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Research Report

Cholinergic projections to the suprachiasmatic nucleus and lower subparaventricular zone of diurnal and nocturnal rodents

Alexandra Castillo-Ruiz[a], *Antonio A. Nunez*[a,b,*]

[a]*Department of Psychology, Behavioral Neuroscience Interest Group, 108 Giltner Hall, Michigan State University, East Lansing, MI 48824, USA*
[b]*Neuroscience Program, Michigan State University, East Lansing, MI 48824, USA*

ARTICLE INFO

Article history:
Accepted 5 March 2007
Available online 12 March 2007

Keywords:
Acetylcholine
Vesicular acetylcholine transporter
Circadian
Suprachiasmatic nucleus
Lower subparaventricular zone

ABSTRACT

In nocturnal species cholinergic agonists alter circadian rhythm phase when injected intraventricularly or directly into the suprachiasmatic nucleus (SCN), but the phase shifts obtained differ depending upon the site being injected. Cholinergic projections reach the SCN of nocturnal laboratory rats, however, nothing is known about these projections in diurnal rodents. The first objective of this study was to evaluate the hypothesis that cholinergic projections to the SCN are only present in nocturnal species. The second objective was to evaluate the hypothesis that the lower part of the subparaventricular zone (LSPV) is a candidate for being a site that mediates the phase shifts observed when cholinergic agonists are injected intraventricularly. These hypotheses were tested in the diurnal unstriped Nile grass rat (*Arvicanthis niloticus*) and the nocturnal laboratory rat. Additionally, we evaluated if the light–dark (LD) cycle had an effect on the expression of the vesicular acetylcholine transporter (VAChT) in the SCN, LSPV, and in two control areas. Animals were kept in a 12:12 LD cycle and perfused at six time points. VAChT immunoreactivity was observed in the SCN, LSPV, and in the control areas of both species. The SCN and LSPV showed a differential distribution and density of cholinergic projections between the two species, but similar temporal patterns of VAChT expression were found across species. These results provide evidence for a differential cholinergic stimulation of the SCN between grass rats and laboratory rats that may reflect a rewiring of neural projections brought about by the adoption of a diurnal activity profile.

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* *Corresponding author*. Department of Psychology, Behavioral Neuroscience Interest Group, 108 Giltner Hall, Michigan State University, East Lansing, MI 48824, USA. Fax: +1 517 432 2744.
E-mail address: nunez@msu.edu (A.A. Nunez).

Abbreviations: 3V, third ventricle; ACh, acetylcholine; CalB, calcium binding protein D-28K; ChAT, choline acetyltransferase; HDB, horizontal limb of the diagonal band of Broca; -ir, immunoreactivity; LD, light–dark cycle; LH, lateral hypothalamus; LSPV, lower subparaventricular zone; OX, optic chiasm; Pir, piriform cortex; SCN, suprachiasmatic nucleus; sPVZ, subparaventricular zone; VAChT, vesicular acetylcholine transporter; VP, vassopresin; ZT, zeitgeber time

0006-8993/$ – see front matter © 2007 Published by Elsevier B.V.
doi:10.1016/j.brainres.2007.03.010

1. Introduction

The suprachiasmatic nucleus (SCN) is the master circadian pacemaker in mammals. In rats, the SCN receives cholinergic projections from the basal forebrain and brainstem (Bina et al., 1993), areas that are active during REM sleep and/or wakefulness (Lydic and Baghdoyan, 2005) and virtually silent during non-REM sleep (Lee et al., 2005a). The functional role of these cholinergic projections to the SCN is not completely understood.

At first it was argued that acetylcholine (ACh) had a role in the mediation of photic inputs to the SCN (Zatz and Brownstein, 1979). The evidence for this hypothesis consisted in the finding that intraventricular infusion of carbachol, a non-selective cholinergic agonist, was able to induce phase shifts in circadian rhythms similar to those produced by light pulses (Zatz and Brownstein, 1979; Zatz and Herkenham, 1981), i.e., phase delays when applied early in the subjective night and phase advances when applied late in the subjective night. Additionally, it was found that light increased ACh concentration in the SCN (Murakami et al., 1984).

In contrast, other findings provided evidence against a role of ACh in photic entrainment of rhythms. The retinal projection for photic entrainment, the retinohypothalamic tract (RHT), lacks cholinergic fibers (Shibata et al., 1986) and the phase shifts induced by carbachol are not accompanied by induction of the expression of the early gene *c-fos* in the SCN, as it is seen with light pulses (Colwell et al., 1993). Moreover, when carbachol was applied during the subjective night directly to the rat SCN brain slice (Liu and Gillette, 1996) or to the mouse SCN *in vitro* or *in vivo* (Buchanan and Gillette, 2005), only injections late in the subjective night produced phase advances.

As argued by Buchanan and Gillette (2005), it could be that the different outcomes obtained following the injection of cholinergic agonists in the ventricle (in early studies) or directed to the SCN (more recent studies) reflect the participation of an extra-SCN site that is only stimulated when the agonists are applied intraventricularly and produce effects similar to those produced by light pulses. This site would have to be located close to the third ventricle, project directly to the SCN, participate in circadian rhythmicity, and be cholinoceptive. The subparaventricular zone (sPVZ) meets most of the criteria for such a site. The sPVZ is located dorsal to the SCN flanking the third ventricle, projects to the SCN (Krout et al., 2002), receives a large proportion of inputs from the SCN (Watts and Swanson, 1987; Watts et al., 1987), and seems to participate in the regulation of circadian patterns of activity (Lu et al., 2001).

If cholinergic fibers reach the sPVZ, then this region could be a site where ACh acts to induce light-like phase shifts. The ventral part of the sPVZ, which we refer to as the lower subparaventricular zone (LSPV), shows remarkably different rhythms of cFos expression when diurnal grass rats are compared to nocturnal laboratory rats (Nunez et al., 1999). In constant darkness this rhythm persists in grass rats but not in laboratory rats (Schwartz et al., 2004). Additionally in grass rats, the LSPV shows a rhythmic expression of the clock genes *per1* and *per2* (Ramanathan et al., 2006). But nothing is known about the presence of cholinergic projections to the LSPV of nocturnal rodents or diurnal grass rats.

All the available data are consistent with the view that the molecular clock of the SCN and its phase relation to the light–dark cycle are the same in diurnal and nocturnal rodents (Caldelas et al., 2003; Lambert et al., 2005). Since nocturnal and diurnal species display sleep and wakefulness at different phases of the light dark cycle, any cholinergic stimulation of the SCN associated with stages of vigilance (i.e., wakefulness, non-REM sleep, and REM sleep) should impact the clock at opposite phases of its cycle depending on whether an animal is diurnal or nocturnal and could result in different physiological and/or behavioral outcomes across species. In diurnal mammals, however, cholinergic inputs to the SCN have not been described, and thus it is possible that any feedback to the SCN mediated by cholinergic inputs is restricted to nocturnal species.

In the present study, we used vesicular acetylcholine transporter immunoreactivity (VAChT-ir) to visualize and quantify the cholinergic projections to the SCN and the LSPV of grass rats and laboratory rats. We also evaluated the density of VAChT immunoreactive fibers in two "control" areas that are not directly involved in the generation of circadian rhythmicity, the lateral hypothalamus (LH) and the piriform cortex (Pir).

The VAChT is a protein that participates in the life cycle of ACh. The role of VAChT is to transport ACh to synaptic vesicles in axon terminals. When used as an immunocytochemical marker of cholinergic neuronal phenotype, VAChT not only stains cell bodies but also stains terminal fields (Arvidsson et al., 1997). The fact that axon terminals are clearly labeled with VAChT is extremely important for this study since cholinergic fibers in the SCN of nocturnal rodents were reported to be of very fine caliber (Bina et al., 1993). Additionally, VAChT is a protein with an amino acid sequence that has been highly conserved in mammalian and non-mammalian species (Usdin et al., 1995) and it is likely to serve the same function across rodent species.

Here we use the density of fibers immunopositive for VAChT as an indirect measure of cholinergic activity across the light–dark cycle in the SCN, LSPV, and two control areas (the LH and Pir) of grass rats and laboratory rats. There is evidence for a close relationship between synthesis of ACh and production of VAChT. The gene that codes for the production of VAChT is contained in the gene that codes for the enzyme involved in the synthesis of ACh (i.e., choline acetyltransferase or ChAT; Usdin et al., 1995), and the expression of both proteins seems to be coregulated (Berrard et al., 1995; Eiden, 1998). Moreover, the expression of ChAT in cholinergic terminal fields in the cingulate cortex of rats was found to be higher when animals were active (Greco et al., 1999). Thus, if the enzyme that synthesizes ACh is most abundant when animals are awake, and ChAT and VAChT are coregulated, it seemed reasonable to infer that more VAChT should be associated with more ACh in axon terminals.

In summary, the present study served to evaluate two hypotheses. First is that cholinergic projections to the SCN are only present in nocturnal species. Second is that the LSPV is a suitable candidate for being a cholinoceptive site that mediates the light-like phase response obtained when cholinergic agonists are delivered to the third ventricle. In addition, we determined if the light–dark cycle had an effect on the abundance of VAChT-ir in the SCN, the LSPV, the LH, and the Pir of laboratory rats and grass rats.

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