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The effects of glutamate on membrane potential and discharge rate of suprachiasmatic neurons

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The suprachiasmatic nucleus (SCN) is a major pacemaker for circadian rhythms in mammals. Photic entrainment of the circadian pacemaker is mediated by the retinohypothalamic tract (RHT). Most likely, excitatory amino acids function as neurotransmitters in this pathway. We have now investigated the effect of glutamate on the membrane potential of cultured SCN cells of the rat with the aid of the patch clamp technique. It was found that 1 mM glutamate depolarizes the cells by about +44 mV. In spontaneously active neurons, the glutamate induced depolarization caused either an increase in discharge or a depolarization block. We then investigated the effect of 1 mM glutamate on SCN discharge in the acutely prepared hypothalamic slice of the hamster. In most cells glutamate induced an increase in discharge whilst in a few cells discharge was suppressed. Both series of experiments indicate that glutamate in the used dosage was effective and its effect reversible. The data are discussed with respect to the failure of 1 mM glutamate injections to mimic the effect of light on the circadian activity rhythm of the hamster.

INTRODUCTION

The circadian pacemaker of the suprachiasmatic nucleus (SCN) entrains to the light–dark cycle via the retina[20]. An important pathway for entrainment is the retinohypothalamic tract (RHT, see ref. 13). Several lines of evidence indicate that excitatory amino acids (EAA) are transmitters of the RHT, which mediate the effects of light on the pacemaker of the SCN. Stimulation of the optic chiasm increases the concentration of glutamate and aspartate in the SCN[12], and SCN field responses to optic nerve stimulation can be blocked by antagonists of EAAs[3,4,18]. Some experiments specifically indicated that EAAs play a role in mediating light (and not dark) information: intraperitoneal injections with antagonists of EAAs in both hamsters and mice block light-induced phase shifts[5] and light-induced c-Fos expression in the SCN[1]. The effect of light on pineal melatonin is mediated by the SCN. This effect can be mimicked by injection of the EAA agonist *N*-methyl-D-aspartate into the SCN and local injections of antagonists block this effect[16,19]. Likewise, SCN glutamate injections mimic the effect of retinohypothalamic tract stimulation in activating interscapular brown adipose tissue thermogenesis whereas EAA blockers prevent this effect[2].

In view of these results one would expect that local injection of EAAs into the SCN would also mimic the phase shifting effect of light. Local injection of 1 mM glutamate and, to a lesser extent aspartate, induces phase shifts of the circadian activity rhythm of hamsters. However, the time at which glutamate induces phase advances (3–6 h before activity onset) differs from the time at which light produces phase advances (6–9 h after activity onset[6,14]). The question arises whether this discrepancy merely reflect the inability to mimic the endogenous glutamate release by microinjections or whether it indicates that the light input pathway that is involved in phase shifting the circadian pacemaker is essentially different from our expectations.

Here we report on electrophysiological experiments on the SCN both in the acute slice preparation of the hamster SCN and in cultured SCN cells of the rat. In

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these studies we established the effect of 1 mM glutamate on (a) the discharge of SCN cells and (b) the membrane potential of SCN cells.

MATERIALS AND METHODS

Experiments on cultured neurons

The suprachiasmatic areas of 18- to 19-day-old foetuses were collected and rinsed in saline and then transferred to 1-ml sterile medium (21°C) containing nerve growth factor (see also ref. 22). Cells were mechanically dissociated using the pasteur pipette method[17]. The cell suspension was enriched and purified by centrifuging three times in fresh sterile CDM (1,000 rpm for 5 min). The final solution contained 10^6–10^7 cells/ml. Two drops of cell suspension were placed on a poly-D-lysine-coated circular glass coverslip (24 mm diameter) which was placed in a plastic petri dish. The dishes were put into an incubator with water saturated air containing 5% CO_2 at 37°C for 2 h to allow the cells to attach to the coverslip. Finally, 0.5 ml sterile medium was added to the culture and renewed after 24 h. In the course of the culture period, the medium was renewed every 2 days.

Electrophysiological recordings were performed at room temperature. The glass cover slip was removed from the culture dish and placed in a special holder[10] for use on an inverted microscope. The bath was continuously perfused with standard control solution containing (in mM): NaCl 150, KCl 5, $CaCl_2$ 1, $MgCl_2$ 1, *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid (HEPES) 10, at pH = 7.2). Dishes were used for at most 2 h. Patch pipettes were pulled from borosillicate glass with a resistance after fire polishing of about 2–5 MΩ. Pipettes were filled with an intracellular-like fluid containing (in mM): NaCl 5.5, KCl 140.5, $CaCl_2$ 4, $MgCl_2$ 1, HEPES 10, ethyleneglycol-bis-(beta-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) 10, at pH = 7.2). The free calcium concentration was 10^{-7}M (ref. 7).

Experiments were carried out at culture day 10–14. Cell types were easily recognisable by use of an 100×oil immersion objective. Measurements were performed in the cell-attached patch mode (CAP) and in the whole cell configuration (WC)[9] using a List Medical EPC-7 patch-clamp amplifier. Glutamate (1 mM) and aspartate (1 mM) were applied to the bath by switching from standard extracellular perfusion medium to a continuous perfusion with the experimental medium. The perfusion system also allowed the wash out of glutamate and aspartate by switching back to the standard solution. The application and wash out of glutamate was usually carried out twice per cell. Data were stored on video tape or FM tape recorder. These data were analyzed off line using an A/D converter. They were further processed with a software package (pClamp).

Slice experiments

Male golden hamsters were purchased from Harlan CPB, Zeist, The Netherlands at the age of 7 weeks. They were entrained at a light–dark cycle (L:D = 14:10). At the beginning of their subjective day, the hamsters were anaesthetized with ether and decapitized after which the brain was quickly placed in ice cold saline solution. Coronal 400 μm slices were transferred to the interface of an incubation chamber. Artificial cerebrospinal fluid (pH 7.4) was maintained at 34.4°C and contained (mM): NaCl 122, KCl 3, $CaCl_2$ 1.5, $MgSO_4$ 1.3, NaH_2PO_4 1.25, $NaHCO_3$ 25, glucose 10. Humidified O_2 (95%) and CO_2 (5%) was blown over the slices. Control and glutamate containing solutions (pH = 7.4) were perfused at a rate of 1.5 ml/min.

Extracellular recordings were performed with glass microelectrodes filled with 4 M NaCl (2–4 MΩ). The electrodes were lowered with a stepmotor controlled micromanipulator. The signal from the recording electrode was passed through a high-impedance amplifier and displayed on an oscilloscope and a chart recorder. The optic chiasm was stimulated with sharpened tungsten bipolar electrode (7.5–15 V, 75 μs). Data were stored on a magnetic tape and could be digitized via a transient recorder (Biomation 802) for analysis.

RESULTS

The hypothalamic cultures consisted of reaggregates of cells which were connected by some of their axons or dendrites. As has been described previously[21,22] cells were morphologically distinguishable by their shape and processes. Most cells were bipolar, others were multipolar or unipolar[21]. Recordings were only performed in the bipolar cells because there is recent evidence that these are neurons[22]. All cells reported in this study were neurons since either they were spontaneously active or action potentials could be elicited by depolarizing current pulses.

From six culture dishes, a total number of 17 cells were recorded. Whole cell recordings in these cells revealed their mean membrane potential (±S.D.) was −40.0 ± 13.5 mV. Membrane potentials less negative than −30 mV were observed in one of the culture dishes. Some of these cells were hyperpolarized before the effect of glutamate on membrane potential was determined.

Glutamate bath application induced a depolarization of membrane potential in all but one cell (Fig. 1A). The mean depolarization in membrane potential induced by glutamate (±S.E.M.) was 44.3 ± 6.4 mV (n = 13). In six cells that were hyperpolarized it was 47.2 ± 17.5 (two of these were also measured without

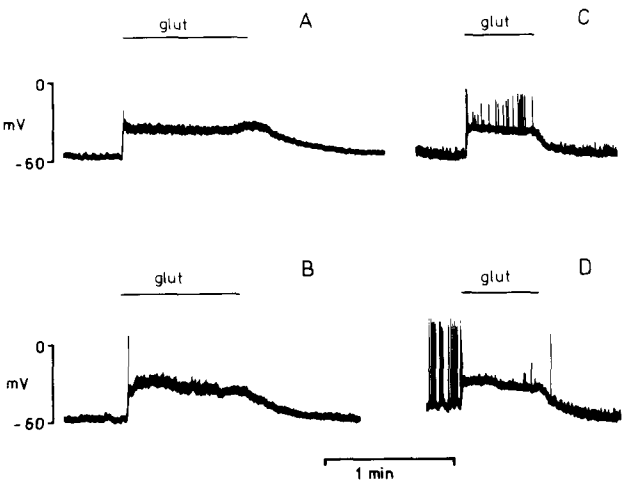

Fig. 1. Whole cell recordings of membrane potential and discharge of two hypothalamic neurons following glutamate bath application. The timing of glutamate application is indicated above the records. In A it is illustrated that glutamate raises the membrane potential from −52 to −30 mV in an otherwise silent cell. An action potential is triggered only at the beginning of the glutamate application. B illustrates the response to glutamate in the same cell in calcium free medium. The membrane potential is raised from −57 to −23 mV. C illustrates an increase in discharge rate induced by glutamate application. The initial membrane potential was −59 mV and depolarized to −38 following glutamate application. D shows the response of the same cell to glutamate some time later. The initial membrane potential of −48 mV was raised to −30 mV which resulted in a depolarization block.

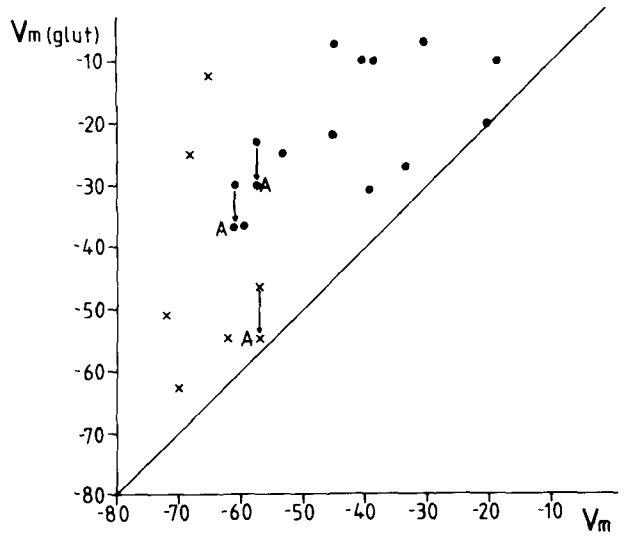

Fig. 2. Summary of the effects of glutamate on the membrane potential of 13 cells (dots) and 6 hyperpolarized cells (crossmarks, two of these were also measured in the non-hyperpolarised condition). On the x-axis, the initial membrane potential is indicated while on the y-axis the membrane potential following glutamate application is shown. In three cells, aspartate was also applied (A). The depolarization induced by aspartate was smaller than the glutamate induced depolarization (arrow).

hyperpolarizing current). No relationship was observed between the initial membrane potential and the magnitude of the depolarization (Fig. 2). In three cases where 1 mM aspartate was applied, qualitatively similar results were obtained but the mean change in membrane potential was smaller (+15 mV).

Responses to 30 s–3 min glutamate applications were sustained and were sometimes preceded by a phasic component. When administered in a low calcium solution, glutamate induced the same depolarization, indicating that the response was not mediated by synaptic input of surrounding cells (Fig. 1B). Additional evidence for membrane depolarization was obtained from a glutamate-induced shift in the reversal potential of spontaneously active channels in the CAP mode.

In spontaneously active neurons, the effect of glutamate bath application on discharge was analyzed. Glutamate induced either an increase in discharge or a total block of discharge. The block of discharge was predictable by the level of depolarization. No discharge was observed from membrane potentials less negative than −38 mV. Even within one cell it could be demonstrated that depending on the level of depolarization induced, an excitation or suppression of discharge was obtained (Fig. 1 C, D). Suppression of discharge was obtained not only in the whole cell configuration but also in the cell attached patch mode.

In 13 hypothalamic slices, a total number of 50 SCN neurons were recorded, of which 22 displayed a steady state discharge rate for at least 20 min. In the latter group the effects of 1 mM glutamate bath application were analyzed between circadian times 3 and 11 (the zone at which glutamate injections are known to produce phase shifts) together with the effects of electrical stimulation of the optic chiasm.

The spontaneous discharge rate of SCN cells ranged from 0.7–6 Hz (mean ± S.D. = 2.7 ± 1.8 Hz). Bath application of glutamate induced a sustained two to four fold increase ($n = 9$), a sustained two- to five-fold decrease ($n = 3$) or a transient decrease ($n = 2$) in discharge (Fig. 3). In the remaining 8 cells, glutamate did not affect the spontaneous discharge rate. Electrical stimulation of the optic chiasm resulted in a change in electrical discharge in eight cases. Five cells were suppressed by stimulation while four were excited. No relation existed with the responsiveness to glutamate: two cells that were excited by optic nerve stimulation were also excited by glutamate while one excited cell

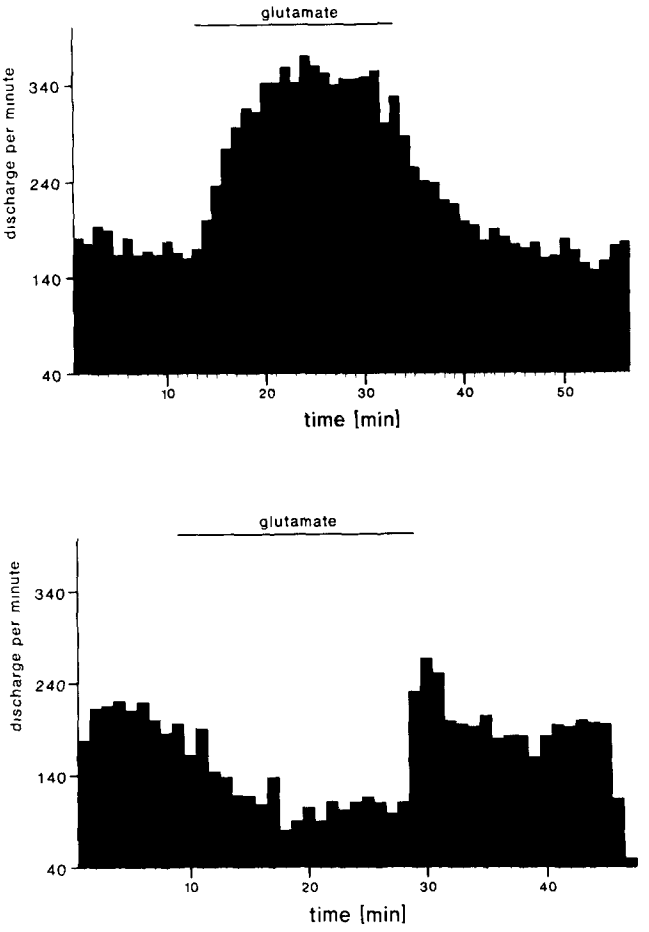

Fig. 3. The effects of glutamate on the single unit activity of two SCN cells that were recorded in the acute slice preparation. The application of glutamate (indicated above the record) resulted in an increase in single cell activity in the upper peristimulus time histogram. In the cell on the bottom, glutamate bath application induced a decrease in single unit activity. Recordings were performed during the subjective day.

was not responsive to glutamate. In one cell the response to glutamate was not tested. Two stimulation-suppressed cells were also suppressed by glutamate, while two others were not responsive to glutamate. One cell that was suppressed by chiasm stimulation was excited by glutamate.

DISCUSSION

The effect of putative neurotransmitters on the membrane potential of SCN cells has not yet been studied in cultured SCN neurones with the patch-clamp technique. Our experiments have shown that this method allows long-term analysis (up to 20 min) of membrane potential of cultured SCN cells which is long enough for investigating the effect of neurotransmitters. Measurements in the whole cell configuration have demonstrated that the main effect of glutamate on membrane potential is a depolarization of about +44 mV. The results are qualitatively consistent with patch clamp experiments on acutely dissociated rat SCN cells[11]. Glutamate was shown to induce an inward current at a concentration 10 times lower than was used in this study. Unfortunately the authors did not describe neurons which were spontaneously active.

In our spontaneously active neurons we could concomitantly follow the action of glutamate on membrane potential and discharge rate. When glutamate depolarized the membrane potential to a level more negative than −38 mV we observed an increase in discharge rate. In those cases where the membrane potential was depolarized above −38 mV, glutamate resulted in a cessation of discharge. Since glutamate also induced decrements in discharge in the cell-attached patch mode, impalement artefacts can be excluded. When glutamate was applied a in calcium-free medium similar depolarizations were obtained, indicating that its effect was direct on the recorded cell and not mediated by synaptic processes. Measurements in the CAP mode indicated that glutamate also had a depolarizing effect in the intact cell, indicating that perfusion artefacts in the whole cell mode are not responsible for the glutamate-induced depolarization.

Cultured cells are a good preparation for studying changes in membrane potential in response to transmitters. However, the number of interactions between cultured neurons is considerably smaller than in slices. Moreover, the number of glia cells is smaller in cultured preparations, which leads to an overestimation of the effect of glutamate in this dosage. Hence, these results cannot legitimately be generalized to a slice or intact preparation. We have extended the experiments by recording the effect of glutamate bath application on single units in SCN slices of the hamster.

In the acute slice experiments it was demonstrated that bath application of glutamate can induce both increases and decreases in discharge rate and that, in other cases, discharge rate was not affected by glutamate. For the latter group the question remains whether these cells are not sensitive to glutamate or whether glutamate did not effectively reach their receptors. The responsiveness to glutamate appeared to be unrelated to the response to electrical stimulation of the optic chiasm.

At least two explanations exist for the suppressions of discharge following application of glutamate to the hypothalamic slice. The most likely one is that intrinsic inhibitory neurons in the SCN may have been stimulated by bath application of glutamate. Their inhibitory effect on the recorded cell may have been stronger than the direct excitatory effect of glutamate. An alternative explanation is that glutamate may have caused a depolarization block. It is unknown whether a depolarization block also occurs upon physiological glutamate release or whether it reflects a pathological response to a high dosage of glutamate.

With respect to the dosage of glutamate, two points should be made. Despite indications that glutamate in the concentration of 1 mM and for an exposure time of 30 min may be toxic[8], we found no indications of toxicity in our experiments. Responses to glutamate, both in cultured cells and in slices, were always reversible, even when the cells were repeatedly exposed to glutamate. Other experiments have indicated that a concentration of 1 mM glutamate may be too low. Injection of 100 mM to 1 M glutamate into the SCN activates interscapular brown adipose tissue thermogenesis, as does retinohypothalamic tract stimulation. Our experiments clearly demonstrate that the dosage used is sufficient to affect SCN discharge, at least in the acute slice preparation of the hamster, and always depolarizes the membrane potential of isolated neurons of the rat.

Not only the effect of RHT stimulation on brown adipose tissue thermogenesis can be mimicked by glutamate injection but also the effect of light on pineal melatonin can be mimicked by injection of EAA agonists into the SCN. Considering that the effect of light on the circadian pacemaker of the SCN cannot be mimicked by SCN glutamate injections in a dosage that was shown to be effective, the results suggest that at some point the pharmacological organisation of the input pathway to the circadian pacemaker differs from the other two pathways. In other words, light input to the SCN pacemaker cannot be mimicked by glutamate

injection whilst light input to the SCN mediating melatonin suppression and brown adipose tissue thermogenesis *can* be replicated by such injections. Since these experiments were all performed in vivo we cannot distinguish whether this difference is attributable to a difference in the light input pathway per se or whether there is an interaction with secondary inputs involved that explains the difference. The possible difference in light input pathways has previously been suggested by Nelson and Takahashi[15] based on a discrepancy in light sensitivity of melatonin suppression vs. phase shifting. Our data support this hypothesis.

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