Low acetylcholine during early sleep is important for motor memory consolidation

Samsoon Inayat[1,3], Qandeel[1,3], Mojtaba Nazariahangarkolaee[1], Surjeet Singh[1], Bruce L. McNaughton[1,2], Ian Q. Whishaw[1], and Majid H. Mohajerani[1,4]

1. Canadian Centre for Behavioural Neuroscience, University of Lethbridge, Lethbridge, Alberta, Canada
2. Center for the Neurobiology of Learning and Memory, University of California, Irvine, California, USA
3. These authors contributed equally
4. Corresponding author: mohajerani@uleth.ca

Abstract: Sleep rejuvenates the body and plays a role in consolidating newly encoded memory for long-term storage. Evidence suggests that early sleep, featuring reduced cholinergic tone and slow-wave activity, is important for consolidating episodic memory. The relationship between procedural memory consolidation, early sleep, and acetylcholine level has not been investigated. We hypothesized that increasing acetylcholine levels during early sleep would disrupt the consolidation of procedural memory as measured by rotarod and skilled reaching tasks. Separate groups of adult mice were trained on the tasks and after training, were given physostigmine, an acetylcholinesterase inhibitor, to increase cholinergic tone in subsequent sleep. Post sleep testing showed that physostigmine impaired motor performance. Home-cage video monitoring and electrophysiology revealed that physostigmine disrupted sleep structure in the first hour after injection, delayed slow-wave sleep onset, and reduced slow-wave power in the hippocampus and cortex. The impaired motor performance with physostigmine, however, was not due to its effects on sleep structure, as one hour of sleep deprivation after training did not impair performance. A reduction in cholinergic tone using chemogenetic inactivation of cholinergic neurons during early sleep also did not affect rotarod performance. Finally, administration of agonists and antagonists of muscarinic and nicotinic acetylcholine receptors revealed that it was the activation of muscarinic receptors during early sleep that impaired rotarod performance. The experiments support the idea that the increased slow wave activity of early sleep and associated inactivation of muscarinic receptors due to reduced acetylcholine contribute to procedural memory consolidation.

Significance statement: Altered acetylcholine levels or modified expression of acetylcholine receptors exist in mental/neurological disorders such as Alzheimer's, Parkinson's, and Huntington disease and schizophrenia. Our study contributes to the understanding of the role of low acetylcholine levels and increased slow-wave EEG sleep for procedural memory consolidation. We show that increasing acetylcholine levels during early sleep dominated by slow waves disrupts motor memory consolidation. An important implication of our study is the importance of sleep onset to the timing of the administration of therapeutic drugs (existing and in development) for diseases that alter acetylcholine levels and impair memory.

INTRODUCTION

Memory formation consists of two processes; encoding which involves stimulus-induced gene-expression within cells and consolidation which happens through formation and strengthening of synaptic connections (Ebbinghaus 1885-Republished Translation 1964, Jenkins and Dallenbach 1924, McGaugh 2000, Dudai 2004, Frankland and Bontempi 2005, Diekelmann and Born 2010, Clopath 2012, Rasch and Born 2013, Yang, Lai et al. 2014, Dudai, Karni et al. 2015, Chambers 2017). Memory consolidation can happen in both awake and sleep states, can last from seconds to years, and might involve different processes for episodic, memory for facts and experiences, and procedural, memory for perceptual conditioning and motor skill (Clopath 2012, Rasch and Born 2013, Chambers 2017). A growing interest is on the sleep effects on memory consolidation. Procedural and episodic memories might be consolidated during rapid eye movement (REM) or non-REM (NREM) phases/stages of sleep respectively (Yaroush, Sullivan et al. 1971, Plihal and Born 1997), or through cyclic succession of NREM and REM (Giuditta, Ambrosini et al. 1995, Ambrosini and Giuditta 2001). Reactivation of memories during sleep (Wilson and McNaughton 1994, Kudrimoti, Barnes et al. 1999, Sutherland and McNaughton 2000, Kali and Dayan 2004, Euston, Tatsuno et al. 2007, Buhry, Azizi et al. 2011, Tononi and Cirelli 2014, Yang, Lai et al. 2014), formation and strengthening of spines during NREM and REM phases of sleep, and synapse pruning during REM sleep might also play a role (Yang, Lai et al. 2014, Li, Ma et al. 2017). The synaptic homeostasis theory of memory consolidation suggests a mechanism through which synaptic strengthening could occur. It proposes that low levels of neuromodulators such as acetylcholine, dopamine, serotonin, during NREM sleep permit overall synaptic downscaling while allowing recently activated synapses to be relatively more active thus improving signal-to-noise and thereby providing a mechanism for synaptic

stabilization, memory replay, and memory consolidation (Tononi and Cirelli 2003, Tononi and Cirelli 2014).

Consistent with the synaptic homeostasis theory, low levels of acetylcholine (ACh) allow synaptic pruning/formation and episodic memory consolidation during NREM sleep (Hasselmo 1999, Walker 2009, Alger, Chambers et al. 2015, Miyamoto, Hirai et al. 2017). Whether procedural memory consolidation is similarly enhanced by low ACh levels is uncertain. Studies on the effects of lesions that lower cortical ACh on motor learning have produced mixed results (Conner, Culberson et al. 2003; Gharbawie and Whishaw 2003). Other evidence suggests that increasing cholinergic tone in humans during post-learning NREM does not affect consolidation of procedural memories (Gais and Born 2004). Nevertheless, disrupting cholinergic levels through sleep deprivation has also been shown to affect motor memory. For example, reactivation of motor task-specific neurons during NREM sleep is involved in forming new synapses after motor learning in mice but consolidation is impaired in sleep deprived animals (Yang, Lai et al. 2014). Furthermore, there is a proposed relationship between the amount of slow-wave activity (SWA) during NREM sleep and motor learning. For example, humans learning a reaching task display local increases in SWA in the parietal cortex (Landsness, Ferrarelli et al. 2011) whereas arm immobilization induces a local decrease in SWA (Huber, Ghilardi et al. 2006). SWA also significantly improves visual texture discrimination skills (Gais, Plihal et al. 2000). In mice, performance of a skilled forelimb reaching for food task increased local SWA in motor cortex (Hanlon, Faraguna et al. 2009). In sum, this evidence points to a role for decreased cholinergic tone during NREM sleep in procedural memory consolidation, but a more definitive conclusion requires a direct test of the idea.

In the present study, we investigated the role of cholinergic activity during early NREM sleep in the consolidation of procedural memory of the motor skills of mice performing a rotarod task and a skilled reaching task. We described the effects of acetylcholine levels on sleep structure and phases, we related acetylcholine levels in post-training sleep to subsequent motor performance, and we investigated the role of muscarinic and nicotinic acetylcholine receptors in motor memory consolidation. In sum, we propose that low acetylcholine levels during SWS are necessary for the consolidation of procedural memory.

RESULTS

Physostigmine injection after a motor learning task impairs the consolidation of motor memories

Two separate groups of mice were trained using two different motor tasks (rotarod and skilled forelimb reaching). The rotarod task was used because learning takes place quickly, in 10-20 trials within a single

training session, allowing assessment of the effects of manipulations during relatively immediate post-training sleep (Yang, Lai et al. 2014). In contrast, skilled forelimb reach task was used because training takes place slowly in multiple training sessions (each with 20 trials) spread over 2-6 days. Thus, it involves multiple cycles of encoding and post-training sleep. For both tasks, we investigated the effect of post-training and pre-sleep injections of physostigmine, which is an acetylcholinesterase inhibitor and prevents the breakdown of ACh thus increasing cholinergic tone (Aquilonius and Hartvig 1986, Pubchem 2018).

Rotarod Task Results

In the rotarod task (Fig. 1A), mice learn to maintain balance on an elevated rotating drum to prevent themselves from falling. The maximum speed they attain just before they fall is used as a measure of their learning during training and performance at a later time. On day 1 (training day) mice were given 10 trials of rotarod early in the morning (8:30am – 10:00am) when sleep pressure is high. They gradually learnt the task as speeds increased with trials (Fig. 1B - left). The increase in speeds was much faster in trials 1, 2 and 3 and became gradually faster in successive trials for 58 mice in the baseline group ($F_{9,513} = 24.52$, $p_{gg} = 5.97e-27$, ANOVA-RM, effect for trials, p_{gg} is Greenhouse-Geisser corrected p value – see methods). Post hoc comparisons with Tukey-Kramer's correction revealed that speeds in trials 2 and 3 were significantly higher than in trial 1 ($p = 0.010$ and $p = 1.103e-5$ respectively) indicating fast learning. Furthermore, speeds in trials 7-10 were significantly higher than in trial 3 ($p<0.006$ for all pairwise comparisons) suggesting continual learning in subsequent trials. At the end of the training sessions, saline or physostigmine was immediately administered in two groups of mice, and they were returned to their home cages allowing them to rest or sleep.

To assess whether mice retained motor memories acquired on the training day, we gave 10 trials of rotarod training on day 5 (probe day) around the same time of light/dark cycle on which initial training had occurred (Fig. 1A). We compared speeds in the first trial of the probe day with speeds in the last trial of the training day. For mice in the saline group, the two sets of speeds were similar (Student's t-test one-tailed), indicating retention of motor memory formed on the training day. For mice in the physostigmine group, speeds in the first trial of the probe day were significantly lower than speeds in the last trial of the training day in the baseline group ($t_{70} = 2.50$, $p =0.007$, Student's t-test one-tailed) suggesting impaired retention of motor memory compared to the saline group (Fig. 1C). We also compared average speeds (over animals) for all trials within the experimental groups using a one-way ANOVA, which revealed a significant difference among groups ($F_{3,568} = 52.01$, $p =1.03e-29$). The average speed of saline group was

significantly greater than that of the baseline group, a composite of all mice given preliminary training on Day 1, (Fig. 1D, p=3.768e-9, post hoc Tukey's correction) as well as physostigmine group (p=3.768e-9, post hoc Tukey's correction). There was no significant difference between average speeds of physostigmine and the baseline group. These results thus suggest that the learned motor memories for mice in the saline group were consolidated better than for mice in the physostigmine group.

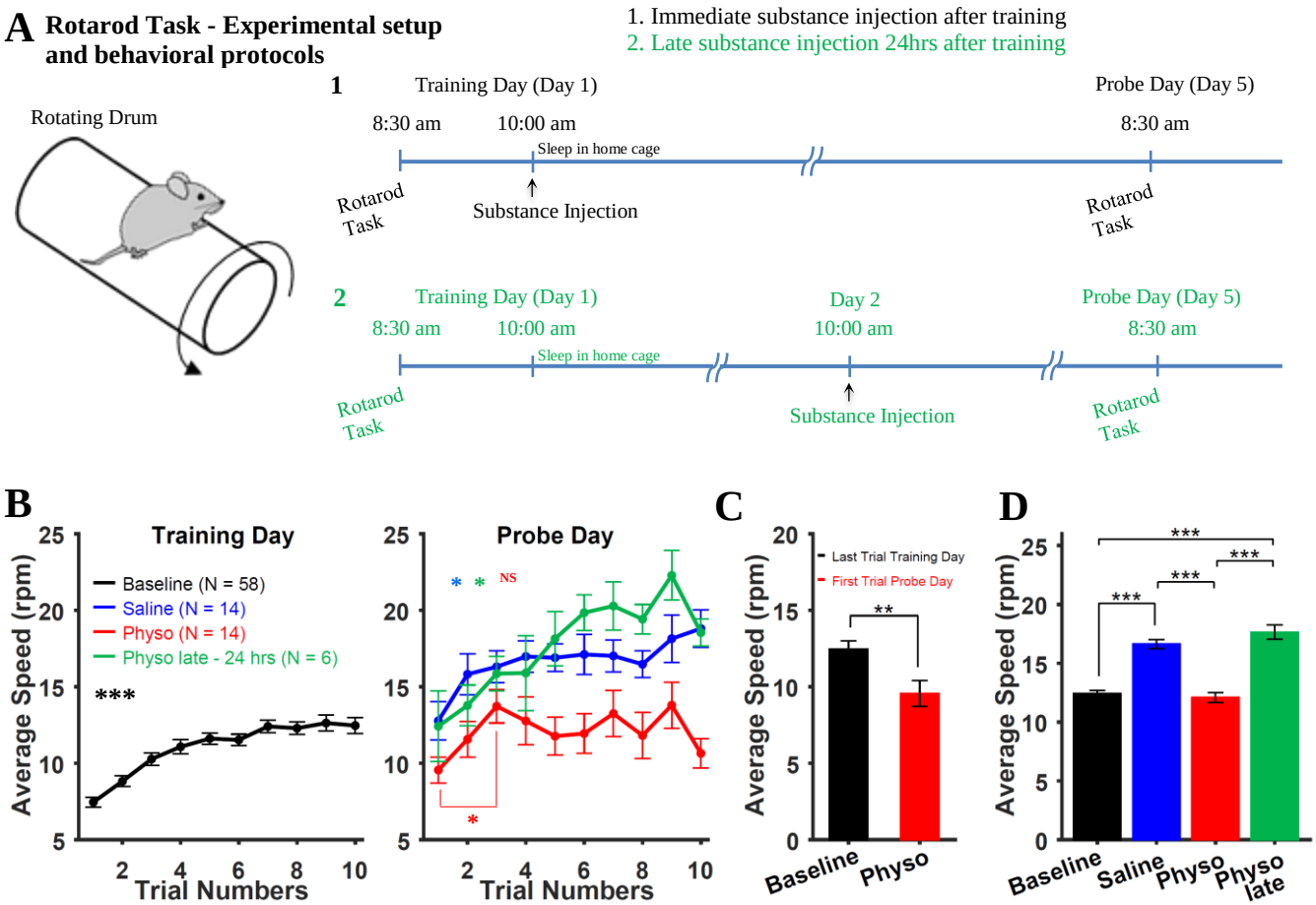

Figure 1. Increasing acetylcholine levels using physostigmine (physo) in early sleep impairs the consolidation of motor memories induced by the rotarod task. (A) Experimental setup and behavioral protocol. In the rotarod task, animals learn to run and keep balance on an elevated rotating drum to prevent themselves from falling. Mice were trained on Day 1 (training day) and retested on Day 5 (probe day). **(B)** Average speeds (over animals) attained on the rotarod versus trials for training and probe days. N indicates number of animals in each group. After fast learning in the first half of trials on training day, mice gradually reached a plateau in performance. *** indicates significant effect of trials on speed ($F_{9,513}$ = 24.52, p_{gg} = 5.97e-27, ANOVA-RM). On probe day, performance of mice in saline and physo-late groups further improved significantly with trials ($F_{9,117}$ = 3.10, p_{gg} = 0.02 for saline, blue *, and $F_{9,45}$ = 5.17, p_{gg} = 0.01 for physo-late, green *, ANOVA-RM). The performance of mice in the physo group did not improve significantly with trials ($F_{9,117}$ = 1.92, p_{gg} = 0.13, ANOVA-RM), however, there was an initial

improvement in the first three trials ($F_{2,26}$ = 6.37, p_{gg} = 0.01, ANOVA-RM with p = 0.03 for comparison of trials 3 and 1 with post-hoc Tukey-Kramer correction, red *). **(C)** Average speeds in trial 1 of physo group were significantly smaller than those in last trial of baseline group suggesting impairment in consolidation (t_{70} = 2.50, p = 0.007, one-tailed Student's t-test). **(D)** Between groups comparison of average speeds over animals using one-way ANOVA ($F_{3,568}$ = 52.01, p = 1.03e-29). Error bars represent SEM. p_{gg} is Greenhouse-Geisser corrected p-value. ANOVA-RM is one-way ANOVA with repeated measures.

Although the half-life of physostigmine is less than 1 hr in rodents (Somani 1989), but physostigmine also produces bradycardia, salivation, and tremors. There was a possibility that these non-mnemonic effects of physostigmine affected performance on the probe day. In a separate cohort of mice (n=6), physostigmine was injected 24 hours after the last trial ended on the training day (physo-late group). Rotarod speeds of these mice in the first trial on the probe day were similar to baseline speeds in the last trial of the training day (Fig. 1B). In addition, average speed (over animals) in all trials was significantly greater compared to baseline and physostigmine groups (Fig. 1D. p = 3.768e-9 for both comparisons, one-way ANOVA with post hoc Tukey's correction). These results suggest that motor memory induced by the rotarod task is consolidated after the first sleep session post training.

Skilled Forelimb Reach Task Results

The single pellet reaching task (Fig. 2A) requires animals to coordinated movements of the arm, wrist, and hand in order to reach through a slot to obtain a food pellet. Mice were trained/tested for 10 days and on each day one group was injected saline and a second group injected with physostigmine after training (Fig. 2A). Mice injected with physostigmine were slower in learning the task, as suggested by a smaller number of animals learning the task within the first 5 days compared to those injected with saline (Fig. 2B, an association between substance injected and success was observed on days 3 and 4, $\chi^2(1)$ = 10.8315, p=0.00099 and $\chi^2(1)$ = 4.492 p=0.0341 respectively, Chi square independence test). A successful animal here was defined as one which completed 20 trials on that particular day with at least one successful reach. Furthermore, we compared the average percentages (over animals) of successful trials over days (Fig. 2C) which increased with time, showing that mice in both groups did learn (for saline group, $F_{9,81}$ = 31.49, p_{gg} = 2.69e-8, for physo group, $F_{9,90}$ = 14.21, p_{gg} = 1.27e-7, ANOVA-RM, effect of days). The average percentages for the physostigmine group were significantly different than those for saline group ($F_{9,171}$ = 2.81, p_{gg} = 0.0169, ANOVA-RM for group-days interactions) with significantly smaller values for physostigmine on days 3,7, and 8 (p=0.041, p=0.009, and p=0.011 respectively, post hoc tukey-kramer

correction, group by days). These results show that the administration of physostigmine immediately after reach training impaired performance.

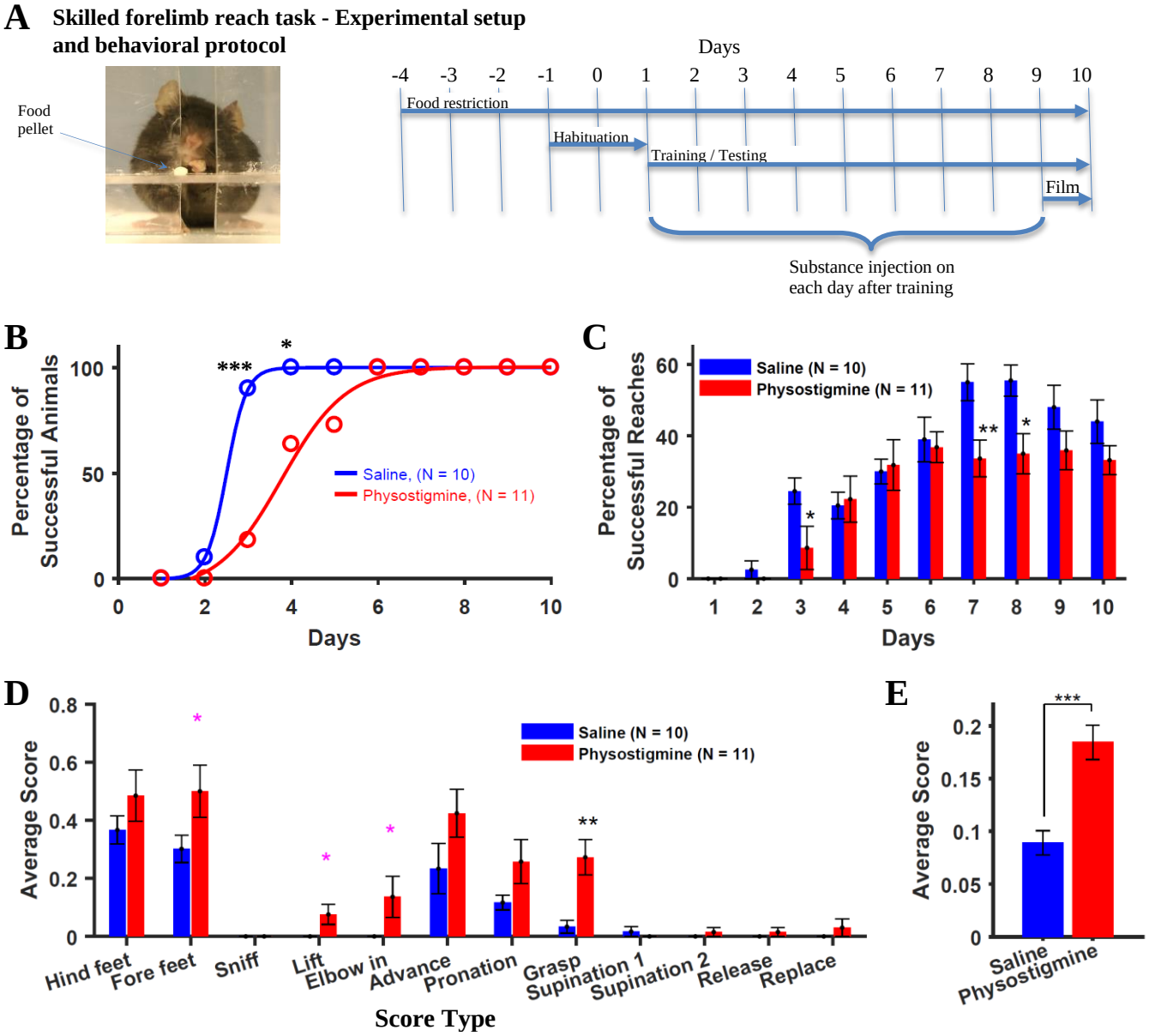

Figure 2. Increasing acetylcholine levels using physostigmine in early sleep impairs the consolidation of motor memories induced by the skilled forelimb reach task. (A) Experimental setup and behavioral protocol. Animals learn to reach through a slot and grasp a food pellet placed on a platform. Mice were trained/tested for 10 days. They were filmed on 9th day and reaches were scored from videos. **(B)** Percentage of successful animal vs days. A "successful" animal completed 20 trials with at least one successful reach and grasp. Firm line is fitted curve (sigmoidal). An association between substance injected and success was observed for days 3 and 4 ($\chi^2(1) = 10.8315$, $p<0.001$ and $\chi^2(1) = 4.492$ $p<0.05$ respectively, Chi square independence test). **(C)** Percent single reach successes (averaged over animals)

vs days. Reaching accuracy improved over the training period for both groups (p_{gg} = 2.69e-8 and 1.27e-7 for saline and physostigmine groups respectively). However, reaching accuracy of physostigmine group was significantly different from that of saline group ($F_{9,171}$ = 2.81, p_{gg} = 0.0169, ANOVA-RM, group-days interaction) and smaller on days 3,7, and 8 (p=0.041, p=0.009, and p=0.011 respectively, post hoc tukey-kramer correction, group by days). **(D)** Quality of reaches was assessed from video recordings done on day 9 for 12 components of reach and grasp motion. A higher score indicates inferior performance. Although group-component interaction was not significant for saline and physostigmine groups ($F_{11,209}$ = 1.83, p_{gg} = 0.129, ANOVA-RM), the grasp scores were significantly higher for physo group (p = 0.002, post hoc tukey-kramer correction, black asterisk) as well as scores for fore-feet, lift, and elbow-in (t_{19} = -1.89, p = 0.037, t_{19} = -2.08, p = 0.025, t_{19} = -1.84, p = 0.040 respectively, magenta asterisks, one-tailed Student's t-test). **(E)** Average component scores (over animals) for the physostigmine group were significantly larger than those for saline group (t_{754} = -4.711, p = 1.464e-6, one-tailed Student's t-test). All Data are presented as Mean±SEM.

To assess the effect of physostigmine injections on the quality of reaches, we scored reaching movements from the video recordings on day 9 by giving a score to each of 12 components of movements that make up the reaching act (Whishaw, Whishaw et al. 2008, Whishaw, Faraji et al. 2017). The first three successful reaches were scored and for a given component movement, a score of 0, 0.5, and 1 was assigned for good, impaired, and abnormal performance respectively. A higher score would thus indicate inferior performance. There was no significant difference between the scores for physostigmine and saline groups determined by one-way ANOVA with repeated measures ($F_{11,209}$ = 1.83, p_{gg} = 0.129, group-components interaction). However, post-hoc tukey-kramer correction revealed a higher mean score for the grasp component for physostigmine (p = 0.002). Mean scores for the fore-feet, lift and elbow-in components were also significantly larger for the physostigmine group compared to saline group revealed with one-tailed student's t-test (t_{19} = -1.89, p = 0.037, t_{19} = -2.08, p = 0.025, t_{19} = -1.84, p = 0.040 respectively). Finally, average component scores (over components and animals) were higher for the physostigmine group (Fig. 1E, t_{754} = -4.711, p = 1.464e-6, one-tailed Student's t-test). These results show that as mice in the physostigmine group acquired the task, their motor performance was impaired compared to the saline group. Larger scores in the physostigmine group for grasp, forefeet, lift, and elbow-in components could indicate impaired consolidation for associated memories compared to the saline group.

Physostigmine injection after rotarod training altered sleep structure

To investigate how physostigmine injection post-training impaired subsequent motor performance, we asked whether physostigmine injection altered post-training activity or sleep. Separate cohorts of mice were injected with saline or physostigmine after rotarod training, and returned to their home cages. Their behavior was monitored using a video monitoring system developed in house (Singh, Bermudez-Contreras

et al. 2018). From the video frames, locomotion (Fig. 3A) was quantified by counting the number of pixels that changed between consecutive video frames (Singh, Bermudez-Contreras et al. 2018). Motion profiles were then transformed to motion/rest traces using the mean motion profile as threshold. Mice in the physostigmine group had increased activity levels and showed delayed onset of rest/sleep. For each mouse, the cumulative history of motion/rest periods was determined. The average cumulative duration of motion for mice in the saline group was significantly right-shifted compared to mice in the physostigmine group (Fig. 3B, p = 9.533e-143, one-tailed two-sample Kolmogorov-Smirnov test) and the immobility periods of mice in the saline group were longer. Mice in the physostigmine group were more active and had shorter episodes of rest. These results thus suggest that physostigmine injections altered sleep/activity in the mice.

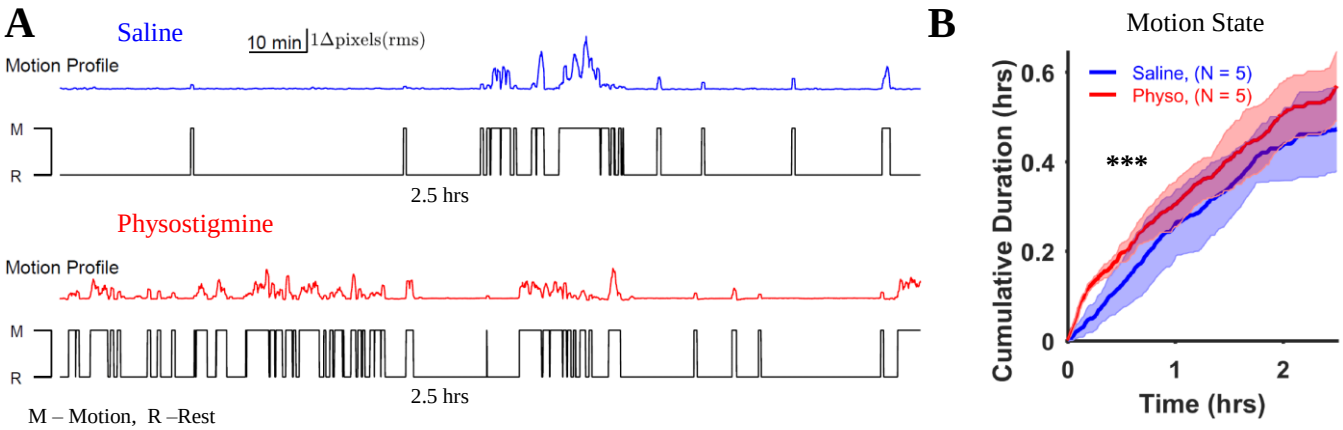

Figure 3. Quantification of motion after rotarod task in home cage using video monitoring. There is larger motion in animals after physostigmine injection. (A) Representative raw traces of rms value of change in number of pixels in video frames versus time. Blue and red traces are for mice injected with saline and physostigmine (physo) respectively. Mean of the whole trace was used as a threshold to classify motion and rest periods shown in black traces below each raw trace. **(B)** Average cumulative motion versus time. For each mouse, the cumulative sum was determined from motion/rest traces. Shaded regions show SEM. The mean curve for saline group is significantly right shifted compared to physo group (p = 9.533e-143, two-sample Kolmogrov-Smirnov test) suggesting delayed motion after injections in mice in saline group.

Physostigmine delays slow wave sleep onset and reduces slow wave sleep power

To further explore how physostigmine might alter sleep structure, we used electrophysiology to monitor EEG in one group of mice (N = 5). We implanted electrodes in the trapezius muscle (in the neck), hippocampus

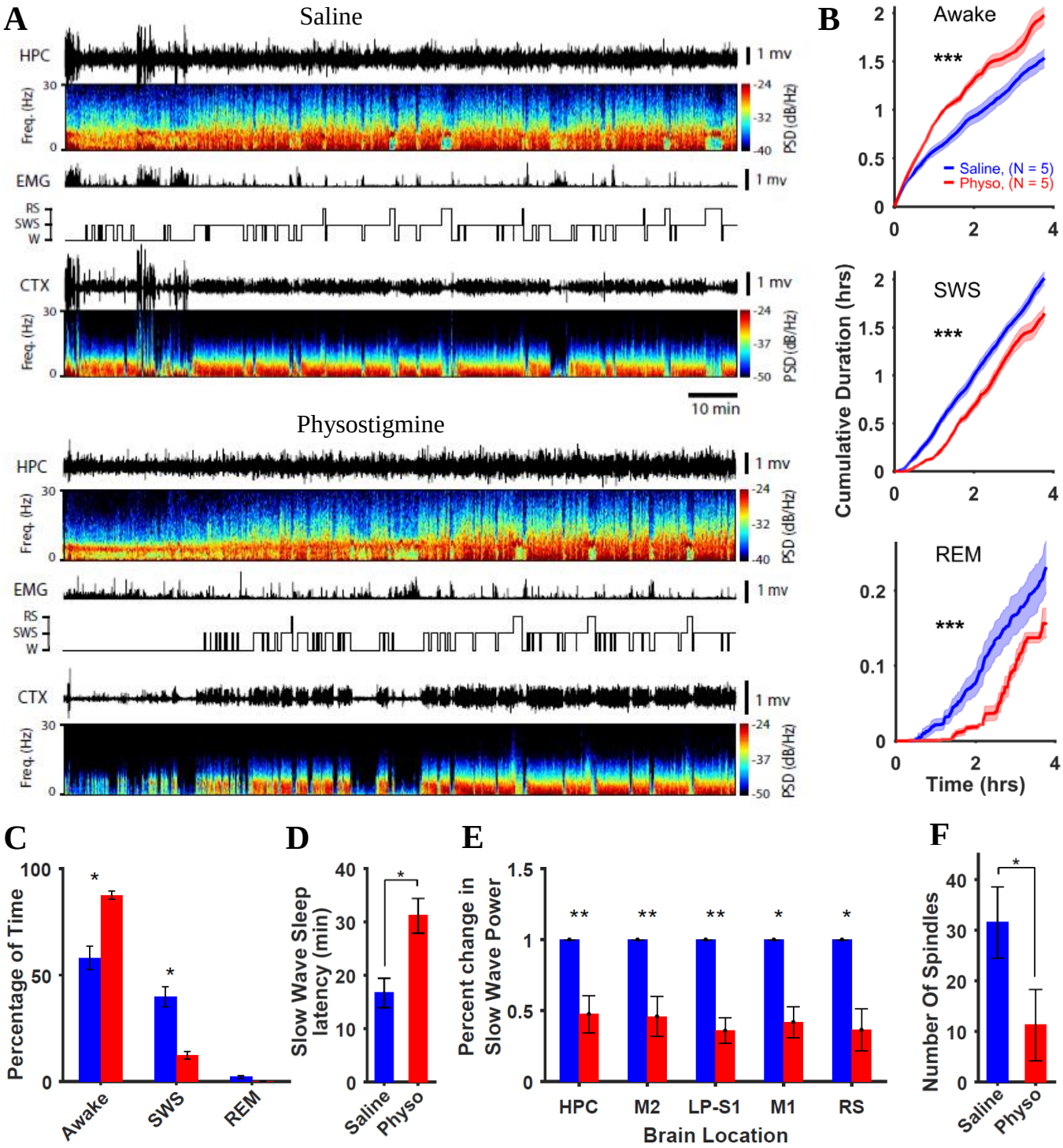

Figure 4. Increasing acetylcholine levels using physostigmine (physo) reduces slow-wave sleep and slow-wave power in the first hour of sleep post injection. (A) Representative hippocampal (HPC) and cortical (CTX – from M2, secondary motor area) local field potentials (LFP), electromyography (EMG) signal, hypnogram, and spectrogram. In the hypnogram, RS, SWS, and W represent states of animals i.e. REM sleep, slow-wave sleep, and wake periods respectively, which were scored from EMG traces and spectrogram of hippocampal and cortical LFPs.

PSD is power spectral density. Note that the hypnogram for physostigmine shows more fragmented sleep structure compared to that for saline. **(B)** Cumulative duration vs time of recording for awake, SWS, and REM sleep states. Difference in states between animals injected with physostigmine and saline lies mostly in the first hour post injection. Mean cumulative curves for all states were significantly shifted between animals injected with saline and physostigmine ($p < 0.0001$ for all comparisons, Kolmogorov-Smirnov test) **(C)** Comparison of sleep structure between saline and physo groups in the first hour after injection. Waking activity in physo group was higher ($t_4 = -4.14$, $p = 0.014$) while SWS was reduced ($t_4 = 4.32$, $p = 0.012$) – paired Student's t-test. **(D)** SWS latency increased in physo group compared to saline group ($t_4 = -3.43$, $p = 0.013$, paired Student's t-test one-tailed). **(E)** Slow wave power during SWS in the first hour of recording reduced in physo group compared to saline group in HPC and cortical areas (M2 – Secondary motor area, LP-S1 – Lip primary sensory area, M1 – primary motor area, and RS – retrosplenial cortex) revealed with one-tailed - paired Student's t-test. **(F)** Number of thalamo-cortical spindles identified from M2 cortical LFP during the SWS period were smaller in physo group compared to saline. All Data are presented as Mean±SEM.

(HPC), primary and secondary motor cortices (M1 and M2 respectively), cortical lip area (LP), and the retrosplenial cortex (RS). Local field potentials (LFPs) were recorded after injecting saline and on a subsequent day after injecting physostigmine (Fig. 4A). Mice were injected at 8:30am and signals were analysed for the first four hours after injection. In both hippocampal and cortical spectrograms, we observed visible reduction in power spectral density (PSD) in slow wave band within the first hour after physostigmine injections (Fig. 4A). For further comparisons, we first determined sleep structure by finding durations of wakefulness, REM sleep, and SWS using EMG signals and spectrogram of hippocampal and cortical (from M2) LFPs (Fig. 4A and 4B). Compared to saline injections, sleep structure after physostigmine injections was altered in the first hour of recording. After the first hour, cumulative duration curves for saline and physostigmine injections were parallel for awake, SWS, and REM sleep conditions (Fig. 4B) suggesting similar dynamics. After physostigmine injections mice had delayed onset of SWS (Fig. 4A and 4B) and time spent awake was increased (Fig. 4C, $t_4 = -4.14$, $p = 0.014$, paired Student's t-test one-tailed) while the SWS period was shorter (Fig. 4C, $t_4 = 4.32$, $p = 0.012$). REM sleep was minimal during the first hour and not different for physostigmine and saline injections. SWS latency was longer after physostigmine (31.17±3.26 mins for physo vs 16.70±2.76 mins for saline, Fig. 4D, $t_4 = -3.43$, $p = 0.013$) and slow-wave power was reduced in the hippocampus (Fig. 4D, $t_4 = 4.00$, $p = 0.008$) and cortex, (M2 - $t_4 = 3.79$, $p = 0.0006$, LP- $t_4 = 7.11$, $p = 0.001$, M1- $t_2 = 5.34$, $p = 0.017$, and RS- $t_2 = 4.34$, $p = 0.025$). The duration of thalamocortical spindles from M2 was significantly reduced after physostigmine injections within the first hour (Fig. 4F) but the spindle occurrence frequency was similar. These results thus suggest that physostigmine injection delay the onset of SWS sleep while reducing its quality (lower slow wave power).

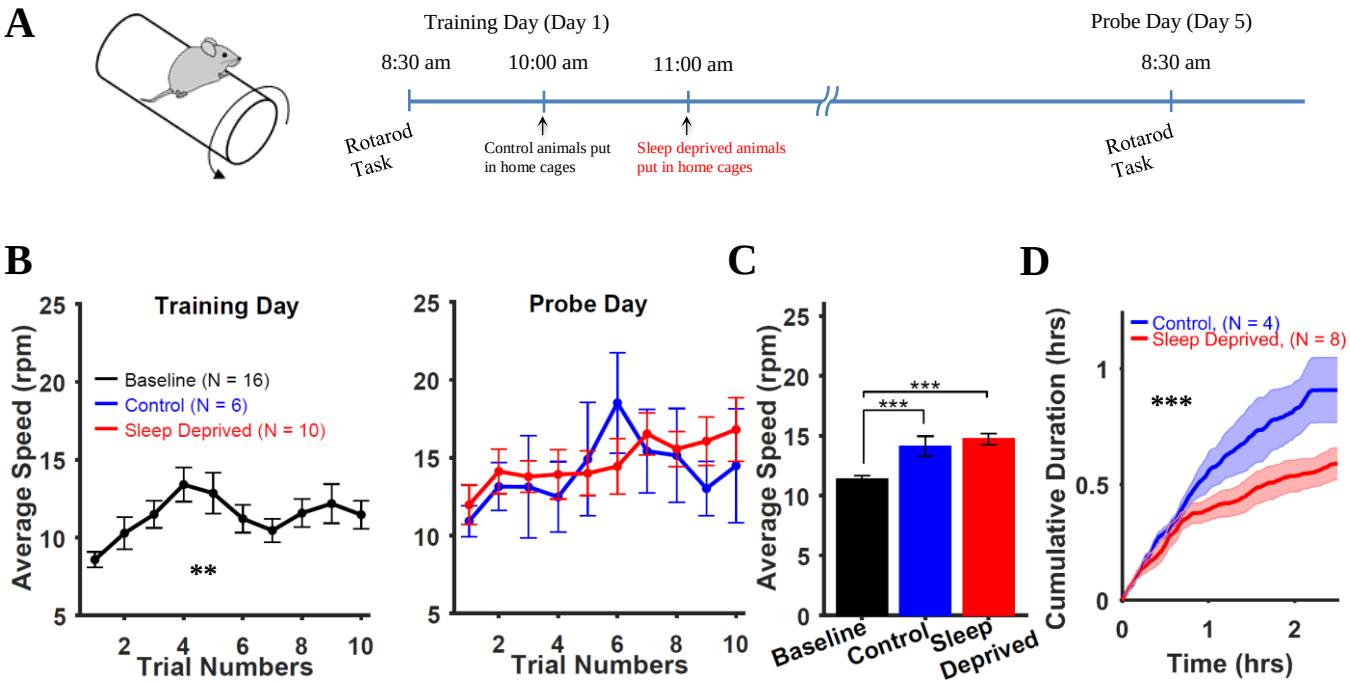

Figure 5. 1-hr sleep deprivation post rotarod training does not impair motor memories induced by the rotarod task. (A) Control animals were put in home cages at 10am while sleep deprived animals were kept awake and put in home cages at 11am **(B)** Average speeds (over animals) attained on the rotarod versus trials. N indicates number of animals in each group. Average speeds of mice in baseline group improved significantly with trials ($F_{9,135}$ = 3.23, p_{gg} = 0.0094, ANOVA-RM, effect of trials). **(C)** Average speeds (over animals) of all trials for control and sleep deprived groups were significantly larger than those of baseline group ($p < 0.0005$ for both control and sleep deprived groups, one-way ANOVA with post-hoc Tukey's correction, $F_{2,317}$ = 17.55, p = 5.90e-8). **(D)** Average cumulative motion versus time. Sleep deprived animals had smaller motion ($p < 0.0005$, two-sample Kolmogrov-Smirnov test). Shaded regions show SEM.

One-hour sleep deprivation after rotarod training does not impair consolidation of motor memories induced by the rotarod task

One might argue that the observed differences in rotarod performance were due to the sleep disruptive effects of physostigmine rather than to increasing acetylcholine levels *per se* (Figs. 3 and 4). To investigate this potentially confounding factor, we performed another set of experiments in a separate cohort of animals in which one group of animals was sleep deprived for the first hour post rotarod training while animals in the control group were put in home cages right after training to allow them to sleep (Fig. 5A). As before, all animals in this set of experiments were combined to obtain "baseline" performance on training day (Fig. 5B, left). The average speed (over animals) increased with trials ($F_{9,135}$ = 3.23, p_{gg} = 0.0094, ANOVA-RM, effect of trials) with a significant increase from trial 1 to trials 3, 4, and 10 (p =

0.045, 0.012, and 0.049 respectively, post-hoc Tukey-Kramer correction). The increase in speeds with trials for control and sleep deprived groups were not significant on the probe day. The average speeds over trials and animals for both control and sleep deprived groups were significantly greater than the baseline (Fig. 5C, $p < 0.0005$ for both control and sleep deprived groups, one-way ANOVA with post-hoc Tukey's correction, $F_{2,137} = 17.55$, $p = 5.90e-8$), however. We also quantified motion of animals post-training with home-cage video recording (Fig. 5D) and observed that sleep deprived animals had lower overall motion ($p < 0.0005$, two-sample Kolmogrov-Smirnov test) suggesting that they perhaps slept more in shorter duration compared to controls. These results suggest that one-hour sleep deprivation post motor training does not impair the consolidation of motor memories.

Chemogenetic inactivation of cholinergic neurons during early sleep does not impair the consolidation of motor memories

Alternatively to increasing acetylcholine levels using physostigmine, here, we investigated the effect of further reducing acetylcholine levels during early sleep on motor memory consolidation using transgenic Chat-Cre::CAG-hM4Di mice (Sternson & Roth , 2014; Madisen et al., 2010). These mice express inhibitory (hM4Di) DREADDs (Zhu, Aryal et al. 2016) within cholinergic neurons, and following the administration of the drug clozapine-*N*-oxide (CNO), presynaptic release of neurotransmitter acetylcholine in cholinergic neurons decreases. Since CNO exerts its maximum effects an hour to an hour-and-half after the administration, we injected CNO just before the rotarod training in mice belonging to the Chat-Cre::CAG-hM4Di CNO group (Fig. 6A). In another group of Chat-Cre::CAG-hM4Di mice, saline was injected to establish a control group. As for C57 mice, all Chat-Cre::CAG-hM4Di mice for the training day were combined to form a baseline group. Mice learnt the task on the training day (Fig. 6B, $F_{9,144} = 3.72$, $p_{gg} = 0.005$, ANOVA-RM, effect of trials) but their learning curve was not as steep as that for the C57 mice (compare with Fig. 1B). Speeds in the first trial on the probe day were similar to speeds in the last trial of training day for both CNO and saline groups (one-tailed Student's t-test), however. Furthermore, average speeds (over animals) for all trials in both CNO and saline groups were significantly higher than the average speeds for the baseline group on the training day (Fig. 6C, $p < 0.001$ for both CNO and Saline, one-way ANOVA with post-hoc Tukey's correction, $F_{2,339} = 31.27$, $p = 3.482e-13$), suggesting that mice improved further on the probe day. The average speeds in the CNO group, however, were smaller than those in saline group (Fig. 6C, $p = 0.0006$). These results suggest that the action of CNO on transgenic

mice during early sleep does not appreciably affect rotarod performance and has a small effect on motor memory consolidation compared to physostigmine injections.

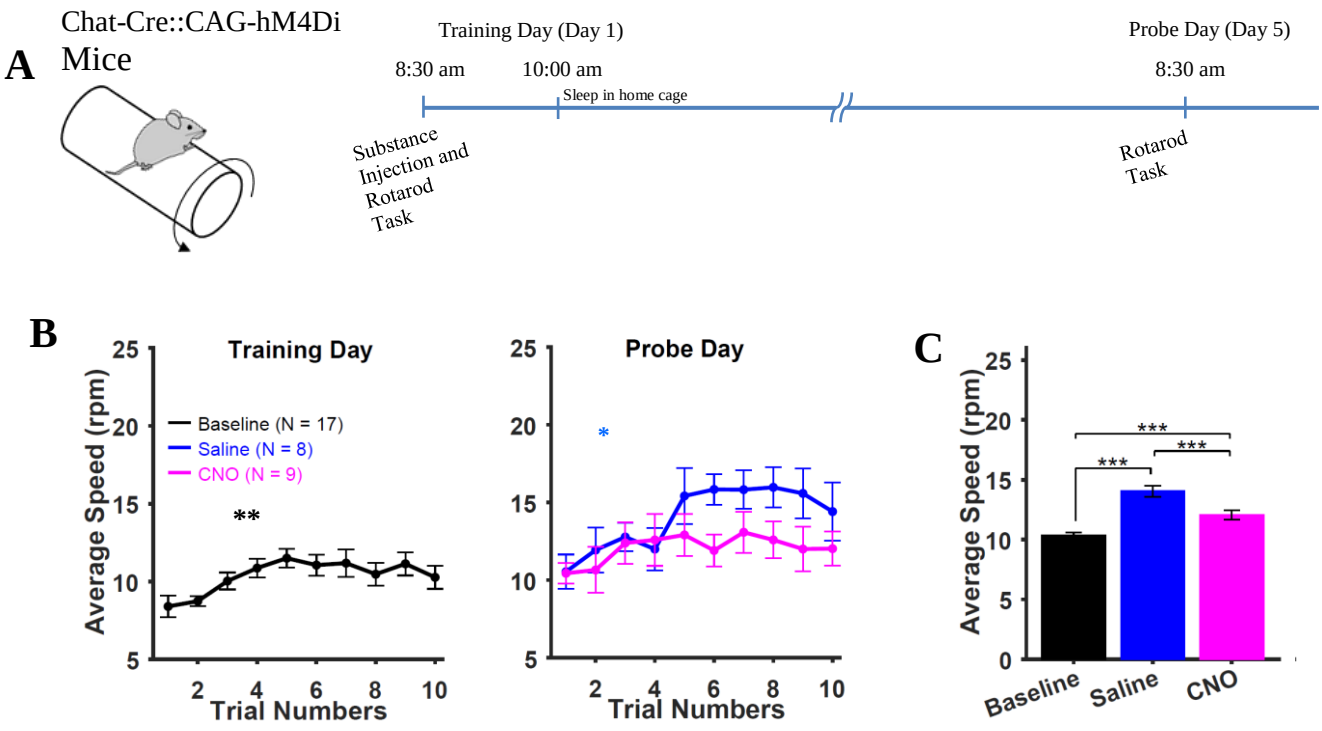

Figure 6. Decreasing acetylcholine levels in early sleep does not impair motor memories induced by the rotarod task. (A) Chat-Cre::CAG-hM4Di transgenic animals were injected with either saline or clozapine-*N*-oxide (CNO) before starting rotarod training because CNO exerts its maximum effect an hour to an hour and a half after administration. **(B)** Average speeds (over animals) attained on the rotarod versus trials. N indicates number of animals in each group. For training day, saline and CNO groups were combined to form the baseline group. Speeds improved with trials for mice in baseline group ($F_{9,144} = 3.72$, $p_{gg} = 0.005$, ANOVA-RM, effect of trials, black **) and saline group ($F_{9,63} = 3.40$, $p_{gg} = 0.029$, blue *, ANOVA-RM). **(C)** Average speeds (over animals) of all trials in both CNO and saline groups were significantly higher than those in baseline group ($p < 0.001$ for both CNO and Saline, one-way ANOVA with post-hoc Tukey's correction, $F_{2,339} = 31.27$, $p = 3.482e-13$).

Activation of muscarinic ACh receptors during early sleep impairs motor memory consolidation

In earlier experiments, post-training administration of physostigmine which indirectly activates both muscarinic and nicotinic ACh receptors impaired post-sleep motor performance. Here, we investigated the individual involvement of ACh receptors in motor memory consolidation. Based on the anatomical distribution of ACh receptors in the brain, we hypothesized that, similar to previous studies, the muscarinic

ACh receptors would play a larger role in motor memory consolidation as compared to nicotinic ACh receptors (Power, Vazdarjanova et al. 2003, Brown 2010, Hut and Van der Zee 2011). To activate muscarinic and nicotinic ACh receptors selectively, the respective agonists oxotremorine and nicotine were injected immediately after rotarod training in two separate groups of mice (behavioral protocol 1, Fig. 1A). For both groups, speeds in the first trials on the probe day (Fig. 7) were similar to speeds in the last trials on the training day (one-tailed Student's t-test) demonstrating performance retention. The average speeds (over animals) for all trials for the nicotine group were significantly faster compared to the baseline group (Fig. 7B, p = 0.0004 one-way ANOVA with post-hoc Tukey's correction, $F_{4,611} = 25.97$, p = 6.823e-20) but slower than the speeds in the saline group (Fig. 7B, p = 0.002). For the oxotremorine group, average speeds were similar to those in the baseline group and were significantly smaller than those in the saline group (Fig. 7B, p = 1.272e-8, one-way ANOVA with post hoc Tukey's correction). These results suggest that oxotremorine has a larger effect on the consolidation of motor memories as compared to nicotine.

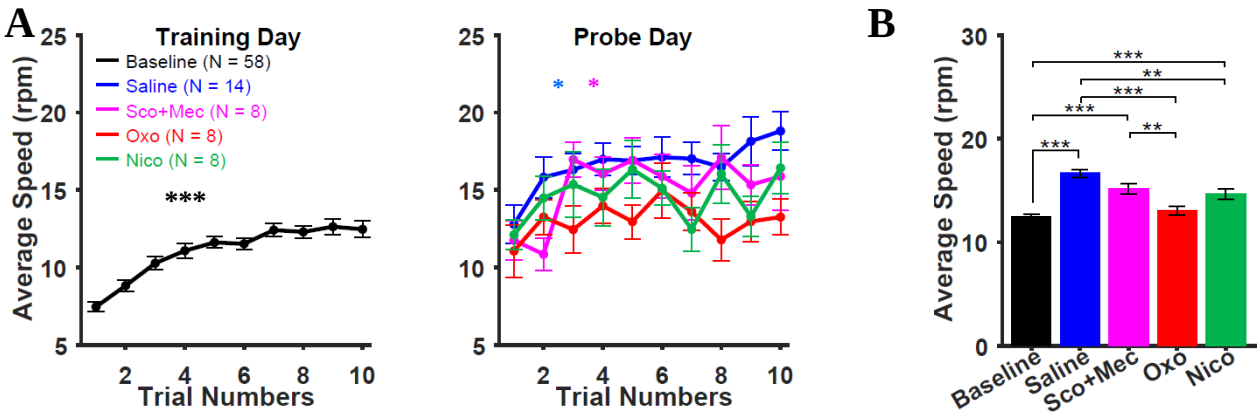

Figure 7. Activation of muscarinic acetylcholine (ACh) receptors during early sleep impairs motor memory consolidation. (A) Average speeds (over animals) attained on the rotarod versus trials. N indicates number of animals in each group. Baseline and saline groups are same as in Fig. 1. Average speeds of mice in groups administered with oxotremorine (oxo) and nicotine (nico) did not significantly improve with trials on probe day (NS, ANOVA-RM) but those of mice in scopolamine-mecamylamine (sco+mec) group improved significantly ($F_{9,63} = 3.67$, $p_{gg} = 0.024$, ANOVA-RM, effect of trials) **(B)** The average speeds (over animals) of all trials for nico and sco+mec groups but not oxo group were significantly larger than those for the baseline group ($p < 0.0005$ for both, one-way ANOVA with post-hoc Tukey's correction, $F_{4,611} = 25.97$, p = 6.823e-20).

As a supporting experiment, we used a combination of two drugs, scopolamine and mecamylamine for combined blockade of muscarinic and nicotinic acetylcholine receptors. Scopolamine and mecamylamine were given immediately after rotarod training (See protocol 1 in Fig. 1A). The speeds obtained by the mice in the first trial on the probe day were not significantly different from the last trial on the training day (one-tailed Student's t-test). There was an increase in speeds on the probe day (Fig. 7A, $F_{9,63} = 3.67$, $p_{gg} = 0.024$, ANOVA-RM, effect of trials). Furthermore, the average speeds (over animals) of all trials were significantly higher than those for baseline and oxotremorine groups (Fig. 7B, p = 3.307e-6 and p = 0.009 respectively, one-way ANOVA with post-hoc Tukey's correction, $F_{4,611} = 25.97$, p = 6.823e-20). Combined blockade of ACh receptors therefore did not alter performance on the rotarod task.

Table 1 shows the significance results of combined one-way ANOVA (with post-hoc tukey's corrections for p values) for all groups of C57 mice (N = 58) used in the rotarod task. The results although slightly different from those reported above when one-way ANOVA was done on subsets of groups, the main conclusions still hold. Significance results from combined ANOVA strongly support the conclusion that reduced muscarinic activity is associated with improved retention.

Table 1 – Significance results from One-way ANOVA. Post hoc Tukey's Correction. *, **, and * for p<0.05, p<0.01, and p<0.001 respectively. ns is not significant**

	Baseline	Saline	Physo	Physo-late	Sco+Mec	Oxo	Nico
Baseline		***	ns	***	***	ns	**
Saline			***	ns	ns	***	*
Physo				***	***	ns	***
Physo-late					*	***	***
Sco+Mec						*	ns
Oxo							ns
Nico							

Abbreviations: Physo – Physostigmine, Sco+Mec – Scopolamine + Mecamylamine, Physo-late – Physostigmine administered 24 hrs later, Oxo – Oxotremorine, Nico – Nicotine

DISCUSSION

We investigated how altering ACh levels during early sleep affects procedural memory consolidation of two motor tasks. Supporting previous studies of episodic memory (Hasselmo 1999, Gais and Born 2004, Hasselmo and McGaughy 2004), we find that low levels of ACh during NREM sleep enhances procedural memory consolidation. Physostigmine, which is expected to increase ACh levels, given immediately after training, delayed the onset of NREM sleep, reduced slow-wave power in the

cortex and hippocampus), and impaired post-sleep motor performance. Physostigmine given 24 hrs after training, after first sleep session was over, did not affect motor performance. To rule out that sleep disruption by physostigmine did not cause impaired motor performance in the initial experiments, supporting experiments confirmed that one-hour sleep deprivation after training does not affect motor performance and CNO injection into transgenic mice, which might be expected to decrease acetylcholine levels, did not affect motor performance. By using agonists and antagonists of muscarinic and nicotinic ACh receptors, we found that only activation of muscarinic receptors during early sleep impairs motor performance. Taken together, these experiments are consistent with the idea that inactivation of muscarinic receptors due to natural lowering of ACh levels during early sleep contributes to the consolidation of procedural memory.

It is well known that motor skills show the greatest improvement if intermittent training is given (Karni, Meyer et al. 1998, Buitrago, Ringer et al. 2004, Peters, Chen et al. 2014). It is suggested that this pattern in which daily improvement is preserved and then enhanced on successive days of training is enabled by a post training period of consolidation. Our results from animals injected with saline after training in both rotarod and reach tasks reflect a similar pattern. For the rotarod task, the improvement obtained on the first day was preserved and augmented on the second day. Similarly, for the skilled reach task there was a day to day improvement in performance which eventually plateaued after a number of days of training. The expectation underlying the experiments was that post training procedural memory consolidation is important for this daily improvement in motor performance.

We obtained support for the idea that lower ACh levels during early sleep has a positive effect in enhancing motor performance suggesting a positive role for lower ACh levels in procedural memory consolidation. First, we used physostigmine to increase ACh levels in post training sleep. We did not measure ACh levels directly but inferred from sleep pattern changes that cholinergic tone was increased. In the first hour of sleep, post injection, SWS sleep onset was delayed and there was a reduction in slow-wave power, consistent with half-life of physostigmine in rodents which is less than an hour (Somani 1989). Previous studies have reported similar changes in sleep patterns following manipulation of cholinergic tone (Sitaram, Wyatt et al. 1976, Vanderwolf, Raithby et al. 1993, Dringenberg and Vanderwolf 1996, Dringenberg and Vanderwolf 1997, Gais and Born 2004, Anaclet, Pedersen et al. 2015). We then asked whether impairment in motor performance resulting from physostigmine injections was due either to an increase in acetylcholine levels or to sleep disruption. We ruled out the later possibility by showing that one hour of sleep deprivation post training does not impair motor performance. This is

consistent with previous studies showing that 3-7 hours (greater than one hour) of sleep deprivation impairs motor memory consolidation (Gais, Plihal et al. 2000, Yang, Lai et al. 2014, Nagai, de Vivo et al. 2017) – but see Nagai et al (2017) who showed that sleep deprivation only affected motor performance on a complex wheel task but not on the rotarod task. We also showed that the first sleep session after motor learning is crucial for memory consolidation in-line with studies of effects of motor learning on SWS, supporting the importance of first hour of sleep post training (Huber, Ghilardi et al. 2006, Aeschbach, Cutler et al. 2008, Hanlon, Faraguna et al. 2009, Landsness, Crupi et al. 2009, Landsness, Ferrarelli et al. 2011). Taken together, these results suggest that impairment in motor performance that we observed with physostigmine injections was due to an increase in ACh levels in the first hour of sleep post training. We therefore conclude that reduced ACh levels during early sleep are crucial for motor memory consolidation parallel to a previous study for declarative memory (Gais and Born 2004). Moreover, we propose that as for declarative memory, ACh levels might function as a switch between brain modes of encoding and consolidation respectively (Hasselmo 1999, Hasselmo and McGaughy 2004, Rasch, Born et al. 2006). We hypothesize that in a future experiment, blocking the action of acetylcholine during wakefulness will impede new motor learning but will enhance motor memory consolidation.

Next, we asked whether further reducing ACh levels during early sleep would enhance motor memory consolidation. Since, reducing ACh levels pharmacologically in wild type mice is a challenging manipulation, we used a chemogenetic approach with transgenic mice to inactivate cholinergic neurons during early sleep. Again, we did not measure ACh levels directly but inferred from behavior that levels were reduced or they remained unaffected. The rotarod speeds of mice in CNO group were significantly higher than those in baseline group but they were also significantly lower than those in saline group suggesting an effect of CNO injection. However, undesired effects of CNO might have adversely affected motor performance in comparison with saline group (Gomez, Bonaventura et al. 2017, Mahler and Aston-Jones 2018, Manvich, Webster et al. 2018). These results suggest that further reducing ACh levels does not affect motor memory consolidation as does increasing ACh levels. There are mixed findings about whether selective ACh reduction affects motor performance (Conner, Culberson et al. 2003, Gharbawie and Whishaw 2003, Ramanathan, Tuszynski et al. 2009, Conner, Kulczycki et al. 2010). Assuming that CNO had the intended action of reducing acetylcholine levels, we conclude that such acute manipulation does not adversely affect motor memory consolidation. These results also support our earlier conclusion regarding the requirement of low acetylcholine levels for memory consolidation.

Finally, we investigated which ACh receptors might play a crucial role in motor memory consolidation. Post-sleep motor performance was reduced by oxotremorine, a muscarinic acetylcholine receptor agonist, suggesting that activation of muscarinic receptors is undesirable for memory consolidation during early sleep. There was also no significant difference between oxotremorine and nicotine groups. In natural early sleep therefore, reduction in ACh on muscarinic receptors is related to motor memory consolidation. Other work has also shown that muscarinic acetylcholine receptors are more involved in cognitive functioning (Hut and Van der Zee 2011, Zwart, Reed et al. 2018) and their activation causes increased REM like EEG whereas their blockade produces slow wave activity (Vanderwolf, Raithby et al. 1993, Dringenberg and Vanderwolf 1996).

The findings of the present study are relevant to contemporary theories of how sleep contributes to motor memory consolidation. Our findings are consistent with the synaptic homeostatic theory of sleep that slow wave sleep (SWS) facilitates memory consolidation by initiating downscaling of synaptic strengths to bring the synaptic load impacting each neuron to baseline levels, thus increasing signal to noise ratio of recently activated synapses (Tononi and Cirelli 2003). It also argues that low levels of ACh present during SWS might mediate the synaptic downscaling process (Tononi and Cirelli 2006). Consistent with this theory, when we increased ACh levels during early sleep, consolidation was impaired. Our findings are not inconsistent with the report that it is the cyclic succession of SWS and REM is important for memory consolidation (Giuditta, Ambrosini et al. 1995, Ambrosini and Giuditta 2001, Rasch and Born 2013, Gais and Schonauer 2017). This finding suggests that SWS causes selective weakening of non-adaptive memories (noise) and strengthening of adaptive memories (useful for survival) while REM sleep is involved in integration of adaptive memories into existing knowledge. In our study, physostigmine likely altered sleep structure disrupting the cyclic succession of SWS and REM and thus causing impaired consolidation in this way. The present results are not consistent with the dual process theory that advocates that SWS supports the consolidation of episodic memories while REM sleep facilitates procedural memory consolidation (Stickgold, Whidbee et al. 2000, Louie and Wilson 2001, Mednick, Nakayama et al. 2003, Peigneux, Laureys et al. 2003, Gais and Born 2004, Mascetti, Muto et al. 2013). We find that low ACh levels during SWS facilitate motor memory consolidation. Thus, our findings are consistent with numerous other studies (see above) that are inconsistent with this theory. Perhaps the REM sleep dependent consolidation of procedural memories is dependent on the type of task (Chambers 2017).

In conclusion, our results suggest that downregulated ACh levels during SWS are associated with motor memory consolidation. We used pharmacological manipulation for altering ACh levels, but future studies might use more refined methods. For example, by activating or silencing cholinergic neurons optogenetically with a feedback system to detect sleep states, one could clamp ACh levels in the desired brain region and study their role in memory consolidation using behavioral measures, electrophysiology or optical imaging (Mohajerani, Chan et al. 2013). Furthermore, measurements to observe synaptic and biochemical changes during sleep would allow the study of structural correlates of memory consolidation.

METHODS

All experiments were performed in accordance with Canadian Council of Animal Care and were approved by the University of Lethbridge Animal Welfare Committee.

Experimental Animals

Adult wild-type (WT) mice (C57 Bl/6J, Jackson laboratories), N=110, and transgenic mice (Chat-Cre::CAG-hM4Di), N=17, 3-6 months old, both male and female, and weighing 20-30g were used in this study. Transgenic mice had inhibitory (hM4Di) DREADDs in cholinergic neurons, which were activated by the inert molecule clozapine-*N*-oxide (CNO) to reduce release of acetylcholine. For generating these mice, CAG-hM4Di mice (B6N.129-Gt(ROSA)26Sortm1(CAG-CHRM4*,-mCitrine) Ute/J) were crossed with Chat-Cre mice (B6;129S6-Chattm2(cre)Lowl/J) to enable expression of CAG hM4Di in cholinergic neurons of the brain (Stachniak, Ghosh et al. 2014, Vardy, Robinson et al. 2015). All mice were housed up to four per cage and were provided food and water ad libitum. They were kept in a controlled temperature (22°C), humidity, and light with a 12:12 light/dark cycle. All testing and training was performed during the light phase of the cycle at the same time each day.

Motor Learning Tasks

Rotarod Task

In the rotarod task, mice learn to balance while running on an elevated rotating drum whose speed is gradually increasing (Fig. 1A). As they learn to keep their balance they can stay on the rotating drum for longer times at higher speeds of the drum. Eighty four C57/BL6 and 17 Chat-Cre::CAG-hM4Dimice were used and were randomly divided into control and experimental groups. We used the modified version of

the rotarod as described by shiotuski et al, (Shiotsuki, Yoshimi et al. 2010) to emphasize the learning aspect of the test. A four lane rotarod with automatic timers and fall sensors (Med Associates Inc.) with a test chamber (74 cm / 84 cm / 50 cm) was used. The Diameter of drum was 7cm and was covered with Duct tape to prevent mouse from gripping on to the surface. Animals were placed on the drum 3 mins before the start of each session to habituate them. An accelerating rotarod paradigm was used in which the rotation speed gradually increased from 4-40 rpm over the course of 5 min. The time latency and rotation speed were automatically recorded by the photo sensors when the animal was unable to keep up with the increasing speed and fell. The rotarod training consisted of one session (1-1.5h) with 10 trials and an inter trial interval of 3-5 mins. Performance was measured as average speed animals achieved during the training session. To evaluate long-term memory, 10 trials were repeated 5 days after the initial session and speeds were compared. All sessions were performed at the same time 8:00-10:00 am in the morning and animals were returned to home cages to allow them to sleep.

Skilled-Forelimb Reach Task

The skilled forelimb reaching task was established by Whishaw et al (Whishaw 2000) and is used to study fine/precise co-ordinated movements of forelimb, arm, hand and digits as they work together to retrieve food. For the skilled forelimb reach task 21 C57/BL6 mice were used and were divided into control and drug groups by a third person making sure the experimenter was blind to the two groups. The animals were placed on food restriction 3-4 days before the beginning of training. Prior to food restriction, mice were weighed three days to obtain an average pre-restriction weight followed by a daily monitoring during the food restriction period to maintain 85% of the average weight.

The animals were placed in a clear plexiglass testing chamber (20 cm long, 9 cm wide, and 20 cm high) with a slit (1 cm wide) located in the center of the front wall. A 3cm wide shelf with two grooves/notches at each side of the slot was mounted 1cm above the floor and outside of the front wall. The grooves were curved out on the shelf to place 14mg food pellets. All mice were handled daily and were habituated to the testing chamber for a week. Two days before training, the mice were given 10 pellets inside the chamber and then 10 pellets very close to the slit on shelf before moving the pellet further on the groove. The training/testing began on the day the mice were presented with food pellets in the grooves located on the shelf. The training/testing consisted of 10 days with one session each day consisting of 20 trials. **“**Reach” was scored when the animal extended its forelimb through the slot. A “success” was scored if

the animal grasped the food, retracted the paw and successfully brought the pellet back to its mouth and consumed it in a single reach. On each day, immediately after training/testing and before sleep all mice received either drug or saline and were allowed to sleep. On 9th day of training/testing all mice were filmed with a Panasonic HDC-SDT750 camera at 60 frames per second with an exposure rate of 1ms. Illumination for filming was obtained from a two-arm cold light source (Nikon Inc.). Quality of reaches was assessed from the videos by the scoring method developed by Whishaw (Whishaw 2000). Components of reach related to, hind feet, fore feet, sniff, lift, elbow in, advance, pronation, grasp, supination 1, supination 2, release, and replace were scored. 0, 0.5, and 1 scores were given for good, impaired, and abnormal reaches respectively. A higher score thus indicated inferior performance. On each day of testing the order of the animals to be tested was randomized and the experimenter was blind to the two groups.

Drugs and Solutions

The drugs Physostigmine Salicylate, Scopolamine Hydrobromide, Mecamylamine Hydrochloride, Oxotremorine M, and Nicotine hydrogen tartrate salt were used in this study. All drugs were obtained from Sigma-aldrich and dissolved in saline for administration. Saline was injected in control animals. Physostigmine was first dissolved in 100% ethyl alcohol to make a stock solution of 10mg/ml and later diluted 1000 times in saline to obtain a final concentration of 0.01mg/ml. All Drugs were prepared just before use or diluted from a stock solution. The drugs were administered intraperitoneally. For rotarod task, mice were assigned to six groups. Each group received one of the drugs; physostigmine (P group), Oxotremorine (Oxo group), Nicotine (Nico Group), Scopolamine and Mecamylamine (SM group), and Saline (S group). For skilled reach task, mice were assigned to two groups with one receiving physostigmine and the other saline.

Pharmacological Treatment.

Physostigmine group (Physo group)

To increase cholinergic tone during SWS 14 mice were administered with physostigmine (an acetylcholine esterase inhibitor which indirectly stimulates both muscarinic and nicotinic receptors) intraperitoneally right after the first rotarod training session (Gais and Born 2004, Jafari-Sabet, Jafari-Sabet et al. 2016, Dhingra and Soni 2018). Since it was a single dose study an acute dosage of physostigmine 0.1mg/kg was

used. After the training session and drug administration, the animals were transferred to their home cages and allowed to sleep. The elimination half-life of physostigmine is documented to be 1.5-2 hours in humans (Hartvig, Wiklund et al. 1986) and less than 1 hour in rodents (Somani 1989) enough to stay in the system for the course of early SWS. No side effects were observed. Probe session was done on fifth day. In the control condition, 14 mice received saline (S group).

Physostigmine-late Group (Physo-late 24hrs)

Next, we wanted to determine whether or not the performance of the animals on probe day was affected because of the side-effects of physostigmine injected on Day 1. We therefore trained 6 mice on the rotarod on Day 1 but instead of giving the drug right after the rotarod session and before sleep, we injected with same dose of physostigmine 24 hours later. Retesting was done similarly on Day 5 as with Physo group.

Scopolamine/mecamylamine group (Sco+Meca group).

To block cholinergic transmission, we administered a combination of the muscarinic receptor antagonist scopolamine (0.4mg/kg) and the nicotinic receptor antagonist mecamylamine (3mg/kg) intraperitoneally to 8 mice. Previous studies have shown that these drugs if not given in combination do not produce any effect on declarative memory encoding and consolidation (Rasch, Born et al. 2006). Therefore, both substances were administered simultaneously, after the end of the rotarod session on Day 1. Animals were then transferred to their home cages and allowed to sleep. We chose relatively low doses to avoid strong side effects of cholinergic blockade and to ensure that the substances had largely washed out at the time of probe testing. The half-life in plasma has been estimated at 4.5 ± 1.7 hour for scopolamine (Putcha, Cintron et al. 1989) and 10.1 ± 2 hour for mecamylamine (Young, Shytle et al. 2001).

Oxotremorine and nicotine Groups (Oxo and Nico respectively)

To observe ACh receptor specificity for mediation of motor memory consolidation, we administered either oxotremorine (0.01mg/kg) or Nicotine (2mg/kg), a selective muscarinic and nicotinic ACh receptor agonist respectively, to two separate groups of animals (oxotremorine n=8, nicotine n=8) after the rotarod task. The doses were chosen based on past studies that examined the effect of nicotine and oxotremorine on rodents (Power, Vazdarjanova et al. 2003). The half-life has been reported as 100 minutes for oxotremorine and 1.5-2 hours for nicotine (Moyer, Charlson et al. 2002, Marchand, Brossard et al. 2017).

Animals were then transferred to their home cages and were allowed to sleep. Probe session was done on Day 5.

Baseline group

All 58 WT mice trained on Day 1 were combined to form one baseline group. The performance of mice in other groups mentioned above was compared to the baseline group.

Transgenic Groups

17 Transgenic mice, 9 in CNO group (Tg-CNO) and 8 in saline group (Tg-Saline) were used. The dosage of CNO used was 5mg/kg (MacLaren, Browne et al. 2016). CNO was obtained from the Thermo-Fisher Scientific and was dissolved in 10% DMSO then diluted to a final concentration of 0.5 mg/ml CNO with saline solution. As the onset of the action of CNO begins 1-hour post administration, it was injected just before the rotarod training session so that the effect of drug will start in the post-learning sleep after the training. Probe session was done on Day 5 as explained above.

Motion quantification with filming in home cage

In a separate cohort of mice (n = 10), sleep recording was done by filming animals in their home cages after the rotarod training session to observe their motion for quantification of post-learning activity. Five mice were given saline while the rest received physostigmine. A Pi camera connected to a Rasp-berry pi computer was attached to the roof of the lid of the home cage and 2.5 hours long videos were recorded post training (Singh, Bermudez-Contreras et al. 2018). Motion estimation was done in Matlab® (Mathworks, Natick, MA) by first identifying difference of consecutive frames and then calculating the mean difference over pixels to get an approximation of the overall change in the values of pixels from one frame to the other. The accuracy of motion estimation was verified with manual observation.

Sleep quantification using electrophysiology

Animal Surgeries

Four adult C57BL6J mice and one Chat-Cre::CAG-hM4Di were anesthetized with isoflurane (2.5% induction, 1-1.5% maintenance) and implanted with cortical, hippocampal, and muscular electrodes using aseptic techniques. For cortical and hippocampal recording of local field potentials, bipolar (tip separation

= 0.6 mm) and monopolar electrodes made from Teflon-coated stainless-steel wire (bare diameter 50.8 µm) were implanted in cortical areas and pyramidal layer of CA1 hippocampal region according to the following coordinates in mm: primary motor cortex (M1): AP: 1.5, ML: 1.7, DV: 1.5, secondary motor cortex (M2): AP: 1.7, ML: 0.6, DV:1.1 mm, Lip cortical sensory area (LP-S1): AP: 0.75, ML: 3.0, DV: 1.6, retrosplenial cortex (RS): AP: -2.5, ML: 0.6, DV: 1.1, and hippocampus (HPC): AP: -2.5, ML: 2.0, DV:1.1 mm. For EMG, a multistranded Teflon-coated stainless-steel wire (gauge 40) was implanted into the neck musculature using a 25 gauge needle. The reference and ground screws were placed on the skull over the cerebellum. The other end of electrode wires were clamped between two receptacle connectors (Mill-Max Mfg. Corp.) and the headpiece was secured to the skull using metabond and dental cement.

Electrophysiology

Animals were allowed to recover for 10 days after surgery and then habituated for 5 to 7 days in the recording setup. On baseline days, animals were injected with saline at 8:25 am and moved to the recording setup where their sleep activity was recorded from 8:30 am for 4 hours using a motorized commutator (NeuroTek Inc.). Baseline recording was repeated for three days. On fourth day, same steps were repeated except animals were injected with physostigmine (dosage 0.1 mg/kg). Local filed potentials and EMG activty were amplified, filtered (0.1-4000 Hz) and digitized at 16 kHz using a Digital Lynx SX Electrophysiology System (Neuralynx, Inc.) and the data were recorded and stored on a local PC using Cheetah software (Neuralynx, Inc.). A Pi camera was used to record animal's behaviour during the recording.

Data Analysis

All the analyses were performed offline. Sleep scoring was performed in 6-sec long epochs. For that, raw EMG activity was filtered between 90 Hz and 1000 Hz and then rectified and integrated using 4-sec moving windows. This signal was thresholded to detect periods of immobility. Slow wave power (0.5 to 4 Hz) of the cortical LFP was calculated in each epoch using taper spectral analysis and thresholded using values between 0.04 and 0.1 mV^2/Hz (depending on the animal) to detect SWA in cortical recording. When the animal was immobile and cortical LFP showed SWA, the epoch was scored as SWS. For detecting REM sleep, the ratio of theta power (6 to 9 Hz) to the total power of hippocampal LFP was calculated in each epoch using taper spectral analysis and when this ratio was above 0.4-0.6 (different for individual animals) and EMG showed immobility, the state was scored as REM sleep. All other epochs

were considered as waking state. These scorings were further confirmed using video recording. To investigate the effect of physostigmine on sleep, duration of each behavioral state in each recording session was normalized to the recording duration and then compared between physostigmine and saline. SWS latency was calculated as the time between the onset of recording and the onset of first SWS epoch longer than 20 seconds. Moreover, slow wave power (0.5 to 4 Hz) of cortical signal was averaged across all SWS epochs in the first hour of recording and then compared between physostigmine and saline. For saline injection, sleep structure, SWS latency and SW power were averaged across three baseline days for each animal.

For detecting spindle events, following Philips et. al., M2 cortical signal was filtered between 8 and 18 Hz and then rectified (Phillips, Bartsch et al. 2012). The envelope of the rectified signal was then calculated using cubic interpolation of its maxima. Putative spindle events were detected where the envelope signal was higher than 3.5 standard deviations (SD) from its mean. Onset and end of spindle events was determined where the envelope signal was higher than 2.5 SD from its mean. Spindles shorter than 500msec or longer than 2sec were rejected. Spindles that occurred within 100 msec of each other were combined to be considered a single event.

Sleep deprivation

Animals were observed for signs of sleep such as little motion and closed eyes and were gently moved with hand to prevent them from sleeping. Place of their nest was also changed sometimes to keep them awake for a little longer period before they would begin to sleep again. Occasionally, their cage was lifted and put down to keep them awake. However, animals were not disturbed when they were already awake.

Statistical Analysis

Statistical analysis of behavioral data was done in SPSS (Version 22) and verified in Matlab R2016 while all other statistical analysis was done in Matlab R2016. All data are presented as mean ± the standard error of mean (S.E.M). Statistical tests, paired and two sample Student's t-test (both two and one tailed), one-way analysis of variance (ANOVA), and one-way ANOVA with repeated measures (ANOVA-RM) were used. Statistical test are reported in the text along with p values. For ANOVA-RM, Greenhouse-Geisser corrected p value (p_{gg}) is reported for tests in which Mauchly's criteria of sphericity failed (Mauchly 1940,

Geisser and Greenhouse 1958, Huynh and Feldt 1976). An alpha value of 0.05 was used for determining significance.

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AUTHOR CONTRIBUTIONS

Q, SI, IQW, BLM and MHM designed the study. Q and SI performed all rotarod and reach task experiments. Q, SI, and IQW analyzed behavioral data. SI and SS collected and analyzed home cage video recording data. For electrophysiology experiments, MN performed animal surgeries for implanting electrodes, SI, Q, and MN collected data, and MN and SI analyzed data. MHM participated in all data analyses. SI, Q, IQW, and MHM wrote the manuscript, which all authors commented on and edited. IQW and MHM supervised the study.

SUBMISSION & CORRESPONDENCE INFORMATION

Dr. Majid Mohajerani, mohajerani@uleth.ca

WORK PERFORMED

All the work was performed at the Canadian Center for Behavioral Neuroscience, University of Lethbridge, Alberta, Canada

DISCLOSURE STATEMENT

None.

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