Mediation of memory permanence: Neural activity versus protein synthesis

by

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Abstract

Memory research has progressed substantially in the recent past, yet the mechanisms responsible for memory formation and permanence are still not well established. Much of this research has been focused on the events that occur at the level of the cell, leading to many molecular theories about the process of learning and memory. Currently, the prevailing explanation for how long-term memories are established is the *de novo* protein synthesis hypothesis, which suggests that new proteins are required to stabilize a memory trace. This hypothesis is primarily supported by studies that demonstrated memory deficits in experimental animals treated with protein synthesis inhibitors (PSIs). Recent work, however, has shown that PSIs suppress spontaneous and evoked neural activity in the hippocampus, suggesting that the memory impairments caused by protein synthesis inhibition may actually be attributed to altered neural activity. If PSIs, such as anisomycin (ANI), function by silencing neural activity, then their effects on behaviour would be functionally similar to that of drugs which temporarily inactivate neural tissue. In this thesis I tested the effect of pre-training microinfusions of ANI, tetrodotoxin (TTX), muscimol (MUSC), or a vehicle (PBS) on unconditioned fear, as well as on short- and long-term memory. TTX and MUSC are commonly used to temporarily inactivate neural tissue by blocking sodium channels and by binding to $GABA_A$ receptors, respectively, and act as positive controls for the neural suppressive effects of ANI. I injected one of these four solutions bilaterally into the basolateral amygdala of rats prior to an unconditioned fear test and training on an auditory fear conditioning task. All animals were then tested for short- and long-term memory of the fear conditioning task as measured by active freezing. The results of this

study indicate that infusions of ANI, TTX, and MUSC result in significantly less freezing to the auditory cue at both short-term (2 hour) and long-term (24 hour) time points as compared to the PBS group. This indicates that ANI can disrupt both short- and long-term memory to the same extent as other neural inactivation techniques. These results challenge the *de novo* protein synthesis hypothesis of memory and suggest it must be carefully reexamined.

Preface

This thesis is an original work by Lisa A. Rimstad. The research projects, of which this this is a part, received research ethics approval from the University of Alberta Research Ethics Board, Project Name “Cellular and Network Dynamics of Neo- and Limbic-Cortical Brain Structures”, AUP00000092.

Some of the research conducted for this thesis forms part of an internal research collaboration, led by Professor Clayton T. Dickson together with Professor Dallas Treit at the University of Alberta.

This thesis is an original work by Lisa A. Rimstad. No part of this thesis has been previously published.

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I would like to extend a special thank you to my supervisor Clayton Dickson for his guidance, support, and humor throughout my graduate school experience. Any time I thought that my experiments could not possibly work out, a brief chat with Clay would convince me otherwise. It is a special skill of his to see the possibilities in every challenge and pass that confidence on to his trainees. In addition to his support within the lab, Clay encourages his students to balance their science lives with their personal lives and to have fun. This led to many instances of late night board gaming, beer drinking, and skiing, resulting in a happy, cohesive lab family that I'm very grateful for.

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AMPA	Alpha-amino-3-hydroxy-methyl-4-isoxazolepropionic acid
ANI	Anisomycin
ANOVA	Analysis of variance
AP	Anterior-posterior
ATP	Adenosine tri-phosphate
BDNF	Brain-derived neurotrophic factor
BLA	Basolateral amygdala
Ca^{2+}	Calcium ion
cAMP	Cyclic adenosine monophosphate
CEA	Central amygdala
CREB	cAMP-response binding element
CS	Conditioned stimulus
dB	Decibels
DREADDs	Designer receptors exclusively activated by designer drugs
DV	Dorsal-ventral
EPM	Elevated plus maze
GABA	Gamma-aminobutyric acid
K^{+}	Potassium ion
kHz	Kilohertz
LA	Lateral amygdala
LTP	Long-term potentiation
mA	Milliamp

Mg^{2+}	Magnesium ion
ML	Medial-lateral
mRNA	Messenger RNA
MUSC	Muscimol
Na^{+}	Sodium ion
NMDA	N-Methyl-D-aspartate
PBS	Phosphate buffer solution
PKA	Protein kinase A
PSI	Protein synthesis inhibitor
RNA	Ribonucleic acid
SEM	Standard error measurement
SPW-R	Sharp-wave ripple
TTX	Tetrodotoxin
US	Unconditioned stimulus
ZIP	Zeta inhibitory peptide

Introduction

Memory

Memory, simply put, is the ability to retain information from prior experiences and use that information to modify future behaviour. This is arguably a critical function for animals to survive and yet it is a process that remains elusive. Although it is clear that memory has been a subject of much interest for thousands of years, it has only been in the last two centuries that people began to test memory empirically. In 1885, Herman Ebbinghaus documented the first forgetting curve by testing his own memory for lists of nonsense syllables. From his research, Ebbinghaus determined that retention is better when there is a temporal gap between study sessions and when material is recited multiple times (Lechner et al., 1999). Ebbinghaus's work was continued on by Georg Muller and Alfons Pilzecker; who are credited with coining the term 'consolidation' to describe how memories are strengthened in the time after learning (Muller and Pilzecker, 1890, as cited in Lechner et al., 1999). The idea that memory requires time to become stronger corresponded well to clinical cases of amnesia following a traumatic head injury or electroconvulsive shock (Lewis and Maher, 1965; Yarnell and Lynch, 1970; Sara and Hars, 2006), and also supported the idea that memory can be divided into short-term and long-term phases (James, 1890; Brown, 1964). From this early research the term consolidation became known as the process by which newly encoded information transitions from its initial labile state into a stable long-term memory (Lechner et al., 1999; McGaugh, 2000).

One widely accepted theory regarding consolidation is that it relies on *de novo* protein synthesis. According to this explanation, long-term memories require the

production of messenger RNA (mRNA) and new proteins, whereas short-term memories do not (Davis and Squire, 1984; Schafe and LeDoux, 2000). Support for the *de novo* protein synthesis hypothesis comes mainly from studies that demonstrated the deleterious effects of protein synthesis inhibitors (PSIs) on long-term, but not short-term, memories in experimental animals (Figure 1; Schafe et al., 1999; Schafe and LeDoux, 2000). Of particular significance is a review of the protein synthesis literature by Davis and Squire (1984), which concluded that short-term memories were unaffected by PSI use and that such substances did not cause other damaging effects. Davis and Squire's report is frequently cited as evidence of the protein synthesis dependence of memory, despite the fact that other researchers were reporting confounding effects of PSIs at the time (Barraco and Stettner, 1976; Flood et al., 1977; Martinez et al., 1981). Recently, the utility of translational inhibition has been called into question again however, with many researchers reporting on the confounding neurobiological effects associated with PSI use (Routtenberg and Rekart, 2005; Gold, 2008; Rudy, 2008b).

Neurobiological substrates for memory traces

In order to explain the premise of the *de novo* protein synthesis hypothesis, it is necessary to have an understanding of the proposed neurobiological substrate of memory traces. The Canadian psychologist Donald Hebb is typically credited with first theoretically describing the mechanism by which memories may be stabilized at the cellular level. Hebb proposed the idea that groups of cells, known as cell assemblies, were activated by a stimulus and that the short-term memory for the stimulus was reliant on this reverberating activity (Hebb, 1949). He further theorized that if this activity was persistent enough, the cells would become more strongly connected to one another and a

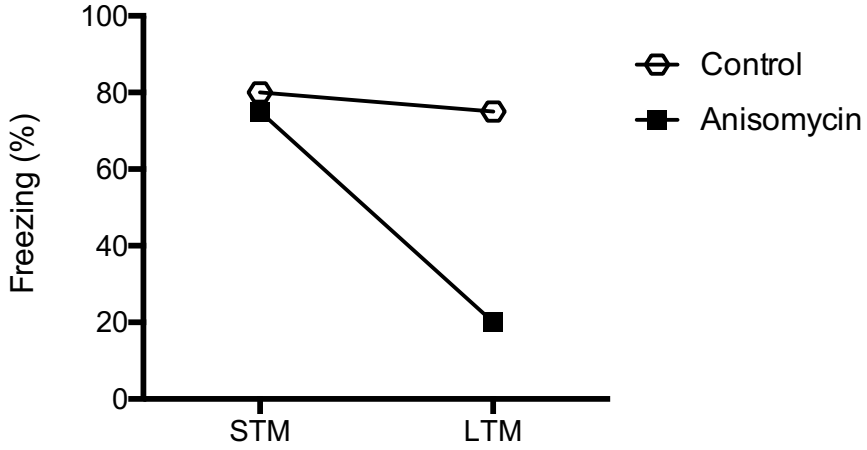

Figure 1: Separation of short- and long-term memory with anisomycin. This figure demonstrates the type of data used to support the *de novo* protein synthesis hypothesis of memory. Animals treated with the protein synthesis inhibitor anisomycin show similar amounts of freezing, indicative of memory, to the control animals at the short-term memory test (STM). At the long-term memory test (LTM), however, the animals treated with anisomycin have disrupted memory as compared to the control animals. The researchers then conclude that long-term memory must require protein synthesis but that short-term memory does not. Adapted from Nader and Hardt (2009).

stable memory could be formed (Hebb, 1949). This phenomenon is often summed up in the phrase “cells that fire together, wire together”.

The mechanisms by which neurons may strengthen their connections were initially investigated using simple models of learning in several invertebrate species, most notably the sea slug *Aplysia.* This research demonstrated that persistent stimulation of a pre-synaptic neuron leads to changes in the responsiveness of the post-synaptic neuron that could be mediated by alterations at the pre- or post-synaptic level (or both) (Bear et al., 2007; Squire et al., 2008). In a parallel approach taken in mammalian tissue, researchers Timothy Bliss and Terje Lømo discovered that applying short, high-frequency pulses of electrical stimulation to the perforant path of the hippocampus resulted in strengthening of the synaptic connections for up to several hours (Bliss and Lømo, 1973). This increase in synaptic efficacy was termed long-term potentiation (LTP) and is often considered to be the cellular analog of memory (Bear et al., 2007). Researchers have now intricately studied LTP using tetanic stimulation in reduced preparations, as well as in anaesthetized and non-anaesthetized animals (Barrionuevo and Brown, 1983; Huang et al., 1994; Nguyen et al., 1994). Other studies have additionally shown that learning stimulates LTP-like states in the brain, further supporting the relationship between this process and memory (McKernan and Shinnick-Gallagher, 1997; Rogan et al., 1997). This work contributed to the development of the idea that memory permanence can be explained by maintained changes in synaptic efficacy.

Molecular mechanisms of LTP

Like memory, it is suggested that two stages of LTP exist, early-LTP and late-LTP (Rudy, 2008a; Squire et al., 2008). In the typical model of LTP induction, a stimulus

will first activate the presynaptic neuron, causing the release of the excitatory neurotransmitter glutamate. Glutamate will bind to α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors on the post-synaptic neuron, which will then allow a mixed cationic (Na^+ and K^+) conductance. If this stimulation is persistent or strong enough, the membrane will depolarize sufficiently to allow relief of a Mg^{2+} block of another ionotropic glutamate receptor complex, the N-Methyl-D-aspartate (NMDA) receptor. Importantly, the cationic conductance allowed via this channel includes Ca^{2+}. The resulting influx of calcium not only acts to strongly depolarize the post-synaptic membrane, but also acts as an important second-messenger. One of the actions of Ca^{2+} is to elicit a signaling cascade that results in an increase in the efficiency and number of AMPA receptors present, thus increasing the receptivity of the post-synaptic membrane to glutamate. These modifications allow for greater post-synaptic effects, even with similar concentrations of pre-synaptic glutamate release (Schafe et al., 2001; Bear et al., 2007; Squire et al., 2008). This initial increase in synaptic excitability, or early-LTP, can be only temporary. If the suprathreshold stimulation continues, however, other signaling cascades are initiated which engage nuclear responses that result in a longer lasting increase in synaptic efficacy (late-LTP). This cascade can involve the production of cyclic adenosine monophosphate (cAMP), which in turn activates other proteins, such as protein kinase A (PKA). Once activated, PKA may travel to the cell nucleus and engage transcription factors, such as cAMP response element binding protein (CREB). CREB will then induce the transcription of genes, for example cAMP response element (CRE)-mediated genes, ultimately leading to new protein synthesis. The newly synthesized proteins are utilized for modification to, or addition of, synapses that allow for a stronger

connection between the pre-synaptic and post-synaptic neurons involved in the memory trace (Kandel et al., 2000; Schafe et al., 2001; Bear et al., 2007; Squire et al., 2008). Although this is not the only pathway by which transcriptional and translational activation might affect synaptic plasticity in a neuron, it is a good example of how protein synthesis may underlie the development of late-LTP.

The role of protein synthesis in memory

According to the *de novo* protein synthesis hypothesis, long-term memories and late-LTP require gene transcription and new materials but short-term memories and early-LTP do not (Nader and Hardt, 2009). Research investigating the role of novel proteins in memory consolidation has relied heavily on PSIs, which are drugs that block the translation of mRNA and prevent the synthesis of new proteins (Garreau de Loubresse et al., 2014). Early research began using systemic injections of PSIs into a variety of animal subjects and showed that learning and/or memory was impaired by these drugs (Barondes and Cohen, 1967b, 1968; Watts and Mark, 1971). Due to many of the issues associated with systemic injections, discussed in detail in the next section of this thesis, researchers soon began using area-specific local injections of PSIs (Schafe et al., 1999; Debiec et al., 2002). These studies also supported the idea that new protein production is a critical aspect of memory consolidation. Other researchers used genetic or molecular techniques to target specific proteins, such as cAMP, PKA, or CREB; all of which had detrimental effects on long-term memory (Guzowski and McGaugh, 1997; Bourtchouladze et al., 1998; Schafe et al., 1999; Quevedo et al., 2004). During this same time frame, it was demonstrated that inhibiting protein synthesis completely, or suppressing the function of CREB or cAMP, also disrupts late-LTP (Huang et al., 1994;

Nguyen et al., 1994). The general consensus of this work is that long-term memory requires changes to the properties of neurons that are reliant on gene transcription and new protein synthesis, similar to process of late-LTP (DeZazzo and Tully, 1995; Dudai, 2004). There are opposing theories, however, which suggest that new protein products are steadily required by the cell and that post-translational modification of proteins may be sufficient for some long-term memories (Routtenberg and Rekart, 2005). Importantly, many of the studies that support the *de novo* protein synthesis hypothesis relied on PSIs, and a number of researchers continue to use PSIs for this purpose (Fukushima et al., 2014; Ryan et al., 2015; Roy et al., 2016).

Problems with the *de novo* protein synthesis hypothesis

Issues with early studies using PSIs

Many of the studies upon which Davis and Squire (1984) based their review provide inconsistent time courses for the disruption of memory by PSIs which do not correspond to those reported in more recent studies. For instance, Barondes and Cohen (1967b, 1968), as well as Squire and Barondes (1972), found that blocking protein synthesis after learning had no effect on future recall, which contradicts the current method of applying PSIs immediately after training (Schafe and LeDoux, 2000; Debiec et al., 2002; Remaud et al., 2014). In other studies, the researchers found that the PSI cycloheximide caused an impairment in both short- and long-term memory (Davis et al., 1976), or that long-term memory was initially impaired but recovered days after the drug injection (Squire and Barondes, 1972; Oliver et al., 1979). Multiple researchers also discovered that the memory deficits imparted by treatment with PSIs could be attenuated with stimulant drugs or enhanced with depressant drugs, a phenomenon that was

reviewed extensively by Martinez et al. (1981) and Flood et al. (1977). More recent work has revisited this phenomenon and demonstrated that alterations in neurotransmitter and neuromodulator release may be partly responsible for such effects (Canal et al., 2007; Qi and Gold, 2009). Such early reports also used PSIs such as puromycin, acetoxycycloheximide, and large doses of systemic cycloheximide that are now known to result in extreme malaise and/or death, as well as many other confounding effects, besides disrupting protein synthesis (Barondes and Cohen, 1966; Cohen et al., 1966; Barondes and Cohen, 1967b, a, 1968; Watts and Mark, 1971).

Another issue in interpreting these prior results is the phenomenon of overshadowing (Biedenkapp and Rudy, 2009; Sutherland et al., 2010; Sparks et al., 2011). This has been specifically shown for contextual fear conditioning, which typically depends on the hippocampus but can also occur through the involvement of extra-hippocampal regions (Maren et al., 1997; Biedenkapp and Rudy, 2009; Sutherland et al., 2010; Sparks et al., 2011). Overshadowing occurs when the learning and memory phases of the contextual paradigm are run with mismatched hippocampal activity states, for example, when the hippocampus is inactivated at the time of learning but is active at the time of testing. In this example, memory expression is impaired, but surprisingly re-appears following re-inactivation of the hippocampus (Sparks et al., 2011). This suggests that there is interference between the hippocampus and these extra-hippocampal areas, which causes the deficits in memory expression. Although this phenomenon has been shown specifically for the hippocampus, it is reasonable to assume that it may occur in other brain structures as well. For instance, there is a dominant route by which auditory fear memory is processed in the amygdala, however, there are redundant pathways as

well (Pape and Pare, 2010; Duvarci and Pare, 2014). This suggests that inactivation prior to training can sometimes result in a different behavioural response than post-training inactivation (Pape and Pare, 2010; Akagi Jordao et al., 2015). These results are suggestive for prior studies that may have had misguided interpretations of the actual neurobiological action of PSIs on consolidation (Davis et al., 1976).

Confounding effects of the PSI anisomycin

The current most commonly utilized PSI is anisomycin (ANI), and as such has been the focus of many of the recent investigations regarding the efficacy of translational inhibition (Gold, 2008; Rudy, 2008b). As a result of these inquiries, many confounds associated with the use of ANI have been exposed, including massive fluctuations in the extracellular concentrations of several neuromodulators (Canal et al., 2007; Qi and Gold, 2009), gene superinduction (Radulovic and Tronson, 2008) and apoptotic cell death (Monaghan et al., 2014). It is reasonable to consider that alterations in neurotransmitter release or immediate early gene expression, and especially neuronal death, could themselves cause impairments in the expression of memory. However, another effect of PSI application that may override these other confounding outcomes is the ability to severely depress, and even eliminate, electrical activity (Sharma et al., 2012). In this study, Sharma et al. (2012) demonstrated that infusions of ANI or cycloheximide resulted in the suppression of spontaneous and evoked hippocampal activity. Evidence for the suppression of neural responses following ANI application has been shown by others as well, both in the motor cortex (Kleim et al., 2003) and in the hippocampus (Shires et al., 2012) of anaesthetized rats. However, Kleim et al. (2003) did not conclude that PSIs may affect neural activity, but rather that the motor map must be reliant on ongoing protein

synthesis. The research done by Sharma et al. (2012) showed that the degree of neural suppression was positively correlated to the extent of protein synthesis inhibition as determined by amino acid incorporation. As a result of this relationship the authors concluded that normal neuronal function is likely reliant on protein synthesis and thus the behavioural deficits caused by ANI may be due to the suppression of neural activity rather than the inhibition of protein synthesis *per se*.

Evidence for neural activity in memory consolidation

The effect of PSIs on electrophysiological processes is consequential, as there is overwhelming evidence for the role of neural activity in memory consolidation both during learning and afterwards during rest or sleep states (Oyanedel et al., 2014; Watson and Buzsaki, 2015). Indeed, many of the earliest studies of consolidation used electroconvulsive shock, a process which seriously disrupts normal activity, to determine the critical time window for memories to become stable (Lewis and Maher, 1965). Following this, many researchers began using microinfusions of local anaesthetics, such as lidocaine, or the sodium channel blocker tetrodotoxin in order to disrupt neural activity in specific regions of the brain, resulting in amnesia for learned events (Ambrogi Lorenzini et al., 1999; Sacchetti et al., 1999). Research has also shown that particular types of oscillatory brain activity, such as the slow oscillation during slow-wave sleep and sharp-wave ripple (SPW-R) events, are increased after a learning event (Skaggs and McNaughton, 1996; Eschenko et al., 2006; Oyanedel et al., 2014). Further studies have demonstrated that memory may be improved by enhancing the slow oscillation (Marshall et al., 2006; Binder et al., 2014) or disrupted by interfering with SPW-Rs after learning (Girardeau et al., 2009; Ego-Stengel and Wilson, 2010). It has been suggested that the

function of SPW-R events may be coordinated neuronal replay, which is the patterned reactivation of the same neuronal ensembles activated by learning in a period after training (Skaggs and McNaughton, 1996; Diba and Buzsaki, 2007). This research provides support for an activity-dependent explanation for the consolidation of memories during SPW-R events that occur during rest or sleep (Ramadan et al., 2009; Carr et al., 2011). Recent advances using the technique of deep brain stimulation have also provided evidence that altering the electrical connections between medial temporal lobe networks can improve spatial memory ability in both rats (Hescham et al., 2016) and humans (Suthana et al., 2012).

In addition to network oscillations, it is becoming increasingly apparent that modulations which were originally perceived to be strictly molecular in nature, such as the effect of CREB on memory, may exert their effects by modulating activity at the neuronal level (Lopez de Armentia et al., 2007; Zhou et al., 2009). For instance, studies have shown that increasing neuronal CREB expression prior to training leads to an enhanced memory for a learning event and that decreasing CREB has the opposite effect on memory (Han et al., 2007; Yiu et al., 2011). This work was originally interpreted to highlight the importance of the transcription factor in memory. However, the results of other research, which has shown that increasing neuronal CREB expression leads to higher excitability (Dong et al., 2006; Lopez de Armentia et al., 2007; Viosca et al., 2009), suggests that these behavioural results may be explained by increased neuronal activity. Similar outcomes have occurred with other molecules that were purported to be critical for memory consolidation, such as brain-derived neurotrophic factor (BDNF) (Suzuki et al., 2011). Although initially suggested to play a specific role in promoting

memory, later research showed that increasing BDNF promotes neuronal excitability, suggesting that the observed memory effects may also be explained by increased activity (Desai et al., 1999; Binder et al., 2001). Further support for this concept comes from a study in which several techniques were used to manipulate neuronal excitability, including modification of ion channels, DREADDs (designer receptors exclusively activated by designer drugs), and optogenetics (Yiu et al., 2014). The results of the work by Yiu et al. (2014) showed that no matter how increased neuronal excitability is achieved, the outcome is enhanced memory.

The amygdala and fear behaviour

I chose to use an auditory fear conditioning paradigm to investigate the action of ANI on short-term memory due to the specific dependence of this task on the amygdala, as well as its frequent use to support the *de novo* protein synthesis hypothesis (Schafe et al., 1999; Nader et al., 2000; Schafe and LeDoux, 2000). The amygdala is a useful target for studying simple, associative memory that is formed via classical conditioning. In classical fear conditioning, the presentation of a neutral stimulus, the conditioned stimulus (CS), is paired with an aversive stimulus, the unconditioned stimulus (US). This pairing causes future presentations of the CS to provoke behaviours normally elicited by the US. One common form of this training is auditory fear conditioning, a paradigm that uses the co-occurrence of an auditory cue (the CS) alongside a mild foot shock (the US) to create an association of fear with the previously neutral tone. The association of the CS with fear is typically measured by the amount of time spent freezing, defined as complete immobility excluding respiratory movement, which is an innate fear response in rodents (LeDoux, 2000; Curzon et al., 2009). Although fear conditioning represents a simplified

version of learning, it is commonly used due to the rapid acquisition of the response, the ability to discretely control the variables, and the specificity of the memory to the amygdala (LeDoux, 2000; Pape and Pare, 2010; Ehrlich and Josselyn, 2015; Josselyn et al., 2015).

Structure and function of the amygdala nuclei

The amygdala is composed of several distinct nuclei that vary by cellular composition, as well as by their connectivity with other neural structures both within the amygdala and throughout the brain (Knapska et al., 2007; Pape and Pare, 2010). Many techniques have been used to determine exactly which amygdala nuclei are critical for the acquisition and expression of an auditory fear memory, including lesioning and temporary inactivation (Phillips and LeDoux, 1992; Muller et al., 1997; Sacchetti et al., 1999). From this work it has been determined that the lateral and basal nuclei, known together as the basolateral amygdala (BLA), are critical for the acquisition and consolidation of auditory fear conditioning (Muller et al., 1997; Sacchetti et al., 1999). It is suggested that the association of the CS-US occurs within the lateral amygdala (LA), which then projects to the basal nucleus in order to reach the medial central nucleus (CEA) for output to the brainstem structures responsible for freezing behaviour (Figure 2; LeDoux, 2000; Schafe et al., 2001; Pape and Pare, 2010). The role of this amygdala pathway is further supported by the induction of LTP-like synaptic plasticity in this structure after training (McKernan and Shinnick-Gallagher, 1997; Rogan et al., 1997). There is an additional pathway by which the LA may reach the medial CEA - through the intercalated cell mass - although the basal nuclei is considered to be the dominant

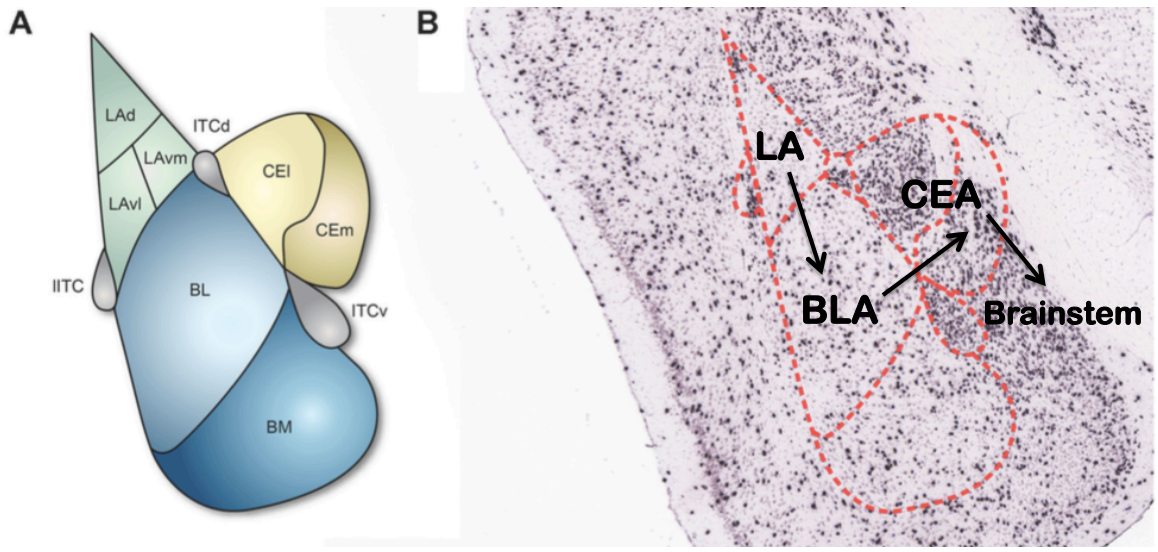

Figure 2: Rat amygdala anatomy as it relates to fear conditioning. A) A schematic diagram of the rat amygdala showing the different nuclei. LA: lateral amygdala, can be subdivided into dorsal (LAd), ventromedial (LAvm), and ventrolateral (LAvl) components. BL: basolateral nucleus. BM: basomedial nucleus. CEA: central amygdala, can be subdivided into lateral (CEl) and medial (CEm) components. Also shown are the intercalated cell masses (ITCs). **B)** The arrows in this figure depict the dominant pathway for auditory fear conditioning. Auditory information (CS) and foot shock information (US) reach the LA first, which projects to the BLA. The BLA then projects to the medial CEA, from which the information is sent to the brainstem areas in control of freezing behaviour. The researchers used *in situ* hybridization for a GABA synthesizing enzyme, glutamic acid decarboxylase (GAD67), to visualize the amygdala nuclei in this image. Modified from Lee et al. (2013).

connection (Pape and Pare, 2010). This specificity contributes to the rationale behind using the amygdala to test the effect of PSIs on memory for a fear conditioning event.

Objectives and hypothesis

The purpose of this thesis is to further investigate the effect of intracerebral ANI on neural activity by comparing the behavioural deficits caused by ANI to ones caused by temporary inactivation of the same brain region. This study employed the use of two reversible inactivating drugs, tetrodotoxin (TTX) and muscimol (MUSC), as positive controls for neural suppression. TTX is a sodium channel blocker that inactivates neural tissue, affecting both cell bodies and axonal transmission (van Duuren et al., 2007). MUSC is a type A gamma-aminobutyric acid ($GABA_A$) agonist that inactivates neural activity at the level of the soma without affecting fibers of passage (van Duuren et al., 2007). The target of the drug microinfusions was the basolateral amygdala (BLA), due to the well-documented involvement of this structure in fear memory (Muller et al., 1997; Sacchetti et al., 1999). In this study I explored the effect of intraamygdalar ANI on unconditioned fear, as well as on short- and long-term memory for a conditioned fear event. My hypothesis was that ANI would affect the expression of fear behaviour to the same extent as neural inactivation. The results indicate that ANI, like MUSC and TTX, disrupted both short- and long-term memory for a conditioned fear event. Overall, these results provide support for the idea that translational inhibition produces its amnestic actions via suppression of neuronal activity rather than acting on protein synthesis specifically.

Materials and Methods

Surgery and handling

Male Sprague-Dawley rats, weighing between 200-250 g, were anesthetized in a gas chamber with isoflurane gas (4% induction, 1.5% maintenance in 67% N_2O and 33% O_2 gas). Surgical plane was maintained by delivering isoflurane through a nose cone attached to the stereotaxic equipment (Kopf Instruments, Tujunga, CA, USA) throughout the procedure. Prior to surgery all animals received injections of 0.9% saline (3 ml, s.c.) to maintain hydration, and bupivacaine (1.5 mg/0.3 ml s.c. near the incision site) as a local anesthetic. Animals were then implanted bilaterally with 22 gauge, 8 mm guide cannulae 1 mm above the basolateral amygdala (BLA; -2.9 mm AP and ±5.1 mm ML relative to bregma; -7.3 mm DV from skull surface) using a brain atlas (Paxinos and Watson, 2007). Cannulae were secured to the skull with dental acrylic and two jeweler's screws. The incision site was closed, carprofen (0.5 mg/0.1 ml s.c.) was administered for reduction of postoperative inflammation, and animals were placed under a heat lamp until they regained consciousness. All animals were allowed to recover for at least four days before being handled for four consecutive days prior to behavioural testing.

General testing procedures

Following handling procedures, each animal was exposed to the conditioning and testing chambers to be used in the fear conditioning paradigm for 5 min each. On the day of testing, animals were infused with 0.5 µl per hemisphere of PBS (4% phosphate buffer solution), ANI (Sigma-Aldrich; 100 µg/µl dissolved in a minimal amount of 1N hydrochloric acid, brought to volume and adjusted to a physiological pH of 7.4 with PBS), TTX (Abcam; 10 ng/µl dissolved in PBS), or MUSC (Sigma-Aldrich; 0.5 µg/µl

dissolved in PBS). Behavioural testing began 30 min after the completion of infusion (Figure 3). The animals were first tested on the elevated plus maze, followed immediately by fear conditioning, and then a short-term cued memory assessment after a 2 h delay. The long-term memory test occurred 24 h after the initial fear conditioning. All testing was conducted between 0800 and 1700 h in a quiet room masked with white noise generators to dampen any extraneous environmental noise.

Elevated plus maze. The testing apparatus is a plus-shaped maze with an open roof, consisting of two 50 cm x 10 cm open arms and two 50 cm x 10 cm enclosed arms, all elevated at a height of 50 cm from the floor. For each trial the animal was placed at the intersection of the four arms and allowed to explore the maze freely for 5 min. All trials were video recorded with only the animal's identification number visible to allow for blind scoring. Four variables were measured: 1) time spent in the open arms; 2) time spent in the closed arms; 3) number of entries into the open arms; 4) number of entries into the closed arms. A rat was considered to have entered an arm only when all four paws were in the respective arm. Proportionately more time spent in the open arms and/or more open arm entries compared to control are indicative of reduced unconditioned fear. For the statistical analyses, I compared the proportion of open arm time, as well as the proportion of open arm entries, between the groups. Proportion of open arm time was calculated by taking the amount of time (s) spent on the open arms divided by the total amount of time spent on the open and closed arms combined. Proportion of open arm entries was calculated by taking the number of entries onto the open arms divided by the total number of entries made onto the open and closed arms.

Auditory fear conditioning. Immediately following exploration of the elevated plus maze, the animal was placed into a metal conditioning chamber (20.0 cm by 30.0 cm wide and 19.0 cm tall) and allowed to explore for a 2 min baseline period. After the 2 min baseline, a speaker played a 5 kHz tone at 75 dB for 29 s, which co-terminated with a 0.65 mA foot shock for 1 s. Following a 60 s delay, this tone-foot shock pairing occurred again for a total of two pairings. The rat then remained in the chamber for an additional 60 s until it was removed and placed back into its home cage. The foot shocks were delivered by a scrambled output shock generator (HSCK1000, Lafayette Instruments) through a grid floor consisting of electrically insulated rods placed 1.75 cm apart. Two test sessions followed conditioning in order to assess for memory of the auditory cue associated with the aversive event. Behaviour was video recorded through a transparent viewing window during conditioning and both testing periods for future scoring.

Cued memory tests. Two hours after conditioning, short-term memory was assessed by playing the same tone used during testing but in a different chamber (20.0 cm by 30.0 cm wide and 26.0 cm tall; composed of colored Plexiglas and one curved wall) which was cleaned using a 5% acetic acid solution to provide a novel scent from that of the 70% ethanol solution used in the conditioning chamber. Following a 2 min baseline period, the tone was played for 2 min with no shock administered at any point. The rat was placed back into its home cage immediately after the cessation of the tone. This procedure was repeated again at 24 h after fear conditioning in order to assess long-term memory.

Scoring. Memory for the conditioned fear paradigm was scored on the basis of the percentage of time spent ‘freezing’: defined as an animal being completely immobile with the exception of respiratory movements. Behaviour was scored every 5 s for

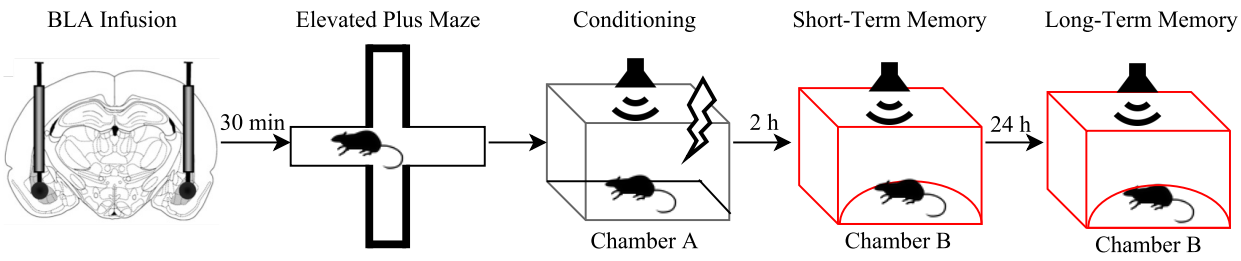

Figure 3: Experimental timeline. All the rats were handled for four consecutive days prior to the onset of behavioural testing. On the day of testing animals received a bilateral infusion of ANI, TTX, MUSC, or PBS into the BLA, and were placed on to the elevated plus maze 30 min later. Fear conditioning commenced immediately after the end of the elevated plus maze trial, where the animals were trained with two CS-US pairings in Chamber A. The animals were tested for short-term memory 2 h after conditioning and long-term memory 24 h after conditioning. Both retention tests occurred in Chamber B where the auditory cue (CS) was played and freezing was assessed as a measure of memory.

'freezing' or 'no freezing'. This method allows for 12 samples per min, from which a total percentage of time spent freezing is obtained. Previous studies have established that freezing is a reliable indicator of fear memory that can easily be scored by an observer who is blind to the treatment groups (Schafe et al., 1999; Curzon et al., 2009).

Euthanasia and histology

Animals were initially anaesthetized in a gas chamber with 4% isoflurane and then injected with urethane (1 ml, 0.67g/ml i.p.) to ensure a deep anesthetic plane before being perfused intracardially with 0.9% saline, followed by 4% formaldehyde. The brain was then removed and placed in a 30% sucrose and 4% formaldehyde solution for at least 48 h before sectioning. Brain tissue was flash frozen with compressed CO_2 before being sectioned coronally into 60 μm slices using a rotary microtome (1320 Microtome; Leica) and mounted on gel-coated microscope slides. All slides were thionin-stained and sections were viewed under a microscope to confirm cannula placements. Only animals with both cannulae in the target area were included in the analysis (Figure 4).

Statistical analyses

The data was analyzed using Prism (GraphPad, San Diego, CA, USA) software. One-way analysis of variance (ANOVA) was used to compare measures between the control (PBS) group and the experimental groups. Post hoc tests were computed using the Tukey HSD. Group sizes for the conditioned fear measures were: PBS (n = 20), ANI (n = 13), TTX (n = 11), and MUSC (n = 10). Three animals had to be excluded from the elevated plus maze data due to video recording errors resulting in the following group sizes: PBS (n = 18), ANI (n = 13), TTX (n = 10), and MUSC (n = 10).

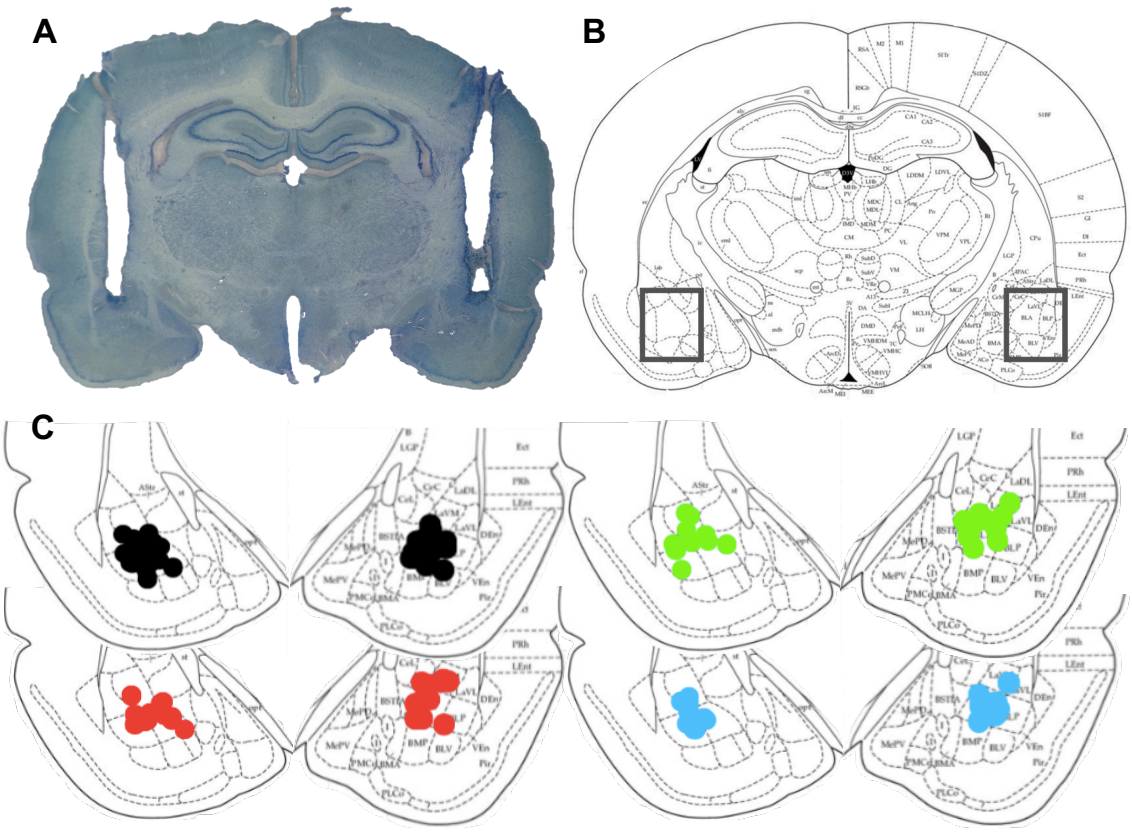

Figure 4: Cannulae placements. Guide cannulae were aimed 1 mm above the basolateral amygdala (BLA) and internal cannulae extended 1 mm past guide. **A)** Representative slice showing cannula tracks. **B)** Grey rectangles surround target area for acceptable guide cannula placements. **C)** Cannula tip placements from rats infused with PBS *(black)*, ANI (*red)*, TTX *(green)*, and MUSC *(blue)*. Figures 4B and 4C adapted from Paxinos and Watson (2007).

For behavioural responding during fear conditioning, I also conducted a mixed-design ANOVA to determine whether there was an increase in freezing during fear conditioning within each of the four groups and to test for interactions between the drug condition and time points. This comparison assessed the percent of time spent freezing prior to the administration of any foot shocks (baseline) to the percent of time spent freezing following the second foot shock (post-shock) within each of the four groups. Bonferroni adjustments for multiple comparisons were made for the repeated measures. Follow up tests across groups used a collapsed ANOVA model with a Tukey HSD correction for post hoc comparisons as previously described.

Results

Effect of intraamygdalar infusions on unconditioned fear

The results for the EPM indicated significant group differences for both open arm time (F (3, 47) = 4.67; p = 0.0062) and open arm entries (F (3, 47) = 6.76; p = 0.0007). As indicated in Figure 5, post hoc tests revealed a significant difference between the PBS and TTX groups for both open arm time (p = 0.0088) and open arm entries (p = 0.0004). There were no significant differences in proportion of open arm time between the PBS and ANI groups (p = 0.23) nor for the PBS and MUSC group comparisons (p > 0.99). Similarly, there were no differences for open arm entries between the PBS and ANI groups (p = 0.46) nor for the PBS and MUSC group comparisons (p = 0.94). These results suggest that unconditioned fear behaviour, as measured by the EPM, is reduced in the TTX group but does not appear to be significantly affected in either the ANI or MUSC groups. Furthermore, the comparable results between these latter groups supports the premise that fear behaviour in general is not affected by the ANI and MUSC infusions.

Effect of intraamygdalar infusions on fear conditioning

As additional measures of general fear responding, I evaluated behaviour throughout the fear conditioning procedure itself, focusing on the unconditioned response to the foot shocks, as well as freezing behaviour post-shock. All animals were assessed for physical reactivity to the shock during fear conditioning. This was scored as flinching, jumping, and/or running across the chamber. All animals across all four conditions displayed a physical response to both foot shocks, suggesting that there were no impairments in the processing of the US.

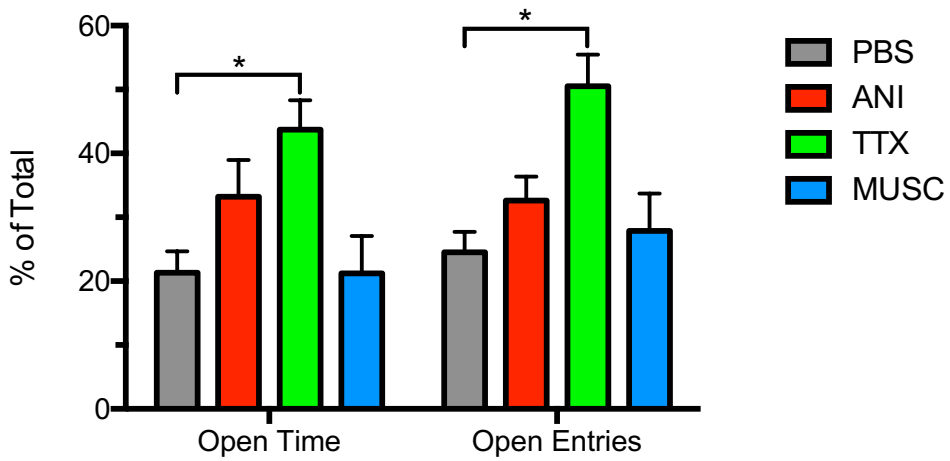

Figure 5: Unconditioned fear behaviour on the elevated plus maze. Open arm exploration displayed as mean percentage (± SEM) of total arm exploration (open / open + closed) of the four groups during a 5 min trial. PBS (n = 18), ANI (n = 13), TTX (n = 10), and MUSC (n = 10). The TTX group spent significantly more time on the open arms than the PBS group and made significantly more entries onto the open arms than the PBS group. *Indicates $p<0.05$.

In order to determine whether the two CS-US pairings had an effect on freezing behaviour directly following the last shock itself, I compared the proportion of time spent freezing at baseline to the proportion of time spent freezing subsequent to the second shock (post-shock) (Figure 6). There were significant main effects for drug condition (F (3, 50) = 3.87, p = 0.0145) and for time point (F (1, 50) = 71.16, p < 0.0001), as well as a significant drug condition and time point interaction (F (3, 50) = 4.70, p = 0.0057). Post hoc analyses showed that there was a significant increase in freezing behaviour from baseline to the post-shock period in the PBS (p < 0.0001), ANI (p < 0.0001), and MUSC (p = 0.018) groups, but not the TTX group (p = 0.20). The main effect for drug condition and the interaction between drug condition and time point can likely be accounted for by the lack of effect of the foot shocks on the freezing behaviour of the TTX group. To confirm this, I examined freezing behaviour during the post-shock period between the groups. The results indicated a significant difference in freezing behaviour across the groups (F (3, 50) = 4.32, p = 0.0087). Post hoc analyses showed that the percentage of time spent freezing during the post-shock period was significantly different between the PBS and TTX groups (p = 0.0089), but not between the PBS and ANI groups (p = 0.31) nor the PBS and MUSC groups (p = 0.082), as shown in Figure 7. This suggests that the PBS, ANI, and MUSC groups all responded fearfully to the foot shocks, but that the TTX group was impaired. Again, these results support the premise that ANI and MUSC do not affect unconditioned responding to painful or fear-inducing stimuli.

Effect of intraamygdalar infusions on short-term and long-term fear memory

Compared to each of the experimental groups, the PBS animals displayed an increased level of freezing behaviour to the auditory cue at both the short-term and long-

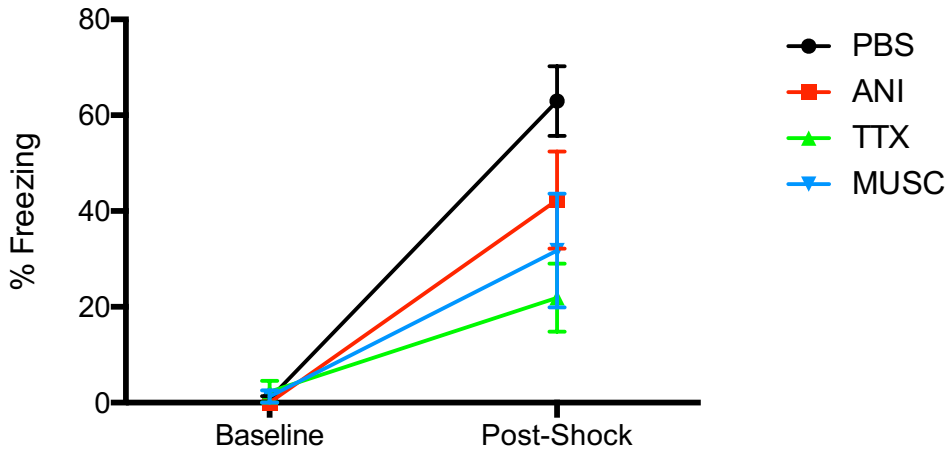

Figure 6: Freezing behaviour during fear conditioning. Mean (± SEM) percent time spent freezing prior to the shock (baseline), and after the two CS-US pairings (post-shock) in each of the four groups: PBS (n = 20), ANI (n = 13), TTX (n = 11), and MUSC (n= 10). The animals in the PBS, ANI and MUSC groups all froze significantly more during the post-shock period than at baseline.

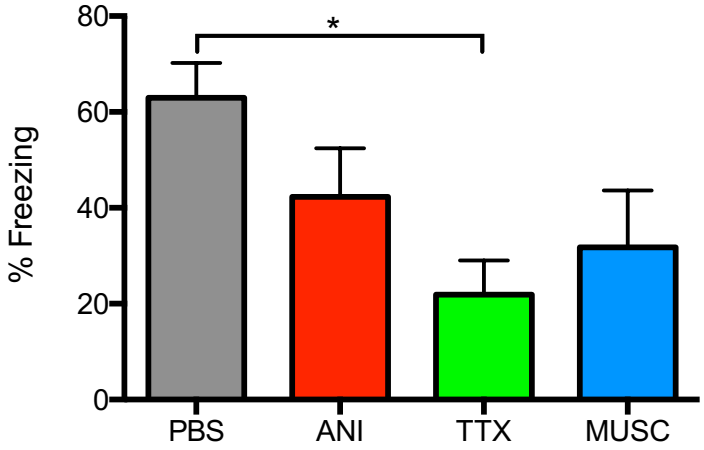

Figure 7: Freezing behaviour during the post-shock period. Mean (± SEM) percent time spent freezing in the 60 s following the two CS-US pairings (post-shock) for the four conditions: PBS (n = 20), ANI (n = 13), TTX (n = 11), and MUSC (n= 10). The animals in the TTX group froze significantly less than the animals in the PBS group. *Indicates $p < 0.05$.

term memory tests. At the short-term test phase, the effect on freezing was significantly different across the four infusion conditions (F (3, 50) = 15.19, $p < 0.0001$). Post hoc analyses revealed that the percentage of time spent freezing was significantly different between the PBS and ANI groups ($p < 0.0001$), as well as between the PBS and TTX groups ($p < 0.0001$) and the PBS and MUSC groups ($p < 0.0001$) (Figure 8). There was no significant difference in freezing between the ANI and TTX groups ($p = 0.86$), the ANI and MUSC groups ($p = 0.91$), nor the TTX and MUSC groups ($p = 0.99$). For the long-term test phase, a similar pattern emerged, with the effect on freezing being significantly different across groups (F (3, 50) = 12.03, $p < 0.0001$) and post hoc tests revealing significant differences between the PBS and ANI groups ($p < 0.0001$), the PBS and TTX groups ($p = 0.0017$), as well as the PBS and MUSC groups ($p < 0.0001$) (Figure 9). Again, there were no differences between the ANI and TTX groups ($p = 0.94$), the ANI and MUSC groups ($p = 0.97$), or the TTX and MUSC groups ($p = 0.78$). These results suggest that auditory fear memory was impaired in the ANI, TTX, and MUSC groups at both the short- and long-term testing points. Such memory deficits would be predicted to occur due to the neural inactivation by TTX and MUSC, however the deficits incurred by the ANI animals at the short-term memory test at are odds with the *de novo* protein synthesis hypothesis.

Relationship between post-shock freezing and memory

Although the ANI and MUSC groups did not show a statistically significant decrease in freezing during the post-shock period in comparison to the control group, both groups did show some evidence of decreased unconditioned fear responding to the shock. It might be expected that future memory performance in terms of a conditioned

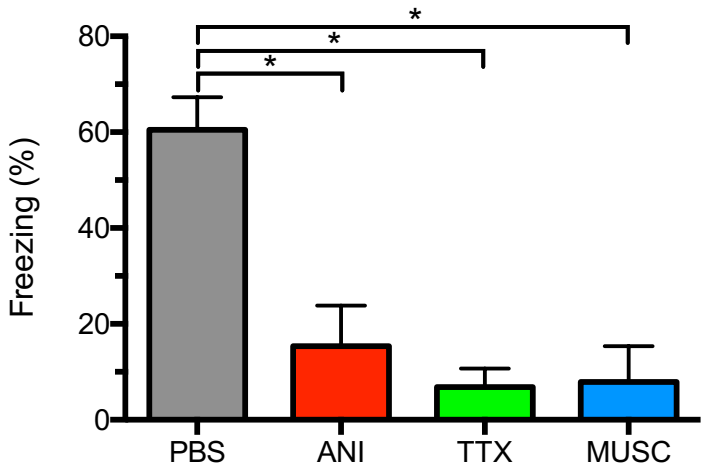

Figure 8: Freezing to the auditory cue at the short-term memory test. Mean (± SEM) percent time spent freezing during a 2 min presentation of the auditory cue for the four conditions during the short-term memory test (2 h after conditioning). PBS (n = 20), ANI (n = 13), TTX (n = 11), and MUSC (n= 10). The ANI, TTX, and MUSC groups spent significantly less time freezing than the PBS control group. *Indicates $p < 0.05$.

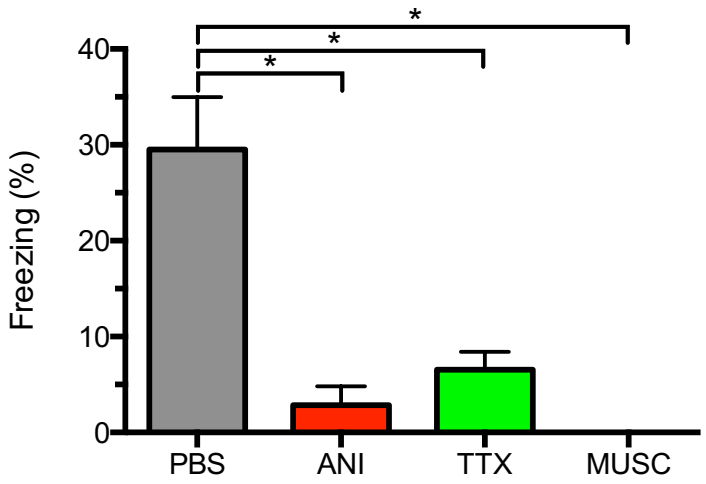

Figure 9: Freezing to the auditory cue at the long-term memory test. Mean (± SEM) percent time spent freezing during a 2 min presentation of the auditory cue for the four conditions during the long-term memory test (24 h after conditioning). PBS (n = 20), ANI (n = 13), TTX (n = 11), and MUSC (n= 10). The ANI, TTX, and MUSC groups froze significantly less than the PBS group. *Indicates $p < 0.05$.

association would have a relationship to the initial responding to the unconditioned stimulus (shock), and that any decrease in memory measures of the experimental groups might be explained by a reduction in unconditioned fear responding. Using regression analyses, I determined that a significant linear relationship did exist between the measures of post-shock and short-term freezing in the control (PBS) group (F (1, 18) = 19.95, p = 0.0003; R^2 = 0.526; Figure 10A). However, using the resulting mathematical relationship (Y = 0.6757*X + 17.96, where X is the percentage of time spent freezing during the post-shock period and Y is the percentage of time spent freezing at the short-term test) to estimate the expected short-term freezing levels in the experimental groups based on their post-shock freezing, we found that short-term freezing measures were much lower than expected (Figure 10B). Furthermore, no significant linear relationship existed between initial fear responding to the shock and the short-term responding to the cue in any of the experimental groups (Figure 10C-E). Thus, it is unlikely that memory disruptions are caused by a lack of unconditioned fear responding.

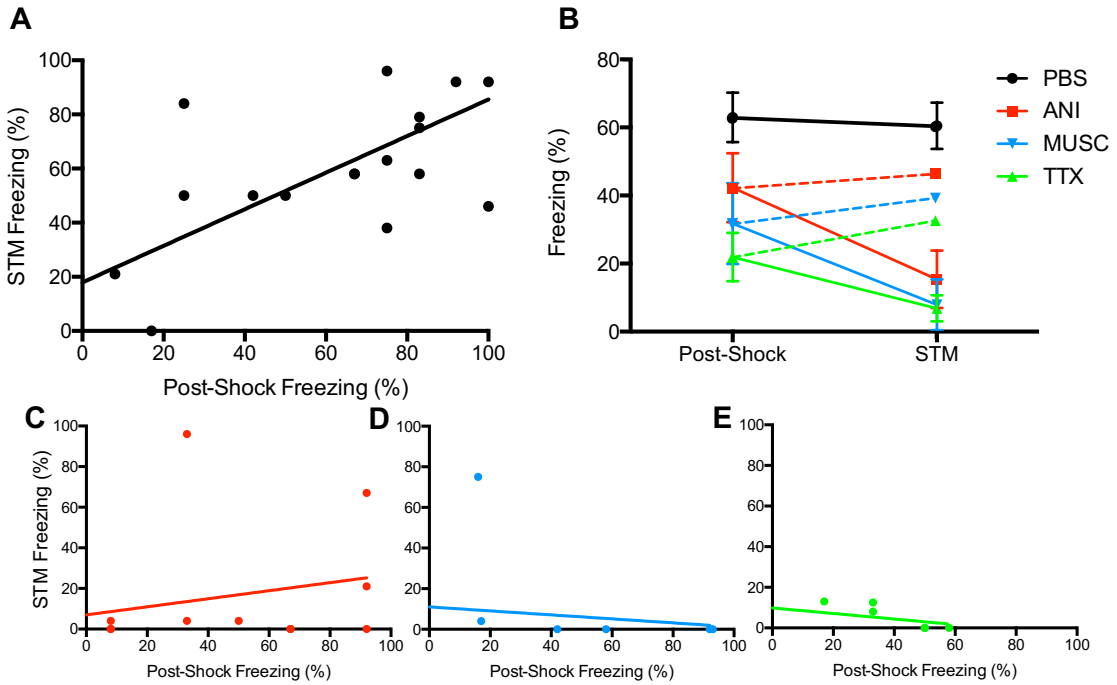

Figure 10: Relationship between post-shock freezing and memory. A) Relationship between post-shock freezing and freezing to the cue during the short-term memory test for the PBS group. Post-shock freezing strongly predicts freezing during the short-term memory test. **B)** Expected values for freezing during the short-term memory test in the experimental groups if predicted by post-shock freezing as in the PBS group. Solid lines represent observed data and dashed lines represent expected values. Expected values are substantially higher than observed values in all three experimental groups. **C-E)** Relationship between post-shock freezing and freezing to the cue during the short-term memory test for the ANI (*C*), TTX (*D*), and MUSC (*E*) groups. Post-shock freezing does not predict freezing during the short-term memory test for any of the three groups.

Discussion

Memory is often described as having two temporal stages that can be separated on the basis of *de novo* protein synthesis (Dudai, 2004; Squire et al., 2008; Nader and Hardt, 2009). This premise has been supported by research that used translational inhibitors to specifically disrupt long-term memory, while sparing short-term memory (Davis and Squire, 1984; Schafe and LeDoux, 2000). Although recent evidence suggests that blocking protein synthesis may actually suppress ongoing neural activity (Sharma et al., 2012), it remained to be shown whether PSIs affect short-term auditory fear conditioning memory; a test which was purportedly unaffected by PSIs (Schafe and LeDoux, 2000). In this thesis, I compared the behavioural deficits caused by ANI to those produced by the sodium channel blocker TTX and the $GABA_A$ agonist MUSC. Each manipulation impaired both short- and long-term memory, a result that is at odds with the premise that blocking protein synthesis, unlike neural inactivation, selectively impairs long-term memory. Together with prior results showing that PSIs impair neural activity and neural function, my present results suggest that the memory impairments previously attributed to translational inhibition may in fact be due to neural suppression.

ANI suppresses short- and long-term associations without affecting fear

The results of the auditory fear conditioning task showed that intra-amygdalar ANI produces detrimental effects on short-term, in addition to, long-term memory. This memory deficit was comparable to the deficit produced by two functionally different drug treatments, both of which temporarily inactivate neural tissue. As it is plausible that a deficit in the ability to express fear behaviour in general could explain the diminished memory, I also examined unconditioned fear and responding during conditioning in each

group. For the elevated plus maze, a measure of unconditioned fear, only the TTX group showed significantly reduced innate fear behaviour, whereas the ANI and MUSC groups were comparable to the PBS group. This suggests that unconditioned fear, at least, is intact in the ANI and MUSC groups.

For fear responding during the conditioning procedure, I examined the behavioural responses of the animals to the foot shocks directly, as well as their freezing behaviour in the post-shock period. During the conditioning trials, all rats across all groups showed evidence of reactivity to the shocks, as shown in previous studies (Gispen et al., 1975; Canal et al., 2007). These physical reactions to the foot shocks included flinching, jumping, and/or running across the chamber. This suggests that all animals, regardless of group, perceived the foot shock.

In order to assess responding following the CS-US pairings, I also characterized freezing behaviour occurring directly following the second post-shock period. I did not include freezing behaviour in response to the tone or for the first post-shock period as other researchers have suggested that these results are difficult to interpret (Phillips and LeDoux, 1992; Rogan et al., 1997; Bailey et al., 1999). Freezing behaviour increased after the two CS-US pairings within the PBS, ANI, and MUSC groups, but not the TTX group. Across groups, there was a significant reduction in fear responding when comparing the control (PBS) and TTX groups, but not the control to ANI or MUSC groups. Although not significant, a decrease in post-shock freezing was observed between the PBS group and the ANI and MUSC groups. This reduction, however, was not sufficient to predict the decreased short-term responding in any of the experimental groups.

Thus, it is unlikely that the memory deficits in the ANI and MUSC groups were due to an inability to process the US, but rather that they were due to impaired learning, and subsequent memory, of the CS-US relationship. The reduced unconditioned fear responding and the impaired freezing during the post-shock period seen in the TTX group may be due to the effects of this drug on axonal transmission, which could result in neuronal suppression in additional areas adjacent to the BLA, such as the CEA (van Duuren et al., 2007). Altogether, these results suggest that in the BLA, the effects of ANI may be more comparable to the effects of MUSC as both treatments allow US responsiveness while also impairing memory. Importantly, the effect of ANI on freezing at the short-term memory test contradicts the selective temporal nature proposed for the role of protein synthesis in long-term memory uniquely. My results thus suggest a review of the *de novo* protein synthesis hypothesis of memory is necessary.

Although this is the first study to show the effect of ANI on short-term memory for auditory fear conditioning, there are other findings supporting this result. For example, Canal and Gold (2007) found that intraamygdalar ANI disrupted both short- and long-term memory for an inhibitory avoidance paradigm, while another group found deficits in short-term contextual fear memory when ANI was injected into the CA1 region of the hippocampus (Remaud et al., 2014). In a recent study, a deficit was found when ANI or rapamycin (another PSI) was applied to the amygdala prior to a cued fear conditioning memory test (Lopez et al., 2015). In addition to memory impairments, several studies have recently shown that infusions of ANI can produce effects on online (moment-to-moment) behaviour in rodents (Greenberg et al., 2014; Dubue et al., 2015). In one study, the researchers found that ANI, when applied to the ventral hippocampus,

resulted in a suppression of unconditioned fear behaviour in rodents tested on the elevated plus maze (Greenberg et al., 2014). In a follow-up study, it was determined that dorsal intrahippocampal ANI impaired navigational ability in a water maze task to the same extent as reversible inactivation by TTX (Dubue et al., 2015). These results provide further behavioural support for the notion that the deficits incurred by translational inhibitors are not simply limited to memory consolidation. When considered together, the resulting behavioural effects of PSI application support the idea that protein synthesis inhibition suppresses ongoing neural activity, as proposed by Sharma et al. (2012).

Mechanism of neural inactivation by translational inhibitors

Although not the topic of this thesis, it is reasonable to question the possible mechanisms by which translational inhibition may produce effects on neural activity. Sharma et al. (2012) demonstrated that the degree of neural inactivation following intracranial application of ANI was directly correlated with the degree of protein synthesis inhibition. This suggests that normal neurobiological function is likely reliant on regular protein synthesis and that any effect of translational inhibition on synaptic plasticity may be less significant by comparison. Indeed, there are many classes of polypeptide products requiring quick turn-over that are likely to have fundamental roles in cellular function and health, including those involved in cytoskeletal maintenance, enzymatic processes, intracellular signaling, as well as the essential operations of cellular organelles. A poignant example of the latter would be mitochondria. There is evidence that inhibiting protein synthesis with emetine or cycloheximide disrupts the mitochondrial membrane potential and the production of ATP (Hillefors et al., 2007). Additional evidence comes from unpublished data from others in my lab which

demonstrated membrane depolarization in hippocampal cells that were treated with ANI *in vitro* (LeBlancq et al., 2014). The author of this work suggested that the inability to maintain membrane potential may be due to reduced ATP production in mitochondria and subsequent failure of the sodium-potassium pump. Although more research is needed to confirm these effects, they do suggest that the disruption of proteins critical for mitochondrial function could directly impact the electrophysiological properties of neurons.

Other researchers have proposed similar ideas, suggesting that depletion of a calcium buffering housekeeping protein may explain the effect of PSIs on LTP (Namgung et al., 1995), or that the effect of PSIs on memory recall is due to suppression of receptor trafficking (Lopez et al., 2015). Regardless, it is likely that multiple systems failure would occur at a cell-wide level following full translational inhibition. In fact, past research has shown that blocking the action of only one specific protein product through the use of antisense oligonucleotides can result in a reduction of neuronal (Neumann et al., 1995) and network excitability (Garcia-Osta et al., 2006). Similarly, it has recently been shown that a compound purported to act only on a specific peptide product, ζ inhibitory peptide (ZIP), results in the suppression of neural activity to the same degree as the topical anaesthetic lidocaine (LeBlancq et al., 2016). These particular studies are important in demonstrating that even targeted protein synthesis inhibition can have dramatic effects on the normal functioning of the brain, suggesting that complete translational inhibition would be even more detrimental to neuronal physiology.

Activity-dependent memory consolidation

As discussed in the introduction of this thesis, it is very likely that neural activity plays a critical role in the maintenance of memory, both on a cellular level, as well as on a network level. In the time after a learning event, the neuronal assemblies that were initially activated by the learning experience itself will spontaneously reactivate in the same pattern, a phenomenon known as replay. These replay events have been studied most extensively in the hippocampus where they have been shown to occur both in sleep and in quiet resting states following learning (Ramadan et al., 2009; Carr et al., 2011; Atherton et al., 2015). Although the hippocampus has been the main target for investigating neuronal oscillations in memory, it is likely that consolidation in the amygdala also relies on coordinated neural activity. Gamma oscillations, for instance, have been suggested to play a role in fear conditioning (Headley and Pare, 2013; Headley and Weinberger, 2013). In support of this notion, a recent study showed that increasing gamma frequency activity in the BLA using optogenetic stimulation results in the enhancement of memory for an inhibitory avoidance task (Huff et al., 2013). In that same study, inhibition of the BLA in the 15 minutes after training resulted in a memory deficit. This suggests that neuronal activity in the BLA in the time shortly after learning is critical for consolidation. Other studies have highlighted the importance of amygdalar theta activity during sleep, suggesting that it promotes coordination between the amygdala, hippocampus, and cortical structures (Pelletier and Pare, 2004; Popa et al., 2010). Indeed, the vast literature suggesting that oscillatory brain activity is critical for memory consolidation supports the proposition that suppressing this activity would be

detrimental to future recall (Inostroza and Born, 2013; Rasch and Born, 2013; Watson and Buzsaki, 2015).

Considering the recent work being done in this field, a unified theory of memory encoding and consolidation could rely on systematic variations in neural activity. For instance, at the cellular level, neurons that have higher excitability are more likely to become allocated to a memory trace (Yiu et al., 2014). Once a subset of cells has participated in a memory trace, they then suppress the activity of surrounding neurons via inhibitory interneurons, and thus are more likely to be involved in a subsequent memory trace (Cai et al., 2016; Rashid et al., 2016). If these cells engage in reactivation, like with what occurs in hippocampal replay, then the trace would be further strengthened following the training event. Importantly, this type of activity would likely be comparable with cellular events that occurred during the initial learning session. This suggests that neuronal replay would re-initiate processes similar to those involved in LTP, such as NMDA receptor activation. Indeed, prior studies have shown that blocking NMDA receptor activation in the consolidation period post-training impairs long-term memory (McDonald et al., 2005). It is likely that this strengthening occurs within a critical time window within 6 hours post-learning that has been suggested to be important by others (Schafe and LeDoux, 2000; Rashid et al., 2016). Additional consolidation events may then occur during sleep as rhythmic activity allows communication between separate brain regions. Based on this research, it would be difficult to explore an independent and unique role for protein synthesis in consolidation, although it is reasonable to assume that this process plays an important role for structural changes that occur in the synaptic connections mediating memory traces.

Inconsistent definitions of short- and long-term memory

In the animal literature, short-term memory is often described as the protein synthesis independent stage of memory, whereas long-term memory is dependent on protein synthesis (Dubnau et al., 2003; Tully et al., 2003; Nader and Hardt, 2009). These definitions of memory, as a result of being dependent on a process that itself is now controversial and not well defined temporally, are impractical. Problematically, in defining memory stages temporally, rather than by their dependence on molecular events, variability still persists. Some researchers define short-term memory as lasting for several hours (Nader and Hardt, 2009), while others propose that it decays within minutes (Dubnau et al., 2003; Tully et al., 2003). In some cases there is a third stage, intermediate-term memory, that is purported to begin after short-term memory has subsided and before consolidation of long-term memory (Squire and Barondes, 1972; Vianna et al., 2000; Dubnau et al., 2003). There is also marked variability in the timing of the experimental testing of memory. In early studies using PSIs, short-term memory was often tested immediately after training, either during the intervals following a CS-US presentation in auditory fear conditioning (Agranoff et al., 1966; Squire and Barondes, 1972), or as soon as 10 or 15 minutes after training. (Barondes and Cohen, 1966; Watts and Mark, 1971). In more recent studies, short-term memory is typically tested between 30 minutes and 4 hours after behavioural training (Schafe and LeDoux, 2000; Quevedo et al., 2004; Canal and Gold, 2007). In these same studies, long-term memory is typically tested 24 or 48 hours after training. With research investigating spatial memory, however, training often occurs over several days in order to produce consistent retention (Vorhees and Williams, 2006; Ego-Stengel and Wilson, 2010; Binder et al., 2013). In spatial

memory studies that do use only one day of training, the retention test will often occur within a few hours after the training session (Mumby et al., 2002; Bolding and Rudy, 2006). Yet another difference in the timing of memory testing comes from studies of sleep deprivation or enhancement. In such studies, retention testing will occur at 80 to 120 minutes after training (Binder et al., 2012; Oyanedel et al., 2014). These contradictions are not limited to memory either, as the temporal characteristics of LTP are highly variable as well, as reviewed in detail by Abbas et al. (2015). Thus, the definition of short- and long-term memory, even within a taxonomic group, is indefinite.

Additional discrepancies are found in the human literature, where the stages of memory are defined quite differently than in animal research. Short- and long-term memories are often dissociated based on the length of time for which they are able to be remembered, such that a short-term memory may last only a day while a long-term memory may last a lifetime (Bear et al., 2007). Much of the understanding about human memory comes from studying people with memory deficits due to illness or injury. For instance, studies focusing on individuals with memory loss for past events, or retrograde amnesia, showed that more recent memories were most vulnerable to disruption (Bear et al., 2007; Pinel, 2011). This research suggested that memories continue to become consolidated over many years, rather than just over several hours or days. Although the disparities in defining memory are far too extensive to explore here, this will hopefully have given the reader an understanding of the problems involved with attempting to separate this complex behaviour into such stages, especially by molecular events.

Conclusion

This thesis demonstrated that inhibiting protein synthesis with ANI affects short-term and long-term memory for an auditory fear conditioning task to the same extent as neural inactivation. Taken in consideration with prior findings that showed suppression of neural activity with PSIs, these results suggest that separating short- and long-term memory using translational inhibitors is untenable. An alternative explanation that can account for many of the contradictions regarding memory research is that consolidation relies on coordinated neural activity during and after a learning event. It is likely that such activity would involve synaptic modifications which would require protein synthesis. However, this also suggests that any manipulation which suppresses ongoing neural activity, such as PSIs, will be detrimental to future recall. Future research investigating the specific role of neural activity in the period shortly after learning using reversible excitation or inactivation, via pharmacological or optogenetic manipulations, would help to elucidate the role of neural activity in consolidation. Additionally, exploring the role of ongoing protein synthesis in the maintenance of cellular respiration and the maintenance of the neuronal membrane potential would clarify the specific mechanism by which translational inhibition suppresses neural activity. Regardless of these outcomes, I would strongly advise against other researchers using PSIs to investigate memory processes in the future due to the serious confound of neural inactivation. More generally, this work emphasizes the importance of assessing the electrophysiological function of the brain as an intervening step between molecular techniques and behavioural outcomes.

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