Novel phase-shifting effects of GABA<sub>A</sub> receptor activation in the suprachiasmatic nucleus of a diurnal rodent

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Novak, C. M., and H. E. Albers. Novel phase-shifting effects of GABA<sub>A</sub> receptor activation in the suprachiasmatic nucleus of a diurnal rodent. Am J Physiol Regul Integr Comp Physiol 286: R820–R825, 2004. First published December 4, 2003; 10.1152/ajpregu.00575.2003.—The vast majority of neurons in the suprachiasmatic nucleus (SCN), the primary circadian pacemaker in mammals, contain the inhibitory neurotransmitter GABA. Most studies investigating the role of GABA in the SCN have been performed using nocturnal rodents. Activation of GABA<sub>A</sub> receptors by microinjection of muscimol into the SCN phase advances the circadian activity rhythm of nocturnal rodents, but only during the subjective day. Nonphotic stimuli that reset the circadian pacemaker of nocturnal rodents also produce phase advances during the subjective day. The role of GABA in the SCN of diurnal animals and how it may differ from nocturnal animals is not known. In the studies described here, the GABA<sub>A</sub> agonist muscimol was microinjected directly into the SCN region of diurnal unstriped Nile grass rats (Arvicanthis niloticus) at various times in their circadian cycle. The results demonstrate that GABA<sub>A</sub> receptor activation produces large phase delays during the subjective day in grass rats. Treatment with TTX did not affect the ability of muscimol to induce phase delays, suggesting that muscimol acts directly on pacemaker cells within the SCN. These data suggest that the circadian pacemakers of nocturnal and diurnal animals respond to the most abundant neuronal metabolic activity in the SCN neurons in opposite ways. These findings are the first to demonstrate a fundamental difference in the functioning of circadian pacemaker cells in diurnal and nocturnal animals.

Experimental Procedures

Male and female A. niloticus (n = 94) were used in the following studies. All animals were raised from a colony at Georgia State University, descendants from the colony at Michigan State University (29). Animals had access to food and water ad libitum. Animals were housed in Plexiglas cages (20 × 40 × 20 cm) in a 12:12-h light/dark cycle and after surgery were individually housed in cages equipped with a running wheel.

Each grass rat underwent surgical implantation of a guide cannula (26 gauge; Plastics One). The animals were deeply anesthetized (pentobarbital sodium, 50 mg/kg dose, 1 ml/kg volume ip) and placed in a stereotaxic frame. The guide cannula was aimed at the SCN region using the following coordinates: 9.5° angle toward the midline, +1.5 mm anterior to bregma, +1.4 mm lateral to bregma, and −3.0 to −3.1 mm ventral to dura mater; the final position of the 32-gauge, 14-mm microinjection needle was 6.0 to 6.1 mm below dura. Cranioplastic cement (Plastics One) was used to secure the cannula to the skull.

Running wheels were outfitted with magnetic switches that recorded each wheel revolution. For each wheel, the number of wheel revolutions per 5-min period was recorded using Mini-mitter equipment (dataports and QA-4s) and VitalView software (Mini-mitter; Bend, OR). After animals were entrained to a 12:12-h light/dark cycle, the costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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they were released into constant darkness (DD). After 7–10 days in DD, animals were given their first of up to three microinjections. At least 10 days separated each set of injections. During microinjections, animals were removed from their cage and gently restrained while the microinjection needle was inserted into the guide cannula. Muscimol (Sigma; 1–10 mM) or saline vehicle (200 nl) were microinjected into the SCN area using a 1-μl Hamilton syringe. Muscimol induced an extended behavioral stupor in grass rats, which usually started within 15 min after microinjection. The change in behavior occurred regardless of what time of day the animal received muscimol, and animals fully recovered within a few hours. Similar effects have been seen in hamsters under the same conditions. Also, microinjections of baclofen (GABA\(_B\) agonist) produce a similar behavioral change with minimal phase alterations (C. M. Novak, unpublished observation). It is therefore unlikely that any phase shifts seen after microinjection of muscimol are due to nonspecific effects (such as changes in body temperature) secondary to the behavioral change.

Linear regression was used to 1) predict the onset of wheel running activity and 2) calculate the phase shift value after each injection (9). A regression line was fitted to the activity onsets (defined as the first 5-min bin with 10 or more revolutions, followed by 3 similar bins) for the 7 days preceding the injection. The resulting predicted onset values were used to calculate the injection time for each animal. A second regression line was fitted to the injection day (the first 2–3 onsets were not included because of transient onsets after phase shifts). This yielded two regression values, both predicting the onset time of the day after injection. The postinjection regression value was subtracted from the preinjection value to calculate the phase shift value. Phase advances were therefore positive numbers, and phase delays were negative numbers. Phase shift values were then calculated from the preinjection values (if injection day was subtracted from preinjection value to calculate the phase shift value. Phase advances were therefore positive numbers, and phase delays were negative numbers. Phase shift values were not included if 1) the injection of muscimol did not induce the typical behavioral stupor (5 data points excluded), 2) the standard error of either regression line was over 20 min (variability in onset times was not related to treatment or dose), 3) the difference between the slopes of the pre- and postinjection regression lines was not within ±0.01 (14.4 min, to account for any possible changes in circadian period due to the treatment; 23 total data points excluded), or 4) the microinjection site was not within 300 μm of the SCN (see below).

Although A. niloticus are strictly diurnal in the field (5), animals in the laboratory show a variety of patterns of wheel running activity. Access to a running wheel induces varying degrees of crepuscular (twilight active) activity in some grass rats, which is likely due to masking. Although this wheel-induced masking presents a limitation to the use of A. niloticus as a diurnal model, there is no better species to use for an experiment of this nature. Because of the crepuscular tendencies exhibited by many grass rats, the morning activity onset could not always be used to calculate a regression line. Depending on which onset (morning associated or evening associated) had least day-to-day variability (i.e., standard error of onset values), that onset was used to calculate injection time and phase shift values (see Ref. 42). At the conclusion of each experiment, an ANOVA was used to determine if either the sex of the animal or the onset used to calculate phase shift or injection time affected the phase shift value; no significant effects were found (\(P > 0.05\)).

The phase-shifting effects of muscimol were determined for each hour over the course of the subjective day to describe the effects of muscimol using a phase-response curve (PRC). In a PRC, the magnitude of the phase shift is plotted on the vertical axis, with phase delays below the vertical axis and phase advances above it. Time of day is plotted as circadian time (CT; hours after morning activity onset) on the horizontal axis, starting with CT 0, the start of subjective day, with CT 12 defining the start of subjective night. In this way, the amount and direction of the phase shifts induced by a stimulus can be described over the course of the day. To determine the phase-response curve for muscimol, the GABA\(_A\) agonist (Sigma; 10 mM in saline vehicle) was microinjected at each hour of the cycle (the morning activity onset was defined as CT 0 if this onset was used to determine phase; if the evening activity onset was used, this was considered CT 12; Ref. 42). The resulting phase shift for each injection was plotted across the circadian cycle. The data were analyzed in CT groups (data points were divided into 8 groups according to injection time: CT 0–2, 3–5, 6–8, 9–11, 12–14, 15–17, 18–20, and 21–23). A one-way ANOVA was used to determine if muscimol-induced phase shifts differed over time of day, and post hoc (least significant difference) tests were used to probe differences between CT groups.

After the time (i.e., CT) of maximal response to muscimol was ascertained, a dose-response curve for muscimol was determined. Grass rats were microinjected with muscimol at CT 4 using the following doses: 0 mM (vehicle microinjection), 1 mM, 5 mM, and 10 mM. A one-way ANOVA was used to determine if muscimol induced a significant phase shift at CT 4, and the least significant difference test was used to reveal significant differences between doses of muscimol.

Lastly, the ability of muscimol to induce phase delays in the presence of TTX (reversibly blocks sodium-dependent action potentials) was assessed. At CT 4, grass rats were microinjected with 200 nl of muscimol (10 mM), either alone or in a cocktail with TTX (Sigma; 5 μM in saline vehicle), TTX alone, or vehicle. Administration of TTX in a cocktail successfully blocks the effects of a GABA receptor agonist (baclofen, but not muscimol) in vitro (3); TTX has also been microinjected into a cocktail with muscimol previously in this lab (14). The resulting phase shifts were calculated, and a two-way ANOVA was used to determine if muscimol-induced phase shifts were affected by the addition of TTX (using muscimol and TTX as the independent variables, phase shift values as the dependent variable). Moreover, in a separate experiment, the effect of TTX on light-induced phase shifts was assessed as a positive control for TTX. In hamsters, SCN microinjections of TTX attenuate the phase-shifting effects of light (38) but not the phase-shifting effects of muscimol (24). Grass rats were exposed to a light pulse (500 lx, 15 min) at CT 14–16, when light induces phase delays; animals were microinjected with either TTX or vehicle immediately (usually within 1–2 min) before light exposure. An independent-samples \(t\)-test was used to determine the effects of TTX on light-induced phase delays in grass rats.

After all activity data were collected, brains were extracted to ascertain cannula placement. Each animal was injected with a lethal dose of pentobarbital sodium (0.5 ml/animal ip; 50 mg/ml) then microinjected with 200 nl India ink through each guide cannula. The brains were removed and placed into 10% buffered formalin for at least 24 h. Brains were sectioned into 100-μm sections using a vibratome, and the SCN sections were evaluated using a microscope. Only animals with injection sites placed within 300 μm of the border of the SCN that did not penetrate the third ventricle were included in the analysis (see Fig. 1).

RESULTS

**Muscimol induces phase delays during the subjective day.** As shown in Fig. 2, microinjections of muscimol induced phase shifts in wheel running activity when microinjected into the SCN region but not at all times of the circadian cycle. During the subjective day, especially between CT 3 and 5, muscimol induced phase delays in the rhythm of wheel running activity. No consistent phase-shifting effect of muscimol was found during the subjective night. An ANOVA revealed a significant effect of time of injection on muscimol-induced phase shifts (\(P < 0.01\)). Muscimol induced significantly greater phase delays at CT 3–5 compared with all other CT groups.

Before the slope criterion was applied, a correlation test was run to determine if the magnitude of the phase shift was correlated to a change on slope (circadian period). No signif-
significant correlation was found \((r = -0.035, P = 0.759)\), indicating that muscimol-induced phase shifts were not associated with a change in circadian period compared with microinjections of muscimol that did not induce phase shifts.

**Muscimol-induced phase delays are dose dependent.** Muscimol induced significant phase delays at CT \(4\) \((P < 0.01)\). As seen in Fig. 3, a post hoc test revealed that 10 mM muscimol induced significantly larger phase delays than the 5 mM, 1 mM, or vehicle groups \((P < 0.01)\).

**Muscimol-induced phase delays are not blocked by TTX.** The two-way ANOVA showed no significant interaction between muscimol and TTX on phase shifts; there was a significant main effect of muscimol \((P < 0.001)\) but not TTX. As illustrated in Fig. 4, muscimol induced phase delays, and TTX had no significant effect on muscimol-induced phase delays. In the control experiment, light-induced phase delays in grass rats at CT \(14\)-16 and TTX significantly attenuated these phase shifts \((P < 0.05)\). The mean phase delay after light + vehicle was \(-72.29\) min \(\pm\) 6.98 SE, whereas the mean phase delay after treatment with light + TTX was significantly lower, \(-48.73\) min \(\pm\) 5.00 SE (33% decrease). These results demonstrate that TTX reduced light-induced but not muscimol-induced phase delays in *A. niloticus*.

**DISCUSSION**

The studies described here demonstrate that the GABA\(_A\) receptor activation in the SCN of a diurnal rodent, *A. niloticus*, induces large phase delays of the circadian wheel running rhythm during the subjective day, with 10 mM as the critical dose to achieve phase delays. These results differ dramatically from the phase-shifting effects of muscimol in nocturnal animals. In hamsters, muscimol produces phase advances during the subjective day (50). The phase-shifting effects of muscimol in grass rats and hamsters may also differ during the subjective night. In hamsters, muscimol induces small phase delays dur-
GABA-induced phase delays in diurnal animal

Muscimol-induced phase shifts are not significantly different between species (50). In addition, in both grass rats and hamsters, shifts produced by muscimol are not strikingly different between species. Muscimol induces a behavioral stupor phase shifts during the day, other effects of muscimol are the level of individual SCN neurons. Activation in diurnal and nocturnal rodents might originate at the level of individual SCN neurons. Taken together, these data suggest that muscimol acts directly on SCN pacemaker cells or cells that communicate with pacemaker cells by mechanisms other than sodium-dependent action potentials in both hamsters and grass rats. These data further suggest that the circadian pacemakers of diurnal and nocturnal animals respond differently to GABA. These findings are the first to demonstrate a significant difference in a fundamental characteristic of the circadian clock mechanism between diurnal and nocturnal animals. Moreover, it is the first indication that there may be diurnal/nocturnal differences within cells housing circadian oscillatory mechanism.

GABA-induced phase resetting in the SCN can be demonstrated at the cellular level. Activation of GABA_A receptors during the subjective day phase advances the circadian rhythm of rat SCN neuronal activity in vitro in a manner similar to its effect on behavioral rhythms (51). Indeed, GABA-induced phase shifts can be found at the level of a single neuron (32). Also, single-unit discharge of most SCN neurons is inhibited by GABA and GABAergic agents (6, 12, 31, 47), as is the daytime peak of metabolic activity within the SCN. GABA may also act as a synchronizing agent within the SCN, entraining each cellular oscillator to the same phase (32). It has also been proposed that GABA is involved in optic nerve stimulation-induced suppressions of neuronal activity in both rats and diurnal Octodon degus (27). Lastly, GABA suppresses regular intracellular Ca^{2+} oscillations in SCN cells (54); this may be one mechanism through which GABA might alter the molecular oscillatory mechanism within SCN neurons. Taken together with previous studies, the data presented here suggest the possibility that the different responses to GABA_A receptor activation in diurnal and nocturnal rodents might originate at the level of individual SCN neurons.

In addition to the opposite effects of muscimol in producing phase shifts during the day, other effects of muscimol are similar between species. Muscimol induces a behavioral stupor in both grass rats and hamsters. The magnitudes of the phase shifts produced by muscimol are not strikingly different between species (50). In addition, in both grass rats and hamsters, muscimol-induced phase shifts are not significantly affected by inhibition of sodium-dependent action potentials by TTX (3, 24). These data strongly suggest that muscimol activates GABA_A receptors on SCN pacemaker cells rather than on neurons that communicate with SCN pacemaker cells via conventional synaptic transmission. Other explanations are possible, however. For example, a nonsynaptic chemical messenger such as nitric oxide (NO) may be involved in mediating phase shifts (10). Phase resetting by NO is restricted to the subjective night, however, making it unlikely that NO mediates muscimol-induced phase shifting during the subjective day (11). Another possibility is that muscimol may act on cells that communicate with pacemaker neurons via gap junctions or other mechanisms not involving sodium-dependent action potentials. In support of this hypothesis is the finding that muscimol has been shown to inhibit dye transfer between SCN neurons, indicating that GABA_A receptor activation may influence the strength of gap junction communication and the resulting spread of depolarization (7, 48). The possible role of gap junctions in GABA-related phase shifting during the day remains unclear. If gap junctions or NO are necessary for muscimol-induced phase shifting to occur, it seems likely that either mechanism affects only local neuronal networks.

One role of GABA within the SCN is thought to be the mediation of nonphotic phase shifts. Specifically, in nocturnal animals, GABA receptor activation within the SCN produces a similar pattern of phase shifting as forced activity or arousal (41), NPY (21), and activation of the serotonin system (36, 43). Both NPY from the intergeniculate leaflet and 5-HT from the dorsal and median raphe nuclei relay information about nonphotic events to the SCN and related brain structures (4, 13, 34). Antagonists to GABA_A receptors microinjected within the SCN also block the phase-shifting effects of NPY (22). GABA receptor activation within the SCN may be the final common pathway for nonphotic stimuli to act on pacemaker mechanisms in nocturnal animals. Whether it serves the same purpose in diurnal animals remains to be determined. In fact, very little is known about the roles of 5-HT or NPY in the rhythms of diurnal animals (18, 49). Taken together with previous results, the data described here demonstrate that the SCN is sensitive to the phase-shifting effects of GABA_A receptor activation during the subjective day, regardless of whether the animal is diurnal or nocturnal. However, phase shifting effects of activation of GABA_A receptors is opposite in nocturnal animals and diurnal grass rats.

It might be assumed that the same stimulus that causes nonphotic phase shifts in nocturnal animals (i.e., arousal or activity) would induce nonphotic phase shifts in diurnal animals. If this were the case, it follows that the circadian system of diurnal animals would be sensitive to these nonphotic stimuli at a different time of day (during the night) than nocturnal animals (during the day). Evidence suggests that scheduled activity has, at most, a modest influence on phase in diurnal rodents (17, 25, 28). From these studies combined with the data presented here, it seems that the phase of the circadian pacemaker, not the phase of the animal’s activity cycle (i.e., diurnal or nocturnal), determines when an animal is sensitive to a nonphotic stimulus (17, 25, 28). On the other hand, the direction of the phase shift induced by GABA_A receptor activation (i.e., phase delays in grass rats but phase advances in hamsters) may be coupled to the daily activity pattern of the species. The environmental stimulus or behavioral change that may ultimately cause an alteration of SCN GABA_A receptor activation in grass rats is unknown. Other possible entraining agents should be considered in addition to induced activity. For example, social contact has been shown to promote rhythm synchronization in other diurnal (44) and nocturnal (8, 35, 40) animals.olfactory cues can entrain and accelerate reentrainment in diurnal Octodon degus (19). The ability of arousal or activity to act as an entraining agent may not be a general property of the circadian system of mammals.

The data presented here suggest that the circadian pacemaker of both nocturnal and diurnal animals is sensitive to nonphotic stimuli during the daytime but that the direction of the phase shift, as well as the stimulus needed to induce the phase shift, may differ according to species. These data imply that information on nonphotic entrainment and the neural mechanisms mediating nonphotic entrainment in nocturnal...
animals may not generalize directly to diurnal animals, including humans.

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