ACETYLCHOLINE IN THE HIPPOCAMPUS MODULATES ASSOCIATIVE LEARNING WITH CORRESPONDING CHANGES IN ADULT NEUROGENESIS AND ENDOGENOUS THETA RHYTHM ACTIVITY

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ABSTRACT OF THE DISSERTATION

Acetylcholine in the hippocampus modulates associative learning with corresponding changes in adult neurogenesis and endogenous theta rhythm activity

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Decades ago, acetylcholine was considered intrinsic to processes related to learning and memory. However, in the last decade or so, this relationship has been questioned and with good reason. That said, only a few studies have addressed the involvement of acetylcholine in tasks that require an animal to associate stimuli separated in time, such as trace eyeblink conditioning. Trace eyeblink conditioning is associated with hippocampal theta rhythmic activity and dependent on adult neurogenesis in the hippocampus, both of which are thought to be mediated by cholinergic activity. In the present study, 192 IgG-Saporin (SAP) was infused into the medial septum diagonal band (MSDB) complex of Sprague-Dawley rats to selectively kill cholinergic neurons and produce bilateral and unilateral lesions. Animals with bilateral, unilateral, or sham lesions

were trained with trace eyeblink conditioning at least 14 days after the SAP infusion. Animals with a sham lesion made more conditioned responses over all conditioning trials compared to animals with bilateral and unilateral lesions. However, conditioned responses increased over time in all groups. Taken together, bilateral and unilateral lesions both retard but do not drastically impair learning. In two separate experiments, the effect of bilateral and unilateral lesions on adult neurogenesis and theta rhythms was assessed. Animals were injected with 5-bromo-2'-deoxyuridine (BrdU) to label dividing cells at least 14 days after the SAP infusion. Seven days later, the number of BrdU-positive cells in the dentate gyrus of the hippocampus of animals with bilateral and unilateral lesions was reduced by ~40% in both hemispheres. Hippocampal local field potentials were recorded from another group of animals. Seven days following the SAP infusion, relative theta power was reduced in the bilateral but not unilateral group to a similar extent in both hemispheres. However, relative theta power was similar in all three groups by Day 14. This data suggests that a reduction in the number of new neurons in the hippocampus may be a contributing factor to a trace learning deficit as a result of a MSDB lesion. Moreover, even incomplete lesions that disrupt septohippocampal cholinergic activity are sufficient to reduce hippocampal adult neurogenesis and retard learning.

DEDICATION

To my mom, dad, and husband

Your support grounded me and provided the foundation that made this research possible.

Your love allowed me to dream things grander than I could have ever imagined alone.

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INTRODUCTION

The hippocampus plays a substantial role in many types of learning. This notion rests upon an enormous body of work - most notably, the studies published on learning and memory in humans and other animals with hippocampal lesions. The most famous of these studies involve the patient H.M., who was unable to retain information about new experiences and events following the extensive removal of his medial temporal lobes, which contains the hippocampus, to treat his epilepsy (Scoville and Milner, 1957). This unfortunate memory deficit that occurred as a result of the surgery set off a wave of focused experiments exploring the specific role of the hippocampus in learning and memory and the underlying processes involved. While the trisynaptic circuit is the most visually obvious and dominant component of hippocampal processing, innervation from the medial septum diagonal band (MSDB) complex is also thought to play an intrinsic role in hippocampal activity. A large proportion of the neurons that comprise the septohippocampal pathway ramify and release acetylcholine into all three subregions of the trisynaptic circuit. While the sheer neuroanatomical position is enough to implicate the cholinergic system in learning and memory formation, the relationship has been question in the last decade or so (Parent and Baxter, 2004). These experiments are aimed at determining whether cholinergic septohippocampal projections are necessary for hippocampal-dependent learning and some of the underlying processes in the hippocampus associated with this type of learning. This research will explore the relationship between acetylcholine and learning, adult neurogenesis and theta rhythm.

EXPERIMENT 1: THE ROLE OF CHOLINERGIC SEPTOHIPPOCAMPAL PROJECTIONS IN TRACE EYEBLINK LEARNING

Introduction

A type of hippocampal-dependent learning

The hippocampus is necessary for many but not all types of learning. This is the case with various subtypes of a form of associative learning called eyeblink conditioning. In eyeblink conditioning, a neutral stimulus typically precedes the onset of another stimulus that naturally causes an eyelid closure (Figure 1 top). For example, the presentation of white noise (conditioned stimulus, CS) precedes stimulation to the eyelid (unconditioned stimulus; US), as is the case in the experiments presented here. Over time as the CS and US are presented together, an animal learns that the white noise predicts the onset of the eyelid stimulation. As a result, animals learn to blink in anticipation of the US. These blinks known as conditioned responses (CRs) develop over hundreds of trials. Two basic types of eyeblink conditioning, delay and trace, differ based on the temporal relationship between the CS and US. In delay conditioning, the CS onset precedes the presentation of the US, but the CS and US co-terminate (Figure 1 top). Neither the acquisition nor the expression of delay CRs are dependent upon the presence of the hippocampus (Schmaltz and Theios, 1972; Berger and Orr, 1983). In trace eyeblink conditioning, the CS and US are separated by a stimulus free period. This temporal gap is referred to as the trace interval because it is assumed that an animal is required to hold a memory “trace” of the CS across that period of time in order to associate it with the US.

In contrast to delay eyeblink conditioning, hippocampal lesions do impair the ability of an animal to acquire trace CRs (Kim, Clark, & Thompson, 1995; Moyer, Deyo, & Disterhoft, 1990; Port, Romano, Steinmetz, Mikhail, & Patterson, 1986; Shors et al., 2001; Solomon, Vander Schaaf, Thompson, & Weisz, 1986; Waddell, Anderson, & Shors, 2011; Weiss, Bouwmeester, Power, & Disterhoft, 1999). Trace eyeblink conditioning is also impaired in humans with hippocampal damage (McGlinchey-Berroth et al., 1997; Clark and Squire, 1998), although not severely in all cases (Woodruff-Pak, 1993). For example, H.M. could learn the trace CR. However, he was previously trained with delay conditioning. This finding is consistent with animal studies. Animals with hippocampal lesions that are trained to learn the CR during delay conditioning can then perform the trace response (Beylin et al., 2001). Taken together, these studies indicate that the hippocampus is necessary for learning the association when it is separated by a trace interval, but once the association is learned, it is no longer necessary. Analysis of hippocampal activity during acquisition of the trace CR corroborates this idea (Solomon et al., 1986; Cheng et al., 2008). Moreover, trace eyeblink conditioning is considered a model for declarative memory formation (the type of memory impaired in medial temporal lobe amnesiacs and in patients with Alzheimer's disease) in animals because it requires awareness of the CS-US temporal relationship and numerous brain structures during consolidation (Clark and Squire, 1998; Clark, 2011).

Learning and acetylcholine: Correlational studies

Humans suffering with Alzheimer's disease express deficits in learning trace memories and these deficits have been associated with a loss of the neurotransmitter

acetylcholine (Woodruff-Pak & Papka, 1996). It was first observed in the 1970s that a loss of cholinergic activity in the brain of patients with Alzheimer's disease correlated with cognitive impairments (for review see Bartus, 2000) and, it was later established that this was the result of degradation of the septohippocampal pathway (Figure 2; Whitehouse et al., 1982). These observations led researchers to speculate that acetylcholine plays an important role in learning and memory, and thus, possibly the development of learning and memory-related diseases. Numerous animal studies support this hypothesis, however, the degree to which acetylcholine is required for learning hippocampal-dependent tasks and how it is involved has recently been called into question because of many conflicting results in the learning and memory literature (Parent and Baxter, 2004).

The hypothesized role of acetylcholine in learning is supported by numerous correlational studies. For example, it is reported that acetylcholine increases in the ventral hippocampus across training sessions of delay conditioning (Meyer et al., 1996). Interestingly, in this study acetylcholine release was significantly higher in animals during training only on days when significant gains in learned responding were observed, suggesting that acetylcholine may be most essential during acquisition. However, as discussed, the hippocampus is not necessary for learning the delay conditioned response, and therefore, acetylcholine in the hippocampus may modulate performance but is not necessary for achievement. It is reasonable to suspect that acetylcholine would increase during training with a hippocampal-dependent version of eyeblink acquisition and others have observed changes in acetylcholine activity during other forms of hippocampal-dependent learning. Specifically, Decker and colleagues (1988) report that following

training on a spatial task referred to as the Morris water maze resulted in a decrease in high affinity choline uptake (HACU; a protein responsible for transporting choline into the cell) in animals trained with the hippocampal-dependent version, but not the hippocampal-independent version. Even though the decrease in HACU indicates that cholinergic activity decreased, HACU measurements were taken post mortem and thus a considerable time after training. As the authors suggest, a decrease in HACU after training may be the result of a compensatory response to an increase in cholinergic activity during training. Indeed, other experiments support this idea. In another more difficult version of the spatial water maze, decreased levels of HACU were found in fast learners following training. However, animals that had not reached asymptotic learning in the more difficult task were more likely to have increased levels of HACU following training. In accordance with the delay eyeblink study, these experiments suggest that cholinergic activity seems to correspond with acquisition and not expression of a learned behavior. In another spatial task where animals must similarly use visual cues to find rewards in a radial arm maze, researchers have also observed that acetylcholine is elevated in the dorsal hippocampus during training (Stancampiano et al., 1999). Notably, the elevation in acetylcholine occurred during a period before each training session when animals were likely anticipating the food reward.

Learning and acetylcholine: Pharmacological manipulations

Numerous experiments provide evidence that acetylcholine and hippocampal-dependent learning are causatively related. Most of these studies have utilized pharmacological agents that temporarily enhance or block cholinergic activity.

Metrifonate is an organophosphate compound that inhibits acetylcholinesterase (AChE), an enzyme that hydrolyzes and thus degrades action of acetylcholine in the synapse. Two studies reported that administration of this compound facilitated acquisition of CRs during trace eyeblink conditioning of aged rabbits compared to performance in aged controls that were not exposed to the compound (Kronforst-Collins et al., 1997a, 1997b). Two other drugs target cholinergic receptors: galantamine and CI-1017. Galantamine, which both modulates nicotinic receptors and inhibits AChE, facilitates early acquisition of CRs in trace eyeblink conditioning in healthy, young animals (Simon et al., 2004). In this study, young animals that did not receive galantamine eventually reach the same level of responding as animals that did received the drug. However, two other reports indicate more drastic responses to the drug in aged animals (Weible, Oh, Lee, & Disterhoft, 2004; Woodruff-Pak & Santos, 2000). In these studies, galantamine drastically improved learning in aged animals resulting in a level of responding similar to young animals. The other drug, CI-1017, is a cholinergic agonist that binds to M1 muscarinic receptors. CI-1017 has been reported to improve acquisition of trace CRs in aged animals to the same extent (Weiss et al., 2000), suggesting that both nicotinic and muscarinic receptor activation are involved in trace eyeblink learning. Overall, compounds that enhance acetylcholine cholinergic activity tend to facilitate learning in young animals and enhance the number CRs emitted by older animals.

The role of muscarinic receptors in trace eyeblink learning has been studied more extensively using the drug scopolamine, a general muscarinic receptor antagonist. Cholinergic blockade using systemic scopolamine injections impair the development of CRs in both trace and delay eyeblink conditioning (Kaneko & Thompson, 1997;

Salvatierra & Berry, 1989). However, scopolamine disrupts trace eyeblink conditioning more severely than delay conditioning. This is an expected outcome because the hippocampus is more critically involved in trace learning. Interestingly, Kaneko and Thompson (1997) also found that a single injection of scopolamine was able to suppress CRs in animals that were previously given saline during training and learned the task well. Others have reported a similar suppression of a well-learned behavior in animals following scopolamine injections (Múnera et al., 2000). Therefore, these findings pose a possible role of muscarinic receptors in both acquisition and the expression of learned responses. This idea complements research showing that an intact hippocampus is necessary for both learning and, for short period of time, expressing trace eyeblink CRs (Kim et al., 1995; Takehara, Kawahara, & Kirino, 2003). Suppression of CRs as a result of scopolamine is likely not due to nonspecific or motor effects. Kaneko and Thompson (1997) report that unconditioned responses to the US were unaltered and animals reach normal levels of responding eventually during delay conditioning. It is important to note, however, that systemic injections of scopolamine ultimately affect all muscarinic cholinergic activity in the brain, including both the basalocortical and the septohippocampal pathways. However, Solomon and colleagues (1983) show that impairments in delay eyeblink conditioning as a result of systemic injections of scopolamine are prevented by hippocampal lesions. Their data suggest that the effects of systemic injections of scopolamine on learning can be completely explained by scopolamine causing dysfunctional hippocampal activity. Others have studied the effects of infusing scopolamine directly into the hippocampus in another type of trace conditioning task (Pang et al., 2010). In this study, a tone and a footshock were paired but

separated in time by a stimulus free trace interval. In this trace fear task, animals learn to fear the tone and freeze in anticipation to the footshock. Pang and colleagues (2010) report that infusions of scopolamine into the dorsal hippocampus impaired acquisition and recall of the conditioned freezing response to the tone. Acquisition was also impaired during the delay version of the task as well, but not recall. It should also be noted that nicotinic antagonists also impair trace fear learning, but not recall, and furthermore, that nicotinic antagonists do not disrupt delay fear conditioning (Raybuck and Gould, 2010).

Learning and acetylcholine: Lesion studies

It has been hypothesized that acetylcholine contributes to learning through projections from the MSDB complex to the hippocampus. For example, electrolytic lesions of the medial septum have also been shown to disrupt delay eyeblink conditioning (Berry and Thompson, 1979). However, these type of lesions kill all neuronal subtypes in the MSDB, and thus, cannot be appropriately used to assess the role of cholinergic MSDB neurons in learning. Conversely, the immunotoxin 192 IgG-Saporin can selectively lesion cholinergic neurons with few side effects when administered in the basal forebrain in controlled doses (Wiley, Oeltmann, & Lappi, 1991). Using 192 IgG-Saporin, Fontán-Lozano and colleagues (2005) showed that cholinergic projections are necessary to learn trace eyeblink conditioning. In fact, this group of researchers report drastic impairments – conditioned responding never increased over any of the training sessions. These researchers were the first to report that cholinergic MSDB neurons have a critical role in trace eyeblink conditioning. However, they used an atypical trace procedure, with an eyelid stimulation as both the CS and US. Most other researchers that

have studied the role of acetylcholine in eyeblink conditioning used a “neutral” stimulus as the CS. Furthermore, less pronounced deficits are reported in other types of hippocampal-dependent learning. Baxter and colleagues have repeatedly reported that animals with cholinergic lesions performed similarly or were not drastically impaired during performance in the Morris water maze compared to performance in animals with sham lesions (Baxter, Bucci, Gorman, Wiley, & Gallagher, 1995; Frick, Kim, & Baxter, 2004). Others report that cholinergic lesions also do not impair spatial learning on a radial arm maze (Dwyer et al., 2007), which contrasts findings from experiments using pharmacological manipulations (Carli, Luschi, & Samanin, 1997; Kim & Levin, 1996). Dwyer and colleagues (2007) also found that while disruption to cholinergic septohippocampal projections had no effect on learning, acquisition was disrupted by the loss of GABAergic MSDB neurons (the other major neuronal type in MSDB complex; Figure 2). These reports have led to researchers questioning the presumed critical role of acetylcholine in learning (Baxter & Bucci, 2013; Parent & Baxter, 2004). Thus, researchers have not yet reached a consensus about the degree of acetylcholine’s involvement in the different phases of learning and recall.

The present experiment aims to independently assess whether cholinergic neurons in the MSDB complex are necessary for a traditional version of trace eyeblink conditioning. Animals with cholinergic MSDB lesions, generated using the immunotoxin 192 IgG-Saporin, are used to determine whether septohippocampal projections are necessary for trace eyeblink conditioning. The development of trace CRs occurs over hundreds of trials (Anderson et al., 2011). Therefore, the task is useful for assessing whether animals can learn and form declarative memories, and also, for observing how manipulations may

disrupt early versus late phases of learning. The present study also evaluates whether cholinergic activity is important for early acquisition, asymptotic responding, or both. In this experiment, animals with bilateral or unilateral lesions were trained with trace or delay eyeblink conditioning. Animals with unilateral lesions were employed for two reasons. First, unilateral hippocampal damage in humans does not always induce deficits in learning (Scoville and Milner, 1957; Baxendale et al., 2013). Hence, unilateral lesions were used in this study to understand whether intact septohippocampal connections in one hemisphere can compensate for the loss of those connections in the other. Second, unilateral lesions were used to make within subject comparisons between the “intact” and “lesion” hemisphere in experiments 2 and 3. Additionally, this experiment explores the relationship between the extent of the lesion and learning. This was done to clarify whether incomplete lesions may account for varied findings in the literature. Overall, experiment 1 was conducted to test the following hypotheses:

1. Cholinergic MSDB neurons are required for trace eyeblink conditioning.
2. Cholinergic MSDB neurons in one hemisphere are sufficient for learning trace eyeblink conditioning.
3. The extent of the loss of cholinergic MSDB neurons correlates with the level of learning impairment.

General Methods

Subjects

Male Sprague-Dawley rats were bred at Rutgers University at the Department of Psychology. After reaching postnatal day 28, they were put into groups of 2-3 in a plastic box cage with a wire top (44.5cm long x 21.59cm wide x 23.32cm high) and kept in a 12 hour light-dark cycle, with the lights turning on at 7 am. All rats were given free access to water and food throughout their life. Prior to experimental manipulations, animals were handled and then separated into individual cages to prevent the animals from interfering with permanent equipment implanted to record eye muscle activity or hippocampal local field potentials (LFPs). All experimental manipulations took place during the light portion of the light-dark cycle. All experiments were conducted in full compliance with the rules and regulations specified by the PHS Policy on Humane Care and Use of Laboratory Animals and the Guide for the Care and Use of Laboratory Animals. A portion of the animals in experiment three were obtained from Harlan Laboratories and housed at the University of Jyvaskyla. Animals were kept on similar light-dark cycles, only lights turned on at 8 am. All other conditions were the same. The experimental procedures conducted on these animals were implemented in accordance with directive 2010/63/EU of the European Parliament and of the Council on the care and use of animals for research purposes. Overall, animals in all groups in experiments 1, 2 and 3 were drawn from at least two discrete rounds of experimentation and litters over the course of three years.

Methods

Design

Animals were used to determine whether a decrease in the number of cholinergic cells in the medial septum/diagonal band complex (MSDB) resulted in a deficit in trace eyeblink conditioning. Animals were assigned to one of three groups: Sham, Bilateral or Unilateral. A full description of the group assignment is below. These animals were also used in an additional coinciding experiment. All animals received a single injection of 5-bromo-2'-deoxyuridine (BrdU) injection (200 mg/kg, i.p.; Sigma-Aldrich, St. Louis, MO, USA) seven days before animals were exposed to the learning context. BrdU data for this experiment is not relevant for the purposes of testing the hypotheses stated in the introduction section and will not be presented in this dissertation. See figure 3 for a summary of the timeline of the experimental manipulations.

Surgery

The animals were assigned to receive the immunotoxin 192 IgG-Saporin (Advanced Targeting Systems, San Diego, CA) or the vehicle, 0.1 M phosphate buffer saline (PBS). 192 IgG-Saporin directs a ribosome-inactivating cytotoxic protein (saporin) to p75, a low-affinity cell surface receptor expressed predominately on cholinergic neurons. As a result, 192 IgG-Saporin selectively kills cholinergic neurons leaving neighboring neurons largely intact (Rossner et al., 1995). Animals were anaesthetized with sodium pentobarbital (60-70 mg/kg, Nembutal; Henry Schein, Indianapolis, IN, USA). Their heads were shaven and then the animals were placed in a stereotaxic

apparatus (David Kopf, Tujunga, CA, USA). After cleaning the skin three times alternating between ethanol and betadine, a local analgesic (2.5 mg/mL, Marcaine; Hospira, Lake Forest, IL, USA) was injected subcutaneously into the site of the incision. After approximately 3 min, an incision was made on the top of the head and tissue was cleared from the skull. The skull was leveled using Bregma and Lambda. Holes were drilled through the skull at each lesion site. Either 192 IgG-Saporin (0.2 μg/μl in sterile 0.1 M PBS) or vehicle (sterile 0.1 M PBS) was infused into the brain at the following sites relative to Bregma: AP: +0.6, ML: ±0.5, DV: -7.8; AP: +0.6, ML: ±0.5, DV: -6.6 (DV measure from the dura). Some animals received four infusions of either 192 IgG-Saporin or vehicle (two in the left hemisphere and two on the right) while others received only two infusions in only either the left or right hemisphere (Figure 4).

Due to the small width of the MSDB (it is less than 1.2mm wide at the target lesion site), it was possible to use two infusions in one hemisphere to produce both bilateral and unilateral lesions. The resulting type of lesion was ultimately dependent on the normal varying nature of using Bregma on the skull to locate the target infusion site inside of the brain, and also the spread of the drug. Indeed, other laboratories have used fewer infusions to produce MSDB lesions using 192 IgG-Saporin (Van der Borght et al., 2005; Yoder and Pang, 2005; Dwyer et al., 2007). When only one hemisphere was targeted, the hemisphere was counterbalanced. The learning data did not differ between animals that received infusions in one hemisphere versus both, Sham [Interaction: $F(11,66) = .67$, $p = .76$, Number of infusions: $F(1,66) = 1.78$, $p = .23$]; Bilateral [Interaction: $F(11,66) = 1.04$, $p = .42$, Number of infusions: $F(1,66) = .002$, $p = .97$]; (Figure 5a,b). Thus, all animals that received vehicle, regardless of whether they received the infusion in one (n = 5) or

both (n = 3) hemispheres, were merged into a single Sham group to further analyze the learning data. Similarly, animals with a bilateral lesion, regardless of whether they received the infusion in one (n = 5) or both (n = 2) hemispheres, were merged into a single Bilateral group for analysis. All animals with a unilateral lesion received infusions in only one hemisphere because targeting both hemispheres never produced a unilateral lesion. A 10 µl Hamilton syringe (Hamilton Company, Reno, NV, USA) attached to an automated infusion pump was used to administer the immunotoxin or vehicle. The syringe was dropped into the brain, then left to rest for 1 min before infusing 0.4 or 0.3 µl (when both or only one hemisphere, respectively, was targeted) at DV:-7.8 at a rate of 0.1 µl/min. The drug was allowed to diffuse for an additional 5 min. Afterwards, the syringe was lifted to the DV -6.6 infusion site, left to rest for 1 min, then 0.3 µl or 0.225 (when both or only one hemisphere, respectively, was targeted) was infused at 0.1 µl/min. The syringe was left undisturbed for 5 min to allow the drug to diffuse. If both hemispheres were targeted, then the same procedure was used to infuse 192 IgG-Saporin or vehicle at the other two sites. The hole in the skull was filled with bone wax.

Following the lesion, four stainless wires were implanted around and through the right upper eyelid. The wires were connected to a four-pin connector by solder and were attached to the skull using skull screws and dental cement. Two electrodes were used to record electromyographic (EMG) activity, to detect eyeblinks, and the other two delivered a periorbital stimulation to the eyelid, which always elicits an eyeblink. Animals were given supplemental booster injections of sodium pentobarbital due to the length of the surgery. Upon awakening, the rats were given 1 mL of acetaminophen orally (32 mg/mL, Children’s Acetaminophen; Rite Aid) and returned to their home

cages. All animals were allowed to recover for a minimum of 14 days and an additional seven days following the BrdU injection referenced above.

Eyeblink conditioning

Thirty nine animals (Sham/Vehicle n = 13, 192 IgG-Saporin n = 26) were trained with eyeblink conditioning. A minimum of 21 days following the surgery, the animals were connected to a cable that allows free movement within a conditioning chamber. Animals were first acclimated to the conditioning apparatus without the presentation of any stimuli for approximately 40 min (100 twenty-five second trials). During the acclimation period, spontaneous blinks were recorded during a 500 ms period for each trial to obtain a baseline blink rate. After acclimation, rats were returned to their home cage. Training began 24 hr after the initial acclimation period.

The next day, animals were exposed to an additional 30 trials to record spontaneous blinks. Immediately following those 30 trials, training commenced. During trace training, a trial consisted of a 250 ms white noise conditioned stimulus (CS; 82 dB) followed by a 500 ms trace interval, and then immediately thereafter by the unconditioned stimulus, a 100 ms shock to the eyelid (US; 65 mA), which always elicits an unconditioned eyelid closure. The inter-trial interval was 25±5 seconds. Eyeblinks were detected by changes in the eyelid EMG, which were bandpass filtered between 0.3 and 1.0 kHz and amplified (10K, Differential AC Amplifier Model 1700; A-M systems, Sequim, WA, USA). EMG during the trace interval was compared to a 250 ms baseline recording before the onset of the CS. Eyeblinks that occurred 500 ms before the presentation of the US, are larger than 0.5 mV, greater than 2.5 standard deviations above

the mean of the baseline recording, and greater than 10 ms in length were considered conditioned responses (CRs). Eight animals (Sham/vehicle n = 5, 192 IgG-Saporin n = 3) were removed from the study because blinks were not detected during the acclimation period or baseline EMG activity was excessively noisy. Data is presented as a percentage of CRs made during each trial block. The average percent CR was calculated across blocks of 100 trials as well and used for analysis in experiments 1, 2 and 3.

Additional animals that were infused with 192 IgG-Saporin (n = 8) were trained with delay conditioning to confirm that animals with bilateral and unilateral lesions were able to learn the easier version of the task. Training was the same as described above, only delay trials consisted of a 750 ms white noise that co-terminated with the 100 ms shock to the eyelid. Eyeblinks that occurred 500 ms before the presentation of the US, are larger than 0.5 mV, greater than 2.5 standard deviations above the mean of the baseline recording, and greater than 10 ms in length were considered CRs.

Immunohistochemistry

Rats were deeply anaesthetized with sodium pentobarbital (0.3 ml, Sleepaway; Butler Schein, Dublin, OH, USA) and transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were extracted and kept in paraformaldehyde for 24 hours and then transferred to 0.1 M PBS until sectioning. A vibratome was used to obtain 40 μm thick coronal sections of the hippocampus and 50 μm thick coronal sections of the MSDB. Slices of the left and right hippocampal formation and MSDB were collected and stored in cryoprotectant at -20^{0}C until staining. Every third MSDB slice (for a total of 10 slices spanning the entire structure) was stained for choline acetyltransferase (ChAT) to

reveal the extent of the lesion. ChAT is an enzyme that forms acetylcholine and is thus found in cholinergic neurons. Ten other adjacent slices of the MSDB were stained for parvalbumin (PARV), a GABAergic neuronal marker, to confirm that the dose of immunotoxin used was selective for cholinergic neurons. To do so, tissue was removed from cryoprotectant and washed with dH2O for 10 min twice and then 0.1 M PBS for an additional 10 min. The tissue was then incubated for 48 hr in either anti-ChAT (1:200, goat anti-ChAT; Millipore, Temecula, CA, USA) or anti-PARV primary antibody (1:1000, mouse anti-PARV; Sigma-Aldrich, St. Louis, MO, USA) with 10% horse serum (Vectors Labs, Burlingame, CA, USA) and 0.05% Triton-X 100 (Sigma-Aldrich, St. Louis, MO, USA). The tissue was then washed with PBS and left in secondary (1:200, biotinylated anti-goat; 1:200, biotinylated anti-mouse; both from Vector Labs, Burlingame, CA, USA) for 1 hr at room temperature. The tissue was then again washed and incubated in avidin–biotin–horseradish peroxidase (1:100, Vectastain ABC elite kit; Vector Labs, Burlingame, CA, USA), and lastly in diaminobenzidine for 1-2 min (DAB SigmaFast tablets; Sigma-Aldrich, St. Louis, MO, USA) with PBS rinses in between. The tissue was then mounted onto superfrost glass slides (Fisher, Suwanne, GA, USA) and left to dry. The tissue was dehydrated and cleared before coverslipping with Permount (Fisher Scientific, Fair Lawn, NJ, USA).

Bilateral and unilateral lesions were also verified by examining acetylcholinesterase (AChE) in the hippocampus. AChE is an enzyme that hydrolyzes acetylcholine and is found in cholinergic synapses. The immunotoxin kills both cholinergic neurons that project within MSDB and also cholinergic neurons that comprise the cholinergic septohippocampal projections. These projections from the MSDB are, for

the most part, unilateral, so that one side of the MSDB projects predominantly to the ipsilateral hippocampal formation (Kiss et al., 1990a, 1990b). Every 12th hippocampal section (both left and right) were rinsed three times with dH2O for 10 min each. The AChE staining protocol previously published by (Paxinos and Watson, 2007) was slightly modified and implemented. Briefly, the tissue was incubated in stock solution containing 116 mg S-acetylthiocholine iodide (Sigma-Aldrich, St. Louis, MO, USA) and 3 mg ethopropazine (Sigma-Aldrich, St. Louis, MO, USA) for every 100 ml. The tissue was rinsed with dH20 for approximately 5 min and developed for 30 min in 1% sodium sulphide (Sigma-Aldrich, St. Louis, MO, USA) at pH 7.5. The tissue was then mounted onto superfrost glass slides (Fisher, Suwanne, GA, USA) and left to dry. The tissue was dehydrated and cleared before coverslipping using Permount.

Quantification

To quantify the extent of the lesion, the number of ChAT-positive cells per unit area was determined. To do so, circles with a diameter of 0.4 mm were placed on microphotographs taken of the MSDB at 40x using a Nikon Eclipse 80i light microscope and Nikon NIS standard imaging software (Melville, NY, USA). Circles were used to fill and sample from all areas of the MSDB. The circle placement was pre-determined using animals with sham lesions and based on their proximity to the medial line, anterior commissure and each other. The circle placements were then used for animals that received infusions of 192 IgG-Saporin keeping the placement consistent between animals (Figure 6). This procedure allowed for direct comparisons between hemispheres in different areas of the MSDB and the counter to account for tissue damaged as a result of

the lesion or sectioning. ChAT-positive cells were counted within each circle with the image zoomed to 100%. Cells were counted if the soma (not necessarily the cell's projections) resided completely within the circle. The counter was blind to experimental condition and the performance during training. The number of ChAT-positive cells was divided by area of the circle, 0.1256 mm2, and then summed to determine a value (ChAT+/mm2) for the left hemisphere, right hemisphere, and both hemispheres combined. The value for ChAT+/mm2 was compared to the values calculated from animals with a sham lesion. Each total brain (left and right combined) ChAT+/mm2 value was divided by the average of ChAT+/mm2 calculated from all animals with a sham lesion, and multiplied by 100. This value is referred as "percent ChAT" remaining in the results section for simplification purposes. The percent ChAT was subtracted from 100 to determine "percent lesion". Each hemisphere was also analyzed separately. "Intact" and "target" refer to whether 192 IgG-Saporin was infused in the hemisphere, not whether cholinergic neurons were intact or targeted. These terms are used for the Sham group and Bilateral group as well. Each ChAT+/mm2 value from the intact and target hemispheres of animals given 192 IgG-Saporin was divided by either the intact or target hemispheric average of ChAT+/mm2 values, calculated from the intact and target hemisphere from the Sham animals respectively, and multiplied by 100. Once again, these values are referred as "percent ChAT" in the results section. Again, percent ChAT was subtracted from 100 to determine "percent lesion" for both the intact and target hemisphere.

Animals that received 192 IgG-Saporin were split into bilateral and unilateral lesion groups based on lesion outcome (percent ChAT), not whether one or both hemispheres were infused. Animals were considered to have a bilateral lesion if the

percent lesion was equal to or greater than 85% and the difference between percent lesion of each hemisphere was less than 30%. An animal was considered to have a unilateral lesion if the difference between percent lesion between each hemisphere was greater than 30%. If an animal had either vehicle or 192 IgG-Saporin injected into both of its hemispheres (Figure 4 top), the left hemisphere was included in the “intact” hemispheres and the right hemisphere was included in the “target” hemispheres. Nine animals that were given 192 IgG-Saporin did not fall into either the bilateral or unilateral category or had excessive damage to other structures and were excluded from the analysis. The same quantification methodology was applied to tissue stained to reveal PARV-positive cells, although only the ChAT data was used to determine whether animals had a bilateral or unilateral lesion.

To quantify AChE expression, microphotographs were taken at 10x of the hippocampal tissue using a Nikon Eclipse 80i light microscope and Nikon NIS standard imaging software (Melville, NY, USA). The microphotographs were converted to 8-bit grayscale images and then the background was subtracted using a rolling ball radius of 1400 pixels to control for variation in the background. The hippocampus at approximately Bregma -2.8, -3.8, -4.8, and -5.8 was traced using ImageJ (National Institutes of Health, USA) and the mean optical density (OD) was calculated. Again, the data handler was blind to experimental condition and performance during training. The average of the four mean OD values obtained from each slice was calculated. The mean optical density was measured for an area of the corpus callosum and subtracted from the average OD. This was done separately for the left and right hemisphere. The resulting left and right average OD was compared between animals.

Analysis

Statistical analysis included Sham (n = 8), Bilateral (n = 7), and Unilateral (n = 7) groups for examining the effect of the lesion on trace eyeblink conditioning. Statistical analysis included Bilateral (n = 4) and Unilateral (n = 4) groups for examining the effect of the lesion on delay eyeblink conditioning. Repeated measures analysis of variance (ANOVA) was used to analyze changes across time and differences between groups for the learning data. Repeated measures ANOVAs were used to analyze differences between hemisphere and between groups for ChAT, PARV, and AChE data. The percent of the positive cells remaining compared to sham animals values were used for ChAT and PARV analysis. The relative optical density (average optical density of lesion animal over average optical density of entire sham group) values were used for the AchE analysis. Separate ANOVAs for each group and post hoc comparisons using the Student–Newman–Keuls method were also used, when appropriate, to further examine differences between groups and time points. All differences were considered statistically significant if the p-value was less than 0.05. Error bars in all graphs represent standard error. A Spearman's correlation was used to examine the relationship between the value for ChAT+/mm2 and the average percent CRs made across all eight 100 trial blocks.

Results

192 IgG-Saporin produces selective cholinergic lesions in the MSDB

The MSDB complex of Sprague-Dawley rats was infused with either vehicle or 192 IgG-Saporin. Animals given 192 IgG-Saporin were split into two groups: animals with a bilateral or unilateral lesion. Whether to categorize the lesion as bilateral or unilateral was determined using the percent lesion and the difference in the percent lesion between intact and target hemispheres, as previously describe in the methods section. A two way repeated measures ANOVA (type of lesion versus hemisphere as the repeated measure) was used to compare percent ChAT (percent of ChAT+/mm2 remaining) between groups and hemispheres (Figure 7a,c,d). A significant interaction was found between the type of lesion and the hemisphere, $F(1, 20) = 91.13$, $p < .00001$. Significant main effects were also found for both type of lesions, $F(1, 20) = 51.23$, $p < .00001$, and hemisphere, $F(1, 20) = 120.21$, $p < .00001$. Post-hoc comparisons revealed that the percent ChAT in the intact hemisphere in animals with a unilateral lesion was significantly different from all other groups, “Unilateral Target” $p = .0002$; “Bilateral Intact” $p = .0001$; “Bilateral Target” $p = .0002$. In addition, the percent ChAT in the target hemisphere in animals with a unilateral lesion also differed from the intact and target hemispheres in animals with a bilateral lesion, $p = .01$ and $p = .01$ respectively. Percent lesion (100-“percent ChAT”) ranged from 82-99% for animals with a bilateral lesion and 10-74% for animals with a unilateral lesion. An independent-samples t-test, comparing the difference in percent ChAT between hemispheres revealed that there was a significant difference between animals with a bilateral and unilateral lesion, $t(14) = -9.49$,

$p < .00001$ (Figure 7b). The difference in percent ChAT between the intact and target hemispheres ranged from 0-27% for animals with a bilateral lesion and 34-76% for animals with a unilateral lesion. Overall, the procedures used for splitting animals into a bilateral and unilateral lesion group led to statistically distinct groups.

192 IgG-Saporin is a selective toxin for cholinergic neurons because it targets the p75 receptor. To determine how selective the toxin was, adjacent MSDB slices were stained to reveal GABAergic PARV-positive cells. A two way repeated measures ANOVA (type of lesion versus hemisphere as the repeated measure) was used to compare percent PARV (percent of PARV+/mm2 remaining) between groups and hemispheres. There was not an interaction between type of lesion and hemisphere $F(1, 20) = .36$, $p = .56$ (Figure 8a,c,d). While there was not a main effect for type of lesion, $F(1,20) = 3.44$, $p = .08$, there was a main effect for hemisphere, $F(1,20) = 4.81$, $p = .04$. In other words, the target hemisphere has significantly fewer PARV-positive cells compared to the intact hemisphere. The difference in percent PARV between hemispheres was not significant between animals with a bilateral and unilateral lesion, $t(20) = .40$, $p = .69$ (Figure 8b). Overall, 192 IgG-Saporin was not perfectly selectively, but did predominantly kill ChAT-positive neurons.

To examine cholinergic activity in each hemisphere of animals with bilateral and unilateral lesions, hippocampal slices were stained to reveal AchE. A two way repeated measures ANOVA was conducted to examine differences between groups (type of lesion versus hemisphere as the repeated measure). There was a significant interaction between type of lesion and hemisphere, $F(1,20) = 45.28$, $p < .0001$ (Figure 9a,b,c). In addition, there were significant main effects both variables, lesion: $F(1,20) = 14.50$, $p = .001$ and

hemisphere: $F(1,20) = 68.96$, $p < .0001$. A post hoc analysis revealed that the intact hemisphere in animals with a unilateral lesion has significantly more AchE expression compared to the target hemisphere, $p = .0002$ (a decrease in AchE expression is indicated by an increase in optical density). In addition, AchE expression in the Unilateral Intact hemisphere was significantly higher than both hemispheres of animals with a bilateral lesion, Bilateral Intact: $p = .0002$, Bilateral Target: $p = .0002$. The AchE expression in the intact and target hemispheres in animals with a bilateral lesion were similar to each other.

Cholinergic lesions retard trace eyeblink conditioning

Following a recovery period, animals were trained with a trace eyeblink conditioning procedure for four consecutive days, 200 trials per day for a total of 800 trials. In a trial, a CS and US are separated by 500 ms trace interval. A learning criterion of making CRs 60% of the trials during any 100 trial block is used often in our laboratory to separate animals into “good” and “poor” learners (Anderson et al., 2011). Seventy-five percent of animals with a sham lesion (6 of 8) reached this learning criterion. Generally, fewer animals with lesions reach this learning criterion. Only 28.6% of animals with a bilateral lesion (2 of 7) and 57.1% of animals with a unilateral lesion (3 of 7) reach the learning criterion. Of the animals that learned well in each group, animals with bilateral and unilateral lesions took longer to reach the learning criterion, Sham: $M = 350.00$, $SE = 55.81$; Bilateral: $M = 533.33$, $SE = 88.19$; Unilateral: $M = 625.00$, $SE = 143.61$. The electrodes that delivered the US and recorded eyeblink responses were always implanted in the right eyelid. Our laboratory has never observed that this influenced behavioral outcomes. However, others report that CA1 neurons ipsilateral to the eyelid electrodes

respond earlier in training compared to CA1 neurons contralateral to the trained eye of rabbits (Weible et al., 2006), so particular attention was paid to animals with unilateral lesions in this experiment. In the unilateral group, 1 of the 3 animals with a left hemispheric lesion and 3 of the 4 animals with a right hemispheric lesion learned well. A two way repeated measures ANOVA confirmed that animals with left lesions and right lesions performed similarly, Interaction between lesion hemisphere and responding across time: $F(11,55) = .64$, $p = 78$; Main effect for lesion hemisphere: $F(1,55) = 1.16$, $p = .33$ (Figure 10). Therefore, all animals with a unilateral lesion were analyzed as one group. The learning data for all groups was further analyzed using the total number of CRs made over the entire 800 trials. A one way ANOVA was used to compare the total number of CRs made between lesion groups. Using the total number of CRs made during the entire trace interval (500-1000 ms; Figure 11a), the ANOVA revealed that there was not a significant difference between the sham, bilateral and unilateral groups, $F(2,19) = 2.33$, $p = .12$.

Learning that the CS predicts the US is seemingly apparent to the animals within the first session of training (i.e. animals freeze and often blink during the CS). As trials continue, blinks occur more often during the trace interval. However, CRs emitted during the last 250 ms reflect a very finely timed response and occur more often during later stages of learning. These late stage CRs are assumed to reflect a more difficult aspect of learning trace eyeblink conditioning, which is learning to make finely timed responses in anticipation of the US. Furthermore, quantifying CRs during the last 250 ms ensures that behavioral responses to the CS (alpha responses) are excluded from the analysis. Using CRs made 250 ms before the US, did not change the number of animals in any of the

groups that reach the learning criterion of 60% CRs in any 100 trial session. However, it did slightly increase the average number of trials it took to reach the learning criteria for each group, Sham: M = 466.67, SE = 49.44; Bilateral: M = 600.00, SE = 57.74; Unilateral: M = 733.33, SE = 66.67. There was a significant difference in the total number of CRs made between groups when only the number of CRs emitted during the last 250ms of the trace interval (750-1000 ms; Figure 11b) was used, $F(2,19) = 4.16$, $p = .03$. A post hoc analysis revealed that animals with a sham lesion emitted significantly more CRs, 250 ms before the onset of the US, than animals with a bilateral or unilateral lesion, $p = .04$ and $p = .03$ respectively. This data suggests that a lesion, regardless of whether it is bilateral or unilateral, somewhat impairs an animal's ability to learn to make finely timed responses.

A two way repeated measures ANOVA (type of lesion versus CRs over time) was used to analyze trace CRs over time between groups. First, learning using CRs emitted within any time during the trace interval (500-1000 ms) in a trial was analyzed. There was not a significant interaction between type of lesion and CRs over time, $F(22,209) = .89$, $p = .60$ (Figure 11c). In addition, there was not a difference between types of lesions, $F(2,209) = 1.57$, $p = .23$. However, the number of CRs did change and generally increased over time, $F(11,209) = 2.60$, $p = .004$.

A two way repeated measures ANOVA (type of lesion versus CRs over time) was used to once again analyze trace eyeblink learning between groups, only this time, using CRs made 250 ms before the US (trace interval 750-1000 ms). There was not a significant interaction between type of lesion and CRs over time, $F(22,209) = 1.03$, $p = .43$ (Figure 11d). There was nearly a significant difference between types of lesions,

$F(2,209) = 3.12$, $p = .06$. Separate one way ANOVAs were used to analyze CRs over time for each type of lesion. Animals in the sham group generally emitted more responses over time $F(11,77) = 4.20$, $p < .001$. Animals with a bilateral or unilateral lesion also emitted significantly more responses over time, Bilateral $F(11,66) = 6.16$, $p < .001$; Unilateral $F(11,66) = 4.24$, $p < .001$. Overall this data suggests that the loss of cholinergic MSDB neurons results in a small, but measureable, learning deficit (regardless of whether it is a bilateral or unilateral lesion).

Ultimately, animals in all three conditions appeared to reach similar levels of learning. Statistical analysis for the last 100 trials using a one way ANOVAs (type of lesion as the dependent variable) support this observation, 500-1000 ms: $F(2,20) = .05$, $p = .95$; 750-1000 ms: $F(2,20) = .41$, $p = .67$. However, animals in both lesion groups seemed to be impaired during early acquisition (first 100 trials). Responding dramatically increases across the first 100 trials in animals with a sham lesion, but not in animals with bilateral or unilateral lesions (Figure 12). To explore this observation, a two way repeated measures ANOVA was conducted for responses made during the last 250 ms of the trace interval for the first 100 trials. It revealed a significant interaction between lesion group and learning, $F(8,76) = 33.29$, $p = .02$. A post hoc analysis revealed that animals with a sham lesion emitted significantly more responses during the last 20 trial block of the 100 trials compared to animals with a bilateral or unilateral lesion, $p = .05$ and $p = .04$ respectively. No differences were found using CRs made during the entire trace interval, Interaction between type of lesion and responding across time: $F(8,76) = 1.69$, $p = .11$; Main effect for type of lesion: $F(2,19) = .57$, $p = .57$. Therefore, differences in learning between animals with sham, bilateral and unilateral lesions could be the result of

impairments in the ability to learn to emit finely timed responses very early during training.

The extent of the lesion does not predict how well an animal learns

The extent of the MSDB cholinergic lesion varied to a large extent in animal given 192 IgG-Saporin. The learning data suggests that a deficit might be a result of a decrease in the number of ChAT-positive cells, regardless of the degree of the decrease and whether the decrease was lateralized or not. To explore this idea, the relationship between percent ChAT and the total number of CRs emitted was analyzed. There was not a significant correlation between the percent ChAT and CRs emitted during entire trace interval, $r = .07$, $p = .82$, nor CRs emitted during the last 250 ms, $r = .27$, $p = .35$ (Figure 13a, b). In addition, the relationship between the degree to which the damage was lateralized and the total number of CRs emitted was also analyzed. There was not a significant correlation found between the difference in percent ChAT between hemispheres and CRs emitted during the entire trace interval, $r = .002$, $p = .995$, nor CRs made during the last 250 ms, $r = .20$, $p = .49$ (Figure 14a, b). Therefore, the extent of the damage did not predict how well an animal would learn.

To confirm that animals with bilateral and unilateral lesions were able to learning any form of eyeblink conditioning, other animals were trained with a delay eyeblink conditioning procedure. In this type of eyeblink conditioning, the CS and US are not separated by a time and is considered less difficult than the trace version (Clark and Squire, 1998). All animals reached the learning criterion of 60% responding. A one way repeated measures ANOVA was used to compare CRs over time between lesion groups

(Figure 15). A significant interaction was not found, $F(11,66) = .22$, $p = .995$. There was not a difference between bilateral and unilateral groups, $F(1,66) = .09$, $p = .78$. CRs did generally increased over time, although not significantly likely because animals reached asymptotic levels of responding quickly, $F(11,66) = 1.70$, $p = .09$.

Discussion

Acetylcholine is considered intrinsic to processes related to learning and memory. Pharmacological agents used to increase cholinergic activity facilitate eyeblink conditioning (Kronforst-Collins et al., 1997a), while drugs that block cholinergic receptors impair the formation of CRs, most severely when the task depends on an intact hippocampus (Kaneko & Thompson, 1997; Raybuck & Gould, 2010). However, systemic injections of these drugs make it difficult to discern which regions of the brain are responsible for these effects. Drugs that increase or decrease cholinergic activity also facilitate or impair spatial learning, respectively (Buresová et al., 1986; Carli et al., 1997), but many studies that selectively remove cholinergic neurons projecting to the hippocampus report a minimal or no disruption to learning (Baxter et al., 1995; Dwyer et al., 2007; Frick et al., 2004). The present study aimed to understand the specific contribution of cholinergic MSDB neurons in learning impairments previously observed during trace eyeblink conditioning as a result of pharmacological manipulations. In experiment 1, vehicle or 192 IgG-Saporin, a toxin that has been previously reported to selectively kill cholinergic neurons that express the p75 receptor, was infused into the MSDB complex of animals. Two weeks following this infusion, animals were trained with trace eyeblink conditioning. Animals with bilateral lesions were impaired during

training, but not drastically so. This finding contrasts one other study that reports that killing cholinergic MSDB neurons severely impairs trace memory formation (Fontán-Lozano et al., 2005). The procedures used by Fontán-Lozano and colleagues differ from the ones used in the present study in a several ways. First, they used a trace eyeblink task that involved using an eyelid stimulation as both the CS and US. Additionally, the intensity of these shocks varied between animals. The authors used the lowest intensity that elicited a small blink for each animal. The average intensity used for each experimental group was not reported, however, it is most likely that it was less intense compared to the one used in the present study. The intensity of the eyelid stimulation in the present study was selected because it has been previously reported to elicit strong, easily measured blinks. It is possible that the task used in experiment 1 was easier to learn than the one used by Fontán-Lozano and colleagues because the CS was more salient. However, Fontán-Lozano and colleagues also used a 250 ms trace interval, which facilitates acquisition (Waddell et al., 2011). Indeed, animals with sham lesions were readily able to learn to blink during the 250 ms trace interval and reached higher levels of responding than reported here. Therefore, it is difficult to distinguish which factors account for differences in the learning deficits. The present study uses parameters that closely match previous reports using pharmacological manipulations. Bilateral lesions that killed cholinergic MSDB neurons retarded the development of well-timed CRs but did not prevent animals from reaching the same level of responding compared to animals with sham lesions by the final day of training. The impairments I find are not as severe as deficits reported using systemic injections of scopolamine (Kaneko & Thompson, 1997). Therefore, disruption to cholinergic septohippocampal activity only partially accounts for

the learning impairments observed in previous studies. I conclude that cholinergic septohippocampal projections are not necessary for trace eyeblink conditioning. However, the small deficit suggests that acetylcholine is still involved in modulating acquisition.

Findings in the present study suggest that acetylcholine is especially important during early phases of learning. Animals with lesions emitted fewer well-timed CRs during all 800 trials compared to animals with sham lesions, but this deficit can largely be attributed to impairments during early acquisition. CRs increased over time at similar rates between each lesion group during later training sessions. In contrast, the rate at which CRs developed during the first training session in the Sham group was higher compared to animals with cholinergic MSDB lesions. It is possible that with a few more training sessions, a greater number of animals with lesions would have been able to reach the learning criterion. In the present study, animals were trained with delay eyeblink conditioning to simply confirm that animals with cholinergic MSDB lesions could learn delay eyeblink conditioning well. All animals reached the learning criterion, but more importantly, they reached an asymptotic level of responding quickly. Therefore, cholinergic septohippocampal projections appear to be involved in early acquisition of trace CRs but not delay CRs. Confirmation of this notion requires a future experiment designed to examine the role of cholinergic septohippocampal projections in delay versus trace eyeblink conditioning, but based on previously reported learning rates in control animals (Maeng et al., 2010), it is likely that animals with lesions would perform similar to animals with sham lesions during early acquisition of delay conditioning.

Disrupting cholinergic activity may impair learning because acetylcholine modulates processes that organize hippocampal activity. Others have reported that pharmacological agents that increase cholinergic activity also increases hippocampal responsiveness, an effect that is blocked by a muscarinic antagonist (Oh et al., 1999). In addition, learning-related CA1 population responses during the CS-US interval develop earlier during delay compared to trace eyeblink conditioning (Green and Arenos, 2007) possibly because the relationship between the CS and US is more difficult to learn when the stimuli are separated by a trace interval (Beylin et al., 2001). Moreover, trace eyeblink conditioning is dependent on hippocampal activity (Waddell et al., 2011). Therefore, a decrease in hippocampal responsiveness might be disrupting learning-related activity as a result of the lesion, and this is particularly detrimental to trace eyeblink conditioning because it requires an intact hippocampus. Thus, trace eyeblink conditioning is more dependent on acetylcholine compared to delay conditioning. Indeed, others confirm that decreasing cholinergic activity more severely impairs trace compared to delay eyeblink conditioning (Kaneko & Thompson, 1997). It should be noted that correlational studies that measure hippocampal population responses do not infer whether learning causes this organized activity or vice versa. Previous studies suggest that trace eyeblink conditioning is dependent on the hippocampus because it is an especially difficult CR to acquire (Beylin et al., 2001). Green and Arenos (2007) have proposed that this difficulty is the result of the stimulus free time gap which make it harder for an animal to resolve whether the CS or other features of the conditioning context is a better predictor of the US. Others have published data that supports the idea that the hippocampus filters important from irrelevant information (McEchron and Disterhoft,

1997). Additionally, my colleagues and I have previously demonstrated that a cue light that signals the end of a trace trial not only facilitates learning but renders the task hippocampal independent (Waddell et al., 2011). It is possible that the cue light makes it easier for animals to dissociate the relationship of the CS and US from the context/inter-trial interval. This idea is also supported by data that shows that once the association between the CS and US is made during delay conditioning, animals, including humans, are able to learn to form trace CRs even without an intact hippocampus (Beylin et al., 2001; Woodruff-Pak, 1993) . Thus, cholinergic activity may be important in processes that allow animals to efficiently learn the relationship between CS and US.

Both muscarinic and nicotinic receptors have been implicated as having a role in hippocampal-dependent learning. These receptors are expressed throughout the hippocampus in range of neuron types including CA1 and CA3 pyramidal cells, DG granule cells and interneurons (Levey et al., 1995; Zoli et al., 1998; Fabian-Fine et al., 2001; Griguoli and Cherubini, 2012). Pharmaceutical agents that enhance cholinergic activity confirm that activating muscarinic and nicotinic receptors facilitates the formation of trace CRs (Weiss et al., 2000; Simon et al., 2004). Furthermore, blocking the activity of these receptors disrupts trace conditioning (Kaneko & Thompson, 1997; Raybuck & Gould, 2010). Cholinergic MSDB lesions probably result in decreased activation of both muscarinic and nicotinic receptors. Therefore, it is impossible to discern which receptor type mediates the effect of the lesion on learning. The role of specific receptor types in learning have been evaluated using other paradigms. Genetically deleting M1 muscarinic receptors resulted in more severe learning deficits during fear conditioning compared to genetic deletions of alpha7 nicotinic receptors

(Paylor et al., 1998; Anagnostaras et al., 2003). However, mice without M1 or alpha7 receptors performed normally during training in the Morris water maze. Additional experiments are required to dissociate the role of muscarinic and nicotinic receptor activation during trace eyeblink conditioning.

It is possible that the degradation of GABAergic afferents to the hippocampus can account for part of the effects observed in experiment 1. There was approximately a 30% decrease in the number of GABAergic neurons in the MSDB following infusion of 192 IgG-Saporin. The decrease was most prominent in the target hemisphere of animals with bilateral lesion. This decrease suggests that 192 IgG-Saporin is not perfectly selective at the dose used. Most other studies using this immunotoxin qualitatively verify the specificity of the lesion and report that PARV staining is similar between groups. However, another recent study also reports that there was some damage to GABAergic neurons in the MSDB after an infusion of 192 IgG-Saporin (Yoder and Pang, 2005). Still, 192 IgG-Saporin caused much greater damage to the cholinergic neuron population. GABAergic MSDB neuronal activity also may have been altered as a result of the loss of cholinergic neurons. GABAergic neurons in the MSDB express cholinergic muscarinic receptors (Van der Zee and Luiten, 1994). A decrease in muscarinic receptor activation on GABAergic MSDB neurons could ultimately contribute to hippocampal dysfunction. Normally, GABAergic MSDB neuronal activity disinhibits GABAergic interneurons that reside in all three subregions of the hippocampus (Freund and Antal, 1988; Tóth et al., 1997). In fact, others report that GABAergic lesions impair early acquisition during delay eyeblink conditioning (Roland et al., 2013). In addition, GABAergic lesions impair spatial working memory tasks to the same degree as fornix lesions which destroy both

cholinergic and GABAergic projections to the hippocampus (Bussey et al., 2000; Roland et al., 2014). It has yet to be established how GABAergic MSDB lesions would affect trace eyeblink conditioning, but I predict that selectively killing GABAergic septohippocampal projections would also impair acquisition. Nevertheless, the present study clearly demonstrates that cholinergic MSDB lesions reduce cholinergic activity in the hippocampus and that cholinergic septohippocampal activity is not necessary for, but modulates acquisition of trace CRs. To completely dismiss that cholinergic input into the hippocampus has a role in learning would be to ignore that cholinergic neuronal terminals exist in the hippocampus and influence neuronal activity. Therefore, it is most likely that both cholinergic and GABAergic septohippocampal projections participate in processes essential for trace eyeblink conditioning.

I also find that bilateral and unilateral lesions equally disrupt learning. Intact septohippocampal projections in one hemisphere did not compensate for the loss in the other hemisphere. Cholinergic MSDB neurons predominantly project to the ipsilateral hippocampal formation (Kiss et al., 1990a, 1990b). However as previously discussed, cholinergic septohippocampal neurons also synapse onto mossy cells in the DG which in turn synapse onto granule cells in the contralateral hemisphere through commissural projections. Mossy cells provide excitatory input that may compensate for damaged cholinergic septohippocampal projections in the other hemisphere. However, these data reject the hypothesis that septohippocampal connections in one hemisphere are sufficient for learning trace eyeblink conditioning. The present study is the first to report that animals with unilateral and bilateral lesions were similarly impaired during trace eyeblink conditioning. It should be noted that while the word “unilateral” has been used to

describe this lesion, unilateral lesions were not perfectly lateralized. In experiment 1, there is approximately a 40% loss of cholinergic neurons in the intact hemisphere and an 80% loss in the target hemisphere. This resulted in a significant loss of cholinergic activity in the hippocampus in the target hemisphere. Analysis of AChE expression revealed that the unilateral intact hemisphere and both hemispheres in animals with sham lesions had similar levels of AChE expression (data not shown), so the data was expressed as ratios (lesion group to sham group). The ratios also confirm that the intact hemisphere of animals with a unilateral lesion is similar to that of the intact hemisphere in animals with sham lesions. Thus, unilateral lesions resulted in one hemisphere with decreased hippocampal cholinergic activity and another with normal levels cholinergic activity. Still, normal cholinergic activity in the hippocampus in one side was not sufficient to compensate for the loss in the other. Correlational analysis also confirms this finding. Animals with more lateralized, and thus ideal, lesions were not more likely to emit more CRs during training. In other words, animals with a greater number of cholinergic neurons remaining in the intact hemisphere were not more likely to learn well. Still, other findings show that some humans with unilateral lesions are able to form declarative memories (Scoville and Milner, 1957; Baxendale et al., 2013). In humans, the brain is more lateralized compared to other animals (Gómez-Robles et al., 2013). In particular, verbal memory is dependent on the left hippocampus, while spatial memory is dependent on the right (Feigenbaum and Morris, 2004; Cipolotti and Bird, 2006). Research in rodents suggests that unilaterally disrupting other brain regions impairs recall of learned behaviors. For example, Tanninen and colleagues (2013) report that unilateral disruption to the lateral entorhinal cortex disrupted expression of trace memories.

Furthermore, Fenton and Bures (1993) demonstrated that unilateral hippocampal inactivation impaired spatial memory recall. The present study is the first to report that unilateral disruption in rodents impairs acquisition in rodents. It is possible that hippocampal activity is disrupted in both hemisphere in animals with unilateral lesions, and as previously proposed by Solomon and Gottfried (1979), dysfunctional hippocampal activity may be worse than not having a hippocampus.

Even though unilateral lesions generally impair learning, there was great variability in the amount of neuronal loss in this lesion group. Thus, the degree of the learning impairment may depend on the extent of the damage to the MSDB. Previous observations suggest that progressive cholinergic damage correlates with the severity of cognitive impairments in humans (Bartus, 2000). I find that the degree of cholinergic MSDB loss does not predict how well an animal learned. Therefore, differences in lesion size likely do not account for discrepancies in the acetylcholine and learning literature as some have suggested. In conclusion, cholinergic MSDB lesions impair early acquisition of trace eyeblink conditioning probably as a result of irregular hippocampal activity.

EXPERIMENT 2: THE ROLE OF ACETYLCHOLINE IN HIPPOCAMPAL ADULT NEUROGENESIS

Introduction

Adult neurogenesis in the hippocampus

New neurons are continuously produced in the brain throughout the entire lifespan of mammals (Altman and Das, 1965; Gould et al., 1997, 1998; Eriksson et al., 1998; van Praag et al., 1999; Siwak-Tapp et al., 2007). Two areas of the adult brain retain a population of neural stem cells with the capability of giving rise to new cells – one being the dentate gyrus (DG) of the hippocampus. In the DG, neural stem cells asymmetrically divide to produce neural progenitors, which divide several more times, to produce thousands of potential new neurons every day (Cameron and McKay, 2001; Encinas et al., 2011). Many of these new cells die shortly after their birth via apoptosis (Cameron et al., 1993; Cameron and McKay, 2001; Sun et al., 2004). However, they can be influenced to survive in response to learning events (Gould et al., 1999; Anderson et al., 2011). If they do survive, new cells extend dendrites and an axon, and most often, become integrated into hippocampal circuitry as DG granule cells (Ge et al., 2006; Overstreet-Wadiche and Westbrook, 2006; Zhao et al., 2006; Toni et al., 2007, 2008).

Adult neurogenesis is important in hippocampal function

The generation of new neurons in the hippocampus throughout one's lifetime suggests that adult neurogenesis contributes to hippocampal function, considering that

most areas of the brain do not normally make new neurons. Adult neurogenesis offers the potential for thousands and thousands of new synapses to form and influence networks that contribute to learning and memory formation. It has been suggested that their maturation contributes to a constant turnover of DG granule cells. This is supported by data which indicate that the DG does not increase in volume over time in young animals, even though new neurons are continuously being added to the subregion (Kitamura and Sugiyama, 2006). Additionally, cells with condensed DNA, an indicator of programmed cell death, can be reliably found in the outer third portion of the DG, an area associated with older granule cells (Heine et al., 2004). Nottebohm (2002) has suggested that neuronal replacement in the adult brain might function to keep circuitry young and malleable. A decrease in cell turnover with age has been associated with age-induced memory deficits (Heine et al., 2004; Eisch, 2008) indicating that the addition and removal of cells is important for efficient hippocampal function. Indeed, research using trace eyeblink conditioning, the Morris water maze, and contextual fear conditioning corroborates this idea (Rondi-Reig et al., 2001; Derkach et al., 2007; Dupret et al., 2007; Epp et al., 2007; Anderson et al., 2011; Akers et al., 2014).

It is becoming increasingly clear that the formation of new neurons is required for some types of learning including trace conditioning. For instance, when neurogenesis was reduced using injections of an anti-mitotic agent methylazoxymethanol acetate (MAM), animals were unable to reach a learning criteria of responding at least 60% of the time during trace eyeblink conditioning (Shors et al., 2001). Recently, this finding has also been confirmed using another anti-mitotic agent called temozolamide (Nokia et al., 2012a). Trace fear conditioning has also been studied in animals with reduced

neurogenesis as a result of various techniques including MAM treatment, irradiation, and genetic alterations. When MAM and irradiation was used to ablate neurogenesis, animals with fewer new neurons did not freeze as much as controls did in response to a conditioned stimulus (Achanta, Fuss, & Martinez, 2009; Shors, Townsend, Zhao, Kozorovitskiy, & Gould, 2002). Conversely, other animals actually responded with an increased fear response to tone presentations as a result of genetic manipulations to reduce the production of new neurons (Jaholkowski et al., 2009). The reduction in neurogenesis was comparable between these three studies, indicating that these various techniques likely cause additional changes that influence learning. Overall, it is clear that normal levels of fear learning depend on the production of new neurons and that this is likely the result of dysfunctional hippocampal processing considering that trace fear memory formation and recall also requires an intact hippocampus (Bangasser et al., 2006). More importantly, these findings are specific to trace conditioning. A reduction in neurogenesis does not cause deficits in delay fear or delay eyeblink conditioning when conditioning stimuli are not separated in time (Shors et al., 2001a; Achanta et al., 2009). As discussed previously, both of these procedures are not dependent on the hippocampus.

It is generally reported that neurogenesis is not required for learning the hippocampal-dependent version of the Morris water maze (Jaholkowski et al., 2009; Madsen, Kristjansen, Bolwig, & Wörtwein, 2003; Saxe et al., 2006; Shors et al., 2002; Snyder, Hong, McDonald, & Wojtowicz, 2005). However, two studies using relatively sophisticated approaches to genetically knock down neurogenesis report that although animals performed similar the control group early in training, late in training they exhibited deficits (Zhang et al., 2008; Deng et al., 2009). These data suggest that new

neurons may be involved in later stages of spatial learning/memory recall. Other studies confirm that a reduction in neurogenesis does impair an animal's ability to recall spatial positioning of an escape platform in the water maze (Snyder et al., 2005; Jessberger et al., 2009). Therefore, it is possible that neurogenesis is required for spatial memory recall rather than spatial learning. This idea is supported by studies that confirm that new neurons express immediate-early genes, markers of cell activity, after memory recall (Kee et al., 2007; Trouche et al., 2009). In these studies, animals have to remember the location of a hidden platform in the Morris water maze.

Adult neurogenesis and acetylcholine

The cholinergic system, which has been previously discussed because of its relation to learning, is well positioned to regulate adult neurogenesis. Cholinergic septohippocampal neurons project into the DG and synapse on inhibitory GABAergic interneurons and excitatory mossy cells in the hilar region between the two blades of the DG (for review see Cobb & Davies, 2005). Both of these cell types, in turn, synapse onto the granule cells that comprise the DG. Additionally, cholinergic fibers also synapse directly onto granule cells. Therefore, newly-generated granule cells likely begin to receive inhibitory and excitatory cholinergic stimulation at some point in their development. Indeed, Kaneko and colleagues (2006) confirm that newly-generated cells around 1-2 weeks old express M1 and M4 muscarinic receptors and alpha7 and beta2 nicotinic receptors. Mohapel and colleagues (2005) report that cells as early as one day old express M1 and M4 muscarinic cholinergic receptors. Additionally, acetylcholine via M1 muscarinic receptors in slice preparations causes an increase in calcium within the

soma of neural stem cells (Itou et al., 2011). While this is too early for a new cell to receive direct input from cholinergic neuronal axon terminals, research suggests that a great deal of acetylcholine is released into extracellular space (Vizi and Kiss, 1998). Newly-generated cells in the DG receive GABAergic input, which is excitatory early during their maturation, from the network of interneurons in the subgranular zone (Overstreet Wadiche et al., 2005). Therefore, cholinergic septohippocampal input can also influence newly-generated cells in the hippocampus through their early association with these interneurons. Additionally, there are numerous other indirect mechanisms through which acetylcholine can have an effect of any stage of new neuron maturation (Bergami and Berninger, 2012).

Manipulations of cholinergic activity through pharmacological agents and lesions of cholinergic MSDB neurons indicate that acetylcholine regulates adult and neurogenesis. However, the findings from research conducted to assess whether acetylcholine mediates the proliferation of neural progenitors conflicts greatly. Recall that newly-generated cells go through several development stages: proliferation, cell fate adoption, survival and maturation. Two studies report that donepezil, an AchE inhibitor used to treat dementia in humans, does not change the number of proliferating cells in the DG of rodents (Kaneko, Okano, & Sawamoto, 2006; Kotani, Yamauchi, Teramoto, & Ogura, 2006). Similarly, two other groups found that the loss of cholinergic MSDB neurons as a result of the immunotoxin 192 IgG-Saporin also fail to change the number of proliferating cells (Ho et al., 2009; Itou et al., 2011). In opposition, others report that the number of proliferating cells decreased significantly following the loss of cholinergic basal forebrain neurons and also cerebellar Purkinje cells as a result of infusing 192 IgG-

Saporin into the ventricles (Mohapel et al., 2005). In this study, the researchers also observed that physostigmine injections, an AChE inhibitor that cause transient increases in extracellular acetylcholine, result in an increase in the number of proliferating cells. Similarly, Itou and colleagues (2011) observed an increase in the number of proliferating cells following injections of eserine, another type of AChE inhibitor, even though they did not observe a decrease in proliferation following a loss of cholinergic MSDB neurons. It should be noted that that the percent of cholinergic neurons lost varied between these lesion studies as a result of differing locations of the 192 IgG-Saporin infusions. Also, most of these lesion studies did not verify the specificity of the immunotoxin by quantifying the number of GABAergic neurons remaining in the MSDB complex (Mohapel et al., 2005; Ho et al., 2009; Itou et al., 2011). High doses of the immunotoxin have the potential to kill other neuronal types. Therefore, it is difficult to discern why they report different findings.

In contrast, numerous studies agree that acetylcholine mediates the survival of newly-generated cells in the hippocampus. However, the exact role of acetylcholine is still open for debate. Ho and colleagues (2009) found no change in survival following only a partial loss (less than 50%) of cholinergic neurons in the MSDB. This finding points to two possibilities: One, small amounts of acetylcholine are sufficient to promote survival, or two; acetylcholine is only minimally involved in survival. Others report that while a lesion does not change the total number of cells that survive, it does increase cell death in the hippocampus (Cooper-Kuhn et al., 2004). Furthermore, in this study fewer of the surviving cells became neurons suggesting that acetylcholine may be important in promoting a neuronal cell fate. In contrast, Mohapel and colleagues (2005) found that a

cholinergic MSDB lesion results in a decrease in survival, and also that increasing cholinergic activity using the AChE inhibitor physostigmine enhanced survival. It is important to note that their data suggest that changes in survival are the result of changes to the number of cells produced in the first place. Other pharmacological manipulations to increase cholinergic activity suggest otherwise. Another type of AChE inhibitor, Donepezil, increases survival without altering the number of proliferating cells (Kaneko et al., 2006; Kotani et al., 2006). Therefore, it has yet to be established how acetylcholine modulates adult neurogenesis, whether through proliferation, survival, or both. Most of the manipulations used in the articles described above alter cholinergic activity in the entire brain, thus there is little understanding for how cholinergic activity specific to the MSDB and hippocampus affect adult neurogenesis.

In the present, animals with bilateral and unilateral lesions were used to evaluate the role of cholinergic activity, specifically from septohippocampal projections, in proliferation and early survival of newly-generated neurons in the hippocampus. This experiment is the first to verify whether cholinergic MSDB lesions change the number of new cells one week after a BrdU injection, which is incorporated into dividing cells. Animals with unilateral lesions were used to decrease cholinergic activity in one hippocampal formation while preserving the activity in the other hemisphere. It is possible that a change in acetylcholine levels causes other changes that also impact adult neurogenesis. Therefore, animals with unilateral lesions were used to evaluate the direct role of acetylcholine on adult neurogenesis in vivo. Moreover, the present study clarifies whether variation in the amount of cholinergic MSDB neurons influences how extensively adult neurogenesis is decreased following an infusion of 192 IgG-Saporin.

Therefore, 192 IgG-Saporin was used to produce bilateral and unilateral lesions to test three hypotheses:

1. Acetylcholine is involved in the proliferation/early survival of newly-generated cells in the DG during the first week after their birth.
2. Acetylcholine primarily acts directly onto new cells to influence adult neurogenesis.
3. The extent of the loss of cholinergic MSDB neurons correlates with the amount of newly-generated cells in the hippocampus.

Methods

Design

Animals received either infusions of 192 IgG-Saporin or vehicle and were used to determine whether a bilateral and/or unilateral decrease in cholinergic cells in the MSDB result in a decrease in neurogenesis. Animals were injected with a single injection of BrdU and sacrificed seven days later to examine the number of new cells made and that survived for one week (Figure 3).

Surgery

Animals were assigned to receive either infusions of 192 IgG-Saporin or vehicle, 0.1 M PBS. Animals were prepared for surgery as described in experiment 1. In experiment 2, all animals received either two infusions in the left hemisphere or two infusions of the right hemisphere (i.e. only one hemisphere was targeted). Holes were

drilled through the skull at the lesion site. The hemisphere targeted was counterbalanced. Either 192 IgG-Saporin (0.2 μg/μl in sterile 0.1 M PBS) or vehicle (0.1 M PBS) was infused into the brain at the following sites relative to Bregma: AP: +0.6, ML: ±0.5, DV: -7.8; AP: +0.6, ML: ±0.5, DV: -6.6 (DV measure from the dura). A 10 μl Hamilton syringe attached to an automated infusion pump was used to administer the immunotoxin or vehicle. The syringe was dropped into the brain, then left to rest for 1 min before infusing 0.3 μl at DV:-7.8 at a rate of 0.1 μl/min. The drug was allowed to diffuse for an additional 5 min. Afterwards, the syringe was lifted to the DV -6.6 infusion site, left to rest for 1 min, then 0.225 μl was infused at 0.1 μl/min. The syringe was left undisturbed for 5 min to allow the drug to diffuse. The hole in the skull was filled with bone wax and the incision was closed using suture. Upon awakening, the rats were given 1 mL of acetaminophen orally and returned to their home cages. All animals were allowed to recover for a minimum of 14 days.

BrdU, Immunohistochemistry, and Quantification

Twenty nine animals (Sham/vehicle n = 8, 192 IgG-Saporin n = 21) were given a single injection of BrdU (200 mg/kg, i.p.) in physiological saline solution. BrdU can be incorporated into replicating DNA in the S phase of the cell cycle and thereby marks cells that are actively proliferating. BrdU is available in the brain for a period of approximately 2 h after a systemic injection (Cameron and McKay, 2001). Animals were then transferred back to their home cage. One week after a BrdU injection, the number of new cells begins to dramatically decline as result of apoptosis (Cameron et al., 1993; Sun et al., 2004). Therefore, seven days following the single BrdU injection, animals were

deeply anaesthetized with sodium pentobarbital (0.3 ml, Sleepaway) and transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were extracted and kept in paraformaldehyde for 24 hours and then transferred to 0.1 M PBS until sectioning. A vibratome was be used to obtain 40 μm thick coronal sections of the hippocampus and 50 μm thick coronal sections of the MSDB. Slices of the hippocampus and the MSDB were collected and stored in cryoprotectant at -20^{0}C until staining. Every 12th slice of the left and right hippocampus was collected and mounted onto charged glass slides and left to air dry.

Hippocampal slices were used for BrdU immunoperoxidase staining procedure previously described by our lab (Anderson et al., 2011). Slides were pretreated with boiling 0.1 M citric acid (pH 6.0; Sigma-Aldrich, St. Louis, MO, USA). After rinsing with 0.1 M PBS, tissue was incubated in trypsin (Sigma-Aldrich, St. Louis, MO, USA) followed by 2 N HCl (Fisher Scientific, Fair Lawn, NJ, USA) with PBS rinses in between. Slides were kept overnight in primary mouse anti-BrdU (1:200; Becton-Dickinson, Franklin Lakes, NJ, USA) and 0.5% Tween 20 (Vector Laboratories, Burlingame, CA, USA). The next day, tissue was rinsed and incubated for 1 hr in biotinylated anti-mouse antibody (1:200, Vector Labs), then in avidin-biotin-horseradish peroxidase (1:100, Vectastain ABC Kit), and lastly in diaminobenzidine (DAB SigmaFast tablets) with PBS rinses in between. After rinsing one last time, slices were counterstained with 0.1% cresyl violet, dehydrated, cleared and then cover-slipped with Permount.

Quantitative analysis was performed blind to experimental group. Estimates of total number of BrdU-labeled cells were determined using a modified unbiased

stereology protocol (West et al., 1991; Gould et al., 1999). BrdU-labeled cells in the subgranular zone/granule cell layer and hilus on every 12th unilateral section throughout the entire rostro-caudal extent of the DG were counted at 1000x on a Nikon Eclipse 80i light microscope, avoiding cells in the outermost focal plane. The number of cells was multiplied by 12 to obtain an estimate of the total number of BrdU-labeled cells in that hemisphere's hippocampal formation. BrdU counts were combined between hemisphere and across groups. Bilateral and unilateral lesions were verified using the ChAT, PARV, and AchE immunohistochemistry and quantification procedures described in experiment 1. The percent of ChAT-positive cells per mm2 remaining was used to categorize the lesions as either bilateral or unilateral. Seven animals that were given 192 IgG-Saporin did not fall into either category or had excessive tissue damage, and were excluded from analysis.

Analysis

Statistical analysis included Sham (n = 8), Bilateral (n = 7), and Unilateral (n = 7) groups for examining the effect of the lesion on the number of new cells in the DG. A repeated measures ANOVA was used to analyze differences between hemisphere and across groups for the theta data. Repeated measures ANOVAs were used to analyze differences between hemisphere and between groups for ChAT, PARV, and AChE data. Planned comparisons were conducted using t-tests when appropriate. Most differences were considered statistically significant if the p-value was less than 0.05. A Bonferroni adjustment was made to the alpha level to control for an increased risk of making type I errors for planned comparisons, therefore differences were considered statistically

significant if the p-value was less than 0.02 for these tests. Error bars in all graphs represent standard error. A Spearman's correlation was used to examine the relationship between the value for ChAT+/mm2 and neurogenesis.

Results

192 IgG-Saporin produces selective cholinergic lesions in the MSDB

As in experiment 1, the MSDB complex of Sprague-Dawley rats was infused with either vehicle or 192 IgG-Saporin. Once again, animals given 192 IgG-Saporin were split into two groups: animals with a bilateral or unilateral lesion. A two way ANOVA (type of lesion versus hemisphere as the repeated measure) was used to compare percent ChAT between groups (Figure 16a,c,d). A significant interaction was found between the type of lesion and the hemisphere, $F(1, 12) = 91.12$, $p < .00001$. Significant main effects were also found for both type of lesions, $F(1, 12) = 33.45$, $p = .00008$, and hemisphere, $F(1, 12) = 167.10$, $p < .00001$. Post-hoc comparisons revealed that the percent ChAT differed between all groups (see Table 1 for all p values). Percent lesion (100-"percent ChAT") ranged from 86-98% for animals with a bilateral lesion and 17-75% for animals with a unilateral lesion. An independent-samples t-test, comparing the difference in percent ChAT, revealed that there was a significant difference between animals with a bilateral and unilateral lesion, $t(14) = -9.55$, $p < .00001$ (Figure 16b). The difference in percent ChAT between the intact and target hemispheres ranged from 2-20% for animals with a bilateral lesion and 33-60% for animals with a unilateral lesion. As in experiment 1, the

procedures used for splitting animals into a bilateral and unilateral lesion group in experiment 2 led to statistically distinct groups.

To determine how selective the toxin was, adjacent MSDB slices were stained to reveal GABAergic PARV-positive cells. A two way repeated measures ANOVA (type of lesion versus hemisphere as the repeated measure) was used to compare percent PARV (percent of PARV+/mm2 remaining) between groups and hemispheres. In experiment 2, there was an interaction between type of lesion and hemisphere $F(1, 12) = 6.64$, $p = .02$ and a main effect for hemisphere $F(1,12) = 9.37$, $p = .01$ (Figure 17a,c,d). However, there was not a main effect for type of lesion, $F(1,12) = .001$, $p = .97$. A post hoc analysis revealed that target hemisphere in animals with a bilateral lesion has significantly fewer cells compared to the intact hemisphere, $p = .009$. The number of PARV-positive cells in the intact and target hemispheres in animals with a unilateral lesion did not significantly differ. The difference in percent PARV between hemispheres differed significantly between animals with a bilateral and unilateral lesion, $t(12) = 2.34$, $p = .04$ (Figure 17b). Overall, 192 IgG-Saporin was not perfectly selectively, but did predominantly kill ChAT-positive neurons. In particular, the target hemisphere in animals with a bilateral lesion had approximately 27% fewer PARV-positive cells compared to the target hemisphere of animals with a sham lesion.

To examine cholinergic activity in each hemisphere of animals with bilateral and unilateral lesions, hippocampal slices were stained to reveal AchE. A two way repeated measures ANOVA was conducted to examine differences between groups (type of lesion versus hemisphere as the repeated measure). There was a significant interaction between type of lesion and hemisphere, $F(1,12) = 29.71$, $p = .0001$ (Figure 18). In addition, there

were significant main effects between hemispheres, lesion: $F(1,12) = 31.10$, $p = .0001$.There was not a significant main effect for type of lesion, $F(1,12) = 2.42$, $p = .15$. A post hoc analysis revealed that the intact hemisphere in animals with a unilateral lesion has significantly more AchE expression compared to the target hemisphere, $p = .0002$ (a decrease in AchE expression is indicated by an increase in optical density). The AchE expression in the intact and target hemispheres in animals with a bilateral lesion were similar to each other.

Cholinergic lesions decrease the number of new cells in the hippocampus

Animals given either vehicle or 192 IgG-Saporin were injected with BrdU at least 14 days after the surgery. Animals were euthanized 7 days after the BrdU injection, the point at which BrdU counts are usually the highest and when the BrdU-positive cells begin to die (Cameron et al., 1993). The number of BrdU-positive cells was quantified to assess the effect of the lesion on the number of new cells that were generated and survived for seven days in the DG of the hippocampus. Bilateral lesions resulted in a substantial decrease in the number of newly-generated cells, 43% decrease in both hemispheres. Additionally, unilateral lesions also resulted in a large decrease in BrdU-positive cells; a 50% decrease in the intact hemisphere and a 44% decrease in the target hemisphere. A two way repeated measures ANOVA (type of lesion versus hemisphere as the repeated measure) was conducted to analyze the differences between groups and hemispheres. There was not an interaction between the type of lesion and hemisphere, $F(2,19) = 1.17$, $p = .33$ (Figure 19). However, there were significant main effects found for type of lesion, $F(2,19) = 8.14$, p .003, and hemisphere, $F(1,19) = 5.87$, $p = .03$. Three

planned comparisons were conducted using t-tests. The difference in the number BrdU-positive cells in the target hemisphere between animals with a sham and bilateral lesion was nearly significant, $t(13) = 2.46$, $p = .03$. The number of BrdU-positive cells in the target hemisphere significantly differed between animals with a sham and unilateral lesion, $t(13) = 2.58$, $p = .02$. The intact and target hemisphere in animals with a unilateral lesion did not differ, $t(12) = .32$, $p = .75$.

The extent of the lesion did not predict the number of new cells in the hippocampus

To further investigate the effect of the lesion on proliferation/early survival, the relationship between percent ChAT and the number of BrdU+ cells was analyzed. The total BrdU counts were used (intact + target) because no differences were found between hemispheres in bilateral and unilateral groups in a post hoc analysis. Total percent ChAT did not predict the number of BrdU cells, $r = .18$, $p = .53$ (Figure 20). In addition, the difference in percent ChAT between the intact and target hemisphere did not correlate with the number of BrdU-positive cells, $r = .14$, $p = .62$ (Figure 21). Similar to experiment 1, the extent of the damage did not predict how drastically neurogenesis was reduced.

Discussion

Newly-generated cells in the DG proliferate for one week before dying or integrating into hippocampal circuitry (Shors et al., 2012). Numerous environmental factors in the hippocampus have the opportunity to influence the activity of new cells, however, it has recently been established that new cells as early as one day after their

birth express cholinergic receptors (Kaneko et al., 2006; Mohapel et al., 2005). In the present study, cholinergic MSDB lesions reduced adult neurogenesis. Specifically, the loss of cholinergic MSDB neurons resulted in a 40% decrease in the number of new cells in the DG seven days after a BrdU injection. This finding complements another study that reports a 25% decrease 24 hours after a BrdU injection in animals with cholinergic MSDB lesions (Mohapel et al., 2005). Even though adult neurogenesis is an ongoing process in the hippocampus with many new cells at different stages of maturation, a single BrdU injection allows a researcher to visually isolate a population of cells of the same age, relatively. BrdU is permanently incorporated into the DNA of a dividing cell, where it remains for the entire lifetime of the cell. Normally, new cells are actively dividing up to one week after a single BrdU injection (Cameron et al., 1993). Therefore, each division increases the number of BrdU-positive cells until the population of cells begin to die. Given that I report a larger decrease in the number of new neurons seven days after a BrdU injection than Mohapel and colleagues (2005) report after 24 hours, it is possible that acetylcholine regulates activity during the entire one week period in which cells are normally proliferating. Indeed, another group reports a similar decrease (around 50%) in animals with cholinergic MSDB lesions following 10 daily BrdU injections (Van Kampen and Eckman, 2010). However, others find that cholinergic lesions do not alter the number of new cells in the hippocampus 24 hours after a BrdU injection (Ho et al., 2009; Itou et al., 2011). Ho and colleagues (2009) report only a 50% loss in cholinergic neurons in the MSDB and Itou and colleagues (2011) do not present data that verifies the lesions. It is reasonable to be concerned that the variation in the amount of cholinergic neurons remaining influences the degree to which neurogenesis is

altered. However, I report that the number of cholinergic neurons remaining does not predict the number of BrdU-positive cells in the hippocampus. Moreover, bilateral and unilateral lesions resulted in similar decreases in the number of new cells in the DG. Importantly, AchE expression was reduced in the target hemisphere of animals with a unilateral lesion to the same degree that it was reduced in animals with bilateral lesions. This was the case even though there was greater variation in the number of cholinergic neurons remaining in the target hemisphere in animals with a unilateral lesion. Therefore, even a small disruption in cholinergic activity can affect the number of new cells in the hippocampus. Overall, it appears that acetylcholine regulates adult neurogenesis in the hippocampus for at least a week after a new cell's birth.

The effect of the cholinergic MSDB lesions is most likely the result of changes in proliferation and not survival. New cells in the DG normally do not begin to die until day 7 after a BrdU injection (Cameron et al., 1993; Waddell and Shors, 2008). However, it is possible that removal of cholinergic input could cause cells to die earlier if acetylcholine is necessary for survival. Two studies indicate, though, that cholinergic MSDB lesions do not influence the survival of newly-generated cells (Cooper-Kuhn et al., 2004; Ho et al., 2009). Thus, MSDB cholinergic lesions are most likely altering the number of new cell made or how many times they divide, not how many cells are dying. That is not to say that acetylcholine is never involved in processes that promote survival. Increasing cholinergic activity using AchE inhibitors has been reported to increase survival (Kaneko et al., 2006; Kotani et al., 2006). Shors and colleagues have repeatedly reported that learning enhances the survival of new neurons in the hippocampus (Gould et al., 1999; Leuner et al., 2004; Sisti et al., 2007; Anderson et al., 2011; Curlik and Shors, 2011).

When animals are trained with trace eyeblink conditioning seven days after a BrdU injection, a greater number of new neurons survive. It may be the case that acetylcholine mediates the effect of learning on survival.

The effect of the lesion on proliferation is most likely mediated by acetylcholine and not GABA. As previously discussed, cholinergic MSDB lesions may also decrease GABAergic activity because GABAergic septohippocampal projections express muscarinic receptors (Van der Zee and Luiten, 1994). In addition, infusing 192 IgG-Saporin also reduced the number of MSDB GABAergic neurons by approximately 10% in experiment 2. Actively dividing neural progenitors lack processes but produce inward currents in response to GABA that can be blocked with a GABA receptor antagonist (Tonuka et al., 2005). However, GABA is more closely associated with promoting survival as opposed to regulating proliferation (Tozuka et al., 2005; Jagasia et al., 2009). It has been proposed that GABA controls exit from the cell cycle because interneuron activation in the DG causes new cells to become quiescent (Song et al., 2012). Furthermore, others report that inhibiting GABA receptors actually increases proliferation (Giachino et al., 2014). Therefore, acetylcholine regulates proliferation of new cells in the hippocampus while GABA modulates the transition to other development stages.

Acetylcholine may be directly stimulating newly-generated cells given that they express nicotinic and muscarinic receptors early during their development. Van Kampen and colleagues (2010) report that a general cholinergic agonist increases the number of new cells in the DG after 10 days of treatment and that this effect is blocked by a muscarinic antagonist, but not a nicotinic antagonist. This study also demonstrates that

cholinergic MSDB lesions reduced the number of proliferating cells and that this effect is reversed by stimulating muscarinic receptors with a specific agonist. Conversely, others found that an AChE inhibitor increased proliferation but this effect was blocked using nicotinic antagonists but not muscarinic antagonists (Kita et al., 2014). Taking these previous reports into consideration, it is possible that cholinergic MSDB lesions decrease adult neurogenesis as a result of reduced activation of both muscarinic and nicotinic receptors. However, the data presented in present experiment suggest that cholinergic MSDB lesions affect proliferation through another mechanism as well. Animals with unilateral lesions had somewhat normal levels of cholinergic activity in the intact hemisphere (as indicated by an optical density ratio near 1.0), but not the target hemisphere. However, this did not result in corresponding changes in the number of new cells in each hemisphere; the number of newly-generated cells in the hippocampus was similarly decreased in the intact and target hemisphere in animals with a unilateral lesion. Therefore, another factor is mediating the lesion's effect on proliferation. A similar story has emerged for the role of acetylcholine and survival of new neurons. Kita and colleagues (2014) observed that administration of the drug galantamine, an AChE inhibitor that also binds to nicotinic receptors, enhanced survival of newly-generated cells. They also observed that galantamine increased hippocampal insulin growth factor 1 (IGF1) and that this increase was dependent on nicotinic receptor activation (Kita et al., 2013). Moreover, the effect of galantamine on the survival of new neurons was blocked by inhibiting either nicotinic receptors or IGF1. Thus, IGF1 appears to mediate galantamine's ability to enhance survival of new neurons (Kita et al., 2014).

There are many mechanisms for how acetylcholine may indirectly regulate proliferation. As previously suggested, perhaps cholinergic MSDB lesions alter hippocampal activity in both the intact and target hippocampus. Cholinergic afferents project onto hilar mossy cells in the DG which in turn project to the contralateral hippocampus along commissural tracks. Therefore, disruption of hilar mossy cells may also disrupt activity in the contralateral hippocampus. A new study reports that new cells between 1-2 weeks old receive input from hilar mossy cells from the contralateral hippocampus (Chancey et al., 2014). Importantly, this study reports that mossy cells drove GABA release onto newly-generated cells. As previously discussed, GABA causes new cells in the DG to stop dividing (Song et al., 2012). Therefore, the loss of GABAergic input via disruption to hilar mossy cells would probably enhance, not reduce proliferation. Nevertheless, these studies demonstrate a pathway by which the MSDB complex could regulate the maturation of new neurons.

There are a number of additional extrinsic and intrinsic factors that have been reported to regulate the proliferation of new cells in the adult hippocampus (van Praag et al., 1999; as examples Fan et al., 2004; Glenn et al., 2007; Leuner et al., 2007; Hodes et al., 2009). The expression of growth factors in both hemispheres may be altered in response the neuronal damage caused by 192 IgG-Saporin. It has been proposed that neurotrophins are ideal candidates for regulating the various maturation stages of adult neurogenesis (Bergami and Berninger, 2012). Neurotrophins are a family of growth factors that can affect various aspects of a neuron’s maturation (Huang and Reichardt, 2003). Lesions caused by 192 IgG-Saporin have been reported to decrease the mRNA expression of the neurotrophin brain-derived neurotrophic factor (BDNF) in the

hippocampus (Berchtold et al., 2002). Furthermore, activities that enhance BDNF, such as voluntary running also enhance the proliferation of new cells in the DG (van Praag et al., 1999; Berchtold et al., 2002). Lee and colleagues (2002) report that decreased BDNF expression, as a result of genetically deleting one allele of the BDNF gene, decreased proliferation in the hippocampus. However, others report that proliferation is enhanced in mice with conditional BDNF knockouts (Chan et al., 2008). Using this latter genetic manipulation, BDNF expression is preserved during development. Another candidate, fibroblast growth factor 2 (FGF2), has also been established as an important regulator of the proliferation of neural progenitors (Palmer et al., 1995). FGF2 stimulates the proliferation of neural progenitors extracted from the adult hippocampus (Kuhn et al., 1997; Tao et al., 1997). In addition, it has been reported that genetic deletions of FGF2 reduced proliferation in mice (Zhao et al., 2007). Furthermore, others have demonstrated that nicotine increased the expression of FGF2 in the DG of rats (Belluardo et al., 2004). Additional experiments are clearly needed to establish whether unilateral lesions decrease these candidate factors in the ipsilateral and contralateral hippocampus in order to ultimately determine if BDNF or FGF2 mediates acetylcholine’s influence on proliferation.

EXPERIMENT 3: THE ROLE OF CHOLINERGIC MEDIAL SEPTAL NEURONS IN HIPPOCAMPAL THETA RHYTHM

Introduction

Theta rhythm is an electrophysiological oscillation ranging from 3-12 Hz that occurs in many brain structures including the mammalian hippocampus (Berry and Seager, 2001; Hasselmo, 2005). This rhythm occurs in all subregions of the hippocampus, however, the phase of the waveform gradually shifts along the dorsal-ventral axis (Buzsáki, 2002). Two different populations of neurons project from the MSDB complex to the hippocampus and release either acetylcholine or GABA (Figure 2; Freund, 1989; Kiss, Maglóczky, Somogyi, & Freund, 1997). The organized release of these neurotransmitters, in addition to the excitatory input from the entorhinal cortex, is thought to be the mechanism for hippocampal theta rhythm generation (Buzsáki, 2002). Traditionally, theta rhythm has been separated into two categories based on the degree to which certain frequencies within the theta band are “sensitive” to acetylcholine antagonists (Kramis et al., 1975). Type I theta, ~7-12 Hz is readily observed in awake and moving animals and was first thought to be mostly unaffected by large doses of cholinergic antagonists. Type II theta, the lower frequencies within the theta band, can be detected even if an animal is under urethane anesthesia. Early experiments indicated that Type II theta is abolished by cholinergic antagonists. Brain rhythms that can be detected using electroencephalographic (EEG) recordings emerge as the result of synchronized neuronal activity. Therefore, oscillations serve to coordinate brain activity and ultimately, brain function. Theta rhythms, in particular, have been associated with a long list of

behaviors (Buzsáki, 2005). One of the first papers relating this frequency range to learning demonstrated a strong correlation between trials to a learning criterion in delay eyeblink conditioning and the extent to which lower frequencies (2-8 Hz) dominant an EEG signal in the hippocampus prior to learning (Berry et al., 1978b). In other words, EEG signals recorded before training predict how quickly an animal will learn eyeblink conditioning. That same year, it was observed that the activity in groups of neurons in all three major regions of the hippocampus were more organized during certain phases of theta rhythmic waves than during desynchronized rhythms (Berry et al., 1978a). These findings, along with others, led to an extensive body of research aimed at understanding the relationship between theta rhythm and learning.

Theta and learning

The correlation between the amount of type II theta before training (i.e. theta power) and the number of trials it takes to reach a learning criterion suggests that theta oscillation is important for efficient encoding during learning. Importantly, others have replicated this correlation in rabbits and rodents (Nokia et al., 2008, 2012b). In particular, Nokia and colleagues (2009) have also shown this predictive relationship is maintained during early learning phases of trace eyeblink conditioning as well. They propose that theta rhythm represents a motivational or attention brain state that prepares the hippocampus for efficient information processing (Nokia et al., 2008). In fact, theta power shortly preceding the presentation of stimuli during learning events also predicts how fast an animal will learn in both delay and trace eyeblink conditioning (Nokia et al., 2008, 2009). Moreover, theta rhythm in the medial temporal lobe of humans directly

before encoding words presented on a screen predicts how well the person recalls those words later (Guderian et al., 2009; Fell et al., 2011). It has also been observed that theta power increases during trace eyeblink conditioning, but not delay conditioning, suggesting that theta rhythm may be more crucial when the task is more difficult to learn (Beylin et al., 2001; Nokia et al., 2008, 2009). More specifically, relative theta power increases early during training in fast learners, but not in slow learners, and vice versa during later training sessions. Additionally, the increase during eyeblink conditioning seems to be exclusive to novel learning experiences. When animals were trained on a different but related hippocampal-dependent eyeblink task, theta power decreased across training (Beylin et al., 2001; Nokia et al., 2012b). Therefore, theta rhythms seem particularly important during acquisition, but not expression of learned responses, in novel situations. This complements the finding that population responsiveness of hippocampal CA1 neurons diminish once asymptotic levels of responding are reached during trace eyeblink conditioning (McEchron and Disterhoft, 1997).

Various experimental manipulations have been used to ascertain whether there is a causative relationship between theta and learning. For instance, water deprivation increases how much theta dominants an EEG signal (Berry and Swain, 1989; Maren et al., 1994). This manipulation facilitates both eyeblink conditioning in rabbits and contextual fear conditioning in rats. This experimental approach, however, carries with it many confounding variables. To solves these issues, Berry and colleagues have conducted a series of studies which explicitly paired learning trials with naturally occurring trains of theta or desynchronized activity (Seager et al., 2002; Griffin et al., 2004; Darling et al., 2011). Animals that were trained with delay eyeblink conditioning in

the presence of theta reach a learning criterion in half as many trials compared to animals presented with trials in the absence of theta (Seager et al., 2002). Additionally, animals given trials paired with theta also acquired CRs faster than “yoked” control animals given trials at the same time but regardless of whether theta was present or not. Importantly, theta power before learning did not predict learning as a result of the manipulation. Therefore, learning during trains of theta facilitated learning for all animals regardless of whether animals had high or low levels of theta power before learning. The same results were found using trace eyeblink conditioning (Griffin et al., 2004; Darling et al., 2011). Building upon their previous work, Berry and colleagues demonstrated that not only did pairing trace trials with theta facilitate learning, but explicitly pairing trace trials with desynchronized activity prevented normal acquisition. Moreover, presenting trials in the absence of theta prevented the development of hippocampal population responses during the trace interval. However, another group was unable to independently verify that pairing trace eyeblink trials with theta trains facilitates learning (Nokia and Wikgren, 2014). They found that animals presented trials during desynchronized activity actually learned better than other groups. In addition, they found that pairing CS alone trials with the opposite hippocampal state used during conditioning facilitated extinction. The authors suggest that theta rhythm might serve as important contextual brain state, much like an environmental context, during learning.

Lesions or nonspecific inactivation of the medial septum to reduce theta activity have also been used to assess whether theta rhythm is necessary for learning (Berry and Thompson, 1979; Asaka et al., 2002). These manipulations impair acquisition of conditioned responses (CRs) in delay and trace eyeblink conditioning. In fact, animals in

these studies never reached the same level of responding compared to control animals, even after hundreds of trials. Additionally, it has been reported that injections of the muscarinic antagonist scopolamine into the medial septum both reduce theta frequency and retard learning during a similar associative task called trace appetitive conditioning (Asaka et al., 2000). Using this technique, which affects both cholinergic and GABAergic septohippocampal projections, Asaka and colleagues demonstrated that animals required more trials of training to reach a learning criterion. It has also been reported that non-specific lesions of the medial septum, which produced a decrease in prevalence of theta rhythm in some animals, impaired recall but not the acquisition of a spatial learning task (Winson, 1978). Notably, only animals with a complete loss of theta rhythm following the lesion were unable to perform normally. It has also been proposed that a decrease in theta rhythm contributes to learning deficits during trace eyeblink conditioning as a result of chemotherapy (Nokia et al., 2012a). Overall, the sum of these studies suggests that the presence of theta during training positively influences learning, and without theta, an animal may not learn well. Furthermore, it appears that theta and learning may have a reciprocal relationship especially during tasks that require the hippocampus (Asaka et al., 2002; Nokia et al., 2009). However, a consensus is yet to be reach for whether theta rhythm is necessary for learning.

Theta and cholinergic activity in the hippocampus

It is generally accepted that hippocampal theta rhythm is not autonomously generated, and furthermore, that the MSDB complex is an important component in generating rhythms in the hippocampus (Petsche et al., 1962; Buzsáki, 2002). As stated

previously, numerous papers have demonstrated that damage to or non-specific inactivation of the medial septum attenuates or abolishes hippocampal theta rhythm (Winson, 1978; Berry and Thompson, 1979; Lawson and Bland, 1993; Asaka et al., 2000). In a slice preparation that preserved septohippocampal projections, theta rhythmic stimulation of the medial septum subsequently caused theta oscillations in the hippocampus (Tóth et al., 1997). In addition, recording from live animals revealed that GABAergic "pacemaker" neurons from the medial septum tended to lead and be phase locked with hippocampal theta rhythm (Hangya et al., 2009). Hangya and colleagues (2009) also demonstrated that activity of interneurons in the hippocampus also precedes theta rhythm in principle hippocampal cells, however not to the same extent as the medial septal pacemaker cells. Cholinergic and GABAergic neurons arising from the MSDB both synapse onto GABAergic interneurons in the hippocampus which in turn synapse onto principle neurons of the CA1, CA3 and dentate gyral principle neurons (Figure 2). Additionally, cholinergic MSDB neurons also innervate mossy cells which excite granule cells in the DG. However, the results from Hangya and colleagues suggest that disinhibition of interneurons, as a result of GABAergic MSDB neuronal activity, has a more crucial role in generating theta rhythm. Indeed, others provide evidence that GABAergic interneurons in the hippocampus rhythmically pace CA1 pyramidal cells (Cobb et al., 1995; Ylinen et al., 1995). However, it should be noted that others have found that presumed cholinergic MSDB neurons are also phase locked to hippocampal oscillations (Brazhnik and Fox, 1999). Still, Hangya and colleagues propose that there is a gradual recruitment of cells, first interneurons, then hippocampal principle cells, into oscillating networks and that this is driven by GABAergic medial septal neurons. The

authors further hypothesize that background excitatory cholinergic activity is crucial for this process. Others have proposed similar models that emphasized the importance on hippocampal interneurons in driving hippocampal theta rhythm (Stewart and Fox, 1990).

Are cholinergic MSDB neurons necessary for hippocampal theta rhythm? Cholinergic neurons from the MSDB provide both excitatory and inhibitory input to principle hippocampal cells. However, it is possible that GABAergic septohippocampal projections and excitatory glutaminergic input from the entorhinal cortex are sufficient to generate theta oscillations in the hippocampus. Type I and type II theta were first distinguished because it was observed that systemic injections of atropine, a muscarinic antagonist, severely disrupts lower frequencies but not higher frequencies in the theta band (Kramis et al., 1975). However, another group reports that systemic injections of scopolamine, another type of muscarinic antagonist, increase theta power (Masuoka et al., 2006). The reasons for these conflicted findings are difficult to assess because systemic injections of these antagonists affect all areas of the brain. Possible confounds have been overcome by bathing particular brain regions with pharmacological agents. Infusions of the general cholinergic agonist carbachol directly into the medial septum induces type II theta in immobile awake animals (Monmaur and Breton, 1991; Lawson and Bland, 1993). This effect is blocked by infusing atropine after carbachol. Both cholinergic and GABAergic septohippocampal neurons express muscarinic receptors, thus, these results confirm that cholinergic MSDB activity is important for inducing theta rhythm, but not specifically whether cholinergic activity in the hippocampus is involved. Other studies demonstrate that application of carbachol in a dish preparation of a hippocampal slice cause slow rhythmic waves in the CA3 region of the hippocampus

(MacVicar and Tse, 1989; Williams and Kauer, 1997; Cobb et al., 1999). Carbachol also causes GABAergic interneurons to oscillate, an effect that is blocked by a muscarinic antagonist (Chapman and Lacaille, 1999; Cobb et al., 1999). Cobb and colleagues (1999) are one group that demonstrated that muscarinic receptors were critically involved in this effect. However, they also report that nicotinic receptors may have a modulatory role in shifting oscillations. In this study, nicotinic antagonist tubocurarine did not block neuronal activity, but it shifted CA3 neurons from firing in a theta rhythm to firing at a much slower rate. In vivo, carbachol and the AChE inhibitor eserine elicits theta rhythms in number regions of the hippocampus in urethane anesthetized animals (Rowntree and Bland, 1986). Similar to in vitro studies, this study demonstrates that the effect was attenuated with atropine.

While it has been known since the 1960s that there is a relationship between acetylcholine and theta rhythm (Petsche et al., 1962; Stumpf et al., 1962), more sophisticated techniques have been developed to examine the specific role of septohippocampal innervation in theta rhythm. 192 IgG-Saporin produces selective loss of cholinergic neurons and thus, models the loss of cholinergic septohippocampal projections in Alzheimer’s disease (Wiley et al., 1991). Using infusions of this immunotoxin in the MSDB and also kainic acid, which selectively kills GABAergic neurons through excitotoxic stimulation of glutamate receptors, Yoder and Pang (2005) evaluated the specific roles of cholinergic and GABAergic MSDB neurons in type II theta. Anesthetizing animals with urethane is a technique used to isolate lower frequencies in the theta band. In urethane anesthetized animals, lesions of both MSDB neuronal types severely attenuated the amplitude of theta rhythm but did not affect other

frequency bands. However, GABAergic lesions, more so than cholinergic lesions, disrupted higher frequency (type I) theta observed in awake, moving animals. In addition, they demonstrated that a combination of GABAergic and cholinergic lesions severely disrupted type I theta. These findings suggest that both cholinergic and GABAergic neurons modulate type I and type II theta. However, Yoder and Pang also found that lesioning excitatory input from the entorhinal cortex in combination with the loss of GABAergic neurons also severely disrupts theta. Therefore, it is possible that theta rhythm requires excitatory input from the entorhinal cortex and inhibitory input from the medial septum, and that acetylcholine merely serves as a modulator. Indeed, entorhinal cortical lesions disrupt hippocampal theta rhythm in urethane anesthetized animals (Ylinen et al., 1995). However, Tai and colleagues (2012) found that infusions of 192 IgG-Saporin also severely reduces, but does not eliminate, the occurrence of lower frequency theta. In these experiments, activating the vestibular system by passively spinning awake animals in a container induced lower frequency theta. Contrary to the findings above, Lee and colleagues (1994) find that cholinergic lesions severely reduced theta power in both the lower and higher frequency range. Lower theta frequency was induced via systemic injections of the AChE inhibitor physiostigmine in awake animals. They also found that carbachol injections into the medial septum induced hippocampal theta and furthermore, that cholinergic lesions attenuate but did not prevent this effect. The authors conclude that the remaining GABAergic connections are able to maintain an observable level of organized theta oscillation, at least in some situations. Furthermore, they propose that a decrease in hippocampal responsiveness and an increase in GABAergic inhibition in response to anesthetics contribute to severe disruptions to theta

as the result of cholinergic manipulations. Therefore, studying the relationship between acetylcholine and the type of theta rhythm that relates to learning in naturally behaving animals is ideal. Notably, theta recordings that first led to the suggestion that theta is important in learning were conducted in awake immobile animals that were not subjected to drugs or other manipulations to induce theta rhythms (Berry et al., 1978b).

In the present study, the relationship between acetylcholine and theta rhythm was studied by selectively killing cholinergic MSDB neurons using 192 IgG-Saporin. This experiment is the first, to my knowledge, that evaluates whether cholinergic activity in the hippocampus is necessary for theta rhythm in awake but immobile and unstimulated animals. Hippocampal activity was recorded in animals with bilateral and unilateral cholinergic MSDB lesions. Previous research suggests that acetylcholine merely modulates hippocampal theta rhythm. Furthermore, cholinergic septohippocampal neurons also synapse onto mossy cells in DG which in turn synapse onto granule cells in the contralateral hemisphere through commissural projections. Therefore, it is possible that intact cholinergic septohippocampal projections can compensate for damaged septohippocampal projections in the other hemisphere. In experiments 1 and 2, it was established that unilateral lesions result in a significant decrease in acetylcholine in the hippocampus of one hemisphere but not the other. Hence, animals with unilateral lesions were used to determine whether direct cholinergic septohippocampal input is necessary. Moreover, this experiment explores the relationship between the how many cholinergic MSDB neurons are lost and theta power. Overall, animals with bilateral and unilateral lesions were used to test the following hypotheses:

1. Cholinergic septohippocampal projections are necessary for hippocampal theta rhythm.
2. Direct cholinergic input into the hippocampus is necessary for hippocampal theta rhythm.
3. The extent of the loss of cholinergic MSDB neurons correlates with theta power.

Methods

Design

Animals received either infusions of 192 IgG-Saporin or vehicle and were used to determine whether a bilateral and/or unilateral decrease in cholinergic cells in the MSDB result in a decrease in theta power in the hippocampus. LFPs in the hippocampus were recorded seven and 14 days following the surgery (Figure 3).

Surgery

Animals were assigned to receive either infusions of 192 IgG-Saporin or vehicle, 0.1 M PBS. Animals were prepared for surgery as described in experiment 1. In experiment 3, all animals received either two infusions in the left hemisphere or two infusions of the right hemisphere (i.e. only one hemisphere was targeted). Holes were drilled through the skull at the lesion site. The hemisphere targeted was counterbalanced. Either 192 IgG-Saporin (0.2 μg/μl in sterile 0.1 M PBS) or vehicle (0.1 M PBS) was infused into the brain at the following sites relative to Bregma: AP: +0.6, ML: ±0.5, DV: -7.8; AP: +0.6, ML: ±0.5, DV: -6.6 (DV measure from the dura). A 10 μl Hamilton

syringe attached to an automated infusion pump was used to administer the immunotoxin or vehicle. The syringe was dropped into the brain, then left to rest for 1 min before infusing 0.3 μl at DV:-7.8 at a rate of 0.1 μl/min. The drug was allowed to diffuse for an additional 5 min. Afterwards, the syringe was lifted to the DV -6.6 infusion site, left to rest for 1 min, then 0.225 μl was infused at 0.1 μl/min. The syringe was left undisturbed for 5 min to allow the drug to diffuse. The hole in the skull was filled with bone wax. Bipolar permanent electrodes were also implanted into the dorsal hippocampus following the infusions. Four holes were drilled into the following coordinates AP: +4.0, ML: ±2.0; AP: -7.0, ML: ±3.0, and skull screws with standard radio wire attached were inserted making sure that the screws touch the top of the brain. The skull screws were attached in pairs and counterbalanced to serve as either the reference or ground during neural recordings. The pairs were each attached to a gold pin and inserted into a headstage. Four small holes were drilled into the skull and electrodes to record LFPs were inserted at the following dentate gyral coordinates AP: -0.4, ML: ±3.5, DV: -3.6; AP: -0.4, ML: ±2.5, DV: -3.4 (DV measured from the skull near Bregma). The target for electrode placed was the DG because the proliferation of new neurons which occurs in that area was examined in experiment 2. Electrodes consisted of two strands of twisted Formvar-insulated nichrome wire (0.002 inches bare, A-M Systems, Carlsboro, WA, USA) reinforced with two coats of super glue and a small segment of polyethylene tubing for handling purposes. The tip of the electrode was trimmed and sterilized prior to implantation. The ends of the electrodes were untwisted and gold pins soldered to each wire (2 per electrode) prior to surgery. These gold pins were also inserted into the headstage. The 10-pin headstage was then mounted to the skull with dental cement. Animals were given

supplemental booster injections of sodium pentobarbital due to the length of the surgery. Upon awakening, the rats were given 1 mL of acetaminophen orally and returned to their home cages. Animals were given a one week recovery.

Note: Some of these surgeries (Sham n = 4, 192 IgG-Saporin n = 8) were conducted in Finland at the University of Jyvaskyla in the laboratory of Dr. Jan Wikren. Thus, some drugs used differed as a result of differences in accessibility and protocols. Animals were injected with carprofen (5 mg/kg, sc, Rimadyl vet; Pfizer Inc. Animal Health, Espoo, Finland) prior to surgery. Animals were anesthetized with sodium pentobarbital (65 mg/kg, ip, Mebunat Vet; Orion-Yhtymä Oyj, Espoo, Finland). Instead of Marcaine, after the incision a gel lidocaine (0.1ml, Xylocain 2% gel; AstraZeneca, Espoo, Finland) was applied to the incision and left for 3 min before proceeding. Following the surgery and upon awakening, animals were given buprenophrine (0.03 mg/kg, sc, Temgesic; Schering-Plough Europe, Brussels, Belgium). All other materials were the same.

Recording and Theta Rhythm Analysis

Hippocampal LFPs were recorded on Day 7 and 14 following the surgery. Thirty four animals (Sham n = 8, Bilateral/Unilateral n = 26) were put into the recording chamber and their headstage attached to a connector that allowed free movement. Animals were allowed approximately 5 min to acclimate to the chamber in order to minimize the amount of locomotion during recording. Hippocampal LFPs were recorded continuously for 30 min on Day 7 and 14. Afterwards, animals were returned to their home cage. Three animals (Sham n = 2, 192 IgG-Saporin n =1) had signals that were

unusable, likely due to damage to the connections between the electrodes and headstage, and were dropped from the analysis.

Continuously recorded LFPs were sampled at a rate of 5000 Hz (Digidata1440 and AxoScope; Molecular Devices, Sunnyvale, CA, USA) and filtered between 1 and 500 Hz (PGA16; Multi-Channel Systems, Reutlingen, Germany). As previously described (Nokia et al., 2012a), data was broken into 3 sec sweeps using MATLAB (MathWorks, Natick, MA, USA). Sweeps with artefacts most commonly caused by rapid large-scale movements were automatically rejected from the analysis by simple amplitude thresholding in MATLAB. The relative power of hippocampal theta activity [theta / (delta+theta)] was calculated using the Fast Fourier Transform to analyze the frequency composition of the signal. From the result, the relative power of hippocampal theta activity was determined as the ratio between the power of the signal at 4.5–10.3 Hz and the power of the signal at 1.5–10.3 Hz (theta ratio).

Histology, Immunohistochemistry, and Quantification

Rats were deeply anaesthetized with sodium pentobarbital (0.3 ml, Sleepaway) and transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were extracted and kept in paraformaldehyde for 24 hours and then transferred to 0.1 M PBS until sectioning. A vibratome was be used to obtain 50 μm thick coronal sections of the hippocampus and 50 μm thick coronal sections of the MSDB. The hippocampal slices were mounted onto charged glass slides and left to air dry to verify electrode placement. Hippocampal slices were counterstained with 0.1% cresyl violet (Sigma-Aldrich, St. Louis, MO, USA), dehydrated, cleared and the coverslipped using Permount. Each

electrode placement for every animal was determined at 100x using a Nikon Eclipse 80i light microscope and Nikon NIS standard imaging software. The best placed electrode from each hemisphere (Figure 22) was used for theta analysis described above. Three animals (Sham/vehicle n = 1, 192 IgG-Saporin n = 2) had electrode placements outside of the acceptable region of interest and were excluded from the analysis.

Bilateral and unilateral lesions were verified using the ChAT and PARV immunohistochemistry and quantification procedures described in experiment 1. The percent of ChAT-positive cells per mm2 remaining was used to categorize the lesions as either bilateral or unilateral. Six animals that were given 192 IgG-Saporin did not fall into either category or had excessive tissue damage, and were excluded from analysis. After using MSDB tissue to stain for ChAT, in three cases (Sham n = 1, Bilateral n = 1, Unilateral n = 1) there was not tissue left over to stain for PARV due to staining complications. AChE staining was not examined because the hippocampal tissue was used to determine electrode placement.

Analysis

Statistical analysis included Sham (n = 5), Bilateral (n = 8), and Unilateral (n = 9) groups for examining the effect of the lesion on theta rhythm. Repeated measures ANOVAs were used to analyze changes across time and differences between groups. Additionally, repeated measures ANOVAs were used to analyze differences between hemisphere and between groups for the ChAT and PARV anaylsis. Planned comparisons were conducted using t-tests when appropriate. All differences were considered statistically significant if the p-value was less than 0.05. A Bonferroni adjustment was

made to the alpha level to control for an increased risk of making type I errors for planned comparisons, therefore differences were considered statistically significant if the p-value was less than 0.02 for these tests. Error bars in all graphs represent standard error. A Spearman's correlation was used to examine the relationship between the value for ChAT+/mm2 and theta power.

Results

192 IgG-Saporin produces selective cholinergic lesions in the MSDB

As in experiment 1, the MSDB complex of Sprague-Dawley rats was infused with either vehicle or 192 IgG-Saporin. Again, animals given 192 IgG-Saporin were split into two groups: animals with a bilateral or unilateral lesion. A two way repeated measures ANOVA (type of lesion versus hemisphere as the repeated measure) was used to compare percent ChAT between groups (Figure 23a,c,d). A significant interaction was found between the type of lesion and the hemisphere, $F(1, 15) = 79.77$, $p < .00001$. Significant main effects were also found for both type of lesions, $F(1, 15) = 40.31$, $p = .00001$, and hemisphere, $F(1, 15) = 137.19$, $p < .00001$. Post-hoc comparisons revealed that the percent ChAT in the intact hemisphere in animals with a unilateral lesion was significantly differently from all other groups, "Unilateral Target" $p = .0002$; "Bilateral Intact" $p = .0001$; "Bilateral Target" $p = .0002$. Percent lesion (100-"percent ChAT") ranged from 81-99% for animals with a bilateral lesion and 35-74% for animals with a unilateral lesion. An independent-samples t-test, comparing the difference in percent ChAT, revealed that there was a significant difference between animals with a bilateral

and unilateral lesion, $t(15) = -8.98$, $p < .00001$ (Figure 23b). The difference in percent ChAT between the intact and target hemispheres ranged from 0-23% for animals with a bilateral lesion and 53-103% for animals with a unilateral lesion. Once again, the procedures used for splitting animals into a bilateral and unilateral lesion group led to statistically distinct groups.

To determine how selective the toxin was, adjacent MSDB slices were stained to reveal GABAergic PARV-positive cells. A two way repeated measures ANOVA (type of lesion versus hemisphere as the repeated measure) was used to compare percent PARV (percent of PARV+/mm2 remaining) between groups and hemispheres. In experiment 3, there was not an interaction between type of lesion and hemisphere $F(1, 13) = 1.36$, $p = .26$ (Figure 24a,c,d). In addition, the main effects were not significant, type of lesion: $F(1,13) = 1.88$, $p = .19$; hemisphere: $F(1,13) = 1.71$, $p = .21$. The difference in percent PARV between hemispheres also did not significantly differ between animals with a bilateral and unilateral lesion, $t(13) = .29$, $p = .78$ (Figure 24b). Overall, 192 IgG-Saporin was not perfectly selectively, but did predominantly kill ChAT-positive neurons. Animals from experiment 3 had the most selective lesions of all the experiments.

Cholinergic MSDB lesions result in a transient decrease in theta power

Hippocampal LFPs in both the right and left hemispheres were recorded in all animals on Day 7 and Day 14 after the surgery to determine the effect of the lesion on theta activity (4.5–10.3 Hz; see Figure 25 for an example of a waveform in this frequency range). In animals with a sham lesion, theta dominates the EEG recordings and a loss of cholinergic neurons in the MSDB clearly attenuates the power (Figure 26). Power for

each frequency was used to determine the relative power of hippocampal theta activity to other frequencies (theta/theta+delta). The relative theta power in each hemisphere of each animal is expressed as a single number and averaged to determine the mean theta power for each hemisphere in all groups (Figure 27). Two independent two way repeated measures ANOVAs were used to compare theta power between lesion groups and hemisphere. There was a significant interaction between type of lesion and hemisphere on Day 7, $F(2,19) = 3.61$, $p = .05$ (Figure 27, left). While there was not a significant difference in theta power between the intact and target hemispheres, $F(1,19) = .87$, $p = .36$, there was a significant difference between lesion groups, $F(2,19) = 9.73$, p .001. Three planned comparisons were conducted using t-tests. The means for theta power in the target hemisphere between animals with a sham lesion and bilateral lesion significantly differed, $t(11) = 5.97$, $p \leq .001$. However, there was not a difference between the target hemisphere of animals with sham and unilateral lesions, $t(12) = 2.26$, $p =.04$ (adjusted alpha level = 0.2). In addition, there was not a difference between the intact and target hemispheres in animals with a unilateral lesion, $t(16) = 1.08$, $p = .30$. On Day 14, there was not an interaction between type of lesion and hemisphere, $F(2,19) = .22$, $p = .80$ (Figure 27, right). In addition, there were not significant differences between hemisphere, $F(1,19) = .04$, $p = .85$, and lesion groups, $F(2,19) = 1.45$, $p = .26$. Given that were no significant differences detected with the ANOVA, planned comparisons were not analyzed for Day 14 because the repeated measures ANOVA revealed no differences. Given that a change in delta may contribute to an observed difference in relative theta power, the separate delta and theta power values were evaluated (Figure 28). A two way repeat measure ANOVA revealed that there was not a significant interaction between

type of lesion and hemisphere on Day 7 for delta power, $F(2,19) = 1.24$, $p = .31$. There was not a main effect for lesion type, $F(2, 19) = .79$, $p = .47$, or hemisphere, $F(1,19) = .05$, $p = .83$. This suggests that a change in delta does not significantly contribute to the observed difference in relative theta power between groups. Overall, this data suggests that a bilateral lesion decreases the relative power of theta activity, while a unilateral lesion does not. However, this effect is gone by Day 14.

The extent of the lesion predicts theta power in animals with bilateral and unilateral lesions

To explore whether the loss of MSDB neurons predicts theta power on day 7, the relationship between percent ChAT and theta was analyzed. Overall, there was a significant relationship between the percent ChAT remaining and the average theta power across hemispheres, $r = .51$, $p = .04$ (Figure 29a). However, the percent ChAT remaining in each hemisphere did not have a significant relationship with the theta power in the target hemisphere, $r = .19$, $p = .47$, and the intact hemisphere, $r = .39$, $p = .13$ (Figure 29b, c). Overall, this suggests that the amount of cholinergic MSDB neurons lost corresponds to how drastically theta is impaired. To further evaluate whether direct cholinergic input into the hippocampus has an important role in hippocampal theta rhythm, the relationship between theta power and the difference in percent ChAT between hemispheres was also analyzed. The difference in percent ChAT between hemispheres also correlated with relative theta power, $r = .58$, $p = .02$ (Figure 30). Therefore, relative theta power tended to be higher when damage was more restricted to one hemisphere. The reduction in theta

power is appears to be dependent on the extent of the loss in the number of ChAT-positive cells in the MSDB complex. Moreover, having one side of the MSDB largely intact compensates for damage in the other side. More importantly, any reduction in theta power appears to recover over time.

Discussion

Spontaneous endogenous theta rhythm power in awake, immobile and unstimulated animals predicts how well animals learn (Berry et al., 1978b; Nokia et al., 2009). In addition, disruption of cholinergic activity disrupts both theta rhythm and trace eyeblink conditioning (Kaneko & Thompson, 1997; Kramis et al., 1975; Lee et al., 1994). However, understanding the specific role of cholinergic septohippocampal activity in hippocampal theta rhythm is prevented by two issues in the previous literature. First, a disruption in cholinergic activity is not restricted to the MSDB in many studies. Second, previous studies only report a disruption in theta rhythm during locomotion or in response to stimuli or drugs. In the present study, spontaneous hippocampal theta rhythms were recorded in awake animals with cholinergic MSDB lesions. Bilateral lesions resulted in approximately a 30% decrease in theta power seven days after infusing 192 IgG-Saporin into the MSDB. Lee and colleagues (1994) report that theta power in animals under the influence of physostigmine, an AChE inhibitor, progressively decreased for up to 14 days after infusing 192 IgG-Saporin. On Day 14, a dose of 0.11 µg, similar to the dose used in the present study, resulted in approximately a 30% decrease in theta power. Therefore, small doses of 192 IgG-Saporin impair theta rhythm, but the current study finds that it is possible for theta power to recover.

I also find that theta rhythm is more severely impaired when the lesion is more complete. A small dose of 192 IgG-Saporin resulted in extensive damage in some cases. However, there was a considerable amount of variation in lesion size depending on the location of the infusion track. Correlational analysis confirmed that the extent of the lesion predicted theta power. Therefore, variability in lesion size accounts for individual differences between animals. Lee and colleagues (1994) reported that higher doses of 192 IgG-Saporin resulted in much greater reductions in theta power compared to smaller doses. They do not report whether higher doses increased the number of cholinergic MSDB neurons lost, but lesion size may account for differences between our findings. Small doses were used here to prevent non-selective death of neurons in the MSBD. However, the dose used resulted in about a 20% loss in GABAergic neurons. Higher doses may result in even greater GABAergic neuronal loss which may account for why higher doses used by Lee and colleagues attenuated theta to a large degree, although, they report based on visual inspection that PARV staining was similar between groups. Even so, the damage caused by 192 IgG-Saporin in the current study did not completely eliminate theta even when most cholinergic neurons were killed. Therefore, GABAergic projections from the MSDB, in conjunction with other excitatory input, are sufficient to maintain theta rhythm.

A disruption in GABAergic MSDB neuronal activity may have contributed to the effects of the lesion on theta rhythm on Day 7. It is possible that the small loss of GABAergic neurons exasperated the effects of the cholinergic MSDB lesion on theta power. In addition, muscarinic receptors are expressed on GABAergic MSDB neurons (Van der Zee and Luiten, 1994) indicating that cholinergic MSDB neurons also regulate

GABAergic projections to the hippocampus. Others report that injecting a muscarinic antagonist directly into the MSDB, which disrupts GABAergic activity in the hippocampus, only partially blocked carbachol-induced theta in immobile but alert animals (Lawson and Bland, 1993). Thus, GABAergic MSDB neurons are not solely responsible for regulating hippocampal theta rhythms. These results are consistent with others demonstrating that both cholinergic and GABAergic septohippocampal projections modulate theta rhythm (Yoder and Pang, 2005). Yoder and Pang report that while the loss of cholinergic or GABAergic MSDB neurons equally decreased the amplitude of the theta wave during locomotion, lesions that kill both cholinergic and GABAergic projections resulted in a more drastic decrease (Yoder and Pang, 2005). I predict that the loss of both cholinergic and GABAergic neurons would result in a much greater decrease in the power of spontaneous theta rhythms as well. Thus, disruption of cholinergic activity in MSDB and the hippocampus are both contributing factors in the effects observed in the present study.

Cholinergic input is well positioned to directly influence oscillations in the hippocampus. Cholinergic septohippocampal projections synapse onto principle cells, interneurons and hilar mossy cells (Cobb and Davies, 2005). In particular, GABAergic interneurons in the hippocampus are thought to play a large role in rhythmically pacing CA1 pyramidal cells (Cobb et al., 1995; Ylinen et al., 1995). In a dish preparation of a hippocampal slice, carbachol, a cholinergic agonist, induces interneurons to oscillate via activation of muscarinic receptors (Chapman and Lacaille, 1999). In turn, stimulation of interneuron increases rhythmicity of CA1 pyramidal cells. Interestingly, theta was also reduced in both the intact and target hemisphere of animals with a unilateral lesion.

Conversely, unilateral lesions produced a significant decrease in hippocampal cholinergic activity in the target hemisphere but not the intact hemisphere. Therefore, this difference in cholinergic activity did not translate into a difference in theta power between hemispheres. Furthermore, correlational analysis revealed that theta power was higher in animals with lesions that were more lateralized. These results suggest that in addition to the direct cholinergic innervation to principle cells and interneurons, another component of the circuitry is involved. For instance, cholinergic MSDB neurons also project onto mossy cells which provide input to the contralateral hemisphere (Deller et al., 1999). In the context of the literature, mossy cells have been an underappreciated component in hippocampal circuitry. Even so, Henze and Buzsáki (2007) have proposed that mossy cells provide excitation to principle cells in the opposition to the rhythmic input from interneurons. In addition, mossy cells provide both excitatory and inhibitory input to DG granule cells (Jinde et al., 2012). It has also been reported that the oscillations of mossy cells are phased locked to principle cells in the contralateral hippocampus (Soltesz et al., 1993). Therefore, mossy cells might serve an important role in generating rhythms in both the ipsilateral and contralateral hippocampus.

GENERAL DISCUSSION

These three experiments demonstrate that cholinergic MSDB lesions retard learning and produce corresponding changes in adult neurogenesis and hippocampal theta rhythm. Many processes are involved in hippocampal-dependent learning. Specifically, reductions in neurogenesis and possibly theta power are contributing to the learning deficits observed in experiment 1. Here, I will discuss how learning, adult neurogenesis and theta rhythm influence each other and approaches and limitations with regards to studying these interrelated factors.

Fewer new neurons disrupts learning

It is well established that intact neurogenesis is necessary for some forms of learning. Shors and colleages have twice demonstrated that reducing neurogenesis with anti-mitotic agents impairs trace eyeblink conditioning (Shors et al., 2001b; Nokia et al., 2012a). A reduction in the number of new cells in the DG as a result of either anti-mitotic agents or irradiation also impairs trace fear learning (Shors et al., 2002; Achanta et al., 2009). In the present study, animals were trained in experiment 1 beginning on the day that the number of new cells was quantified in experiment 2. Therefore, the effect of cholinergic MSDB lesions is mediated, in part, through reduced neurogenesis. Cholinergic MSDB lesions did not completely ablate neurogenesis, however, a previous study has demonstrated that a 50% reduction is sufficient to impair trace learning. Conversely, a reduction in the number of new cells in the DG does not impair delay eyeblink conditioning. An impairment in adult neurogenesis may therefore be a factor for

why cholinergic MSDB lesions more severely impairs trace compared to delay eyeblink conditioning (Kaneko & Thompson, 1997).

Research definitively indicates that the role of acetylcholine in learning is dissociated from dependence on the hippocampus. Similarly, learning's dependence on adult neurogenesis is also not a matter of whether the task is dependent on the hippocampus or not. My colleagues and I have established that one type of eyeblink task that requires an intact hippocampus is not impaired when neurogenesis reduced (Beylin et al., 2001; Nokia et al., 2012a). In this very long delay eyeblink task, the CS and US are contiguous in time, however, the onset of each stimuli are separated by a large gap in time because of the CS is much longer than the CS used in a traditional delay task. One important feature of very long delay conditioning is that it takes longer for animals to acquire CRs compared to a traditional delay task (Leuner, Waddell, Gould, & Shors, 2006). Therefore, very long delay is more difficult to learn (Beylin et al., 2001; Leuner et al., 2006). As previously discussed, task difficulty may be an important feature in why cholinergic MSDB lesions impair trace eyeblink conditioning. Future studies should use various eyeblink procedures including short trace, very long delay and trace with a cue light to experimentally confirm that task difficulty is an important component in why reduced cholinergic activity impairs learning.

Others have suggested that neurogenesis mediates the relationship between acetylcholine and other types of learning and memory as well. Mohapel and colleagues (2005) have reported that cholinergic MSDB lesions reduced proliferation of new cells and also impaired learning during training with the Morris water maze task. Similarly, Van Kampen and colleagues (2010) demonstrated the cholinergic MSDB lesions

decreased neurogenesis and impaired working memory during a spatial radial arm maze task. Van Kampen and colleagues additionally reported that a muscarinic agonist reversed both of these effects. Furthermore, others have reported that AchE inhibitors increased proliferation and also facilitated trace eyeblink conditioning in aged animals (Kronforst-Collins et al., 1997a; Itou et al., 2011). It has been suggested that adult neurogenesis is in important factor in both progression and treatment of Alzheimer's disease (Mu and Gage, 2011). Taken together, adult neurogenesis probably mediates the effectiveness of cholinergic enhancing drugs as a treatment for mild to moderate Alzheimer's disease.

Producing new neurons critically contributes to learning because it creates a dynamic, plastic circuitry in the hippocampus. Generating new neurons leads to thousands of new impressionable synaptic connections that can change neuronal circuitry (Toni et al., 2008). Even though most cells die in normal situations, learning can enhance the number of cells that survive and integrate into neuronal circuitry (Anderson et al., 2011; Leuner et al., 2004). New cells that survive begin extending axons into the CA3 between 4 to 10 days after their birth (Hastings and Gould, 1999). Between the period when axons begins to grow and when the reach their targets in the CA3, newly-generated cells receive GABAergic, glutaminergic and cholinergic input at differing points in their maturation (Overstreet Wadiche et al., 2005; Tozuka et al., 2005; Overstreet-Wadiche and Westbrook, 2006; Itou et al., 2011). Furthermore, activity from CA3 neurons may attract or repel these forming synaptic terminals. Indeed, others have proposed that this activity-dependent maturation influences the formation of networks (Aimone et al., 2009). Additionally, several reports indicate that synaptic input of new neurons can

continue to change beyond that of older mature neurons through their dendritic spines enhanced motility (Hastings and Gould, 1999; Zhao et al., 2006; Toni et al., 2007). Moreover, new neurons are more excitable during the first few months of their life (Aimone, Deng, & Gage, 2010; Overstreet Wadiche et al., 2005; Snyder, Kee, & Wojtowicz, 2001). This allows the strength of synapses in newer neurons to change more readily compared to older ones. Through the incorporation of information during their development, new neurons may change hippocampal circuitry so that it is better wired to respond to future situations similar in nature to the conditions under which they were formed (Aimone et al., 2009). Therefore, learning depends on neurogenesis and new neurons create more efficient circuitry in the hippocampus. There is some evidence that supports this hypothesis. In one study, a higher proportional of cells that were 6-8 weeks old at the time of training on the Morris water maze were reactivated during recall (Kee et al., 2007). In addition, others have demonstrated that cells rescued from death by the water maze spatial task are reactivated during relearning (Trouche et al., 2009). In conclusion, learning and adult neurogenesis have a reciprocal relationship. If one therapeutic approach to treating Alzheimer's disease is to create more cells in the hippocampus through the use of drugs that enhance cholinergic activity, it is probably best for a patient to use their medication and engage in activities that promote a neuron's integration into hippocampal circuitry.

Another variable in learning: Theta

How much theta dominants hippocampal activity is an important variable for whether an animal learns well (Berry et al., 1978b; Guderian et al., 2009; Nokia et al.,

2009). In particular, the presence of theta immediately before a learning event seems particularly important for encoding memories (Griffin et al., 2004). Therefore, there is something intrinsically different between a brain state with and without theta. Some researchers have attributed this difference to being related to attention. In one of the first publications to propose a relationship between theta and attention, Holmes and Adey (1960) report that slow oscillations in the entorhinal cortex seemed to correlate with alertness and attention in cats learning an associative task. A recent report indicates that theta increases in slow learners during trace eyeblink conditioning which corresponds to a change in attentional effort (Nokia et al., 2009). The amount of attention directed to environmental stimuli is a critical component of whether an animal learns well and can be manipulated by pre-exposing animals to the CS. This phenomenon impairs associative conditioning, presumably because an animal first learns that the CS is irrelevant. Thus, this learning paradigm is used as a model for attention. Using this model, others have demonstrated that cholinergic MSDB neurons are important is attention modulation (Baxter, Holland, & Gallagher, 1997). Animals with lesions as a result of 192 IgG-Saporin are not impaired during associative conditioning following pre-exposure to the CS, indicating that animals were initially unable to shift their attention away from the CS. As discussed previously, cholinergic lesions may impair trace learning because animals are unable to attend appropriately to the relevant stimuli (i.e. CS versus context). If the presence of theta indicates a heightened level of attention, this idea is corroborated by the decreased theta power observed on a Day 7 following cholinergic MSDB lesions.

However, theta rhythm is not always necessary for learning (Nokia and Wikgren, 2014) and the present study establishes that learning impairments are not solely

dependent on a disruption in theta rhythm. Moreover, the presence or absence of theta does not necessarily indicate that an animal will not learn, just that they possibly will not learn well. These caveats lead to questions about what roles theta rhythm serve in the hippocampus. Oscillations are obviously the flux of ions at a particularly frequency, but they emerge as a result of organized activity among a population of cells. Therefore, an oscillation serves to coordinate neurons with the frequency of that oscillation determining the distance and time of that synchronization. Slower rhythms travel further distances and have a larger time span though which different cell populations can coordinate. It has been demonstrated that cholinergic-induced theta rhythm in hippocampal cells increases synaptic plasticity in CA1 pyramidal cells (Huerta and Lisman, 1993). Importantly, enhanced post synaptic potentiation is dependent on both theta and additional stimulation of CA1 afferents during the positive phase of the theta waveform. Others report that stimulating CA1 afferents during the trough of the theta waveform induces long term depression (Stanton and Sejnowski, 1989). Therefore, theta enhances the likelihood that the strength of synapses can change in response to incoming information. Moreover, theta organizes activity of cell assemblies. Particular groups of neurons in all three subregions of the hippocampus are more likely to fire together in relation to the phase of the theta waveform (Berry et al., 1978a; Harris et al., 2003). Harris and colleagues have proposed that this organized activity facilitates information transmission. Indeed, a few studies indicate that theta rhythm coordinates different brain regions during learning. For instance, when there is higher theta power in the hippocampus, there is also higher theta power in the cerebellar cortical area HVI, an area important in the development of CRs during delay eyeblink conditioning (Wikgren et al., 2010). In this study, theta rhythm in

each brain region was phase locked during all delay eyeblink conditioning sessions. In addition, theta rhythm is important is coupling activity in downstream brain regions as well. Adey and colleagues (1960) have reported that early in training, the phase of hippocampal theta rhythms preceded the phase of theta rhythms in the entorhinal cortex. However, the entorhinal cortex led the hippocampus during later phases of learning and predicted when the animal correctly responded. A recent report confirms that when hippocampal theta was phase locked to the CS during trials late in training, animals did not emit a CR during very long eyeblink conditioning (Nokia and Wikgren, 2013). Others have reported that pairing trace trials with trains of hippocampal theta facilitated learning-related population responses in the medial prefrontal cortex, a structure downstream of the hippocampus that is required for trace eyblink conditioning (Kalmbach et al., 2009; Darling et al., 2011). Importantly, the development of medial prefrontal cortical responses appear to shift from being associated with an attentional role to a retrieval role (Hattori et al., 2014). Overall, it appears that theta facilitates the transfer of information between structures so that brain regions can appropriately encode information and ultimately contribute to the expression of learned responses. Thus, while the presence of theta rhythm does not always indicate that learning will or will not occur, theta rhythm appears to contribute to efficient information transfer.

It is also possible that theta rhythm primes newly-generated cells in the hippocampus for encoding new information similarly to other mature neurons. It has been established that new cells sense rhythmic stimulation early during their maturation and that this promotes their survival (Tozuka et al., 2005). Conversely, new neurons may play an important role in organizing cell oscillatory activity. My colleagues and I have

previously demonstrated that anti-mitotic agents that reduced adult neurogenesis in the hippocampus also reduced spontaneous theta rhythm (Nokia et al., 2012a). Moreover, animals given this anti-mitotic agent were impaired during trace eyeblink conditioning. Therefore, disrupted theta rhythm could exasperate the effects of reduced neurogenesis on learning. However, the results presented in the current study oppose this latter possibility because adult neurogenesis and theta rhythm are dissociated. Adult neurogenesis is impaired 14 days after infusing 192 IgG-Saporin, but theta is not. In addition, another study reports that a reduction in adult neurogenesis actually enhances rhythmicity in the hippocampus (Lacefield et al., 2012). The authors of this study propose that new neurons serve to destabilize neuronal networks. This theory complements the idea that new neurons are ideal for encoding new information because they remain more excitable and plastic for months after their birth. Therefore, theta rhythm and neurogenesis may be competing organizing and disorganizing forces during learning.

In order to understand the role of theta rhythm in the relationship between acetylcholine and learning, it will be important to record hippocampal oscillations and neuronal population activity during trace eyeblink conditioning in future experiments. Even though theta power recovers by day 14 in the current experiment, it is still possible that theta is impaired during training and that this disrupts the development of normal population activity in response to the CS and trace interval. Most of the studies on theta rhythm referenced above and the new experiment I propose are correlational in nature and do not demonstrate that theta mediates the relationship between acetylcholine and learning. This will likely remain the case because removing oscillations the way a researcher removes brain structures, proteins or genes is not possible. Therefore, it is

difficult to assess whether changes in theta precede or follow behavioral changes. Instead of removing theta, it might be useful to shift activity to another oscillation or to desynchronized activity, but this will also have indirect consequences in a brain structure that has many reciprocal processes.

Studying a complex structure

The many reciprocal relationships described highlight an important feature of the hippocampus – its circuitry, processes, and function are all components of an incredibly interconnected system. The hippocampus has been appreciated for its obvious striking unidirectional organization. Even Santiago Ramon y Cajal's drawings of the hippocampus include arrows that propose that information flows in one direction. However, years of research reveal many interconnected visually obscured features within this brain structure. It is important to appreciate that from this complexity comes complex behavior. However, complexity also means that there is great difficulty in isolating one factor from another. Unilateral lesions were used in the present study as an attempt to study the direct role of acetylcholine on hippocampal adult neurogenesis and theta rhythm. However, cholinergic MSDB lesions equally disrupted adult neurogenesis and theta rhythm in both hemispheres. These results indicate that cholinergic activity is both directly and indirectly influencing adult neurogenesis and theta rhythms, and thus, learning. As previously discussed, there are many factors that could mediate acetylcholine's roles in the hippocampus. In particular, hilar mossy cells are a component of the circuitry that is well positioned to coordinate hippocampal activity between each hemisphere. I propose that cholinergic input to hilar mossy cells regulates both

hippocampal adult neurogenesis and theta rhythm and furthermore, are important for learning. To test this possibility, it is necessary to selectively remove cholinergic receptors from mossy cells during adulthood. Mossy cells express both muscarinic and nicotinic receptors (Frazier et al., 2003; Hofmann and Frazier, 2010) as do many cells in the hippocampus. However, a recent study has developed a genetic approach to selectively kill mossy cells (Jinde et al., 2012). It may be possible to adapt their techniques to selectively delete cholinergic receptors in order to study the relationship between mossy cells, neurogenesis, theta rhythm and learning. However, it is important to point out that selectively killing cholinergic MSDB neurons resulted in smaller effects in all three experiments compared to non-selective pharmaceutical manipulations. As researchers attempt to isolate increasingly smaller components of this complex system, we will likely find smaller effects on processes in the hippocampus and behavior. Thus, these differences will then be harder to statistically detect. Future experiments should take these concerns into consideration in hopes that the results might reveal the importance of underappreciated components of hippocampal circuitry.

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		1	2	3	4
		Bilateral Intact	Bilateral Target	Unilateral Intact	Unilateral Target
1	Bilateral Intact		0.03	0.0002	0.02
2	Bilateral Target	0.03		0.0002	0.01
3	Unilateral Intact	0.0001	0.0002		0.0001
4	Unilateral Target	0.02	0.01	0.0001	

Table 1. The p values from the post hoc analysis conducted in experiment 2 comparing the percent of ChAT+ cells/mm^2 remaining between all groups. All groups differed from each other.

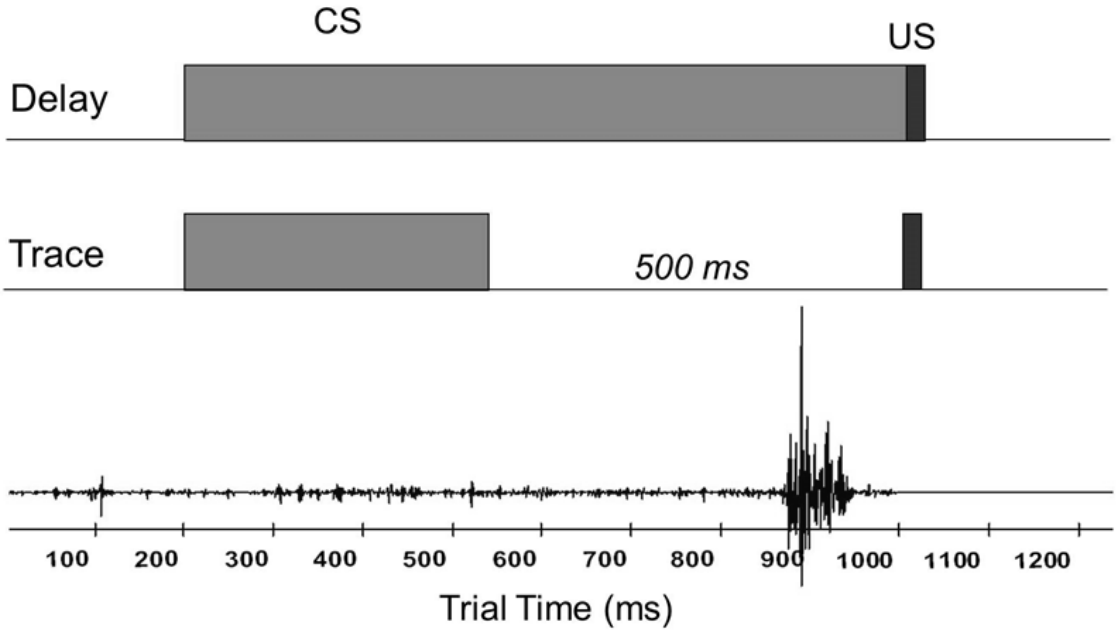
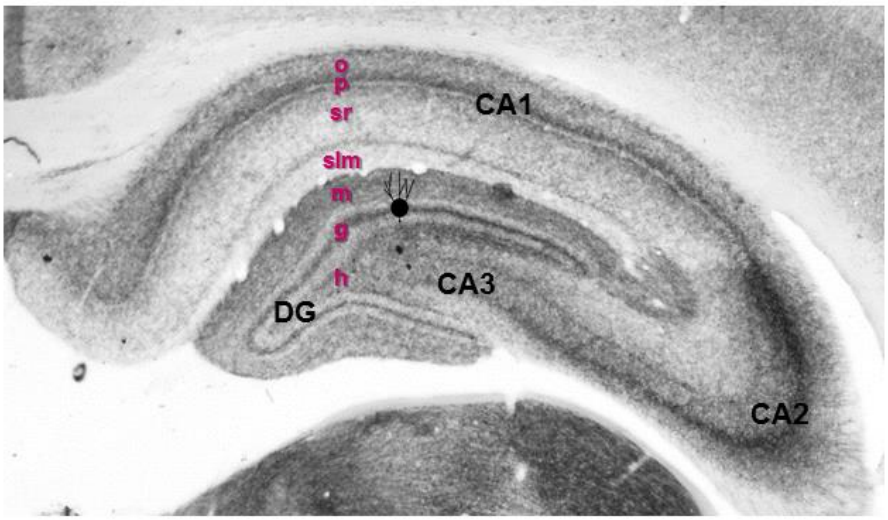


Figure 1. A schematic showing the temporal relationship between the CS and US in delay versus trace conditioning. Animals that learn these tasks well blink directly before the onset of the US as indicated by the example electromyographic recording.



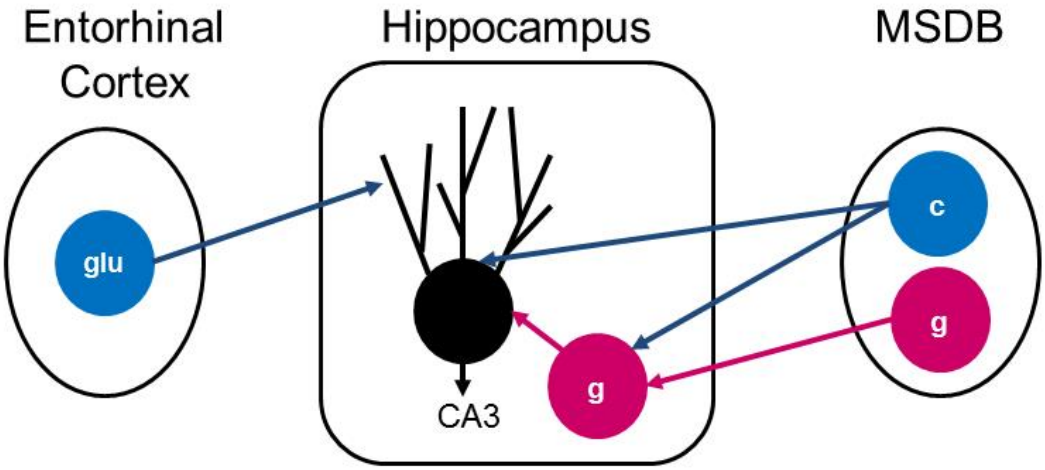


Figure 2. A microphotograph of the dorsal hippocampus stained to visualize AchE (top). The subregions (black) and the layers (pink) are labeled. Key: DG, dentate gyrus; CA, cornu ammonus; o, stratum oriens; p, stratum pyramidal; sr, stratum radiatum; slm, stratum lacunosum-moleculare; m, stratum moleculare; g, stratum granulosum (granule cell layer); h, hilus/polymorphic layer. A cartoon of a granule cell is placed in the dentate gyrus. A schematic of the septohippocampal and entorhinal cortical projections to a granule cell (bottom). Key: c, cholinergic; g, GABAergic; glu, glutaminergic.

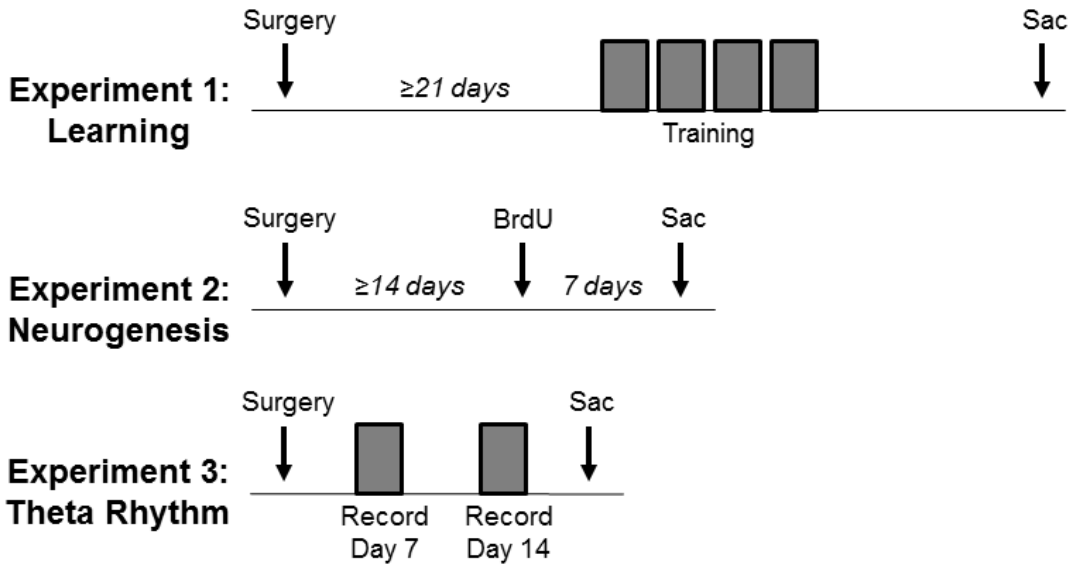


Figure 3. A timeline for all experiments. In the first experiment, a sham or lesion surgery was performed on all animals to assess whether cholinergic neurons in the hippocampus are necessary for trace eyeblink conditioning (top). All animals were exposed to four days of training with trace or delay eyeblink conditioning before being sacrificed. In experiment 2, a sham or lesion surgery was performed to determine whether acetylcholine influences proliferation/early survival of newly-generated cells in the hippocampus (middle). All animals were given a single BrdU injection and sacrificed seven days later. In the third experiment, a sham or lesion surgery was performed to assess the effect of the loss of cholinergic MSDB neurons on hippocampal theta rhythm (bottom). On Day 7 and Day 14 following the surgery, hippocampal neural activity was recorded.

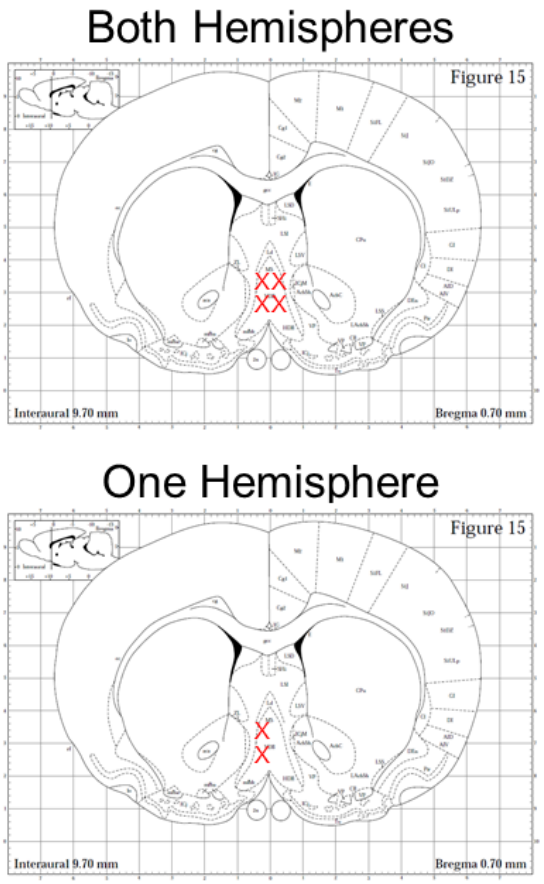


Figure 4. Diagrams of the MSDB complex with marks showing the approximate infusion sites for animals that received 4 infusions (top) and animals that received only 2 infusions (bottom). Infusions in one hemisphere were sufficient to produce both bilateral and unilateral lesions. The red crosses reflect the approximate position of the infusion sites. The infusion sites were located at AP 0.6, not AP 0.7, however, this brain atlas slide was used because it was the closest position available.

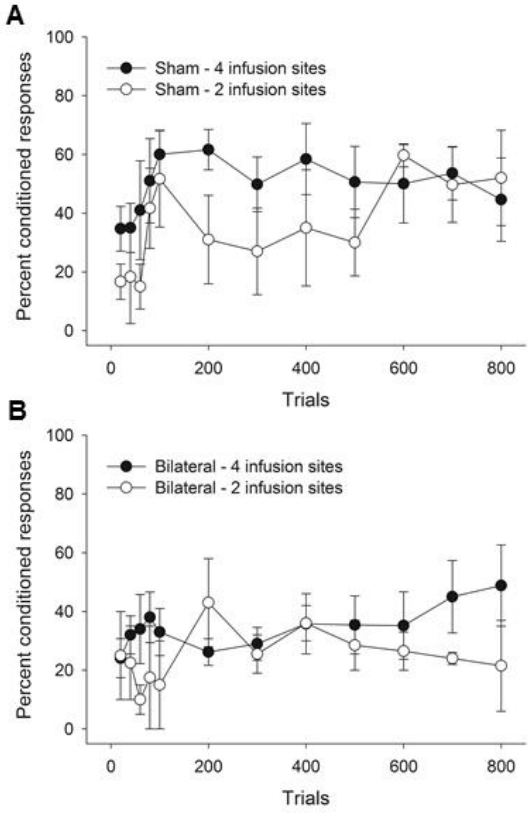


Figure 5. Trace eyeblink conditioning data for animals after they were given infusions of vehicle (a) or 192 IgG-Saporin (b) in two or four sites in the MSDB. Animals with a sham lesion after 4 infusions of vehicle (n=5) reached the same level of percent conditioned responses as animals with a sham lesion produced using 2 infusions (n=3). Animals with a bilateral lesion after 4 infusions of 192 IgG-Saporin (n=5) reached the same level of percent conditioned responses as animals with a sham lesion produced using 2 infusions (n=3).

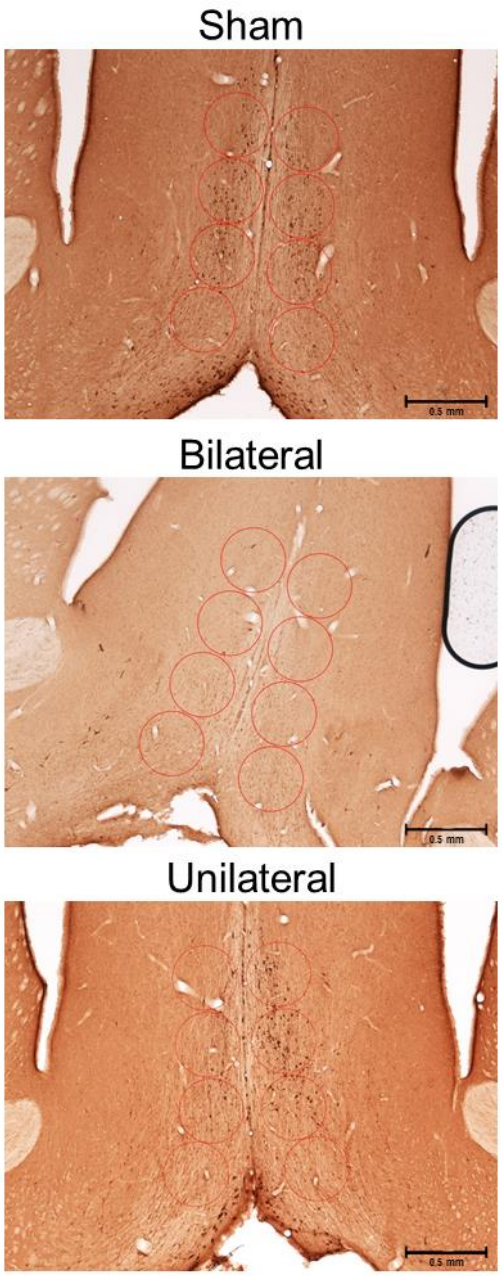


Figure 6. Example microphotographs of coronal slices of the MSDB showing the placement of circles used to quantify ChAT- and PARV-positive cells. Circles with a diameter of 0.4mm were placed within the medial septal diagonal band region in the microphotographs. The number of ChAT-and PARV-positive cells were quantified including only cells whose somas resided completely within the circle. Microphotographs in this diagram were taken at a total magnification of 40x at approximately Bregma 0.25.

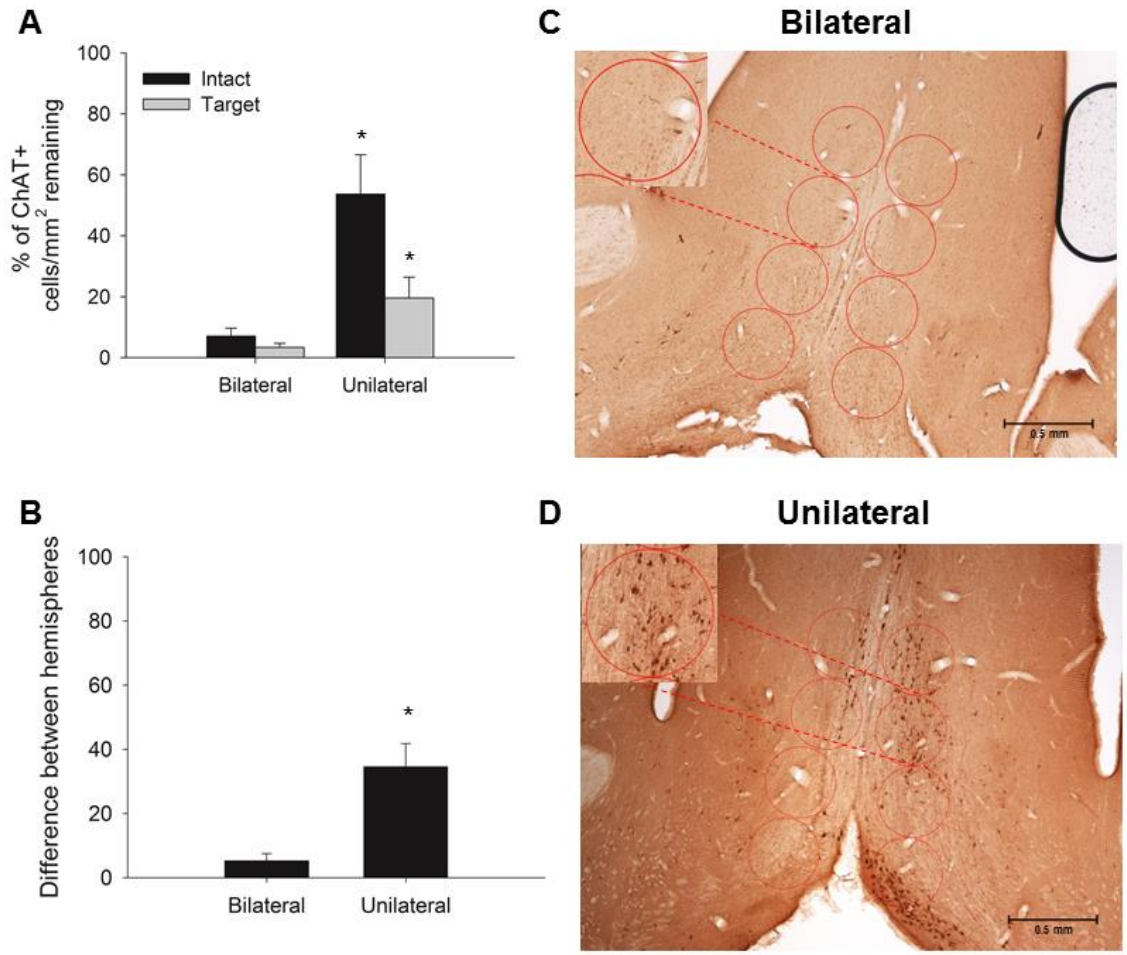


Figure 7. Animals given 192 IgG-Saporin in experiment 1 were split into Bilateral (n = 11) and Unilateral (n = 11) lesion groups. There was a significantly higher percent of ChAT+ cells/mm^2 remaining in the Unilateral Intact hemisphere compared to all other groups. In addition, the percent of ChAT+ cells/mm^2 remaining in the Unilateral Target hemisphere was different compared to all other groups (a). The difference in the percent of ChAT+ cells/mm^2 between intact and target hemispheres was greater in the Unilateral group compared to the Bilateral group (b). Representative microphotographs of ChAT expression in a coronal slice of the MSDB from an animal with a bilateral lesion (c) and another with a unilateral lesion (d). Microphotographs in this diagram were taken at a total magnification of 40x at approximately Bregma 0.25. * indicate groups that were significantly different from all other groups within each analysis.

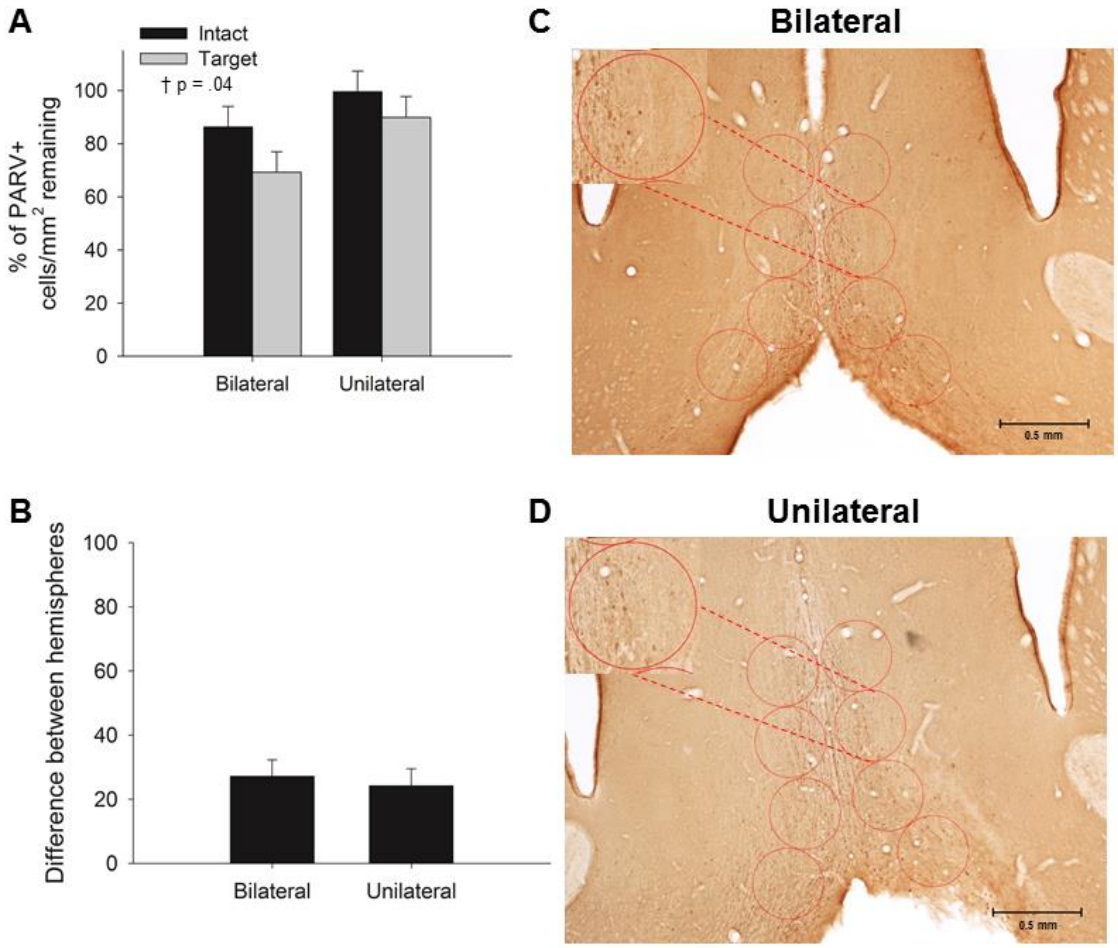


Figure 8. The percent PARV+ cells/mm^2 remaining in animals with bilateral and unilateral lesions compared to animals with sham lesions from experiment 1. There was a significantly higher percent of PARV+ cells/mm^2 remaining in the intact hemispheres compared to the target hemispheres (a). Animals with bilateral and unilateral lesions had a similar difference in the percent of PARV+ cells/mm^2 remaining between intact and target hemispheres (b). Representative microphotographs of PARV expression in a coronal slice of the MSDB from an animal with a bilateral lesion (c) and another with a unilateral lesion (d). Microphotographs in this diagram were taken at a total magnification of 40x at approximately Bregma 0.25. † indicates that there was a significant difference between intact and target hemispheres.

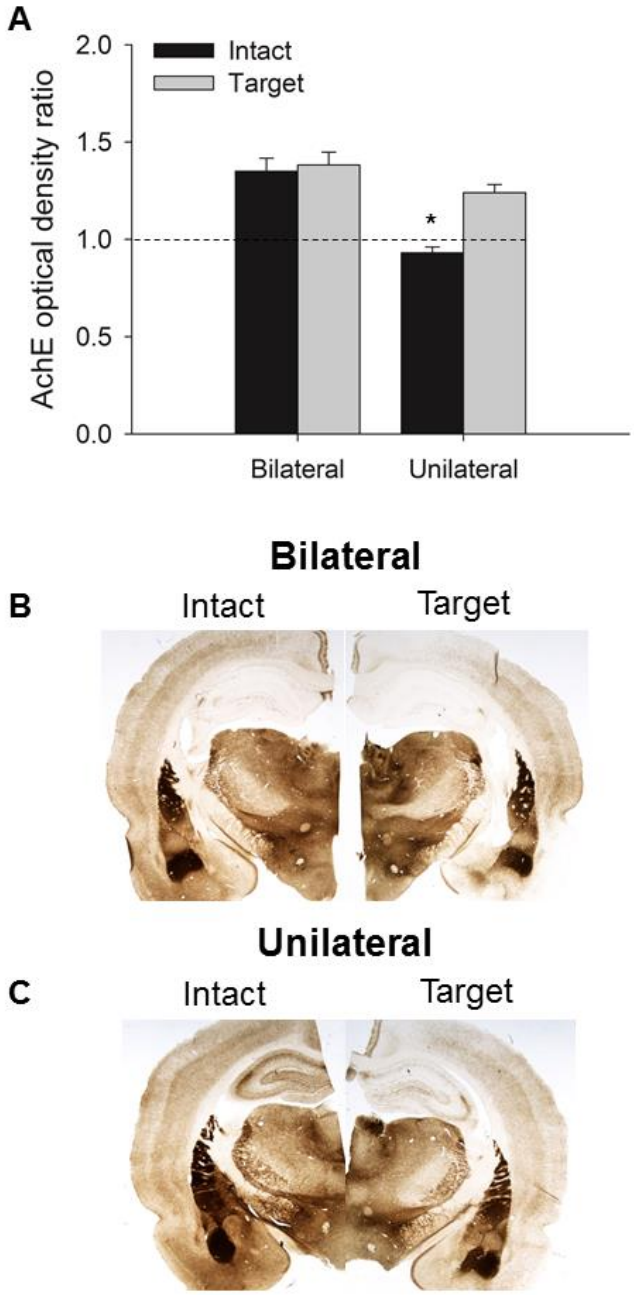


Figure 9. Average optical density of AchE staining in the hippocampus expressed as a ratio (lesion to sham) for each hemisphere in animals with bilateral and unilateral lesions in experiment 1. An increase above an optical density of 1 represents a decrease in AchE activity (a). Representative microphotographs of AchE expression in a coronal slice of the dorsal hippocampus from an animal with a bilateral lesion (c) and another with a unilateral lesion (d). Microphotographs in this diagram were taken at a total magnification of 10x at approximately Bregma -2.80. * indicate groups that were significantly different from all other groups within each analysis.

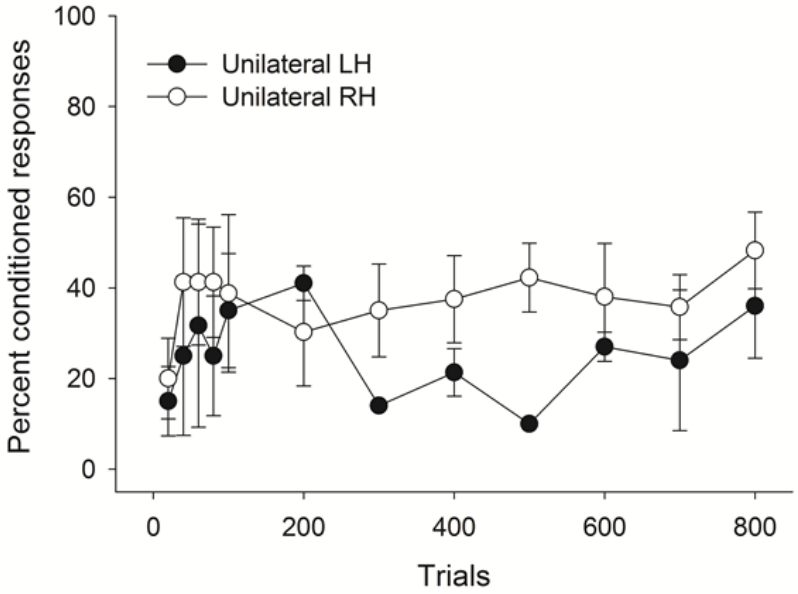


Figure 10. Trace eyeblink conditioning data for animals with unilateral left and right lesions. Animals with Unilateral LH (n = 3) lesions performed similar to animal with Unilateral RH lesions (n = 4).

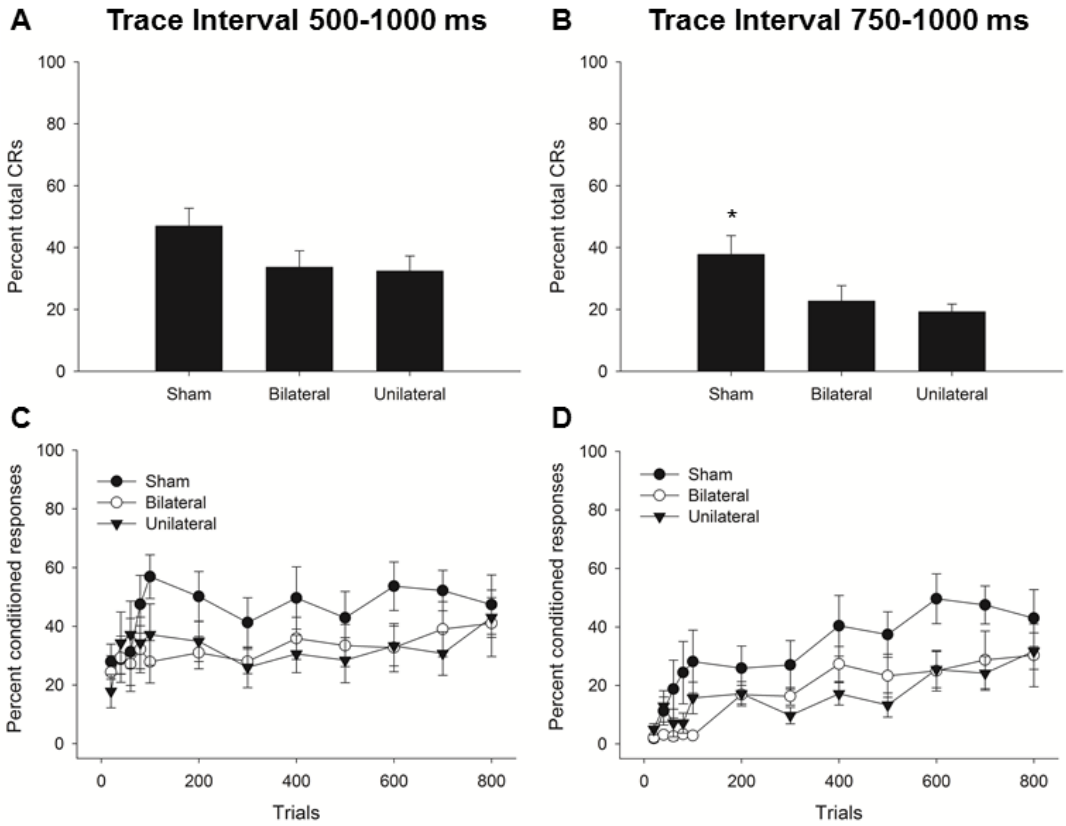


Figure 11. The loss of cholinergic MSDB neurons resulted in a small deficit in trace eyeblink learning. The percentage of total of CRs emitted during the entire trace interval did not differ between groups, Sham n = 8, Bilateral n = 7, Unilateral n = 7 (a). However, animals with lesion were impaired in emitting CRs during the last 250 ms of the trace interval. Animals in the Sham group emitted more CRs compared to the Bilateral and Unilateral groups (b). The sham group emitted more CRs over time during the entire trace interval (near significance) while the bilateral and unilateral groups did not (c). All groups emitted significantly more CRs over time during the last 250 ms of the trace interval, but there was a near significant difference between groups (d). * indicate groups that were significantly different from all other groups within each analysis.

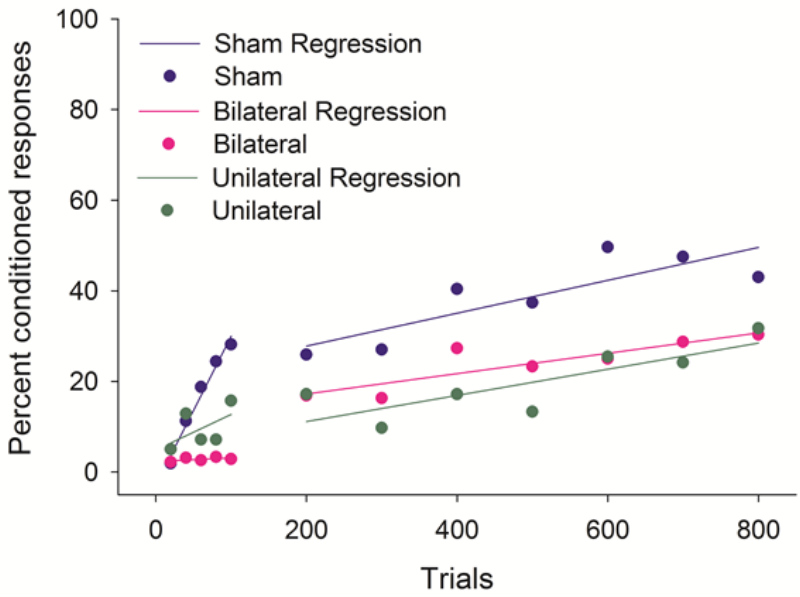


Figure 12. Early acquisition of finely time CRs is impaired in animals with bilateral and unilateral lesions. CRs emitted by animals with sham lesions increase dramatically across the 1st 100 trials, but not in animals with bilateral or unilateral lesions. The increase in CRs during trials 200-800 are more similar between groups. First 100 trials: Sham, slope = .32, Bilateral slope = .001, Unilateral slope = .08; Trial 200-800: Sham slope = .04, Bilateral slope = .006, Unilateral slope = .009.

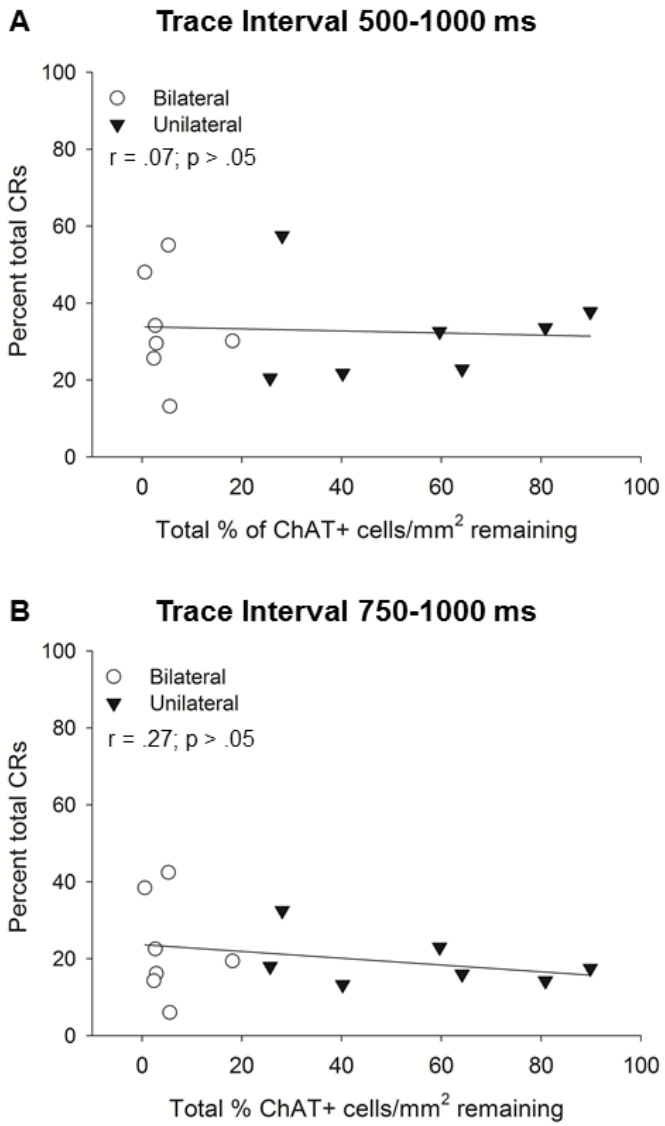


Figure 13. The extent of the lesion does not predict how well an animal learns. The number of ChAT+ cells/mm2 remaining did not predict the total number of CRs made during the entire trace interval (a) nor during the last 250 ms of the trace interval (b).

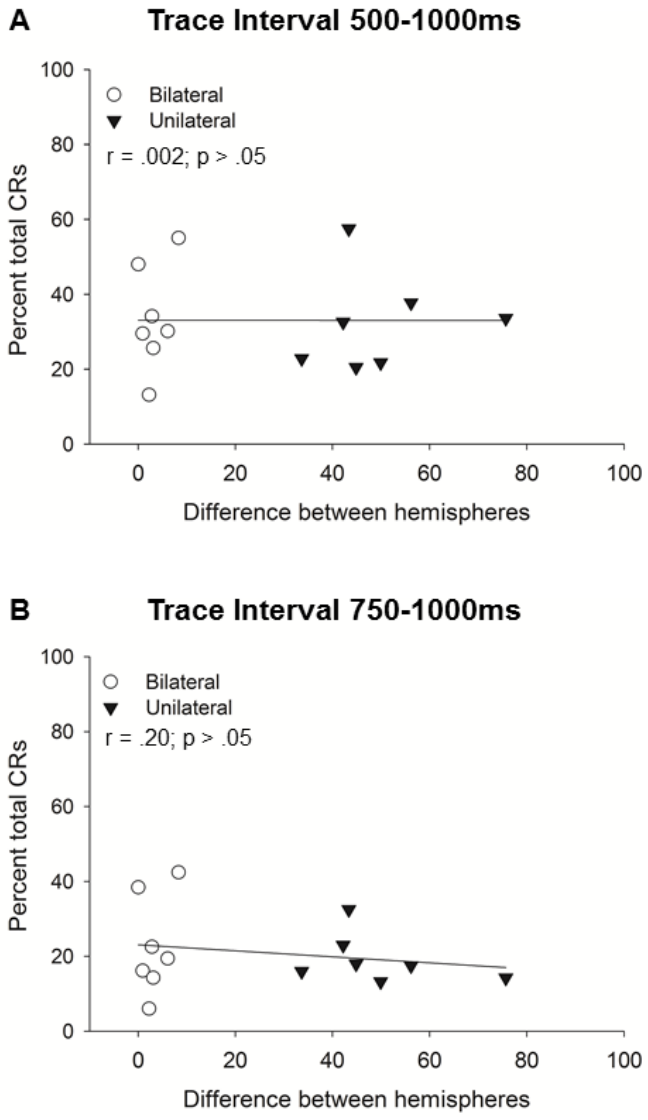


Figure 14. The extent to which the lesion was lateralized also does not predict how well an animal learns. The difference in percent ChAT between hemispheres did not predict the total number of CRs made during the entire trace interval (a) nor during the last 250 ms of the trace interval (b).

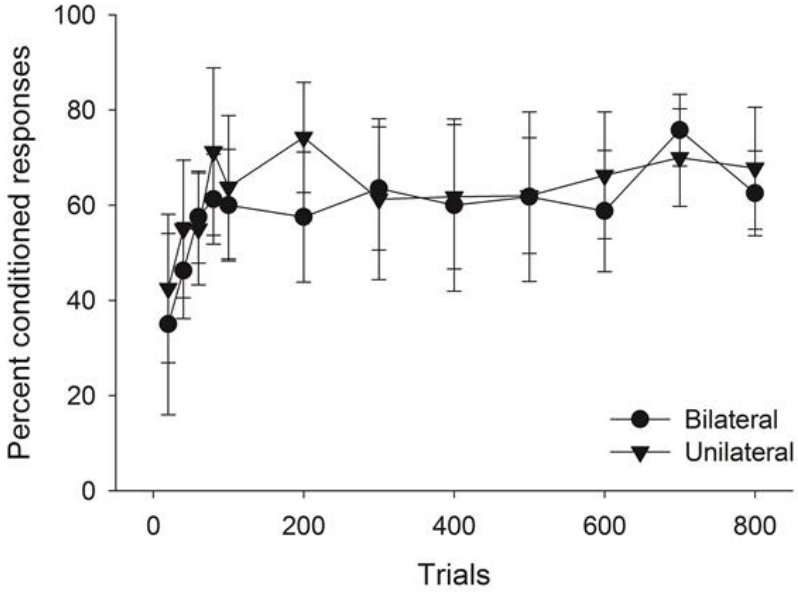


Figure 15. Both the Bilateral and Unilateral groups were able to reach the learning criterion of 60% during delay eyeblink conditioning.

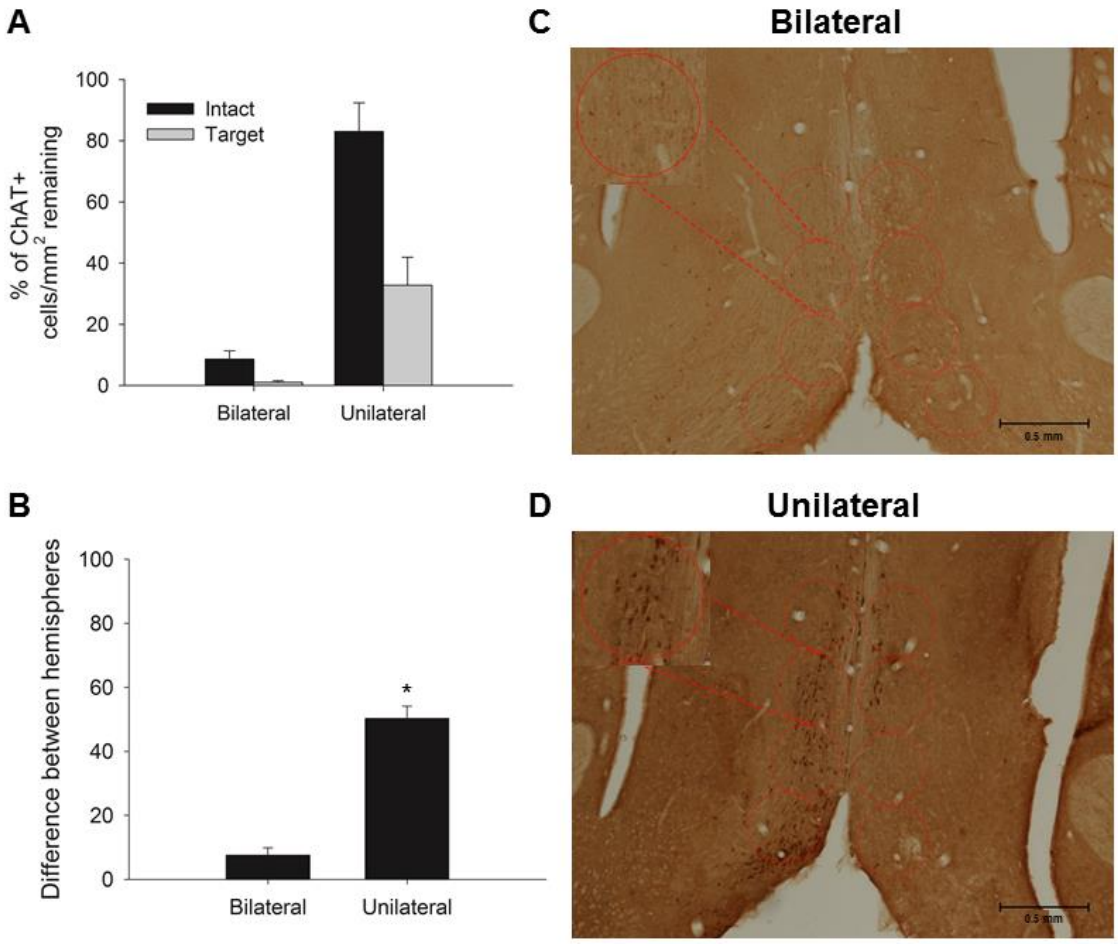


Figure 16. Animals given 192 IgG-Saporin in experiment 2 were split into Bilateral (n = 7) and Unilateral (n = 7) lesion groups. The percent of ChAT+ cells/mm^2 remaining differed between all groups (a). The difference in the percent of ChAT+ cells/mm^2 remaining between intact and target hemispheres was greater in the Unilateral group compared to the Bilateral group (b). Representative microphotographs of ChAT expression in a coronal slice of the MSDB from an animal with a bilateral lesion (c) and another with a unilateral lesion (d). Microphotographs in this diagram were taken at a total magnification of 40x at approximately Bregma 0.25. * indicate groups that were significantly different from all other groups within each analysis.

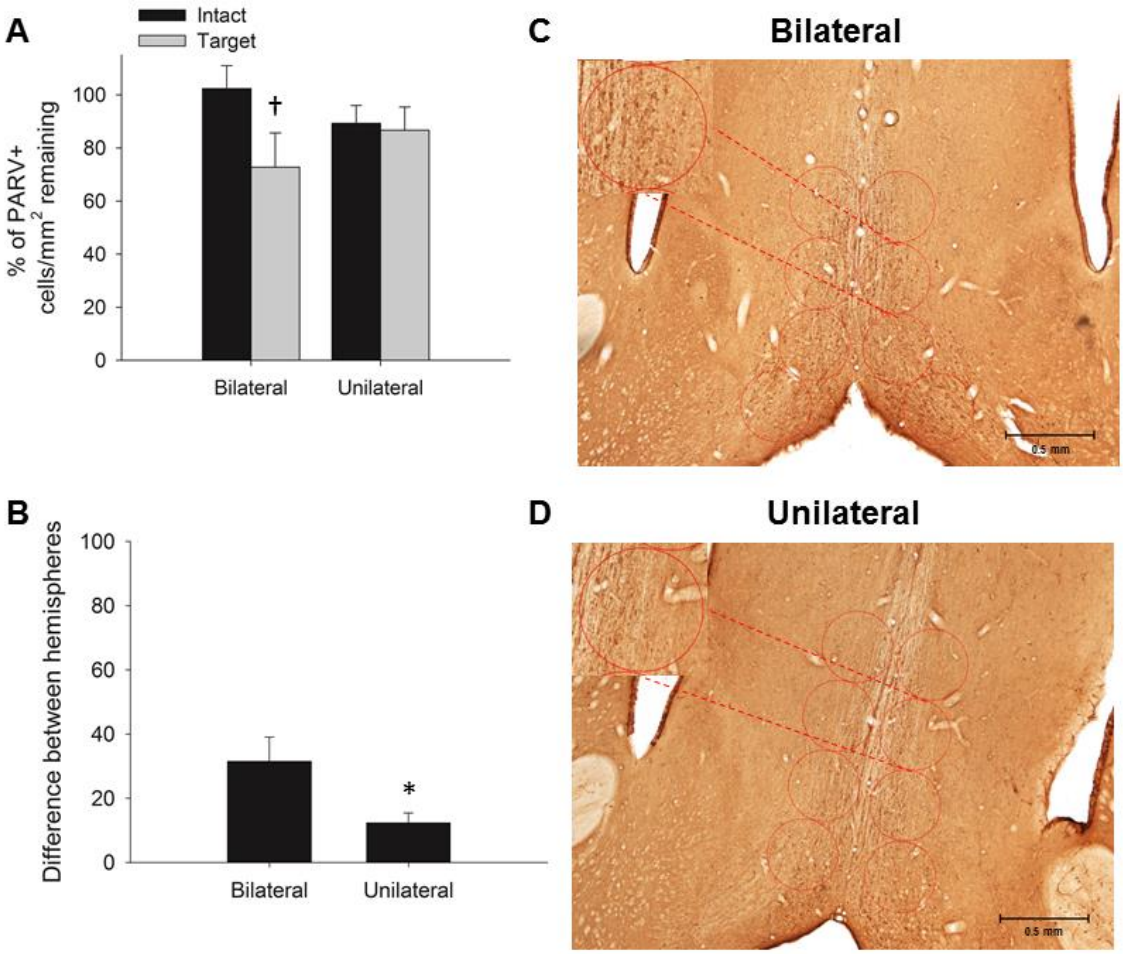


Figure 17. The percent PARV remaining in animals with bilateral and unilateral lesions compared to animals with sham lesions from experiment 2. There was a significant difference between the intact and target hemisphere in the Bilateral group but not the Unilateral group (a). The difference in the percent of PARV+ cells/mm^2 between intact and target hemispheres was greater in the Bilateral group compared to the Unilateral group (b). Representative microphotographs of PARV expression in a coronal slice of the MSDB from an animal with a bilateral lesion (c) and another with a unilateral lesion (d). Microphotographs in this diagram were taken at a total magnification of 40x at approximately Bregma 0.25. † indicates that the group significantly differed from one other group. * indicate groups that were significantly different from all other groups within each analysis.

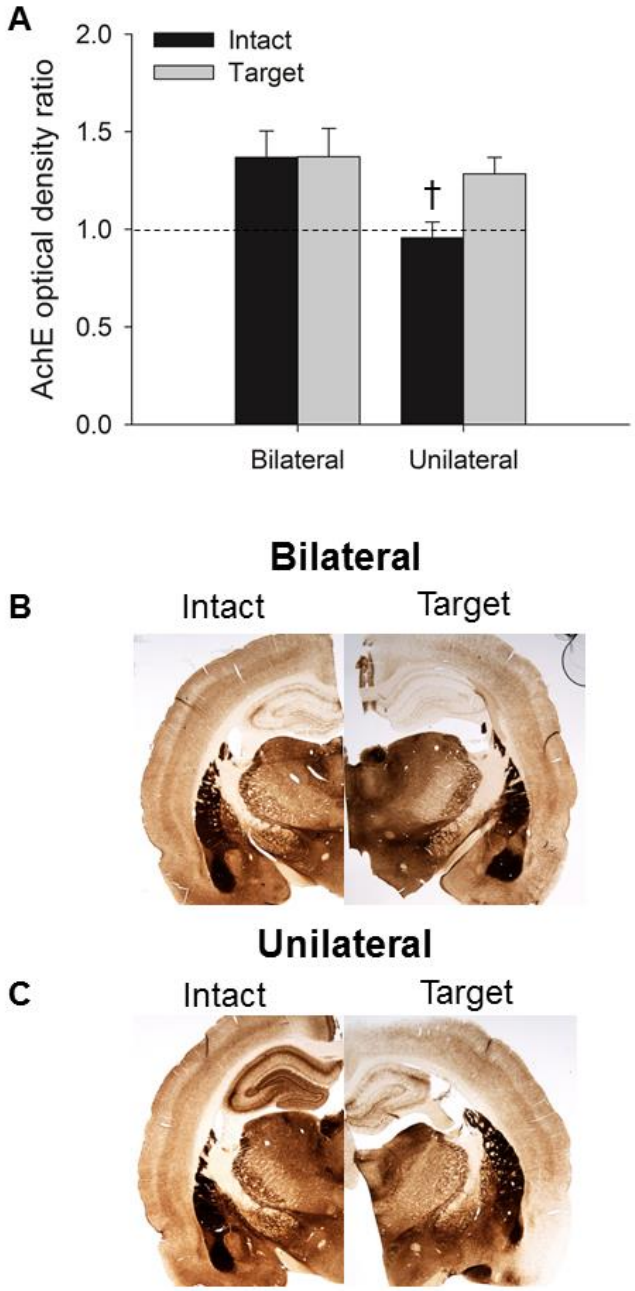


Figure 18. Average optical density of AchE staining in the hippocampus expressed as a ratio (lesion to sham) for each hemisphere of animals with a bilateral and unilateral lesion in experiment 2. An increase above an optical density of 1 represents a decrease in AchE activity. Representative microphotographs of AchE expression in a coronal slice of the dorsal hippocampus from an animal with a bilateral lesion (c) and another with a unilateral lesion (d). Microphotographs in this diagram were taken at a total magnification of 10x at approximately Bregma -2.80. † indicates that Unilateral Intact and Unilateral Target were significantly different.

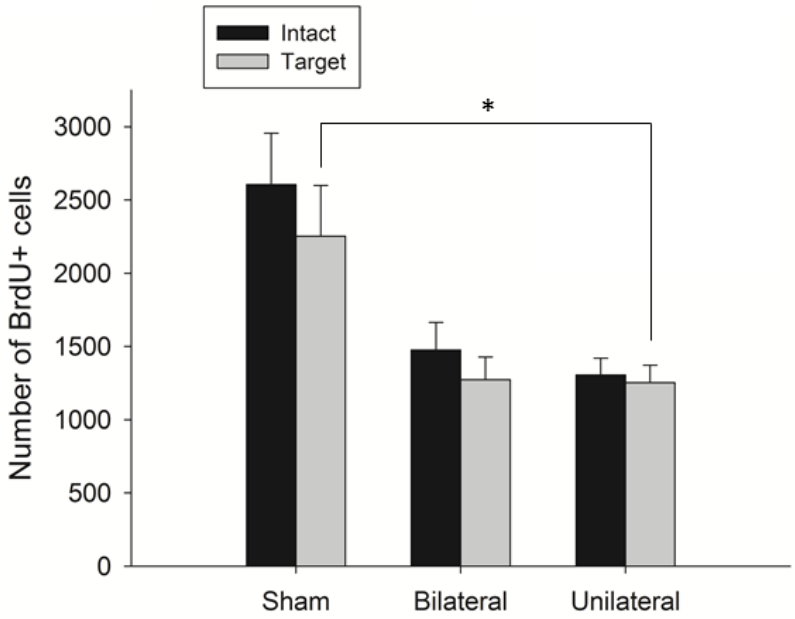


Figure 19. A reduction of acetylcholine release in the hippocampus reduces the number of newly-generated cells in the dentate gyrus. The number of BrdU+ cells in that target hemisphere was significantly different between animals with sham (n = 8) and unilateral (n = 7) lesions, and nearly significantly different between animals with sham and bilateral (n =7) lesions.

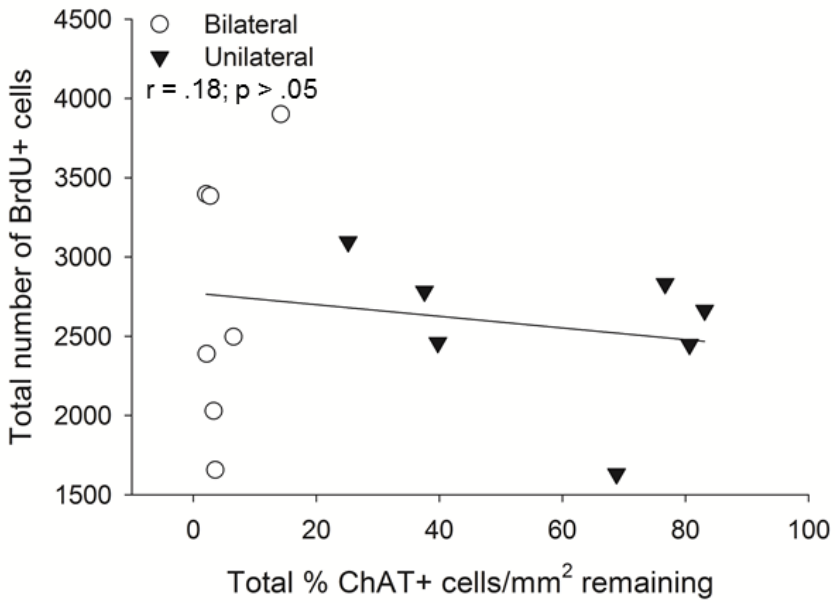


Figure 20. The extent of the lesion does not predict how many new cells are made and survive for 7 days in the dentate gyrus. In experiment 2, the percent ChAT remaining did not have a significant relationship with the number of BrdU+ cells.

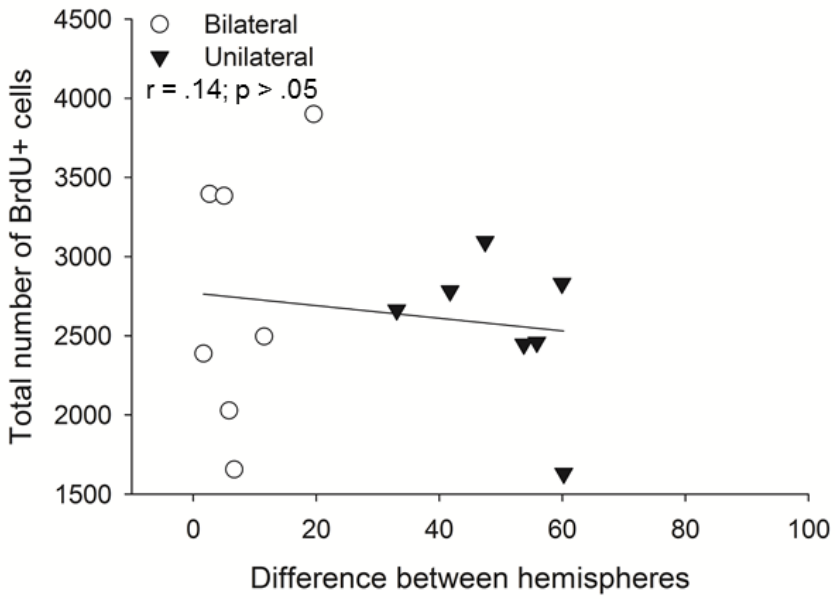


Figure 21. The extent to which the lesion was lateralized also does not predict how many new cells are made and survive for 7 days in the dentate gyrus. In experiment 2, the difference in percent ChAT remaining between hemispheres did not have a significant relationship with the number of BrdU+ cells.

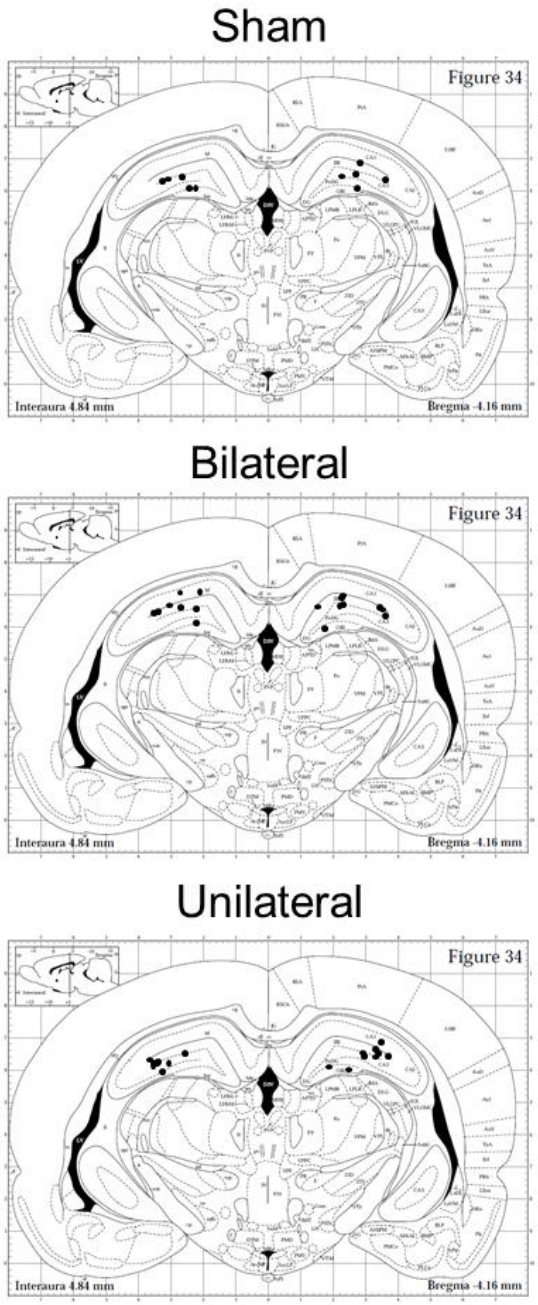


Figure 22. Representative marks showing the approximate electrode placement in the dorsal hippocampus of animals with sham (top), bilateral (middle) or unilateral (bottom) lesions. Hippocampal recordings taken with these electrodes were used for the analysis for experiment 3.

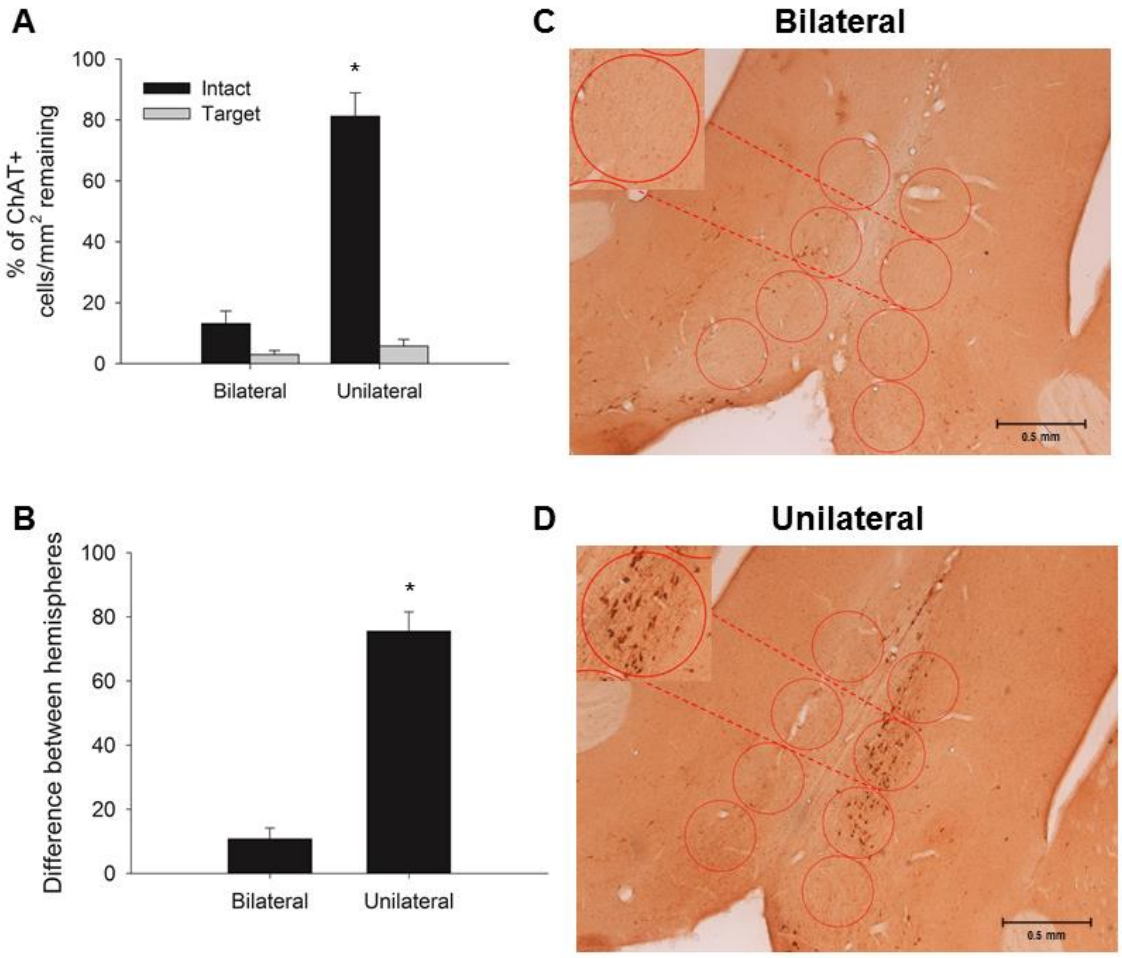


Figure 23. Animals given 192 IgG-Saporin in experiment 3 were split into Bilateral (n = 8) and Unilateral (n = 9) lesion groups. There was a significantly higher percent of ChAT+ cells/mm^2 remaining in the Unilateral Intact hemisphere compared to all other groups (a). The difference in the percent of ChAT+ cells/mm^2 remaining between intact and target hemispheres was greater in the Unilateral group compared to the Bilateral group (b). Representative microphotographs of ChAT expression in a coronal slice of the MSDB from an animal with a bilateral lesion (c) and another with a unilateral lesion (d). Microphotographs in this diagram were taken at a total magnification of 40x at approximately Bregma 0.25. * indicate groups that were significantly different from all other groups within each analysis.

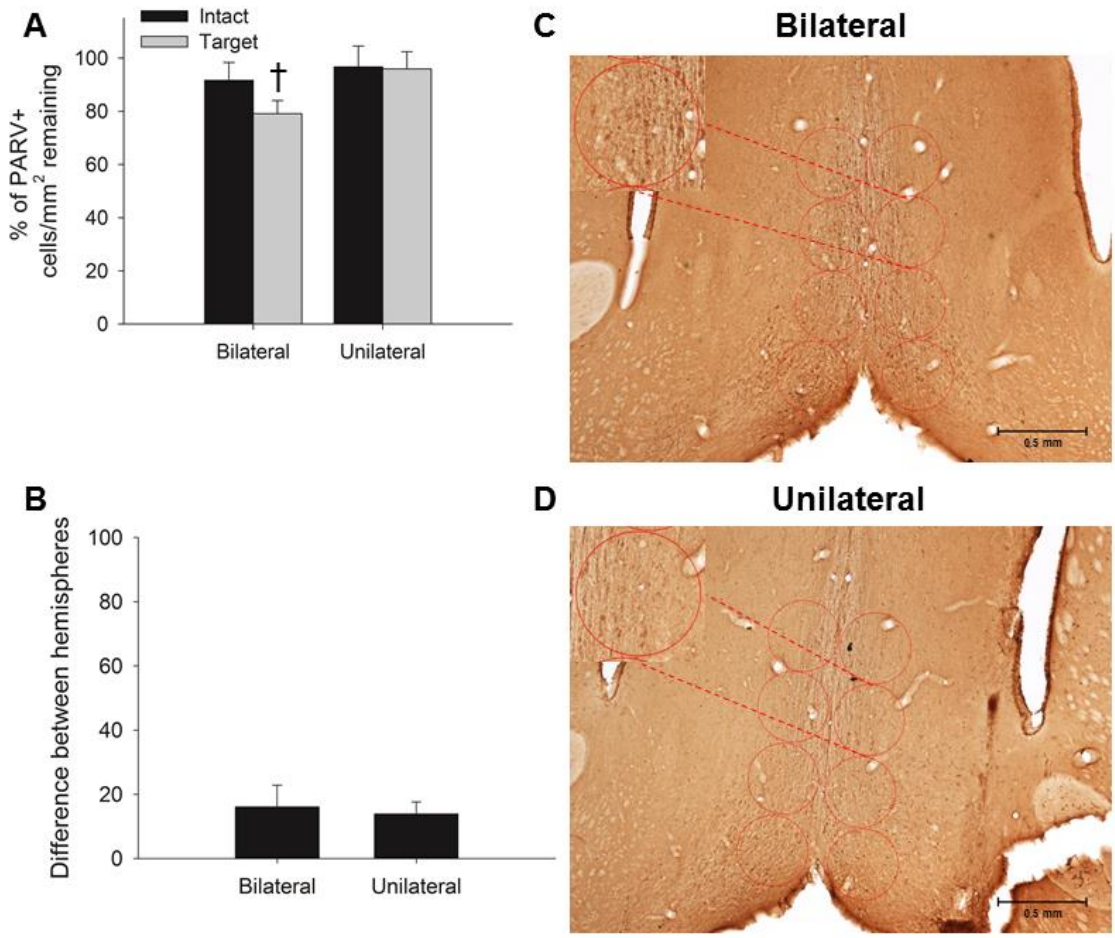


Figure 24. The percent PARV remaining in animals with bilateral (n = 7) and unilateral (n = 8) lesion compared to animals with sham lesions in experiment 3. There was a significant difference in the percent of PARV+ cells/mm^2 remaining between the intact and target hemisphere in the Bilateral group but not the unilateral group (a). Animals with bilateral and unilateral lesions had a similar difference in the percent of PARV+ cells/mm^2 between intact and target hemispheres (b). Representative microphotographs of PARV expression in a coronal slice of the MSDB from an animal with a bilateral lesion (c) and another with a unilateral lesion (d). Microphotographs in this diagram were taken at a total magnification of 40x at approximately Bregma 0.25. † indicates that Bilateral Intact and Unilateral Target were significantly different.

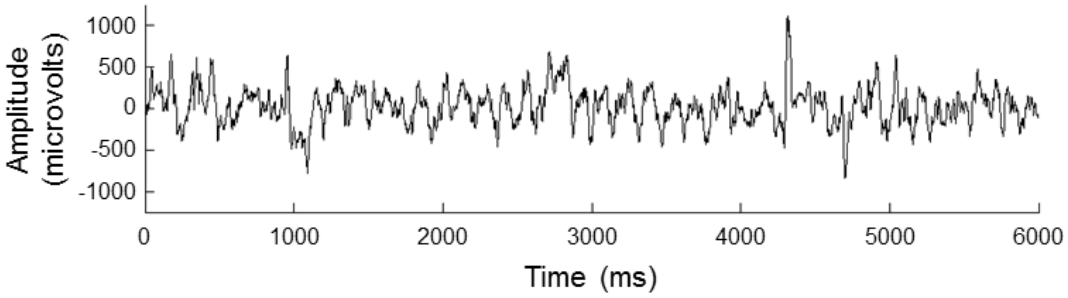


Figure 25. An example of theta activity from an animal with a sham lesion.

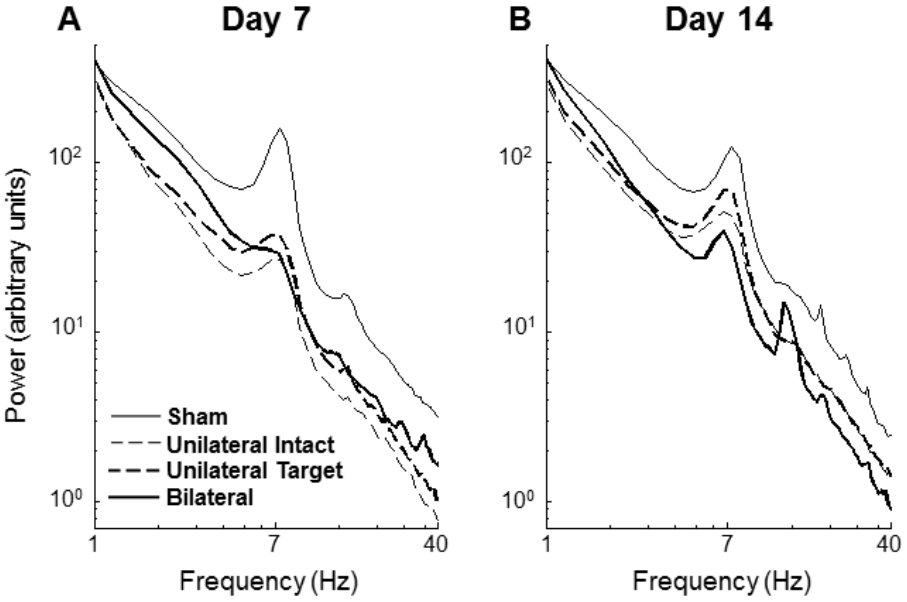


Figure 26. Power spectrum showing power for each frequency for Day 7 (a) and Day 14 (b). Peaks during the frequency range for theta indicate a higher power. Power spectrums from the intact and target hemispheres in animals with sham and bilateral lesions were consolidated into one spectrum. Sham n = 5, Unilateral n = 9, Bilateral n = 8.

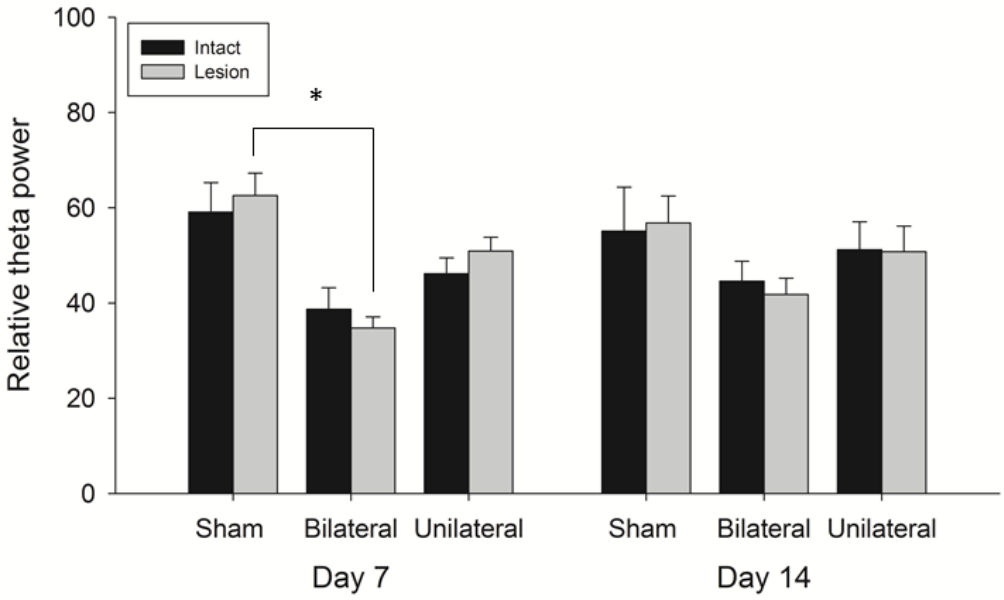


Figure 27. The loss of cholinergic MSDB neurons results in a transient decrease in relative hippocampal theta power. On Day 7, theta power in the target hemisphere differed between animals with a sham and bilateral lesions (left). No differences were detected on Day 14 (right). Sham n = 5, Bilateral n = 8, Unilateral n = 9.

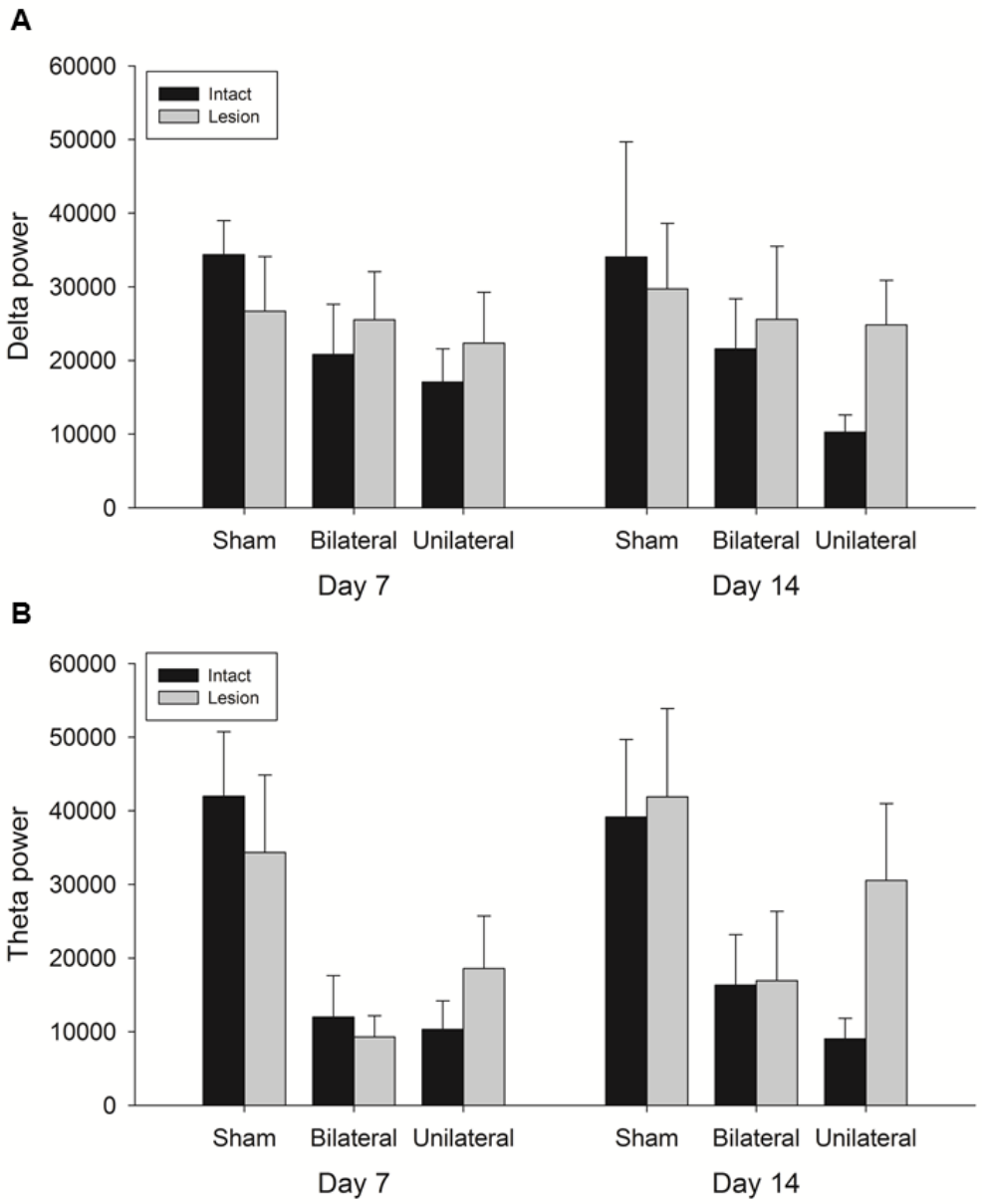


Figure 28. Delta power did not differ between groups (a). However, theta power in animals with sham lesions differed from animals with bilateral and unilateral lesions (b).

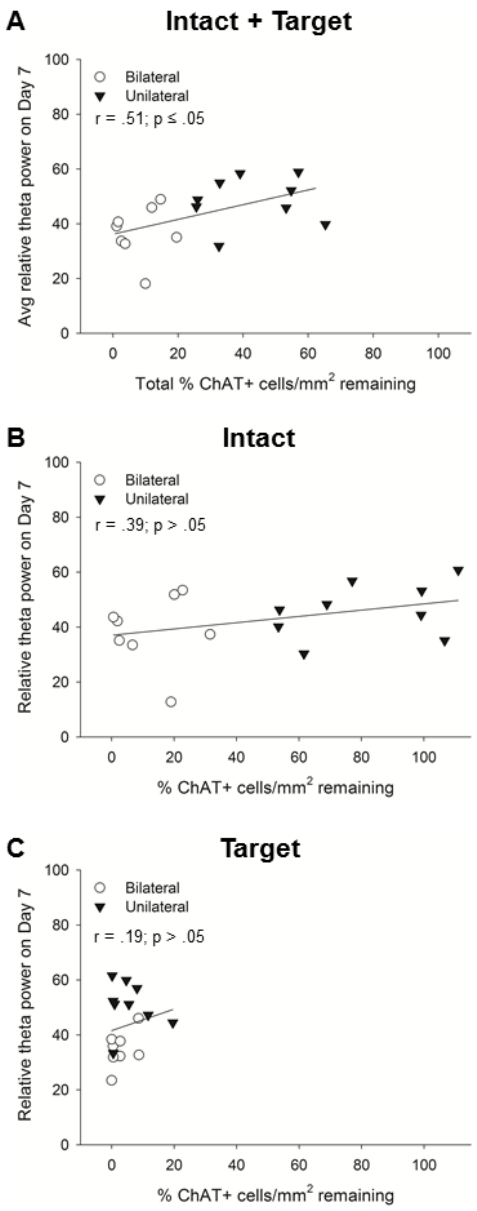


Figure 29. The extent of the lesion predicts relative theta power. There was a significant relationship between the total number of ChAT+ cells/mm^2 remaining and relative theta power across hemispheres (a). Conversely, when the variables for the intact (b) and target (c) hemisphere were considered separately, there were no significant relationships found.

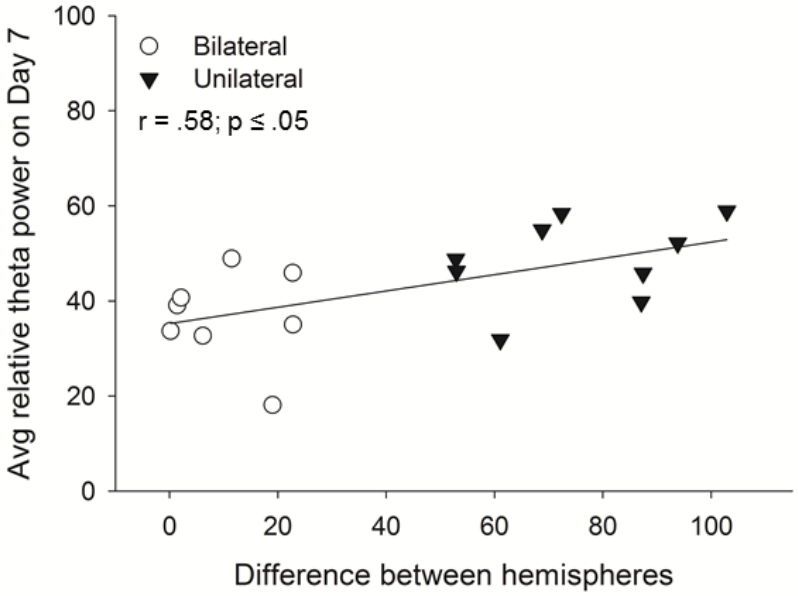


Figure 30. There was a significant relationship between the difference in total number of ChAT+ cells/mm^2 between hemispheres and relative theta power.