LOW ACETYLCHOLINE DURING EARLY SLEEP IS CRUCIAL FOR MOTOR MEMORY CONSOLIDATION

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Abstract

There has been no evidence that manipulation of acetylcholine levels in association with sleep treatments can influence motor memory storage. The purpose of the present study was to investigate a potential relationship between motor memory and acetylcholine activity during early part of sleep. We propose that during the early phase of sleep, characterized by slow wave electroencephalographic activity and reduced cholinergic tone, a downscaling of overall synaptic strength happens that improves signal-to-noise ratio and facilitates motor memory consolidation. Therefore, altering acetylcholine levels during early sleep would disrupt the consolidation of motor memories. To test this hypothesis, 93 C57 Black/6J and 17 transgenic (CAG-hM4Di x Chat-Cre) adult mice were trained for motor tasks either rotarod or skilled-forelimb reach. Immediately after motor training, ACh was either increased or decreased in the subsequent post-learning sleep. Retesting showed that increasing ACh in post-learning sleep impaired motor memory consolidation however, decreasing ACh levels did not have an effect. Furthermore, a relatively larger involvement of muscarinic ACh receptors compared to nicotinic ones was observed in motor memory consolidation. Home-cage filming suggested that mice in both control and experimental conditions had similar sleep cycles while electrophysiology showed that slow wave power in early sleep was reduced with increasing ACh levels. These results suggest that motor memories are consolidated during slow wave sleep when cholinergic levels are low. The early phase of sleep therefore may contribute to motor memory consolidation by providing a homeostatic state characterized by slow wave activity and reduced ACh.

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List of Abbreviations

EEG	Electroencephalography
EMG	Electromygraphy
LTP	Long term potentiation
LTD	Long term depression
NREM	Non-rapid eye movement sleep
REM	Rapid eye movement sleep
SWS	Slow wave sleep
SWA	Slow wave activity
LFPs	Local field potentials
GABA	Gamma-aminobutyric acid
SWRs	Sharp wave ripples
PGO	Ponto-geniculo-occipital
ACh	Acetylcholine
SI	Substantia inominata
NBM	Nucleus basalis magnocellularis
MS	Medial septum
mAChRs	Muscarinic acetylcholine receptors
nAChRs	Nicotinic acetylcholine receptors
DREADDs	Designer receptors exclusively activated by designer drugs
CNO	Clozapine N-oxide
P	Physostigmine
O	Oxotremorine
N	Nicotine
SM	Scopolamine + Mecamylamine
S	Saline
Tg	Transgenic
WT	Wild-type

1. Introduction

> *"Remarkable it may fitly be called; for what more singular than that nearly a third part of existence should be passed in a state thus far separate from the external world! – a state in which consciousness and sense of identity are scarcely maintained; where memory and reason are equally disturbed; and yet, with all this, where the fancy works variously and boldly, creating images and impressions which are carried forwards into waking life, and blend themselves deeply and strongly with every part of our mental existence"*
>
> *(Holland, 1839)*

Sleep is a mysterious state the explanation of which has eluded neuroscientists. Nevertheless, over nearly a century of investigation, with a waxing and waning of interest, a large body of research has grown to show that sleep facilitates memory processing and consolidation. Beginning with Ebbinghaus's (1885) well known self-report of "forgetting curve" and role of sleep in reducing forgetfulness (Ebbinghaus, 2013), a substantial literature has developed showing that sleep benefits memory retention. Jenkins and Dallenbach and Van Omer provided the first experimental evidence in 1924 (Jenkins & Dallenbach, 1924; Rasch & Born, 2013; Van Ormer, 1932) and revealed similar effects of sleep as it acts by passively protecting memory from retroactive interferences. They showed that memory retention was better in the morning after a night of sleep than in the evening after an equivalent amount of time awake. However, in the latter half of the 20th century, many studies advocated for an active role of sleep in memory consolidation. They proposed that an active redistribution of recently encoded memory representations within hippocampal and neocortical networks takes place during sleep and could be a basis for long-term memory consolidation (Rasch & Born, 2013). However, questions remain as to

how different phases of sleep contribute to the consolidation of different types of memories under the influence of neuromodulation. My study highlights and addresses some of these questions and presents a background related to memory processes, types of memories, the role of sleep, phases of sleep, and neuromodulation with acetylcholine.

1.1 Memory processes

Memory formation and retrieval is the most fundamental ability of multicellular organisms with nervous systems and it allows them to adapt to the challenges of the ever-changing environment. The memory function is composed of three basic subprocesses 1) encoding, 2) consolidation, and 3) retrieval. Mechanisms underlying the formation, storage, and retrieval of long-term memories are still under investigation. It is suggested that memory encoding and retrieval happens during wakefulness, while memory consolidation happens during sleep. At this time initially fragile newly encoded memories become stabilized or consolidated for long-term retention (McGaugh, 2000).

Memory formation at the neuronal level is thought to be mediated by the strength of synaptic connections. When a synapse is repeatedly activated or inhibited, its strength increases or decreases through the processes of long-term potentiation (LTP) or long-term depression (LTD) respectively. LTP or LTD represent major forms of learning-induced synaptic plasticity induced by encoding (Collingridge, Peineau, Howland, & Wang, 2010; Kandel, 2001; Kemp & Manahan-Vaughan, 2007). During LTP and LTD, the density of membrane receptors in the synapse is changed. After memory formation, the corresponding neuronal activity that encodes the memory reverberates in the neuronal networks for either synaptic or systems consolidation (Dudai, 2004).

1.1.1 Synaptic consolidation

In synaptic consolidation (synaptic LTP and LTD), the memory is consolidated in place where it was encoded i.e. (synapses and spines of the neurons in the network contributing to the memory trace). Synaptic consolidation leads to remodeling of these synapses and spines through LTP and LTD, and eventually stabilizes the reverberating neuronal representation and augments the efficacy of the participating synapses through strengthening of synaptic connections (Hofer, 2010; Kandel, 2001; Redondo & Morris, 2011). Synaptic consolidation happens at a fast temporal pace i.e. (milliseconds to minutes) and involves changes in early gene expression, transcription factors, and synaptic proteins that occur in a limited time window during and immediately after training (Dudai, 2004). As mentioned below system's consolidation is based on synaptic consolidation.

1.1.2 System consolidation

System consolidation is a processes through which labile memory traces in newly encoded representations are reorganized and perhaps transferred to other brain regions for the formation of permanent memory (Frankland & Bontempi, 2005). Memory is first formed and stored in the hippocampus and later transferred to cortical regions for long-term storage. While the memory resides in the hippocampus, reactivation can facilitate synaptic consolidation in the hippocampus as well as system consolidation for transfer to cortical regions (Euston, Tatsuno, & McNaughton, 2007; Sutherland & McNaughton, 2000; Wilson & McNaughton, 1994). After system consolidation, the memory retrieval becomes independent of hippocampus (Takehara, Kawahara, & Kirino, 2003). Note that system consolidation is dependent upon synaptic consolidation.

1.2 Types of memories

An extensive list of the types of memories is beyond the scope of this thesis. Therefore only long-term memories are introduced here which are classified into two major types; Declarative and Non-declarative (Rasch & Born, 2013).

1.2.1 Declarative memories

Declarative memories are explicitly or consciously acquired and are proposed to require the integrity of hippocampal circuitry. They are sometimes referred to as hippocampus dependent memories. Declarative memories are further divided into 1) episodic memories, memories for autobiographical material and memories related to spatial and temporal context and 2) semantic memories i.e., memories of facts. Declarative memory is relatively encoded quickly and can be demonstrated through recall of a single trial and require medial temporal lobe structures (hippocampus and surrounding brain regions) for formation and consolidation (Alger, Chambers, Cunningham, & Payne, 2015; Squire & Zola, 1996; Winocur, Moscovitch, & Bontempi, 2010).

1.2.2 Non-declarative memories

Non-declarative memories are implicitly or unconsciously acquired, e.g. (perceptual skills), procedural learning and certain forms of conditioning. Non-declarative memories do not require medial temporal lobe structures and hippocampal circuitry for acquisition and storage (Squire & Zola, 1996). In this thesis we have focused on a type of procedural memory called motor memory.

1.2.2.1 Motor memories

Motor memories are learnt sequences of movements e.g. (riding a bicycle, driving a car, or playing a piano) and are stored in brain areas such as motor cortex, striatum, and cerebellum (Kandel, 2013; Squire & Zola, 1996). Motor memories are gradually acquired during multiple trials of motion sequences through an online i.e. (inter trial) as well as offline i.e. (inter session) mechanism characterized by absence of training (Buitrago, Ringer, Schulz, Dichgans, & Luft, 2004; Karni et al., 1998). This offline period implies a limited but susceptible time window for consolidation as motor memory trace is prone to get conflicted with interferences until it gets stabilized (Krakauer & Shadmehr, 2006). Our brain can form multiple long-term (> 24 hours) motor memories, in as much that they become well-characterized reflexes and we seemingly never forget them. Interestingly, where declarative memories require attention or awareness for their storage, motor memories can be acquired and recalled without explicit attention (Krakauer & Shadmehr, 2006).

1.3 Functions of sleep and memory consolidation

In modern text books, sleep is defined as a naturally recurring, active and reversible physiological state characterized by loss of consciousness and relative reduction in responsiveness to external stimuli. Several hypotheses have been proposed regarding functions of sleep as to why sleep is essential. These hypotheses focus on aspects of homeostasis, such as energy conservation (Berger & Phillips, 1995; Rasch & Born, 2013; Webb, 1988), metabolic regulation (Knutson, Spiegel, Penev, & Van Cauter, 2007), thermoregulation, cell tissue restoration (Oswald, 1980), detoxification of free radicals (Inoue, Honda, & Komoda, 1995; Reimund, 1994), and immune function (Lange,

Dimitrov, & Born, 2010). The most exciting hypothesis is that sleep contributes to cognition and promotes cognitive functions such as memory, language ability, decision making, categorization etc. (Chambers, 2017; Diekelmann, 2014). Because of the vital role of learning and memory in various cognitive processes that promote brain plasticity, recent research has focused on influence of sleep on memory. With the use of amnesic interventions such as sleep deprivation and pharmacological agents causing inactivation of neural activity, it has been proposed that memory consolidation happens during sleep (Dudai, Karni, & Born, 2015). Both declarative and motor memories are improved by sleep (Chambers, 2017) but how phases of sleep transform newly assimilated fragile memories into enhanced and stable memory traces is not understood.

1.4 Sleep stages or phases

Sleep in mammals consists of two basic phases which alternate in a cyclic manner. One phase is called is called rapid-eye-movement (REM) sleep, because eye movements are observed at this time. This phase of sleep is also characterized by an activated (fast wave) electrical activity in the neocortex and the absence of muscle tone. The other phase is Non rapid eye movement sleep (NREM) during which no eye movement occur. This phase is also called slow wave sleep (SWS) because slow waves are recorded from the neocortex (Diekelmann & Born, 2010).

1.4.1 NREM sleep

NREM sleep can be differentiated from wakefulness and REM sleep by a generalized reduction of physiological markers like heart rate, respiratory rate, blood pressure. As is noted above it is marked by slow high-amplitude electroencephalographic (EEG)

oscillations (slow wave activity, SWA). NREM sleep occurs in the early part of the sleep and is subdivided into lighter N1, N2 and deeper N3 stage in humans (Fig. 1.1). However, a clear subcategorization has not been shown in rodents although several attempts have been made (Jarosiewicz, McNaughton, & Skaggs, 2002; Sharma, Wolansky, & Dickson, 2010). For example, Jarosiewicz et al 2002, characterized sleep in rats and found a second physiological state within SWS called small amplitude irregular activity in addition to the well characterized large amplitude irregular activity. Three major types of electric oscillatory activity described below occur during NREM sleep. The focus of this thesis however is the slow oscillatory activity which occur during early sleep (Shore, Millman, Silage, Chung, & Pack, 1985; Siegel, 2009; Zemaityte, Varoneckas, & Sokolov, 1984).

1.4.1.1 Slow oscillations

The early part of sleep, SWS, is dominated by slow oscillatory, <1-Hz, synchronized activity evident in EEG (Diekelmann & Born, 2010). During SWS, neurons get depolarized and hyperpolarized periodically giving rise to slow waves. Depolarized and hyperpolarized states of neurons are also called up and down states respectively (Vyazovskiy & Harris, 2013). Slow waves originate from neurons in layer 5 of the cortex and later spread to superficial cortical layers (Chauvette, Volgushev, & Timofeev, 2010). In humans, slow waves typically originate from the frontal cortex and spread as travelling waves to other cortices (Massimini, Huber, Ferrarelli, Hill, & Tononi, 2004). The amount of SWA during SWS characterizes sleep homeostasis (Hanlon, Faraguna, Vyazovskiy, Tononi, & Cirelli, 2009).

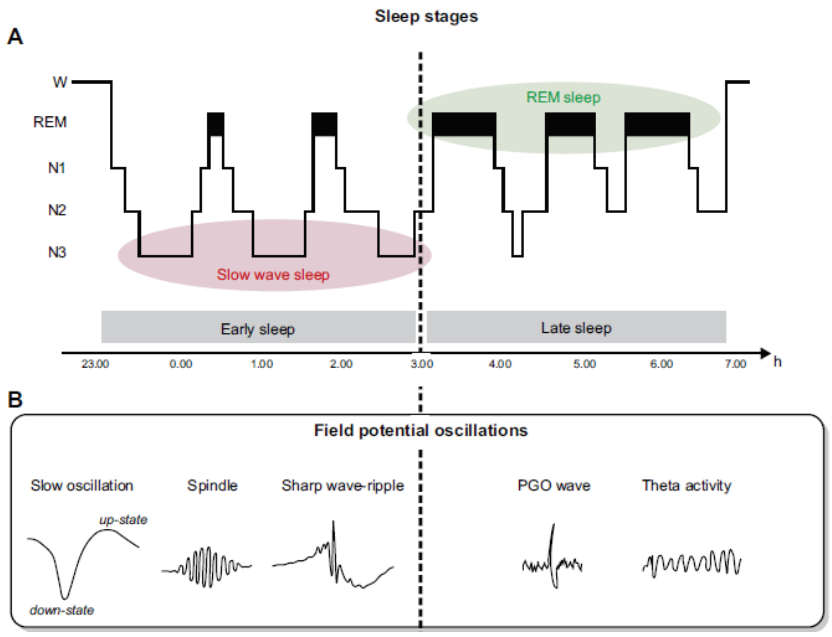

Figure 1.1 The composition of sleep stages. (**A**).The brain cycles through early non-rapid eye movement (NREM) sleep which progresses from lighter N1, N2 and deeper N3 stages and rapid-eye-movement and the later (REM) sleep in roughly 90-min intervals. The deepest stage of NREM sleep is slow wave sleep (SWS), and majority of it is obtained during the first half of the night, while more REM sleep is dominant during the second half of the night. (**B**). During NREM sleep three major electric field potential occur, neocortical slow oscillations, thalamocortical spindles and hippocampal sharp wave ripples. REM sleep is characterized by pontogeniculo-occipital waves (PGO) and hippocampal theta activity. Reprinted with permission from The American Physiological Society, (Rasch & Born), copyright (2013).

1.4.1.2 Thalamocortical Spindles

In the lighter stage of NREM sleep, local field potentials (LFPs) recorded from the thalamus and filtered between 10-15 Hz reveal waxing and waning activity that originates in the thalamus and spreads to cortical and hippocampal networks. This activity is referred to as thalamocortical spindles (Fig. 1.1) (Steriade, 2003). Studies have shown that spindles are produced when thalamocortical cells interact with gamma-aminobutyric acid (GABA) releasing interneurons in the reticular nucleus (Halassa et al., 2011; Steriade, Deschenes, Domich, & Mulle, 1985; Steriade, Domich, Oakson, & Deschenes, 1987). The

synchronization of spindles within the thalamic areas is controlled by projections from the cortex to the thalamus (Contreras & Steriade, 1996; Steriade, 2003).

1.4.1.3 Hippocampal sharp wave ripples

Sharp wave ripples (SWRs) are seen in hippocampal LFPs and represent high frequency 100-300Hz ripples riding on top of fast depolarization waves. SWRs originate in the CA3 region and may be locked to cortical up states and thalamocortical spindles. It has been proposed that SWRs mediate the memory consolidation process through hippocampal-cortical interactions (Rothschild, Eban, & Frank, 2017; Siapas & Wilson, 1998; Sirota, Csicsvari, Buhl, & Buzsaki, 2003).

1.4.2 REM sleep

REM sleep, (also termed paradoxical sleep), is dominated by fast and low-amplitude oscillatory brain activity (desynchronized) and increase in physiological markers like heart rate, respiratory rate etc., (similar to wakefulness) and muscular atonia. REM sleep tends to occur in the later part of the sleep and is characterized by rapid movement of the eyes. It is also associated with vivid dreams as well (Kahn, Stickgold, Pace-Schott, & Hobson, 2000). During REM sleep, memory consolidation is dependent on 7-19 Hz theta-1 and 4–6 Hz theta-2 oscillations (Pavlova, 2009) in the hippocampus generated by GABAergic interneurons projecting from the medial septum (Boyce, Glasgow, Williams, & Adamantidis, 2016). REM sleep, in animals, is also characterized by ponto-geniculo-occipital (PGO) waves, which are generated from pontine brainstem and propagate to lateral geniculate nucleus and visual cortex (Fig. 1.1). PGO waves are characterized by

strong bursts of synchronized activity that occur in temporal association in rodent EEG but are not readily identified in human EEG (Rasch & Born, 2013).

Among these sleep oscillations, recent research emphasizes a relationship between SWS and memory consolidation. Several studies have remarked on the clinical implications of SWS phenomena by enhancing memory consolidation through triggering of slow waves in humans and animals (Marshall, Helgadottir, Molle, & Born, 2006; Miyamoto et al., 2016; Rembado, Zanos, & Fetz, 2017). SWS likely modulates synaptic connections that are important for long-term memory formation through a complex neuro-chemical interplay of various neuromodulators such as acetylcholine (ACh) (Tononi & Cirelli, 2006).

1.5 Sleep and acetylcholine neuromodulation

As mentioned above sleep and sleep stages were originally defined by frequency signatures in electroencephalograms and local field potentials recorded from different brain regions. Chemical measurements have revealed the composition of neurotransmitters and hormones during sleep can also be used to characterize sleep stages. In general, neurotransmitter activity is more likely to be upregulated and down-regulated during REM and NREM sleep respectively (Diekelmann & Born, 2010; Tononi & Cirelli, 2006). How sleep stages and associated neurochemical activity contribute to memory consolidation is still debated. However, some of the neurotransmitters and hormones create a specific neurochemical milieu that contribute to memory consolidation through synaptic and system consolidation (Rasch & Born, 2013; Tononi & Cirelli, 2006). It has been proposed that these neurochemical changes facilitate either replay of the learning experience (Euston et al., 2007; Kali & Dayan, 2004; Wang, Grone, Colas, Appelbaum, & Mourrain, 2011) or

synaptic changes that were initiated during learning (Abraham & Williams, 2008; Polyn & Kahana, 2008). A number of studies also suggest that the activity of acetylcholine, dopamine, serotonin, noradrenaline, GABA, and so forth could be related to memory storage (Connors, Malenka, & Silva, 1988; Eschenko, Magri, Panzeri, & Sara, 2012; Gais & Born, 2004; Ghoneim & Mewaldt, 1990; Lena et al., 2005). In this thesis however, we focus on the neuromodulatory role of neurotransmitter acetylcholine and how it facilitates motor memory consolidation during sleep.

ACh is a key neurotransmitter that carries out an array of functions and is involved in attention, arousal, circadian rhythmicity and memory formation and consolidation (Hut & Van der Zee, 2011; Micheau & Marighetto, 2011; Obermayer, Verhoog, Luchicchi, & Mansvelder, 2017). SWS is characterized by dramatic decline in cholinergic activity compared with the alternatively high cholinergic tone during wakefulness and REM sleep (Hasselmo, 1999; Marrosu et al., 1995; Pace-Schott & Hobson, 2002). ACh release in the neocortex, hippocampus and tegmental area is activated over projections from nuclei in the basal forebrain and it has been shown that this system is related to cortical plasticity and motor learning (Conner, Culberson, Packowski, Chiba, & Tuszynski, 2003; Conner, Kulczycki, & Tuszynski, 2010; Ramanathan, Tuszynski, & Conner, 2009).

Acetylcholine is released in cortical and subcortical areas via two groups of projections: the magnocellular basal forebrain cholinergic system and the brainstem cholinergic system (Newman, Gupta, Climer, Monaghan, & Hasselmo, 2012; Paul et al., 2015).

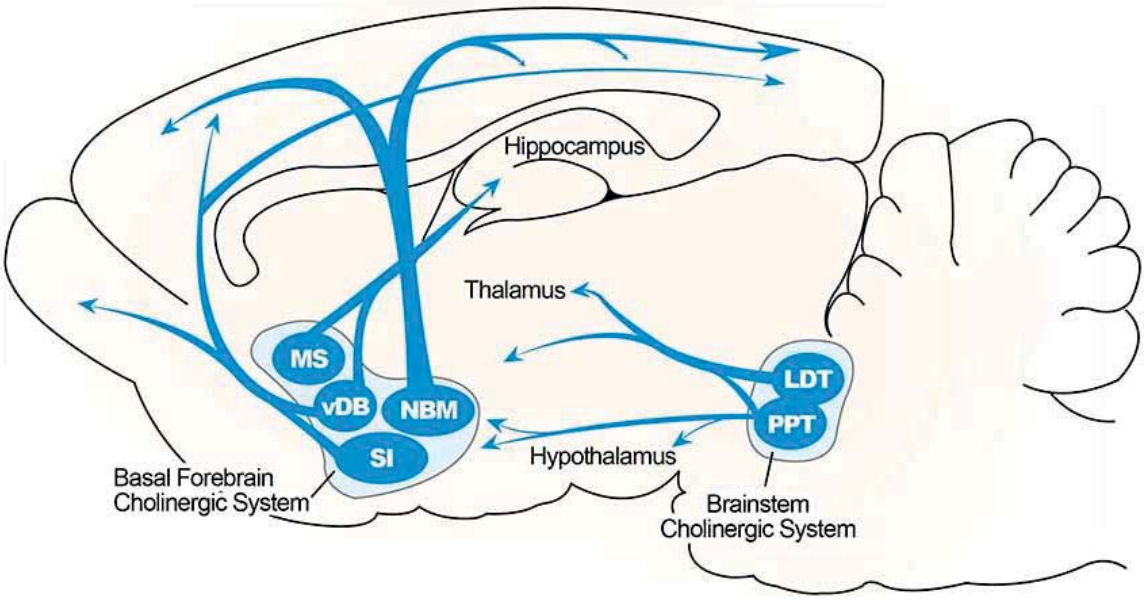

Figure 1.2 Anatomy of major cholinergic projections to central nervous system. Basal Forebrain Cholinergic system through various nuclei (SI substantia inominata, NBM Nucleus basalis magnocellularis, MS medial septum and vDB vertical limb of diagonal band of broca) sends afferent projections to the entire neocortex. Brainstem Cholinergic system sends projections to thalamus and basal forebrain via LDT latero-dorsal tegmental nucleus and PPT pendunclo-pontine tegmental nucleus. Reprinted with permission from the Frontiers in Aging Neuroscience, (Paul, Jeon, Bizon, & Han, 2015), copyright (2015).

1.5.1 The magnocellular basal forebrain cholinergic system

The magnocellular basal forebrain cholinergic system serves as a major component of the cholinergic projections innervating the neocortex. This system includes cells located in the medial septal nucleus (MS), the vertical and horizontal limbs of the diagonal band of Broca (DB), and the nucleus basalis magnocellularis (nBM). The horizontal limb of the DB and nBM extensively innervates neocortex as well as send projections to basolateral amygdala and olfactory bulb. The MS and vertical limb of the DB mainly innervate the limbic cortices including hippocampus and entorhinal cortices (Mesulam, Mufson, Wainer, & Levey, 1983; Mike, Castro, & Albuquerque, 2000; Mufson, Bothwell, Hersh, & Kordower, 1989).

1.5.2 The brainstem cholinergic system

The brainstem cholinergic system serves as a minor component of the cholinergic projections innervating the neocortex. This system includes neurons located in the pedunculopontine tegmental nucleus (PPT) and laterodorsal pontine tegmentum (LDT), which predominantly projects to the thalamus and basal ganglia but also innervates the basal forebrain (Mesulam et al., 1983; Rye, Saper, Lee, & Wainer, 1987).

1.6 Acetylcholine receptors and subtypes

Once ACh is released, it can bind to two types of receptors, muscarinic receptors (mAChRs), and/or nicotinic receptors (nAChRs) (Kolb & Whishaw, 2010).

1.6.1 Muscarinic acetylcholine receptors (mAChRs)

Muscarinic acetylcholine receptors (mAChRs) are metabotropic (g-protein coupled) receptors and are activated by muscarine but not nicotine. Muscarinic receptors are categorized into five subtypes M1-M5 which are differentiable via amino acid structures and immunohistochemistry. The M1 receptors are functionally related to M3 and M5 receptors and mediate postsynaptic depolarization due to their location at the post-synaptic site. M2 receptors are functionally related to M4 receptors which mediate cholinergic modulation of glutamatergic synaptic transmission and are located at both pre- and post-synaptic sites (Dasari & Gulledge, 2011; Mrzljak, Levey, & Goldman-Rakic, 1993).

1.6.2 Nicotinic acetylcholine receptors (nAChRs)

Nicotinic acetylcholine receptors (nAChRs) are ligand gated, ionotropic, cholinergic receptors that are activated by nicotine but not muscarine. They are diversely distributed in

brain as either heteropentamers or as homopentamers of alpha and beta subunits and carry out an array of functions ranging from their role in development and control of cell cycle to brain plasticity (Barbagallo, Prescott, Boyle, Climer, & Francis, 2010; Bear & Singer, 1986; Soderman, Mikkelsen, West, Christensen, & Jensen, 2011).

A substantial literature suggests that mAChRs are responsible for mediation of most of the effects of ACh released from cholinergic neurons to the cerebral cortex, hippocampus and basal ganglia (Brown, 2010; Power, Vazdarjanova, & McGaugh, 2003). It has also been shown that the encoding and consolidation of episodic (time-place association) memories which are type of declarative memories are also predominantly influenced by mACHRs (Hut & Van der Zee, 2011). Furthermore, administration of cholinergic agonists, particularly with affinity to mAChRs seems to mimic the effect of wakefulness in EEG whereas cholinergic antagonists induce slow wave activity in anesthetic preparations (Dringenberg & Vanderwolf, 1997; Vanderwolf, Raithby, Snider, Cristi, & Tanner, 1993).

1.7 Role of slow-wave sleep and acetylcholine in memory consolidation

1.7.1 Declarative memory and system consolidation

The role of cholinergic activity in the brain for declarative memory consolidation has been proposed by Buzaski and Hasselmo as follows (Fig. 1.3). During awake and awake–like conditions such as REM sleep, elevated levels of acetylcholine enable information encoding initially in the neocortical structures. The encoded information/memory then flows through entorhinal cortex and dentate gyrus to CA3 region of the hippocampus. High acetylcholine levels suppress the hippocampal-cortical connections therefore the information is stored in CA3 region temporarily. During SWS the minimal cholinergic

activity thereby releases the tonic inhibition of hippocampal CA3 and CA1 feedback neurons (from cholinergic influence) and enables the transfer and reactivation of encoded information to the neocortical structures for long-term consolidation (Hasselmo, 1999).

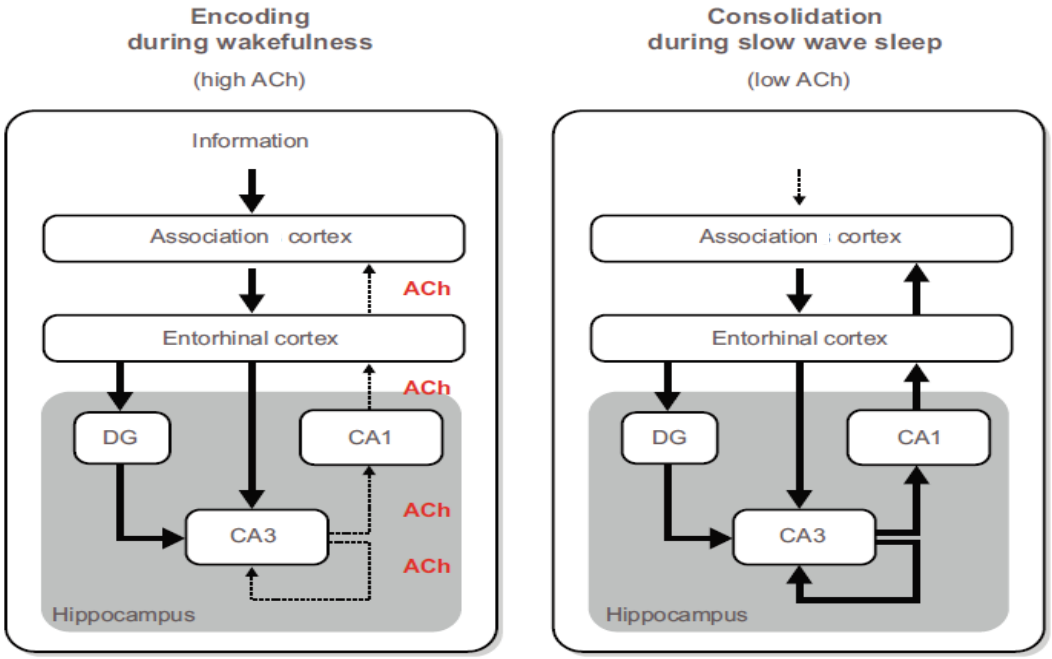

Figure 1.3 Two-stage model of declarative memory formation and system consolidation. Increased levels of acetylcholine during wakefulness results in memory encoding, while decreased levels during slow wave sleep results in consolidation. Reprinted with permission from the American Physiological Society, (Rasch & Born, 2013), copyright (2013).

Consistent with this model, increasing the cholinergic tone in humans during post-learning slow wave rich sleep by administration of a choline-esterase inhibitor impairs the consolidation of declarative memories (Gais & Born, 2004). Conversely, combined blockade of both muscarinic and nicotinic acetylcholine receptors during post learning SWS enhanced consolidation of declarative memories but impaired encoding of new memories (Hasselmo & McGaughy, 2004; B. Rasch, Born, & Gais, 2006). These studies concluded that SWS may not be involved in non-declarative memory consolidation.

1.7.2 Procedural/motor memory and synaptic consolidation

An ongoing debate among the neuroscience community is whether non-declarative memories are consolidated during REM sleep or SWS corresponding to low and high acetylcholine levels respectively (Gais, Plihal, Wagner, & Born, 2000; Gais, Rasch, Wagner, & Born, 2008; Krakauer & Shadmehr, 2006; Luo, Phan, Yang, Garelick, & Storm, 2013; Mascetti et al., 2013; Pennartz et al., 2004; Schonauer, Geisler, & Gais, 2014). No convincing evidence suggests the specificity of distinct phases of sleep for non-declarative memory consolidation. Several animal and human studies have directly or indirectly concentrated on the role of SWS for non-declarative memory consolidation. Reactivation of motor task-specific neurons during NREM sleep i.e. (SWS) is involved in forming new synapses after motor learning in mice (Yang et al., 2014). In rats skilled motor learning increased the SWA during post-learning sleep and augmented the cortical protein levels of the *c-fos* and *Arc-2* experience dependent genes in same region in the motor cortex (Hanlon et al., 2009). In humans, visual texture discrimination skills significantly improve after early sleep, characterized by slow wave activity, but did not improve after REM sleep (Gais et al., 2000). Visuomotor learning improves motor task performance in a reaching task and induces local increases in sleep slow wave activity (SWA) (Landsness et al., 2009). Arm immobilization induces deterioration in motor performance, a decrease in cortical plasticity, and a local decrease in sleep SWA (Huber et al., 2006). Moreover, studies have shown that the basal forebrain cholinergic system is involved specifically in forms of learning and memory that induce cortical plasticity e.g. (motor learning and memory). Selective lesions of cholinergic neurons in basal forebrain not only impair motor task acquisition but also eliminate cortical plasticity associated with motor skill learning lesions (Conner et al., 2003).

In light of the above-mentioned literature review, it is less known how SWS and ACh levels facilitate motor memory consolidation. However, evidence suggests that contrary to declarative memory consolidation, which happens at the system level, motor memories are encoded and consolidated at the synaptic level (Tononi & Cirelli, 2006). Therefore, we propose that the synaptic homeostasis theory of sleep (explained next) suggested by Tononi and Cirelli might explain the combined role of SWS and ACh for motor memory consolidation.

1.7.2.1 Synaptic homeostasis theory of sleep

According to this model, wakefulness is characterized by upregulation of plastic processes, synaptic proteins, increased release of neuromodulators, overall fast pattern of EEG activity, and net increase in synaptic strength in the cortical networks. SWS is characterized by slow EEG activity, therefore plays a role in a downscaling of overall synaptic strength to maintain a stable homeostatic state (indicated by Process S in Fig. 1.4). The unique neuromodulatory milieu with reduced ACh, noradrenaline, serotonin, and histamine during SWS is also an important factor promoting downregulation. Sleep dependent downscaling benefits learning and memory consolidation by conserving the energy, promoting synaptic competition, and increasing the signal-to-noise ratio in the relevant brain structures.

The increase in signal-to-noise (SNR) ratio might occur due to 1) an increase in signal i.e. (efficacious synapses), 2) decrease in noise i.e. (erroneous synapses) or 3) both an increase in signal and decrease in noise. A mechanism for increase in signal might be “reactivation” of memory which has been clearly shown for declarative memories (Euston et al., 2007; Sutherland & McNaughton, 2000; Wilson & McNaughton, 1994). During sleep,

reactivation of brain areas which were involved during learning would strengthen the signal and improve SNR. Decrease in noise could happen due to reverse learning in the neuronal networks as suggested by Crick and Mitchison (1983). According to them, when learning happens, parasitic modes i.e. (noise) might also appear due to strengthening of unwanted synapses. Parasitic modes must be removed to reduce noise from the network for memory encoding and consolidation. Crick and Mitchison however argued that REM sleep plays the role of reverse learning and cleaning of parasitic modes by perhaps dreaming-like activation of the cortex (dreaming for forgetting). Increase in SNR by both an increase in signal and decrease in noise might also occur when reactivation and dreaming-like activation would happen in a sequential/cyclic fashion (Ambrosini & Giuditta, 2001; Giuditta et al., 1995).

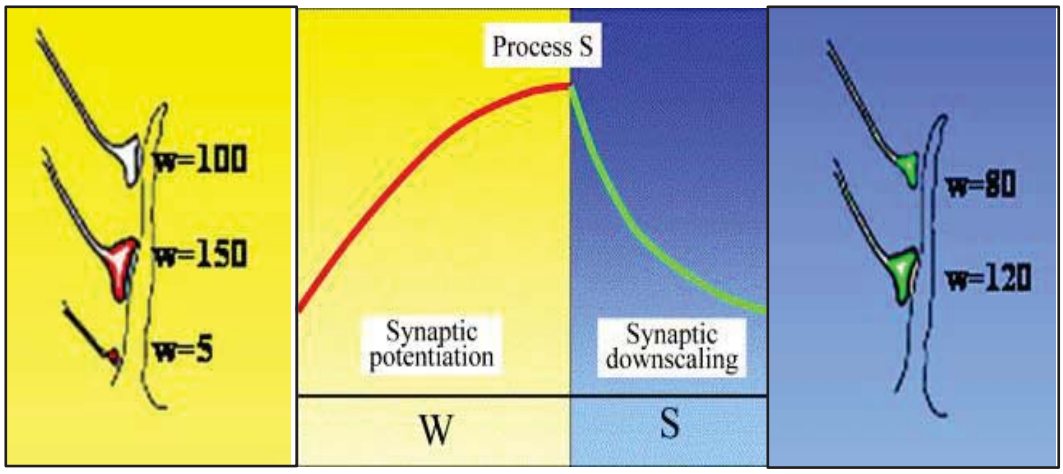

Figure 1.4 The synaptic homeostasis model involving synaptic potentiation during wakefulness and synaptic downscaling during sleep. Process "S" indicates homeostasis. "W" and "S" indicate wake and sleep periods shown in yellow and blue backgrounds respectively. "w" indicates synaptic weight. Red colored synapse with w = 150 is strengthened while one with w = 5 is newly formed during learning. After synaptic downscaling, the synapse with weights w=150 and 100 are downscaled to 120 and 80 (green color) respectively. The synapse with w = 5 is eliminated. Reprinted with permission from Elsevier, (Tononi & Cirelli, 2006), copyright (2006).

Following these arguments, during motor learning, motor memories are induced by plastic changes in a subset of synapses through upregulation of postsynaptic receptors. As a result, an increase in the potentiation of synaptic strengths is observed. For homeostasis, SWS instigates downscaling of overall synaptic strength to help differentiate non-allocated or poorly allocated synapses (noise) from efficacious and strong synapses (signal) improving signal to noise and facilitating motor memory consolidation. Synaptic downscaling might be mediated by neuromodulation e.g. (by low levels of ACh). However, like declarative memories, SNR can also be improved for motor memory consolidation by post-learning reactivation of motor memories during sleep (Fogel et al., 2017).

Synaptic homeostasis theory accounts for the role of cortical slow wave activity and attributes it to memory consolidation. However, this theory does not consider the role of REM sleep in the homeostasis process since it assumes that like wakefulness, REM sleep corresponds to synaptic potentiation and not downscaling. However, Grosmark et al (2012) showed a contrasting evidence in rats that REM sleep is important for decreasing hippocampal excitability and increasing synchrony of CA1 neurons during the total sleep period (Grosmark, Mizuseki, Pastalkova, Diba, & Buzsaki, 2012).

1.8 The Present Study

Here, we investigated the role of cholinergic activity during early sleep in the consolidation of motor memories. As mentioned above, it has been shown that low levels of ACh in conjunction with SWS enable hippocampal-neocortical dialogue allowing "system consolidation" of declarative memories. However, robust evidence suggesting synaptic

consolidation of motor memories influenced by SWS and cholinergic activity is lacking (Fig. 1.5).

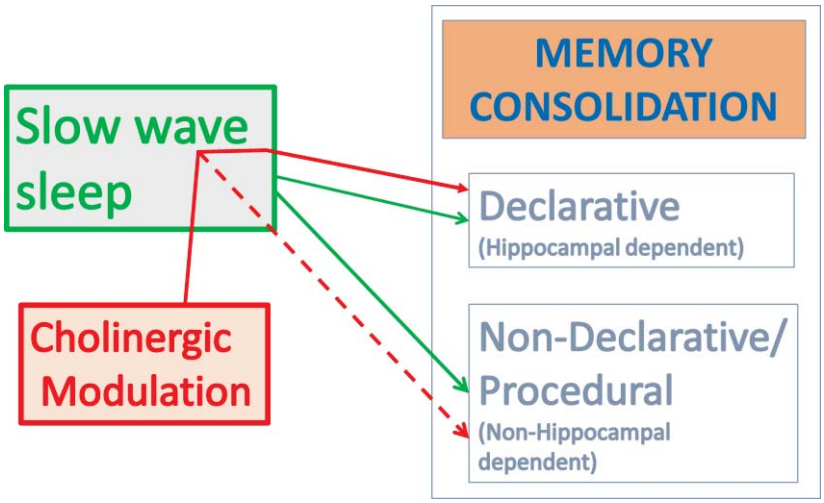

Figure 1.5 The Present Study addresses what is missing in memory consolidation research is how cholinergic neuromodulation plays a role in the consolidation of non-declarative memories including motor memories (shown with dotted arrow).

Theory

In accordance with the synaptic homeostatic theory of sleep, we propose that motor memory consolidation happens at the synaptic level during slow wave sleep under a decreased cholinergic tone. For consolidation, slow wave sleep promotes overall downscaling of synaptic strengths facilitated by low levels of acetylcholine to decrease noise and improves signal-to-noise ratio.

Hypotheses

I hypothesize that

1) Increasing ACh levels during post learning sleep will impair consolidation of motor

memories.

2) Further decreasing ACh levels during post learning sleep will not impair motor memory consolidation.

To test these hypotheses, I induced motor memories in mice using rotarod and Whishaw's skilled-forelimb reach task while altering cholinergic tone during early sleep using pharmacological or chemogenetic manipulation (Dhingra & Soni, 2018; Jafari-Sabet, Jafari-Sabet, & Dizaji-Ghadim, 2016; MacLaren et al., 2016). By elevating acetylcholine levels during early sleep, I demonstrate impairment of motor memory consolidation while I do not see an effect of decreasing acetylcholine levels on motor memory consolidation. Furthermore, I provide evidence that muscarinic ACh receptors play a larger role in motor memory consolidation.

2. METHODS

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

2.1 Experimental Animals

Adult wild-type (WT) mice (C57 Bl/6J, Jackson laboratories), N=93, and transgenic (Tg) mice (CAG hM4Di Chat-cre), N=17, 3-6 months old, both male and female,and weighing 20-30g were used in this study. Transgenic mice had inhibitory 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, & Sternson, 2014; Vardy 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.

2.2 Motor Learning Tasks

2.2.1 Rotarod Task

In the rotarod task, mice learn to balance while running on a rotating drum whose speed is gradually increasing. As they learn to keep their balance they can stay on the rotating drum for longer times at higher speeds of the drum. Fifty eight C57/BL6 and 17 CAG hM4Di

Chat-cre mice 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 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 (Fig. 2.1A). 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.

2.2.2 Whishaw´s 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.

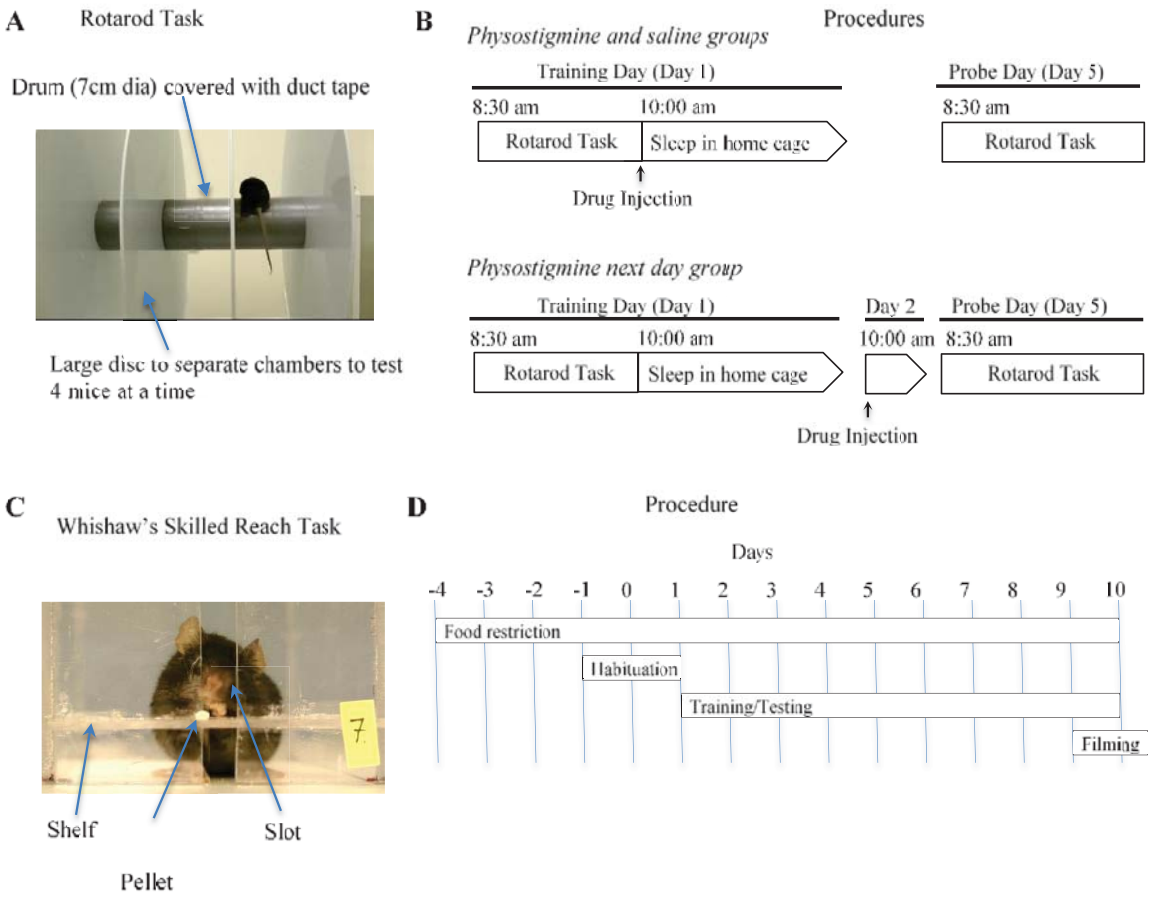

Figure 2.1 Experimental setups and procedures. **(A)** A rotarod consists of a rotating drum on which a mouse is placed, and it must keep balance in order to run **(B).** Rotarod protocols. Animals were trained on Day 1 and retested on Day 5. **(C)** Whishaw's skilled reach task. Mouse in a clear plexiglass chamber is advancing its forelimb through the slot to retrieve food pellet on the shelf. **(D)** Protocol for skilled reach task. Food restriction was started 3 days and habituation to food pellets 2 days before training and testing. Training/testing was done for 10 days consisting of one session (20trials) followed by substance administration.

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 9^{th} day of training/testing all mice were filmed with a 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.

2.3 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. 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.

2.3.1 Pharmacological Treatment.

2.3.1.1 Physostigmine group (P 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 (Dhingra & Soni, 2018; Gais & Born, 2004; Jafari-Sabet et al., 2016). 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, & Lindstrom, 1986) and upto 1 hour in rodents (Somani, 1989) enough to stay in the system for the course of early SWS. No side effects were observed, and no animal died because of the drug intervention. Probe session was done on fifth day. In the control condition, 14 mice received saline (S group).

2.3.1.2 Physostigmine next day Group (P next day group)

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 P group.

2.3.1.3 Scopolamine/mecamylamine group (SM 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 (B. Rasch 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, Tsui, Vanderploeg, & Kramer, 1989) and 10.1 ± 2 hour for mecamylamine (Young, Shytle, Sanberg, & George, 2001).

2.3.1.4 Oxotremorine and nicotine Groups

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 et al., 2003). The half-life has been reported as 100 minutes for oxotremorine and 1.5-2 hours for nicotine (Marchand et al., 2017; Moyer et al., 2002). Animals were then transferred to their home cages and were allowed to sleep. Probe session was done on Day 5.

2.3.1.5 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.

2.3.1.6 Transgenic Groups

17 Transgenic mice, 9 in CNO group (Tg-C) and 8 in saline group (Tg-S) were used. The dosage of CNO used was 5mg/kg (MacLaren 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.

2.4 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 3 hours long videos were recorded post training. Motion estimation was done in Matlab using block matching algorithm with a block size of 33x33 pixels (Matlab, 2006). Briefly, motion of a block is estimated using two consecutive frames and optical flow analysis by finding where the block moved from one frame to the next and its displacement vector is obtained. The accuracy of motion estimation was verified with manual observation.

2.5 Sleep quantification using electrophysiology

2.5.1 Animal Surgeries

Four adult C57BL6J mice 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, bipolar (tip separation = .6 mm) and monopolar electrodes made from Teflon-coated stainless-steel wire (bare diameter 50.8 μm) were inserted in the secondary motor cortex and pyramidal layer of CA1 according to the following coordinates respectively : CTX: AP: 1.7, ML: .6, DV (deep pole):1.1 mm and hippocampus (HPC): AP: -2.5, ML: 2, 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.

2.5.2 Electrophysiology

Animals were allowed to recover for 7 days after surgery and then habituated for 3 to 5 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 (Fig. 2.2). 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.

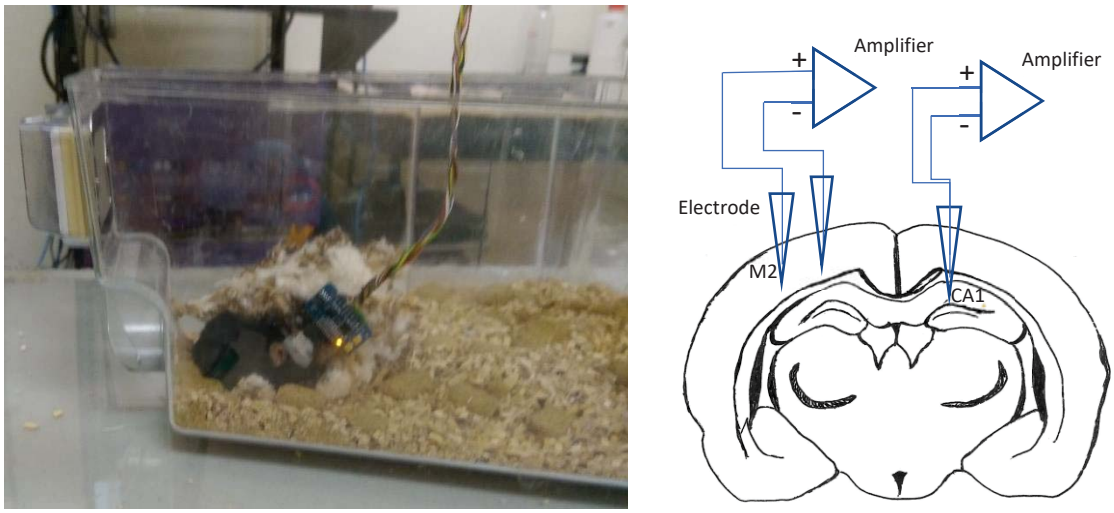

Figure 2.2 Experimental setup for sleep quantification using electrophysiology. (Left) animal sleeping with pre-amplifier installed on head. (Right) configuration of electrodes. CA1, Hippocampal CA1 region, M2, Motor cortex.

2.5.3 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.

2.6 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, two sample student's t-test, one-way analysis of variance (ANOVA), and one-way ANOVA with repeated measures were used and reported in the text along with p values. An alpha value of 0.05 was used for determining significance.

3. RESULTS

3.1 Increasing the levels of acetylcholine during early sleep after a motor learning task impairs the consolidation of motor memories

It is still controversial how distinct phases of sleep such as slow-wave-sleep (SWS) and rapid-eye-movement (REM) sleep play a role in the consolidation of motor memories. Here, we investigated how altering SWS characterized by reduced acetylcholine (ACh) levels might affect motor memory consolidation. Increasing ACh levels during early sleep to disrupt SWS has been shown to impair episodic memory consolidation (Gais & Born, 2004). We therefore hypothesized the same effect for motor memories. To test this hypothesis, we induced motor memories in mice using two different motor tasks (rotarod and Whishaw's skilled reaching tasks) while we increased ACh levels during early sleep through pharmacological manipulation using the drug physostigmine.

3.1.1 Rotarod Task Results

For this task (Fig. 2.1A and 2.1B), the quantification of motor memories was done by measuring the maximum speed mice attained while running and keeping their balance on the rotating drum. On day 1 (training day) mice gradually learnt the task as speeds increased with trials (Fig. 3.1A). The increase in speeds was much faster in trials 1, 2 and 3 and became gradual in successive trials for 58 mice in the control group ($F_{9,504}$ = 23.79, Greenhouse-Geisser corrected $p<0.00001$, one-way ANOVA with repeated measures for trials). Post hoc comparisons with Tukey-Kramer's correction revealed that speeds in trials 2 and 3 were significantly larger than those in trial 1 ($p<0.05$ and $p<0.0001$ respectively) suggesting fast motor learning. Furthermore, speeds in trials 7-10 were significantly larger

than those in trial 3 ($p<0.01$) suggesting gradual motor learning in subsequent trials. At the end of learning sessions on the training day, we immediately administered either saline or physostigmine in two groups of mice separately and allowed the animals to sleep in their home cages.

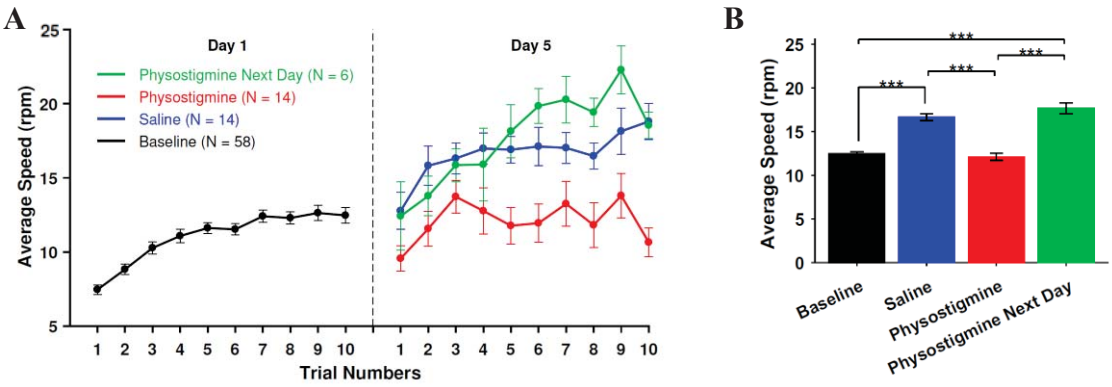

Figure 3.1 Increasing acetylcholine levels in early sleep impairs motor memories induced by rotarod task. **(A).** Average (mean) speeds (over animals) attained on the rotarod before the mice fell versus trials. N indicates number of animals. **(B)** Average speeds of saline group (blue) were significantly larger than baseline (black) ($F_{6,63} = 15.98$, $p<0.0001$, one-way ANOVA with post hoc Tukey's correction) as well as physostigmine group (red). For Physostigmine next day group (green) average speeds (over animals) in all trials were significantly larger compared to baseline and physostigmine groups ($p<0.0001$ for both comparisons, one-way ANOVA with post hoc Tukey's correction). Error bars represent SEM. *** indicate $p<0.001$, one-way ANOVA with posthoc Tukey's correction.

To assess whether mice retained motor memories learnt on the training day, we executed 10 trials of rotarod on Day 5 (probe day) around the same time of light/dark cycle (Fig. 3.1A). We hypothesized that mice in the saline group will retain motor memories better than mice in the physostigmine group. Firstly, 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 (two sample t-test) suggesting retention of motor memories formed on the training day. For mice in the physostigmine group however, speeds in the first trial of the probe day were significantly smaller and larger than speeds in the last and

first trial of the training day respectively ($p<0.01$ for both comparisons, two sample t-test) suggesting impaired retention of motor memories compared to saline group. Secondly, we compared average speeds (over animals) for all trials within the experimental groups using one-way ANOVA (Fig. 3.1B). One-way ANOVA revealed significant difference among groups ($F_{6,63} = 15.98$, $p<0.00000001$) with average speeds of saline group significantly larger than those of baseline (Fig. 3.1B, $p<0.001$, post hoc Tukey's correction) as well as physostigmine group ($p<0.001$). However, there was no significant difference between average speeds of physostigmine and baseline groups. These results thus suggest that the learnt motor memories for mice in the saline group were consolidated better than for mice in the physostigmine group.

Since the half-life of physostigmine is about 1-2 hours (see methods), we asked whether the effects of physostigmine we observed earlier were exclusive to the first sleep session immediately after the motor task. Furthermore, since physostigmine has side effects such as bradycardia, salivation, and tremors (although we observed none), we wondered whether performance on the probe day was affected by the peripheral effects of physostigmine per se. To address these concerns, in a separate cohort of mice (n=6), we injected physostigmine 24 hours after (instead of administering immediately after) the last trial ended on the training day. We hypothesized that the retention of motor memories in these mice would be comparable to those in the saline group. 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. 3.1A). Also, average speeds (over animals) in all trials were significantly larger compared to baseline and physostigmine groups (Fig. 3.1B, $p<0.001$ for both comparisons, one-way ANOVA with post hoc Tukey's correction). We therefore concluded from these

observations that motor memories induced by the rotarod task are consolidated after the first sleep session post training. Furthermore, all the above-mentioned results clearly suggest that increased levels of acetylcholine in early sleep (mentioned next) with the administration of physostigmine impairs consolidation of motor memories induced with the rotarod task.

3.1.1.1 Sleep characterization after rotarod task and physostigmine administration

To explore how increasing acetylcholine levels during early sleep disrupt the consolidation of motor memories, we characterized motion using home cage filming (in n = 10 mice) and sleep patterns using electrophysiology (in n=3 mice). With home cage filming, we quantified the motion of mice for three hours after rotarod training to assess whether the animal was sleeping after physostigmine injection. The average motion profiles for three hours post-training was not different between groups injected with saline or physostigmine (Fig. 3.2). With electrophysiology, signals were recorded from electrodes implanted in the neck muscle, hippocampus, and the motor cortex (Fig. 2.2). Signals were first recorded by injecting saline to obtain baseline values and on a later day by injecting physostigmine (after rotarod training to mimic previous experimental conditions). We analyzed signals within the first hour after injection and hypothesized that increasing acetylcholine levels would disrupt SWS. First, using EMG signals, we determined the durations of wakefulness or sleep and found longer waking period for physostigmine compared to saline ($p<0.05$, Fig. 3.3A and B). The hippocampal signal was then used to characterize SWS or REM sleep and we observed that SWS duration for physostigmine was significantly smaller than that for saline group ($p<0.05$). SWS latency was also longer for physostigmine group compared to saline group ($p>0.05$). Slow wave power (0.3 to 4 Hz) during SWS in the

motor cortex was smaller for physostigmine compared to saline (Fig. 3.3C) but not significantly different (p=0.0581). This result suggests that SWS is disrupted by increasing acetylcholine levels and thus could be responsible for impairment in memory consolidation in the earlier rotarod experiments.

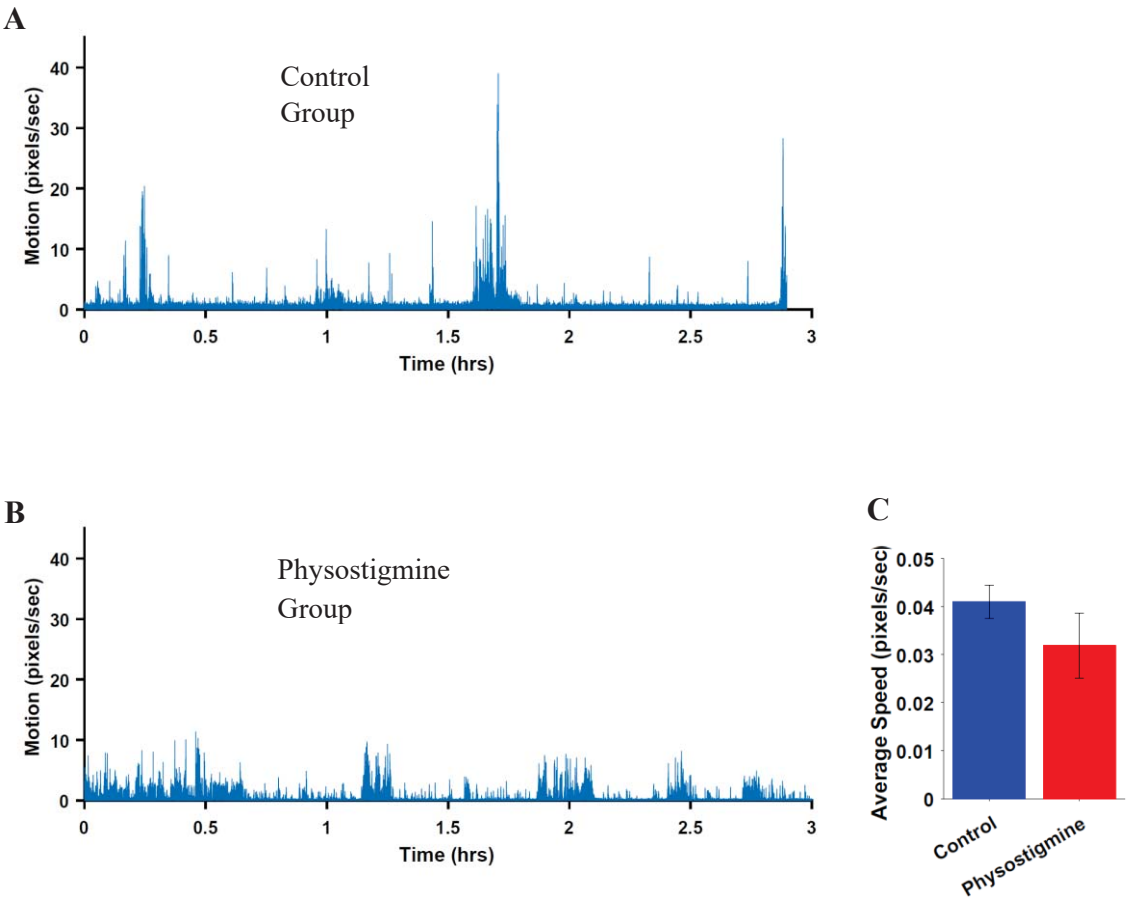

Figure 3.2 Quantification of motion post training using filming in home cage. **(A)** and **(B)** show representative motion profiles of mice in control and physostigmine groups respectively for three hours post-training. **(C).** Mean ±SEM of average motion (N=5 mice in each group). The mean for one animal was over the three hours period. There was no significant difference between the means (p>0.05, two-sample t-test)

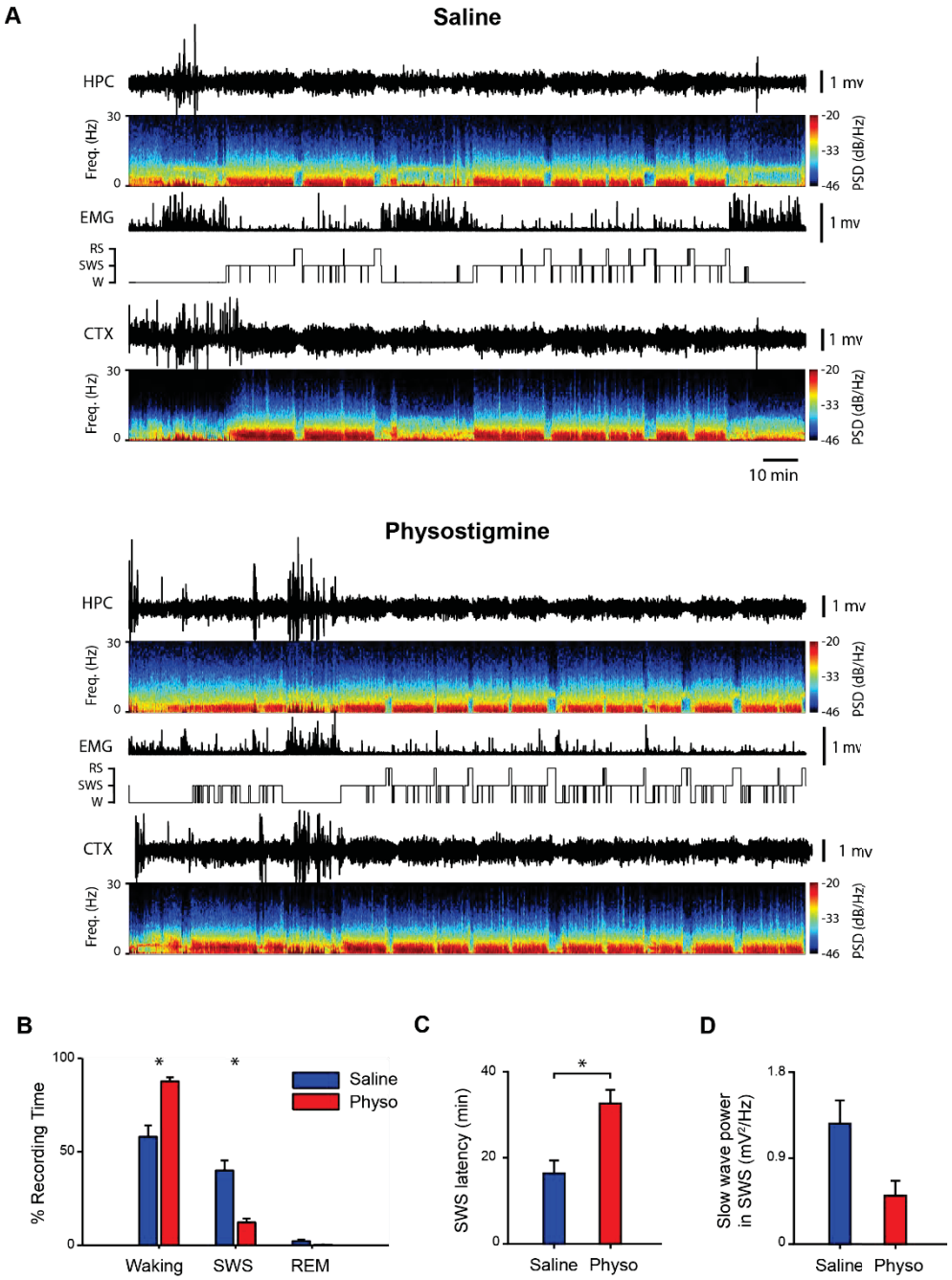

Figure 3.3 Quantification of sleep using electrophysiology. **(A)** Example of hippocampal, EMG, hypnogram and cortical traces after saline and physostigmine injection. Spectrogram of hippocampal and cortical LFP which were used for state scoring and spectral analysis is shown below each trace. PSD is power spectral density **(B)** Comparison of sleep structure (relative duration of waking, SWS and REM sleep) between saline and physostigmine in the first hour after injection showed increase of waking activity in expense of SWS (paired student's t-test, n=4). **(C)** Injection of physostigmine (0.1 mg/kg) significantly increased sleep latency compared to control group (paired student's t-test, n=4). **(D)** Comparison of power of slow wave activity (0.5-4 Hz) during SWS happening in the first hour of recording between saline and physostigmine. (P-value=0.0581, paired student's t-test, n=4).

3.1.2 Whishaw's Skilled-forelimb Reach Task Results

This task (Fig. 2.1B) requires animals to accurately control coordinated movements of arm, wrist, hand, and fingers to obtain food rewards and thus constitute a separate set of motor memories as compared to those for the rotarod task. Here also, we expected disruption of motor memories in mice injected with physostigmine compared to those injected with saline. We trained/tested mice for 10 days and on each day injected saline or physostigmine in respective groups (Fig. 2.1B). Mice injected with physostigmine were slower in learning the task as suggested by a smaller number of successful animals within the first 5 days compared to those injected with saline (Fig. 3.4A). A successful animal here was defined as the one which successfully completed 20 trials on that particular day. Furthermore, we compared the average percentages (over animals) of successful trials over days (Fig. 3.3B) which increased with time suggesting that mice in both groups showed learning (for saline group $F_{9,81} = 31.49$, Greenhouse-Geisser corrected $p<0.000001$ and for physostigmine group $F_{9,90} = 14.21$, Greenhouse-Geisser corrected $p<0.000001$, one-way ANOVA with repeated measures). The average percentages for the physostigmine group were significantly different than those for saline group ($F_{9,171} = 2.81$, Greenhouse-Geisser corrected $p<0.05$, repeated measures ANOVA for group-time interaction) with significantly smaller values for physostigmine on days 3,7, and 8 ($p<0.05$, $p<0.01$, and $p<0.05$ respectively, post hoc tukey-kramer correction, group by days). These results thus suggest that the administration of physostigmine immediately after the reaching task impairs behavioral performance of mice. Thus, increasing acetylcholine levels during early sleep can hinder the consolidation of motor memories induced by the reaching task.

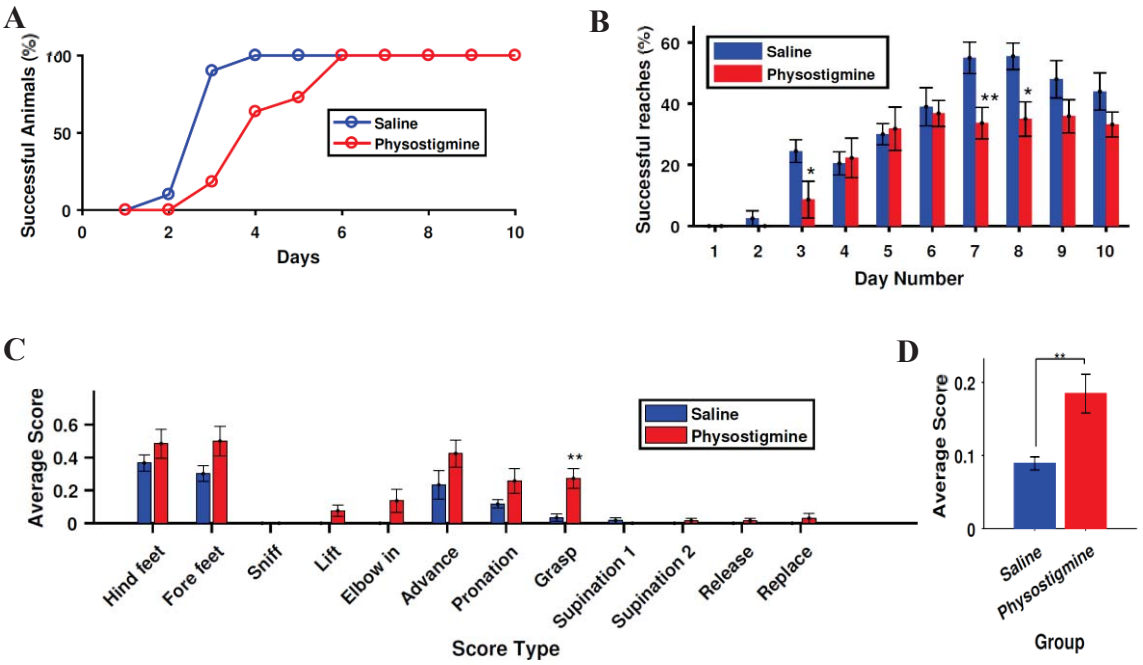

Figure 3.4 Increasing acetylcholine levels in early sleep impairs motor memories induced by Whishaw's skilled-forelimb reach task. **(A) Acquisition.** Percentage of successful animal across 10 days. An animal was considered successful if it completed all 20 trials with reaches greater than zero on that day. All animals in control group were successful on day 4 whereas all animals in Physostigmine group were successful on day 6. **(B) Performance.** Percent single reach successes of all animals averaged across 10 days. Each animal made 20 reaches. While both groups of mice improved in reaching accuracy over the training period ($p<0.000001$, one-way ANOVA with repeated measures) percentage of single reaches was impaired for physostigmine group (n=11) as compared to control group (n=10) overall ($p<0.05$, repeated measures ANOVA for group-time interaction) and particulary on days 3,7, and 8 ($p<0.05$, $p<0.01$, and $p<0.05$ respectively, post hoc tukey-kramer correction, group by days). **(C) Movement scores.** Quality of reach as assessed by the 12-component system of reach (score type). The first three successes out of 20 reaches were scored and averaged across 10 days. 0, 0.5, and 1 scores were given for good, impaired, and abnormal reaches. A higher score thus indicates inferior performance. The two groups had similar scores ($F_{11,209} = 1.8314$, Greenhouse-Geisser corrected $p>0.05$, one-way ANOVA with repeated measures, group-components effect). Scores for grasp were significantly different ($p<0.01$, posthoc Tukey-Kramer's correction), (F) Average score for the physostigmine group was also significantly larger than that for saline group ($p<0.001$, two sample t-test). All Data are presented as Means±SEM.

From the video recordings on day 9, we also scored the quality of reaches by observing 12 components of movements. Although, the average score for the physostigmine group was significantly larger than that for saline group ($p<0.001$, two sample t-test) and the majority

of the mean scores were greater for the physostigmine group (Fig. 3.4C and 3.4D), 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=0.13, group-components interaction). These results imply that the quality of reaches might only be slightly different in physostigmine and saline groups indicating that when mice in the physostigmine group learn the task, their motor memories are similar to those in the saline group.

3.2 Decreasing the levels of acetylcholine during early sleep after a motor learning task does not impair consolidation of motor memories

We hypothesized that further decreasing ACh levels during SWS would not alter the memory consolidation process. Here also we used the rotarod task to induce motor memories. However, to reduce Ach levels during early sleep, we used two approaches; pharmacological and chemogenetic. In the first approach we used a combination of two drugs, scopolamine and mecamylamine which are muscarinic and nicotinic acetylcholine receptor blockers respectively and were given immediately after rotarod training. Animals were then allowed to sleep in their home cages and their performance was assessed on the probe day. The speeds of mice in the first trial on the probe day were not significantly different from the last trial on the training day (two sample t-test) while there was an increase in speeds with trials on the probe day (Fig. 3.5A, $F_{9,63}$ = 3.67, Greenhouse-Geisser corrected $p<0.05$, one-way ANOVA with repeated measures for trials). Furthermore, the average speeds (over animals) of all trials were significantly larger than those for the baseline and physostigmine groups (Fig. 3.5B, $p<0.001$) but were similar to those for the saline group (one-way ANOVA with post hoc Tukey's correction). Thus, pharmacological

manipulation to reduce ACh levels during early sleep does not alter memory consolidation of motor memories induced by the rotarod task.

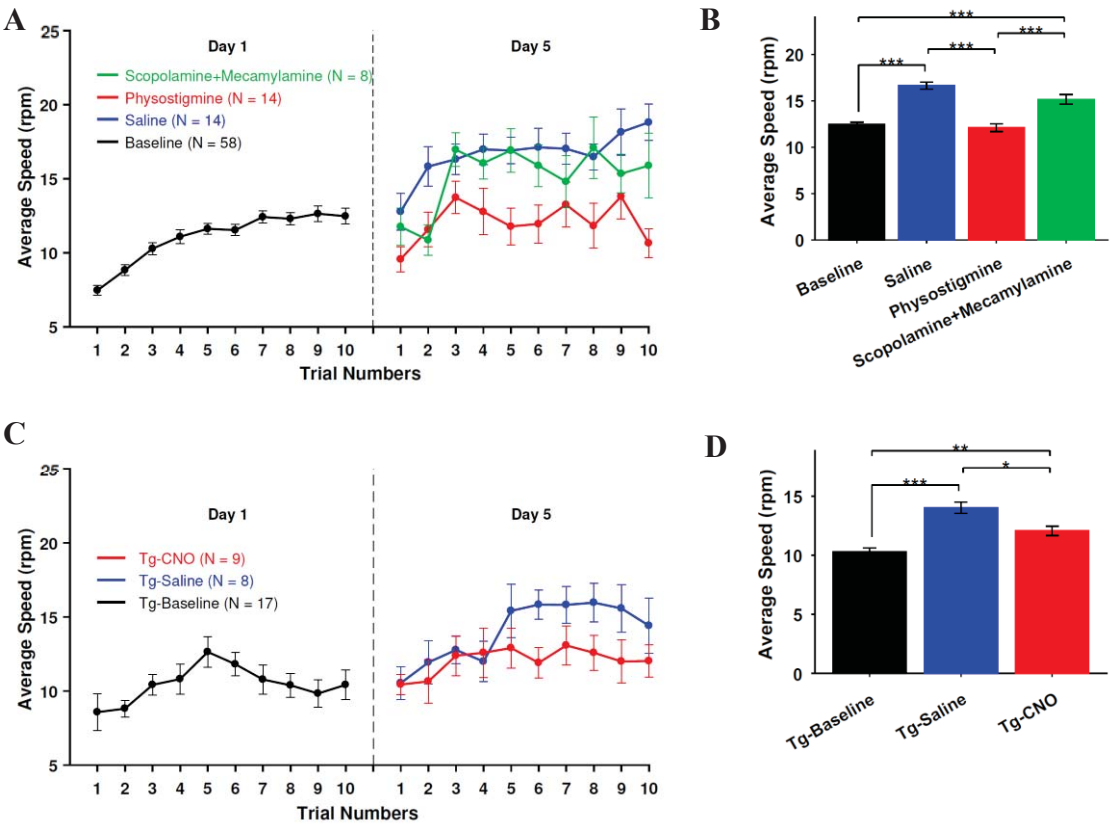

Figure 3.5 Decreasing acetylcholine levels in early sleep does not impair motor memories induced by rotarod task. **(A). C57 mice**. Average (mean) speeds (over animals) attained on the rotarod before the mice fell versus trials. N indicates number of animals. **(B).** The average speeds (over animals) of all trials for scopolamine+Mecamylamine (green) group were significantly larger than those for the baseline (black) and physostigmine (red) groups ($p<0.001$ and $p<0.05$ respectively) but were similar to those for the saline (blue) group (one-way ANOVA with post hoc Tukey's correction). **(C). Transgenic Mice.** Average (mean) speeds (over animals) attained on the rotarod before the mice fell versus trials. N indicates number of animals. **(D).** Average speeds (over animals) in both Tg-C (red) and Tg-S (blue) groups on probe day were significantly higher than the average speeds on the training day. Baseline, saline, and physostigmine groups are same as in Fig. 3.1.

In the chemogenetic approach, we used transgenic CAG Hmdi4 xChat cre mice. These mice have inhibitory DREADDs in their cholinergic neurons. Following administration of

the drug clozapine-*N*-oxide (CNO), presynaptic release of neurotransmitter acetylcholine in cholinergic neurons decreases resulting in overall reduction of cholinergic tone throughout the brain. Since CNO exerts its maximum effects an hour to hour-and-half after the administration, we injected CNO one hour before sleep session i.e. just before the rotarod training in mice belonging to the transgenic CNO group (Tg-C). In another group of mice, saline was injected similarly to establish a saline group (Tg-S). As for C57 mice, all transgenic mice for the training day were combined to form a control group (baseline). Mice learnt the task on the training day (Fig. 3.5C, $F_{9,144} = 3.72$, Greenhouse-Geisser corrected $p<0.01$, one-way ANOVA with repeated measures for trials) but their learning curve was not as steep as for C57 control mice (compare with Fig. 3.1A). Post hoc tukey-cramer correction revealed that there was a significant difference between trial 2 and trial 5 ($p<0.01$) and trial 2 and trial 6 ($p<0.05$) suggesting an initial learning. The decrease in average speed following trial 5 might be attributed to the fact that animals could have become tired as they are transgenic. To assess retention of motor memories, 10 trials were conducted to assess speeds on the probe day (day 5). Speeds in the first trial on the probe day were similar to speeds in the last trial of training day for both Tg-C and Tg-S groups (two sample t-test) suggesting motor memory consolidation on training day. Furthermore, average speeds (over animals) for all trials in both Tg-C and Tg-S groups were significantly higher than the average speeds for the control group on the training day (Fig. 3.5D, $p<0.001$ and $p<0.05$ respectively, one-way ANOVA, post hoc Tukey's correction) suggesting that mice further learnt the task on the probe day. We conclude therefore that reducing ACh levels with chemogenetic approach does not affect consolidation of rotarod motor memories.

3.3 Muscarinic ACh receptors are predominantly involved in motor memory consolidation compared to nicotinic ACh receptors

Previously with the administration of physostigmine, we observed the effect of simultaneous activation of both ACh receptors i.e. (of muscarinic and nicotinic types) on motor memory consolidation during SWS. Here we investigated whether one or the other or both types of ACh receptors mediate motor memory consolidation. Based on previous studies, we hypothesized that muscarinic ACh receptors would play a stronger role in motor memory consolidation as compared to nicotinic ACh receptors (Brown, 2010; Hut & Van der Zee, 2011; Power et al., 2003).

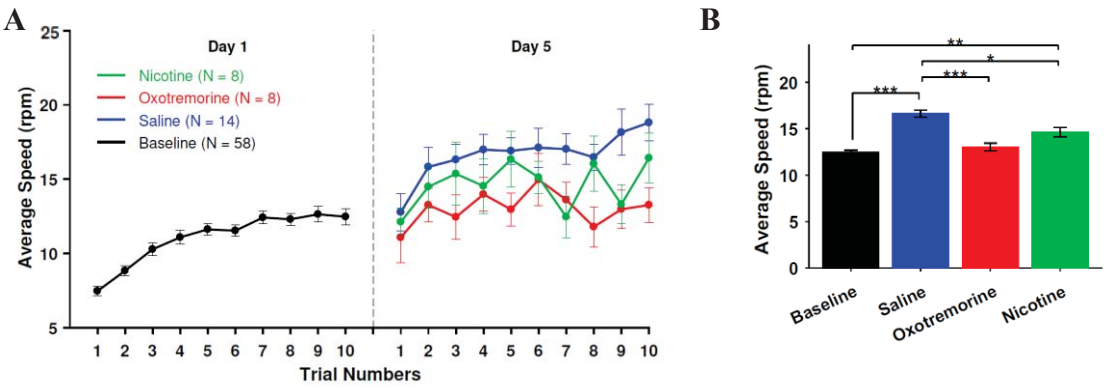

Figure 3.6 Muscarinic ACh receptors are predominantly involved in motor memory consolidation compared to nicotinic ACh receptors. **(A)** Average (mean) speeds (over animals) attained on the rotarod before the mice fell versus trials. N indicates number of animals. **(B).** Average speeds (over animals) in all trials for the nicotine group (green) were significantly larger compared to the baseline group (black) ($p<0.01$, one-way ANOVA with post hoc Tukey's correction) and were similar to those in the saline group (blue). On the contrary for the oxotremorine group (red), average speeds were similar to those in the baseline group but were significantly smaller than those in the saline group ($p<0.01$, one-way ANOVA with post hoc Tukey's correction). Baseline, saline, and physostigmine groups are same as in Fig. 3.1.

For activating muscarinic and nicotinic ACh receptors selectively, we used respective agonists oxotremorine and nicotine which were injected immediately after rotarod training

in two separate groups of mice. For both groups, speeds in the first trials on the probe day (Fig. 3.6) were not significantly different from speeds in the last trials on the training day (two sample t-test). However, average speeds (over animals) for all trials for the nicotine group were significantly larger compared to the baseline group (Fig. 3.6B, $p<0.01$, one-way ANOVA with post hoc Tukey's correction) and were similar to those in the saline group. On the contrary for the oxotremorine group, average speeds were similar to those in the baseline group but were significantly smaller than those in the saline group (Fig. 3.6B, $p<0.01$, one-way ANOVA with post hoc Tukey's correction). We deduce from these results that oxotremorine has a larger effect on the consolidation of motor memories as compared to nicotine. Hence, muscarinic ACh receptors largely mediate the motor memory consolidation process as compared to nicotinic ACh receptors.

Table 3.1 – 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	P Group	P Next Day	SM	Oxo	Nico
Baseline		***	ns	***	***	ns	**
Saline			***	ns	ns	***	*
P Group				***	***	ns	***
P Next Day					*	***	***
SM						*	ns
Oxo							ns
Nico							

Abbreviations: P – Physostigmine, SM – Scopolamine + Mecamylamine, P Next Day – Physostigmine Next Day, Oxo – Oxotremorine, Nico – Nicotine

4. Discussion

The synaptic homeostatic theory of sleep states that slow wave sleep (SWS) facilitates motor memory consolidation by initiating downscaling of overall synaptic strengths and bringing the synaptic load impacting on each neuron to baseline levels (increasing signal to noise ratio). Low levels of acetylcholine (ACh) present during SWS might mediate the synaptic downscaling process. Therefore, when we increased cholinergic tone during early sleep by administering physostigmine (Dhingra & Soni, 2018; Gais & Born, 2004; Jafari-Sabet et al., 2016) right after the induction of motor memories and just before animals went to sleep, motor memory consolidation was disrupted (Fig. 3.1, 3.4, and Table 3.1). Alternatively, further reducing ACh levels during SWS by either administering scopolamine and mecamylamine in WT mice or using a chemogenetic approach with transgenic mice, did not impair motor memory consolidation (Fig. 3.5).

Our findings are also inline with the sequential hypothesis of function of sleep which emphasizes on the complementary role of cyclic succession of SWS and REM for long term memory storage (Ambrosini & Giuditta, 2001; Giuditta et al., 1995; Rasch & Born, 2013). According to this hypothesis, 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, motor memories induced by rotarod and skilled reach task are both adaptive as the animal adapts to the exposed environment in case of rotarod and must obtain food to survive in the reach task. When SWS was disrupted with an increase in levels of ACh in our study, perhaps the normal cyclic succession of SWS and REM was also disrupted and thus the

strengthening of motor memories (adaptive memories) was disturbed and decreased behavioral performance was observed.

The pattern of acquisition of motor memories in our study is similar to what has been reported previously. The learning of a motor task is sequential and requires repetition and practice. Performance of a skilled motor task such as reaching to a target requires extensive training before being mastered to perform effortlessly. It has been shown that skilled motor performance is acquired in an initial "fast" learning phase followed by a period of consolidation and an incremental "slow" learning phase (Buitrago et al., 2004; Karni et al., 1998). Our results reflect the same pattern of motor learning. In both rotarod as well as skilled forelimb reach task the learning was incremental and sequential. In rotarod the average speeds in the first few trials showed fast acquisition phase, whereas the probe day reflected a more incremental and gradual gain (Fig. 3.1A). Similarly, for skilled reach task mice were trained/tested for 10 days and a steep curve was observed for percentage of successful animals as well as percentage of successful reaches for initial 4 days which then plateaued (Fig. 3.4A).

After the acquisition of motor memories, we administered drugs to the animals for altering cholinergic levels and later allowed them to sleep. Although we did not directly measure cholinergic levels, previous studies have demonstrated the effect of drugs (we chose in our study) in altering cholinergic levels as described in the methods section. Furthermore, altering cholinergic levels disrupts sleep patterns (Dringenberg & Vanderwolf, 1996, 1997; Sitaram, Wyatt, Dawson, & Gillin, 1976; Vanderwolf et al., 1993) e.g. (a simultaneous change in sleep stage from non-REM to REM sleep and to wakefulness) was observed subsequent to increasing central cholinergic tone (Sitaram et al., 1976). In a more recent

study (Anaclet et al., 2015), basal forebrain cholinergic neurons were activated by genetically targeted chemogenetic activation, and a reduction in slow wave activity was observed. Similarly, in our electrophysiological recordings, we observed disruption of slow-wave activity in the motor cortex with the administration of physostigmine (Fig. 3.3) and hence we believe that physostigmine increased ACh levels.

With physostigmine administration, we show that SWS is disrupted in the motor cortex. However, this might not be the case in other cortical areas because SWS sleep period was detected from hippocampal LFP and might not be present globally in all cortical areas. Previously our lab has shown that during REM-like sleep state characterized from hippocampal LFP in urethane anesthetized mice, all cortical areas do not exhibit REM-like state globally rather some cortical areas exhibit SWS (Nazariahangarkolaee, 2017). Although a direct comparison with this study is not possible (natural sleep vs anesthetized), we speculate that when we increase ACh levels with physostigmine, SWS sleep might be disrupted non-uniformly in cortical areas and might also disrupt REM sleep (see sequential hypothesis above). Therefore, the impairment we observe in behavioral tasks after physostigmine administration might not be solely due to disruption of SWS but perhaps involve other unknown factors.

The increase in cholinergic levels with physostigmine and subsequent disruption of SWS impaired motor memory consolidation in both rotarod and skilled reaching motor tasks (Fig 3.1 and 3.4). To the best of our knowledge, our study is the first one which demonstrates the combined effect of altering cholinergic levels during early sleep on motor memory consolidation. Our findings are consistent with studies discussed below in which either

sleep or the cholinergic system was disrupted and impairments in motor learning were observed.

Sleep deprivation studies have shown that disrupting sleep particularly early sleep impairs consolidation of motor/procedural memories e.g. those formed in visual texture discrimination task, rotarod and complex wheel tasks (Gais et al., 2000; Nagai et al., 2017; Yang et al., 2014). Alternatively, it has also been shown that motor learning during wakefulness affects the amplitude of slow wave (SWA) activity in subsequent SWS. An improved SWA amplitude was observed in subsequent SWS after training in a visual texture discrimination and visuomotor adaptation task (Aeschbach, Cutler, & Ronda, 2008; Landsness et al., 2009; Landsness et al., 2011), whereas a reduction in SWA and synaptic depression was observed in SWS following arm immobilization (Huber et al., 2006). The most prominent study in mice is by Hanlon et al which showed an increase in local SWA in motor cortex after training in skilled forelimb reach task (Hanlon et al., 2009). This increase was most prominent during the first hour of the post-learning NREM sleep. Our results also suggest that an immediate sleep session after learning is most important for motor memory consolidation. When we administered physostigmine after the first sleep session i.e. 24 hours later (physostigmine-next-day), no impairment in motor memory consolidation was observed (Fig. 3.1).

Studies have also shown that disrupting cholinergic levels in the cortex impair motor learning and memory consolidation. For example, cutting the afferent transmission of basal forebrain cholinergic neurons to the cortical areas including the motor cortex impeded motor learning in a reach task (Conner et al., 2003; Conner et al., 2010; Ramanathan et al., 2009). Our results from the rotarod and reach tasks are in harmony with these results.

Consistent with our initial hypothesis, decreasing cholinergic levels during early sleep do not impair motor memory consolidation. It has been speculated that during wakefulness and sleep ACh levels function as a switch between brain modes of encoding and consolidation respectively (Hasselmo, 1999; Hasselmo & McGaughy, 2004). Our results support this conjecture for motor memories which has been previously confirmed for declarative memories i.e. (formation/encoding requires high while consolidation is facilitated by low ACh levels) (B. Rasch et al., 2006). In our study by using wildtype and transgenic animals in rotarod task and further decreasing ACh levels in the post learning sleep, we found that the consolidation process was unaffected (Fig. 3.5). With a combined blockade of muscarinic and nicotinic cholinergic receptors (using scopolamine and mecamylamine) during wake period, Rasch et al (2006) showed enhanced consolidation of declarative memories but impaired new encoding. Our results demonstrate similar effects but during the sleep interval rather than awake state. This is unique as to the best of our knowledge; no study has yet compared the effect of a further reduction in ACh levels during sleep on motor memory consolidation. We propose that due to basement effect, further decreasing ACh levels when they are naturally reduced would not affect memory consolidation.

Although both nicotinic and muscarinic ACh receptors carry out an array of functions both centrally and peripherally, mAChRs are particularly associated with mediating central cholinergic tone and are involved in many aspects of cognitive functioning (Hut & Van der Zee, 2011; Zwart, Reed, & Sher, 2018). Memory function is also greatly influenced by the activity of mAChRs in the amygdala, hippocampus, striatum, and cortex as shown by several studies (Brown, 2010; Power et al., 2003). Furthermore, muscarinic receptor

activation causes production of low voltage fast wave like activity whereas muscarinic receptor blockade produces more slow wave like activity (Dringenberg & Vanderwolf, 1996; Vanderwolf et al., 1993). In line with these studies, our results suggest that mAChRs are predominantly involved in motor memory consolidation (Fig. 3.6).

Our results are in contrast with studies advocating the dual process hypothesis which states that SWS supports the consolidation of declarative memories while REM sleep and not SWS facilitates motor memory consolidation (Gais & Born, 2004; Louie & Wilson, 2001; Mascetti et al., 2013; Mednick, Nakayama, & Stickgold, 2003; Peigneux et al., 2003; Stickgold, Whidbee, Schirmer, Patel, & Hobson, 2000). For example, Gais and Born, showed that post learning decrease of ACh during SWS enhances declarative (hippocampus dependent) memory consolidation but does not affect the REM sleep dependent consolidation of procedural learning (mirror tracing task) (Gais & Born, 2004). However, numerous other studies (see above) also contradict their conclusions. Some other studies also suggest that cholinergic receptor agonists may have an enhancing effect on post-learning long-term memory. For example, Power et al (2003) used a passive avoidance task to show enhancement of long-term memories when muscarinic receptor agonists were given post-learning. However, they did not specifically control for sleep or wakefulness in this study and it has been proposed that memories induced by passive avoidance task are hippocampus dependent (Power et al., 2003). Nagai et al (2017) reported that post-learning sleep deprivation affected performance of mice on a complex wheel task and not on the rotarod task (Nagai et al., 2017) which is contradictory to our results as well as those of Yang et al (2014) which showed improvement on rotarod task when animals were allowed to sleep after training instead of sleep deprivation (Yang et al., 2014). To resolve this

conflict, we used a modified version of rotarod as reported by Shiotsuki et al to enhance the learning aspect of the rotarod (Shiotsuki et al., 2010).

5. Conclusion and Future Directions

In conclusion, we have convincing results that increased ACh levels during SWS impair motor memory consolidation. Nevertheless, with crude pharmacological manipulation, we non-specifically, increased ACh levels in brain areas. A refined approach e.g. using optogenetics with transgenic mice is recommended for future experiments in which ACh levels can be altered specifically by exciting or inhibiting cholinergic neurons in chosen nuclei in the basal forebrain or the brain stem. For example, local ACh release in the neocortex has been achieved by expressing channelrhodopsin2 in cholinergic neurons in the basal forebrain and stimulating their axons in the neocortex in urethane anesthetized mice (Kalmbach, Hedrick, & Waters, 2012). Furthermore, selective activation of muscarinic or nicotinic receptors could be achieved by optogenetics combined with pharmacological manipulation (Kalmbach & Waters, 2014). For silencing cholinergic neurons, an approach similar to that of Boyce et al (2016) can be used where medial septal GABAergic neurons were silenced during REM sleep episodes (Boyce et al., 2016). With the capability of activating or silencing cholinergic neurons, and with a feedback system to detect sleep states i.e. (REM or NREM), one can accurately manipulate ACh levels in the desired brain region and study their role in memory consolidation. For example, the extension of our study could be the selective alteration of cholinergic levels in the motor cortex with simultaneous detection of post learning NREM sleep online with electrophysiology. Alternatively, to study the role of motor memory encoding during specific phases of behavioral tasks, ACh levels could be selectively increased or decreased

in the motor cortex e.g. (when the animal extends its arm to reach in a skilled forelimb reaching task) (Mohammad et al., 2017). The experiments that we have proposed here can also be done with chemogenetics in order to study the role of various ACh receptors in memory encoding and consolidation. For example, with chemogenetics, one can selectively activate specific receptors (muscarinic, nicotinic, GABAergic, or glutamatergic) in the neocortex (Anaclet et al., 2015).

We propose that hippocampal-independent (non-declarative) memories are consolidated at a synaptic level in relevant brain areas with motor memories in the motor cortex consistent with the model describing synaptic homeostasis function of SWS (Bellesi, Riedner, Garcia-Molina, Cirelli, & Tononi, 2014; Tononi & Cirelli, 2006, 2016). To study the anatomical and physiological changes happening at the synaptic level that accompany memory encoding and consolidation, we propose, optical imaging (using two-photon and three-photon microscopes) of cells and synapses. For anatomical and physiological studies, one can use yellow fluorescent protein (YFP) and Thy1-GCaMP6 transgenic animals respectively. Cortical neurons can be easily imaged in YFP animals for structural studies while calcium imaging of Thy1-GCaMP6 animals can be used for functional studies. An extension of our study is to observe anatomical correlates e.g. (changes in spine density, synaptic structure, dendritic lengths etc.) during post-learning sleep using two-photon imaging (Yang et al., 2014) and after altering cholinergic levels. Physiological changes could also be observed with calcium imaging of somas as well as axonal synaptic boutons, dendrites, and spines (Chen et al., 2013). These proposed studies will allow the dissection of anatomical and physiological correlates of memory encoding and consolidation during

post-learning sleep. Furthermore, imaging techniques could be combined with optogenetics for fine control of neuromodulator levels during behavior or post-learning sleep.

Finally, we also propose that since altered cholinergic levels are one of the biomarkers in Alzheimer's disease (AD), the role of altering cholinergic levels during behavior (on memory encoding) and in post-learning sleep (on memory consolidation) should be studied in mouse models of AD and other neurodegenerative disease models. Our study suggests that the timing of administration of drugs to elevate cholinergic levels in Alzheimer's disease patients must be re-evaluated to improve the efficacy of respective drugs. Currently, patients are prescribed with cholinesterase inhibitors such as drugs (Aricept®, Exelon®, and Razadyne®) for increasing acetylcholine levels, with once a day dosage but without mention of the time of day (National Institute of Aging, 2018). We propose that in order to facilitate efficient memory encoding and consolidation, these drugs should be advised to be taken preferably in the morning compared to being taken after dinner or before sleep to improve their respective potency and efficacy.

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