

NSL 07822

## Aspartate injections into the suprachiasmatic region of the Syrian hamster do not mimic the effects of light on the circadian activity rhythm

Martinus J. De Vries and Johanna H. Meijer

*Department of Physiology and Physiological Physics, University of Leiden, Leiden (The Netherlands)*

(Received 3 January 1991; Revised version received 24 March 1991; Accepted 28 March 1991)

**Key words:** Aspartate; Circadian rhythm; Retinohypothalamic tract; Suprachiasmatic nucleus

The excitatory amino acids glutamate and aspartate are thought to be involved in the photic entrainment of the circadian pacemaker of the suprachiasmatic nuclei (SCN). When applied to the SCN region glutamate imitates the effects of dark pulses on the circadian activity rhythm rather than those of light. We have now injected aspartate into the SCN region of Syrian hamsters. These injections mimicked the effects of dark pulses as well, in so far as slight advances of the activity rhythm were obtained during the subjective day. However, the mean phase shift was not significantly different from the shift obtained with control injections. It is concluded that (1) aspartate has little or no effect on the phase of the circadian activity rhythm and (2) none of the putative transmitters of the photic afferents of the SCN produces the effects of light upon injection.

The suprachiasmatic nuclei (SCN) of the anterior hypothalamus serve as a major pacemaker for the circadian rhythms in mammalian behavior and physiology [15]. This pacemaker generates rhythms of approximately 24 h, which can be entrained to the environmental light–dark cycle. Photic entrainment is mediated by a direct retinal projection to the SCN: the retinohypothalamic tract (RHT) [10]. The geniculohypothalamic tract (GHT) is an indirect photic afferent projection to the SCN, originating in the ventral lateral geniculate nucleus and the intergeniculate leaflet of the thalamus. The GHT may contribute to the process of entrainment [12, 20].

Several studies suggest that excitatory amino acids function as neurotransmitters of the RHT. The release of glutamate and aspartate in the SCN of brain slices increases following optic nerve stimulation [13]. The large negative field potentials that are generated in the SCN of such slices by electrical stimulation of the optic nerve [24] are absent after bath application of antagonists of excitatory amino acids [4, 5, 23]. The phase shifting effects of light *in vivo* can be blocked by intraperitoneal injections with antagonists of excitatory amino acids both in hamsters and mice [6, 7]. Moreover, application of the excitatory amino acid antagonist MK-801 directly to the SCN reduces the light induced phase advances [14]. Finally, neurons of the SCN are excited by ionto-

phoretic injections of glutamate and aspartate [19, 22, 23]. Whilst these experiments suggest that glutamate and aspartate are transmitters of the RHT, immunocytochemical studies have indicated that neuropeptide Y is the transmitter of the GHT [11, 17].

Dark pulses induce phase advances of the free-running activity rhythm when applied during the animals' subjective day, whereas small phase delays are obtained during the subjective night [3, 9]. Microinjection of neuropeptide Y into the SCN region has the same phase shifting effects as dark pulses [1]. Light is the major entraining agent of the circadian pacemaker. The effects of light pulses differ from those obtained by dark pulses in that light produces phase delays during the early part of the subjective night whilst towards the end it produces advances [8, 9]. Surprisingly, microinjection of glutamate into the SCN region fails to mimic the effects of light pulses but has similar phase shifting effects as dark pulses have [16]. The question arises whether the amino acid aspartate can produce the effects of light. Therefore we injected aspartate into the SCN region of hamsters with free-running activity rhythms.

Adult male Syrian hamsters (*Mesocricetus auratus*, Harlan/CPB, Zeist, The Netherlands) were individually housed in cages (1 × w × h = 36.5 × 25.0 × 16.0 cm) containing a running wheel with a diameter of either 14.5 or 24.0 cm. Groups of 6 cages were kept in continuously illuminated boxes. The boxes were placed in a sound attenuating ventilated room at a temperature of 23°C. Running wheel activity was recorded by a computer sys-

tem which accumulated the amount of activity over either 6- or 30-min epochs. Food (Hope farms B.V.) and water were continuously available.

Under pentobarbital sodium anaesthesia (75 mg/kg b.w) the animals were stereotaxically implanted with a stainless steel cannula (o.d. = 0.30 mm, i.d. = 0.15 mm). The cannula was aimed at the SCN at an angle of  $5^\circ$  to the vertical (coordinates: 0.7 mm anterior and 0.7 mm lateral to bregma and 7.35–7.50 mm ventral to dura) with the toothbar 2.0 mm below the interaural line. The cannula was fixed to the skull with jeweller screws and dental acrylic. A removable cap protected the cannula, which was kept open by an inserted wire. Thirty-four animals were placed under continuous dim light (Intensity < 0.1 lux) and 9 implanted animals were maintained under continuous bright light (LL, Intensity > 100 lux).

At intervals of 9–12 days the animals were injected with either 0.5  $\mu$ l of a 1 mM L-aspartic acid (J.T. Baker Chemicals B.V.) solution or vehicle artificial cerebrospinal fluid (CSF). An aspartate concentration of 1 mM was used because bath application at this concentration is able to alter the discharge rate of SCN neurons in brain slices (Meijer, unpublished data). Moreover, a number of injections were performed with 0.1 and 10 mM aspartate solutions to animals kept in dim light. Before each injection the animals were anaesthetized with ether in order to attach a plastic tube to the cannula. The tube was connected to a 10  $\mu$ l Hamilton syringe. The injections to animals kept in dim light were performed under a red darkroom lamp while the animals' eyes were covered with a black cap. Injections were completed within 2 min. Aspartate injections were performed at dif-

ferent circadian times (CT). The injections between CT4.5–10.5 had the strongest effect. Therefore, CSF control injections were clustered during these hours. We also aimed the aspartate injections in LL at these circadian hours. These injections were performed in bright light.

A straight line was eye-fitted through the 7–10 activity onsets before the injection. The two activity onsets immediately following the injection were excluded from the data analysis to reduce the influence of transients in estimating the phase shift. A second line was fitted by eye through the next 7–10 activity onsets. Phase shifts were assessed by measuring the difference between the pre- and post-injection lines on the day of the first activity onset following the injection. At the end of the experiment brain sections (50  $\mu$ m) were stained with Cresyl violet to verify the location of the cannulas. The SCN region was defined as the area within 500  $\mu$ m from the border of the SCN, because this area corresponds with the diameter of the injected volume.

In 31 animals kept in dim light the tip of the cannula was situated inside the SCN region. In these animals most injections with 1 mM aspartate between CT4.5–10.5 induced slight phase advances (Figs. 1a, 2A), the mean being 0.50 h (S.E.M. = 0.12). Most CSF injections between CT4.5–10.5 induced phase shifts in the same direction as those with aspartate (Fig. 2C), the mean advance being 0.35 h (S.E.M. = 0.16). Between CT10.5–16.5, CT16.5–22.5 and CT22.5–4.5 delays were mainly obtained with aspartate injections (Fig. 2A). The mean phase delays were respectively 0.38 (S.E.M. = 0.17), 0.31 (S.E.M. = 0.15) and 0.13 h (S.E.M. = 0.15). No phase ad-

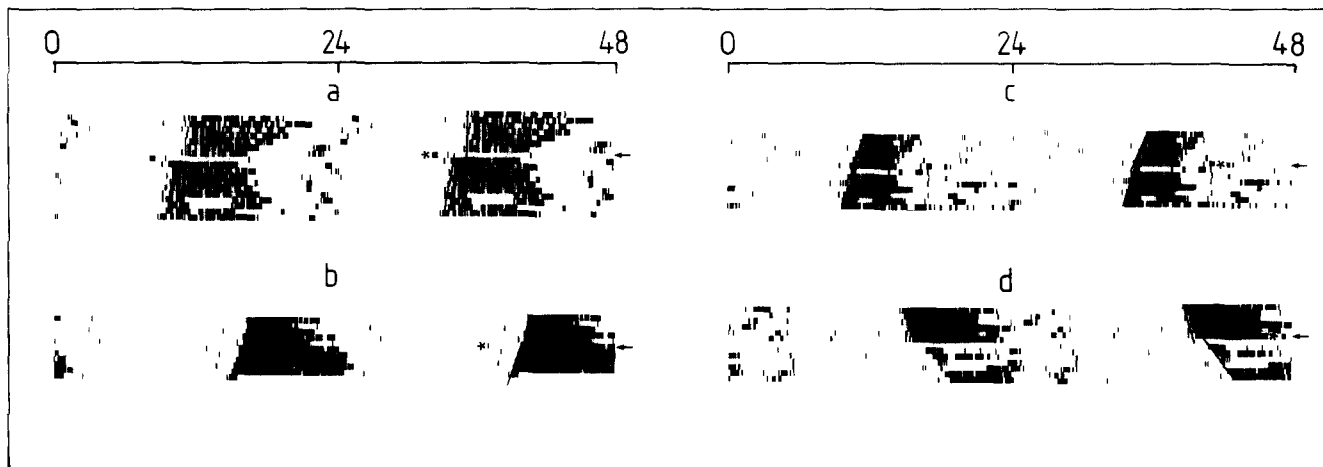


Fig. 1. Double plotted activity records of hamsters in dim light with the cannula placed inside the SCN region. The time of day (in hours) is indicated above the actograms, the successive days are plotted beneath one another. The days of aspartate injections are marked by arrows, the time of injection is indicated by an asterisk. A small mean phase advance was obtained with 1 mM aspartate injections between CT4.5–10.5. One of the larger phase shifts obtained is shown in a. The mean phase advance did not increase when a dose of 10 mM was used (b). Aspartate does not mimic the phase shifting effects of light when injected around CT19 (c; injection with 0.1 mM and d; injection with 10 mM aspartate).

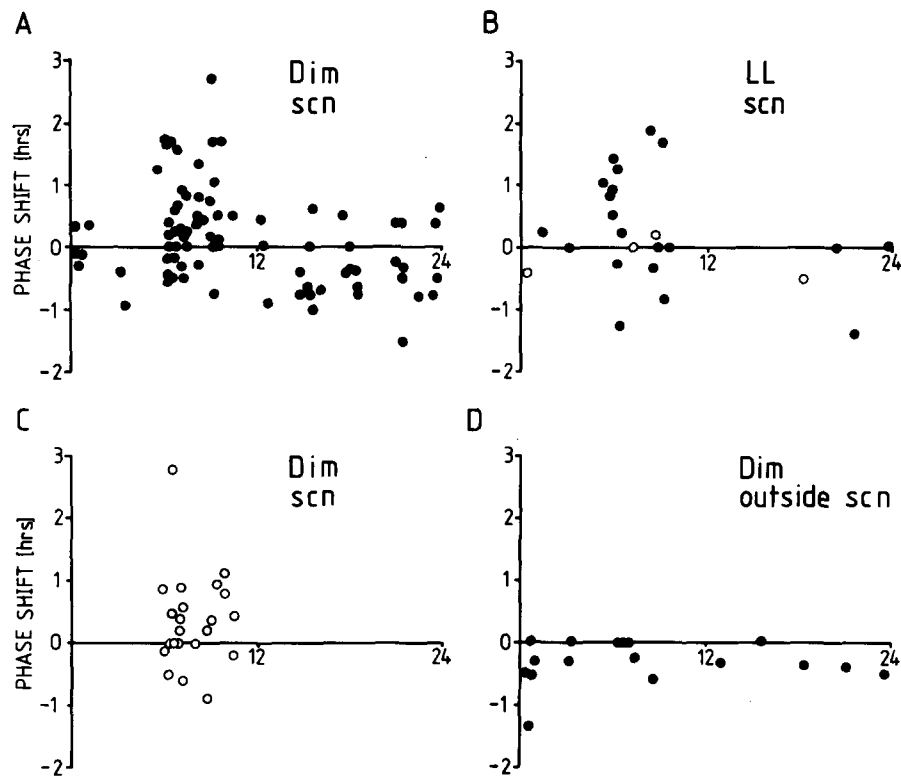


Fig. 2. Phase shifts induced by 1 mM aspartate (●) (A in dim light and B in LL) and artificial CSF (○) (B in LL and C in dim light) injections into the SCN region. In D, the phase shifts produced by 1 mM aspartate injections outside the SCN region in dim light are shown. The circadian time is indicated on the abscissa, with the activity onset defined as CT12. Phase advances and delays are plotted in positive and negative direction respectively.

vances were obtained with aspartate injections in 3 animals with the cannula placed outside the SCN region (Fig. 2D).

In all animals maintained in LL the cannula was situated inside the SCN region. Most aspartate injections in these animals induced phase advances between CT4.5–10.5 as well (Fig. 2B). The mean phase advance was 0.49 h (S.E.M. = 0.24). Four CSF injections were performed in these animals (Fig. 2B). Injections neither with 0.1 nor with 10 mM aspartate to animals in dim light were able to mimic the effects of light (Fig. 1c, d). Injections of both concentrations induced mainly phase delays around CT19; the mean delay with 0.1 mM aspartate injections was 0.53 h ( $n=4$ , S.E.M. = 0.12) and 0.47 h ( $n=6$ , S.E.M. = 0.22) with 10 mM injections. Injections with 0.1 mM aspartate around CT8 induced a mean phase delay of 0.09 h ( $n=2$ , S.E.M. = 0.09) and with 10 mM a mean advance of 0.28 h ( $n=4$ , S.E.M. = 0.09) was obtained (Fig. 1b).

In conclusion, aspartate injections of 1 mM into the SCN region of hamsters kept in dim light tended to induce small phase delays during the hours of the subjective night whilst injections between CT4.5–10.5 in dim light and LL induced a slight phase advance. Since injections with 0.1 and 10 mM aspartate around CT19

induced small phase delays as well, the effects resemble those of dark but not of light pulses [3, 8, 9]. The phase advances induced by 1 mM aspartate between CT4.5–10.5 in dim light are significantly different from zero ( $P \leq 0.001$ , Student's *t*-test) but do not differ from those induced by CSF ( $P = 0.47$ ). The phase shifts induced by CSF are of the same magnitude as those obtained with control injections in other studies [1, 16]. Therefore, the phase shifts obtained with aspartate cannot be ascribed to the excitatory properties of aspartate. The lack of effect of aspartate on the activity rhythm cannot be due to the concentrations used, since these included a large range which was well above the aspartate concentration in the interstitial fluid (for rat: 0.4–8.8  $\mu\text{M}$ ) [2].

Non-photic stimuli such as induced wheel running [21], cage cleaning or social interaction [18] can phase advance the circadian pacemaker when applied during the subjective day. Recent studies have indicated that dark pulses may phase shift the pacemaker as a consequence of evoked activity, rather than by a photic mechanism [25]. Injections applied between CT4.5–10.5 are often accompanied by locomotor activity, while normally no activity is observed during these hours. The question arises whether the induced activity may have caused the phase advances between CT4.5–10.5. For all

injections that were performed during these circadian hours we assessed the amount of running wheel activity between the moment of injection and CT10.5. Since we found no significant correlation between the amount of induced activity and the magnitude of the phase shift we have no indications that the phase advances are caused by evoked locomotor activity. Arousing stimuli which are associated with the injection procedure, such as ether anaesthesia or handling the animal, may have caused the small phase advances between CT4.5–10.5 in our study. However, this is an unlikely explanation for our results because animals which were exposed to the same injection procedure but received the aspartate injection outside the SCN did not show phase advances (Fig. 2D).

Neuropeptide Y injections produce significant phase shifts which resemble the effects of dark but not of light pulses [1]. The same effect is obtained by 1 mM glutamate injections [16]. From this study it is obvious that also aspartate fails to mimic the phase shifting effects of light. It is surprising that none of the putative transmitters of the photic afferents, when applied to the SCN, have mimicked the effects of light since the circadian system is extremely sensitive to light. Moreover, antagonists of excitatory amino acids block the phase shifting effects of light [6, 7, 14]. The present results suggest that the circuitry of the photic input is such that steady exposure to putative photic afferent neurotransmitters is unable to activate the phase shifting mechanism which is normally triggered by light.

We thank Hans Duindam, Simon Ploeger and Ton De Vries for their technical assistance and Mervyn Wise for critically reading the manuscript. This work was supported by Psychon (Grant 560-258-046) which is subsidized by the Netherlands Organisation for Scientific Research (NWO).

- 1 Albers, H.E. and Ferris, C.F., Neuropeptide Y: role in light-dark cycle entrainment of hamster circadian rhythms, *Neurosci. Lett.*, 50 (1984) 163–168.
- 2 Benveniste, H., Brain microdialysis, *J. Neurochem.*, 52 (1989) 1667–1679.
- 3 Boulos, Z. and Rusak, B., Circadian phase response curves for dark pulses in the hamster, *J. Comp. Physiol.*, 146 (1982) 411–417.
- 4 Cahill, G.M. and Menaker, M., Kynurenic acid blocks suprachiasmatic nucleus responses to optic nerve stimulation, *Brain Res.*, 410 (1987) 125–129.
- 5 Cahill, G.M. and Menaker, M., Effects of excitatory amino acid receptor antagonists and agonists on suprachiasmatic nucleus responses to retinohypothalamic tract volleys, *Brain Res.*, 479 (1989) 76–82.
- 6 Colwell, C.S., Ralph, M.R. and Menaker, M., Do NMDA receptors mediate the effects of light on circadian behavior, *Brain Res.*, 523 (1990) 117–120.
- 7 Colwell, C.S., Foster, R. and Menaker, M., NMDA receptor antagonists block the effects of light on circadian behavior in the

- mouse, *Soc. Neurosci. Abstr.*, 16 (1990) 270.3.
- 8 Daan, S. and Pittendrigh, C.S., A functional analysis of circadian pacemaker in nocturnal rodents, II The variability of phase response curves, *J. Comp. Physiol.*, 106 (1976) 253–266.
- 9 Ellis, G.B., McKlveen, R.E. and Turek, F.W., Dark pulses affect the circadian rhythm of activity in hamsters kept in constant light, *Am. J. Physiol.*, 242 (1982) R44–R50.
- 10 Groos, G.A., The neurophysiology of the mammalian suprachiasmatic nucleus and its visual afferents. In J. Aschoff, S. Daan and G. Groos (Eds.), *Vertebrate Circadian Systems*, Springer, Berlin, 1982, pp. 96–105.
- 11 Harrington, M.E., Nance, D.M. and Rusak, B., Neuropeptide Y immunoreactivity in the hamster geniculate-suprachiasmatic tract, *Brain Res. Bull.*, 15 (1985) 465–472.
- 12 Harrington, M.E. and Rusak, B., Lesions of the thalamic intergeniculate leaflet alter hamster circadian rhythms, *J. Biol. Rhythms*, 1 (1986) 309–325.
- 13 Liou, S.Y., Shibata, S., Iwasaki, K. and Ueki, S., Optic nerve stimulation-induced increase of release of [<sup>3</sup>H]glutamate and [<sup>3</sup>H]aspartate but not [<sup>3</sup>H]GABA from the suprachiasmatic nucleus in slices of rat hypothalamus, *Brain Res. Bull.*, 16 (1986) 527–531.
- 14 Lord, A.B., Turek, F.W. and Takahashi, J.S., Application of MK-801 into the suprachiasmatic region attenuates the phase-shifting effects of light on the circadian activity rhythm of the hamster, *Soc. Neurosci. Abstr.*, 16 (1990) 317.16.
- 15 Meijer, J.H. and Rietveld, W.J., Neurophysiology of the suprachiasmatic circadian pacemaker in rodents, *Physiol. Rev.*, 69 (1989) 671–707.
- 16 Meijer, J.H., Van der Zee, E.A. and Dietz, M., Glutamate phase shifts circadian activity rhythms in hamsters, *Neurosci. Lett.*, 86 (1988) 177–183.
- 17 Moore, R.Y., Gustafson, E.L. and Card, J.P., Identical immunoreactivity of afferents to the rat suprachiasmatic nucleus with antisera against pancreatic polypeptide, molluscan cardioexcitatory peptide and neuropeptide Y, *Cell Tissue Res.*, 236 (1984) 41–46.
- 18 Mrosovsky, N., Phase response curves for social entrainment, *J. Comp. Physiol. A*, 162 (1988) 35–46.
- 19 Nishino, H. and Koizumi, K., Responses of neurons in the suprachiasmatic nuclei of the hypothalamus to putative transmitters, *Brain Res.*, 120 (1977) 167–172.
- 20 Pickard, G.E., Ralph, M.R. and Menaker, M., The intergeniculate leaflet partially mediates effects of light on circadian rhythms, *J. Biol. Rhythms*, 2 (1987) 35–56.
- 21 Reeb, S.G. and Mrosovsky, N., Effects of induced wheel running on the circadian activity rhythms of Syrian hamsters: entrainment and phase response curve, *J. Biol. Rhythms*, 4 (1989) 39–48.
- 22 Shibata, S., Liou, S.Y. and Ueki, S., Different effects of amino acids, acetylcholine and monoamines on neuronal activity of suprachiasmatic nucleus in rat pups and adults, *Neurosci. Lett.*, 39 (1982) 187–192.
- 23 Shibata, S., Liou, S.Y. and Ueki, S., Influence of excitatory amino acid receptor antagonists and of baclofen on synaptic transmission in the optic nerve to the suprachiasmatic nucleus in slices of rat hypothalamus, *Neuropharmacology*, 25 (1986) 403–409.
- 24 Shibata, S., Oomura, Y., Kita, H., Liou, S.Y. and Ueki, S., Field potentials in the suprachiasmatic nucleus of rat hypothalamic slice produced by optic nerve stimulation, *Brain Res. Bull.*, 12 (1984) 377–379.
- 25 Van Reeth, O. and Turek F.W., Stimulated activity mediates phase shifts in the hamster circadian clock induced by dark pulses or benzodiazepines, *Nature*, 339 (1989) 49–51.