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# DAY–NIGHT DIFFERENCES IN NEURAL ACTIVATION IN HISTAMINERGIC AND SEROTONERGIC AREAS WITH PUTATIVE PROJECTIONS TO THE CEREBROSPINAL FLUID IN A DIURNAL BRAIN

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**Abstract—In nocturnal rodents, brain areas that promote wakefulness have a circadian pattern of neural activation that mirrors the sleep/wake cycle, with more neural activation during the active phase than during the rest phase. To investigate whether differences in temporal patterns of neural activity in wake-promoting regions contribute to differences in daily patterns of wakefulness between nocturnal and diurnal species, we assessed Fos expression patterns in the tuberomammillary (TMM), supramammillary (SUM), and raphe nuclei of male grass rats maintained in a 12:12 h light–dark cycle. Day–night profiles of Fos expression were observed in the ventral and dorsal TMM, in the SUM, and in specific subpopulations of the raphe, including serotonergic cells, with higher Fos expression during the day than during the night. Next, to explore whether the cerebrospinal fluid is an avenue used by the TMM and raphe in the regulation of target areas, we injected the retrograde tracer cholera toxin subunit beta (CTB) into the ventricular system of male grass rats. While CTB labeling was scarce in the TMM and other hypothalamic areas including the suprachiasmatic nucleus, which contains the main circadian pacemaker, a dense cluster of CTB-positive neurons was evident in the caudal dorsal raphe, and the majority of these neurons appeared to be serotonergic. Since these findings are in agreement with reports for nocturnal rodents, our results suggest that the evolution of diurnality did not involve a change in the overall distribution of neuronal connections between systems that support wakefulness and their target areas, but produced a complete temporal reversal in the functioning of those systems.** 

**Key words: Fos, serotonin, histamine, cerebrospinal fluid, circadian, grass rat.**

## INTRODUCTION

Wakefulness is supported by several systems distributed throughout the mammalian brain, including the serotonergic raphe and the histaminergic tuberomammillary nuclei (TMM; reviewed in Jones, 2005). In nocturnal rodents these brain areas exhibit increased activation during the active phase in comparison to the inactive phase of the animals' activity/rest cycle (Guzman-Marin et al., 2000; Ko et al., 2003; Takahashi et al., 2006). Likewise, in the diurnal grass rat, *Arvicanthis niloticus*, the ventral tuberomammillary nucleus (VTM) exhibits a day–night difference in neural activation (as assessed by Fos expression) that resembles the species daily activity/rest cycle (Novak et al., 2000), but that pattern has not been documented for this rodent's dorsal tuberomammillary nucleus (DTM). Also, it remains to be determined whether the raphe nuclei of diurnal species exhibit daily patterns of neural activation and whether the phase of such rhythms differs between diurnal and nocturnal animals. The implications of these observations are important for understanding the differential roles that histaminergic and serotonergic brain areas have on the circadian regulation of behavior in diurnal and nocturnal species.

Given that neural activity in histaminergic and serotonergic areas appears to be regulated by circadian mechanisms, and that in other arousal systems increased neural activity is accompanied by release of neurotransmitters at terminal buttons (Greco et al., 1999, 2000), it is likely that the release of histamine (HA) and serotonin (5HT) into target areas also follows a circadian pattern. Of particular interest is the choroid plexus (CP) as a possible target for circadian modulation by histaminergic and/or serotonergic inputs. The CP, located at different sites of the third (3V), fourth (4V) and lateral ventricles (LV; Wolburg and Paulus, 2010), is the site of production of the cerebrospinal fluid

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*Abbreviations:* 3V, third ventricle; 4V, fourth ventricle; 5HT, serotonin; CP, choroid plexus; CSF, cerebrospinal fluid; CTB, cholera toxin subunit beta; DR, dorsal raphe; DTM, dorsal tuberomammillary nucleus; HA, histamine; ICC, immunocytochemistry; lDR, lateral dorsal raphe; LSd, lateral septal nucleus, dorsal part; LV, lateral ventricle; mDR3–5, medial dorsal raphe levels 3 through 5; ME, median eminence; MR, median raphe; NDS, normal donkey serum; NGS, normal goat serum; PB, phosphate buffer; PBS, phosphate-buffered saline; PLP, paraformaldehyde–lysine–sodium periodate; PVN, paraventricular nucleus of the hypothalamus; SCN, suprachiasmatic nucleus; SUM, supramammillary nucleus; TBS, tris-buffered saline; TMM, tuberomammillary nucleus; TX, triton-X; vSPZ, ventral subparaventricular zone; VTM, ventral tuberomammillary nucleus; ZT, zeitgeber time.

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(CSF), which transports essential molecules throughout the nervous system. The CP contains receptors for 5HT (Watson et al., 1995) and HA (Crook et al., 1986), and both neurotransmitters influence metabolism in this area (Crook et al., 1984; Watson et al., 1995); the route may be through their direct release into the CSF as suggested by studies that report the presence of histaminergic (Ericson et al., 1987; Tillet et al., 1998) and serotonergic (Lorez and Richards, 1973; Chan-Palay, 1976) axons or dendrites in close proximity to the ventricles.

Here we examine the hypothesis that differences in temporal patterns of neural activity in histaminergic and serotonergic areas could contribute to differences in daily patterns of arousal between nocturnal and diurnal species. To this end, we used Fos expression to examine functional changes in cells of the DTM, VTM, and dorsal (DR) and median (MR) raphe nuclei of male grass rats across the 24 h/day. All of these regions not only support wakefulness, but also have been implicated in the modulation of circadian entrainment (Meyer-Bernstein and Morin, 1999; Harrington et al., 2000). We also measured Fos expression in the supramammillary nucleus (SUM) because of its role in arousal and reward (reviewed in Pan and McNaughton, 2004; Ikemoto, 2010). Additionally, to examine whether serotonergic and histaminergic areas of the grass rat brain have access to the CSF, we injected the retrograde tracer cholera toxin subunit beta (CTB) into the 3V or LV and examined the pertinent areas for the presence of CTB labeled neurons. In the DR we combined the analysis of CTB with identification of 5HT-positive neurons.

## EXPERIMENTAL PROCEDURES

Adult male grass rats, born in our laboratory and derived from a group brought from Kenya (Katona and Smale, 1997), were used in this study. Animals were kept on a 12:12 h light–dark cycle [lights on at zeitgeber time (ZT) 0] and provided with *ad libitum* access to water and food. All experiments were approved by the Michigan State University Institutional Animal Care and Use Committee in compliance with guidelines established by that institution as well as the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

### Experiment 1: Neural activation in TMM, SUM and raphe

*Immunocytochemistry (ICC).* Animals ($n$ = 6 per ZT) were perfused at ZTs 2, 6, 10, 14, 18, and 22. Intraperitoneal injections of sodium pentobarbital (Ovation Pharmaceutical, Deerfield, IL, USA) were used to deeply anesthetize the animals. To prevent exposure to light, animals that were perfused during the dark phase were injected under a red light ( < 5 lux) and were fit with an aluminum foil hood upon anesthesia onset. All animals were intracardially perfused with 0.9% saline, followed by 4% *N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (Sigma–Aldrich, St. Louis, MO, USA) in 0.1 M phosphate buffer (PB; note that this step was called for by the HA antibody used, AB5885; Millipore, Temecula, CA, USA), and then by 4% paraformaldehyde with 75 mM lysine and 10 mM sodium periodate (PLP) in 0.1 M PB. Following perfusion, brains were removed, post-fixed for approximately 4 h in PLP, and transferred to 20% sucrose in 0.1 M PB at 4 °C until they sunk to the base of the vial. Then, coronal sections (30 μm) were cut on a freezing sliding microtome. Alternate sections were collected in three series in cryoprotectant and stored at −20 °C until further processing. One series was used for the colocalization of Fos and 5HT and another for Fos and HA.

Since the ICC for Fos and HA did not produce adequate double labeling (data not shown), we limited our study to the analysis of Fos expression in the TMM and SUM and of Fos and 5HT expression in the raphe. Unless indicated otherwise, all ICC procedures were carried out at room temperature, and all incubations and rinses involved gentle agitation. In addition, with the exception of experiment 2, sections were rinsed three times (5 min/rinse) in 0.01 M phosphate-buffered saline (PBS) between all ICC steps and all incubations included 0.3% Triton X-100 (TX) and PBS. For Fos staining, free-floating sections containing the TMM, SUM, and raphe were washed in PBS (six times, 10 min/rinse), blocked (1 h) using 5% normal donkey serum (NDS; 017-000-121; Jackson ImmunoResearch Laboratories, West Grove, PA, USA), and incubated overnight in a rabbit anti-Fos antibody (1:20,000 in 3% NDS; SC-52; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4 °C. Then, sections were treated with a donkey anti-rabbit biotinylated antibody (1:200 in 3% NDS for 1 h; 711-065-152; Jackson ImmunoResearch Laboratories) and the avidin–biotin peroxidase method (0.9% each avidin and biotin solutions for 1 h; PK-6100; Vector Laboratories, Burlingame, CA, USA) with 0.025% diaminobenzidine enhanced with 2.5% nickel sulfate as the chromogen. For 5HT staining, the same sections were blocked (1 h) in 5% normal goat serum (NGS; S-1000; Vector Laboratories), incubated overnight in a rabbit anti-5HT antibody (1:10,000 in 3% NGS; NT-102; Protos Biotech Corp., New York, NY, USA) at 4 °C and treated with a goat anti-rabbit biotinylated antibody (1:200 in 3% NGS for 1 h; BA-1000; Vector Laboratories), after which the avidin–biotin peroxidase method (0.9% each avidin and biotin solutions for 1 h) was used with 0.02% diaminobenzidine as the chromogen. All sections were mounted on slides, dehydrated, and coverslipped with DPX mountant for histology (Sigma–Aldrich).

*Quantification.* For Fos quantification in the TMM, we selected one section that contained both the VTM and DTM. For the quantification of Fos and 5HT expression in the raphe, we selected three sections containing the medial subdivision of the dorsal raphe (mDR), one containing the lateral subdivision of this nucleus (lDR), and one of the MR. In addition, we quantified the expression of Fos in one section containing the SUM.

For the DTM, cells expressing Fos were counted within a region defined by a 120 $\mu m^2$ box placed on