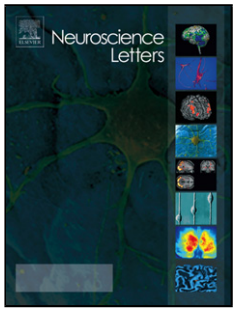

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Activation of M1/4 receptors phase advances the hamster circadian clock during the day

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Highlights

- The cholinergic agonist carbachol produces phase advances in the circadian clock
- These phase shifts are mimicked by the M1/4 selective agonist McN-A-343
- The M2/3 muscarinic agonist bethanechol does not affect clock phase
- Daytime cholinergic advances of the circadian clock are mediated through M1/4 receptors

Abstract

The mammalian circadian clock in the suprachiasmatic nucleus (SCN) can be reset by the cholinergic agonist carbachol. In hamsters, intraSCN carbachol produces phase advances during the day. This phenomenon has previously been attributed to the muscarinic receptors, as carbachol-induced phase shifts are blocked by pretreatment with the muscarinic antagonist atropine. The SCN contains all five muscarinic receptors, leaving open the question as to which muscarinic receptors mediate these shifts. Here we test two selective muscarinic agonists, the M1/4 agonist McN-A-343 and the M2/3 agonist bethanechol, in addition to the non-selective cholinergic agonist carbachol. Consistent with previous reports, carbachol produced significant phase advances when injected to the SCN during the mid-subjective day. At the doses used here, McN-A-343, but not bethanechol, also produced significant phase shifts when injected to the SCN during the mid-subjective day. Phase shifts to McN-A-343 were as large as those produced by carbachol, suggesting that activation of the M1/4 receptors alone can fully account for the daytime phase advances produced by cholinergic agonists. Given acetylcholine's role in arousal, and the similarity between phase advances to carbachol/McN-A-343 and to exercise and arousal manipulations, it is possible that acetylcholine may contribute to non-photic resetting of the circadian clock.

Keywords: circadian; suprachiasmatic nucleus; acetylcholine; non-photic; muscarinic

Abbreviations:

CT	circadian time
i.c.v.	intracerebroventricular
IGL	intergeniculate leaflet
NPY	Neuropeptide Y
PBS	phosphate buffered saline
RM ANOVA	Repeated Measures Analysis of Variance
SCN	suprachiasmatic nucleus

1.0 Introduction

The suprachiasmatic nucleus (SCN) is the master circadian pacemaker in mammals that regulates daily rhythms in physiology and behavior [3, 4]. The phase of the circadian clock can be reset by photic [4] and non-photic cues [1, 32, 39]. Key among the non-photic cues are manipulations that increase arousal. In hamsters these manipulations include wheel running [10], dark pulses [11] and sleep deprivation [1]. These treatments elicit large phase advances during the mid-subjective day and small phase delays during the late-subjective night (i.e., the normal rest phase for hamsters).

The neurochemical basis for non-photic phase shifting has been investigated and a number of neurotransmitters have been implicated as contributing to these phase shifts. Notably, neuropeptide Y (NPY) innervation from the intergeniculate leaflet (IGL) [6, 20] appears to contribute to non-photic phase shifts. However, other neurotransmitters may also contribute. One neurotransmitter that has received very little attention in this respect is acetylcholine. The SCN receives cholinergic innervation from a number of cholinergic areas including the basal forebrain [8]. Cholinergic neurons in the basal forebrain are involved in producing wakefulness and arousal [23]. The SCN contains numerous cholinergic receptors, including α7 and α4 nicotinic receptors [35] and M1, M2, M3, M4 and M5 muscarinic receptors [9, 40]. The non-selective cholinergic agonist carbachol has a wide range of effects on the circadian clock, with results differing according to injection site (intracerebroventricular (i.c.v.) or intraSCN), species (rats, mice or hamsters) and technique (shifting locomotor rhythms *in vivo* versus shifting electrical rhythms *in vitro*) [12]. In hamsters, intraSCN or i.c.v. carbachol produces phase advances when injected during the mid-subjective day [7, 30], consistent with it playing a non-photic role at this time of the day. Carbachol also produces non-photic type phase shifts when injected to the IGL

[13], an area which gives rise to NPY input to the SCN and has been implicated in non-photic phase shifting [21, 22, 27, 33, 34]. These data suggest that one role of cholinergic input to the circadian system, particularly during the rest phase, may be to mediate non-photic responses. If this hypothesis is true, then determining the receptors underlying these responses would be useful. The phase advances to daytime intraSCN carbachol are blocked by pretreatment with the muscarinic antagonist atropine, but not by the nicotinic antagonist mecamylamine [7] indicating that these daytime advances are mediated through muscarinic receptors.

Since all the muscarinic receptors are found in the SCN [40], and since atropine blocks all muscarinic receptor subtypes, it is not clear which receptors might mediate these non-photic phase shifts. Electrophysiological evidence from the rat suggests that the M1 and M4 receptors may play a role in carbachol's effects on electrical activity in the SCN. When applied to the SCN, more than half of neurons exhibit inhibition of their firing while a quarter exhibit enhanced firing [40]. These effects are blocked to a large extent by M4 antagonists and to a lesser extent by M1 antagonists, but not by M2/3 antagonists. Furthermore, the effects of carbachol on SCN electrical activity are mimicked by the M1/4 agonist McN-A-343 [40]. Here we test the hypothesis that activation of M1/4 muscarinic receptors, but not of M2/3 receptors, in the hamster SCN will elicit phase advances of the circadian clock.

2.0 Materials and Methods

2.1 Animals and Housing

Male Syrian hamsters (n=37; *Mesocricetus auratus*, ~90 g upon arrival in the lab) obtained from Charles River Labs (Kingston, NY) were used in this study. Hamsters were initially housed in groups of two or three and maintained under a 14:10 light:dark cycle. Cage level illuminance was approximately 300 lux. Animals had access to food and water *ad libitum*.

Following cannula implantation, each animal was allowed to recover on its own for a minimum of one week. After this recovery period, hamsters were transferred to individual polycarbonate cages (20 x 45 x 22 cm) containing running wheels (14 cm in diameter), and were maintained in constant darkness for the duration of the studies. All protocols were approved by the Life and Environmental Sciences Animal Care Committee at the University of Calgary and adhered to the Canadian Council on Animal Care guidelines.

2.2 Behavioral Phase Shifts

Wheel-running activity detected by closure of magnetic switches mounted to running wheels was continuously recorded by a computer running the Clocklab data collection software (Coulbourn Instruments, Allentown, PA). Actograms were generated and analyzed using Clocklab Analysis software. Prior to the start of manipulations, the hamsters were allowed to free run for a minimum of 10 days.

Clocklab's automatic fitting function was used to create best fit regression lines to activity onsets for the 10 days prior to and including the manipulation day, and the 10 days following the manipulation day, beginning on the fourth day so as not to be affected by transient onsets following treatment. The horizontal difference between the predicted onsets from both regression lines on the day following the manipulation day was used to determine the degree to which each hamster phase shifted. Night-vision goggles (BG15Alista, Richmond Hill, Ontario, Canada) were used during all injections.

2.3 Cannula Implantation

Cannula implantation to permit injections directly to the SCN took place approximately one week after hamsters arrived into their new environment, or when the animal weighed roughly 110 g. A subcutaneous injection of the analgesic butorphanol (2 mg/kg; Wyeth) was

administered prior to surgery, followed by an intraperitoneal injection of sodium pentobarbital (120 mg/kg; CEVA) for anesthesia. Hamsters were stereotaxically implanted with a 22 gauge stainless steel cannula (9 mm long, Plastics One, Roanoke, VA) cemented to the skull with dental acrylic. The cannula was aimed at the SCN (coordinates: at the same rostral caudal level as bregma, 0.3 mm lateral to the midline, and 7.0 mm ventral to the skull surface, with the incisor bar set at 2 mm below the interaural level). The tip of the injection cannula extended 1 mm beyond the end of the guide cannula.

Cannula placements were verified for all experiments via histology. Hamsters were deeply anesthetized with sodium pentobarbital and were perfused intracardially using 100 ml of phosphate buffered saline (PBS), followed by 100 ml of buffered 4% paraformaldehyde. Brains were postfixed in 4% paraformaldehyde overnight, and then transferred to 20% sucrose for a minimum of 24 h. Alternate cryostat sections (35 μm) of the third ventricle were collected and stained with cresyl violet. Animals were excluded if the tip of the cannula was >500 μm from the SCN, within the third ventricle, or caused excessive SCN damage.

2.4 Drugs and Injections

All drugs were obtained from Sigma-Aldrich (St. Louis, MO, USA) and were dissolved in sterile phosphate buffered saline. The non-selective cholinergic agonist carbachol was dissolved to a final concentration of 10 mM. The selective M1/4 muscarinic receptor agonist McN-A-343 (C7041, (4-Hydroxy-2-butynyl)-1-trimethylammonium-3-chlorocarbanilate chloride) [16, 18, 29] was dissolved to a final concentration of 79 mM. The selective M2/3 muscarinic receptor agonist bethanechol (C5259, carbamyl-β-methylcholine chloride) [16, 29, 36] was dissolved to a final concentration of 212 mM.

Animals received a treatment approximately once every two weeks. A regression line was fit to activity onset times for 10 days to predict activity onset on the manipulation day. Injections were given 6 hours prior to projected activity onset (i.e., at circadian time (CT) 6, where CT12 is defined as activity onset by convention). Animals were removed from their home-cages, and were gently restrained. The dummy cannula was removed and an injection cannula inserted into the implanted guide cannula. They then received a 0.5 µl injection using a 1 µl Hamilton syringe connected to the injector by PE20 tubing. The injection was performed slowly at a rate of 1 µl/min, and the injector was left in place for 1 minute following the injection to permit diffusion of the bolus. The dummy cannula was then replaced and the animal was returned to its home-cage.

2.5 Experiment #1

To confirm the phase shifting properties of carbachol during the mid-subjective day, hamsters (n=18) were implanted with a cannula aimed at the SCN. Each animal received injections of carbachol (0.5 µl of 10 mM solution) or vehicle control (sterile PBS) in a counterbalanced fashion separated by approximately 2 weeks.

A separate set of hamsters (n=8) were used to investigate phase shifting properties of the selective M1/4 muscarinic receptor agonist McN-A-343 [16, 18, 29]. Each animal received injections of McN-A-343 (0.5 µl of a 79 mM solution) or vehicle control (sterile PBS) in a counterbalanced fashion separated by approximately 2 weeks.

2.6 Experiment #2

To investigate possible interactions between M1/4 and M2/3 muscarinic receptors, we used a 2 X 2 repeated measures design where animals (n=11) received 4 different treatments, each separated by approximately 2 weeks and delivered in a counterbalanced fashion.

Treatments were: 1) vehicle control (sterile PBS), 2) bethanechol alone (0.5 µl, 212 mM), 3) McN-A-343 alone (0.5 µl, 79 mM), and 4) a 0.5 µl cocktail of McN-A-343 (79 mM) and bethanechol (212 mM).

2.7 Statistical Analysis

In experiment #1, results for each drug relative to its vehicle control were analyzed with paired *t*-tests. Effects of the two drugs were compared using an independent-samples *t*-test. For experiment #2, a 2 X 2 (McN-A-343 or vehicle X bethanechol or vehicle) repeated measures analysis of variance (RM ANOVA) was used to examine the results. To ensure that questions central to addressing the hypotheses were examined, the following planned comparisons were included: drug versus its vehicle, and each drug versus one-another. Animals were excluded from analysis if their phase shifts to the control treatment was aberrant (>2 standard deviations different that the mean phase shift for the control). In no cases were animals excluded based on their response to the drug treatments. All means are reported ± standard error of the mean.

3.0 Results

3.1 Experiment #1

Of the 18 animals to complete the carbachol experiment, six animals were excluded from the carbachol analysis due to missed cannula placements, three were excluded due to excessive damage to the SCN, and two were excluded due to a large phase shift to their PBS control injections (2.1h and 1.58h), thus leaving a sample size of n=7. Of the eight animals to complete the McN-A-343 experiment, one animal was excluded from the McN-A-343 analysis due to a missed placement, thus leaving a final sample size of n=7. In each case, all animals received both a drug and vehicle control treatment in a counterbalanced fashion.

Consistent with previous reports [7, 30], carbachol elicited significant phase advances when injected to the SCN at CT6 (paired *t*-test, $t_{(6)}$=5.57, p=0.0014, Figure 1A,C). The selective M1/4 receptor agonist McN-A-343 [16] also induced significant phase advances of circadian locomotor rhythms when injected to the SCN at CT6 (paired *t*-test, $t_{(6)}$=2.52, p=0.045, Figure 1B,C). Phase shifts elicited by our doses of McN-A-343 were not significantly smaller than those to carbachol (independent samples *t*-test, $t_{(12)}$=0.944, p=0.364, Figure 1C).

3.2 Experiment #2

Of the eleven hamsters to complete all treatment conditions, six animals were excluded due to missed placements, leaving a sample size of n=5. All animals received all 4 treatment conditions in a counterbalanced fashion.

To investigate the role of activation of M2/3 receptors, alone or in addition to M1/4 activation by McN-A-343, animals were treated with the selective M2/3 agonist bethanechol [16], alone or in combination with the M1/4 agonist McN-A-343. Similar to experiment #1, McN-A-343 elicited significant phase advances when injected to the SCN at CT6 (two-way RM ANOVA, main effect of McN-A-343, $F_{(1,7)}$=13.682, p=0.008; Figure 2A,B). Bethanechol did not elicite significant phase shifts on its own at the dose employed here (two-way RM ANOVA, main effect of bethanechol, $F_{(1,7)}$=2.986, p=0.128; Figure 2A,B). Similarly, the planned comparison of bethanechol versus its PBS control revealed no significant effect (p=0.946). Furthermore, there was no significant interaction between McN-A-343 and bethanechol ($F_{(1,7)}$=2.94, p=0.13; Figure 2A,B).

4.0 Discussion

Consistent with previous reports [7, 30], we observed that the non-selective cholinergic agonist carbachol produced phase advances of the circadian clock when applied to the hamster

SCN during the mid-subjective day. This phenomenon had been previously narrowed down to the muscarinic class of receptors [7]. Here we present evidence consistent with these daytime advances being produced by activation of the M1/4 muscarinic receptors. Specifically, at the doses examined here, the M1/4 agonist McN-A-343, but not the M2/3 agonist bethanechol, was able to produce phase advances of circadian locomotor rhythms when applied to the SCN during the mid-subjective day. This matches electrophysiological evidence for the rat, when the effects of carbachol on SCN electrical activity are blocked by M1 and M4 antagonists, but not by M2/3 antagonists, and are mimicked by the M1/4 agonist McN-A-343 [40].

Non-photic phase shifts are characterized by large advances during the subjective day and small delays in the subjective night [10]. The fact that phase shifts to exercise or sleep deprivation only occur when they applied during the rest phase [1, 10] suggest the involvement of an arousal system such as the cholinergic basal forebrain [23]. In fact, entrainment to another non-photic manipulation, a cognitive task, in rats requires the cholinergic basal forebrain [19].

Both McN-A-343 and carbachol produce inhibition of SCN electrical activity in over half of SCN cells [40]. Other chemicals implicated in non-photic phase shifts produce similar effects. NPY predominantly inhibits spontaneous firing of SCN neurons [24]. Serotonin agonists can also inhibit the spontaneous firing rate of SCN neurons [37, 41]. This inhibition of firing rate mirrors an inhibition of immediate-early gene expression (i.e., cFOS [1, 31]), and inhibition of *period* gene expression [28, 39] and a downregulation of the mitogen-activated protein kinase pathway [5, 15] in the SCN by non-photic manipulations. It has been suggested that inhibition of SCN activity at the electrical, biochemical and molecular levels at times of the day when the SCN has a high level of activity may be the final common pathway underlying the various forms of non-photic phase shifts [32].

Daytime exercise or sleep deprivation procedures typically elicit phase advances of up to 3h [1, 10]. This is in contrast to much smaller non-photic phase shifts elicited by cholinergic agonists observed here and elsewhere [7, 30], NPY [6, 20], and with serotonin agonists *in vivo* [2, 38]. It is possible that simultaneous release of acetylcholine, NPY and serotonin at the SCN may be necessary to elicit full-magnitude phase shifts. Cocktail application of agonists to these various neurotransmitters may help resolve this question. Additionally, while a dose response curve for daytime carbachol demonstrates that phase shifts plateau at about 1h [30], no such dose response curve exists for McN-A-343, and thus the role of the M1/4 receptors in these daytime advances should be interpreted cautiously until more doses have been examined.

While the current results suggest that M1 and/or M4 receptors underlie the observed phase advances, other complimentary approaches should be applied to confirm this. Selective antagonists could be applied prior to carbachol application, similar to how atropine was used previously [7]. Furthermore, if acetylcholine is necessary for non-photic phase shifting, then application of antagonists to the SCN prior to non-photic manipulations should attenuate the resulting phase shifts. Additionally, the effects of intraSCN McN-A-343 should be examined at other phases to determine how well it matches other non-photic phase response curves (PRC). The PRC to carbachol does differ from some other non-photic PRCs [30], and even resembles the photic PRC in some studies [17], possibly reflecting its indiscriminate pharmacology.

The applicability of these findings to other species may be limited. The investigation of acetylcholine's role in the circadian system has a complicated history. Early reports suggested that acetylcholine might mediate photic responses in the circadian system [42-44]. *In vitro* work from the rat confirmed this [25, 26]. However, other groups have called this suggestion into question [14]. More recently, it has been suggested that carbachol does not produce photic-like

responses by directly activating the SCN, but rather by acting outside the SCN to produce photic-like effects [12]. Future *in vivo* studies aimed at investigating the role of acetylcholine in circadian rhythms may benefit from using McN-A-343 as a more specific agonist instead of carbachol, depending on the results from a McN-A-343 PRC.

5.0 Conclusions

Acetylcholine a major neurotransmitter underlying arousal in the mammalian brain [23]. Given that the SCN receives cholinergic innervation from the basal forebrain [7], and that carbachol and McN-A-343 produce non-photic like phase-advances when injected to the SCN during the mid-subjective day, it is possible that acetylcholine may play a larger role in regulating the effects of arousal on the circadian system, and that this may depend more on the M1/4 muscarinic receptors than M2/3 subtypes.

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References

[1] M.C. Antle, R.E. Mistlberger, Circadian clock resetting by sleep deprivation without exercise in the Syrian hamster, J Neurosci 20 (2000) 9326-9332.

[2] M.C. Antle, M.D. Ogilvie, G.E. Pickard, R.E. Mistlberger, Response of the mouse circadian system to serotonin 1A/2/7 agonists in vivo: surprisingly little, J Biol Rhythm 18 (2003) 145-158.

[3] M.C. Antle, R. Silver, Orchestrating time: arrangements of the brain circadian clock, Trends Neurosci 28 (2005) 145-151.

[4] M.C. Antle, V.M. Smith, R. Sterniczuk, G.R. Yamakawa, B.D. Rakai, Physiological responses of the circadian clock to acute light exposure at night, Rev Endocr Metab Disord 10 (2009) 279-291.

[5] M.C. Antle, F. Tse, S.J. Koke, R. Sterniczuk, K. Hagel, Non-photic phase shifting of the circadian clock: role of the extracellular signal-responsive kinases I/II/mitogen-activated protein kinase pathway, Eur J Neurosci 28 (2008) 2511-2518.

[6] S.M. Biello, D. Janik, N. Mrosovsky, Neuropeptide Y and behaviorally induced phase shifts, Neuroscience 62 (1994) 273-279.

[7] K.G. Bina, B. Rusak, Muscarinic receptors mediate carbachol-induced phase shifts of circadian activity rhythms in Syrian hamsters, Brain Res 743 (1996) 202-211.

[8] K.G. Bina, B. Rusak, K. Semba, Localization of cholinergic neurons in the forebrain and brainstem that project to the suprachiasmatic nucleus of the hypothalamus in rat, J Comp Neurol 335 (1993) 295-307.

[9] K.G. Bina, B. Rusak, M. Wilkinson, Daily variation of muscarinic receptors in visual cortex but not suprachiasmatic nucleus of Syrian hamsters, Brain Res 797 (1998) 143-153.

[10] K.J. Bobrzynska, N. Mrosovsky, Phase shifting by novelty-induced running: activity dose-response curves at different circadian times, J Comp Physiol A 182 (1998) 251-258.

[11] Z. Boulos, B. Rusak, Circadian phase response curves for dark pulses in the hamster, J Comp Physiol 146 (1982) 411-417.

[12] G.F. Buchanan, M.U. Gillette, New light on an old paradox: site-dependent effects of carbachol on circadian rhythms, Exp Neurol 193 (2005) 489-496.

[13] S.W. Cain, M. Verwey, M. Szybowska, M.R. Ralph, J.S. Yeomans, Carbachol injections into the intergeniculate leaflet induce nonphotic phase shifts, Brain Res 1177 (2007) 59-65.

[14] C.S. Colwell, C.M. Kaufman, M. Menaker, Phase-shifting mechanisms in the mammalian circadian system: new light on the carbachol paradox, J Neurosci 13 (1993) 1454-1459.

[15] A.N. Coogan, H.D. Piggins, Dark pulse suppression of P-ERK and c-Fos in the hamster suprachiasmatic nuclei, Eur J Neurosci 22 (2005) 158-168.

[16] C. Dallanoce, M. De Amici, E. Barocelli, S. Bertoni, B.L. Roth, P. Ernsberger, C. De Micheli, Novel oxotremorine-related heterocyclic derivatives: Synthesis and in vitro pharmacology at the muscarinic receptor subtypes, Bioorg Med Chem 15 (2007) 7626-7637.

[17] D.J. Earnest, F.W. Turek, Neurochemical basis for the photic control of circadian rhythms and seasonal reproductive cycles: role for acetylcholine, Proc Natl Acad Sci U S A 82 (1985) 4277-4281.

[18] K.W. Figueroa, M.T. Griffin, F.J. Ehlert, Selectivity of agonists for the active state of M1 to M4 muscarinic receptor subtypes, J Pharmacol Exp Ther 328 (2009) 331-342.

[19] H.J. Gritton, A.M. Stasiak, M. Sarter, T.M. Lee, Cognitive performance as a zeitgeber: cognitive oscillators and cholinergic modulation of the SCN entrain circadian rhythms, PLoS One 8 (2013) e56206.

[20] K.L. Huhman, H.E. Albers, Neuropeptide Y microinjected into the suprachiasmatic region phase shifts circadian rhythms in constant darkness, Peptides 15 (1994) 1475-1478.

[21] D. Janik, N. Mrosovsky, Gene expression in the geniculate induced by a nonphotic circadian phase shifting stimulus, Neuroreport 3 (1992) 575-578.

[22] D. Janik, N. Mrosovsky, Intergeniculate leaflet lesions and behaviorally-induced shifts of circadian rhythms, Brain Res 651 (1994) 174-182.

[23] B.E. Jones, Modulation of cortical activation and behavioral arousal by cholinergic and orexinergic systems, Ann N Y Acad Sci 1129 (2008) 26-34.

[24] S.Y. Liou, H.E. Albers, Single unit response of neurons within the hamster suprachiasmatic nucleus to neuropeptide Y, Brain Res Bull 27 (1991) 825-828.

[25] C. Liu, J.M. Ding, L.E. Faiman, M.U. Gillette, Coupling of muscarinic cholinergic receptors and cGMP in nocturnal regulation of the suprachiasmatic circadian clock, J Neurosci 17 (1997) 659-666.

[26] C. Liu, M.U. Gillette, Cholinergic regulation of the suprachiasmatic nucleus circadian rhythm via a muscarinic mechanism at night, J Neurosci 16 (1996) 744-751.

[27] E.G. Marchant, N.V. Watson, R.E. Mistlberger, Both neuropeptide Y and serotonin are necessary for entrainment of circadian rhythms in mice by daily treadmill running schedules, J. Neurosci. 17 (1997) 7974-7987.

[28] E.S. Maywood, N. Mrosovsky, M.D. Field, M.H. Hastings, Rapid down-regulation of mammalian period genes during behavioral resetting of the circadian clock, Proc Natl Acad Sci U S A 96 (1999) 15211-15216.

[29] M. McKinney, J.H. Miller, V.A. Gibson, L. Nickelson, S. Aksoy, Interactions of agonists with M2 and M4 muscarinic receptor subtypes mediating cyclic AMP inhibition, Mol Pharmacol 40 (1991) 1014-1022.

[30] J.H. Meijer, E. van der Zee, M. Dietz, The effects of intraventricular carbachol injections on the free-running activity rhythm of the hamster, J Biol Rhythms 3 (1988) 333-348.

[31] J.D. Mikkelsen, N. Vrang, N. Mrosovsky, Expression of Fos in the circadian system following nonphotic stimulation, Brain Res Bull 47 (1998) 367-376.

[32] R.E. Mistlberger, M.C. Antle, Entrainment of circadian clocks in mammals by arousal and food, Essays Biochem 49 (2011) 119-136.

[33] N. Mrosovsky, Locomotor activity and non-photic influences on circadian clocks, Biol Rev Camb Philos Soc 71 (1996) 343-372.

[34] N. Mrosovsky, A non-photic gateway to the circadian clock of hamsters, Ciba Found Symp 183 (1995) 154-167.

[35] B.F. O'Hara, D.M. Edgar, V.H. Cao, S.W. Wiler, H.C. Heller, T.S. Kilduff, J.D. Miller, Nicotine and nicotinic receptors in the circadian system, Psychoneuroendocrinology 23 (1998) 161-173.

[36] N.A. Sharif, Z.P. To, K.H. Wong, R.E. Delmendo, R.L. Whiting, R.M. Eglen, M3 muscarinic receptors on murine HSDM1C1 cells: Further functional, regulatory, and receptor binding studies, Neurochem Res 20 (1995) 61-68.

[37] S. Shibata, S.Y. Liou, S. Ueki, Effect of amino acids and monoamines on the neuronal activity of suprachiasmatic nucleus in hypothalamic slice preparations, Jpn J Pharmacol 33 (1983) 1225-1231.

[38] K. Tominaga, S. Shibata, S. Ueki, S. Watanabe, Effects of 5-HT_{1A} receptor agonists on the circadian rhythm of wheel- running activity in hamsters, Eur J Pharmacol 214 (1992) 79-84.

[39] I.C. Webb, M.C. Antle, R.E. Mistlberger, Regulation of circadian rhythms in mammals by behavioral arousal, Behav Neurosci 128 (2014) 304-325.

[40] J.J. Yang, Y.T. Wang, P.C. Cheng, Y.J. Kuo, R.C. Huang, Cholinergic modulation of neuronal excitability in the rat suprachiasmatic nucleus, J Neurophysiol 103 (2010) 1397-1409.

[41] S.-W. Ying, B. Rusak, Effects of serotonergic agonists on firing rates of photically responsive cells in the hamster suprachiasmatic nucleus, Brain Res 651 (1994) 37-46.

[42] M. Zatz, M.J. Brownstein, Injection of alpha-bungarotoxin near the suprachiasmatic nucleus blocks the effects of light on nocturnal pineal enzyme activity, Brain Res 213 (1981) 438-442.

[43] M. Zatz, M.J. Brownstein, Intraventricular carbachol mimics the effects of light on the circadian rhythm in the rat pineal gland, Science 203 (1979) 358-361.

[44] M. Zatz, M.A. Herkenham, Intraventricular carbachol mimics the phase-shifting effect of light on the circadian rhythm of wheel-running activity, Brain Res 212 (1981) 234-238.

Figure Captions

Figure 1 A) Representative actogram depicting running wheel activity from an animal given an intraSCN injection of either vehicle (white circle) or carbachol (Carb, grey circle, 0.5 µl, 10 mM) at CT6. Vertical black marks reflect proportional amount of wheel running in a 10 minute bin. Each horizontal line depicts the activity for a particular day, with the subsequent day plotted both below. Diagonal lines depict regression lines fit to activity onsets both before and after the manipulation, and the horizontal distance between these lines on the day following the manipulation was used to calculate the magnitude of the resulting phase shift. B) Representative actograms from an animal given an intraSCN injection of either vehicle (white circle) or McN-A343 (McN, grey circle, 0.5 µl, 79 mM) at CT6. C) Mean (±SEM) phase shifts for carbachol (Carb) and McN-A-343 (McN) relative to their own vehicle control treatments. ** $p>0.01$, * $p>0.05$

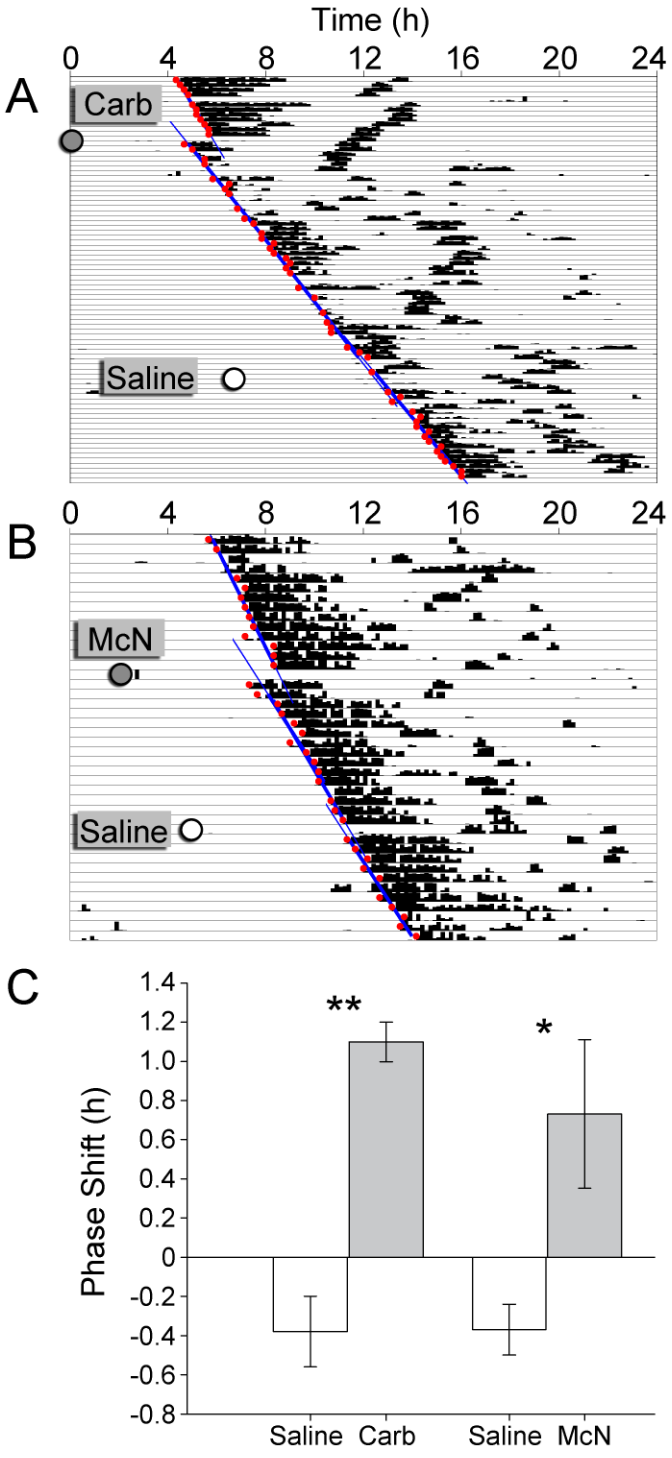

Figure 2 A) Representative actogram depicting running wheel activity from an animal given an intraSCN injection of vehicle (white circle), bethanechol (Beth, light gray circle, 0.5 µl, 212 nM), McN-A-343 (McN, medium grey circle, 0.5 µl, 79 mM) or a cocktail of McN-A-343 and bethanechol (McN+Beth, dark grey circle) at CT6. B) Mean (±SEM) phase shifts for the various treatments. * $p>0.01$