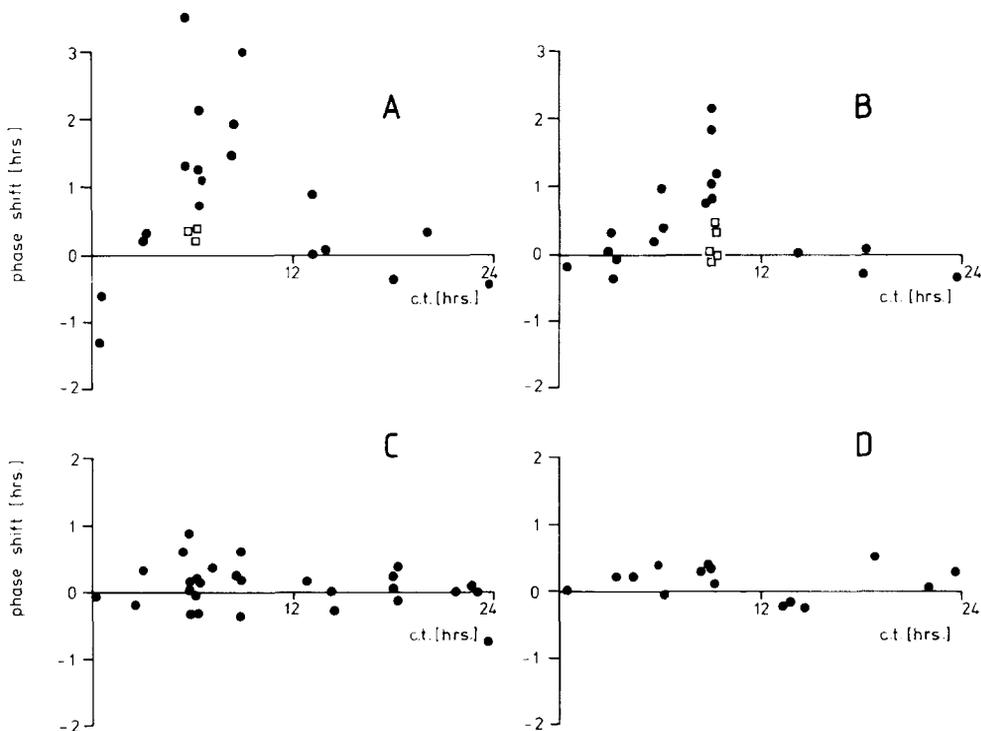


Fig. 2. Phase-response curves for glutamate injections in the SCN (A in LL and B in DD) and outside (more than $500\ \mu\text{m}$) the SCN (C in LL and D in DD). On the horizontal axis, the circadian time (c.t.) is indicated in hours. The activity onset is defined as c.t. 12. The open squares represent phase shifts induced by artificial CSF injections in the SCN.

were observed ($n=5$ in DD, $\Delta\phi \pm \text{S.E.M.} = -0.10 \pm 0.04$ h). These shifts are not included in Fig. 2. Four hamsters were excluded from the data analysis because the histological picture was not clear or because the SCN was considerably damaged.

The freerunning period of the circadian activity rhythm did not alter significantly following glutamate injections in the SCN. The mean (S.E.M.) change in LL is 0.030 h (0.034) while the mean change in the dark is 0.009 h (0.037). Levels of activity were sometimes markedly decreased immediately following an injection (see Fig. 1B). In such cases the presence of transients could not be assessed. Most phase shifts, however, did not appear to be accompanied by transients.

The present experiments demonstrate that glutamate injections in the suprachiasmatic nucleus cause phase shifts that are dependent on the circadian time at which glutamate is applied. Significant phase advances were generated by injections in the mid-late subjective day. Phase shifts of this kind were never encountered following vehicle injections in the SCN (see Fig. 2A,B) nor were they caused by glutamate injections more than 500 μm outside the suprachiasmatic area (Fig. 2C,D). Therefore, we attribute the effects of the injection to the action of glutamate on the neurons in and near the suprachiasmatic area.

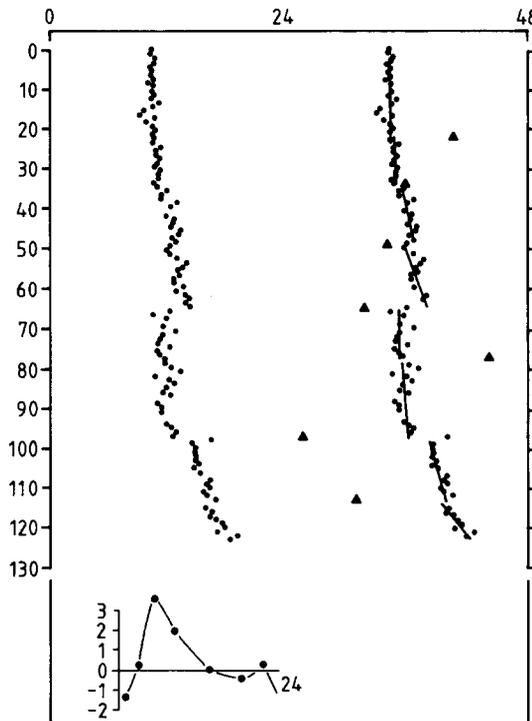


Fig. 3. Double-plotted wheel running activity record of a hamster housed in LL. The time of day is indicated on the horizontal axis, while the consecutive days are numbered along the vertical axis. The beginning of activity is marked with black dots, glutamate injections are indicated by triangles. The phase-response curve at the bottom summarizes the glutamate-induced phase shifts for this animal. The circadian time of the injection is indicated on the abscissa and the phase shift on the ordinate.

In two animals glutamate injections dorsal to the SCN at CT 3, 6 and 18 caused phase delays. Possibly these injections also reached the dorsal SCN. The physiological significance of these phase shifts is not clear since the terminals of the RHP end predominantly in the ventral SCN. However, the possibility that subdivisions of the SCN may be differently involved in phase shifts of the circadian pacemaker should be further investigated.

The effects of glutamate injections resemble the phase response curve generated by exposing animals in LL to dark pulses [2]. Moreover, the phase dependency of the shifts resembles the PRC for neuropeptide Y injections in the SCN [1] and for electrical stimulation of the ventral lateral geniculate nucleus, by which endogenous neuropeptide Y is presumably released in the SCN [11]. Neuropeptide Y, like glutamate, excites most SCN neurons [10]. The question arises whether the PRCs generated by injections of NPY and glutamate can be produced by any excitatory transmitter, or whether they reflect a specific involvement of these transmitters in phase shifting by light. Since the excitatory drug carbachol does not produce similar phase shifts it seems unlikely that the glutamate PRC would be obtainable by non-specific excitation [5]. On the other hand carbachol injected in the lateral ventricle will mainly reach the dorsal and medial SCN via the third ventricle, while our injections affected also the ventral SCN. PRCs for different transmitters may not be comparable without considering the locus of stimulation.

Recently, a projection from the SCN to the ventral lateral geniculate nucleus has been identified [18]. Therefore, our glutamate injection (and glutamate release from the RHT) may result in stimulation of this indirect pathway. Phase shifts induced by GHT stimulation may have contributed to the PRC for glutamate injections.

Another finding in our study was that glutamate caused a larger phase advance when the animals were housed in LL than when they were housed in DD. The pooled mean phase advance (S.E.M.) at CT 6 and CT 9 in LL is 1.8 h (0.3) and in DD 1.01 h (0.21). These data differ significantly ($P=0.022$) as was indicated by a one-tailed *t*-test. When the assumption is dropped that no differences between animals exist the means for individual animals have to be weighed. The corresponding result in DD is then negligibly different but that for LL is 1.9 ± 0.55 h, thus with an appreciably larger standard error. This is because the variation between animals is much larger for LL than for DD although this could be a chance effect. Shibata et al. [16] have shown that glutamate perfusion of a SCN-containing slice preparation desensitizes SCN neurons to optic nerve stimulation. The similarity of the glutamate PRC with the dark pulse PRC suggests that glutamate is released in the dark. Therefore, one might expect a relative insensitivity to exogenous glutamate in DD as compared to LL housed animals. Our results are consistent with this expectation.

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