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INTRACELLULAR ELECTROPHYSIOLOGICAL STUDY OF SUPRACHIASMATIC NUCLEUS NEURONS IN RODENTS: EXCITATORY SYNAPTIC MECHANISMS

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SUMMARY

1. To study the synaptic mechanisms of excitatory transmission in the suprachiasmatic nucleus (SCN), we assessed the effects of excitatory amino acid receptor antagonists on excitatory postsynaptic potentials (EPSPs) recorded from SCN neurons in horizontal and parasagittal hypothalamic slice preparations from rats and guinea-pigs. The EPSPs were evoked by electrical stimulation of either optic nerve or a site near the SCN.

2. When evoked at membrane potentials between −60 and −100 mV, the EPSPs from optic nerve stimulation were conventional in shape; they rose to the peak quickly ($6{\cdot}2 \pm 0{\cdot}5$ ms, mean ± S.E.M.; $n = 45$) and decayed gradually over 50–250 ms. When evoked at membrane potentials between −20 and −55 mV after blockade of outward K^+ currents and fast Na^+ spikes by intracellular injection of Cs^+ and QX-314 ($n = 5$ neurons), a slow depolarizing potential emerged near the fast peak of the EPSP. This slow potential, unlike the fast peak, was not linearly related to membrane potential.

3. An antagonist for kainate- and quisqualate-type excitatory amino acid receptors, 6,7-dinitroquinoxaline-2,3-dione (DNQX 1–10 μM), depressed in a concentration-dependent and reversible manner the EPSPs evoked by optic nerve stimulation at membrane potentials between −60 and −100 mV ($n = 9$). The effects of DNQX were not associated with any significant changes in the baseline input resistance or membrane potential of the postsynaptic neurons. The selective *N*-methyl-D-aspartate (NMDA) receptor antagonist, DL-2-amino-5-phosphonopentanoic acid (AP5, 50–100 μM), did not affect significantly and consistently the EPSPs evoked at these membrane potentials ($n = 7$). On the other hand, AP5 (50 μM) blocked or depressed the slow depolarizing component of the EPSPs evoked at membrane potentials between −20 and −55 mV ($n = 4$). No significant changes in baseline input resistance or membrane potential accompanied the effects of AP5.

4. Stimulation of a site lateral or dorsocaudal to the SCN evoked EPSPs distinct from those evoked by optic nerve stimulation. Again, DNQX (0·3–10 μM) depressed the EPSPs evoked at membrane potentials between −60 and −100 mV ($n = 4$) whereas AP5 (50μM) had no effect ($n = 5$). When evoked at less negative membrane potentials (i.e. −20 to −55 mV) after intracellular injection of Cs^+ and QX-314, the

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EPSPs had a slow depolarizing potential, similar to the EPSPs from optic nerve stimulation. The slow potential, which often triggered a presumed high-threshold Ca^{2+} spike, was blocked or depressed significantly by AP5 (50 μM, $n = 3$).

5. The present results suggest that, at resting or more negative membrane potentials, non-NMDA receptors mediate excitatory synaptic transmission for both retinal and non-retinal inputs to SCN neurons; at less negative potentials, however, NMDA receptors may also play a role in excitatory synaptic transmission.

INTRODUCTION

Increasing evidence suggests that the suprachiasmatic nucleus (SCN) of the hypothalamus is the major pacemaker for circadian rhythms in mammals (Rusak & Zucker, 1979; Takahashi & Zatz, 1982; Turek, 1985; Meijer & Rietveld, 1989). Bilateral lesion of the SCN eliminates endogenous behavioural and hormonal rhythms (Moore & Eichler, 1972; Stephan & Zucker, 1972; Reppert, Perlow, Ungerleider, Mishkin, Tamarkin, Orloff, Hoffman & Klein, 1981), whereas transplantation of neural tissue containing SCN neurons to the third ventricle of the SCN-lesioned arrhythmic host restores disrupted circadian rhythms (Ralph, Foster, Davis & Menaker, 1990).

Most of the circadian rhythms in mammals are entrained to the environmental light–dark cycle via the retina. Two well-defined pathways delivering photic information from the retina to the SCN are the retinohypothalamic tract (Hendrickson, Wagoner & Cowan, 1972; Moore & Lenn, 1972), a direct projection from the retinal ganglion cells to SCN neurons, and the geniculohypothalamic tract, an indirect photic afferent arising from the intergeniculate leaflet of the thalamus (Swanson, Cowan & Jones, 1974; Ribak & Peters, 1975). While the geniculohypothalamic tract does not appear essential for photic entrainment (Albers, Liou, Ferris, Stopa & Zoeller, 1991), the retinohypothalamic tract seems to be critical. This direct projection by itself is sufficient for photic entrainment (Moore, 1983).

The neurotransmitter(s) in the retinohypothalamic tract is unknown. A key experimental approach for identifying the neurotransmitter(s) is to determine which specific antagonists block the postsynaptic potentials (PSPs) of SCN neurons following activation of retinal afferents. Due to the difficulty of obtaining intracellular recordings from small SCN neurons (mean diameter: about 8–10 μm; van den Pol, 1980), only three intracellular electrophysiological studies have been published. Although they describe some electrical properties of SCN neurons (Wheal & Thomson, 1984; Sugimori, Shibata & Oomura, 1986; Thomson & West, 1990), only one of these reports mentioned briefly the excitatory postsynaptic potentials (EPSPs) following optic nerve stimulation, and the synaptic mechanism(s) responsible for the EPSPs was not investigated.

Recently, from the results obtained with extracellular recording and brain slice techniques, Shibata, Liou & Ueka (1986) suggested that excitatory amino acid (EAA) receptors of the *N*-methyl-D-aspartate (NMDA) subtype mediate retinohypothalamic transmission, whereas Cahill & Menaker (1989*b*) argued that non-NMDA receptors are responsible. The potential problems associated with these extracellular electrophysiological studies are that (1) it is not clear whether the evoked field potentials were actually a *direct* reflection of the synaptic events, and

(2) possible non-specific effects of the employed EAA receptor antagonists (e.g. effects on the baseline membrane potential or input resistance of postsynaptic neurons) were not tested.

In the present study, by using intracellular recording techniques, we could avoid these problems. In addition, we could assess the effects of employed antagonists on evoked EPSPs at various membrane potentials. Although the main focus of this study was on the EPSPs from optic nerve stimulation (i.e. retinal input), we also studied the EPSPs from the stimulation of non-retinal afferents, to test the hypothesis that all the fast EPSPs in the SCN are mediated by EAA receptors. These data support the hypothesis that, at resting or more negative membrane potentials, non-NMDA receptors mediate excitatory synaptic transmission for both the retinal and non-retinal inputs to SCN; at less negative membrane potentials, NMDA receptors also play a role. Preliminary accounts of these results have been published (Kim & Dudek, 1989).

METHODS

Animals

Male Sprague–Dawley rats (120–350 g) and guinea-pigs (150–450 g) were kept under a 12 h light–12 h dark cycle (light on at 07.00 h) for at least 1 week (mostly more than 2 weeks).

Preparation and maintenance of hypothalamic slices

In the morning (after 07.00 h) of the day of the experiment, the animals were injected with sodium pentobarbitone (I.P., 100 mg/kg wt) or were left untreated before being decapitated with a guillotine. After decapitation, the skull was opened and the cranial nerves were carefully cut. The brain was removed from the skull, put in ice-cold artificial cerebrospinal fluid (ACSF) (composition in mM: 124 NaCl, 1·4 NaH_2PO_4, 3 KCl, 2·4 $CaCl_2$, 26 $NaHCO_3$, 1·3 $MgSO_4$, 11 glucose) for about 1 min, and then blocked to contain the hypothalamus. Using a vibroslicer (Campden Instruments, USA), a horizontal or parasagittal slice(s) (450–750 μm) containing the SCN and the optic nerve(s) (rat: 2–5 mm, guinea-pig: 1–3 mm) was cut from the block. In one case, a sagittal slice was cut obliquely to contain the contralateral optic nerve. The slice was transferred to an interface-type recording chamber, which was constantly perfused with warmed ACSF (32–35 °C) at 0·7–1·0 ml/min. A humidified atmosphere of 95% O_2 and 5% CO_2 was maintained above the slice, which was equilibrated to the experimental conditions for at least 2 h before the commencement of experiments.

Intracellular recording

Intracellular micropipettes were pulled with a Flaming–Brown puller (Sutter Instruments, USA) and filled with 2–4 M-potassium acetate (pH = 7·4) or 5 mM-HEPES buffer containing 2 M-CsCl and 150 mM-QX-314, a quaternary lidocaine derivative (pH = 7·4). SCN neurons were impaled by advancing the micropipette recording electrode (90–360 MΩ) through the slice in 3 μm steps with a piezoelectric microdrive (Nano-stepper, List Medical) and oscillating the negative capacitance feedback. The signal from the recording electrode was passed through a high-impedance amplifier with a bridge circuit (Axoclamp-2A, Axon Instruments, USA). Voltage and current traces were displayed on an oscilloscope and a chart recorder. In addition, the traces were digitized (Neurocorder, Neurodata Instruments, USA) and stored on videotape for off-line data analysis with the ISC67AVE system (RC electronics, USA). Each of the voltage traces presented in this paper is an average of ten to thirty, unless stated otherwise.

Electrical stimulation

For electrical stimulation of the optic nerve (ipsi- or contralateral), a bipolar electrode made with Teflon-coated, 90% platinum–10% iridium wires (76 μm in diameter) was placed on the cut end of the nerve. The stimulation was with single monophasic pulses (0·5 ms) of intensities $\leqslant$ 70 V or $\leqslant$ 0·8 mA. To ensure the specificity of the stimulation, the regular bipolar electrode was

occasionally replaced by a suction electrode, which normally required much lower intensity stimulation (as low as 3–5 V), but tended to cause some physical damage to the nerve in long-term experiments. Despite the difference in the stimulus intensity, the onset latency and general shape of the PSPs evoked were similar, suggesting that the stimulation was specific in both cases.

In some experiments a second, bipolar electrode was placed in a site lateral or dorsocaudal to the SCN. By stimulating the site ($\leqslant$ 1·0 mA) simultaneously with the optic nerve, the specificity of the optic nerve stimulation was further assessed. This test was based on the assumption that if a postsynaptic response evoked by optic nerve stimulation is due to current spread to the site near the SCN, the postsynaptic response evoked by stimulation of this nearby site should block the response to optic nerve stimulation. In no case (none of eight neurons from seven slice preparations) was such a blockade observed, further suggesting the specificity of optic nerve stimulation. Finally, any uncertain response, such as immediate depolarization without reasonable onset latency, was excluded from the data analysis.

Pharmacological agents

The antagonists employed in the present study were applied to the brain slices by bath perfusion (> 15 min). Bicuculline methiodide (BIC, γ-aminobutyric $acid_A$ ($GABA_A$) receptor antagonist) and DL-2-amino-5-phosphonopentanoic acid (AP5, NMDA receptor antagonist) were dissolved in the ACSF to the final concentrations, while 6,7-dinitroquinoxaline-2,3-dione (DNQX, non-NMDA receptor antagonist) was pre-dissolved in dimethyl sulphoxide (DMSO) before the final dilution with the ACSF. The final concentration of DMSO was 0·01–0·1 %, which had no apparent effect on the passive membrane properties and synaptic transmission. BIC and AP5 were obtained from Sigma (USA) and DNQX was from Tocris Neuramin (UK).

RESULTS

The data presented in this paper were based on intracellular recordings (10 min to 6 h) from over 100 SCN neurons. Approximately three-quarters of the neurons were from rat slices and the remaining from guinea-pig slices. Since there was no obvious difference between rat and guinea-pig neurons in terms of the postsynaptic response to optic nerve stimulation (except the difference in the onset latency, which was presumably due to the difference in the length of the attached optic nerve; see Methods), the data from the two species were combined. Most of the SCN neurons were spontaneously active (up to 34 Hz). Therefore, isolation of synaptic potentials normally required removal of action potentials either by hyperpolarizing current injection or by direct blockade of the channels responsible for fast Na^+ currents (see below). For the neurons that were recorded with potassium acetate-filled electrodes and which exhibited a postsynaptic response to optic nerve stimulation, the input resistance and the action potential amplitude were 263 ± 21 MΩ (mean ± S.E.M., $n = 43$) and 58 ± 1 mV ($n = 45$), respectively. The input resistance was calculated from the slope of the linear portion of the current–voltage plot, and the action potential amplitude was measured from spike threshold.

Postsynaptic response of SCN neurons to optic nerve stimulation

Optic nerve stimulation evoked fast EPSPs (Fig. 1*A*) in thirty-one neurons impaled in BIC-free medium. The stimulation never evoked a pure, fast or slow inhibitory postsynaptic potential (IPSP) in these neurons or other neurons recorded in the absence of BIC. A fast IPSP followed an EPSP in only one neuron (Fig. 1*B*). BIC (50 μM) completely blocked this IPSP, whereas it had no significant effect on the EPSP (Fig. 1*B*). The EPSPs evoked in other neurons were also not affected by BIC

($n = 9$, Fig. 1*A*). In the presence of BIC and at membrane potentials more negative than -75 mV (i.e. below the reversal potential for $GABA_A$-receptor-mediated IPSPs), the amplitude and duration (at half-amplitude) of the EPSP were 98 ± 5 % and 103 ± 10 % of the values obtained in the BIC-free condition, respectively ($n = 4$).

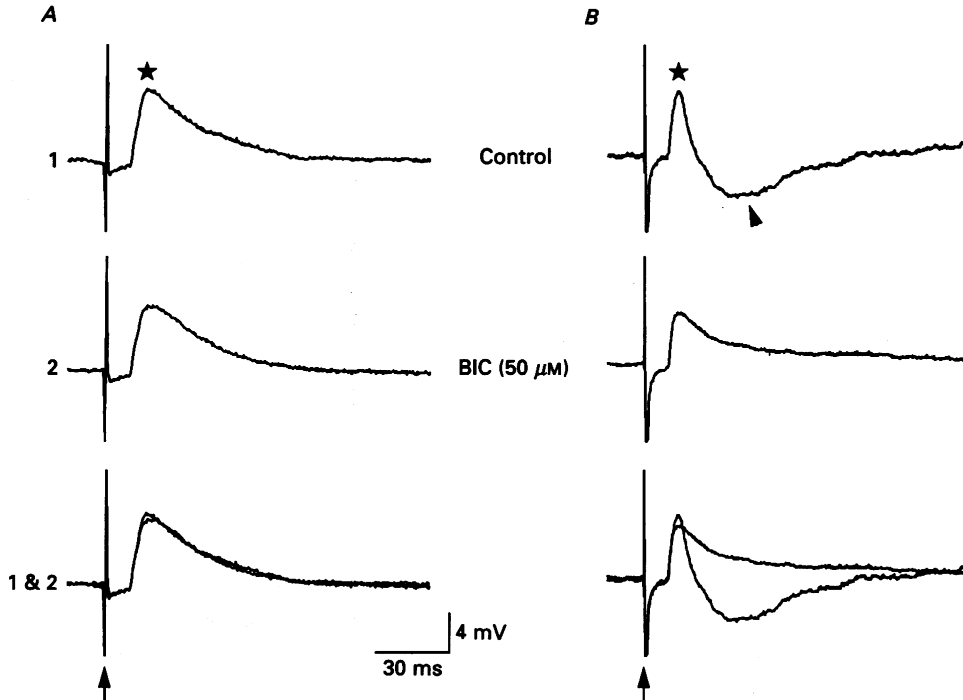

Fig. 1. PSPs from optic nerve stimulation (arrow), and the effects of BIC. Traces in *A* and *B* are from two different neurons recorded with potassium acetate-filled electrodes. Unless stated otherwise, the traces presented in all of the following figures were obtained with potassium acetate-filled electrodes. In *A* the evoked EPSP (★) was not associated with an IPSP, but the EPSP (★) in *B* was followed by an (IPSP) (arrow-head). In *A* and *B*, the PSPs before (1) and during (2) application of BIC are shown in the top and middle panels. Superimposed traces (1 & 2) are also provided in the bottom panel for direct comparison. In *B*, the IPSP following the EPSP was selectively blocked by BIC; the slight decrease in EPSP amplitude during BIC application was associated with and presumably due to a slight decrease in baseline input resistance (data not shown). Cells were current clamped to -60 mV (*A*) and -55 mV (*B*).

At membrane potentials less negative than -75 mV, the amplitude and duration were 94 ± 3 and 108 ± 9 % of the values obtained in BIC-free medium, respectively ($n = 5$). Therefore, these EPSPs were not associated with a BIC-sensitive IPSP.

To insure that the EPSPs under study were not contaminated by a $GABA_A$-receptor-mediated synaptic component, we included BIC (50 μM) in the perfusing medium. Once a slice was bathed in BIC, no attempt was made to wash the BIC out of the bath, because a complete wash-out normally took > 1 h. As a result, several neurons were impaled in the continued presence of BIC. The PSP evoked in these neurons was always a pure EPSP ($n = 20$).

When evoked at membrane potentials between −60 and −100 mV, the EPSPs from optic nerve stimulation were of a conventional shape. In general, the EPSPs rose to their peaks quickly ($6{\cdot}2 \pm 0{\cdot}5$ ms, $n = 45$) (Fig. 2*A*), and decayed gradually over 50–250 ms. In many cases, the decay could be fitted reasonably well with a

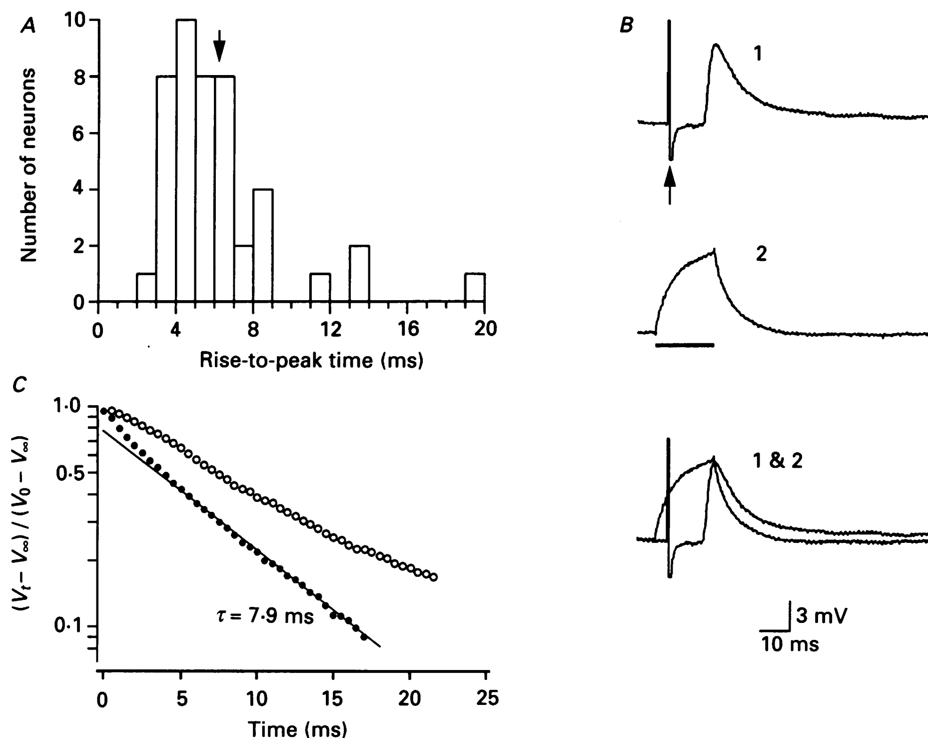

Fig. 2. Time course of the EPSPs evoked by optic nerve stimulation. *A*, frequency histogram for the rise-to-peak time of the EPSPs from forty-five neurons. Arrow indicates the mean. *B*, top and middle panels: EPSP evoked by optic nerve stimulation (arrow) and electrotonic potential evoked by a weak depolarizing current pulse (horizontal line), respectively. These potentials were evoked at the same baseline membrane potential (−72 mV). Bottom panel: superimposition of the top (1) and middle (2) traces for direct comparison. *C*, for the decay of the EPSP and electrotonic potential in *B*, the values of $(V_t - V_\infty)/(V_0 - V_\infty)$, taken at 0·5 ms intervals and integrated over ±0·1 ms, were plotted against time, where V_t is the voltage at time t, V_0 is the voltage at the beginning of the decay and V_∞ is the voltage at the baseline. A straight line was fitted by eye to the latter, linear portion of the plot for the electrotonic potential decay (●), and the apparent membrane time constant (τ) was calculated with the equation $\tau = -0{\cdot}4343/\text{slope}$ of the line (Rall, 1969). The plot for the decay of the EPSP (○) illustrates that the decay had a time constant greater than the apparent membrane time constant.

single exponential. The estimated time constant for the decay of the EPSPs was $20{\cdot}2 \pm 1{\cdot}8$ ms ($n = 14$), and was always larger than the membrane time constant ($12{\cdot}0 \pm 1{\cdot}2$ ms), which was estimated from the latter portion of the charging phase of the averaged electrotonic potential elicited by a hyperpolarizing current pulse (delivered in the linear range of the current–voltage plot). Figure 2*B* and *C* shows that the decay of an evoked EPSP was slower than that of a depolarizing electrotonic

potential. Since these potentials were evoked at the same baseline membrane potential and were similar in amplitude, it is likely that the slower decay of the EPSP is due primarily to a mechanism other than a pure voltage-dependent conductance.

When evoked at membrane potentials between −20 and −55 mV after blockade of outward K^+ currents and fast Na^+ spikes by intracellular injection of Cs^+ and

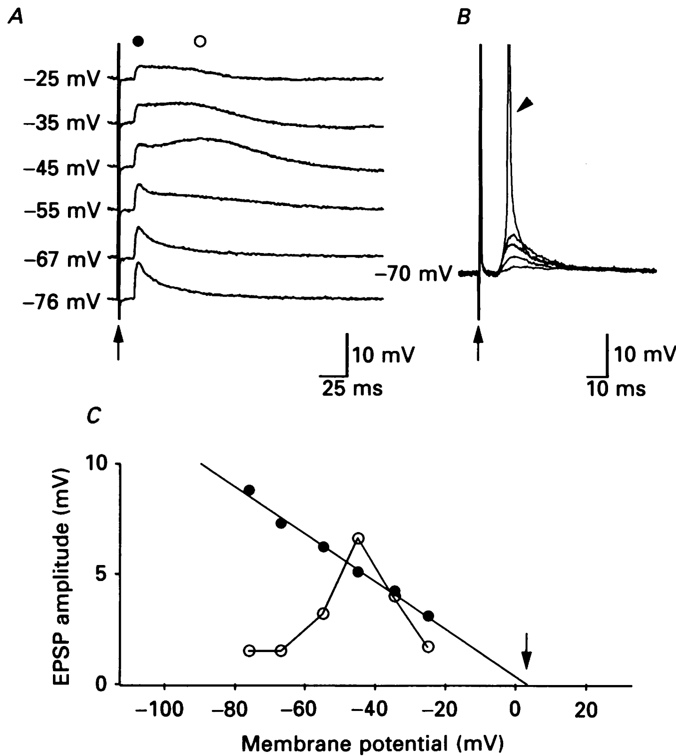

Fig. 3. Amplitude of the EPSPs from optic nerve stimulation (arrows in *A* and *B*) as a function of membrane potential and stimulus intensity. *A*, EPSPs evoked at different membrane potentials. Cs^+ and QX-314 were injected intracellularly to block outward K^+ currents and fast Na^+ spikes. Note the slow depolarizing potentials on the falling phase of the EPSPs evoked at membrane potentials between −25 and −55 mV. *B*, EPSPs evoked at a fixed baseline membrane potential (−70 mV) with different stimulus intensities (0·35–0·5 mA). At the highest intensity, the stimulus evoked an action potential (arrow-head; truncated). Note the same onset latency of the EPSPs. Each superimposed trace is an average of two to eight. Recorded with a pipette filled with potassium acetate (Cs^+ and QX-314 were absent). *C*, plot of the amplitude of the EPSPs in *A* against membrane potential. The amplitude was measured at 18·5 ms (●) and 76·5 ms (○) from the time of stimulation. These time points corresponded to the fast peak of the EPSP and the peak of the slow depolarizing potential, respectively. A linear regression line was fitted through the data points for the fast peaks to estimate the reversal potential (+3 mV; arrow).

QX-314, the EPSPs from optic nerve stimulation ($n = 5$) were quite different in shape from those evoked at more negative membrane potentials (Fig. 3*A*). Near the fast peak of the EPSP, a slow depolarizing potential was present; this component

was apparently absent in the responses evoked at membrane potentials more negative than −60 mV. The amplitude of the slow potential was non-linearly related to membrane potential, and was maximum at membrane potentials of around −45 mV (Fig. 3*A* and *C*).

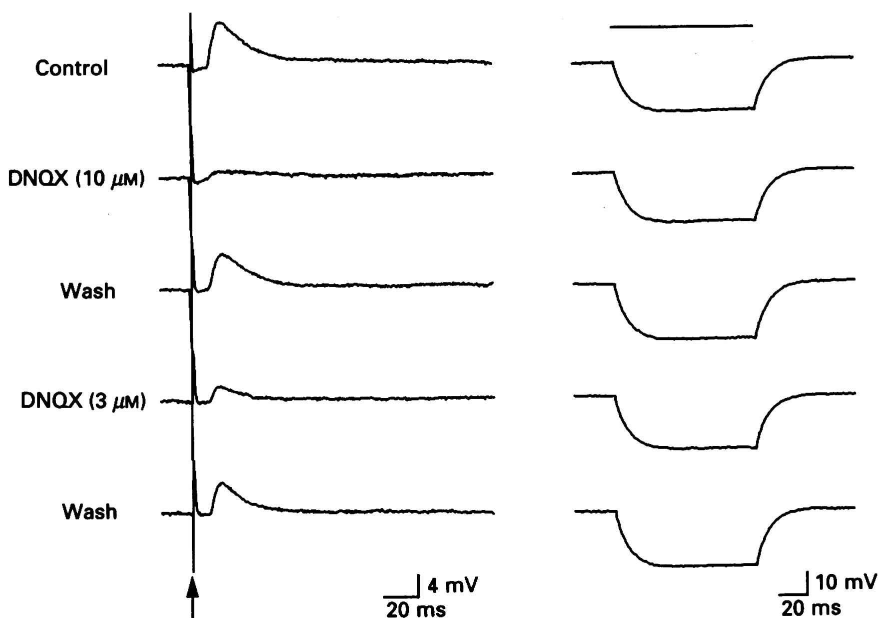

Fig. 4. Depression by DNQX of the EPSPs from optic nerve stimulation. Left panel, EPSPs from optic nerve stimulation (arrow) under each experimental condition. Note the concentration-dependent and reversible depression of EPSPs by DNQX. Also, note that DNQX, even at 10 μM concentration, did not completely block the early part of the EPSP. This residual response could be due to the competition between DNQX and endogenous ligand(s) for non-NMDA receptors. Right panel, electrotonic potentials induced by a hyperpolarizing current pulse (−50 pA, 100 ms; horizontal line) under each experimental condition. Note the lack of DNQX effect on the baseline input resistance. Current clamped to −65 mV.

The EPSP amplitude was a function of stimulus intensity (Fig. 3*B*). In addition, the amplitude of the fast peak of the EPSP was linearly related to membrane potential (Fig. 3*A* and *C*). The reversal potential of the fast peak was -1 ± 6 mV ($n = 7$ neurons) (Fig. 3*C*).

The EPSPs of SCN neurons did not follow high-frequency optic nerve stimuli. In about 20–30% of the cases, they failed to follow stimuli presented even at < 1 Hz. The mean onset latency of evoked EPSPs was variable across neurons (range: 6·5–12·1 ms, $n = 14$ neurons from three guinea-pig and eight rat preparations). Nevertheless, the onset latency of evoked EPSPs in a given neuron was virtually constant: the standard deviation of the onset latency of the EPSPs ($n = 10$–16) was 0·25 ms on average (range of the standard deviation: 0·14–0·53 ms). Across different neurons in a given slice preparation, the EPSPs were similar in onset latency; the difference in the mean onset latency between neurons was < 1 ms. Based on length

of the stimulated optic nerve and the mean onset latency of the evoked EPSPs, the conduction velocity of the optic nerve fibres innervating the SCN was estimated to be 0·39 ± 0·02 m/s (range: 0·31–0·56 m/s, n = 12 neurons from ten rat preparations).

Effects of DNQX and AP5 on the EPSPs from optic nerve stimulation

The effects of DNQX, a non-NMDA receptor antagonist, on the EPSPs evoked at membrane potentials between −60 and −100 mV were examined in nine neurons. In

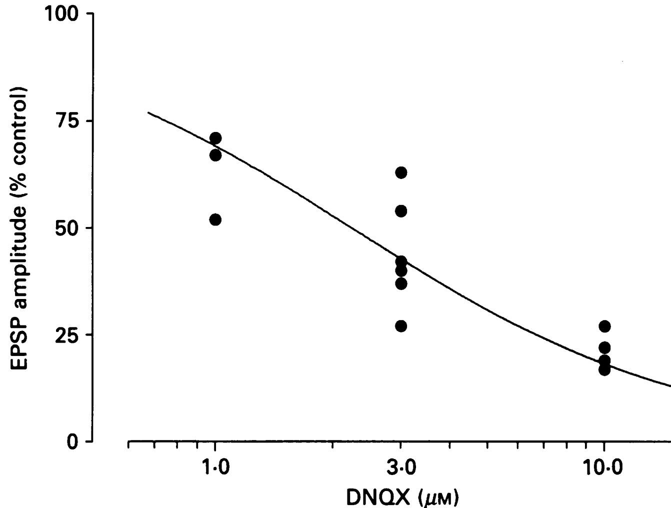

Fig. 5. Concentration–response curve for the DNQX effects on the EPSPs from optic nerve stimulation. This semilogarithmic plot is based on thirteen tests in nine neurons. Four of the nine neurons were tested with two different concentrations, while the remaining were with one. The concentration at which DNQX would produce half-maximal inhibition (IC_{50}) of the EPSP was calculated from the non-linear regression line fitted to the data points, assuming the following relationship: % EPSP = 100 IC_{50}/(IC_{50} + [DNQX]). In this figure the IC_{50} is 2·2 μM.

all of the nine neurons, application of DNQX (1–10 μM) resulted in a significant depression of the evoked EPSPs (Fig. 4). The maximum effect of DNQX was observed 15–60 min after the onset of application. The effects of DNQX were not associated with any significant changes in the baseline input resistance (105 ± 4 % of the control, n = 11 tests in eight neurons) or membrane potential (−3·6 ± 1·7 mV change from the control level, n = 12 tests in eight neurons) of the postsynaptic neurons, and were partially or almost completely reversible following 30–120 min wash (n = 8 tests in six neurons) (Fig. 4). The effects of DNQX were concentration dependent, as illustrated in Figs 4 and 5. The concentration at which DNQX would depress the EPSPs by 50 % (IC_{50}) was estimated to be about 2 μM (Fig. 5).

In contrast to the strong effects of DNQX, AP5 had no consistent effects on the EPSPs evoked at membrane potentials between −60 and −100 mV (Fig. 6). The EPSP amplitude in 50–100 μM-AP5 was 89 ± 9 % (n = 7) of control. On the other

hand, AP5 clearly affected the EPSPs that were evoked at membrane potentials between −20 and −55 mV. In each of the four neurons tested, AP5 (50 μM) blocked or depressed significantly the slow depolarizing potential that emerged at the falling phase of the EPSP, with little or no effect on the fast peak (Fig. 7). The AP5 effects

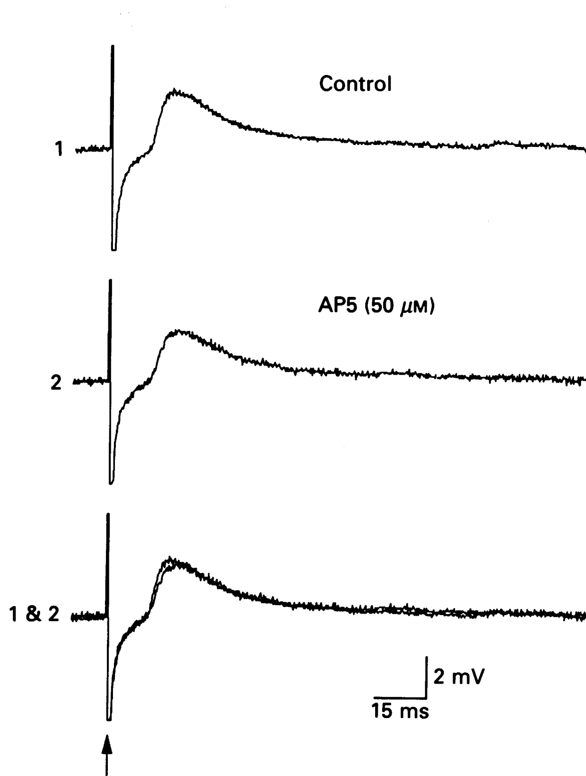

Fig. 6. Lack of effect of AP5 on the EPSPs from optic nerve stimulation (arrow). Top and middle panels, EPSPs before and during application of AP5. Bottom panel, superimposition of the traces in the top and middle panels. Current clamped to −78 mV.

were close to maximum at 10–15 min after the onset of application, and partially reversible after about 30 min wash ($n = 1$). The rapid effects of AP5 (as compared to the DNQX effects) might be due to the hydrophilic property of AP5. The effects of AP5 were not associated with any significant changes in the baseline input resistance (101 ± 13% of the control, $n = 3$) or membrane potential ($-1{\cdot}8 \pm 2{\cdot}3$ mV change from the control level, $n = 4$) of the postsynaptic neurons.

Effects of DNQX and AP5 on the EPSPs from stimulation of a neighbouring site of the SCN

Stimulation of a site lateral or dorsocaudal to the SCN in BIC-treated slices often evoked fast EPSPs distinct from those from optic nerve stimulation. Here again, DNQX (0·3–10 μM) depressed by 15–90% the EPSPs evoked at membrane potentials between −60 and −100 mV ($n = 4$; Fig. 8). The concentration-dependent DNQX

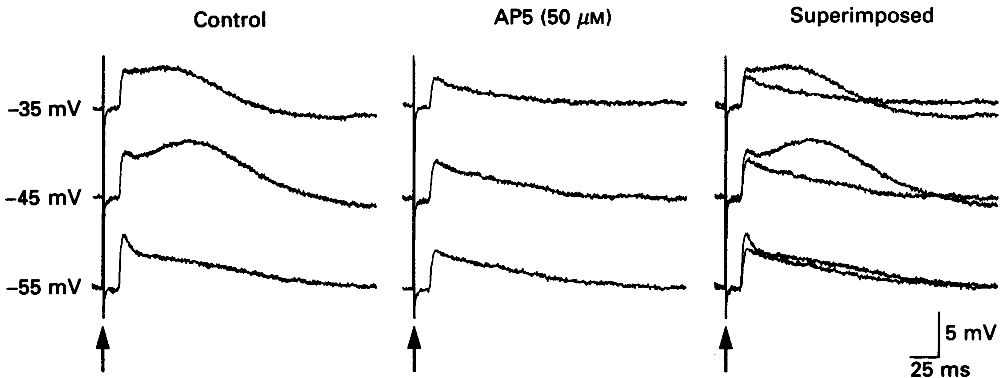

Fig. 7. Blockade by AP5 of the slow depolarizing potentials as a component of the EPSPs evoked by optic nerve stimulation (arrow). Left and middle panels, EPSPs evoked at −35, −45 and −55 mV membrane potentials before and during application of AP5. Right panel, the traces in the left and middle panels are superimposed. Recorded with CsCl- and QX-314-filled electrode.

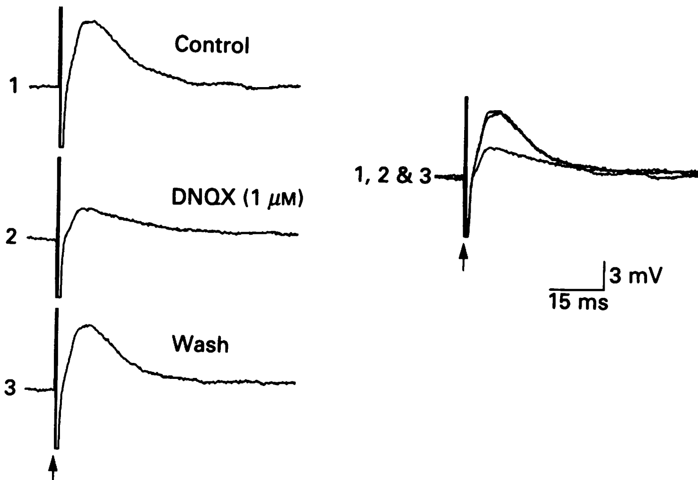

Fig. 8. Depression by DNQX of the EPSP from stimulation of a site lateral to the SCN. Left panel, EPSPs following stimulation (arrow) of the neighbouring site under each experimental condition. Right panel, the traces in the left panel are superimposed. Current clamped to −82 mV.

effect was reversible ($n = 2$), and was not associated with any significant changes in the baseline input resistance (99 ± 1 % of the control, $n = 3$) or membrane potential ($-2{\cdot}0 \pm 1{\cdot}2$ mV change from the control level, $n = 3$) of the postsynaptic neurons. In contrast to the consistent DNQX effects, AP5 had no significant effect on the EPSPs evoked at these membrane potentials (Fig. 9); in 50–100 μM-AP5 the EPSP amplitude was 103 ± 10 % ($n = 5$) of the control.

The EPSPs evoked at membrane potentials between −20 and −55 mV after

blockade of outward K^+ currents and fast Na^+ spikes with Cs^+ and QX-314 contained a slow depolarizing potential, similar to the EPSPs from optic nerve stimulation. A presumed high-threshold Ca^{2+} spike (Llinás & Yarom, 1981; Connors & Prince, 1982) often arose from the slow potential (Fig. 9). As expected, AP5 (50 μM) blocked

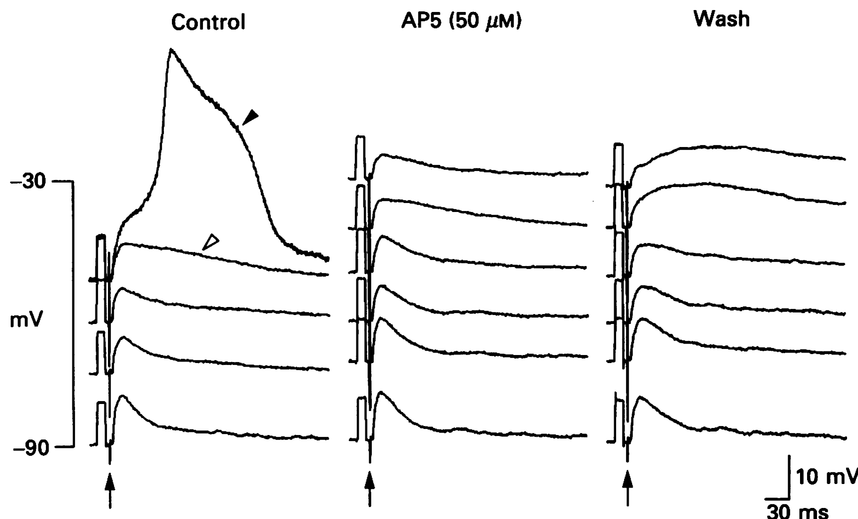

Fig. 9. Effects of AP5 on the EPSPs evoked by stimulation (arrow) of a site dorsocaudal to the SCN. For each experimental condition, several EPSPs were evoked at membrane potentials between −30 and −90 mV. A calibration pulse (10 mV, 10 ms) preceded the dorsocaudal stimulation by 5 ms. The top trace in the left panel exhibiting a presumed high-threshold Ca^{2+} spike (arrow-head) is an individual trace (i.e. not averaged), and each of the remaining traces is an average of six to ten. During the control period (left panel), the EPSPs evoked at a membrane potential of about −50 mV contained a slow depolarizing potential (open arrow-head) at the falling phase. This slow potential often triggered a presumed high-threshold Ca^{2+} spike. During application of AP5 (middle panel), the slow potential was almost completely blocked. After wash-out AP5 (right panel), the slow potential reappeared without triggering the presumed Ca^{2+} spikes. Recovery from the AP5 blockade was more apparent for the EPSPs evoked at less negative membrane potentials. Recorded with CsCl- and QX-314-filled electrode.

or attenuated the slow potential ($n = 3$; Fig. 9). Again, the effect was partially reversible ($n = 2$), and was not associated with any significant changes in baseline input resistance (103 ± 13% of the control, $n = 3$) or baseline membrane potential ($-1{\cdot}0 \pm 2{\cdot}1$ mV change from the control level, $n = 3$).

Depression of spontaneously occurring EPSPs by DNQX

Because SCN neurons in these slice preparations rarely exhibited spontaneous EPSPs of an amplitude and frequency adequate for testing of the antagonist, the effects of DNQX on spontaneous EPSPs were examined in only two neurons, recorded with potassium acetate-filled electrodes. At concentrations of 1–10 μM, DNQX depressed the amplitude of the spontaneous EPSPs by over 50% with no significant effect on the passive membrane properties (Fig. 10). In one neuron, a

concentration dependence of the DNQX effect was observed (data not shown). We did not attempt to assess the possible effects of DNQX on the frequency of the EPSPs.

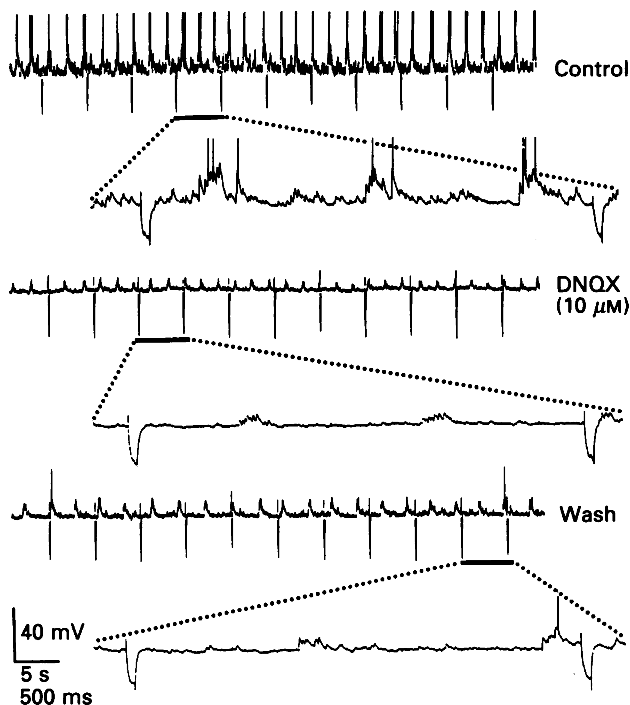

Fig. 10. Depression by DNQX of spontaneously occurring EPSPs. In each panel the underlined portion of the upper trace is shown at a 10 times faster speed (the lower trace). The downward deflections of the traces are electrotonic potentials induced by hyperpolarizing current pulses (−100 pA, 100 ms). During control period large compound EPSPs gave rise to action potentials (truncated). Between the large compound EPSPs were interposed smaller EPSPs. DNQX greatly depressed the EPSPs. The effect of DNQX was only partially reversible after 2 h wash. DNQX did not affect the baseline input resistance. Over the course of the experiment the interval of the large compound EPSPs increased gradually. All of the traces in this figure are individual (not averaged). Current clamped to a membrane potential of about 25 mV below threshold (accurate membrane potential not available).

DISCUSSION

Postsynaptic response of SCN neurons to optic nerve stimulation

Whether inputs from retinal ganglion cells to SCN neurons are excitatory or inhibitory has been an unsettled issue. In anaesthetized rats and hamsters the firing rate of a subpopulation of SCN neurons was shown either to increase or to decrease in response to either retinal illumination or direct electrical stimulation of optic nerve (Nishino, Koizumi & Brooks, 1976; Groos & Mason, 1978; Sawaki, 1979; Groos, Mason & Meijer, 1983; Groos & Meijer, 1985; Meijer, Groos & Rusak, 1986).

Although the prevalent type of response was excitation (i.e. an increase in firing rate in about 75%), inhibition was also present in some units, even after bilateral lesion of the lateral geniculate nucleus (Groos & Mason, 1978). On the other hand, *in vitro* studies with rat and mouse brain slice preparations (Shibata, Oomura, Hattori & Kita, 1984; Cahill & Menaker, 1989*a*) have suggested that an initial increase in firing rate is the predominant type of response immediately after optic nerve stimulation. In these *in vitro* studies a pure decrease in firing rate after optic nerve stimulation was rarely observed, although some neurons following the initial excitation showed an inhibitory or oscillatory firing pattern. Our results agree relatively well with those from other *in vitro* studies, because all the PSPs of SCN neurons to optic nerve stimulation were EPSPs, as indicated by the following: (1) The estimated reversal potential of the PSPs was near 0 mV, similar to the reported values for EPSPs of other central nervous system neurons (Brown & Johnston, 1983; Crunelli, Kelly, Leresche & Pirchio, 1987; Gallagher & Hasuo, 1989) and (2) EAA receptor antagonists blocked the PSPs in a concentration-dependent and reversible manner (see below). The discrepancy between the present results and those from the previous *in vivo* studies may be due to the difference in the type of stimulation (i.e. retinal illumination *vs.* optic nerve stimulation), stimulation parameters (e.g. repetitive *vs.* discrete single shocks to the optic nerve) and/or anaesthesia. Alternatively, it may be due to the fact that in the present study many of the non-retinal afferents to the SCN were severed in the course of hypothalamic slice preparation and, as a result, complications associated with indirect inputs were reduced or eliminated.

The results that optic nerve stimulation never evoked pure IPSPs in any of the neurons recorded in BIC-free medium, and that BIC had no effect on the EPSPs from optic nerve stimulation, strongly suggest GABA is not a neurotransmitter directly responsible for retinohypothalamic transmission. In addition, the absence of pure evoked IPSPs also suggests that the local GABAergic neurons in SCN are not relay neurons between retinal ganglion cells and other SCN neurons. Nevertheless, our data do not exclude the possibility that GABAergic neurons participate in photic information processing. The observation that one of the thirty-one neurons showing an EPSP to optic nerve stimulation also showed a subsequent BIC-sensitive IPSP rather argues for such a possibility.

The EPSPs evoked by optic nerve stimulation appeared to be monosynaptic, because the evoked EPSPs in a given neuron and also across different neurons in a given slice preparation were virtually constant in onset latency; in a given neuron the standard deviation of the EPSP onset latency was only 0·25 ms on average, and across different neurons in a given slice preparation the difference in the mean onset latency was < 1 ms. On the other hand, the failure of SCN neurons to follow high-frequency optic nerve stimuli with EPSPs is consistent with polysynaptic connections, and could imply that some EPSPs are mediated through local interneurons. However, the fragility of the EPSPs could simply be due to axonal conduction block in presynaptic terminals and may be a characteristic specific for the retinal input to SCN neurons (i.e. a consistent following with EPSPs may not be as critical for photic information processing in SCN as in thalamus and cortex).

The estimated conduction velocity of the retinohypothalamic tract in the present study roughly matches that from the previous extracellular electrophysiological

studies conducted with rat and mouse slice preparations (Shibata *et al.* 1984; Cahill & Menaker, 1989*a*), and suggests that the optic nerve fibres innervating SCN neurons are unmyelinated. This conclusion is consistent with the anatomical evidence that optic nerve fibres within rat SCN are unmyelinated (Güldner, 1978).

Mediation by non-NMDA receptors of retinohypothalamic transmission at resting or more negative membrane potentials

The present results demonstrate that DNQX, a non-NMDA receptor antagonist, reversibly depressed the EPSPs, evoked by optic nerve stimulation at membrane potentials between −60 and −100 mV. Also, the data illustrate that the effects of DNQX were not associated with any significant changes in the baseline input resistance or membrane potential of the postsynaptic neurons, suggesting a specific action of DNQX. The estimated concentration at which DNQX depressed the EPSPs by 50% roughly matches the concentrations that were reported to inhibit by 50% the binding of [^{3}H]kainate and [^{3}H]AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid), a quisqualate agonist, to a rat cortical membrane preparation (Honoré, Davies, Drejer, Fletcher, Jacobsen, Lodge & Nielsen, 1988). The match in the DNQX concentrations again suggests that the DNQX effects in the present study were via action at the specific binding sites. In addition, the effects of DNQX were not likely due to its interaction with the strychnine-insensitive, glycine binding site on the NMDA receptor complex (Birch, Grossman & Hayes, 1988; Lester, Quarum, Parker, Weber & Jahr, 1989), because AP5 had no significant and consistent effects on the EPSPs even at high concentrations.

Our results confirm the general conclusion of the previous extracellular electrophysiological studies that EAA receptors mediate retinohypothalamic transmission (Shibata *et al.* 1986; Cahill & Menaker, 1987, 1989*b*), and further provide *direct* intracellular electrophysiological evidence that at resting or more negative membrane potentials retinohypothalamic transmission is mediated by non-NMDA receptors.

Involvement of NMDA receptors in retinohypothalamic transmission at less negative membrane potentials

The role of NMDA receptors in retinohypothalamic transmission is controversial. In support of a role, Shibata *et al.* (1986) showed that the field potentials recorded in the SCN of rat hypothalamic slice preparations following optic nerve stimulation were depressed by a moderately selective NMDA receptor antagonist, DL-2-amino-adipic acid (Watkins & Evans, 1981). However, the antagonist concentration that resulted in approximately 50% depression of the field responses was 1 mM. At this concentration the antagonist may not be selective for NMDA receptors, as pointed out by Cahill & Menaker (1989*b*). Recently, Rusak & Robertson (1989) reported that pre-treatment with MK-801, a non-competitive NMDA receptor antagonist, blocked the light-induced increase in the immunoreactivity of *fos*, the product of the *c-fos* proto-oncogene, in the SCN of hamsters. Also, Colwell, Ralph & Menaker (1990) demonstrated that intraperitoneal injection of MK-801 blocked in a dose-dependent manner the phase-shifting effects of light on the circadian rhythm of wheel-running

activity in hamsters. Although the results from these studies suggest a possible role for NMDA receptors in photic information processing, they do not provide information on the exact location of the NMDA receptors.

A recent *in vitro* extracellular electrophysiological study by Cahill & Menaker (1989*b*) does not support the hypothesis that NMDA receptors participate in retinohypothalamic transmission. According to these investigators, omission of Mg^{2+} from the superfusate did not reveal any AP5-sensitive component (i.e. NMDA component) in the postsynaptic field potentials recorded in the SCN following optic nerve stimulation. Since the absence of an NMDA component could be due to the residual Mg^{2+} in the tissue, which could have been enough to maintain the voltage-dependent block of the NMDA receptor-linked channels (Mayer, Westbrook & Guthrie. 1984; Nowak, Bregestovski, Ascher, Herbet & Prochiantz, 1984), we took the strategy of altering baseline membrane potential, instead of using Mg^{2+}-free medium, in testing for the presence of an NMDA component. In our experiments, at membrane potentials between -20 and -55 mV, optic nerve stimulation evoked slow, as well as fast, depolarizing potentials, whose relationship to membrane potential was non-linear, as expected for NMDA receptor-mediated events. Since these experiments were conducted under a condition where Ca^{2+} channels were not blocked, pure voltage-dependent Ca^{2+} conductances activated by the fast depolarizing potentials might have initiated the slow depolarizing potentials. The selective blockade of the slow potentials by AP5, however, suggests that this is not the case. When coupled with the observation that AP5 had little or no effect on the EPSPs evoked at membrane potentials between -60 and -100 mV, these results suggest that at resting or more negative membrane potentials, NMDA receptors do not contribute or contribute only minimally to retinohypothalamic transmission, whereas at less negative membrane potentials NMDA receptors play a role.

Despite the evidence in the present study for involvement of NMDA receptors in retinohypothalamic transmission at less negative membrane potentials, the extent to which NMDA receptors contribute to excitatory synaptic transmission is unclear. In the current experiments we did not add to the perfusing medium glycine, which is known to allosterically modulate the NMDA receptors (Johnson & Ascher, 1987). We assumed that the sub-synaptic concentration of glycine was high enough for the modulation (Thomson, 1989). If this assumption was incorrect, we might have underestimated the contribution of the NMDA component. In the future, whole-cell voltage-clamp experiments with patch pipettes (Blanton, Lo Turco & Kriegstein, 1989) in medium with different concentrations of glycine and Mg^{2+} are expected to allow a more rigorous and quantitative analysis of the NMDA component.

Contribution of both NMDA and non-NMDA receptors to non-retinal synaptic transmission to SCN

The EPSPs evoked by stimulation of a site near to the SCN at membrane potentials between -60 and -100 mV were sensitive to DNQX, but not to AP5. On the other hand, those evoked at less negative membrane potentials contained an AP5-sensitive component. These results, coupled with the fact that the spontaneous EPSPs in hyperpolarized cells were also sensitive to DNQX, support the hypothesis that the synaptic mechanisms underlying other excitatory inputs to SCN are basically the same as those of the retinohypothalamic tract (i.e. at resting or more

negative membrane potentials, mainly non-NMDA receptors mediate excitatory synaptic transmission, while at less negative membrane potentials NMDA receptors also play a role).

Up to now, no serious attention has been paid to the potential role of the EAAs in non-retinal neurotransmission in the SCN, probably because more emphasis has been put on other putative neurotransmitters (e.g. neuropeptide Y). Many of the non-retinal afferents containing other putative neurotransmitters may also utilize glutamate or similarly related EAAs for fast excitatory neurotransmission. In this case, the other putative transmitters may have modulatory roles.

In conclusion, the data obtained from these experiments suggest that: (1) the direct retinal input to SCN is excitatory, and GABA is not the neurotransmitter, and (2) at resting or more negative membrane potentials, non-NMDA receptors mediate excitatory synaptic transmission for both retinal and non-retinal inputs to SCN neurons. At less negative membrane potentials, however, NMDA receptors may also play a role.

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