Epigenetic Involvement in Heterosynaptic (Tagged) LTP Following Beta-Adrenergic Receptor Activation in the Mouse Hippocampus

by

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A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

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Abstract

Synapses experience long-lasting plastic changes following neuromodulatory action in the brain. One natural modulator is noradrenaline (NA). Beta-adrenergic receptor (b-AR) activation by noradrenaline enhances memory formation and long-term potentiation (LTP), a form of synaptic plasticity characterized by an activity-dependent increase in synaptic strength. Since LTP is believed to be a cellular correlate of learning and memory, understanding the mechanisms by which synapses undergo LTP is necessary for grasping how the brain encodes information. In the mammalian hippocampus, a brain structure responsible for new memory formation, LTP can be observed at multiple synaptic sites after strong stimulation of a single synaptic pathway. This phenomenon, referred to as *synaptic tagging*, may permit distinct synaptic pathways to associate information from separate, convergent synaptic inputs.

Previous research has revealed that synaptic tagging requires protein synthesis, and that both transcription and epigenetic modifications are necessary for eliciting LTP at a single *homosynaptic* site (Maity et al., 2016). However, it is unclear whether transfer of LTP to a secondary *heterosynaptic* site involves b-ARs signalling to the nucleus. The present thesis uses electrophysiological protocols to show that pharmacological inhibition of b-ARs, mRNA synthesis or histone acetyltransferase prevents heterosynaptic plasticity in mouse CA1 hippocampal neurons. Thus, heterosynaptic "tagged" LTP must recruit nuclear signalling by engaging transcription and histone acetylation. Future research should investigate how these intracellular mechanisms modulate memory consolidation in combination with *in vivo* behavioural models. This will allow for a more complete characterization of how mRNA and proteins enable the endurance of long-term memories.

Preface

This thesis is an original work by Nathan Brandwein. The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board, Project Name: “Epigenetic Involvement in Heterosynaptic (Tagged) LTP Following Beta-Adrenergic Receptor Activation in the Mouse Hippocampus.” February 2019.

Acknowledgements

It would not have been possible to write this thesis without the endless support from a number of incredibly special people in my life who I will give particular mention here. I need to begin by thanking my parents for their helpfulness, patience and love. To my father: words cannot describe how grateful I am for all that you have done. You are a true hero in every sense of the word. The number of sacrifices you have made to give me the freedom to study what I wish and the ability to pursue a high-quality education have not gone unnoticed – these are sacrifices that I will never take for granted. To my mother: your unbridled commitment to look out for my well-being has helped me tremendously during my education. You always made sure to direct my focus towards building a strong work ethic while also encouraging me to follow other professional and non-professional interests. By taking special care of me during the rougher times, I knew I could count on you to lift me up when my spirits were low. You've taught me to be more forceful, to ask questions and to combat obstacles with unflinching determination.

I would be remiss if I did not mention my twin brother, Daniel, who has been my best friend since day one. The fact that we are different in so many ways (personality, appearance, life-goals, etc.) is what makes our relationship unique. Although we regularly bond over our shared interests of frisbee, food and music, our differences are what bring us closer together. Living with my not-so-similar doppelganger in an *Odd Couples*-esque apartment makes life exponentially more enjoyable. He brings both goofiness and emotional support to my life, two qualities that make my studies more tolerable. As a master's student himself, he can understandably relate to the near-daily frustrations and tribulations I experience from botched

experiments to writers block to troubleshooting. His moral support outside the lab has impacted me greatly.

I also need to thank Sean Rah, a master's graduate from the lab who assisted me throughout the spring and summer of my arrival. He gave me the necessary tools and skills to help foster confidence and knowledge. Not only was I prepared at performing the essential hard skills, I was also able to work under fluid conditions by applying problem solving skills. His patience, guidance and tactfulness never went unnoticed. And for that, I am honoured to call him a friend.

From the second week I stepped foot in the lab, an undergraduate student has been by my side to offer unconditional support while learning the same techniques as me to eventually tackle her own research project. Her name is Janlyn Hoffman and she deserves a special thanks for keeping me level-headed and sensible whenever uncertainty struck. She created an exceptional work environment while also offering her friendship. With many successful collaborative experiments under our belts, we made a productive and unforgettable team. I am forever indebted.

I would also like to thank the physiology office staff for the kindness they radiate every time I walk through their door. You would be hard-pressed to find nicer and more caring people than Kim, Donna, Dancy and Linda. Their knowledge, compassion and authenticity gave me the comfort to speak to them for any advice and issues I would have.

Last but certainly not least, I would like to pass on my sincerest gratitude to my supervisor, Dr. Peter Nguyen, for offering me a position in his lab. He was supportive of my being with him

since the day we met and is one of the reasons why joining his lab was an easy decision. Throughout my degree, he gave me a clear direction with minimal interference and a space to grow. At the same time, he pushed me to think about my project with profound thought and would regularly provide feedback and new ideas. He genuinely cared about my welfare, as he would frequently inquire about my life outside the lab. We had many fascinating conversations about our shared adoration for classic songs and movies. He challenged me to expand my horizons in areas of music, art and travel by lending me non-fiction novels and classical CDs of Beethoven and Mahler. He taught me to always be curious, as curiosity is the appetite for knowledge.

Before I end, I would like to thank my committee members, Dr. Declan Ali and Dr. Bradley Kerr for providing constructive feedback and critical insights during my proposal meeting as well as for my thesis. I am also extending a thank you to my external examiner, Dr. Fred Tse for taking time out of his busy schedule to read and support my thesis.

Additionally, I would like to thank NSERC for financially backing my research.

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List of Abbreviations

Act-D	Actinomycin-D
ACSF	Artificial cerebrospinal fluid
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors
ANOVA	Analysis of variance between groups
b-AR	Beta-adrenergic receptor
CA1	Cornu ammonis-1
CAMKII	Calcium/calmodulin-dependent protein kinase II
CBP	CREB binding protein
CCAC	Canadian Council on Animal Care guidelines
CREB	Cyclic AMP response element binding protein
DRB	5,6- Dichlorobenzi-midazole 1-β-D- ribofuranoside
E-LTP	Early phase of LTP
EPSP	Excitatory post-synaptic potential
fEPSP	Field excitatory postsynaptic potential
HAT	Histone acetyl transferase
HDAC	Histone deacetylase
HFS	High-frequency stimulation
ISO	Isopreterenol
LC	Locus coeruleus
LTP	Long-term potentiation
L-LTP	Late phase of LTP
mRNA	Messenger RNA

NA	Noradrenaline
NA-LTP	NA induced LTP
NMDAR	N-methyl-D-aspartate receptors
PKA	cAMP-dependent protein kinase
PRP	Plasticity-related products
S1/2	Stimulating electrode 1/2
SEM	Standard error of the mean
TSA	Trichostatn

Chapter 1: Introduction

1.1 Learning and Memory

The brain functions to store and retrieve information about our experiences in order to influence our behaviours. When we learn, we gain new information which we store in our memory systems for future retrieval. Since memory is contingent upon prior learning, both concepts need to be carefully demarcated. Learning is defined as the acquisition of knowledge by an organism with respect to its environment, while memory involves the storage or retention of that knowledge. Extracting the stored information comprises the retrieval aspect of memory. Memory traces are stored in the brain as "engrams," which are likely distributed along different neural systems. The location of these engrams may or may not be contained within a singular structure. Karl Lashley believed that engrams were diffusely patterned (Eichenbaum et al., 2016). However, our current understanding of different memory systems has radically affected the mechanistic validity of the engram and how memories are stored.

The notion that the brain expresses multiple memory processes gained significant traction in the mid-20th century following studies of amnesic patients. Psychological evidence came from research on patient Henry Molaison (HM), whose medial temporal lobes (including the hippocampi) were bilaterally resected to relieve his epileptic seizures (Scoville & Milner, 1957). Although his remote memories and procedural learning were largely unaffected, HM was unable to form new long-term memories, the hallmark of anterograde amnesia. The fact that he was unable to recall factual or episodic memories indicated that he exhibited a pronounced deficit in his ability to form explicit (i.e. declarative) memories. It was clear that explicit memories were

linked to the medial temporal lobe, whereas motor memories were affected by other brain structures outside of the medial temporal lobes.

The case of HM provided new insight into how different types of memories are localized (Cohen and Squire, 1980; Tulving and Schacter, 1990). While most aspects of his memory were poor, he exhibited normal implicit memory (e.g. on procedural memory tasks), illustrating a dissociation between explicit and implicit memories. HM's loss of function confirmed to neuroscientists that the brain's primary memory structure, the hippocampus, is necessary for certain memory processes rather than merely acting as a generic memory repository. For example, the hippocampus consolidates short-term memories into long term memories; this does not mean that memories are themselves permanently stored in the hippocampus. It was Eric Kandel who would later provide physiological evidence that memories are, in fact, stored in cellular networks as changes in synaptic strength among identifiable neurons (reviewed by Kandel, 2001).

It is clear that the hippocampus plays a crucial role in memory formation. The hippocampus receives and sends projections to a variety of brain regions, including higher order association areas. This suggests that the hippocampus performs a number of complex roles. Indeed, O'Keefe & Dostrovsky (1971) showed how hippocampal "place cells" activate differentially based on spatial orientation, and that the hippocampus codes and stores this spatial information as "cognitive maps". The predictions of the cognitive map theory were subsequently tested by Morris (1981) using a now prominent spatial navigation task. Experimental evidence presented above has widely contributed to the belief that hippocampal *neurons* are likely the cellular correlate of learning and memory.

1.2 Neuronal anatomy and physiology

The information that underlies cognitive processes is transmitted at synapses. When a neuron is stimulated by a preceding neuron, it fires an action potential. A subsequent rise in intracellular calcium levels leads to the movement of neurotransmitter-containing vesicles to the presynaptic membrane. Each vesicle possesses a quantum of previously synthesized and packaged neurotransmitter of a singular type (e.g. glutamate). A neuron may be classified as either excitatory or inhibitory, depending on the postsynaptic receptor channels that are recruited by the transmitter. Upon exocytosis, the transmitters rapidly move across the synaptic cleft to bind to receptor sites on the post-synaptic neuron. Ligand-bound receptors will open their channels leading to excitatory or inhibitory postsynaptic potentials, which will alter the membrane potential and the probability of that neuron reaching the voltage threshold needed to fire an action potential.

1.3 Hippocampal tri-synaptic circuit

The hippocampus is a limbic system structure comprised of three major subregions: Ammon's horn, the subiculum and the dentate gyrus. Ammon's horn contains distinct subfields, including CA1 and CA3. Transverse sectioning of the hippocampus produces tissue slices that have a clearly defined anatomical layout. Due to its identifiable configuration, electrophysiological experiments are often conducted directly on these slices (see “Electrophysiology”). Information is routed into the hippocampal formation along the perforant path from the entorhinal cortex and subiculum to the dentate gyrus (DG) and then onto pyramidal cells in CA3 and then CA1. The synaptic connections between DG-CA3-CA1 is collectively referred to as the trisynaptic circuit (**Figure 1.1**).

1.4 Glutamate Receptors

Hippocampal pyramidal neurons release excitatory glutamate. Glutamate will bind to different classes of glutamate receptors, including α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPAR) and N-methyl-D-aspartate receptors (NMDAR). AMPARs are activated by glutamate binding, which leads to membrane depolarization by sodium influx. NMDAR activation requires both glutamate binding and a depolarized membrane to remove the magnesium block from the pore. As a result of their dual activation mechanism, NMDARs are often termed coincidence detectors. Importantly, both receptor classes are subdivided into different types based on their subunit combination. These isoforms provide the receptor with different phosphorylation sites, thereby altering function. NMDARs, for instance, are necessary for memory formation but only for certain isoforms (Sakimura et al., 1995).

1.5 Electrophysiology & Field Potentials

Carbon fibre stimulating electrodes are positioned in the stratum radiatum layer of the hippocampus, where they can stimulate the Schaffer Collateral pathway fibres from CA3 to CA1. Since there are multiple synaptic inputs onto the population of pyramidal cells in CA1, independent paths can be simultaneously recorded (**Figure 1.2**). The two stimulating electrodes are oriented in a manner so that they flank the recording glass electrode on either side, while the recording electrode is placed near the population of CA1 pyramidal cells. Recording two paths allow researchers to study effects on neurons when two converging inputs receive different patterns or time courses of stimulation. The two independent synaptic pathways which converge onto pyramidal neurons are referred to as S1 (the homosynaptic pathway) and S2 (the heterosynaptic pathway). One can study heterosynaptic plasticity using this method, as well as

observing tissue viability during a live LTP experiment. The present thesis will examine CA1 exclusively, as it is a widely known and studied region in neuroplasticity research.

Stimulating the CA3-CA1 pathway permits researchers to record field potentials (fEPSPs) from the apical dendrites of postsynaptic cells from which CA3 pyramidal axons synapse onto. As the recording electrode is placed in apposition to pyramidal cells in an extracellular fashion, the researcher collects information about the activity of the neuronal population. Shortly after stimulating a single path, postsynaptic neurons depolarize, leading to the captured waveform displaying a broad, downward deflection indicative of positive charge flowing away from the electrode tip into the neurons. The extracellular recording will have a corresponding negative slope because the potential difference between a distant grounding electrode and the extracellular fluid is negative.

1.6 Synaptic plasticity

It has long been postulated that memory formation in the mammalian brain involves long-lasting changes in synaptic efficacy of excited neurons (Cajal, 1893; Hebb, 1949). This activity-dependent change, referred to as synaptic plasticity, serves as the underlying mechanism for the plastic modifications occurring at neuronal connections.

One form of synaptic plasticity, long-term potentiation (LTP), is the most widely studied cellular model for learning and memory (Bliss and Collingridge, 1993; reviewed by Martin et al., 2000). It is worth noting that synaptic plasticity is bidirectional, meaning that the strength of synapses can be weakened or strengthened in order to modulate neuronal excitability. The weakening

process is termed long-term depression (LTD) and is induced by low frequency stimulation (Dudek & Bear, 1992; Artola & Singer, 1993).

1.7 Long-term potentiation

Donald Hebb's pioneering work on theorizing a neural basis to learning and memory laid the foundation for the discovery of a phenomenon called long-term potentiation (LTP) (Douglas & Goddard, 1975). Bliss & Lømo (1973) demonstrated that applying high-frequency stimulation to presynaptic neurons led to enduring changes in the synaptic efficacy of hippocampal granule cells. This finding supported Hebb's hypothesis, since the synaptic changes underlying LTP appeared to resemble the changes underlying learning and memory (Lisman et al., 2011).

The associative firing pattern of connected neurons is a key property of LTP, and results in the strengthening of synapses (Levy and Steward, 1979; Gustafsson et al., 1987). LTP is specific to the stimulated pathway and is sustained for an extended duration of time. LTP is marked by neuronal modifications, including molecular changes such as protein kinase activation, regulation of transcription factors and protein synthesis (see "Phases of LTP" section).

1.7.1 Early and late LTP protocols

Importantly, different forms of LTP can be evoked in the hippocampus depending on the protocol that is administered by way of electrical stimulation. Applying a weak LTP induction protocol (early; E-LTP), such as one train at 100 Hz for 1 second will result in LTP that decays to baseline levels. To establish a long-lasting form of hippocampal LTP, a strong LTP protocol (late; L-LTP) is delivered. L-LTP is elicited by applying a strong tetanus (e.g. 4 trains at 100 Hz; each pulse separated by an interval of one minute) (Abraham et al., 2002). This robust response

will result in a field potential slope that is maintained significantly above baseline levels, and it is dependent on the upregulation of transcription and translation (Krug et al., 1984; Frey et al., 1996; Nguyen et al., 1994) (**Figure 1.3**).

1.7.2 Phases of LTP

LTP is defined as a three-part process, whereby stimulation to a neural pathway results in induction, expression and maintenance in post-synaptic neurons. Immediately after applying a train of stimuli to a neuron, that cell will undergo post-tetanic potentiation, a form of short-term plasticity that is maintained for several minutes. This is due to the action of calcium, which increases the probability of transmitter release (Delaney et al., 1989).

The induction phase requires NMDAR activation, which is linked to the influx of positively-charged ions, including calcium. Calcium entry leads to potentiated synapses by action on AMPA receptors, as well as presynaptic transmitter release (Kauer et al., 1988; Perkel et al., 1993). Calcium will activate an enriched synaptic pool of calcium-calmodulin kinase II (CAMKII), which will phosphorylate AMPARs, leading to an increase in the probability of receptor opening (Kristensen et al., 2011). This phosphorylation event also induces AMPAR trafficking to the synaptic membrane (Makino and Malinow, 2009; Lu et al., 2010). AMPAR insertion reduces the number of NMDAR-expressing silent synapses, which is a hallmark of LTP expression (Isaac et al., 1995). During the expression phase, local proteins are synthesized at dendritic spines allowing for efficient and specific receptor transport (reviewed by Steward and Schuman, 2001). In mutant mouse strains lacking CAMKII, LTP is not expressed (Giese et al., 1998). CaMKII also engages the actin cytoskeleton to increase spine size, resulting in the spine

enlargement (Okamoto et al., 2009). In summary, increasing channel number and opening results in a larger synaptic current that is critical for the of expression of LTP.

The latest of phases is LTP maintenance, which recruits the nucleus to cause more permanent changes. This phase is also translation-dependent and may even involve structural changes. For instance, the post-synaptic spine may dramatically increase in volume and number (Matsuzaki et al., 2004). Transcription factors such as cyclic AMP response element binding protein (CREB) translocate to the nucleus to associate with DNA and cause expression of gene products (Lee and Masson, 1993; Tian et al., 1996) to ultimately grow the synapse.

1.7.3 LTP and memory

Although LTP recordings from artificially-stimulated hippocampal slices do not constitute a memory (from an intact brain), LTP provides the most suitable cellular hypothesis to model memory formation in the brain. LTP and memory are both long-lasting, found in the same brain structures and affected by pharmacological manipulations. There are a number of studies that provide corroborating evidence for why LTP is a suitable mechanism for the cellular processes underlying memory. Blocking NMDA receptors in the hippocampus disrupts learning (Morris et al., 1986) and prevents LTP expression (Giese et al., 1998), while NMDAR mutant mice have impaired LTP and spatial memory (Sakimura et al., 1995). Further, avoidance learning in rats produces changes in glutamate receptors that parallel LTP induction in CA1 neurons (Whitlock et al., 2006). This finding is especially significant since AMPA receptor trafficking, induced by conducting a learning task, is believed to be the molecular basis of LTP.

1.8 Noradrenergic neuromodulation

The locus coeruleus (LC) is a brainstem nucleus that synthesizes and secretes noradrenaline (NA) into regions with which its neurons innervate. One prominent structure that the LC sends projections to is the hippocampus. Noradrenaline (also referred to as norepinephrine) binds to noradrenergic receptors, which are expressed globally in the brain. NA influences a variety of CNS functions, including arousal, sleep and memory. Interestingly, arousing experiences may aid in memory storage by strengthening synaptic networks. Since NA can modulate the strength and plasticity of synapses, it is a key neuromodulator in the hippocampus. (Harley, 2007; Lemon et al., 2009; Sara, 2009).

1.8.1 Beta-adrenergic receptors

There are two main classes of noradrenergic receptors: alpha (a) and beta (b). These classes can be further divided into subtypes, each of which exhibit distinct functional and expression patterns. b1- and b2-ARs are functionally expressed in cells of the hippocampus (Hillman et al., 2005a). Furthermore, single cell real time RT-PCR indicates expression of both receptor subtypes in CA1 pyramidal neurons (Hillman et al., 2005b). Electrophysiological evidence has revealed that, b-ARs, as opposed to a-ARs, influence LTP, as blocking a-AR activation has no deleterious effect on homosynaptic plasticity (Maity et al., 2016).

It has been well-established that the b-AR agonist, isoproterenol (ISO), can induce long-lasting LTP when paired with a weak HFS protocol. ISO-induced b-AR activation facilitates LTP persistence and appears to play a major role in the formation of memories (Gelinas & Nguyen 2005; Hu et al., 2007). More recent studies have demonstrated that NA, the endogenous ligand of

the beta-adrenergic receptor, can facilitate late-LTP when overlapped with tetanic stimulation (Thomas et al., 1996; Gelinas et al., 2008). Although b-AR activation alone is not necessary for LTP, pairing NA in *in vitro* slices with a weak HFS protocol (1 train at 100 Hz for 1 second) leads to a long-lasting form of LTP referred to as "NA-LTP" (Thomas et al., 1996; Winder et al., 1999; Gelinas & Nguyen, 2005; Ma et al., 2011).

Coupling b-AR activation with excitatory events lead to potentiated synapses because the activation of downstream molecular targets initiates communication between the receptor and the nucleus (Maity et al., 2016). Preventing NA-b-AR binding in CA1 leads to impaired spatial and long-term memory (Ji et al., 2003a; Ji et al., 2003b).

b-ARs are intrinsically linked to the cAMP secondary messenger system. Upon activation, b-ARs cause the Gs protein to dissociate from an inactivated complex and bind to adenylyl cyclase, an enzyme that catalyzes the conversion of ATP to cAMP. cAMP then binds to the regulatory subunits of PKA, which triggers the two catalytic subunits of PKA to dissociate and translocate into the nucleus where they affect the activity of transcription factors and, ultimately, the expression of LTP-related genes.

NA is also critical for the enhanced activity and trafficking of GluR1-containing AMPARs. PKA activation results in GluR1 phosphorylation at serine-845 (Ehlers, 2000; Banke et al., 2000). Additionally, b-AR activity leads to increased phosphorylation of serine 831 sites by CAMKII. Both phosphorylation events enhance AMPAR function by altering their kinetics, leading to increased ionic conductance, and by promoting insertion of GluR1 into postsynaptic membranes

(Tenorio et al. 2010). Such "metaplastic" events prime the synapse to boost LTP only after an inducing stimulus occurs proximally in time (Tenorio et al., 2010). Therefore, b-ARs alone cannot elicit LTP; rather, it is believed that tetanic stimulation acts as a spark to express long-lasting NA-LTP by clustering extra-synaptic AMPARs into dendritic spines (Oh et al., 2005). Additionally, b-ARs signal to the nucleus (through the cAMP system) to upregulate GluR1-containing vesicle trafficking and receptor membrane insertion by recruiting regulatory proteins (e.g. transmembrane AMPA receptor regulatory proteins; TARPs). Thus, the noradrenergic system exerts modulatory effects on hippocampal synapses to promote LTP expression and stabilize memory formation.

1.9 Synaptic tagging

In order for mRNAs to know which synapses to strengthen, a stimulated synaptic site must be marked by a biological tag. This is the basis of synaptic tagging, a process proposed by Frey and Morris (1997) to putatively explain heterosynaptic plasticity. According to the synaptic tagging and capture hypothesis (**Figure 1.4**), inducing early LTP (E-LTP) or late long-lasting LTP (L-LTP) at a synapse generates a "tag" specific to that synaptic site. For proteins to be specifically captured and sequestered at activated synapses, a previously produced tag must mark these sites. A tag is defined as any molecule that is generated locally (synapse specific) in response to synaptic activity. This activity may be weak or strong. For instance, a dendritic spine which received strong input by four successive trains of HFS will be tagged, as would a spine that received one train of HFS. However, applying a subtheshold stimulus, such as 0.02 Hz test stimulation, is not sufficient for producing a tag. Tags, therefore, are not placed during basal extracellular field potential recordings. Another stipulation regarding tags is that they have a

transient lifespan, likely degrading within one hour. The exact time window varies depending on the half-lives of the tags (Frey & Morris, 1997). The candidate tags are wide-ranging and may include PKA and adhesion molecules (Martin & Kosik, 2002). Since evidence of tags have been subject to debate, researchers have toyed with the idea that tags may even extend to intracellular processes occurring at dendritic spines themselves (e.g. cytoskeletal remodeling, mRNA stability, etc.).

Note that the tag functions to capture plasticity-related proteins/products (PRPs) that are created at another synaptic ("heterosynaptic") site by activation from an independent but converging input. The tags specifically mark sites which have received sufficient stimulation, in order for plasticity products to be captured only at activated, soon-to-be strengthened synapses. Normally, applying an E-LTP protocol (1x100 Hz) to one synaptic pathway is not sufficient for activating translation or transcription; however, inducing L-LTP at another site will "transfer" the LTP to the weakly stimulated site, leading to persistent LTP at both locations. Therefore, LTP-inducing synaptic activity in one pathway may affect synaptic activity presented by another pathway.

1.9.1. PRPs & synapse specificity

Although each neuron forms connections with the dendrites of many adjacent neurons, only the contacts that are activated by strong stimulation will be persistently strengthened (Andersen et al., 1977). The activity-dependent specificity of synapses is key to ensuring that input stimuli will only signal the strengthening of activated synapses. But how does this strengthening manifest itself at a molecular level? Plasticity-related proteins (PRPs) are produced in response to neural activity by genomic signaling, which communicate to the nucleus to transcribe certain

genes (Frey & Morris, 1997). These newly-synthesized products are translated to protein and moved to activated "tagged" synapses where they exert their effects (Frey and Morris, 1997). Proteins may also be synthesized locally at or near dendritic spines, meaning that these mRNA products are already present at the time of stimulation (Kang & Schuman, 1996; Casadio et al., 1999). Indeed, transcripts are known to exist in pools near spines, contained within RNA granules, where they can subsequently become translated and captured by tags (reviewed by Pfeiffer & Huber, 2006). Key LTP-related mRNAs, such as AMPAR subunits, have been observed to undergo local dendritic translation, illustrating the importance of synapse specificity following neural activity (Ju et al., 2004). Lastly, although *de novo* protein synthesis is required for heterosynaptic LTP, whether mRNA transcription is needed has not yet been resolved. There is a case to be made that perhaps previously-synthesized dendritic mRNAs can utilize the local translational machinery in response to neural activity, rather than recruiting the soma to produce new transcripts for subsequent trafficking and capture. Observing changes to neurotransmission if transcription is silenced can provide insight into whether *de novo* mRNAs need to be synthesized in the nucleus for heterosynaptic sites to express persistent LTP.

1.10 Transcription

In addition to long-lasting LTP requiring protein synthesis (Stanton & Sarvey, 1984; reviewed by Klann et al., 2004; Costa-Mattioli et al., 2009), L-LTP at stimulated synapses also requires transcription (Nguyen et al., 1994; Maity et al., 2016). Early experiments looking at changes in neuronal activity following somatic excision revealed a need for the nucleus in LTP expression (Frey et al., 1989). In later years, the use of pharmaceutical inhibitors of transcription would

serve as a more sophisticated means of blocking LTP maintenance, illustrating how a nuclear process is required for eliciting a sustained form of LTP (Nguyen et al., 1994).

Gene expression in the nucleus has been implicated in various studies examining long-term synaptic plasticity (Abraham et al., 1993; Sossin, 1996). The transcription factor, CREB, may initiate the production of memory-related genes, as activating CREB-mediated transcription in CA1 produces a remembered behavioural experience (Impey et al., 1998). Furthermore, NA-induced b-AR activation, when paired with HFS, boosts LTP endurance by signaling to the nucleus to engage transcription (Maity et al., 2016). However, whether enhanced NA-LTP persistence at heterosynaptic tagged synapses depends on *de novo* transcription remains elusive. Since heterosynaptic transfer of LTP likely requires the capture of somatically-produced plasticity-related gene products at tagged synapses (by strong homosynaptic activity), investigating the role of nuclear activity can allow for future research to determine which genes are involved in strengthening heterosynaptic sites.

1.11 Histone Acetylation

Epigenetic mechanisms, defined as alterations to gene expression without affecting the DNA sequence (Jaenisch & Bird, 2003), are key regulators of cellular function. DNA is amenable to epigenetic marks through processes such as DNA methylation and DNA phosphorylation. Epigenetic markers also modify histone proteins, which the DNA wraps itself around.

The fundamental unit of eukaryotic chromatin is the nucleosome, which is composed of two H2A-H2B dimers and one H3-H4 tetramer to form a histone octamer. DNA is wound around

histone protein cores to promote DNA compaction. This interaction is mediated by the N-terminal tail of histones. Epigenetic marks interact with the tail to modify histones (Luger et al., 1997). Histones are subject to post-translational modifications, such as acetylation, methylation and phosphorylation. These modifiers regulate gene expression by altering chromatin structure and accessibility. One key modification of histones is acetylation. Histone acetyl transferase (HAT) adds acetyl groups to the lysine residues of histone tails to loosen chromatin structure, resulting in increased transcriptional activity (**Figure 1.5**). For example, mRNA expression increases upon PKA activation via subsequent CREB activation in the nucleus. Phosphorylated CREB then associates with CBP, which has intrinsic HAT activity, to regulate CREs in promoter regions of genes implicated in learning and memory (Korzus et al., 2004; Wood et al., 2006).

1.11.1 Histone Acetylation: LTP and memory

Epigenetic modifications play a major role in facilitating synaptic potentiation by regulating transcription (Levenson et al., 2004). Histone acetylation is essential for synaptic plasticity and the persistence of memory (reviewed by Peixoto & Abel, 2013). Studies using rodents and pharmacological inhibitors have assessed the involvement of histone acetylation on the modulation of genes underlying LTP and memory processes (Vescey et al, 2007; Bredy et al., 2007; Tian et al., 2010).

Among the most researched epigenetic modifications is acetylation of histone 3 (H3) (Luger et al., 1997), recognized for its role in hippocampal memory. Performance of a spatial memory task increases hippocampal H3 acetylation, and enhanced cAMP signaling leads to hyper-acetylated H3 at lysine 14 (Bousiges et al., 2010). Likewise, addition of histone deacetylase inhibitors to

increase histone H3 acetylation subsequently enhances CA1 LTP induction *in vivo*, underscoring the relationship between epigenetic regulation of H3 and long-term memory formation (Levenson et al., 2004).

While HATs promote chromatin relaxation, histone deacetylases (HDACs) act to condense chromatin, leading to decreased expression of genes (**Figure 1.5**). HDAC inhibition by drug application strengthens both LTP and memory in murine models. In fact, there has been a proliferation of studies over the last 15 years investigating the role of HDAC inhibitors (which act to enhance acetylation and transcription) on memory formation and storage. For example, Sharma et al., (2015) showed how inhibiting HDAC in aging mice (with a corresponding LTP deficit) can restore synaptic tagging and capture by re-establishing heterosynaptic late-LTP. HDACs are widely expressed in the brain, especially in hippocampal and cortical cells (Broide et al., 2007), and are targeted by broad spectrum HDAC inhibitors, including trichostatin A (TSA). TSA modulates LTP at stimulated synapses to induce homosynaptic plasticity (Yeh et al., 2004; Vecsey et al., 2007).

Previous research by Maity et al. (2016) determined that NA triggers histone acetylation to enhance homosynaptic LTP maintenance. Therefore, b-AR activation requires histone acetylation to enhance LTP maintenance, since adding a HAT inhibitor reduced the magnitude of NA-LTP to baseline levels. Subsequent Western blot analysis supports the electrophysiological data, as CA1 showed a marked elevation in H3 (Lys14) acetylation following NA+HFS pairing, but not in the presence of the inhibitor. Thus, b-AR activation (by NA) enhances homosynaptic NA-LTP due to recruitment of histone acetylation. However, NA-LTP expression at "tagged"

synaptic sites by histone acetylation has not been studied. Since histone acetylation is a critical epigenetic mechanism that is upregulated during homosynaptic NA-LTP, perhaps heterosynaptic capture of LTP also involves similar players.

1.12 Thesis objectives

Studying the neuromodulatory influences of synaptic plasticity is critical to our understanding of how genes and proteins work to produce resilient long-term memories. Noradrenaline is a key mediator of long-term hippocampal memory formation, as b-AR activation boosts synaptic strength. These enduring synaptic changes may bolster weakly-activated sites by capturing strong activity from associated synapses.

Additionally, the nucleus is known to mediate LTP enhancement at homosynaptic sites following b-AR stimulation through the activation of intracellular signaling molecules. The central objective of my thesis is to determine whether the nucleus is necessary for boosting b-AR-induced heterosynaptic LTP. Homosynaptic NA-LTP enhancement requires the nucleus to engage transcriptional programs (Maity et al., 2016). It is not clear if NA-LTP persistence at heterosynaptic tagged synapses depends on *de novo* transcription. Furthermore, pharmacologically inhibiting histone acetyl transferase (required for histone H3 acetylation) blocks homosynaptic NA-LTP expression. Whether b-ARs signal via histone acetylation to gate heterosynaptic LTP had not been examined.

My thesis sought to address the following questions:

1. Can the endogenous ligand of b-ARs, NA, induce heterosynaptic transfer of LTP? Additionally, are b-ARs necessary for capture of NA-LTP at heterosynaptic tagged sites?
2. Is transcription required for heterosynaptic NA-LTP?
3. Is histone acetylation required for heterosynaptic NA-LTP and does blocking histone deacetylase boost this form of LTP?

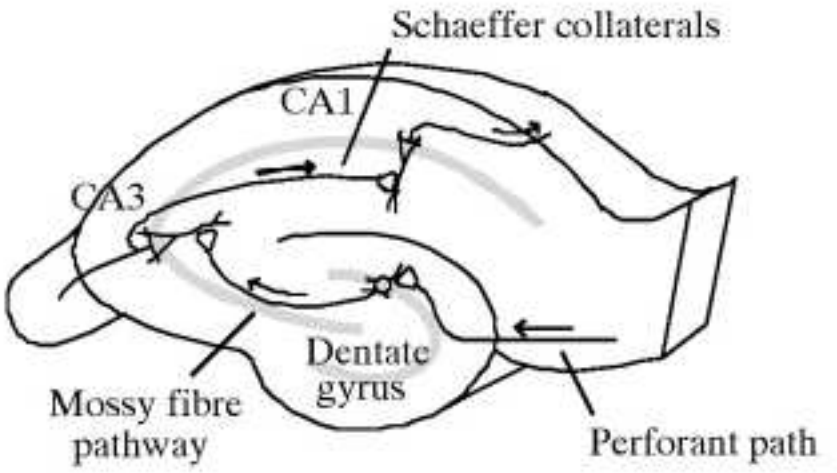

Figure 1.1. Tri-synaptic circuit of a mouse hippocampus section. Nguyen (2006).

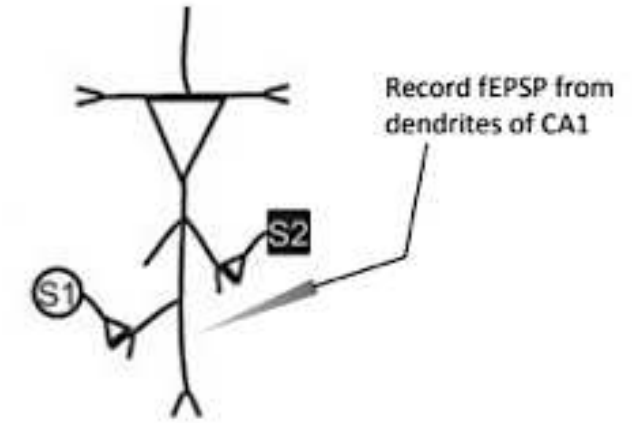

Figure 1.2. Schematic of a CA1 pyramidal neuron, with S1 and S2 synaptic inputs being stimulated, while population field potentials (fEPSPs) are recorded by extracellular implantation of a glass micropipette electrode near apical dendrites in CA1. From P.V. Nguyen.

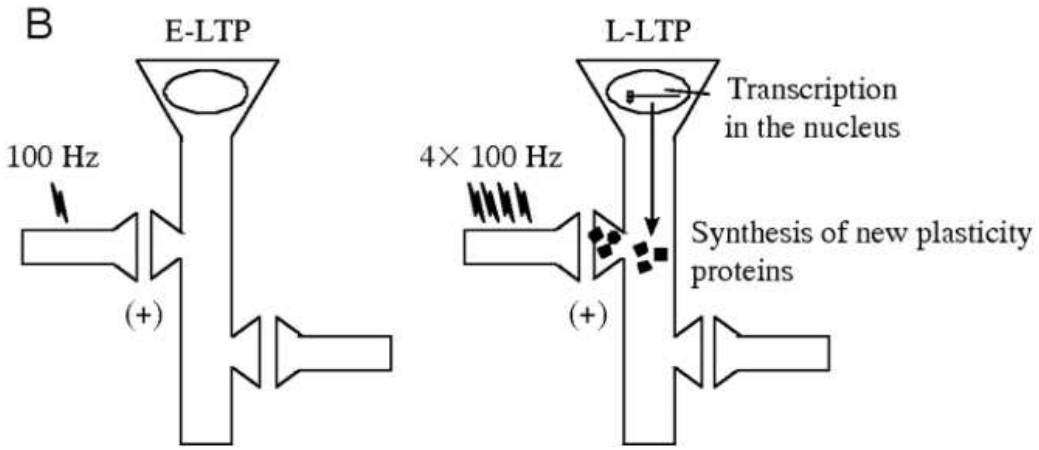

Figure 1.3. Different phases of long-term potentiation (LTP). *A*. Applying one train of high-frequency stimulation (HFS) at 100 Hz elicits a transient, early phase of LTP (E-LTP). *B*. A stronger stimulation (e.g. 4 trains of HFS at 100 Hz) evokes a more persistent, late phase of LTP (L-LTP) that requires transcription and protein synthesis. From Nguyen (2006).

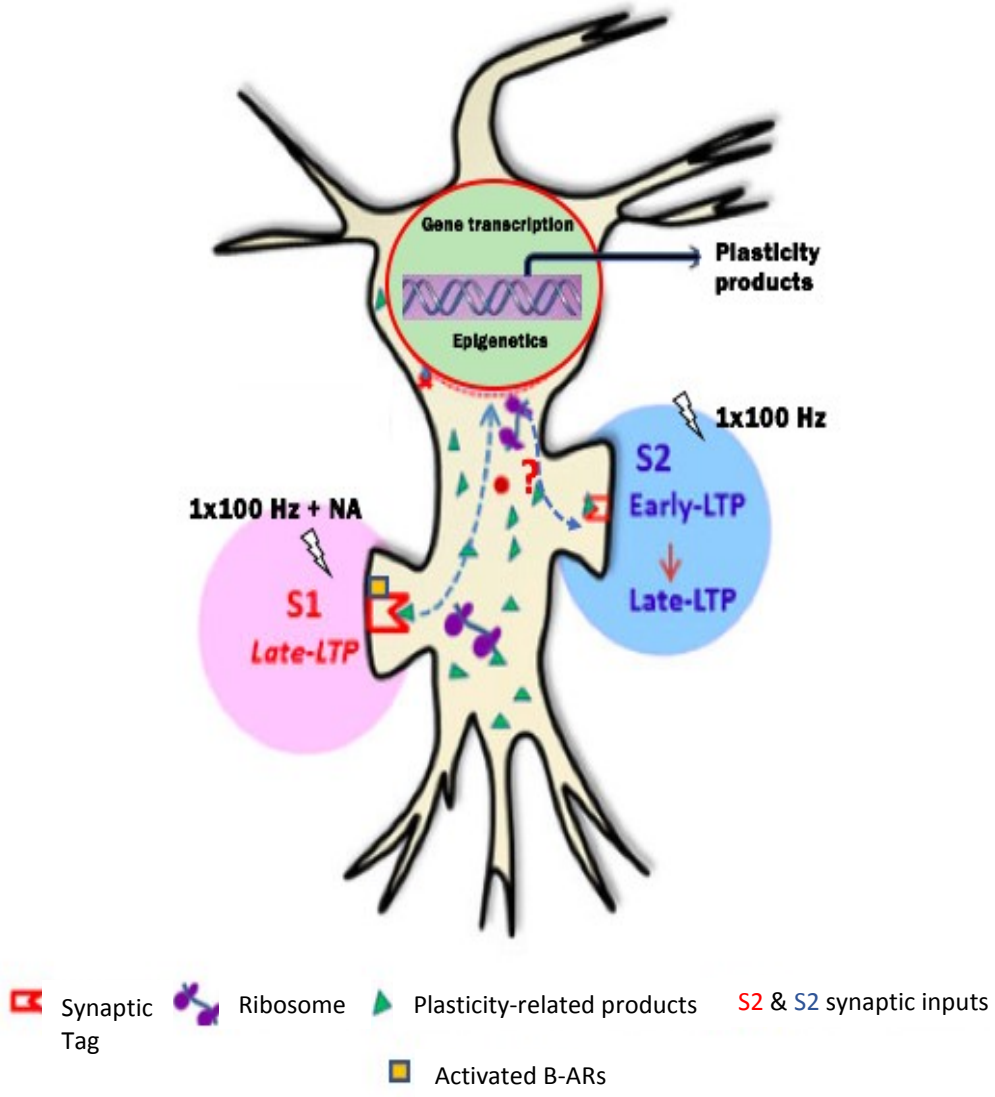

Figure 1.4. Putative model for synaptic tagging and capture, involving the setting of a synaptic tag and the capture of plasticity-related products. Pairing 1 train of high frequency stimulation with NA to S1 induces b-AR-dependent homosynaptic (S1) LTP that depends on transcription and epigenetic mechanisms. It is unknown whether setting a tag at S2 by applying a transient LTP protocol leads to heterosynaptic (S2) LTP that is nucleus-dependent. Adapted from Sharma et al. (2016).

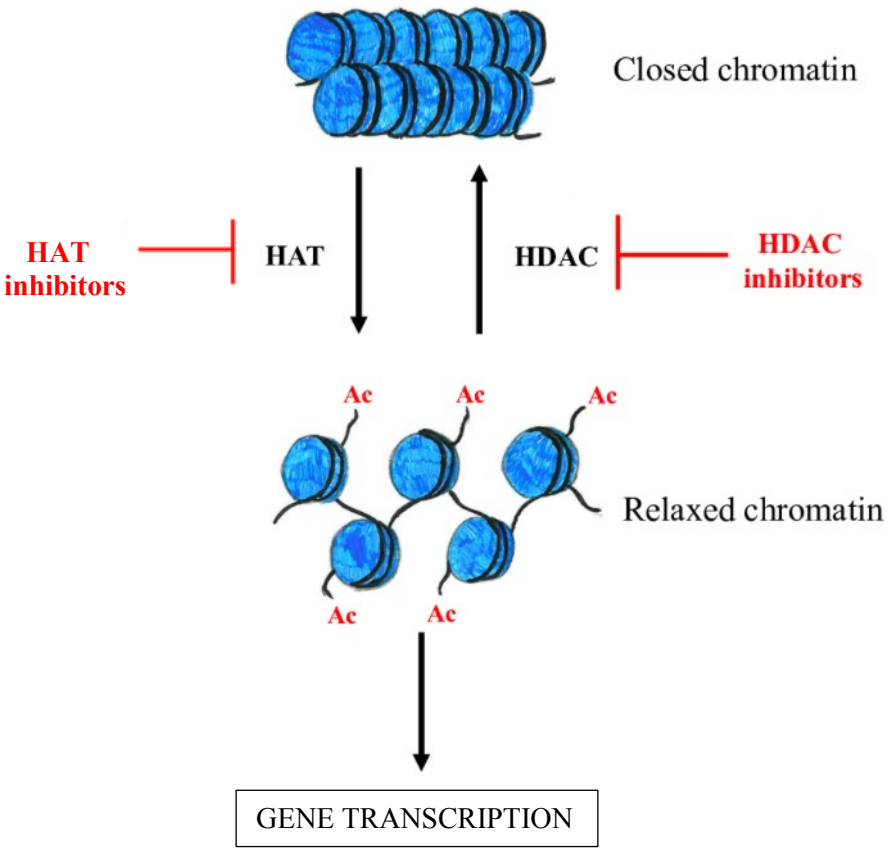

Figure 1.5. Chromatin remodeling by enzymatic catalysts of histone acetylation and deacetylation, and their targets of inhibition. Adapted from Laval & Nauwynck (2016).

Chapter 2: Materials and methods

2.1 Animals

Male C57BL/6 mice (Charles River Canada) were used for all experiments. Mice (aged 7-12 weeks) were fed *ad libitum* and housed at the University of Alberta in accordance with Canadian Council on Animal Care (CCAC) guidelines. All reported data were obtained from *in vitro* hippocampal slices. Proper procedures were taken to minimize animal pain and suffering, as approved by the University of Alberta's Heath Sciences Laboratory Animal Services ethics committee.

2.2 Hippocampal Slice Preparation

Following cervical dislocation and decapitation, the intact mouse brain was removed and placed in ice-cold artificial cerebrospinal fluid (aCSF). Both hippocampi were extracted and sliced transversely (at 400 μm thickness) using a tissue chopper. Slices were then transferred to an interface chamber (Fine Science Tools, Canada), where they recovered for approximately 90 minutes at 30°C and aerated with carbogen (95%O_2/5%CO_2) (**Figure 2.2**). Slices were continuously perfused with aCSF at a rate of 1 mL per min. aCSF was composed of (in mM): 124 NaCl, 4.4 KCl, 1.3 $MgSO_4$, 1 NaH_2PO_4, 26.2 $NaHCO_3$, 2.5 $CaCl_2$ and 10 glucose.

2.3 Electrophysiology

Field excitatory postsynaptic potentials (fEPSPs) were recorded from the CA1 area by placing stimulating and recording electrodes in the *stratum radiatum*. Recording glass microelectrodes were filled with aCSF (resistance of 2-3 MΩ), and bipolar nickel-chromium electrodes were used for stimulating the Schaeffer collateral-commissural pathway. Two independent synaptic paths

(S1 [homosynaptic pathway] and S2 [heterosynaptic pathway]) were stimulated; both converge onto the same population of CA1 pyramidal neurons. Both pathways were confirmed to be separate because they did not exhibit inter-pathway paired-pulse facilitation (Connor et al., 2011b). Field potentials were evoked at a stimulus intensity of 40% of their maximum amplitude, measured in mV. fEPSPs were elicited at a test stimulation frequency of once per minute (0.08 ms pulse duration) by stimulating both pathways 200 ms apart.

2.4 Drugs

Following twenty minutes of baseline recordings, different protocols were administered involving drug application and/or high-frequency stimulation (HFS; 1x100 Hz at 1s duration, to either or both pathways). "Weak HFS" refers to administering 1x100 Hz alone, while "strong HFS" refers to co-application of HFS (1x100 Hz) with NA (10 μM). Induction of homosynaptic and heterosynaptic LTP by pairing 1x100 Hz with ISO has been previously shown (Gelinas & Nguyen, 2005; Connor et al., 2011a). However, mine is the first study to use the natural transmitter, NA, instead of isoproterenol to investigate synaptic tagging. NA was applied 10 min before 100 Hz stimulation at S1, followed by an additional 5 min. Every drug added to overlap with NA was applied 20 minutes before NA application and remained present until 5 or 10 min after NA washout (40-45 min total drug application). Drugs which overlapped with HFS at S2 were applied starting at 20 min before 100 Hz and continued for an additional 5 or 10 min. A complete listing of drugs used for my experiments is catalogued in **Table 2.1**. Experiments were conducted under dimmed light conditions to minimize photolysis of light-sensitive drugs such as NA and some inhibitors.

2.5 Statistical Analysis

Data were analyzed offline using pCLAMP 10 software (Axon Instrument Inc., Union City, CA, USA). Initial fEPSP slopes were measured as an index of synaptic strength (Johnston & Wu, 1997). Slopes were averaged from 20 min of steady baseline recording (prior to HFS) to obtain a "baseline" mean value for each experiment. All subsequent slopes were expressed as percentages of these baseline mean slopes. Mean fEPSP slopes, measured at 90 minutes after HFS to S2, were used for inter-group comparisons of synaptic strength. Student's *t*-test was used for statistical comparisons of mean fEPSP slopes between two groups. One-way ANOVA and Tukey-Kramer *post-hoc* tests were used for comparing the significance between three or more groups (significance level of $p < 0.05$, [*]). All values were standardized to the average baseline slope and reported as mean ± SEM.

Table 2.1: Drugs Used For Experiments

Name	Action	Abbreviation	Company	Stock concentration	Applied in bath
L-(-)- norepinephrine bitartrate salt monohydrate	adrenergic receptor (AR) agonist	NA	Sigma	1 mM in aCSF	10 µM
Propranolol	beta-AR inhibitor	PROP	Sigma	10 mM in aCSF	50 µM
Actinomycin D	transcription blocker	Act-D	Sigma	25 mM in DMSO	25 µM

5,6- Dichlorobenzi-midazole 1-β-D-ribofuranoside	transcription blocker	DRB	Sigma	50 mM in DMSO	50 μM
C646	p300/CBP (HAT) inhibitor	C646	Sigma	10 mM in DMSO	5 μM
Trichostatin A	HDAC inhibitor	TSA	Sigma	5 mM in DMSO	5 μM

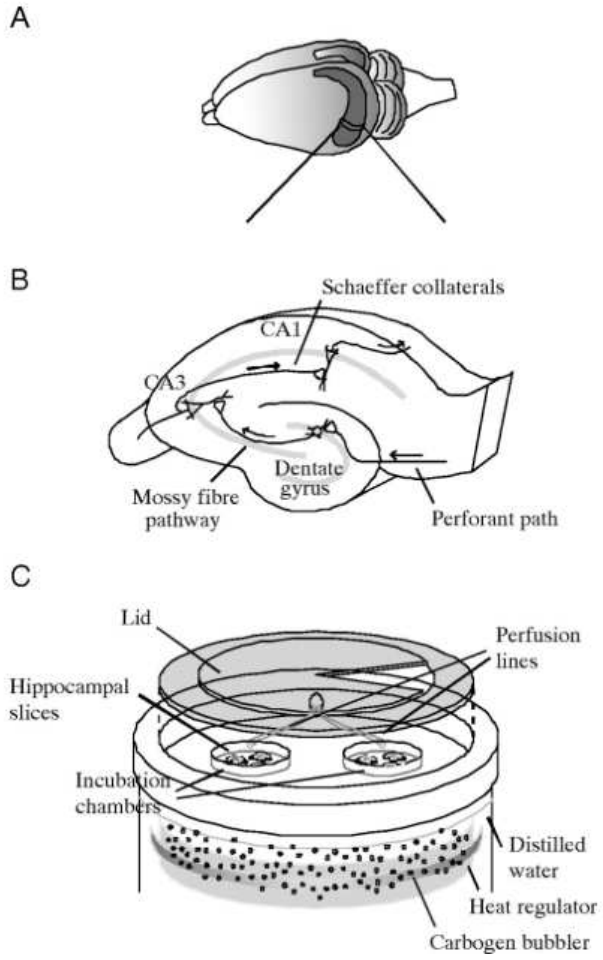

Figure 2.2. Hippocampal slice preparation. *A*. Illustration of a mouse brain prior to bihippocampal excision (in dark grey). *B.* A transverse hippocampal slice with labelled subregions and axonal pathways. *C.* Schematic of an interface recording chamber. Hippocampal slices rest above perfusing artificial cerebrospinal fluid (aCSF) on mesh rings embedded in individual wells. The chamber is fitted with a temperature-regulating system and a bubbler to keep slices in a consistently heated and oxygenated environment. Adapted from Nguyen (2006).

Chapter 3: Results

3.1 b-AR activation facilitates heterosynaptic NA-LTP

Noradrenaline (NA) acts upon b-ARs to boost LTP at excitatory hippocampal synapses (Stanton & Sarvey, 1984; Harley et al., 1996; Katsuki et al., 1997). NA enables the induction of long-lasting LTP when applied together with an inducing stimulus that would otherwise only elicit transient LTP (Thomas et al., 1996; Gelinas et al., 2008). Applying a single train at 100 Hz for one second can induce long-lasting LTP if b-ARs are coincidentally activated (Gelinas & Nguyen 2005; Gelinas et al., 2007; Ma et al. 2011).

Addition of a beta-receptor activator, isoproterenol (ISO), facilitates homosynaptic LTP (ISO-LTP), as does noradrenaline (henceforth referred to as NA-LTP). Heterosynaptic plasticity has also been observed in the presence of ISO (Connor et al., 2011b). However, NA, the natural ligand of adrenoreceptors in the brain, has not been tested in hippocampal slices while examining synaptic tagging and capture.

Upon weakly stimulating S1 and S2 pathways thirty minutes apart, E-LTP induction at S1 did not produce persistent LTP at a heterosynaptic site (S2) (**Figure 1A**). This finding is supported by previous evidence, in which weak stimulation protocols, of 1 train at 100 Hz and 5 Hz to two independent pathways, respectively, was insufficient for inducing lasting LTP (Connor et al, 2011b). Thus, high-frequency stimulation (HFS; 1x100Hz) applied homosynaptically to S1 and S2 did not induce L-LTP, as S1 only received weak HFS, likely preventing subsequent capture of late-LTP by S2 tags.

In contrast, applying repeated trains of HFS elicits homosynaptic and heterosynaptic LTP (Scharfman & Sarvey, 1985; Huang & Kandel, 1994; reviewed by Lynch, 2004). Furthermore, ISO-induced LTP can transfer to a second pathway whose input received weak stimulation (5 Hz) thirty minutes later (Connor et al., 2011b). To determine if NA can facilitate heterosynaptic LTP, I treated slices with NA for 10 minutes prior to weak HFS (1x100 Hz) at S1. NA continued to perfuse the slices for 5 additional minutes before washout. Weak HFS (i.e. 100 Hz only) was similarly administered to S2 30 minutes after stimulation to S1 **(Figure 3.2).** Student's *t*-test comparisons between control and NA-treated slices revealed significant differences in the magnitude of LTP both homosynaptically ($p < 0.01$; mean fEPSP slopes in treated slices were 162 ± 7% of baseline, which is significantly elevated relative to control slices (controls: 112 ± 13% of baseline)) and heterosynaptically ($p < 0.01$; 165 ± 15% in treated slices vs. 95 ± 13% in controls; **Figure 3.1C**). Therefore, NA-LTP generated by pairing NA with weak HFS at one synaptic site leads to LTP capture at a second pathway, as confirmed by LTP enhancement at S2.

The augmentation of heterosynaptic LTP by NA suggests a role for b-ARs in mediating the expression of long-lasting LTP at tagged synaptic sites. If NA acts specifically through binding and activation of b-ARs, then applying a b-AR inhibitor (50 μM propranolol) to CA1 at the same time that S1 receives HFS should prevent late-LTP and subsequent heterosynaptic LTP. To assess the importance of b-AR activity on synaptic tagging, I treated slices with 50 μM propranolol for 30 minutes immediately following 20 minutes of baseline recording. HFS was administered to S1 20 minutes after initial application of drug, and propranolol application remained for 10 additional minutes. S2 was later given weak HFS without co-applying the drug. Comparisons between propranolol and NA-treated slices revealed significant differences in LTP

maintenance both homosynaptically ($p < 0.05$; mean fEPSP slope in propranolol- treated slices was 103 ± 10% of baseline, which was significantly reduced relative to NA-treated slices (151 ± 15% of baseline); **Figure 3.2A**) and heterosynaptically ($p < 0.01$; 105 ± 9% in propranolol slices vs. 166 ± 10% in the positive control; **Figure 3.2B**). In addition to the finding that propranolol precludes NA-LTP at S1 (Maity et al., 2016), blocking b-ARs prevented heterosynaptic NA-LTP expression, presumably because no PRPs were generated for S2 capture.

This does not, however, exclude the possibility that previously applied NA (at S1) remained in the bath and affected slices at low concentrations after washout. If enough residual NA remained, there is a chance that it may have bound to b-ARs at S2 during HFS to S2, leading to homosynaptic LTP under the guise of "heterosynaptic" transfer of NA-LTP (since their observed field potential responses would be indistinguishable). To demonstrate that heterosynaptic LTP does, indeed, occur because of S1 b-AR activation at an earlier time point and not because of b-AR activation at S2 by residual NA, I applied the NA-LTP protocol at S1, consisting of 1 x 100 Hz overlapping with 10 μM NA treatment. NA was washed out for 5 minutes prior to addition of 10 μM propranolol. The b-AR inhibitor perfused the slice for 30 minutes, with HFS administered after 20 minutes of inhibitor application. Therefore, propranolol had ample time to saturate receptors, and continued to antagonize b-AR until 10 minutes post-HFS. Student's *t*-test comparisons between NA+propranolol and NA-treated slices revealed no differences in mean slopes both homosynaptically ($p > 0.05$; mean fEPSP slope in NA+propranolol treated slices was 135 ± 7% of baseline) and heterosynaptically ($p > 0.05$; 133 ± 9% in treated slices; **Figure 3.2C**). These results mitigate against the possibility that b-AR activation at S2 by residual NA was the cause of heterosynaptic L-LTP. Thus, heterosynaptic transfer of NA-LTP from S1 is

likely the reason for LTP maintenance. In short, NA LTP at S2 depends on S1 b-AR activation from 30 minutes earlier, and not b-AR activation at S2.

Although the reversible and competitive binding properties of propranolol have been well-documented (Kaiser, 1980), adding propranolol to overlap with S2 is insufficient for concluding that NA-LTP was transferred from S1, since it assumes that propranolol out-competed enough NA to have blocked induction of homosynaptic LTP at S2. Administering an NA-LTP protocol in the presence of propranolol at S1, therefore, seeks to demonstrate whether the antagonist displaces NA, thereby precluding heterosynaptic LTP. Following baseline recording for 20 minutes, propranolol was added for 45 minutes. During this time period, NA was co-applied for 15 minutes, coinciding with HFS at S1 five minutes prior to washout. Comparisons between NA-treated and propranolol-NA co-application revealed significant differences in the magnitude of LTP both homosynaptically (**Figure 3.2D**; $p < 0.05$; mean fEPSP slope in propranolol-NA overlapping slices was 112 ± 6% of baseline, which was significantly lower than NA-treated slices) and heterosynaptically ($p < 0.01$; 1211 ± 6% in slices treated with overlapping drugs). Thus, applying an NA-LTP protocol to S1 in the presence of a b-AR inhibitor did not induce NA-LTP at S1 nor at an S2 tagged site. This strengthens the earlier finding (**Figure 3.2C**) that propranolol can displace residual NA. Therefore, b-AR activation at S1 is necessary not only for enhanced homosynaptic transmission, but also for heterosynaptic capture of L-LTP. A histogram comparing mean fEPSP slopes for all propranolol-related experiments is depicted in **Figure 3.2E**.

3.2 A requirement for transcription in NA-LTP

Transcription and proteins play a vital role in the expression of long-lasting LTP. In addition to L-LTP requiring protein synthesis, L-LTP at stimulated synapses also relies on transcription (Nguyen et al., 1994). More specifically, NA stimulation of b-ARs initiates signalling to the nucleus to homosynaptically boost LTP endurance (Maity et al., 2016). While long-lasting forms of synaptic plasticity engage translation (Costa-Mattioli et al., 2009; Connor et al., 2011b), the role of somatic transcription on synaptic tagging has not been addressed.

To determine if enhanced NA-LTP persistence at heterosynaptically-tagged synapses depends on *de novo* transcription, I exposed slices to two commonly used inhibitors of transcription. Actinomycin-D (Act-D, 25 μM) binds to DNA to block RNA polymerase from synthesizing mRNA, and 5,6- Dichlorobenzimidazole 1-β-D-ribofuranoside (DRB, 50 μM) inhibits the elongation phase of transcription (Nguyen et al., 1994). Addition of Act-D (**Figure 3B**; mean fEPSPs were 106 ± 4% of baseline) or DRB (**Figure 3.3C**: mean fEPSPs were 98 ± 9% of baseline) inhibited NA-LTP at S1 relative to inhibitor-free controls (**Figure 3A**; means fEPSPs were 147 ± 6% of baseline). Act-D also prevented heterosynaptic NA-LTP (**Figure 3.3B**; mean slopes were 101 ± 17% of baseline), as did DRB (**Figure 3.3C**; 100 ± 9% of baseline) compared to controls. An ANOVA comparing fEPSPs of both transcription inhibitor treatments showed a significant difference between groups ($F(2,16) = 12.52$; $p < 0.001$) (**Figure 3.3D**). A subsequent Tukey-Kramer *post-hoc* test revealed that both Act-D and DRB prevented homosynaptic ($p < 0.005$) and heterosynaptic ($p < 0.05$) NA-LTP, implicating transcription in the expression of NA-LTP at tagged synapses. No significant differences were observed between Act-D and DRB treated groups ($p > 0.05$ in both S1 and S2).

3.3 Histone Acetylation is necessary for heterosynaptic NA-LTP

The nucleus may also regulate synaptic transmission at tagged locations by modifying chromatin and histones (Levenson et al., 2004; Chwang et al., 2007; Biergans et al., 2012). Histone acetylation, a prominent epigenetic process catalyzed by histone acetyl transferase (HAT), has been widely probed in the study of synapse-specific synaptic plasticity and memory formation (Levenson et al., 2004; Oliveira et al., 2007; Peixoto & Abel, 2013). Research has shown that intrinsic HAT activity of CRE-binding protein (CBP) and its homolog (p300) potentiate activated synapses and modulate memory formation (Korzus et al., 2004; Wood et al., 2006). Noradrenergic receptors have also been shown to regulate epigenetic modifiers, including the acetylation and deacetylation of histones proteins (Chang et al., 2012; Maity et al., 2016; Lim et al., 2016). By using a potent and specific CBP/p300 inhibitor, C646, I probed the requirement of histone acetylation for NA-induced LTP. Treating slices with NA and HFS in the continued presence of C646 (5 μM: this concentration blocks H3 acetylation in CA1 of mouse hippocampal slices (Maity et al. 2016)) at S1 occluded NA-LTP expression (**Figure 3.4B**; 96 ± 6% of baseline) compared to controls (**Figure 3.4A**; 149 ± 11% of baseline) as well as its transfer to S2 (fEPSP means were 92 ± 6% of baseline in treated slices compared to 148 ± 10% in inhibitor-free controls). A Student's *t*-test revealed that applying C646 significantly decreased fEPSP mean slopes compared to positive controls at S1 (**Figure 3.4C**; $t(11) = 3.45$, $p < 0.01$) and S2 ($t(11) = 5.04$, $p < 0.001$), indicative of significantly reduced NA-LTP expression and capture.

3.4 Inhibition of HDACs does not boost NA-LTP at tagged sites

While HATs promote gene expression, histone deacetylases (HDACs) repress acetylation and reduce transcription. Broad spectrum inhibitors of HDACs such as Trichostatin A (TSA) boost memory consolidation and stabilize homosynaptic LTP at stimulated synapses, making them a prime agent for memory research (Yeh et al., 2004; Vecsey et al., 2007). Intriguingly, TSA fails to further enhance homosynaptic NA-LTP (Maity et al., 2016), suggesting that NA-LTP requires engagement of histone acetylation. Additionally, HDACs can restore heterosynaptic LTP in age-impaired hippocampi (Sharma et al., 2015), highlighting a potential role for histone acetylation in synaptic tagging.

Since my results with C646 suggest that histone acetylation gates, or enables, heterosynaptic NA-LTP, I wanted to see if NA-LTP expression would strengthen when deacetylation was pharmacologically inhibited. This would provide support for HDAC working synergistically with HAT to regulate heterosynaptic facilitation. NA-LTP was expressed at both S1 (**Figure 3.5B**; fEPSP mean slopes were 123 ± 5% of baseline) and S2 (fEPSP mean slopes were 119 ± 7% of baseline), and potentiated slopes 2 hrs post-HFS at S2 did not significant differ from when NA was paired with HFS alone (**Figure 3.5A**; fEPSP mean slopes were 137 ± 4 vs. 140 ± 11%, respectively). This lack of statistical difference was corroborated using Student's *t*-test (**Figure 3.5C**; $t(9) = 2.14$, $p > 0.05$ at S1 and $t(9)=1.76$, $p > 0.05$ at S2). Thus, blocking deacetylation did not further facilitate heterosynaptic LTP. It is likely that, in transferring NA-LTP, b-AR signals to the nucleus to recruit histone acetylation independent of HDAC activity.

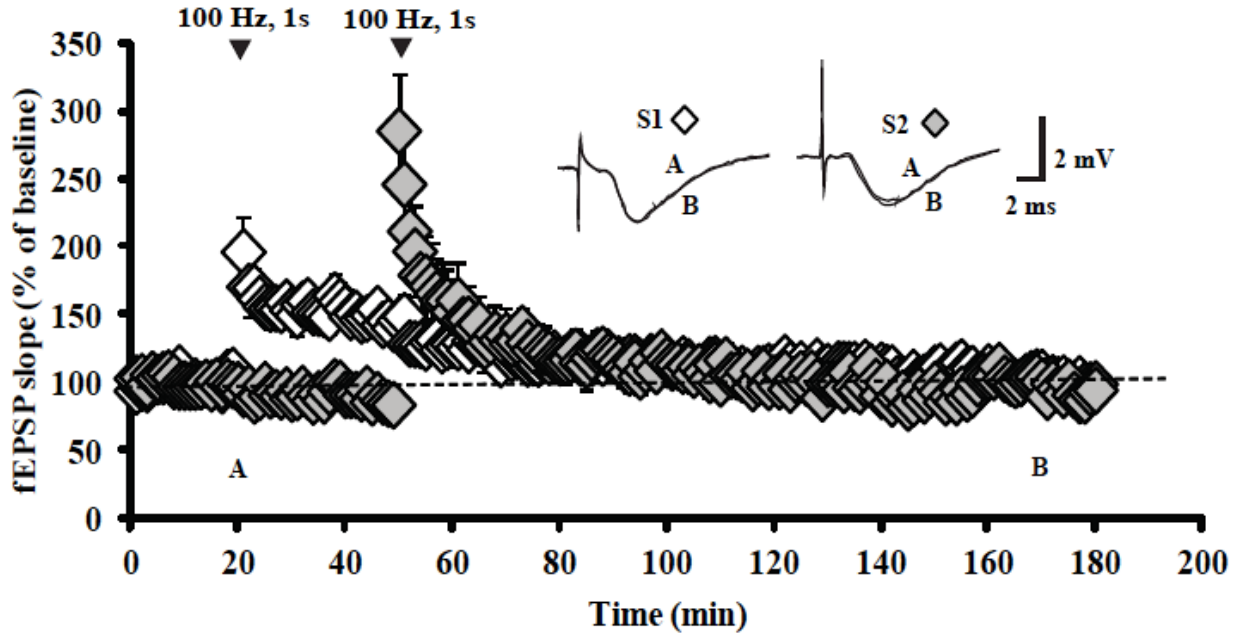

Figure. 3.1. Beta-adrenergic receptor activation promotes heterosynaptic NA-LTP. *A.* Stimulation consisting of 1 train at 100 Hz to the homosynaptic pathway (S1; open symbols) induced transient (< 2 h) LTP which did not promote heterosynaptic LTP following application of an identical stimulation protocol to a second synaptic pathway (S2; filled symbols) (n = 7). HFS alone did not persistently strengthen synapses, as fEPSP slopes at S1 and S2 returned to baseline (within 1 h).

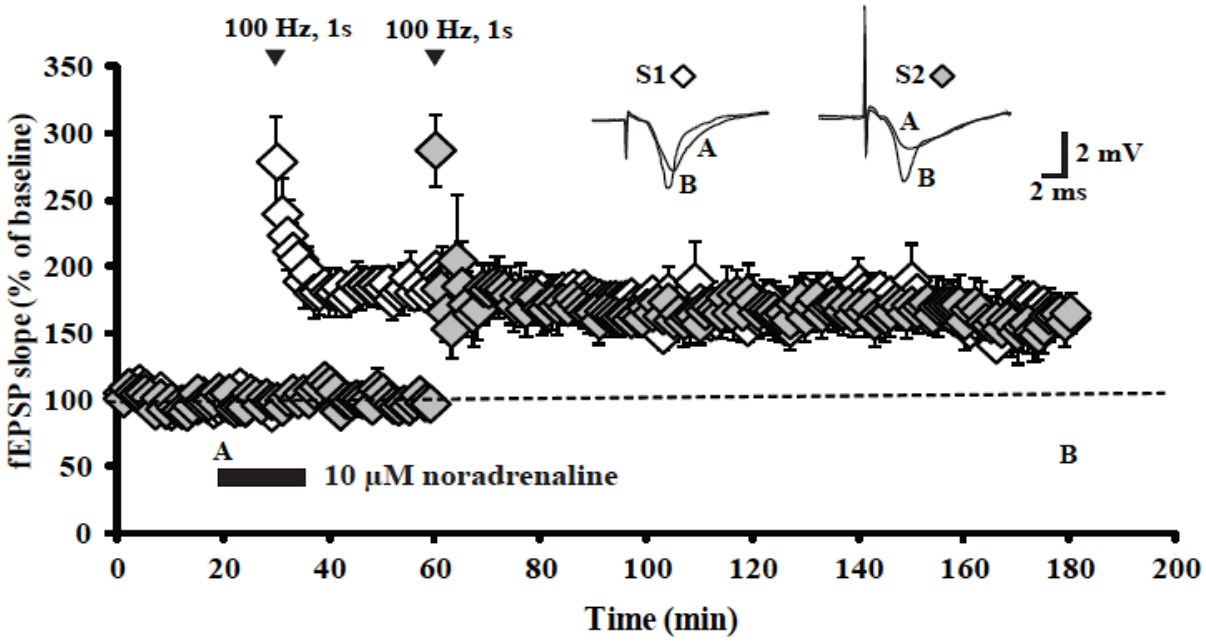

Figure 3.1. *B*. HFS to S1 overlapping with noradrenaline (NA) enhanced LTP. This form of lasting LTP (NA-LTP) can be captured by HFS given 30 minutes later at S2 (n = 6).

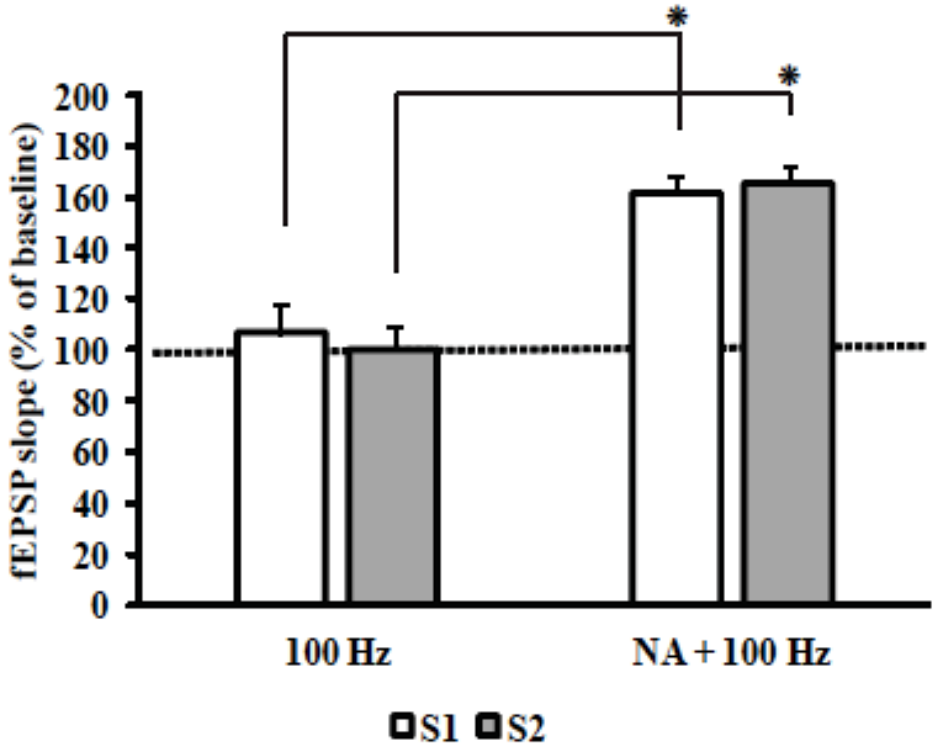

Figure 3.1. *C.* Summary histogram comparing mean fEPSP slopes obtained 150 min after HFS at S1 (white bars) and 120 min after HFS at S2 (grey bars). Representative fEPSP traces were sampled 20 min after commencement of baseline recordings and 150 and 120 min after HFS at S1 and S2, respectively. * specifies statistical significance between treatment groups. Results in C denote means ± SEM, *$p < 0.05$.

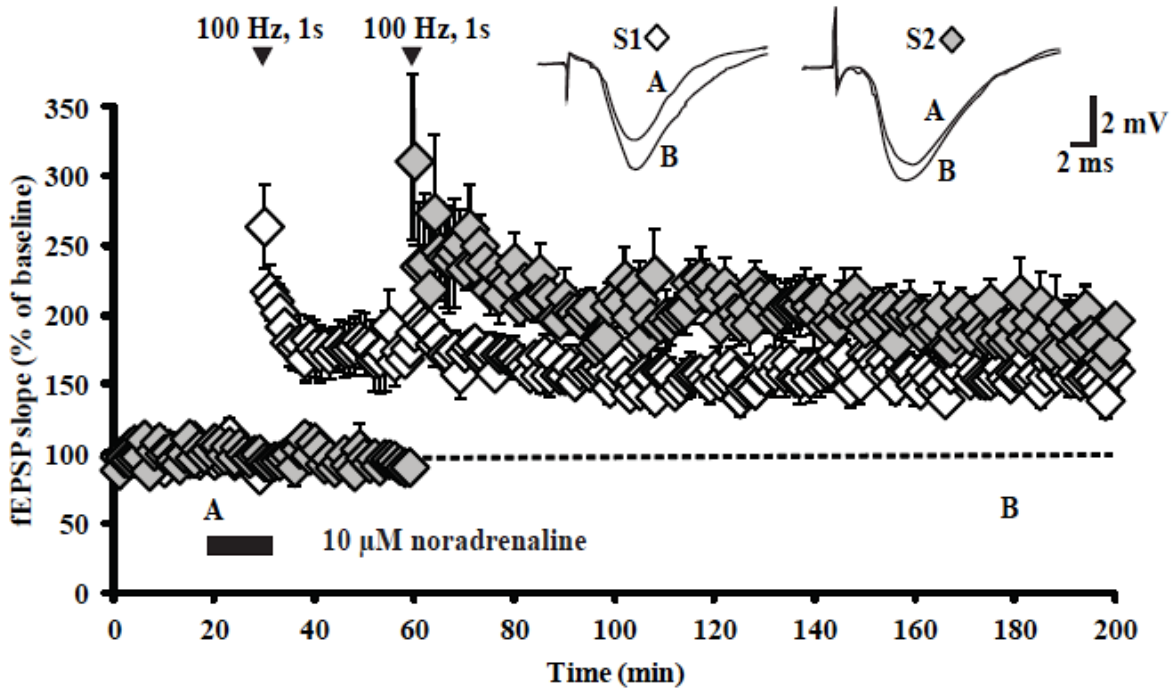

Figure. 3.2. b-ARs are required for heterosynaptic NA-LTP expression. *A*. NA-induced late-LTP maintenance at S1 (open markers) and S2 (filled markers) (n = 5).

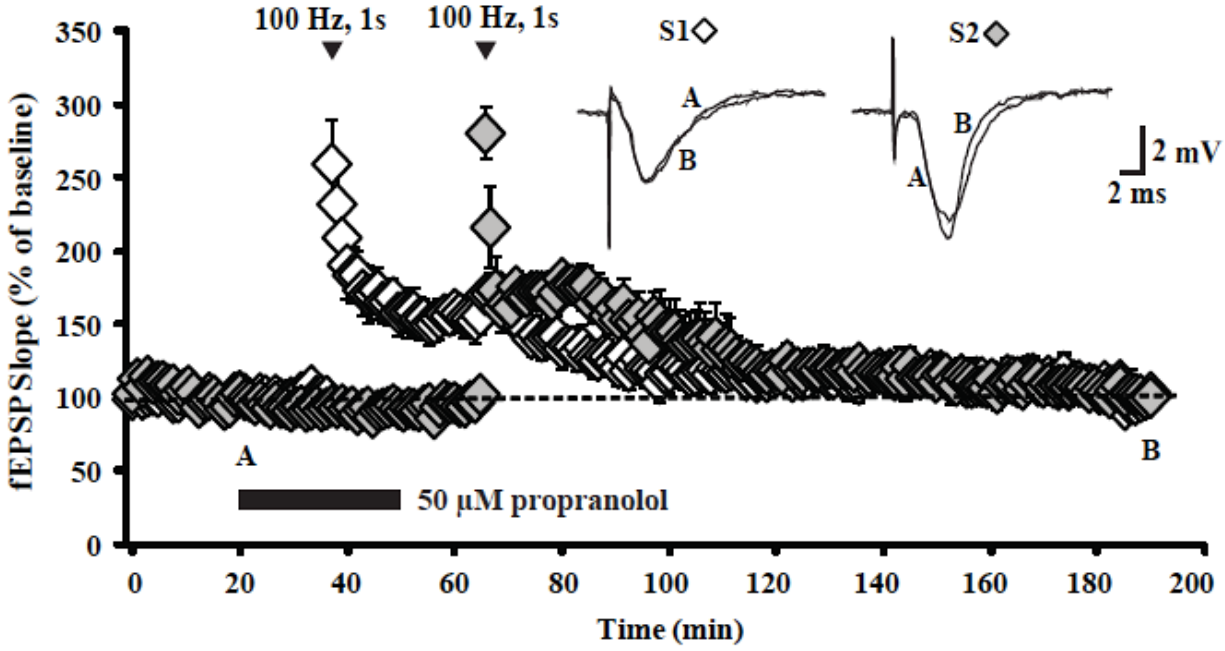

Figure 3.2. *B.* Applying the b-AR inhibitor, propranolol, rather than NA, overlapping HFS at S1 prevented expression of both late-LTP and heterosynaptic LTP (n = 6).

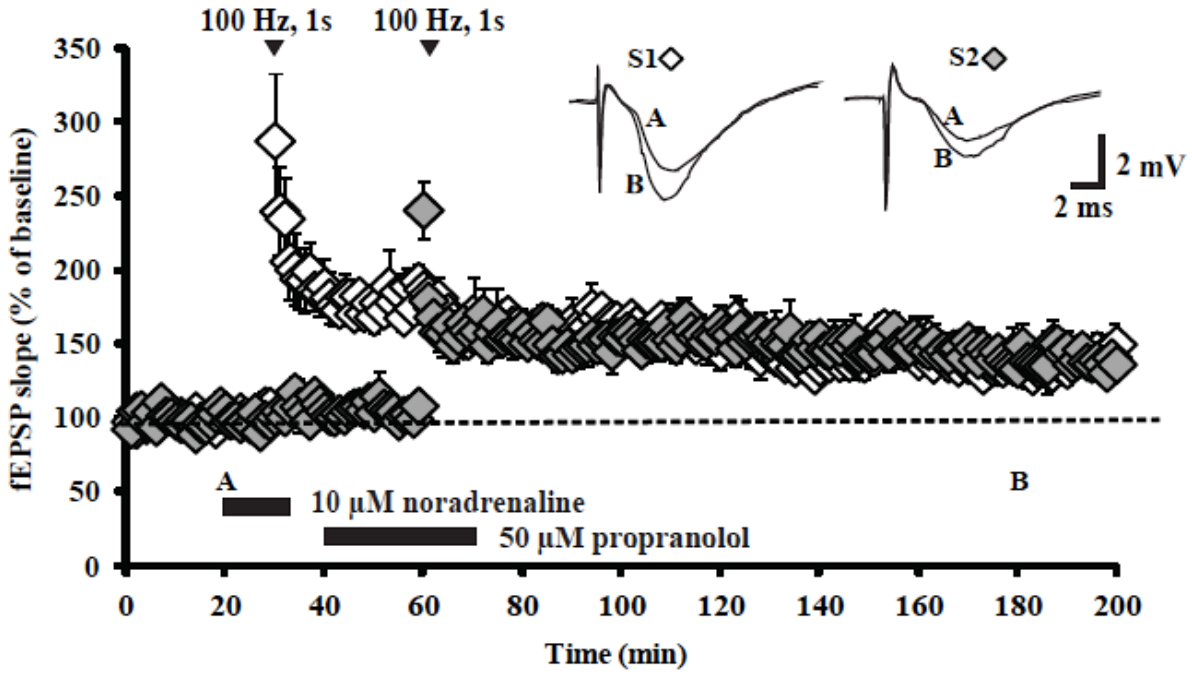

Figure 3.2. *C.* Shifting propranolol to overlap with HFS at S2, while S1 receives an NA-LTP stimulation protocol, boosted LTP longevity homo- and heterosynaptically. Mean fEPSPs remained persistently above baseline values, indicative of enhanced synaptic strength (n = 7).

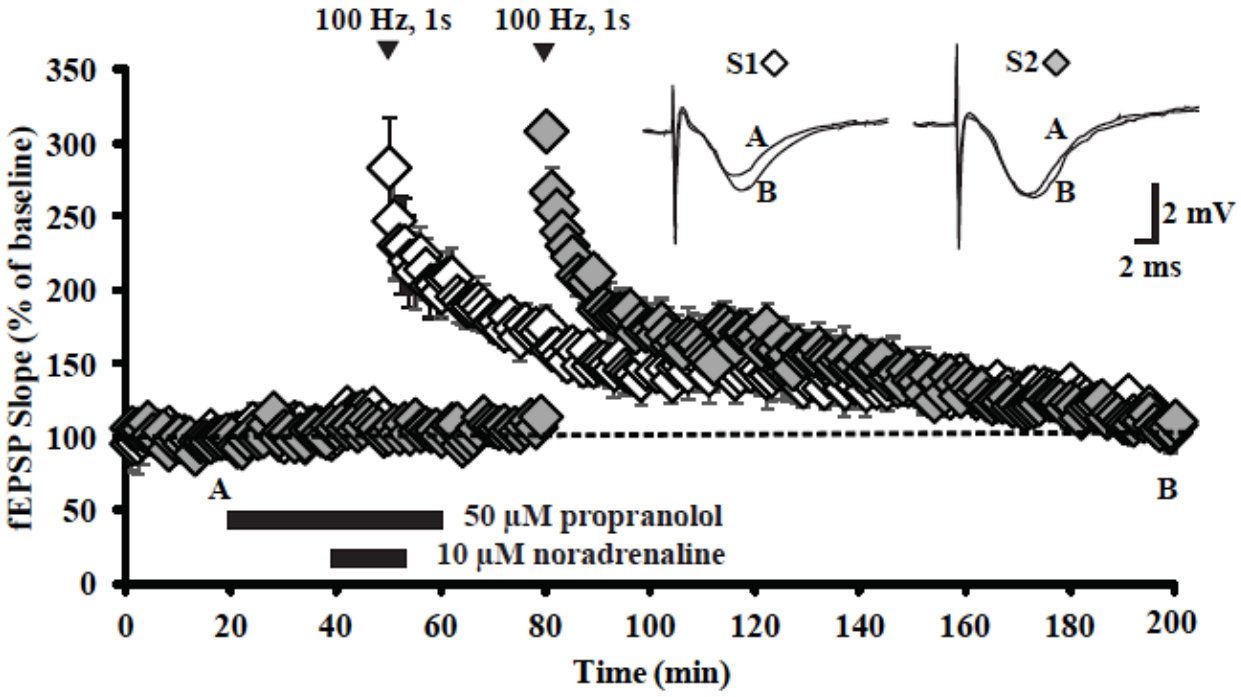

Figure 3.2. *D*. When propranolol was co-applied with NA at S1 (i.e. a late-LTP-inducing protocol), both homosynaptic and heterosynaptic LTP were transiently expressed and return to basal levels (n = 7).

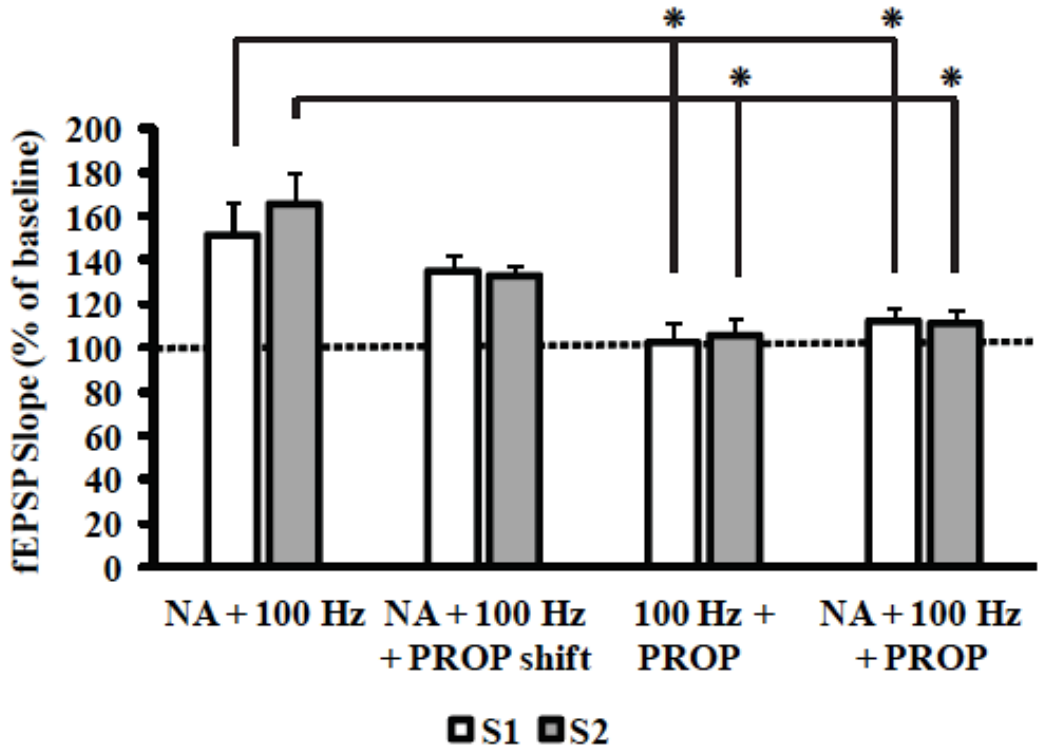

Figure 3.2. *E.* Summary histogram comparing mean fEPSP slopes obtained 150 min after HFS at S1 (white bars) and 120 min after HFS at S2 (grey bars). Representative traces were sampled 20 min after commencement of baseline recordings and 150 and 120 min after HFS at S1 and S2, respectively. * specifies statistical significance between treatment groups. Results in *E* denote means ± SEM, *$p < 0.05$.

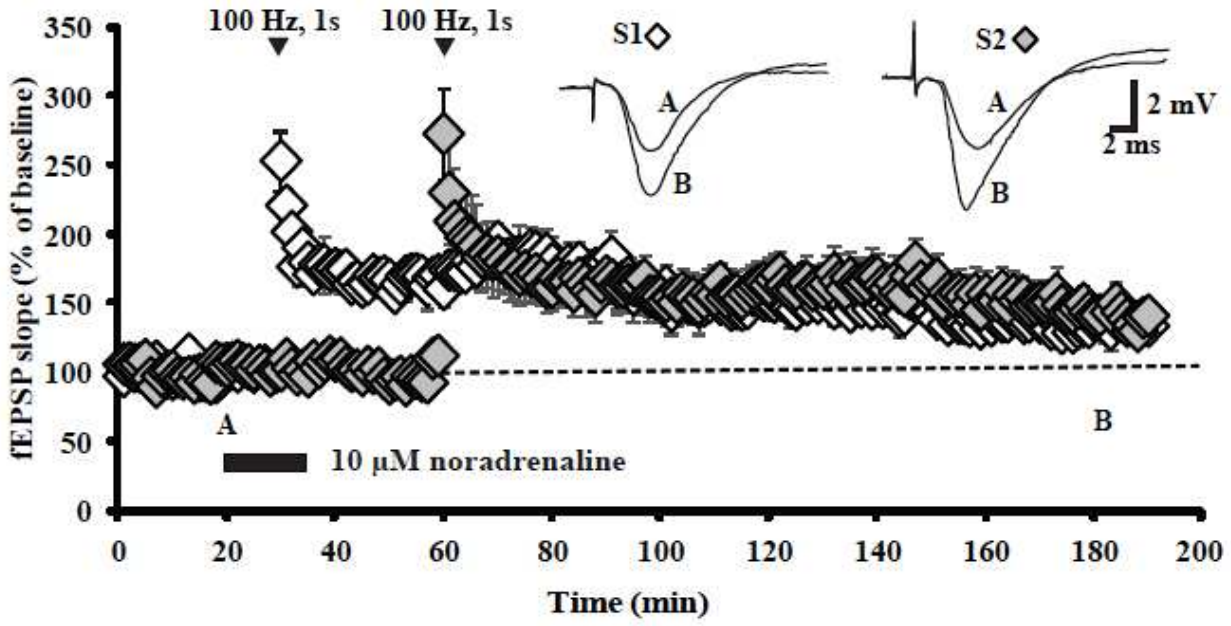

Figure 3.3. Transcription is necessary for heterosynaptic transfer of b-AR-induced LTP. *A*. NA-LTP is elicited at S1 (open markers) and subsequently captured at a second pathway (filled markers) (n = 5).

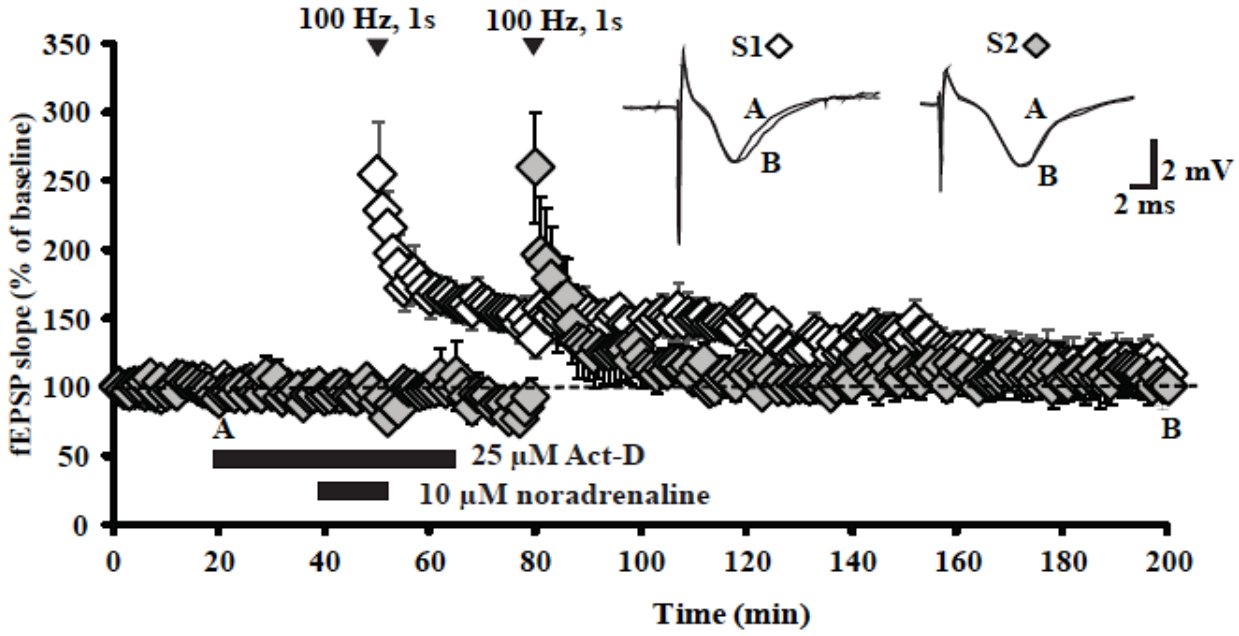

Figure 3.3. *B*. Slices treated with a transcription inhibitor, actinomycin-D (Act-D), during b-AR activation paired with HFS did not express LTP at S1 (white marker) or S2 (grey marker) (n = 7).

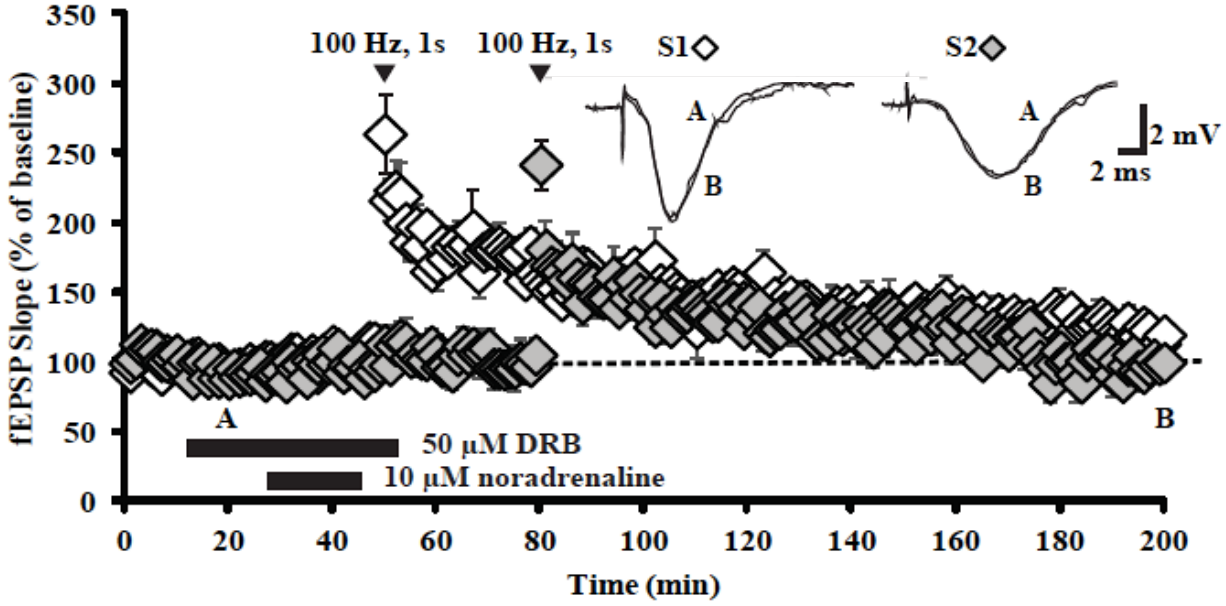

Figure. 3.3. *C.* Applying a different transcription blocker, DRB, produced a similar effect to Act-D, as NA-LTP was not significantly above baseline at S1 or S2 (n = 7).

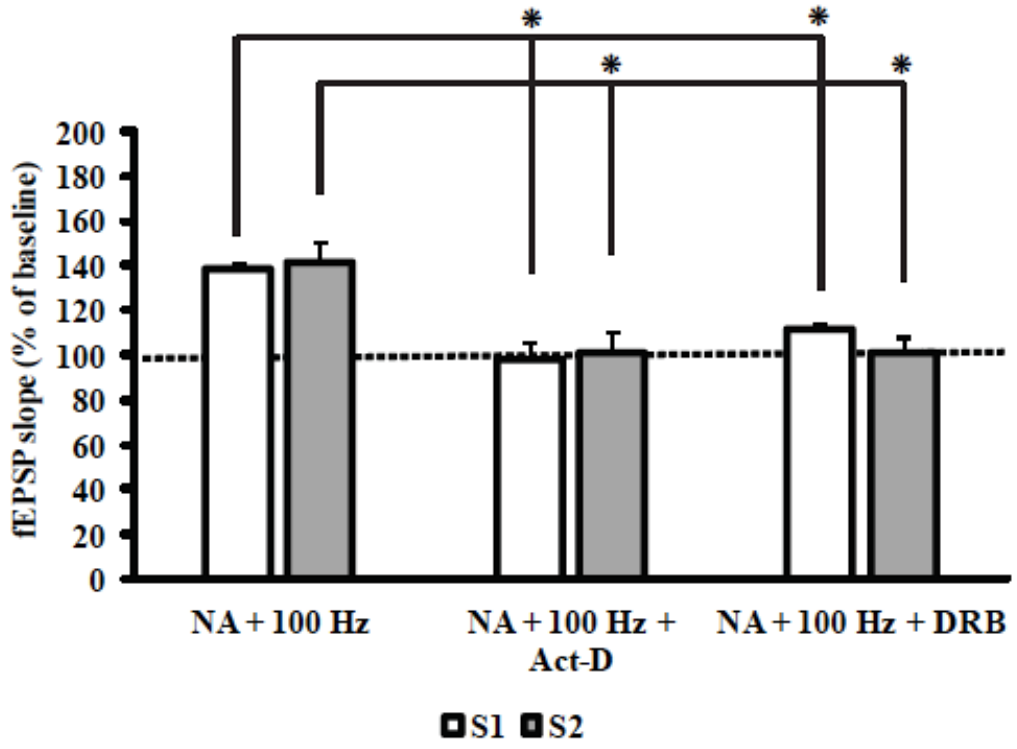

Figure. 3.3. *D*. Summary histogram comparing mean fEPSP slopes obtained 150 min after HFS at S1 (white bars) and 120 min after HFS at S2 (grey bars). Representative traces were sampled 20 min after commencement of baseline recordings and 150 and 120 min after HFS at S1 and S2, respectively. * specifies statistical significance between treatment groups. Results in *D* denote means ± SEM, *$p < 0.05$.

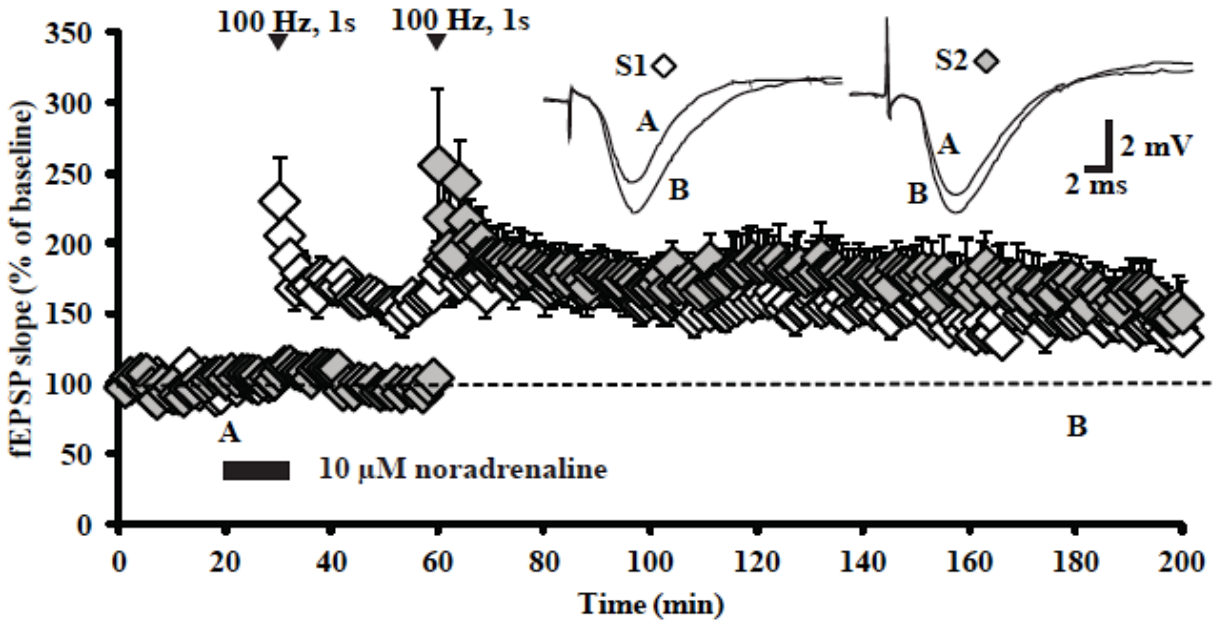

Figure 3.4. Histone acetylation is necessary for heterosynaptic NA-LTP. *A*. Slices were perfused with NA, and HFS at S1 induced lasting LTP that was maintained homosynaptically and heterosynaptically (filled markers) (n = 5).

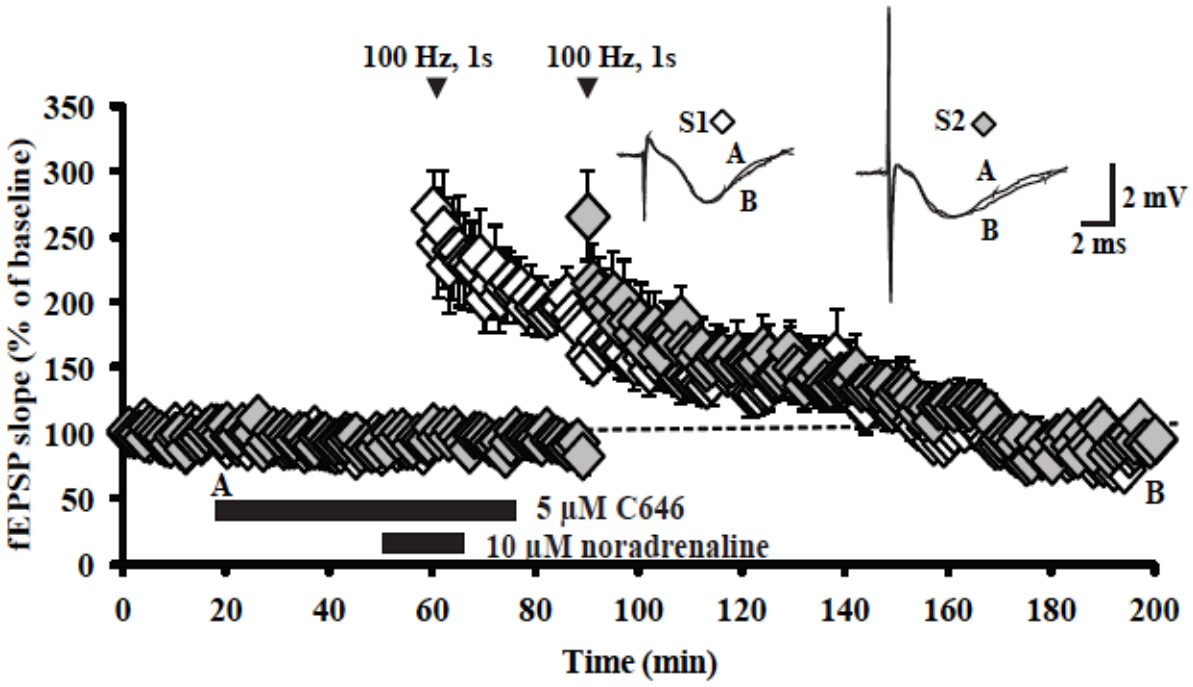

Figure 3.4. *B*. Bath application of C646, a histone acetyl transferase (HAT) inhibitor, significantly reduced LTP longevity at S1 and S2 when co-applied with NA + HFS at S1 (n = 7).

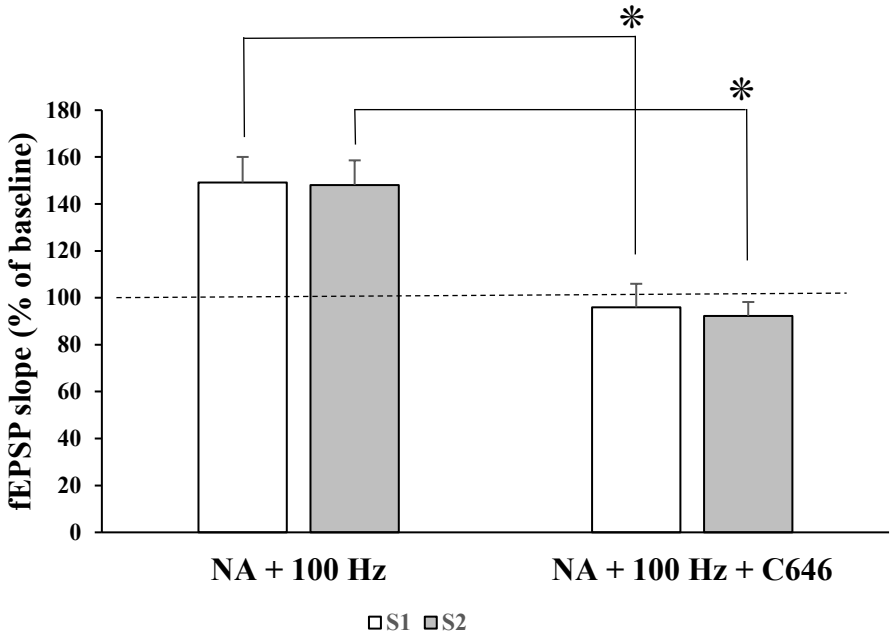

Figure 3.4. *C*. Summary histogram comparing mean fEPSP slopes obtained 150 min after HFS at S1 (white bars) and 120 min after HFS at S2 (grey bars). Representative traces were sampled 20 min after commencement of baseline recordings and 150 and 120 min after HFS at S1 and S2, respectively. * specifies statistical significance between treatment groups. Results in *C* denote means ± SEM, *$p < 0.05$.

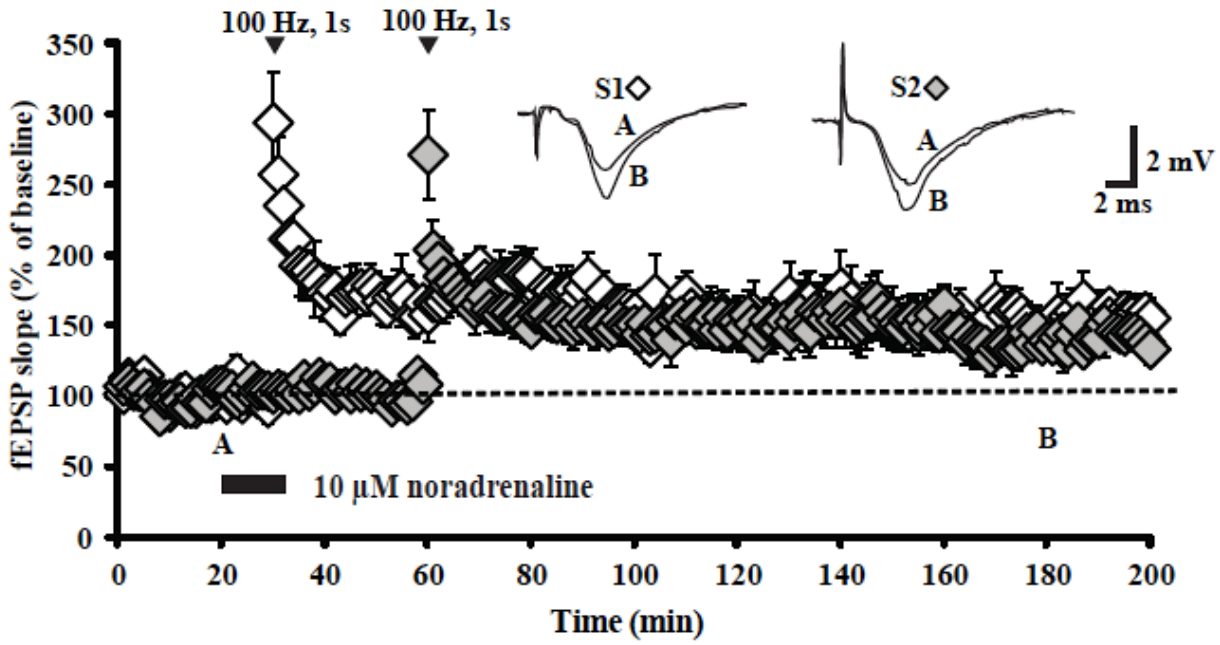

Figure 3.5. HDAC inhibition does not boost b-AR-dependent-LTP at tagged sites. *A*. Both homosynaptic (open symbols) and heterosynaptic (filled symbols) NA-LTP were persistently expressed following HFS +NA to S1 (n = 5).

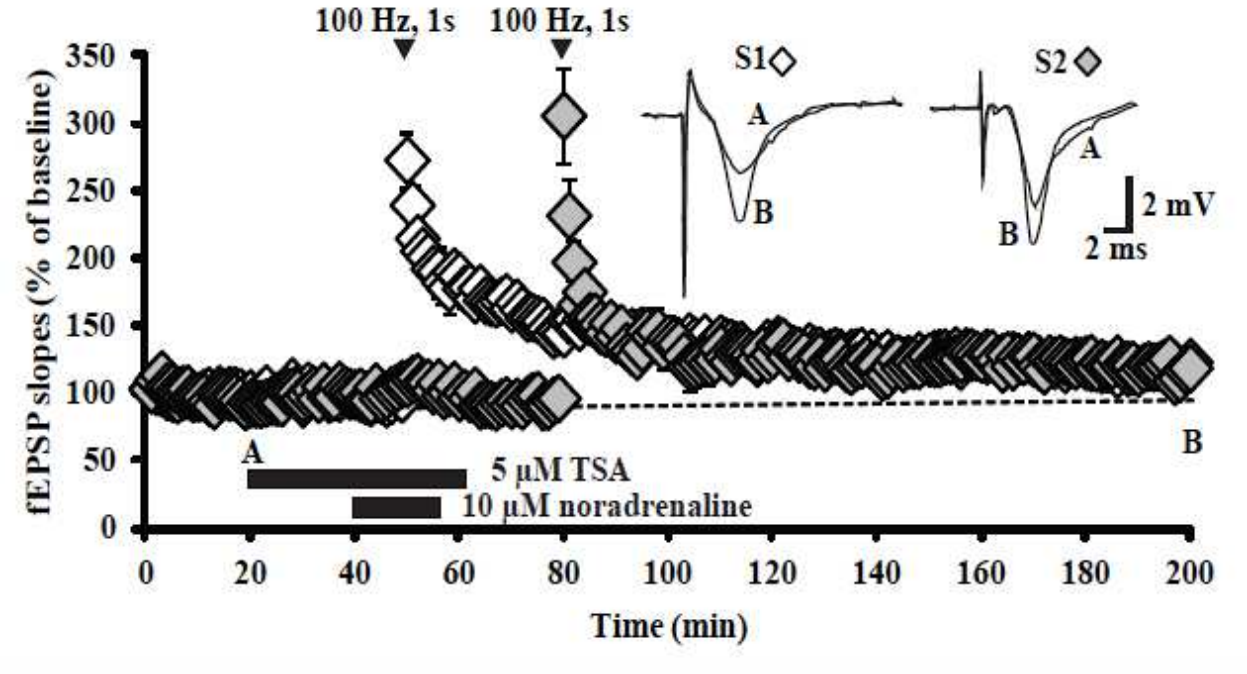

Figure. 3.5. *B*. Application of the HDAC inhibitor, trichostatin A (TSA), to coincide with NA at S1, blocked capture of heterosynaptic LTP (n = 6).

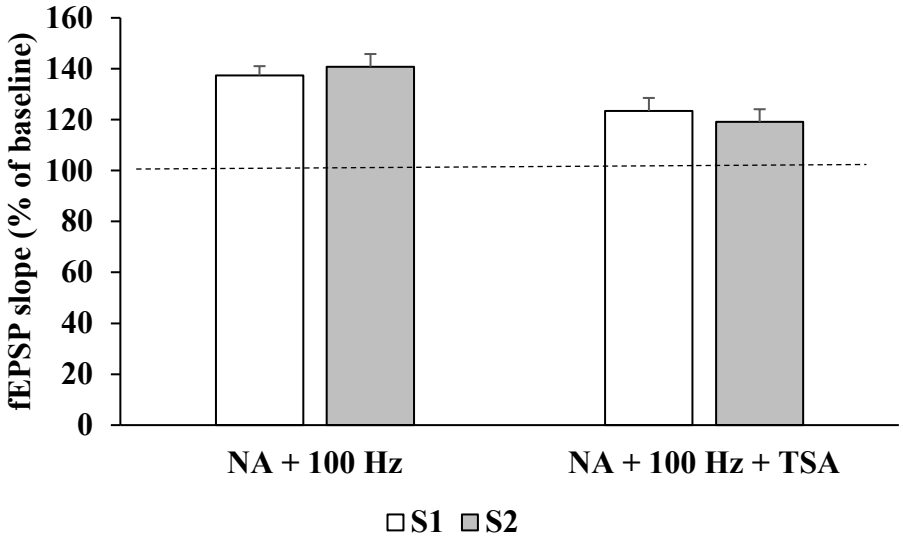

Figure 3.5. *C*. Summary histogram comparing mean fEPSP slopes obtained 150 min after HFS at S1 (white bars) and 120 min after HFS at S2 (grey bars). Representative traces were sampled 20 min after commencement of baseline recordings and 150 and 120 min after HFS at S1 and S2, respectively. * specifies statistical significance between treatment groups. Results in *C* denote means ± SEM, *$p < 0.05$.

Chapter 4: Discussion

4.1 Discussion

In the present study, I identified a requirement for b-ARs in expressing enduring LTP at tagged synapses (see Figure 3.2). This is supported by previous research, in which overlapping 100 Hz with the beta-selective agonist isoproterenol (and not 100 Hz alone) induces late-LTP that can transfer to a second pathway whose input received subthreshold stimulation thirty minutes later (Connor et al., 2011b). Indeed, my data revealed that b-AR-induced LTP can be transferred to a second synaptic pathway, so long as the weak stimulus was presented to the second path within a fixed window of time. That b-AR activation is a requirement for this LTP transfer stems from the finding in which a b-AR inhibitor (propranolol) was applied at S2. Overlapping propranolol administration with S2 stimulation did not affect heterosynaptic transfer of NA-LTP (Figure 3.2C). It can be inferred, therefore, that the intracellular mechanisms needed to elicit late-LTP are already initiated by earlier action of NA, and that b-ARs need not be engaged for expression of NA-LTP at S2. As a result, b-AR activity at homosynaptic sites is responsible for promoting transfer of NA-LTP, since 100 Hz stimulation at S1 alone is insufficient for eliciting late-LTP at S2 (Figure 3.1A).

As a modulator of LTP, b-ARs do not alter synaptic potentiation unless accompanied by an inducing stimulus. Likewise, b-ARs need not be activated for LTP induction by strong stimulation protocols (Swanson-Park et al., 1999). However, b-AR activation can modulate LTP longevity at a single synaptic pathway when inputs receive weaker patterns of stimulation. Applying a single train at 100 Hz can induce long-lasting LTP if b-ARs are coincidentally activated (Gelinas et al., 2005; 2007), as can weaker patterns of theta pulse stimulation (Winder

et al., 1999; Gelinas et al., 2005; Qian et al., 2012). Understanding how different electrical stimuli "couple" to neuromodulatory receptors to alter LTP expression will be the next logical step in further characterizing the synaptic tagging process.

Much of the aforementioned electrophysiology experiments are supported by behavioural data linking b-ARs with hippocampal memory formation. *In vivo* studies show that injecting NA into the hippocampus of rodents leads to spatial memory retrieval, and that b-AR activation by NA is a requirement for this (Sara et al., 1999). Furthermore, NA knockouts display memory deficits, which can be reduced by NA infusion (Murchison et al., 2004). Stimulating the locus coeculeus to secrete natural NA elicits similar outcomes (Devauges & Sara, 1991). That external infusion and physiological release of NA independently produce identical memory enhancements provide robust support to the noradrenergic system's enhancing effects on hippocampal memory.

Although there is ample evidence to suggest that mRNAs from local dendritic pools are translated and trafficked to nearby activated synapses to strengthen synaptic transmission, few studies have focused on nuclear involvement in heterosynaptic synaptic potentiation. The present finding that transcription is required for heterosynaptic NA-LTP identifies a communication network between the beta-adrenergic receptor and the nucleus (Figure 3.3). Different receptor-mediated signaling cascades likely engage transcription of key LTP-promoting genes. The transcripts are then translated into plasticity-related protein products and these are then captured by activity-induced tags. Therefore, protein production and *de novo* transcription are likely necessary – and work synergistically – to express long-lasting heterosynaptic LTP. My data suggest that the heterosynaptic "transfer" of NA-LTP is modulated by the nucleus (see Figure

3.3). The translated products (plasticity-related proteins, PRPs) are then captured at tagged dendritic sites, leading to their specific strengthening (as observed by an increase in fEPSP slope). Confounding presynaptic mechanisms may also contribute to heterosynaptic plasticity, including signalling molecules like PKA, which may interact with protein products to regulate presynaptic transmitter release (Park et al., 2014). Nonetheless, the exact identities of these PRPs remain controversial. Yao et al. (2008) found that PKMζ is necessary for maintaining late-LTP, particular through GluR2-dependent AMPAR trafficking. Furthermore, inducing L-LTP increases expression of PKMζ mRNA and protein in area CA1 (Yao et al., 2008). Additionally, PKA has been noted as an attractive candidate protein for its metaplastic influence on increasing the time window for synaptic transfer of LTP by as much as 1 hour following its activation (Tenorio et al., 2010; Connor et al., 2011b). Activating PKA promotes tagging and capture of late-LTP, whereas inhibiting or mutating PKA impairs this process (Young et al., 2006). CaMKII has also been recognized as a candidate, as its increased activity is necessary for maintaining LTP and strengthening synapses (through AMPAR modification) (Blitzer et al., 1998). Even if PRPs are correctly identified, future research must be done to see if the mRNA transcripts upregulated during heterosynaptic LTP are the same products that are subsequently translated and trafficked to tagged synapses.

It is well established that late-LTP, elicited in numerous ways, requires protein synthesis (Krug et al., 1984; Frey et al., 1988). The base of dendritic processes contains mRNAs which can be locally translated by ribosomes to mediate synaptic plasticity (reviewed by Steward, 1997). Blocking such translational machinery impairs LTP expression by preventing newly-synthesized proteins from being locally produced and trafficked (Kang & Schuman, 1996). Therefore, local

protein production can putatively explain why synapses have the ability to alter their strength in a relatively short time frame. While local translation permits rapid and adaptive responses to stimulation, recruiting the soma affords its own advantages. Gene regulation via nuclear signalling allows the neuron to control expression of specific genes using transcription factors (for review, see Alberini, 2009). For example, b-AR activation engages cAMP to initiate signaling cascades, leading to the phosphorylation of the transcription factor, CREB. CREB, along with p300/CBP HAT activity, collectively alter promoter activity, thereby affecting the transcription of LTP-related genes.

Why does the expression of late NA-LTP, and presumably the capture of proteins at these tagged sites, require transcription, and not translation alone? It is plausible that protein synthesis is an inadequate long-term means of altering heterosynaptic transmission, and that *de novo* transcription helps to facilitate protein trafficking, and local translation at active dendrites. While it is unclear how much local translation of mRNA (perhaps from local mRNA granule pools) is contributing to LTP at S2, PRPs required for heterosynaptic enhancement are shared between synapses in a competitive manner, which may become limited if multiple synapses are activated contemporaneously (Fonseca et al., 2004). Engaging transcription may act to upregulate synthesis of PRPs in order for tagged sites to receive the proteins necessary to stabilize LTP.

More likely, a combination of both local protein synthesis and trafficked protein from *de novo* nuclear transcription leads to expression of heterosynaptic LTP. This interplay may also depend on physical proximity of the S2 site to the soma, as nearby synapses may rely on somatic products more than distal ones. The stochastic capture by tags may also be probabilistically

higher when tags are set in closer proximity to each other. The maximum distance postulated for synaptic tagging and capture to occur between dendritic spines is 70 μm (Govindarajan et al., 2011). Therefore, ribosomes translating *de novo* LTP-promoting mRNAs may be using machinery near dendritic sites, implying that *de novo* mRNAs are being pooled with existing mRNAs from dendritic granule stores to strengthen nearby synapses (Raymond et al., 2000).

Since transcription is required for NA-LTP, other somatic processes, including epigenetic modifications, may also be contributing to LTP. b-AR-induced transcription and epigenetic mechanisms must also share a common signaling pathway, which suggests that certain epigenetic modifiers may be interacting with somatic transcription. My results reveal for the first time a requirement for acetylation of histones in expressing heterosynaptic LTP. This long-lasting response is mediated by b-ARs and is primed to regulate transcription when NA is paired with HFS. Identifying which genes are expressed by histone acetylation to promote synaptic plasticity will be paramount for future study. Certain genes such as *bdnf* and *Arc* have already been proposed as targets of epigenetic modifiers in memory models (Lubin et al., 2008; Penner et al., 2011). Additionally, acetylation of genes by CBP-HATs disengages repressors to boost transcription, synaptic activity and memory formation (Nelson & Monteggia, 2011). As a result, histone acetylation has a direct role in controlling which genes are expressed during synaptic activity.

LTP is enhanced in TSA-treated slices after an E-LTP protocol of 1 train at 100 Hz to CA1 neurons (Vecsey et al., 2007). Thus, TSA modulates LTP at stimulated synapses to promote homosynaptic plasticity (Yeh et al., 2004; Vecsey et al., 2007). However, Maity et al. (2016)

reported no further increase in LTP maintenance when NA+HFS was paired with TSA application. Indeed, I showed no apparent rise in heterosynaptic NA-LTP when TSA was presented concurrently. Therefore, HDACs do not appear to have an enhancing role in either homosynaptic, or heterosynaptic, plasticity. It could be that HDAC inhibitors work to augment transient forms of LTP (e.g. E-LTP) to a certain level and may even support robust LTP. But blocking HDACs is evidently not relevant in boosting LTP at stimulated tagged sites. This may explain why E-LTP is enhanced with TSA addition, as Vecsey et al. (2007) demonstrated. Inhibiting HDAC likely promotes acetylation to the point where LTP at all tagged sites is enhanced to a certain point, but not beyond that induced by NA-LTP. In short, NA-LTP recruits histone acetylation which regulates transcription and translation of products that are captured at previously tagged synapses. Preventing histone deacetylation permits transfer of NA-LTP to tagged sites in much the same way as promoting acetylation. Furthermore, experiments involving the co-application of TSA and actinomycin D (transcription inhibitor) revealed that transcription was necessary for generating late-LTP during HDAC inhibition (Vecsey et al., 2007). Blocking deacetylation appears to maintain strengthened synapses in a transcription-dependent manner by activating key LTP-genes to, ultimately, enhance memory consolidation.

4.2 Future direction

A theoretical model for synaptic tagging and capture of NA-LTP must involve the nucleus. Activating b-ARs leads to recruitment of transcription. At the same time, marking the synapse by HFS allows products of transcription (PRPs) to be recruited to those activated synapses in order to strengthen them. While my data indicated that acetylation of core histones was necessary for

heterosynaptically enhancing NA-LTP, future research must investigate the roles of specific histones. For instance, TSA increases H3K14 acetylation to rescue hippocampal LTP in aging rats (Tian et al., 2010). Meanwhile, addition of histone deacetylase inhibitors to increase H3 acetylation subsequently enhances LTP induction in *in vivo* CA1 hippocampi, underscoring the relationship between epigenetic regulation of H3 and long-term memory formation (Levenson et al., 2004). Performing a chromatin immunoprecipitation could help to identify changes in acetylation at particular histone residues during synaptic plasticity.

Furthermore, both timing and independence are key tenants of the tagging and capture model, as synapse-specific, activity-dependent NA-LTP is required for products to be captured at a secondary site. Late-LTP can rescue decaying potentiation at S2 when weak tetanization precedes or follows strong HFS in *in vitro* and *in vivo* models, so long as the induction of both events occur within a finite window of time (Frey & Morris, 1998; Shires et al., 2012). Quantifying the precise time window for protein capture is necessary, since such time constraints should coincide with decay of the synaptic tag. Demonstrating that both metrics are coincidental in time would further strengthen the tagging hypothesis.

The secondary site, which did not receive strong stimulation, expresses NA-LTP because stimulation above a particular threshold frequency marked the site for future PRP capture. Although it is unlikely that translation has occurred at this site (as the stimulus was too weak to evoke protein synthesis-dependent late-LTP), it cannot be ruled out that tagging a site leads to local synthesis of proteins and their subsequent trafficking to the spine. However, such speculation is dubious since only homosynaptic NA-LTP induced by b-AR activity and HFS, and

not 1 train of HFS alone, recruits protein synthesis. How the newly-synthesized proteins reach marked dendritic sites (i.e. activity-specific synapses) is less understood. Perhaps they are shuttled by colocalizing with molecules, such as Staufen – a protein that is implicated in somatodendritic transport of mRNA – until they find a synaptic signal or "tag", where they are placed into their functionally-specific locations (i.e. "captured") (Kiebler et al., 1999). It could be that transport vesicles, containing synapse-specific proteins, are marked for dendritic spine localization and prompted to these active synaptic locations by tags. Furthermore, if multiple sites require proteins, how do these vesicles know to send the adequate requirements of proteins to each active synapse so that these potentiated synapses stabilize? Understanding the cross-talk between the nucleus, proteins and synapses will provide insight into the capturing mechanisms of LTP-promoting products.

Neuromodulation by NA can boost LTP at excitatory synapses through specific action on adrenoreceptors. Although both major isoforms of the beta-adrenergic receptor – b1 and b2 – lead to enhancement of LTP, their relative contributions are unclear, as research has assigned importance to both (Winder et al., 1999; Qian et al., 2012). Further defining the roles of b-AR subtypes will be key in determining how they function at a cellular level during learning and memory. Their dynamic nature, for instance, is not properly understood. Are they themselves participants in membrane trafficking during LTP? If they are upregulated during plasticity, is it because of increased synaptic activity, in a manner similar to the GluR1 subunits of AMPA receptors?

Noradrenaline is not the only neuromodulator influencing hippocampal function. Dopamine is another neuromodulator which may play an important role in the stabilization of learning-induced synaptic changes triggered by physiological patterns of stimulation (Li et al., 2003). Dopaminergic neurons release dopamine into the hippocampus following exploration of a novel environment (Harley, 2004). Furthermore, novelty exposure in close proximity to an unrelated memory leads to a strengthening of that memory, in accordance with the synaptic tagging model (Morris, 2006). For this reason, novelty appears to stabilize recently-encoded memories, perhaps because memory encoding involves the setting of synaptic tags, which can capture PRPs induced by a novel task, leading to stabilized synapses and memory consolidation. Optogenetically activating these neurons in a temporally-relevant fashion, as one would do to stimulate adrenergic neurons from the locus coeruleus, may help to clarify the behavioural data and further strengthen the synaptic tagging model.

My experiments used extracellular field potential recordings to extrapolate information regarding mechanisms occurring at an intracellular level. This method is extremely useful in studying synaptic plasticity, as the hippocampal tissue contains semi-intact neuronal connections. These connections also happen to be structurally well-organized and largely homogeneous in identity, allowing for clear interpretation of electrophysiologic recordings. Moreover, recording from a population of neurons provides insight into the changes occurring over an extended period of time. In saying that, the cellular mechanisms being delineated are based on acute applications of drugs. These were delivered into the bath. How can one be certain that these drugs are reaching their desired targets and that they are acting specifically on these sites? While every drug used has been widely characterized in the literature, I do not know with certainty that they are acting

on their intended molecular targets. It is, therefore, imperative that multiple drugs known to target the same process are used to corroborate a finding. These drugs may target a process in a slightly different way which may provide stronger confirmation that the drugs are acting with specificity and efficacy. While extracellular recordings are a robust method of studying LTP from a macroscopic perspective, it is not sufficient in characterizing biochemical and mechanistic events occurring at a molecular level. Other approaches should be used to confirm these findings, including intracellular recordings, optogenetic stimulation, and knockout mice. For example, studying mice that lack the HAT domain could shed light on the heteroassociative functions of specific epigenetic regulators (Wood et al., 2006; Oliveira et al., 2007). Examining other NA-mediated epigenetic mechanisms, such as DNA methyltransferase activity, may further elucidate the complex nature of nuclear signalling in modulating heterosynaptic plasticity, as well as learning and memory. A hypothetical model summarizing the nucleus' role in synaptic tagging is illustrated in **Figure 4.1**.

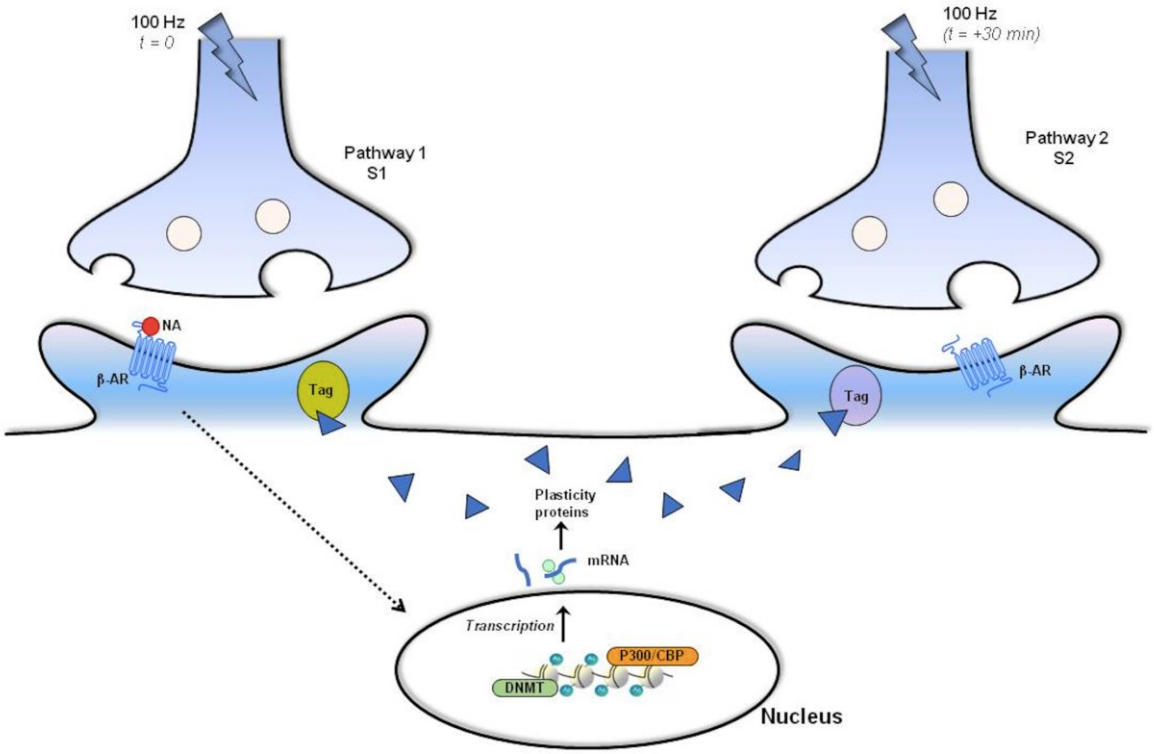

Figure 4.1. Hypothetical schematic representation of the soma's role in heterosynaptic plasticity.

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