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# The role of GABAergic neuron on NMDA- and SP-induced phase delays in the suprachiasmatic nucleus neuronal activity rhythm *in vitro*

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ABSTRACT

Gamma-aminobutyric acid (GABA), and its biosynthetic enzyme, glutamic decarboxylase, are widely distributed in the suprachiasmatic nucleus (SCN). In the present study, we examined the role of the $GABA_A$ receptor on *in vitro* SCN responses to photic-like signals. We found that 100 μM $GABA_A$ receptor antagonist bicuculline partially blocked field potentials evoked by optic nerve stimulation. NMDA- and SP-induced phase shifts of SCN neuronal activity rhythms, were blocked with 10 μM bicuculline. Application of 100 μM bicuculline alone induced phase advance of SCN neuronal activity rhythm. These results show that NMDA- and SP-induced phase shifts are blocked by bicuculline and suggest GABA has an important role as neurotransmitter in the neuronal network regulating phase shifts of the circadian clock.



The suprachiasmatic nucleus (SCN) has been identified as a pacemaker for many circadian rhythms in mammals. The daily light–dark cycle, acting through light-induced advances and delays of the endogenous oscillation, is the primary environmental entraining agent. The retinohypothalamic tract (RHT), which is a direct projection from retinal ganglion cells, appears to provide sufficient information about environmental lighting to maintain the synchronization of circadian rhythms. We previously reported that both *N*-methyl-D-aspartate (NMDA) and substance P (SP) receptors were involved in photic signalling in the SCN *in vitro* [13,22,23]. Treatments with NMDA or SP produced phase shifts of circadian rhythm in neuronal activity in the SCN neuron *in vitro*, with phase response curves similar to that for light pulse *in vivo*.

Most SCN cells are GABAergic neurons [16]. Within the SCN, GABA plays a role in the synchronization of the dorsal and ventral regions of the SCN [1], can synchronize the population of cellular oscillators in culture [15] and evokes excitatory responses in a subset of adult SCN neurons [4]. These results suggest that GABA is the neurotransmitter of neuronal communication within the neuronal network of the SCN. Muscimol, a $GABA_A$ receptor agonist, suppresses SCN neuronal activity and induced phase advance during day time [14,24]. Recent studies show muscimol suppresses *Period1* (*Per 1*) and *Period2* (*Per 2*) mRNA expression in the SCN during day time *in vivo* [6,17]. During night time, $GABA_A$ receptor agonists block light-induced increases in *Per1* and *Per2* mRNA in the early night *in vivo* [7,17]. In contrast, the $GABA_A$ receptor antagonist, bicuculline is reported to block light-induced phase delays in the golden hamster *in vivo* when given systemically [19] and to slightly reduce the optic nerve stimulation-evoked field potentials in the SCN *in vitro* [9]. These effects of bicuculline on the photic entrainment mechanism of SCN neurons are not yet understood in detail. In this study, we examine the effects of bicuculline on SCN responses to photic-like treatments.

Rats (120–350 g) were kept under a 12 h light–12 h dark cycle for at least 1 week (typically more than 2 weeks) for preparation of hypothalamic slices. In the morning of the day of the experiment, the animals were injected with sodium pentobarbital (100 mg/kg i.p.) before being decapitated with a guillotine. After decapitation, the skull was opened and the optical nerves were cut. The brain was removed from the skull, put in ice-cold Krebs–Ringer as previously reported [20,21,25]. The brain was blocked to allow preparation of a hypothalamic slice. A horizontal slice containing the SCN (400–500 μm), both optic nerves (3–5 mm) and an intact optic chiasm was cut from the block using a vibraslicer (Candin Instruments, USA). After 1 h pre-incubation, the slices were transferred into a recording chamber, filled with warmed Krebs–Ringer solution. Krebs–Ringer solution was equilibrated with 95% $O_2$/5% $CO_2$ at 36 °C. Preparations were perfuse continuously at 5 ml/min with Krebs–Ringer solution at 36 ± 1 °C. The stimulation and recording electrodes and recording chamber used here were described previously [20,21,25]. Evoked potentials were recorded in the ventrolateral SCN using glass pipette

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microelectrodes (0.5–1 MΩ) filled with 0.9% NaCl. Insulated bipolar stainless steel wires were placed on the optic nerve approximately 1 mm rostral to the optic chiasm. A single pulse stimulation with a duration of 0.08 ms at 0.5–1.5 mA was applied to the optic nerve with a rate of 0.1 Hz. This stimulus condition has previously been shown to produce a field potential in the ventrolateral SCN [21]. Bicuculline was added to the Krebs–Ringer solution in concentrations ranging 1–100 μM, and this was perfused for up to 15 min through the experimental chamber. Field potentials were quantified a measure of the peak amplitude in mV of the large negative wave as previously reported [21]. The amplitude of the postsynaptic field potential before application of bicuculline was set as 100%.

To examine phase shifts of SCN neuronal firing rhythms to putative RHT neurotransmitters, coronal hypothalamic slices (450 μm thickness) of rat brain were obtained 2 h before application of agents at a specified projected zeitgeber times (ZT). The slices were placed on disk-type chambers, and continuously perfuse with warmed Krebs–Ringer solution equilibrated with 95% $O_2$/5% $CO_2$ at 36 °C [8,12,13]. This buffer was maintained at pH 7.3–7.4. Between ZT 13 and 14 during the first day *in vitro* perfusion was stopped and the medium supplemented with drugs. After 60 min exposure, the drug containing medium was replaced with normal medium and perfusion was reinstated. The spontaneous activity of single SCN cells was recorded extracellularly through glass electrodes filled with 2 M NaCl (DC resistance, 2–10 MΩ in Krebs–Ringer solution). During the second day, *in vitro* single units were averaged into 2 h intervals using 1 h lags [12,13]. Previous studies have shown that this procedure yields a pattern of electrical activity for a population of SCN neurons which varies slightly between animals, and that the time of peak electrical activity is a reliable marker of the phase of the SCN pacemaker. Bicuculline was added to the Krebs–Ringer solution for 5 min before addition of NMDA or SP and was present throughout exposure to NMDA or SP for 60 min.

As shown in Fig. 1A and B, optic nerve stimulation-evoked field potentials in SCN were recorded as previously reported [21,25]. The amplitude of the SCN field potential before application of bicuculline was set as 100% (Fig. 1A and B). Bicuculline dose-dependently reduced the negative wave of the SCN field potential (Fig. 1C). 100 μM bicuculline partially inhibited field potentials (46% inhibition) ($53.3 \pm 9.99\%$, $n = 4$, $P < 0.05$, Student's *t*-test). These effects were completely reversed after 20–60 min of washing with normal Krebs–Ringer solution.

In order to see the effect of bicuculline on phase resetting of the SCN, we used a rat SCN brain slice preparation maintained *in vitro* for 2 days. We previously reported slices treated with 10 μM NMDA or 1 μM SP for 1 h on day 1 between ZT 13 and ZT 15 showed a robust phase delay {10 μM NMDA ($-4.13 \pm 0.24$ h, $n = 4$), 1 μM SP ($-4.63 \pm 0.47$ h, $n = 4$)}[13,22,23]. In this experiment, bicuculline dose-dependently blocked NMDA-induced phase delay [23] (Fig. 2A) {100 μM bicuculline ($-0.67 \pm 0.17$ h, $n = 3$, $P < 0.05$, Student's *t*-test), 10 μM bicuculline ($-1.13 \pm 0.70$ h, $n = 3$, $P < 0.05$, Student's *t*-test)}. Similarly, bicuculline dose-dependently blocked SP-induced phase delay [22] (Fig. 2B) {100 μM bicuculline ($-0.17 \pm 1.10$ h, $n = 3$, $P < 0.05$, Student's *t*-test), 10 μM bicuculline ($-1.17 \pm 0.73$ h, $n = 3$, $P < 0.05$, Student's *t*-test)}. 1 μM bicuculline had no effect on NMDA ($-4.0 \pm 0.29$ h, $n = 3$)- or SP ($-4.3 \pm 0.17$ h, $n = 3$)-induced phase delay. In control experiment where slices were treated with bicuculline (1 or 10 μM) alone for 65 min between ZT 13 and ZT 15, there was no phase-shifting effect on the SCN firing rhythm (data not shown). However, 100 μM bicuculline significantly induced phase advances of the SCN firing rhythm (Figs. 2C and 3) ($1.67 \pm 0.60$ h, $n = 3$, $P < 0.01$, Student's *t*-test).

Most SCN neurons are GABAergic and GABA may be the principal neurotransmitter in the rat SCN circadian system [16]. Both $GABA_A$ and $GABA_B$ agonists inhibit SCN neuronal activity. The distribution of GABA responsive neurons is not localized within any subdivision

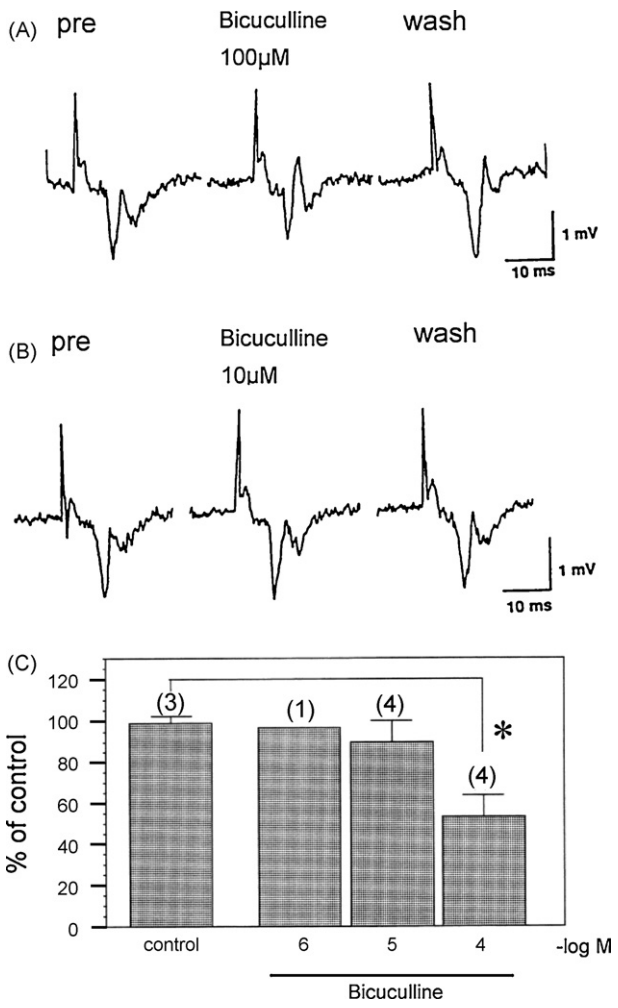


**Fig. 1.** Effect of bicuculline on optic nerve stimulation-evoked field potentials in the rat SCN slice. Representative examples. 100 μM bicuculline (A) and 10 μM bicuculline (B) were applied for 15 min followed with washout. (C) Inhibitory effect of bicuculline on optic nerve stimulation-evoked field potentials in the rat SCN slice. Each column indicates the mean ± S.E.M. Numbers in parentheses are the number of slices used. *$P < 0.05$ from control, Student's *t*-test.

of the SCN [14]. $GABA_B$ receptors agonist, baclofen has been found to depress the excitability of central neurons and central neurotransmission by a presynaptic action which involve a reduction in calcium entry. $GABA_A$ receptors exhibit a high affinity for muscimol and act by increasing the chloride permeability of the membrane. Previous studies have shown that the $GABA_A$ receptor has an important role in both photic entrainment and non-photic entrainment *in vitro* and *in vivo* [6,7,10,11,17,19,24]. A recent report suggests that the different populations of $GABA_A$ receptors are involved in photic and non-photic entrainment of the SCN [7].

In the present study, we found that 100 μM bicuculline partially blocked the optic nerve stimulation-evoked field potentials in the rat SCN slice (46% inhibition) while 10 μM bicuculline had no effect (10% inhibition). Gannon et al. have shown that 10 μM bicuculline has a slight inhibitory effect on SCN field potentials (22%) [9]. This report is consistent with our result that bicuculline partially inhibits on the SCN field potentials. This difference in the concentration of bicuculline may be due to the different experimental condition such as optic nerve stimulation.

Bicuculline blocked both NMDA- and SP-induced phase delay of SCN neuronal activity rhythms. This effect was dose-dependent with significant attenuation observed at concentrations as low as 10 μM (Fig. 2). At present, the mechanism by which bicuculline