Novel phase-shifting effects of GABA$_A$ receptor activation in the suprachiasmatic nucleus of a diurnal rodent

C. M. Novak and H. E. Albers
Departments of Biology and Psychology, Center for Behavioral Neuroscience, Georgia State University, Atlanta, Georgia 30302-4010

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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$_A$ 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$_A$ 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$_A$ 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 neurochemical signal found in 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, descended from the colony at Michigan State University (29). Animals had access to food and water ad libitum. Animals were housed in Plexiglas cages ($20 	imes 40 	imes 20$ 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. Cranio-plastic 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, antisera within the SCN (4) and NPY-induced phase shifts are inhibited by GABA$_A$ receptor antagonists (22), it is likely that activation of GABA$_A$ receptors within the SCN is necessary for nonphotic stimuli to induce phase shifts of circadian rhythms.

The great majority of studies on the neuronal mechanisms controlling circadian rhythms have been conducted using nocturnal animals. The few studies that have examined circadian control in diurnal species have found that the patterns of neuronal metabolic activity in the SCN, as well as the responses to light, are very similar to nocturnal animals (26, 30, 33, 45, 46). There is reason to believe, however, that the role of GABA in the SCN may differ between diurnal and nocturnal species. As described above, GABA appears to mediate the effects of nonphotic stimuli on rhythms (20, 22). In nocturnal animals, arousal during the subjective day, when nocturnal animals are normally quiescent, is crucial for the induction of nonphotic phase shifts (41). It is therefore possible that the phase-shifting effects of nonphotic stimuli, as well as the neural mechanisms that mediate these effects, may differ in diurnal animals. The present study determined if the phase-shifting actions of GABA in the SCN of a diurnal rodent species, *Arvicanthis niloticus* (unstriped Nile grass rat), differ from those reported in nocturnal rodents.

Address for reprints requests and other correspondence: C. M. Novak, Center for Behavioral Neuroscience, Georgia State Univ., PO Box 4010, Biology Dept., Atlanta, GA 30302-4010 (E-mail: biocmn@lanigate.gsu.edu).

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THE SUPRACHIASMATIC NUCLEUS (SCN) OF THE HYPOTHALAMUS IS THE PRIMARY CIRCADIAN PACEMAKER IN MAMMALS. NEURONS WITHIN THE SCN CONTAIN BOTH GABA AND ITS SYNTHETIC ENZYME (GLUTAMIC ACID DECARBOXYLASE; REF. 23) AS WELL AS GABA$_A$ RECEPTORS (15, 39). THE SCN SHOWS A DAILY RHYTHM IN GABA CONTENT, AND IT CONTAINS BOTH GABA AND ITS SYNTHETIC ENZYME (GLUTAMIC ACID DECARBOXYLASE; Ref. 23) AS WELL AS GABA$_A$ RECEPTORS (15, 39). THE SCN SHOWS A DAILY RHYTHM IN GABA CONTENT, AND IT CONTAINS BOTH GABA AND ITS SYNTHETIC ENZYME (GLUTAMIC ACID DECARBOXYLASE; Ref. 23) AS WELL AS GABA$_A$ RECEPTORS (15, 39). THE SCN SHOWS A DAILY RHYTHM IN GABA CONTENT, AND IT CONTAINS BOTH GABA AND ITS SYNTHETIC ENZYME (GLUTAMIC ACID DECARBOXYLASE; Ref. 23) AS WELL AS GABA$_A$ RECEPTORS (15, 39).
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 after 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 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 differences between the slopes of the pre- and postinjection regression lines was ≥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 values; 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 in the SCN of A. niloticus 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-
Fig. 1. Photomicrograph of a microinjection site in the region of the suprachiasmatic nucleus (SCN; anterior SCN pictured here). Arrow points to the ink injection, delineating final placement of microinjection needle. If the tip of the needle was placed within 300 μm of the SCN border without penetrating the wall of the 3rd ventricle (3V), data from that animal were included in the analysis. oc, optic chiasm.

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 ($P < 0.01$).

DISCUSSION

The studies described here demonstrate that the GABA<sub>A</sub> receptor activation in the SCN of a diurnal rodent, <i>A. niloticus</i>, 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, with 10 mM as the critical dose to achieve phase delays. These results demonstrate that TTX reduced light-induced but not muscimol-induced phase delays in <i>A. niloticus</i>.

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 $-72.29 \text{ min} \pm 6.98 \text{ SE}$, whereas the mean phase delay after treatment with light + TTX was significantly lower, $-48.73 \text{ min} \pm 5.00 \text{ SE}$ (33% decrease). These results demonstrate that TTX reduced light-induced but not muscimol-induced phase delays in <i>A. niloticus</i>.
muscimol-induced phase shifts are not significant between species (50). In addition, in both grass rats and hamsters, shifts produced by muscimol are not strikingly different between species. The level of individual SCN neurons, indicating that GABA_A receptors 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. Offactory 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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