Activity-dependent Suppression of Synaptic Strength in the Mouse Hippocampus

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Abstract

This thesis focuses on understanding how synapses are weakened, which represents the cellular basis for information erasure within the brain. In **Project 1**, age-dependent changes in mechanisms supporting "depotentiation," an activity-dependent weakening of recently strengthened synapses, was assessed in aged mice. Ageing is associated with exaggerated forgetting, which may be due to impaired memory consolidation or rapid degradation of recently generated memories.

Memory consolidation is regulated by neuromodulators, neurochemicals that modify the ability of synapses to undergo activity-dependent changes. Noradrenaline is a neuromodulator secreted during arousal and novelty detection which boosts long-term potentiation (LTP). Accordingly, I sought to determine if 1) depotentiation is altered in aged hippocampus synapses and 2) if noradrenaline provides immunity against depotentiation during ageing. I found that aging increases the temporal window for depotentiating synapses which was reversed by treating aged brain slices with noradrenaline. Mechanistically, noradrenaline prevented depotentiation through activation of beta-adrenergic receptors and recruitment of protein synthesis, suggesting that activating beta-adrenergic receptors reverses aged-related synaptic deficits through boosting translation.

In **Project 2**, I further explored how synapses are weakened through characterizing an alternative form of synaptic depression: the phenomenon of LTP decay. LTP is a leading cellular model for the synaptic changes that encode memories. However, not all forms of LTP last, and there is growing evidence that intrinsic neuronal mechanisms actively oppose synaptic potentiation. In this study, I probed how gains in synaptic strength during potentiation are actively

reversed. My approach was to block select cellular signals after inducing LTP, when synaptic strength “decays” or returns back to baseline.

Using this strategy, I discovered that LTP decays in an activity-dependent manner, which requires extrasynaptic GluN2B-containing NMDA receptors and downstream recruitment of Rac1. Interestingly, inhibition of Rac1 facilitated the recruitment of ERK and converted decaying LTP into an enduring, translation-dependent form. These findings suggest that intrinsic mechanisms within the hippocampus may actively promote forgetting by recruiting Rac1-mediated signaling pathways that suppress parallel molecular pathways that normally promote memory consolidation. Taken together, this thesis revealed novel cellular processes that actively reduce synapse potentiation, providing new insights into the neural basis for forgetting.

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Statement of Contribution

Experiments were conducted by Parisa Karimi Tari. Manuscripts and text were written by PKT and edited by Dr. Steven Connor.

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Abbreviations

AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid
BOLD	Blood-Oxygen-Level-Dependent
CA	Cornu Ammonis
CaM	Calmodulin
CaMKII	Calcium/Calmodulin-Dependent Protein Kinase II
cAMP	Cyclic Adenosine Monophosphate
CFC	Cued Fear Conditioning
CP-AMPAR	Calcium-Permeable AMPA Receptor
cryo-EM	Cryogenic Electron Microscopy
E-LTP	Early Long-Term Potentiation
EGTA	Ethylene Glycol-bis(β-aminoethyl ether)-N,N,N′,N′-Tetraacetic Acid
eIF4E	Eukaryotic Initiation Factor 4E
Epac	Exchange Protein Directly Activated by cAMP
ERK	Extracellular Signal-Regulated Kinase
fEPSP	Field Excitatory Postsynaptic Potential
fMRI	Functional Magnetic Resonance Imaging
GEF	Guanine Nucleotide Exchange Factor
GluA	Glutamate Receptor Subunit (AMPA receptor subunit)
GluN	Glutamate Receptor Subunit (NMDA receptor subunit)
HFS	High-Frequency Stimulation
L-LTP	Late Long-Term Potentiation
LC	Locus Coeruleus

LFS	Low-Frequency Stimulation
LTD	Long-Term Depression
LTP	Long-Term Potentiation
MAPK	Mitogen-Activated Protein Kinase
mGluR	Metabotropic Glutamate Receptor
mTOR	Mammalian Target of Rapamycin
NMDA	N-Methyl-D-Aspartic Acid
NSF	N-Ethylmaleimide-Sensitive Fusion Protein
PAK	p21-Activated Kinase
PKA	cAMP-dependent Protein Kinase A
PKC	Protein Kinase C
PKMζ	Protein Kinase M Zeta
PSD	Postsynaptic Density
PSD-95	Postsynaptic Density Protein 95
PTSD	Post-Traumatic Stress Disorder
Rac1	Ras-Related C3 Botulinum Toxin Substrate 1
REM	Rapid Eye Movement
SC	Schaffer Collateral
STC	Synaptic Tagging and Capture
TBS	Theta-Burst Stimulation
Tiam1	T-Cell Lymphoma Invasion and Metastasis 1
TMS	Transcranial Magnetic Stimulation
ZIP	Zeta Inhibitory Peptide

CHAPTER 1: BACKGROUND INFORMATION

Introduction

This ability to adaptively alter neuronal functions, connections and networks is collectively known as brain plasticity. These processes are intimately tied to changes at synapses, which are specialized structures that allow signal transfer between neurons. Experiences or simulated neural activity promote changes in synaptic plasticity, which is defined as the strengthening or weakening of neural connections (Abraham et al., 2024; Collingridge et al., 2010). The importance of this process is borne out by synaptic changes and associated molecular events which support major forms of cognition, including perception, learning, memory, decision making and others. Accordingly, impaired synaptic plasticity is a hallmark feature of neuropsychiatric and neurodevelopmental disorders including Alzheimer's disease, Parkinson's disease, major depression, schizophrenia, autism spectrum disorder and others (Taoufik et al., 2018; Wang et al., 2018).

1.1 An overview of forgetting

Our life experience as humans is shaped by our memories, both retained and lost. Forgetting is the inability to recall something now that could be recalled on an earlier occasion, given the same retrieval cues (Richards and Frankland, 2017; Wixted, 2004). While much is known about how memories are formed and maintained, much less is known regarding the neurophysiological mechanisms that underlie forgetting. This is surprising since forgetting has a rich history in the field of human psychology, starting with the work of Hermann Ebbinghaus (1850-1909) in which he first described the forgetting curve (Ebbinghaus, 2013). Ebbinghaus'

influential work in 1885 established a forgetting curve depicting the decline in retention of nonsense syllables over time (Ebbinghaus, 2013).

Memory loss or forgetting can manifest pathologically or naturally, with a variety of causes leading to permanent or temporary loss (Davis and Zhong, 2017; Ryan and Frankland, 2022). For example, memory loss can be induced by mechanisms that directly weaken potentiated (strengthened) or naïve synapses, such as depotentiation or long-term depression (LTD), which respectively involve the reduction of synaptic transmission strength (Bashir and Collingridge, 1994; Collingridge et al., 2010; Connor and Wang, 2016; Dudek and Bear, 1992; Fujii et al., 1991).

Alternatively, forgetting can happen as the neural correlates of memory, the cellular or molecular events that constitute the biological basis of the memory, *actively* decay. Evidence supporting this mechanism includes observations of the decay of long-term potentiation (LTP), a sustained increase in synaptic transmission strength associated with learning and memory (Villarreal et al., 2002; Xiao et al., 1996). Active decay is believed to be driven by inherent mechanisms within our biological systems that dynamically eliminate synaptic correlates of memory-supporting events (Hardt et al., 2013). Although various mechanisms capable of compromising the integrity of memory traces promote forgetting at the behavioral level (Davis and Zhong, 2017), these processes remain poorly defined.

1.2 Forgetting as an adaptive process

Previous theories on the nature of forgetting postulated that it was a passive process, a time-dependent degradation of memory (Davis and Zhong, 2017; Hardt et al., 2013; Wixted, 2004). However, forgetting is an adaptive and critical aspect to memory optimization that facilitates memory acquisition by liberating neural resources for future encoding (Epp et al., 2016; Nørby,

2015). For example, forgetting outdated or irrelevant information allows the brain to update memories in response to changing environments, a process critical for adaptive decision-making. By weakening or erasing obsolete memory traces, the brain prioritizes new, contextually relevant information, preventing interference from conflicting memories.

Richards and Frankland (2017) argue that forgetting facilitates cognitive flexibility by clearing outdated associations, enabling new learning. More specifically, this group has produced evidence that hippocampal neurogenesis promotes forgetting of old memories by integrating new neurons, thereby disrupting existing memory traces (Akers et al., 2014; Epp et al., 2016). Similarly, intentional forgetting, such as suppression of unwanted memories, enhances memory updating by reducing interference (Anderson and Hanslmayr, 2014).

Active forgetting may also prevent memory overload as previously proposed (Hardt et al., 2013; Wixted, 2004). Accordingly, the brain's limited processing capacity necessitates selective retention of information. Forgetting reduces memory overload by pruning irrelevant details, ensuring that cognitive resources are allocated to high-priority information. fMRI studies previously revealed that intentional forgetting in humans reduces cortical reinstatement of competing memories, thereby improving recall of target information (Wimber et al., 2015).

In a more pathological context, forgetting traumatic or negative memories is adaptive for emotional regulation, reducing the psychological burden of distressing experiences. Active suppression or erasure of emotional memories can mitigate anxiety and post-traumatic stress disorder (PTSD) symptoms, promoting mental health. In line with this contention, suppressing unwanted emotional memories via prefrontal cortex-mediated inhibition reduces amygdala activity, facilitating emotional regulation (Engen and Anderson, 2018). Similarly, rats in which

memory reconsolidation was blocked, fear memories were selectively erased, supporting the adaptive role of forgetting in emotional resilience (Nader et al., 2000).

At a cellular level, studies indicate that sleep-dependent synaptic downscaling erases weak or irrelevant synapses, enhancing neural efficiency (Tononi and Cirelli, 2014), supporting a synaptic homeostasis hypothesis in which selective downregulation of weak synaptic connections optimizes future encoding. Further supporting the notion that information erasure through synapse downregulation may enhance encoding of new information, Hardt et al. (2014) found that persistent LTP in the hippocampus is counteracted by active depotentiation, which prevents neural circuit saturation and enabled new learning (Hardt et al., 2014, 2013).

Beyond the pro-mnemonic functions forgetting may serve, understanding the nature of this phenomenon has profound implications for understanding neurological disorders. Dysregulated forgetting is implicated in conditions like posttraumatic stress disorder (PTSD, characterized by excessive and involuntary memory recall) and dementia (neurodegenerative condition characterized by memory loss) (Berry et al., 2024; Wu et al., 2019). A clearer understanding of the cellular and molecular underpinnings of defective or exaggerated forgetting should therefore reveal novel targets for tuning memory, with major clinical implications.

Before further expanding on the cellular and molecular mechanisms that have thus far been implicated in information erasure mediated by LTP decay, I provide further background on the brain structure that is the focus of my thesis (the hippocampus) and the cellular basis of information communication (the synapse), as outlined below.

1.3 A canonical memory structure: The hippocampus

The hippocampus is a bilateral, medial temporal lobe brain structure located below the thalamus and above the brainstem. As part of the limbic system, a group of brain structures, including the amygdala, thalamus, and hypothalamus, the hippocampus participates in emotional regulation and memory formation. When viewed in the coronal plain, the hippocampus resembles a ram's horn, giving rise to the Latin nomenclature of cornu ammonis (CA) or "ram horn", which is still used in subregions within the hippocampus today (CA1, CA2 and CA3) (Andersen, 2007; Duque-Colorado et al., 2025).

The name "hippocampus" meaning seahorse, originated with the recognition that the unique structural shape of the hippocampus resembles that of a seahorse (Andersen, 2007). The hippocampal formation consists of different subregions including the entorhinal cortex, dentate gyrus, hippocampus (CA3-CA1), and subiculum (Anderson et al., 2007). The hippocampal formation contains specific pathways that form the "tri-synaptic circuit", including the entorhinal cortex which provides inputs into the dentate gyrus known as the perforant pathway. The dentate gyrus in turn projects to area CA3 through mossy fiber projections, and Schaffer collateral (SC) pathway consists of connections between areas CA3 and CA1 (Andersen, 2007).

The different subregions are heterogenous in terms of their cell types and microcircuits. The primary cell type within the dentate gyrus are granule cells, denoted by small, round somas, whereas the hippocampus (CA1-CA3) is comprised mainly of neurons with larger, pyramid shaped cell bodies with extensive basal and apical dendrites, known as pyramidal cells. (Johns, 2014). The hippocampus is a phylogenetically and structurally conserved structure found throughout other vertebrates (Allen and Fortin, 2013). In species ranging from fish to humans, the hippocampus plays a central role in forming spatial and contextual memories (Zola-Morgan et al., 1986).

In addition to encoding spatial memories, the hippocampus is essential for creating new memories about facts and events, known as declarative memories, as these are memories that can be stated or "declared". Insights into the function of the hippocampus in memory are predicated on the case study of patient Henry Molaison (H.M.). Henry presented with severe seizures that may have originated from a bicycle accident as a child (Squire, 2009). To provide relief from the seizures, he underwent an experimental surgery which removed large sections of his medial temporal lobes, including the amygdala and hippocampus form both hemispheres (Scoville and Milner, 1957).

Although the surgery reduced the severity of his seizures, H.M. was left with a profound anterograde memory deficit, meaning he was not longer able to form new memories. For example, without rehearsal, he was only able to retain new memories for facts or events for the span of ~30 seconds. However, memories formed prior to the surgery were mostly intact. Moreover, further testing carried out by Dr. Brenda Milner revealed that although H.M. presented with severe anterograde amnesia for declarative memories, his procedural/implicit memory function was spared. Sadly, the memory deficits limited H.M.'s ability to lead a normal life, and he was dependent upon assisted living until his death in 2008.

As mentioned above, H.M. was capable of learning new implicit, procedural tasks, and showed marked improvement over time in a mirror drawing task which requires motor skill-learning (Squire, 2009). This was a seminal finding in that it demonstrated that there are multiple types of memory systems encoded by distinct parts of the brain, and that the hippocampus is specialized for encoding and storage of declarative memories (Squire, 2009). Similar memory deficits have been noted in other cases of hippocampal ablation in humans (Zola-Morgan et al.,

1986). In summary, the hippocampus has a central role in forming new spatial and declarative memories.

1.4 Synaptic transmission

Neurons are specialized, bioelectric cells that constitute the building blocks of vertebrate and invertebrate central nervous systems. Neurons communicate through both electrical synapses, composed of channels that allow diffusion of ions and small molecules, and chemical synapses, which are protein enriched junctions that transduce presynaptic electrical signals into chemical signals that act on postsynaptic neurons.

Chemical messengers released at synapses are called neurotransmitters, as they "transmit" signals by diffusing across the synaptic cleft to activate receptors on the postsynaptic membrane. The most common excitatory neurotransmitter in the brain is L-glutamic acid or glutamate. (Collingridge et al., 2004; Lisman et al., 2007). Glutamate activates ion channels on the postsynaptic cell, leading to membrane depolarization. This depolarization propagates towards the soma of the postsynaptic neuron, where with sufficient summation, an action potential is generated.

1.5 Excitatory synaptic receptors: AMPA and NMDA receptors

The primary channels activated by glutamate during basal synaptic transmission are know as ionotropic receptors. These receptors are membrane proteins that undergo conformational (shape) changes when bound by glutamate. These shape changes open transmembrane pores, allowing ions to flow across the postsynaptic membrane, changing its local "potential" or complement of ion charges. Two central glutamatergic ionotropic receptors mediating moment-

to-moment synaptic transmission are α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and N-methyl D-Aspartic acid (NMDA) receptors. These receptors, named for their specific agonists (AMPA, NMDA), allow positively charged ions to flux across neuronal membranes. The AMPA receptor is a tetrameric membrane protein composed of four subunits, GluA1-4, which endow the receptor with unique properties including activation kinetics and ion species specificity (Dingledine et al., 1999; Henley and Wilkinson, 2016). For example, although all AMPA receptors are permeable to sodium and potassium, only GluA2-lacking AMPA receptors are permeable to calcium (Park et al., 2018). AMPA receptors are believed to mediate "fast", ongoing synaptic transmission as they rapidly open and desensitize in the presence of their ligand, glutamate (Henley and Wilkinson, 2016; Park et al., 2018; Shepherd and Huganir, 2007).

The NMDA receptor is also permeable to sodium, potassium and calcium. However, it requires the binding of glutamate and glycine and/or the co-agonist D-serine and is blocked at resting membrane potentials by external Mg^{2+} ions. Depolarization ejects Mg^{2+} from NMDA receptor pores, allowing calcium influx to the postsynaptic terminal (MacDermott et al., 1986). This feature allows NMDA receptors to serve as "coincidence detectors", simultaneously "detecting" presynaptically released glutamate and postsynaptic membrane depolarization which allows association of cellular events critical for synaptic plasticity (Hansen et al., 2017; Paoletti et al., 2013; Volianskis et al., 2013).

NMDA receptors are comprised of combinations of different subunits (obligatory GluN1 and combinations of GluN2A-2D and/or GluN3A or 3B), which determine the functional diversity of the receptor, including key properties such as conductance, mean open time, receptor sensitivity, deactivation rates, and sensitization kinetics (Collingridge and Monaghan, 2022; Hansen et al., 2017; Paoletti et al., 2013). Accordingly, NMDA receptors are endowed with the

necessary qualities that facilitate the induction of a diverse range of intracellular signals that mediate synaptic plasticity.

1.6 Synaptic plasticity

The concept of synaptic plasticity was originally put forth by Santiago Ramon y Cajal, a Spanish neuroscientist, who used Camillo Golgi's (a contemporary and fellow Nobel Laureate) stain to approximate features of neural networks, notably their lack of a "syncytium", meaning the cells were individual units and not part of an undifferentiated cellular matrix. Cajal formalized this theory in 1892, through his *cerebral gymnastics hypothesis*, which proposed that exposure to particular stimuli could increase connectivity between neurons (Cajal, 1892). Despite this simple yet elegant explanation for activity-dependent changes as a means for encoding experiences, broader acceptance of this theory was lacking until the paradigm shifting work of Donald Hebb, a Canadian psychologist.

In 1949, Hebb outlined his theory on synaptic plasticity as a cellular model for memory in his book *The Organization of Behaviour.* His classical interpretation of this phenomenon is perhaps best demonstrated by the often cited quote: "When an axon of cell *A* is near enough to excite a cell *B* and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that *A*'s efficiency, as one of the cells firing *B*, is increased" (Hebb, 1949; page 62). This theory provided further theoretical support for changes in synaptic efficacy as a means for neuronal encoding of experiences (Brown, 2020).

Experimental support for this concept was generated in 1973 in a landmark study by neuroscientists Tim Bliss and Terje Lømo. Under the tutelage of Per Anderson, Bliss and Lømo published their seminal findings based on hippocampi recordings in anesthetized rabbits,

demonstrating that brief trains of electrical stimulation increased or "potentiated" postsynaptic currents at perforant pathway-dentate granule cell synapses, which lasted for several hours (Bliss and Lomo, 1973; Lømo, 2025; Nguyen and Kandel, 1997). This activity-dependent, enduring increase in synaptic strength was eventually dubbed long-term potentiation (LTP), which is widely considered the primary cellular mechanism supporting learning and memory (Bliss and Collingridge, 1993; Nabavi et al., 2014; Whitlock et al., 2006).

A key biochemical determinant for the induction of LTP was identified by injection of a Ca^{2+} chelator EGTA (ethylene glycol-bis(β- aminoethyl ether)- N,N,N′,N′- tetraacetic acid) into postsynaptic CA1 pyramidal cells, which blocked LTP (Lynch et al., 1983). The source of this Ca^{2+} was subsequently demonstrated through application of a specific NMDA receptor antagonist, AP5 (also called APV: 2-amino-5-phosphonopentanoic acid), which completely blocked the induction of LTP in CA1 pyramidal cells (Collingridge et al., 1983).

NMDA receptors exhibit a voltage-dependent conductance, meaning the channel requires membrane depolarization to be fully opened. This property is due to block by voltage-dependent inhibition of NMDA receptors at negative membrane potentials by Mg^{2+} ions, which is progressively relieved following membrane depolarization (Mayer et al., 1984; Nowak et al., 1984). Definitive evidence linking NMDA receptors to the induction of LTP was the demonstration that the channel is highly permeable to Ca^{2+} ions (Jahr and Stevens, 1993; MacDermott et al., 1986).

NMDA receptors are comprised of multiple subtypes which uniquely impact LTP properties. Heterotrimeric NMDA receptors comprising GluN1, GluN2A, and GluN2B subunits have been identified as the major subtypes involved in the induction of LTP in rats (Volianskis et al., 2013). The relative contribution of these receptors to different types (theta-burst stimulation;

TBS; high-frequency stimulation; HFS) or components (short-term potentiation, early- LTP, late-LTP) of LTP are also diverse (France et al., 2022; Ingram et al., 2024; Nguyen and Kandel, 1997; Shipton and Paulsen, 2014). Taken together, these studies elegantly confirmed that NMDA receptors mediate the induction (triggering) of increases in postsynaptic Ca^{2+}, essential for initiating LTP, and that magnitude and duration of associated synaptic changes depend upon the contribution of the different subunits.

1.7 LTP expression

Signal transduction supporting LTP differs as a function of multiple variables, including the amount and intensity of the stimulus applied, and thus can be divided into at least two distinct phases – early (E-LTP), and late (L-LTP) (Frey et al., 1993; Huang et al., 1996; Nguyen and Kandel, 1997). Long-lasting forms of LTP typically require multiple rounds of stimulation, and last hours *in vitro* and up to a year *in vivo* (Abraham et al., 2002; Nguyen et al., 1994). Most studies have focused on the Schaffer collateral CA3 to CA1 connection in the hippocampus, using field excitatory postsynaptic potential (fEPSP) recordings. These recordings capture the aggregate responses of thousands of synapses, detected as voltage changes as synapses open and current flows into neurons.

Typical induction protocols consist of spaced, high frequency trains, typically comprised of 100 Hz (100 pulses per second) trains with a duration of 1 second separated by several minutes (Huang et al., 1996; Nguyen et al., 1994), or theta burst stimulation (TBS), consisting of short bursts of high frequency stimulation applied at a theta (5 Hz) frequency (Cao and Harris, 2014; Hoffman et al., 2002; Nguyen and Kandel, 1997; Winder et al., 1999). Accordingly, a transient, decaying E-LTP that lasts ~2 hrs can be induced by one train of high frequency stimuli whereas

L-LTP requires repeated, spaced high frequency trains and lasts > 3 hrs in *ex vivo* slices (Huang et al., 1996; Nguyen et al., 1994).

The question of what links activation of the NMDA subunits to the expression mechanisms of LTP, for example, an increase in presynaptic neurotransmitter release probability, changes in AMPA receptor properties, and/or synapse and spine structural alterations, has its origins in the late 1970s, with the suggestion that phosphorylation may be involved (Lynch, 1989; Malinow et al., 1988). Accordingly, many kinases have been identified as mediators of LTP, including protein kinase C (PKC) and its isoforms (Abeliovich et al., 1993; Hsieh et al., 2021, 2021; Malenka et al., 1986; Malinow et al., 1989; Osten et al., 1996; Wang and Kelly, 1995), Ca^{2+}/calmodulin (CaM)-dependent protein kinases (CaMKs) such as CaMKII (Malinow et al., 1989; Mayford et al., 1995; Nicoll and Schulman, 2023; Rumian et al., 2024; Silva et al., 1992; Wang and Kelly, 1995), cAMP-dependent protein kinase A (PKA) (Abel et al., 1997; Frey et al., 1993; Huang and Kandel, 1994; Nguyen et al., 1994; Park et al., 2021) and many others.

PKA and CaMKII are required for several forms of L-LTP (Park et al., 2021). The initial source of Ca^{2+} that activates CaMKII for E-LTP is provided by NMDA receptors. CaMKII then triggers adenylyl cyclase 1 and/or 8 (AC1/8) and cAMP (cyclic adenosine monophosphate), to activate PKA required for L-LTP. Repeated stimulation typical of L-LTP induction protocols then promotes further NMDA receptor-mediated CaMK activation which drives CP-AMPARs into the perisynaptic regions of the synapse (Park et al., 2018).

CP-AMPARs provide additional Ca^{2+} influx into the synapse, providing highly localized Ca^{2+} signals within distinct nanodomains within synapses (Morita et al., 2014; Park et al., 2018). CP-AMPARs contribute additional Ca^{2+} to further engage molecular signals that drive translation and transcription (Asrar et al., 2009). This provides a means for generating more robust neuronal

responses that reflect and encode the spaced-induction protocols required for recruiting macromolecular synthesis. Interestingly, it is well established that, in terms of memory performance, spaced learning is more effective than massed (i.e., compressed) learning (Cepeda et al., 2006). This type of repetition-induced memory mirrors the tetanization protocols that recruit CP-AMPARs during multiple trains of spaced stimulation.

1.8 Molecules of memory

Interestingly, several molecules associated with LTP maintenance have been lauded as so called "memory molecules". One canonical example is CaMKII. Following the induction of LTP, CaMKII demonstrates constitutive activity theoretically required for maintaining potentiation indefinitely. Autophosphorylation of CaMKII on threonine-286 (T286) and subsequent autonomous activity of the enzyme maintains LTP (Lisman et al., 2002). Application of a cell-permeable CaMKII inhibitor, CN21, prevents LTP when applied during strong afferent stimulation that triggers LTP (Malinow et al., 1989). Similar to CaMKII's activation during LTP, numerous types of memory trace formation within the hippocampus are contingent upon constitutive CaMKII activity (Lisman et al., 2002; Nicoll and Schulman, 2023; Rumian et al., 2024; Silva et al., 1992; Yasuda et al., 2022). The constitutive activation of CaMKII is observed at synapses where GluN2B-containing NMDA receptors anchor and stabilize CaMKII, which enhances LTP (Rumian et al., 2024). Consistent with a role in memory maintenance, overexpression of a dominant negative K42-mutated form of CaMKII erodes established memories, indicating an ongoing requirement for enzyme activity to preserve cellular changes associated with memory acquisition (Rossetti et al., 2017).

Strong supporting evidence has also identified protein kinase Mζ (PKMζ) as an alternative memory molecule. In line with this, synaptic potentiation is associated with upregulated PKMζ activity, as both LTP induction and the acquisition of new memories promotes the rapid translation of PKMζ from dendritically located mRNA, following which PKMζ remains persistently active (Hsieh et al., 2021; Migues et al., 2010). PKMζ stabilizes GluA2-N-ethylmaleimide–sensitive fusion protein (NSF) interactions promoting retention of GluA2-containing AMPA receptors required for the expression of LTP and the maintenance of memory (Hsieh et al., 2021; Migues et al., 2010; Serrano et al., 2005; Yao et al., 2008). Conversely, application of an inhibitory peptide (zeta inhibitory peptide; ZIP) that decreases PKMζ days *after* enduring memories are formed can erase these memories (Pastalkova et al., 2006), providing evidence that PKMζ maintains the synaptic engram required for memory maintenance.

1.9 L-LTP expression

Mechanistically, a major defining feature of enduring L-LTP is a requirement for translation (Connor et al., 2011; Gelinas et al., 2007; Hoeffer et al., 2013; Santini et al., 2014). Many of the aforementioned kinases couple to intracellular signaling pathways that regulate protein synthesis within neurons. Studies of isolated CA1 dendrites (Cracco et al., 2005), demonstrated that synthesis of new proteins required for maintaining LTP can take place locally (within dendritic and/or synaptic subregions). Some forms of L-LTP also engage transcription via signals initiated at synapses which translocate or mediate secondary signals that reach the nucleus to initiate gene transcription (Barco et al., 2005; Nguyen et al., 1994; Nguyen and Kandel, 1997; Zhai et al., 2013). This process provides the messenger ribonucleic acid (mRNA) destined for translation into proteins by protein synthesizing machinery available at the synapse. Thus, major

canonical distinctions between E- and L-LTP are that E-LTP requires transient and reversible modifications of pre-existing proteins (ex. phosphorylation of glutamate receptors), while L-LTP involves protein synthesis, gene activation and new synapse formation (Costa-Mattioli et al., 2007; Guzowski et al., 2000; Hoeffer et al., 2013; Kandel, 2001; Nguyen et al., 1994; Scharf et al., 2002).

Given the distributed nature of synapses forming on neurons, how do neurons selectively target newly synthesized proteins only to those synapses undergoing potentiation? Evidence suggests that newly synthesized transcripts are indiscriminately distributed throughout dendrites and these are subsequently captured by molecular tags established at activated synapses, a process known as "synaptic tagging and capture; STC" (Frey and Morris, 1997, 1998; Koek et al., 2024; Sreedharan Sajikumar and Frey, 2004; Shires et al., 2012). In accord, although both early and late forms of LTP set a synaptic tag, only synaptic pathways exposed to protocols that induce robust LTP engage somatic transcription and translation of plasticity proteins necessary for enduring synaptic potentiation. Interestingly, neuromodulators such as dopamine (Moncada and Viola, 2007; Sreedharan Sajikumar and Frey, 2004) or noradrenaline (Connor et al., 2011) which are known to facilitate the conversion of E-LTP to L-LTP appear capable of similarly upregulating translation to facilitate the generation of plasticity-related proteins that are captured at recently activated synapses.

1.10 AMPA receptor regulation in the expression of LTP

Transcription- and translation-dependent events stabilize LTP through multiple mechanisms, many of which converge on AMPA receptors. Dendritic spines contain and traffic additional AMPA receptors to the postsynaptic density (PSD) via exocytosis from organelles to the plasma membrane. Subsequently, these receptors enter the perisynaptic membrane via lateral

diffusion (Nowacka et al., 2024; Penn et al., 2017). AMPA receptors are highly mobile in the plasma membrane unless anchored to postsynaptic density (PSD) proteins (Nowacka et al., 2024). Anchoring is regulated through accessory subunits of the AMPA receptor, such as γ2 and γ8,which bind tightly to PSD-95 thereby stabilizing AMPA receptors within synapses (Yamasaki et al., 2016). The accessory subunits are in turn regulated by phosphorylation by CaMKII and other kinases. AMPA receptor trafficking has been observed in response to new learning, demonstrated by synaptic accumulation of AMPA receptors within neurons of the lateral amygdala following cued fear conditioning (CFC) (Rumpel et al., 2005). These findings suggest that AMPA receptors accumulate at the synapse following the induction of LTP and *in vivo* during new learning.

The C-terminal tail of the GluA1 subunit serves as a regulatory site for phosphorylation that mediates LTP. Specifically, the phosphorylation of GluA1 at Ser831 on the C-terminal tail is a key step in the rapid trafficking of AMPA receptors into the neuronal membrane following LTP induction (Lee et al., 2007). Recent studies using cryogenic electron microscopy (cryo-EM) have further refined our understanding of the molecular events through which AMPA receptor subunits confer the distinct physiological properties of AMPA receptors during synaptic plasticity. Using this approach, NSF was shown to directly bind the C-terminal tail of the GluA2 subunit which stabilizes and maintains AMPA receptors at synapses during the expression of LTP (Zhang et al., 2023).

As described above, L-LTP requires *de novo* protein synthesis. Upregulation of translation plays multiple roles that enhance potentiation, including generating of additional AMPA receptors and providing the cellular constituents required for rapidly increasing spine size (Yang and Liu, 2022). These processes work in tandem with increasing growth providing additional synaptic space for incorporating the growing pool of AMPA receptors.

Taken together, these studies indicate that LTP induces lasting biochemical, function and structural changes at synapses that would allow memories to persist indefinitely. Accordingly, LTP has revealed the molecular events that underlie memory formation and retention within neurons and allowed for comprehensive analysis of key cellular substrates for neuronal changes capable of acting as cellular memory traces.

1.11 Clinical relevance of synaptic plasticity

In terms of nonhuman primate studies, NMDA receptor-dependent LTP has been observed in area CA3 of hippocampal slices from *Macaca fascicularis* (Urban et al., 1996). In humans, hippocampal tissue removed from patients suffering from intractable epilepsy could readily undergo NMDA receptor-dependent LTP, confirming that this phenomenon extrapolates throughout the phylogenetic tree (Beck et al., 2000). NMDA receptor-LTP was elicited by high-frequency stimulation of the perforant path input to the dentate gyrus at the primary pathway into the hippocampal trisynaptic circuit, and the site initially used in the seminal studies of Bliss and Lomo in the 1970s.

Use of non-invasive Transcranial Magnetic Stimulation (TMS) yielded convincing evidence for activity-dependent plasticity in the human neocortex (Bliss and Cooke, 2011). This was also observed in the hippocampus following theta-burst stimulation TMS targeted to the CA1 region, which significantly increased the functional magnetic resonance imaging (fMRI) blood-oxygen- level-dependent (BOLD) signal in this region and enhanced subsequent memory recollection (Hermiller et al., 2020). These studies demonstrate that NHPs, human brain tissue and neural circuits *in situ* respond to LTP-like stimulation, mirroring neuronal responses observed in

rodent models and bolstering memory performance, indicating that synaptic plasticity is physiologically relevant in a clinical context.

1.12 Neurobiological mechanisms of forgetting

To avoid forgetting and form a memory, synaptic potentiation must be consolidated into a stable long-lasting state. The transformation of transient potentiation (analogous to a short-term memory) into an enduring form (long-term memory) requires gene expression and de novo protein synthesis to stabilize synapses, consistent with LTP studies mentioned above (Kandel, 2001).

The transformation of a short-term memory into one that persists long-term requires gene expression and *de novo* protein synthesis that together culminate in the structural growth that stabilizes memory-supporting synapses (Moncada and Viola, 2007; Nguyen et al., 1994). For example, insertion of GluA2-containing AMPA receptors in the hippocampus is associated with both synaptic strengthening and memory persistence (Migues et al., 2016, 2010).

If the presupposition that forgetting is an "active" process is correct, there must be cellular correlates of ongoing activity that drive the supporting mechanisms. Consistent with the idea that memory degradation involves weakening of synaptic connections, it has been demonstrated that hippocampal-dependent forgetting that occurs naturally over 1-14 days is mediated by the loss of synaptic GluA2 AMPA receptors (Dong et al., 2015; Migues et al., 2016). GluA2 AMPA receptor endocytosis likely mediates forgetting by regulating both the induction of long-term depression (LTD), an activity-dependent weakening of synaptic strength (Collingridge et al., 2010; Connor and Wang, 2016), and the depotentiation or direct reversal of LTP (Dong et al., 2015; Migues et al., 2016).

Similar to LTD or depotentiation, NMDA receptor activity has a related role in regulating active erasure of neuronally stored memories. In a pioneering study, it was shown that NMDA receptor antagonism administered daily after LTP induction blocks the natural decay of LTP in the dentate gyrus of rats (Villarreal et al., 2002). This same NMDA receptor antagonism blocked the natural forgetting of a radial arm maze spatial memory over a 5-day period. That NMDA antagonism blocks the decay of LTP and concomitantly blocks forgetting over the period of ~1 week has been replicated in other hippocampal-dependent tasks including both the water maze (Shinohara and Hata, 2018) and object location memory tasks (Sachser et al., 2016).

Taken together, these experiments suggest that blocking GluA2 AMPA receptor endocytosis or NMDA receptor activity likely prevents forgetting by 'locking' the current state of synaptic connectivity, preserving potentiation, and thereby halting the loss of information stored in the neural circuit (i.e., forgetting). Thus, whereas degradation of stimulus-specific persistent neural activity is a mechanism of forgetting over a period of seconds to minutes, NMDA and AMPA receptor-mediated synaptic decay is thought to be a mechanism of forgetting over more protracted periods (minutes-hours-days).

1.13 LTP decay

A growing body of research supports the proposition that forgetting is not the passive process it was once believed to be (Wixted, 2004), but rather is active, meaning there are dedicated cellular and molecular mechanisms that endow neural circuits with a capacity for reducing synaptic strength to promote memory erasure (**Figure 1.1**) (Awasthi et al., 2019; Davis and Zhong, 2017; Hardt et al., 2014; Sachser et al., 2016). This leads to the key questions of what are the neuronal

events that initiate memory erasure, and what are the molecular events that underlie the associated changes in synaptic weight?

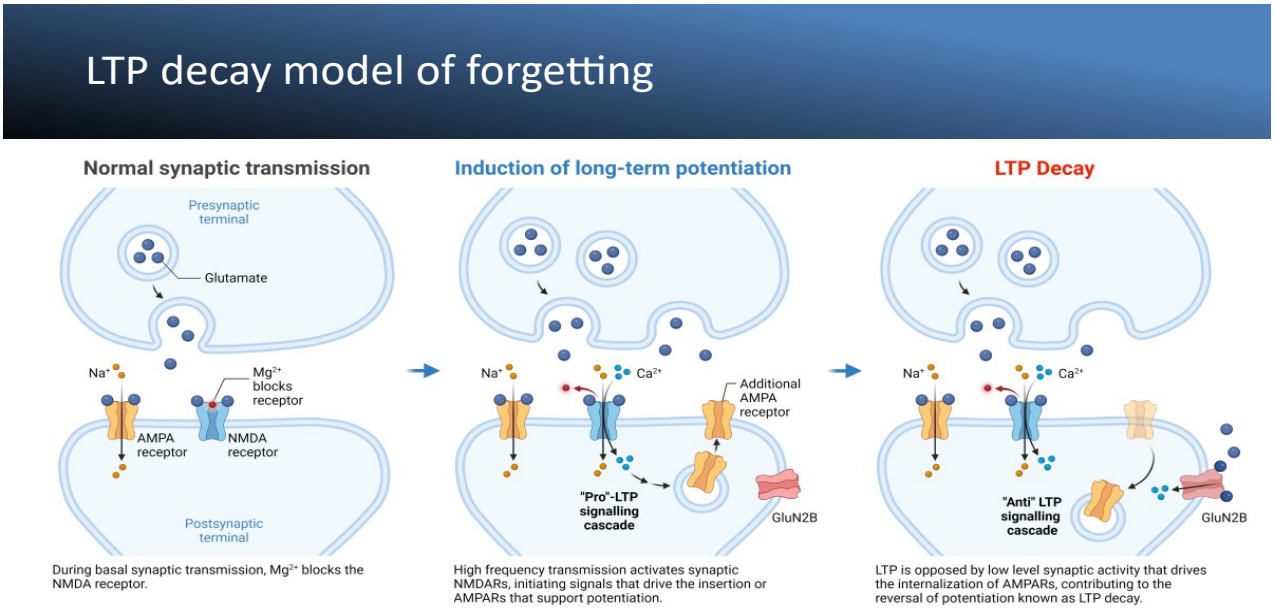

Figure 1.1. LTP decay model of forgetting at the synaptic level. (Left) Glutamate release from the presynaptic terminal binds postsynaptic AMPA and NMDA receptors, with AMPA receptors mediating basal synaptic responses, whereas Mg^{2+} blockade prevents significant NMDA receptor activation. (Center) Induction of LTP. High-frequency synaptic transmission relieves the Mg^{2+} block, allowing Ca^{2+} influx through NMDA receptors. This triggers a pro-LTP signaling cascade, including the membrane insertion of additional AMPA receptors, enhancing synaptic strength. (Right) LTP decay. Low-level synaptic activity opposes LTP by driving ongoing glutamate release which activates extrasynaptic NMDA receptors. This initiates an anti-LTP signaling cascade, leading to the internalization of AMPA receptors and reversal of potentiation. These dynamic changes underpin the molecular dynamics underlying the balance between memory formation and forgetting. Figure made with BioRender.

Initial evidence linking LTP decay to forgetting was provided by Carol Barnes's observation of a correlation between the decay of LTP *in vivo* and forgetting of spatial memory (Barnes, 1979). Weak or "decaying" LTP, refers to potentiation that returns to baseline within ~1-2 hours (Villarreal et al., 2002; Xiao et al., 1996). The corresponding active erasure of synaptic strength observed in these studies required ongoing NMDA receptor activity, as NMDA receptor antagonism administered daily after LTP induction prevented the natural decay of LTP (Villarreal et al., 2002). Similarly, NMDA receptor blockade prevented LTP decay within the hippocampus *in vivo* (Migues et al., 2016) and *in vitro* which appeared to be mediated by GluN2B-containing NMDA receptors (Sachser et al., 2016).

NMDA receptor antagonism also prevents behavioral measures of forgetting over ~1 week in hippocampal-dependent tasks including both the water maze (Shinohara and Hata, 2018, 2014) and object location memory tasks (Sachser et al., 2016). Others have confirmed NMDA receptor-dependent forgetting of long-term memories (Migues et al., 2016; Sachser et al., 2016; Villarreal et al., 2002). Studies indicating that NMDA receptor activation after induction of LTP can initiate countervailing mechanism that oppose LTP leads to an additional question: Which molecular events downstream from NMDA receptors reduce synaptic strength?

Critically, the maintenance of synaptic strength depends on the number of GluA2-containing AMPA receptors present at synapses (Migues et al., 2010; Zhou et al., 2018). Accordingly, preventing the removal of GluA2-containing receptors from the postsynaptic membrane prevents forgetting of long-term memories and associated synaptic weakening. For example, application of a peptide that prevents GluA2 endocytosis converts decaying forms of LTP into enduring forms *in vivo* (Dong et al., 2015; Hardt et al., 2014). Similarly, blocking GluA2

endocytosis prevents forgetting of inhibitory avoidance (Dong et al., 2015), place preference (Migues et al., 2016) and object-location (Migues et al., 2016) memories in mice.

Further evidence for the central role of GluA2 in mediating forgetting was shown through genetic deletion of synaptotagmin 3 (syt3), a protein involved in AMPA receptor internalization, which prevented both GluA2 endocytosis and forgetting of spatial memories (Awasthi et al., 2019). Collectively, these findings suggest that endocytosis of GluA2 is a primary mechanism mediating forgetting of long-term memories. This further supports that elucidating mechanisms that actively drive synaptic weakening are likely to identify key cellular processes that promote forgetting.

1.14 Canonical "forgetting molecules": Rac1

Several studies focused on the intracellular signalling mechanisms that initiate forgetting have identified Rac1, a Rho GTPase that regulates cytoskeletal actin polymerization, as a candidate forgetting molecule. Findings in both flies and mice have established that Rac1 regulates various forms of forgetting (Cervantes-Sandoval et al., 2020; Cui et al., 2021; Jiang et al., 2016; Liu et al., 2018).

In flies, the effects of Rac1 activation of memory suppression appears to begin after initial memory encoding, indicating that signalling mechanisms that support memory maintenance or forgetting may act in parallel (Shuai et al., 2010). Accordingly, interfering with Rac1 modulates forgetting rates, with expression of dominant negative versions of Rac1 reducing forgetting in various memory tasks (Shuai et al., 2010), whereas expression of constitutively active forms of Rac1 accelerate forgetting (Shuai et al., 2011, 2010; Shuai and Zhong, 2010).

In mice, similar effects of Rac1 manipulation have been reported, wherein pharmacological inhibition of Rac1 or dominant negative Rac1 expression prevents forgetting of contextual memories, whereas expression of a constitutively active form of Rac1 promotes forgetting (Jiang et al., 2016; Liu et al., 2016a). The synaptic effects of Rac1 inhibition are similarly observed in brain slices, with Rac1 inhibition prolonging the maintenance of LTP, and Rac1 activation inducing depotentiation (Liu et al., 2016b; Lv et al., 2019). Furthermore, optogenetic activation of Rac1 in hippocampal CA1 neurons following establishment of a contextual memory was sufficient for erasing these memories (Lv et al., 2019). Interestingly, optogenetically-induced memory erasure could be reversed through pharmacological inhibition of Rac1 activity (Lv et al., 2019). This suggests that Rac1-mediated forgetting of established memories may be reversible.

Although NMDA receptors may be able to initiate downstream engagement of Rac1, other receptors capable of responding to changes in animal brain state or environmental triggers may also regulate Rac1 activation. The potent neuromodulator dopamine is also known to modulate Rac1 activity (Berry et al., 2018, 2015, 2012; Himmelreich et al., 2017). DAMB, a type of dopamine receptor in fly mushroom body neurons, appear to be essential for forgetting (Himmelreich et al., 2017). Stimulation of dopaminergic cells converging on these mushroom body neurons induces forgetting of previously consolidated memories (Berry et al., 2012).

Mechanistically, it may be the case that Rac1 signalling regulates synapse structure or connectivity, as Rac1 signalling is known to mediate the serine/threonine-protein kinase (PAK)–LIM-domain kinase (LIMK)–cofilin signalling complex, which alter cytoskeletal structure through actin depolymerization (Cervantes-Sandoval et al., 2016). Therefore, Rac1–mediated cytoskeletal regulation may promote forgetting in response to environmental stimuli that engage dopaminergic activity.

1.15 Long-term depression

To differentiate LTP decay from LTD or depotentiation, I have provided a brief overview of these phenomena below. LTD, a persistent weakening of naïve (unstimulated) synapses was first discovered in the cerebellum in 1980 (Collingridge et al., 2010; Ito and Kano, 1982). NMDA receptor-LTD is an activity-dependent depression or "weakening" of naïve synapses (Connor and Wang, 2016; Mulkey and Malenka, 1992). Given the proposed roles for synaptic strengthening as a cellular analog for new memory formation, LTD has been put forth as a cellular model for forgetting (Tsumoto, 1993).

NMDA receptor-LTD involves the activation of a Ser/Thr protein phosphatase cascade (Mulkey et al., 1993), indicating that LTD requires the dephosphorylation of AMPA receptors. Accordingly, the surface delivery of AMPA receptors decreases following LTD induction (Fujii et al., 2018; Unoki et al., 2012; Zhou et al., 2018), consistent with a downregulation of synaptic AMPA receptors as a core mechanism driving synaptic depression (Collingridge et al., 2010; Connor and Wang, 2016; Pinar et al., 2017).

Early studies suggested that LTP and LTD involve different subtypes of NMDA receptors, but this remains highly controversial (Collingridge et al., 2010; Wong and Gray, 2018). In terms of LTD, there is evidence for a selective requirement for GluN2B-containing (France et al., 2017) and GluN2D-containing NMDA receptors (Bartlett et al., 2007), although this may depend on slice orientation (coronal versus sagittal) which alters the relative dependence on NMDA receptor subtypes (Bartlett et al., 2011).

An interesting development was the finding that during the induction of LTD, NMDA receptors may act through metabotropic mechanisms to suppress synaptic strength (Nabavi et al., 2013). Accordingly, blocking NMDA receptor function with a glycine site antagonist or selective

channel blocker did not prevent NMDA receptors from inducing LTD, leading to the conclusion that binding of glutamate to the NMDA receptor was able to signal to the neuron without flux through the receptor (Nabavi et al., 2013). The mechanism seems to involve a conformational change in the C-terminal region of the NMDA receptor (Dore et al., 2016). However, this result is not obtained uniformly, with examples of NMDA receptor-LTD fully blocked by antagonists that are not competitive in nature (Babiec et al., 2014). One likely explanation for these disparate findings is that NMDA receptors can trigger LTD via both ionotropic and metabotropic mechanisms, the extent to which varies according to a number of factors, such as the developmental stage of the animal (Dore and Malinow, 2021).

1.16 Depotentiation

Depotentiation and *de novo* LTD are mechanisms by which synaptic efficacy is decreased. In the former case, this is a reversal of LTP to (or toward) the basal state, whereas in the latter case it is the depression of synaptic transmission from baseline to below the basal state. These two forms of plasticity have both overlapping and distinct mechanisms and are likely to serve differing functions in the brain.

Depotentiation, the reversal of LTP by low-frequency trains of stimulation (Fujii et al., 1991), is generally assumed to reset recently enhanced synaptic strength. It may enable forgetting (Moreno, 2021), or may be part of the process of memory reconsolidation, and can also reset synaptic tags (S. Sajikumar and Frey, 2004). There are both NMDA receptor-dependent and NMDA receptor-independent (principally metabotropic glutamate receptor-dependent) forms of depotentiation (Sanderson, 2012), and the efficiency of depotentiation depends on the underlying form of synaptic potentiation (Park et al., 2019).

Depotentiation may also vary according to the animal's age. For example, in contrast to LTD, depotentiation is more readily induced in the dentate gyrus of adult rodents relative to juvenile animals; an effect attributable to a switch from GluN2B- to GluN2A-containing NMDA receptors (Ge et al., 2019). This data suggests that LTD may play a more prominent role in synapse weaking and elimination early in development, whereas depotentiation of weak LTP may feature more prominently in aged neural circuits.

1.17 Neuromodulation of LTP

Neuromodulation regulates critical functions of CNS synapses, ranging from neural circuit development to high-order cognitive processes, including learning and memory. This broad scope of action is generally mediated through alterations of the strength of synaptic transmission (i.e. synaptic plasticity) or altering the threshold for synaptic adaptations (metaplasticity). Major differences between canonical neurotransmitters (ex. glutamate, GABA) and neuromodulators (ex. dopamine, noradrenaline) include the engagement of metabotropic signaling, expanded times scales of action, and the sources of neuromodulatory inputs. More specifically, neuromodulators act through metabotropic receptors to modulate the ability of synapses to undergo synaptic plasticity at time scales far exceeding GABAergic and glutamatergic synaptic transmission.

For example, the conversion of E-LTP to L-LTP is due in part to engagement of the dopaminergic system, operating via dopamine D1 and D5 receptors (Sreedharan Sajikumar and Frey, 2004). Activation of D1/D5 receptors enables cAMP-mediated activation of PKA which promotes downstream synthesis of mRNA or somatically translated proteins to bolster LTP (Fuchsberger et al., 2025; Navakkode et al., 2007). Dopamine, acting via D1/D5 receptors also reduces the threshold for LTP induction, enabling weaker stimuli to induce robust potentiation.

This mechanism is linked to increased calcium influx and activation of calcium/calmodulin-dependent kinase II (CaMKII) and extracellular signal regulated kinase-1 and -2 (ERK1/2) (Shivarama Shetty et al., 2016). Collectively, these results indicate that dopamine and associated receptors modify the inducibility of LTP within the hippocampus, through mechanisms that alter synaptic strength in an enduring fashion.

1.18 Noradrenergic modulation of plasticity

An alternative neuromodulator, noradrenaline (NA), is secreted throughout the brain in response to novelty or increased arousal (Nguyen and Connor, 2019; Sara, 2009). Once released, noradrenaline activates adrenergic receptors which demonstrate metabotropic properties, initiating intracellular signaling cascades that promote changes in synaptic strength which facilitate memory storage (O'Dell et al., 2015, 2010). The primary source of noradrenaline in the CNS is the locus coeruleus (LC), a brainstem nucleus located in the caudal pons. The LC projects diffusely throughout the brain, including cortical (cerebral cortex) and subcortical (amygdala, cerebellum, hippocampus and thalamus) structures (Poe et al., 2020; Sara, 2009).

Noradrenergic receptors are G-protein coupled receptors which initiate signaling cascades depending upon the particular G-protein that they couple to. These receptors fall into 2 categories (α or β) that can be further subdivided based on their respective structures (α1, α2 and β1, β2, β3). Noradrenergic receptors modulate a plethora of neuronal properties including cellular excitability,

synaptic transmission and plasticity, protein synthesis and epigenetic modifications (Maity et al., 2020; Nguyen and Connor, 2019; O'Dell et al., 2015, 2010).

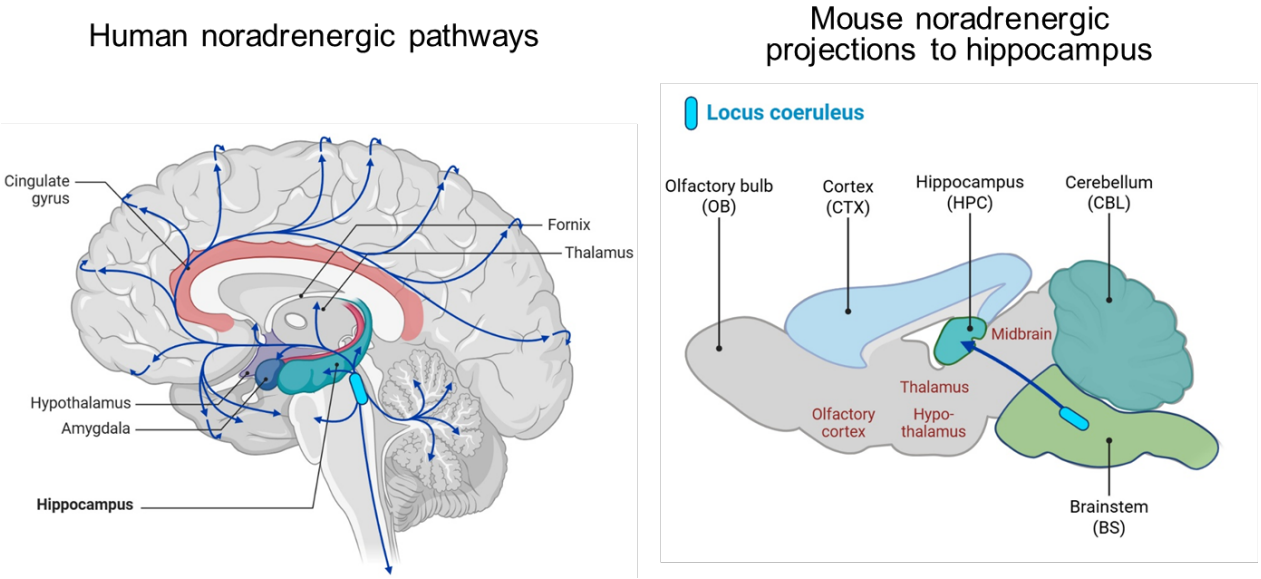

Figure 1.2. Noradrenergic pathways in the human and mouse brain. (Left) Noradrenergic outputs from the locus coeruleus (shown in light blue with projections shown in dark blue) project throughout the human brain, innervating the hippocampus, amygdala and cortex. (Right) Sagittal section of a mouse brain demonstrating locus coeruleus projections into the hippocampus.

The effects of noradrenaline on synaptic function are dependent upon the receptor subtype to which it binds. For example, activation of α1-adrenergic receptors has a modest effect on the induction and maintenance of LTP when paired with weak electrical stimulation in area CA1 (Man et al., 2023; Pussinen and Sirviö, 1998). Depotentiation, an activity-dependent reversal of LTP (Bashir and Collingridge, 1994), can also be induced by noradrenaline acting partially through α1-adrenergic receptors. α2-AR's potentiating effects on plasticity are restricted to structures outside

of the hippocampus. Within the accessory olfactory bulb, noradrenaline application promotes LTP at glutamatergic synapses between mitral cells and GABAergic granule cell interneurons, an effect mediated by α_2-ARs (Huang et al., 2018). Overall, α-adrenergic receptors have a modest, indirect effect on synaptic plasticity and likely play homeostatic or neuroprotective roles.

In contrast with the modest effects of α-adrenergic receptors, β-adrenergic receptors have well-established and substantive impacts on plasticity. Within the human brain, the concentration of β-ARs is highest in the subregions (DG-CA1) of the hippocampus (Reznikoff et al., 1986), and analysis of cell-type expression suggests a predominant localization to pyramidal cells (Atlas and Melamed, 1978). Through coupling to Gs proteins, β-adrenergic receptors increase cell excitability and enhance LTP (O'Dell et al., 2015, 2010). Pairing β-adrenergic receptor stimulation with subthreshold tetanization protocol that normally elicits a transient, decaying form of E-LTP, converts this form of LTP to an enduring, L-LTP that requires mammalian target of rapamycin (mTOR), extracellular signal regulated kinase (ERK) and protein synthesis (Gelinas et al., 2007; Gelinas and Nguyen, 2005; Maity et al., 2020). Additionally, activation of both β1- and β2-adrenergic receptors enhances theta-stimulation induced LTP through phosphorylation of serine 845 (s845) on GluA1-containing AMPA receptors (Tenorio et al., 2010). Consistent with these data, theta LTP was impaired in both β1- and β2-adrenergic receptor knockout mouse acute hippocampus slices (Qian et al., 2012; Winder et al., 1999). Accordingly, the metabotropic effects of β-adrenergic receptor stimulation serve as a permissive gate, allowing for the induction of enduring forms of LTP by subthreshold stimulation protocols.

1.19 Noradrenergic modulation of plasticity: downstream effectors

Substantial progress had been made in characterizing the molecular mechanisms through which β-adrenergic receptors regulate glutamatergic synapse plasticity. β2-adrenergic receptors have been observed in multi-protein complexes containing GluA1, L-type calcium channels and A-kinase anchoring protein 5 (AKAP5; known as AKAP150 in mice) (Larsen et al., 2023), which serves as a molecular scaffold that brings β-adrenergic receptors into close proximity to its downstream effectors, including PKA and AMPA receptors. Stimulation of β-adrenergic receptors increases the phosphorylation of GluA1 on Ser-845, which was dramatically reduced in the hippocampi of AKAP5 knockout mice (Zhang et al., 2013). Prolonged theta LTP was also diminished in AKAP5 knockouts, consistent with a loss of PKA anchoring which otherwise promotes GluA1 phosphorylation and synaptic insertion, key mechanisms supporting this form of β-adrenergic receptor-dependent LTP (Qian et al., 2012). These data suggest that signaling complexes engaged during β-adrenergic receptor-mediated synaptic plasticity in hippocampus enhance synaptic localization of PKA, thereby facilitating subsequent regulation of AMPA receptor trafficking required for bolstering synaptic strength.

Further experiments have revealed specific proteins that are upregulated during noradrenaline-induced plasticity. Puromycin incorporation confirmed an upregulation of translation following noradrenaline treatment in the hippocampal CA1 region. Polysomal profiling identified GluA1 and GluA2 AMPA receptor subunits as translation products that were specifically upregulated during noradrenaline-induced metaplasticity (Maity et al., 2015).

Together with previous findings linking β-adrenergic receptor activation to increased surface expression of GluA1 (Larsen et al., 2023; Tenorio et al., 2010), these data reveal a model in which noradrenaline, acting through β-adrenergic receptors, promotes both the synthesis of new

AMPA receptor subunits and their insertion and retention at synapses. The selective upregulation GluA1 and GluA2 provide supporting evidence against the contention that inhibition of translation has global, non-specific effects on protein synthesis rates that would indirectly impede potentiation. Further research is required to determine if translation rates for other proteins implicated in synaptic plasticity are upregulated by noradrenaline and to further identify mechanisms that promote the selective synthesis of GluA1/GluA2 during noradrenergic-mediated forms of plasticity.

1.20 Summary

This overview was intended to provide the background and rationale for my thesis, which has the overarching goal of advancing our understanding into the synaptic mechanisms that promote synaptic weakening as a proxy for understanding the nature of forgetting. The hippocampus is a key brain region for memory formation and consolidation, and its synaptic plasticity mechanisms, such as LTP, are well-established correlates of learning and memory. However, the processes that govern memory loss, or forgetting, remain poorly understood. Depotentiation, the reversal of LTP, and LTP decay, the gradual weakening of potentiated synapses, are hypothesized to represent cellular mechanisms that contribute to the natural attenuation of memories over time. As such, this thesis was designed to investigate the synaptic mechanisms underlying depotentiation and long-term potentiation (LTP) decay in the hippocampus as these phenomenon model cellular properties important for understanding the neurobiological basis of forgetting.

The rationale for this study stems from the need to understand forgetting not as a passive failure of memory systems but as an active, regulated process essential for cognitive flexibility and

efficient memory management. By examining the molecular, cellular, and network-level dynamics of depotentiation and LTP decay, this research aims to elucidate how these synaptic processes regulate hippocampal information loss, providing insights into the balance between memory persistence and erasure.

These findings have implications for understanding normal cognitive function, as well as disorders characterized by aberrant memory processing, such as Alzheimer's disease and post-traumatic stress disorder. Through a combination of electrophysiological, pharmacological and molecular approaches, this work aims to advance the framework for active synaptic weakening as a fundamental component of memory erasure in mammalian brain circuits, providing the foundation for novel therapeutics that selectively target forgetting mechanisms to advance human health.

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CHAPTER 2: OBJECTIVES AND METHODS

2.1 Project 1, Objectives: Exploring resilience to depotentiation in aged mouse hippocampus synapses

In contrast to models predicated on an inability to initially form memories, the robustness of memory maintenance may be determined by the ability to resist depotentiation, however this postulate remains poorly explored. My first project sought to investigate whether aging in mice compromises the resilience of hippocampal synapses to depotentiation, a synaptic plasticity mechanism implicated in memory erasure (Bashir and Collingridge, 1994; Fujii et al., 1991).

The ability to acquire and consolidate memories changes across the lifespan. A dramatic example of this is age related memory loss – an aged-dependent gradual decline of aged adult's ability to retain memories (Burke and Barnes, 2006; Leal and Yassa, 2015; Lister and Barnes, 2009).

The ability of aged mice and rats to form enduring memories appears to decline as a function of age, with results varying based on strain and methodologies employed (Barnes, 2003; Burke and Barnes, 2006; Gray and Barnes, 2015; Small et al., 2011). Additionally, studies investigating the properties of LTP within aged neural circuits have generated disparate results, with data indicating decreased, increased, or unchanged LTP magnitude in aged synapses (Barnes, 2003; Burke and Barnes, 2006; Huang and Kandel, 2006; Lister and Barnes, 2009; Schimanski and Barnes, 2010). Given the noted variability in results associated with aged rodent neural plasticity, and the lack of insight into how resistant aged synapses are to depotentiation, I sought to determine if aged synapses were more or less resilient to a depotentiating stimulus.

Accordingly, my hypothesis for this component of my thesis is: Aging in mice increases the susceptibility of hippocampal synapses in the CA1 region to depotentiation, a process characterized by the activity-dependent reversal of synaptic potentiation. Specifically, I predict that aged CA1 synapses will exhibit enhanced depotentiation due to alterations in molecular pathways regulating synaptic stability, including disrupted translational control, leading to a diminished capacity to maintain potentiated synaptic states compared to younger mice.

An additional factor likely determining the durability of synaptic potentiation is the presence of neuromodulators known to alter synaptic transmission and plasticity (Dahl et al., 2023). Interestingly, memory deficits during ageing overlap with a general downregulation of CNS neuromodulators, including cholinergic, dopaminergic and noradrenergic systems (Dahl et al., 2023). The importance of these neuromodulators in memory is demonstrated by the neurodegenerative conditions that are associated with their dysfunction, including PD, AD and dementia.

The noradrenergic system plays a prominent role in enhancing memory, primarily through activation of β-adrenergic receptors (Connor et al., 2011; Gelinas et al., 2007; Gelinas and Nguyen, 2005; Maity et al., 2020; O'Dell et al., 2015). However, whether noradrenergic inputs alter depotentiation in aged neural circuits has yet to be established. I sought to fill these gaps in knowledge and characterize age-dependent changes in resistance to depotentiation in mouse hippocampus, area CA1 and how noradrenaline impacts these processes. Accordingly, ***Objective 2 was to explore whether the application of noradrenaline could restore immunity to depotentiation in aged synapses***, focusing on its role in modulating molecular pathways governing translation regulation within neurons.

By integrating electrophysiological recordings, molecular biology techniques, and pharmacological interventions, this Objective will test whether aging disrupts synaptic resilience to depotentiation through altered translational control, while assessing noradrenaline's potential to rescue these deficits via signaling pathways that couple to protein synthesis. Findings associated with this study will provide insights into age-related memory decline and potential therapeutic strategies.

2.2 Project 2, Objectives: Characterize synaptic mechanisms of LTP decay

LTP decay is an established model for cellular events that mediate forgetting in rodent models (Davis and Zhong, 2017; Hardt et al., 2013; Villarreal et al., 2002). Recently, we found that LTP decay requires NMDA receptors as blocking these receptors prevented LTP decay, similar to previous findings (Sachser et al., 2016; Villarreal et al., 2002). This result indicates that NMDA receptors are required for LTP decay, however, fails to differentiate which NMDA receptor subunits mediate these effects, nor the downstream intracellular mechanisms that are recruited during the decay of LTP.

Given the prominent role of GluN2B-containing NMDA receptors in synaptic weakening, including LTD (Delgado et al., 2018; Massey et al., 2004; Shipton and Paulsen, 2014), I hypothesized that: GluN2B-containing NMDA receptors mediate LTP decay which varies as a function of subcellular (synaptic vs. extrasynaptic) location. Accordingly, we asked whether GluN2A- or GluN2B-containing synaptic or extrasynaptic NMDA receptor inhibition alters LTP decay in mouse hippocampus.

Forgetting is also associated with Rac1-mediated signaling, as blocking or knocking down Rac1 appears to extend the duration of memories in flies (Berry et al., 2018; Cervantes-Sandoval

et al., 2020; Shuai and Zhong, 2010) and rodents (Liu et al., 2018, 2016). Given these results, we also examined whether NMDA receptors recruit Rac1 to mediate LTP decay, and whether this depended upon the subcellular localization of these receptors.

Others have shown that Rac1 inhibition prevents LTP decay, allowing the conversion of a transient LTP or short-term memories into enduring forms (Cui et al., 2021). To explain this phenomenon mechanistically, I further theorized that protecting synaptic potentiation from LTP decay unmasks a memory promoting signaling cascade within CA1, within which hippocampal contextual memories are normally formed (Andersen, 2007; Bliss and Collingridge, 1993). This mechanistic "switch" allows the recruitment of signaling pathways that are normally suppressed by Rac1, which may maintain synaptic strength during memory genesis.

Therefore, I next wanted to test this model by probing the signaling cascades coupling to translation regulation that are recruited following the suppression of LTP decay. Given this model, I hypothesized that suppressing translation during Rac1 inhibition should allow the re-expression of LTP decay. Conversely, I predict that Rac1 inhibition should upregulate signaling cascades that mediate protein synthesis, thereby converting decaying, translation-independent LTP into an enduring protein synthesis-dependent type of plasticity.

2.3 General Methods

2.3.1 Mouse hippocampus slices

Hippocampal slices offer distinct advantages that provide an excellent model for probing synaptic changes and intracellular mechanisms associated with synaptic plasticity. These include a well characterized tri-synaptic circuit which is highly amenable to simulation of synaptic events that recapitulate synaptic mechanisms observed during learning and memory in the intact brain.

Slices also afford unimpeded physical and pharmacological access to neurons, which is more difficult to target and manipulate within intact brain tissue.

2.3.2 Mouse model

All animal experiments were carried out in accordance with Canadian Council on Animal Care guidelines and approved by York University Animal Care Committee. Male C57BL/6 mice (Charles River, Montreal, Quebec, Canada) were used for all experiments. Mice were group-housed (two to five per cage) and maintained on a 12-h light/dark cycle with food and water available ad libitum. Mice were acclimatized to the facility for at least one week upon arrival before use in experiments. Mice were sacrificed by cervical dislocation followed by decapitation. The brain was removed and rapidly cooled for ~45 seconds in ice-cold recovery solution comprised of artificial cerebral spinal fluid (aCSF; Table 1) bubbled with carbogen (95% O2, 5% CO2). The brain was then hemisected, and hippocampi were removed from both hemispheres. Hippocampi were sliced using a manual tissue slicer to a width of 400 μm.

Table 1: Recording and slicing solution (aCSF) for mouse hippocampal brain slices. Ingredients added to 1L MilliQ water and pH adjusted to 7.35 with bubbling of carbogen.

Component	Molarity (mM)	Amount for 1L	Molecular Weight (g/mol)
NaCl	124	7.25 g	58.44
KCl	3	220 mg	74.56
CaCl2	2	1000uL	147
MgCl2	1	500uL	202
NaH2PO4	1.25	173 mg	138
NaHCO3	26	2.18 g	84
Glucose	15	2.7 g	180
pH 7.35			
mOsm 310-320			

2.3.3 Mouse hippocampal slice recordings

When recording from the Axon Instruments rig, ~10-20 slices were placed in a heated interface chamber (BSC1-2; Scientific Systems Design Inc) and allowed to recover undisturbed for 1.5 hours. The interface chamber was heated to 30°C using a PTC03 Scientific Systems Design Inc. proportional temperature control unit. The stimulating electrode was constructed from 0.002-inch nichrome wire (80% nickel/20% chromium; A-M Systems), threaded through a 1.5mm width borosilicate glass capillary (TW150F-4; World Precision Instruments), with ends sealed using ArmorCoat© quick setting epoxy.

The stimulating electrode was connected to a DS3 Isolated Current Stimulator (Digitimer, LLC) to control the strength and duration of the electrical stimulus. The recording microelectrode was also constructed from a 1.5mm width borosilicate glass capillary (TW150F-4; World Precision Instruments), pulled to a fine tip using a P-97 Flaming/Brown type micropipette puller. The recording electrode was backfilled with aCSF and secured to an Axon Instruments CV-7B current/voltage clamp headstage. A new recording electrode was constructed for each slice recording measurement, with an acceptable resistance of 1-3MΩ. Signal information from the recording electrode was relayed from the headstage to an Axon© Digidata® 1550B low-noise data acquisition system with HumSilencer©, and MultiClamp© 700B computer-controlled current and voltage clamp amplifier. Signal information was converted to readable output data using the computer software Axon© pCLAMP 11. Mechanical noise reduction was achieved by using a Newport air table to support the brain slice interface chamber and surrounding equipment, including the electrode micromanipulators (M3301; World Precision Instruments) and Laxco© LMS-Z200 Stereo Zoom microscope.

2.3.4 fEPSP recordings

Transverse hippocampal slices (400 μm thickness) of mouse hippocampus (**Figure 2.1**) were collected and transferred to an interface recording chamber maintained at 30°C. Slices were continuously perfused (1–2 ml/min) with artificial cerebrospinal fluid (aCSF) 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, aerated with 95% O_2 and 5% CO_2. Electrophysiological recordings of extracellular field excitatory postsynaptic potentials (fEPSPs) began following a minimum 90 min recovery period. A glass microelectrode (resistance of 2–3 MΩ) filled for fEPSP recording with aCSF was positioned in the stratum radiatum of area CA1. The hippocampal Schaeffer collateral (SC) commissural pathway was stimulated using two bipolar nickel–chromium electrodes placed ~200 μm from the recording electrode. fEPSPs were set to 40% of maximal evoked amplitudes by adjusting the stimulus intensity (0.1 ms pulse duration) to establish baseline responses. fEPSPs were obtained at the rate of once per minute at this test stimulation intensity.

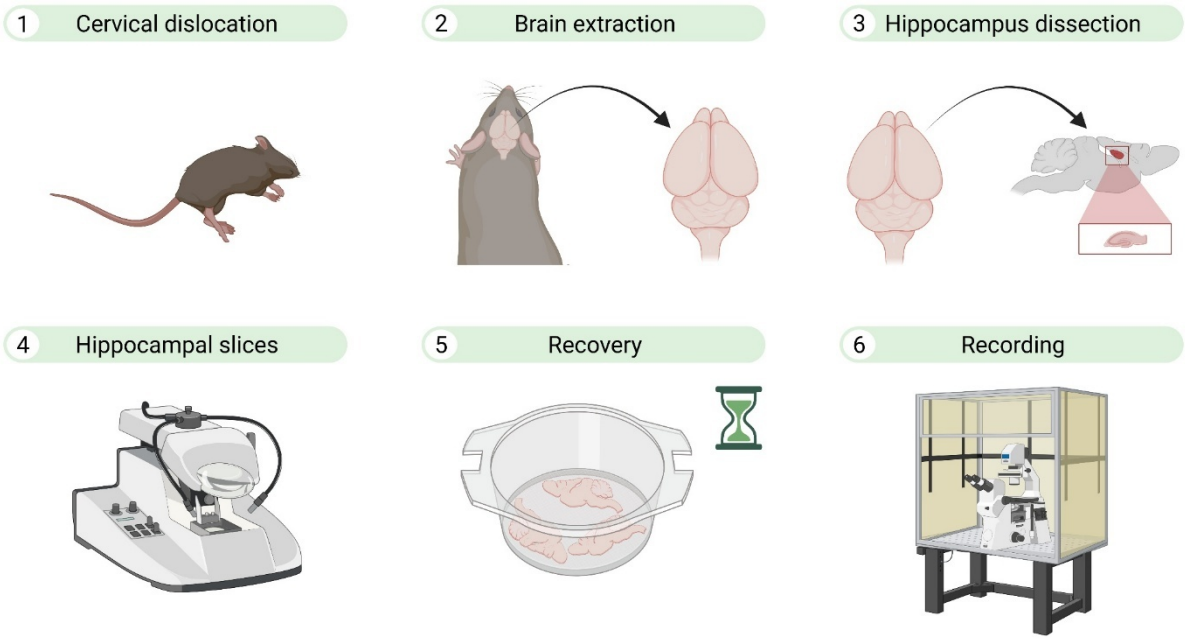

Figure 2.1. Generation of hippocampus slices for fEPSP recordings. This figure illustrates the process of generating mouse hippocampus slices for fEPSP (field excitatory postsynaptic potential) recordings. Following cervical dislocation (1) a mouse brain is rapidly extracted from the skull (2). Each hippocampus is then isolated (3) and sliced (4) to produce an isolated hippocampal slice (400 µm thick). After recovering in the interface chamber (5; minimum 90 min) fEPSPs are recorded using an electrophysiology rig (6). Image generated using BioRender.

2.3.5 Data Analysis

Extracellular field excitatory postsynaptic potentials (fEPSPs) were analyzed using Axon Clampex 11.2 (Molecular Devices, Sunnyvale, CA, USA). The initial slope of the fEPSP (**Figure 3**) was measured as an index of synaptic strength. fEPSP slopes were averaged from 20 min of stable baseline recording to obtain a baseline value for each experiment. All subsequent slopes were expressed as percentage of these baseline slopes. Analysis was performed by using GraphPad

Prism 10.1 (GraphPad Software). Statistical comparisons were made with two-tailed Student's unpaired t-tests, with Welch correction for different standard deviations between groups. For multiple comparisons, one-way ANOVA with Tukey–Kramer post hoc tests were performed to determine which groups were significantly different from others. No statistical significance (ns) was defined as $p > 0.05$, while statistical significance was defined as $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$. All data are reported as the mean ± standard error of the mean (SEM), with n being the number of slices.

2.3.6 Synaptoneurosomes preparation

Hippocampus slices were prepared as described for fEPSP recording experiments outlined above. After recovering for at least 90 min, slices underwent 2 trains of HFS (100 Hz, 1 sec, 20 sec intertrain interval) and drug treatments outlined in Chapter 4. Synaptoneurosomes were isolated from mouse hippocampus tissue (C57BL/6, 8–12 weeks old) using the differential filtration method. Briefly, CA1 hippocampus was microdissected on ice in ice-cold oxygenated artificial cerebrospinal fluid supplemented with protease inhibitor cocktail (Roche, 1 tablet per 10 mL buffer). Microdissected slices were homogenized in 1 mL glass homogenizing tube containing 200 µL of chilled modified Krebs-Henseleit buffer (mKRBS) [containing (in mM): 118.50 NaCl, 4.70 KCl, 1.18 $MgCl2·6H2O$, 2.50 $CaCl2·2H2O$, 1.18 $KH2PO4$, 24.90 $NaHCO3$, 10.00 glucose, pH adjusted to ~7.40 using 1.0 N HCl]. A protease inhibitor cocktail (104mM AEBSF, 80µM aprotinin, 4mM bestatin, 1.4 mM E-64, 2mM leupeptin and 1.5mM pepstatin) was included in the buffer to limit proteolysis activity. Slices were then homogenized with 20 manual pestle strokes over ice, and the homogenate placed in a 1.5 mL microtube for 10 min on ice to allow for gravity sedimentation.

Synaptoneurosomes were prepared by drawing the supernatant into a 1 cc Luer lock syringe and then passing it through a 13 mm filter holder (Millipore Sigma, Ontario, Canada) containing 3 layers of pre-wetted (mKRBS) 100 µm pore nylon filters (Millipore Sigma). Filtrate was then collected in a small Petri dish on ice and air was passed through the filter holder to ensure all filtrate was collected. The filtrate was than drawn into a new 1 cc Luer lock syringe and passed through a 13 mm filter holder containing a single pre-wetted (mKRBS) 5 µm nitrocellulose Durapore membrane filter (Millipore Sigma) into a 1.5 mL microtube; after this, 1 mL of air was passed through the filter holder to ensure all the filtrate was collected. Microtubes were spun at 1000 × g for 15 min at 4°C, supernatants were discarded, and the pellet was re-suspended in 100 µL non- ionizing lysis buffer (10 mM Tris, 25 mM EDTA, 100 mM NaCl, 1 % [v/v] Triton X-100, and 1 % [v/v] NP-40, pH 7.4) containing protease and phosphatase inhibitors and stored at -80°C.

2.3.7 Protein Quantification

Protein concentrations were determined using the Pierce Rapid Gold BCA Protein Assay Kit (Thermo Fisher Scientific) according to the manufacturer's recommended protocol. A bovine serum albumin (BSA) standard curve was prepared by diluting the provided 2 mg/mL BSA standard in ultrapure water to generate a series of standards ranging from 0 to 2,000 µg/mL (0, 20, 40, 125, 250, 500, 750, 1,000, 1,500, and 2,000 µg/mL). For each sample and standard, 10 µL was added in duplicate to a 96-well microplate (Thermo Fisher Scientific). The working reagent was prepared by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B (50:1 ratio). A total of 200 µL of the working reagent was added to each well containing samples or standards. The microplate was covered, gently mixed on a plate shaker for 30 seconds, and incubated at room

temperature (25°C) for 5 minutes. Absorbance was measured at 480 nm using a microplate reader within 10 minutes of incubation to ensure stability of the colorimetric reaction. A standard curve was generated by plotting the absorbance values of the BSA standards (corrected for the blank) against their known concentrations using GraphPad Prism (version 9.0). A second-order polynomial (quadratic) fit was applied to the standard curve to account for the non-linear response of the Pierce Rapid Gold BCA assay. Protein concentrations of unknown samples were calculated by interpolating their absorbance values against the standard curve. Each sample was measured in duplicate, and the average concentration was used.

2.3.8 Immunoblotting

All samples were thawed on ice and the synaptoneurosome samples were then denatured in Laemmli sample buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue) at 95°C for 5 min. Protein samples were then loaded into either 10, 12, or 4-20 % SDS-polyacrylamide gels. After this, proteins were electrophoretically separated at 100 V for 1.5 h in 1× Tris-Glycine-SDS running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3) using a Bio-Rad Mini-PROTEAN Tetra system. Proteins were transferred from the gel onto PVDF membranes via dry transfer using the Trans-Blot Turbo system (Bio-Rad). Membranes were blocked using either 5 % (w/v) bovine serum albumin (BSA, BioShop, Ontario, Canada), or 5 % (w/v) non-fat milk in Tris-buffered saline with Tween-20 (TBST) for 1 h at room temperature and incubated with relevant antibodies overnight at 4°C with gentle agitation. All primary antibodies were diluted in blocking buffer specific to the application. Membranes were imaged using a ChemiDoc Imaging System (Bio-Rad). Exposure

times were optimized (typically 10–60 seconds) to avoid signal saturation while ensuring detection of faint bands.

All synaptoneurosome preparations and Western blot experiments were performed in duplicates to ensure reproducibility. Antibody dilutions and incubation conditions were optimized to minimize non-specific binding. When reprobing was necessary, membranes were stripped using a mild stripping buffer (200 mM glycine, 0.1% SDS, 1% Tween-20, pH 2.2) for 10 minutes, followed by extensive washing in TBST.

Band intensities were quantified using ImageJ software (NIH). The intensity of each target protein band was normalized to the intensity of a loading control (β-actin) to account for variations in protein loading across synaptoneurosome samples. Relative protein expression or phosphorylation levels were calculated by comparing normalized band intensities to a control condition (baseline).

2.3.9 Statistical analysis for Western blots

Data were expressed as mean ± standard error (SEM) from four independent biological replicates and two technical replicates. Statistical analysis was performed using GraphPad Prism (version 9.0). Significant differences were assessed using one-way ANOVA with post-hoc Šidák's test for multiple comparisons, with a p-value < 0.05 considered statistically significant. Normality was tested for using the Shapiro-Wild test, with alpha set to 0.05.

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CHAPTER 3: DEPOTENTIATION

Reversal of aging-related susceptibility to synaptic depotentiation in mouse CA1 through noradrenaline*

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Abstract

Cognitive decline associated with aging has been attributed to deficient synaptic plasticity. These deficits may be the result of a reduced capacity for macromolecular synthesis in aging neurons due to a general downregulation of neural neuromodulatory inputs. Studies of aging-related plasticity have mainly focused on long-term potentiation (LTP) deficits, whereas the ability of potentiated synapses to retain activity-induced synaptic strength is rarely explored. We sought to determine if LTP is less resistant to erasure in the aged hippocampus. Depotentiation, an activity-dependent reversal of LTP, was used to determine if synaptic potentiation in aged neural circuits is more readily reversed in aged mice. Here we show that comparable levels of LTP are observed in both aged and young mouse hippocampus CA1 *ex vivo* slices, which are both depotentiated when low-frequency stimulation is rapidly applied. Conversely, LTP in aged slices could undergo depotentiation at time frames that fall within the synaptic consolidation window during which synapses from young mice are immune to depotentiation. Resistance to depotentiation could be re-established in aged slices through application of noradrenaline, which required beta- but not alpha-adrenergic receptors. This effect was dependent on protein synthesis as application of emetine prevented the rescuing effects of noradrenaline. Collectively, our data show that aging synapses within the hippocampus demonstrate a protracted period of susceptibility to depotentiation that can be reversed through beta-adrenergic receptor stimulation and downstream

recruitment of translation. These findings have implications for the clinical application of noradrenergic receptor activation as a prophylactic strategy for maintaining the temporal window for synaptic consolidation associated with memory formation.

3.1 Introduction

Despite intensive investigation, the cellular mechanisms underlying age-based cognitive decline remain poorly understood (Burke and Barnes, 2010, 2006; Samson and Barnes, 2013). Cognitive deficits associated with advancing age are often linked to dysfunction of the hippocampus (Gray and Barnes, 2015; Lister and Barnes, 2009; Small et al., 2011), a brain structure required for contextual and spatial memory formation (Scoville and Milner, 1957). The hippocampus appears to be particularly susceptible to age-dependent alterations (Small et al., 2011), as both rodents (Mota et al., 2019; Radulescu et al., 2021) and humans (Lister and Barnes, 2009; O'Shea et al., 2016) show compromised hippocampal function during aging which parallels behavioral measures of impaired memory. Accordingly, spatial navigation deficits observed in older individuals are associated with reduced information transmission resulting from deficient hippocampal function (Zheng et al., 2023).

Within the hippocampus, long-term potentiation (LTP) is widely considered a cellular model for the synaptic modifications supporting the acquisition of spatial and contextual memories (Bliss and Collingridge, 1993; Bliss and Lomo, 1973; Dringenberg, 2020), which is compromised in aged hippocampal circuits (Barnes, 2003; Kumar, 2011; Kumar et al., 2007; Ryan et al., 2015). However, studies conducted within the rodent hippocampus examining aging-related changes in LTP have yielded inconsistent results (Costenla et al., 1999; Huang and Kandel, 2006; Kumar et al., 2007; Rex et al., 2005) which may be in part due to the particular LTP induction protocols used, or whether the experiments were performed *in vivo* or *in vitro* (Barnes, 2003; Burke and Barnes, 2006; Costenla et al., 1999; Huang and Kandel, 2006; Kumar, 2011)

Studies reporting modest or null effects of aging on LTP suggest that alternative cellular

mechanisms may underlie age-related memory loss. Depotentiation (Dpt) is an activity-dependent reversal of established LTP typically induced using low-frequency stimulation (LFS) (Bashir and Collingridge, 1994; Huang et al., 1999; O'Dell and Kandel, 1994; Park et al., 2019; Zhang et al., 2009; Zhuo et al., 1999). Unlike long-term depression (LTD), an activity dependent decrease in synaptic strength induced at naïve synapses (Collingridge et al., 2010; Connor and Wang, 2016; Dudek and Bear, 1992; Mulkey and Malenka, 1992), Dpt is more readily induced in aged neural circuits (Dudek and Bear, 1992; Mulkey and Malenka, 1992; Norris et al., 1996), suggesting this phenomenon may recapitulate cellular events that interfere with memory formation or promote memory erasure in the adult brain. Critically, LTP can become "immune" to depotentiation if there is a significant delay between LTP induction and the subsequent application of Dpt stimuli (Fujii et al., 1991; O'Dell and Kandel, 1994; Park et al., 2019). It has been established that this immunity to Dpt is due to synaptic consolidation, a process of stabilization that requires translation-dependent mechanisms (Woo and Nguyen, 2003). Increased susceptibility to Dpt associated with aging may therefore reflect a general downregulation of activity-induced translation regulation normally required for synaptic consolidation (Fando et al., 1980; Ryan et al., 2015; Schimanski and Barnes, 2010).

Interestingly, the reduction in protein synthesis capacity associated with aging diminishes in parallel with a general downregulation of CNS neuromodulation (dopamine, noradrenaline), which mediate key intracellular signaling pathways that couple to translation (Connor et al., 2011; Huang and Kandel, 1995; Maity et al., 2020; Navakkode et al., 2007; Nguyen and Connor, 2019). Accordingly, a causal pathway to impaired synaptic plasticity underlying cognitive decline in old age may be the result of decreases in neuromodulatory inputs (Dahl et al., 2023; Karrer et al., 2017;

Liu et al., 2020; Rieckmann et al., 2011). Major neuromodulatory nuclei including the locus coeruleus project to the hippocampus where secretion of noradrenaline (NA) and subsequent binding to noradrenergic receptors induces potent LTP-promoting effects that bolster the consolidation of synaptic strength gains (Jami et al., 2023; Nguyen and Connor, 2019; O'Dell et al., 2015). NA acts through both α- and β-adrenergic receptors (ARs) to modulate LTP through activation of G-protein coupled intracellular signaling cascades that link adrenergic receptor stimulation to molecular plasticity signals downstream. β-AR stimulation enhances synaptic plasticity through myriad mechanisms, including the activation of extracellular signal-regulated kinase (ERK) and mammalian target or rapamycin (mTOR) pathways which converge to regulate protein synthesis within neurons (Connor et al., 2011; Gelinas and Nguyen, 2005; Maity et al., 2020; Winder et al., 1999). Notably, aberrant translation regulation is observed in multiple models for aging (Schimanski and Barnes, 2010) and has been implicated in the pathology of Alzheimer's disease (Ghosh et al., 2020).

As activation of noradrenergic receptors initiates intracellular signals that enhance synaptic strength through translation-dependent mechanisms (Connor et al., 2011; Tenorio et al., 2010), this suggests that stimulation of these receptors may confer resistance to Dpt. Indeed, pairing LTP induction with noradrenergic receptor stimulation induces a form of LTP that is resistant to Dpt (Katsuki et al., 1997). Given the notable decrease in translation regulation that parallels these age-related changes (Schimanski and Barnes, 2010), this raises the question of whether synaptic consolidation takes longer in aged neural circuits which would extend the temporal window for depotentiating synapses. Accordingly, we tested the hypothesis that aged hippocampus area CA1 synapses are more susceptible to Dpt due to prolonged synaptic

consolidation.

Our results indicate that although transient and robust forms of LTP appear intact and comparable in young and aged mouse hippocampus, the temporal window for Dpt is protracted in aged mouse CA1. We next sought to determine if activation of noradrenergic receptors restores synaptic immunity to depotentiation. We found that application of NA to hippocampus slices restored immunity to Dpt through activation of β- but not α-ARs. Finally, we observed that upregulation of translation following NA application was required for restoring immunity to Dpt. Collectively, our findings suggest that synaptic consolidation and associated resistance to Dpt take longer in aged hippocampal circuits, which can be restored to youthful states through noradrenergic system-mediated recruitment of protein synthesis.

3.2 Materials and Methods

3.2.1 Animal and ethics statement

All animal experiments were carried out in accordance with Canadian Council on Animal Care guidelines and approved by York University Animal Care Committee. Young (6-10 weeks) or aged (12-15 months) male C57BL/6 mice (Charles River, Montreal, Quebec, Canada) were used for all experiments. Mice were group-housed (two to five per cage) and maintained on a 12-h light/dark cycle with food and water available *ad libitum*.

3.2.2 Electrophysiology

Transverse hippocampal slices (400 μm thickness) of mouse hippocampus were collected and transferred to an interface recording chamber maintained at 30°C. Slices were continuously

perfused (1–2 ml/min) with artificial cerebrospinal fluid (aCSF) 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, aerated with 95% O2 and 5% CO^2. Electrophysiological recordings of extracellular field excitatory postsynaptic potentials (fEPSPs) began following a 90 min recovery period. A glass microelectrode (resistance of 2–3 MΩ) filled for fEPSP recording with aCSF was positioned in the stratum radiatum of area CA1. The hippocampal Schaeffer collateral commissural pathway was stimulated using two bipolar nickel–chromium electrodes placed ~200 μm from the recording electrode. fEPSPs were set to 40% of maximal evoked amplitudes by adjusting the stimulus intensity (0.1 ms pulse duration) to establish baseline responses. fEPSPs were obtained at the rate of once per minute at this test stimulation intensity.

3.2.3 Synaptic plasticity protocols

After establishing a stable baseline for 20 min, high-frequency stimulation (100 Hz, 1 sec) was applied to induce LTP. For 2 or 4 train LTP, the interval between trains of HFS was 20 sec. DPT consisted of theta frequency stimulation (5 Hz, 3 min) either 5 or 20 min after inducing LTP.

3.2.4 Drugs

For noradrenaline-induced LTP, bath application of NA (l-(–)-Noradrenaline bitartrate salt monohydrate prepared as 1 mM stock with final concentration of 10 μM) commenced after 10 min of baseline recordings, for a total of 15 min. Experiments were done under dimmed light conditions to prevent photolysis of light-sensitive drugs. The protein synthesis inhibitor emetine (20 μM; Sigma-Aldrich; prepared as 20 mM stock in dimethyl sulfoxide [DMSO]) was bath-applied at

concentrations of 20 μM. The β-AR antagonist (+)-propranolol hydrochloride (Sigma) was also prepared daily in distilled water as a 50 mM stock solution. Prazosin hydrochloride (Sigma), an α1 antagonist was prepared at a stock concentration 150 μM in distilled water and applied at 10 μM. Yohimbine hydrochloride (Sigma), an α2 antagonist was prepared in distilled water at 1 mM stock and applied at 3 μM.

3.2.5 Data Analysis

Extracellular field excitatory postsynaptic potentials (fEPSPs) were recorded using an interface-chamber (Scientific Instruments) and a MultiClamp 700B amplifier. Axon Clampex 11.2 (Molecular Devices, Sunnyvale, CA, USA) was used for fEPSP analysis. The initial slope of the fEPSP was measured as an index of synaptic strength. fEPSP slopes were averaged from 20 min of stable baseline recording to obtain a baseline value for each experiment. All subsequent slopes were expressed as percentages of these baseline slopes. To compare LTP levels between two groups, we used data points at 50-60 min after LTP induction. Analysis was performed by using GraphPad Prism 10.1 (GraphPad Software). Statistical comparisons were made with two-tailed Student's unpaired t-tests, with Welch correction for different standard deviations between groups. For multiple comparisons, one-way ANOVA with Tukey–Kramer post hoc tests were performed to determine which groups were significantly different from others. No statistical significance (ns) was defined as $p > 0.05$, while statistical significance was defined as $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$. All data are reported as the mean ± standard error of the mean (SEM), with n being the number of slices.

3.3 Results

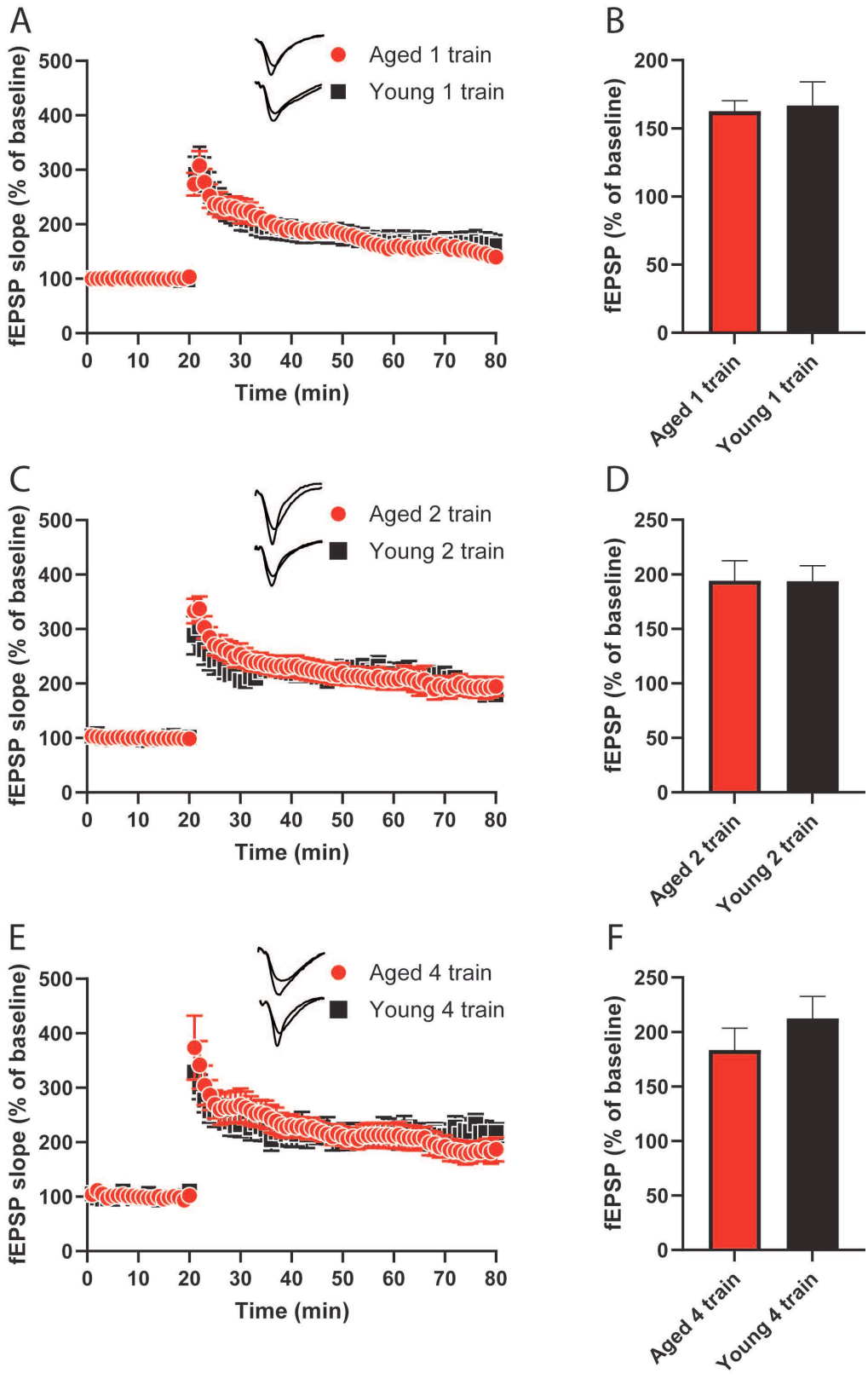
3.3.1 Canonical forms of LTP are similarly induced and expressed in young and aged mouse CA1

Given reports suggesting that LTP may be altered as a function of advanced age (Barnes, 2003; Burke and Barnes, 2006), we first sought to determine if canonical forms of LTP were different in young (6-10 weeks) relative to aged (12-15 months) mouse hippocampus slices. Single train (1 x 100Hz, 1 sec) LTP at Schaffer collateral (SC)-CA1 synapses was induced following a 20 min baseline which generated LTP that was comparable in amplitude in both young and aged mouse CA1 (**Fig. 3.1A**). Single train stimulation generated potentiation that was similar between groups (mean fEPSP slopes were 162.6 ± 7.8% and 166.8 ± 17.3% for aged and young slices respectively, 50-60 min after 1 x 100 Hz stimulation; $p > 0.05$; **Fig. 3.1B**).

We next tested if multiple trains of stimulation induced LTP of differing amplitudes in young and aged slices. Initially, two trains of high frequency stimulation (1 x 100 Hz, 1 sec) were applied at a 20 sec interval, which induced LTP in both groups (mean fEPSP slopes were 194.1 ± 18.3% and 193.8 ± 14.1% for aged and young slices, respectively 50-60 min after 2 × 100 Hz stimulation) (**Fig. 3.1C**). Comparison of LTP 50-60 min post-HFS revealed that 2 train LTP was not significantly different between groups ($p > 0.05$; **Fig. 3.1D**).

Next, we sought to determine if 4 trains of LTP which induces an enduring form of LTP that requires macromolecular synthesis (Nguyen et al., 1994) differs in young and aged slices. Four trains of stimulation (1 x 100 Hz, 1 sec) were applied at 20 sec intervals which induced robust LTP in both young and aged slices (mean fEPSP slopes were 183.5 ± 19.9% and 212.5 ± 20.0% for aged and young slices, respectively 50-60 min after 4 × 100 Hz stimulation) (**Fig. 3.1D**).

Comparison of LTP 1 hour following 4 train LTP induction failed to detect differences between young and aged hippocampal LTP ($p > 0.05$; **Fig. 3.1E**). Collectively, these results suggest that canonical forms of LTP are intact within the first hour post-induction and comparable within the SC-CA1 synaptic pathway in young and aged mouse hippocampus slices, demonstrating preserved synaptic plasticity during aging in response to single, double, or 4 train LTP protocols in this mouse model.


A
fEPSP slope (% of baseline)
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Time (min)
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B
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Aged 2 train
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Aged 2 train
Young 2 train
E
fEPSP slope (% of baseline)
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Time (min)
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fEPSP (% of baseline)
250
200
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0
Aged 4 train
Young 4 train

Figure 3.1. Canonical forms of LTP are similarly expressed in young and aged mouse CA1

(**A**) Application of HFS (1 x 100 Hz, 1 sec) induced LTP in both aged (n = 13, red circles) and young (n = 15, black circles) within the SC-CA1 pathway that was similar in amplitude. (**B**) Summary histogram for these experiments comparing levels of potentiation 50-60 min after HFS (Student's t-test with Welch correction; $p > 0.05$). (**C**) 2 trains of HFS produced LTP in both aged (n = 6, red circles) and young (n = 6, black circles) slices that did not significantly differ. (**D**) Summary histogram for these experiments comparing levels of potentiation 50-60 min after HFS (Student's t-test with Welch correction; $p > 0.05$). (**E**) Four trains of HFS applied at 20 sec intervals induced LTP in both aged (n = 6, red circle) and young (n = 6 black circles) hippocampus slices that were similar in amplitude. (**F**) Summary histogram for these experiments comparing levels of potentiation 50-60 min after the application of multiple trains of HFS (Student's t-test with Welch correction; $p > 0.05$). Induction stimulation not shown. All fEPSP traces (insets) were sampled at 10 min into baseline and 55 min post HFS. Calibration bars: 2 mV, 2 msec.

3.3.2 Application of a depotentiation stimuli shortly after LTP induction reverses LTP in both young and aged mouse CA1

Previous *in vitro* studies have shown that Dpt is most effective at reversing potentiation when applied shortly following LTP induction (Woo and Nguyen, 2003; Zhang et al., 2009). To determine the ability of Dpt to reduce LTP across different ages, we induced 4 train LTP, followed 5 min later by application of a depotentiating stimulation protocol (Dpt; 5 Hz, 3 min). 4 train LTP induced robust synaptic potentiation in young slices which was reversed by Dpt applied 5 min after HFS (**Fig. 3.2A**). Specifically, 4 train LTP was significantly elevated relative to slices in

which 4 train LTP was paired with Dpt (mean fEPSP slopes were 217.7 ± 18.4% and111.7 ± 17.7% on 4 train and 4 train + Dpt treated slices, respectively 50-60 min after 4 x 100 Hz stimulation) (**Fig. 3.2C**).

Similarly, 4 x 100 Hz stimulation generated robust LTP in young slices that was reversed by Dpt applied 5 min after LTP induction (**Fig. 3.2B**). fEPSPs were 194.0 ± 18.7% and 115.6 ± 22.2 in slices undergoing 4 train LTP or Dpt, respectively (**Fig. 3.2C**). An ANOVA comparing fEPSP amplitudes across group from 50-60 min post HFS revealed significant differences ($F_{(3, 21)} = 7.530$, $p < 0.01$). Tukey's posthoc comparisons showed that Dpt reversed LTP in both young ($p < 0.01$) and aged ($p < 0.05$) slices (**Fig. 3.2C**). There were no differences in the relative amount of depotentiation across young and aged slices ($p > 0.05$). Taken together, these data indicate that 4 train LTP is readily depotentiated in both young and aged slices when the Dpt stimulation is applied within 5 min of inducing LTP.

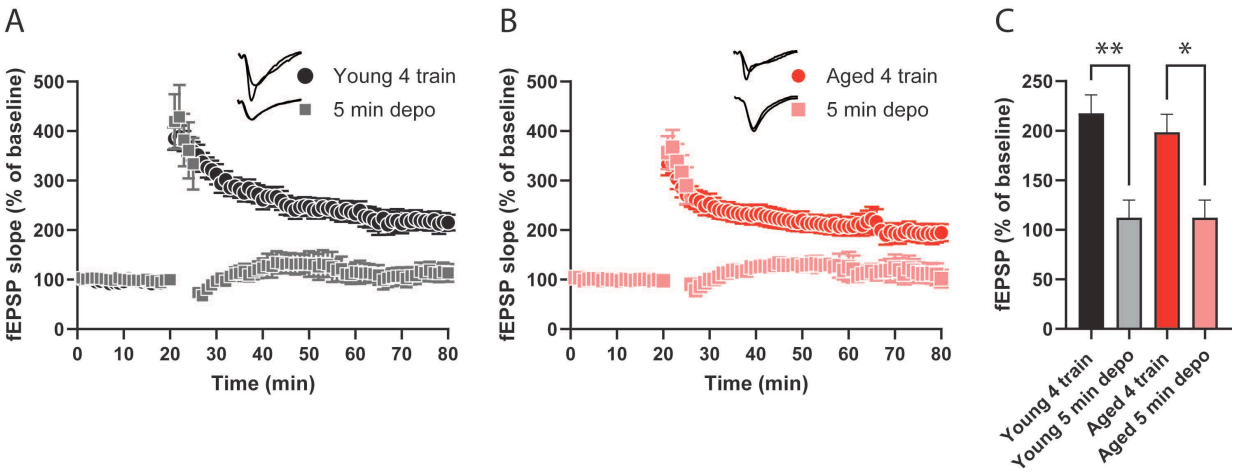

Figure 3.2. Application of a depotentiation stimuli shortly after LTP induction reverses LTP in both young and aged mouse CA1

(**A**) Four trains of HFS applied at 20 sec intervals induced LTP in young slices (n = 6, black circles), which was depotentiated when 5 Hz, 3 min was applied after 5 min (n = 6, grey squares) (**B**) Similar to young slices, 4 trains of HFS induced LTP (n =6; red circles) which was reversed by Dpt (n =7; pink squares). (**C**) Summary histogram for these experiments comparing levels of potentiation 50-60 min after the application of multiple trains of HFS (ANOVA with Tukey's posthoc test; $*p < 0.05$, $**p < 0.01$). Induction stimulation not shown. All fEPSP traces (insets) were sampled at 10 min into baseline and 55 min post HFS. Calibration bars: 2 mV, 2 msec.

3.3.3 Aging increases the temporal window for depotentiation of LTP in mouse CA1

Following LTP induction, there is a period of synaptic consolidation during which synapses become immune to depotentiation (Woo and Nguyen, 2003). This immunity to Dpt requires the synthesis of proteins that bolster LTP, a process that is compromised in aged neurons (Fando et al., 1980; Schimanski and Barnes, 2010). This raises the question of whether aging extends the temporal window for depotentiation of synaptic strength. To test this, we used the same LTP + Dpt protocol as described above, however the delay between LTP induction and Dpt was extended from 5 min to 20 min to allow the onset of synaptic consolidation. We first assessed depotentiation in young mouse slices applied 20 min after induction of 4 train LTP. Dpt resulted in a transient reduction in potentiated fEPSPs that returned to potentiated values in ~15 mins (**Fig. 3.3A**). fEPSPs were 198.9 ± 21.9% and 189.7 ± 20.1% in young slices undergoing 4 train LTP or Dpt, respectively.

Conversely, depotentiation was observed in aged slices as demonstrated by a sustained

reduction in potentiation following Dpt stimulation (**Fig. 3.3B**). Whereas fEPSPs were potentiated to 199.1 ± 18.8% following 4 train stimulation in aged slices, Dpt reset synaptic strength to 119.1 ± 10.1%. An ANOVA indicated significant differences between groups ($F_{(3, 20)} = 4.51$, $p < 0.05$), and subsequent Tukey's posthoc comparison showed that LTP was immune to depotentiation in young slices as demonstrated by a lack of difference between 4 train and 4 train + Dpt fEPSP amplitudes at 50-60 min post HFS ($p > 0.05$; **Fig. 3.3C**). Alternatively, when compared to either young 4 train + Dpt or aged 4 train groups, Dpt stimulation applied 20 min after inducing 4 train LTP significantly reduced potentiation selectively in aged slices ($p < 0.05$) (**Fig. 3.3C**). Accordingly, rapid consolidation of synaptic strength following LTP induction appears to protect synapses from Dpt in the young mouse hippocampus. This data further suggests that aging extends the time window for synaptic consolidation in mice, rendering aged synapses more susceptible to depotentiation.

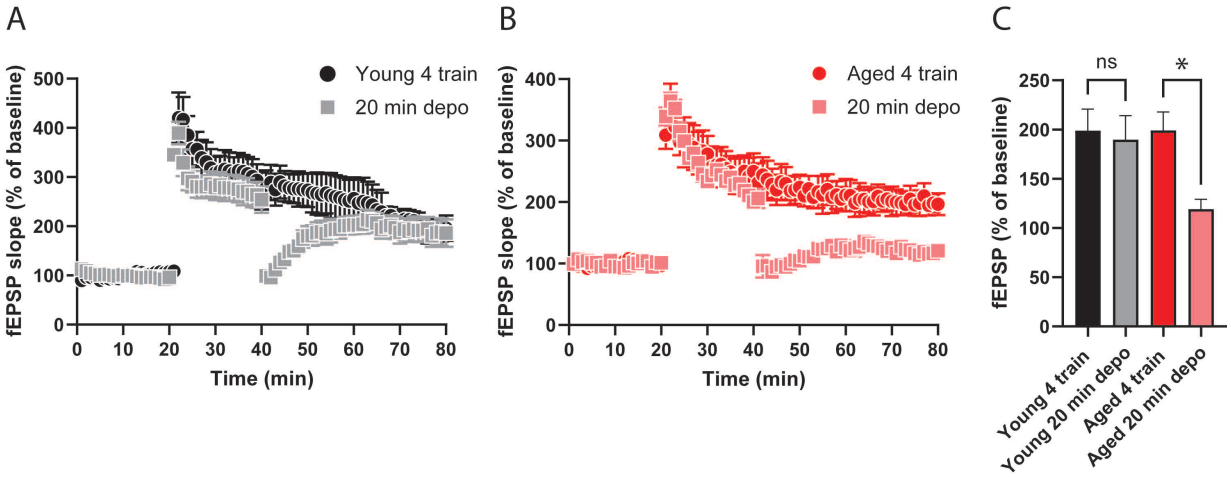

Figure 3.3. Aging increases the temporal window for depotentiation of LTP in mouse CA1

(**A**) Four trains of HFS applied at 20 sec intervals induced LTP in young slices (n = 6, black circles), which was resistant to depotentiation when 5 Hz, 3 min was applied after 20 min (n = 6, grey

squares) (**B**) 4 trains of HFS induced LTP (n = 5; red circles) which was reversible by slices in which Dpt was applied 20 min after LTP induction (n =7; pink squares). (**C**) Summary histogram for these experiments comparing levels of potentiation 50-60 min after the application of multiple trains of HFS (ANOVA with Tukey's posthoc test; $*p < 0.05$). ns = not significant. Induction stimulation not shown. All fEPSP traces (inset) were sampled at 10 min into baseline and 55 min post HFS. Calibration bars: 2 mV, 2 msec.

3.3.4 Noradrenaline restores the temporal window for depotentiation in aged slices through activation of beta-adrenergic receptors

Noradrenaline is a neuromodulatory transmitter that binds adrenergic receptors to engage metabotropic signals that bolster LTP (Connor et al., 2011; Maity et al., 2020, 2015; O'Dell et al., 2015). Activation of β-ARs enhances LTP through upregulation of protein synthesis (Connor et al., 2011; Gelinas and Nguyen, 2005; Maity et al., 2020), and translation regulation during synaptic plasticity renders synapses immune to depotentiation (Woo and Nguyen, 2003). Accordingly, we sought to determine if activation of noradrenergic receptors restores the temporal window for depotentiation in aged mouse slices. To test if activation of noradrenergic receptors confers resistance to Dpt, NA (10 μM) was applied prior to the induction of 4 train LTP, which was followed by the Dpt (5 Hz, 3 min) stimulation. Relative to 4 train stimulation alone, application of NA prevented the reversal of LTP by Dpt stimulation applied 20 min later (mean fEPSP slopes were 192.6 ± 19.7% and 236.7 ± 24.3% for 4 train and 4 train + NA + Dpt treated slices, respectively 50-60 min after HFS) (**Fig. 3.4A**). This data indicates that activating noradrenergic receptors with NA is sufficient for restoring resistance to Dpt in aged slices.

We next sought to determine which noradrenergic receptors mediate immunity to depotentiation in aged hippocampus slices. Given the prominent role of β-ARs in enhancing LTP, we first tested if β-ARs are required for the depotentiation blocking effects of NA. Co-application of the broad-spectrum β-AR antagonist, propranolol (50 μM), prevented NA from blocking depotentiation (**Fig. 3.4B**). Mean fEPSP slopes were 196.7 ± 18.6% and 114.7 ± 17.5% for 4 train LTP and 4 train LTP + NA + propranolol treated slices, respectively 50-60 min after HFS) (**Fig. 3.4B**). An ANOVA revealed significant differences between groups ($F_{(3, 22)} = 6.502$, $p < 0.01$), and subsequent Tukey's posthoc comparisons found that treatment with NA enhanced LTP immunity to depotentiation in aged slices as demonstrated by a lack of difference between 4 train and 4 train + NA fEPSP amplitudes at 50-60 min post HFS ($p > 0.05$; **Fig. 3.4C**). Conversely, co-application of propranolol overlapping with NA prevented the protective effects of NA against Dpt ($p < 0.05$).

We next tested if blocking α-ARs alters NA-mediated resetting of resistance to depotentiation (**Fig. 3.6**). Slices were treated with NA co-applied with the α2-AR receptor antagonist yohimbine (3 μM) and the α1-AR antagonist prazosin (10 μM) and then stimulated with HFS followed 20 min later with the Dpt stimulation protocol. LTP recovered to comparable levels in NA alone and NA + yohimbine + prazosin treated slices (**Fig. 3.6A**). Comparison of fEPSP amplitudes 50-60 min post HFS failed to detect differences in LTP ($p > 0.05$; **Fig. 3.6B**). Collectively, these results indicate that NA re-establishes immunity to Dpt in aged slices through activation of β- but not α-adrenergic receptors.

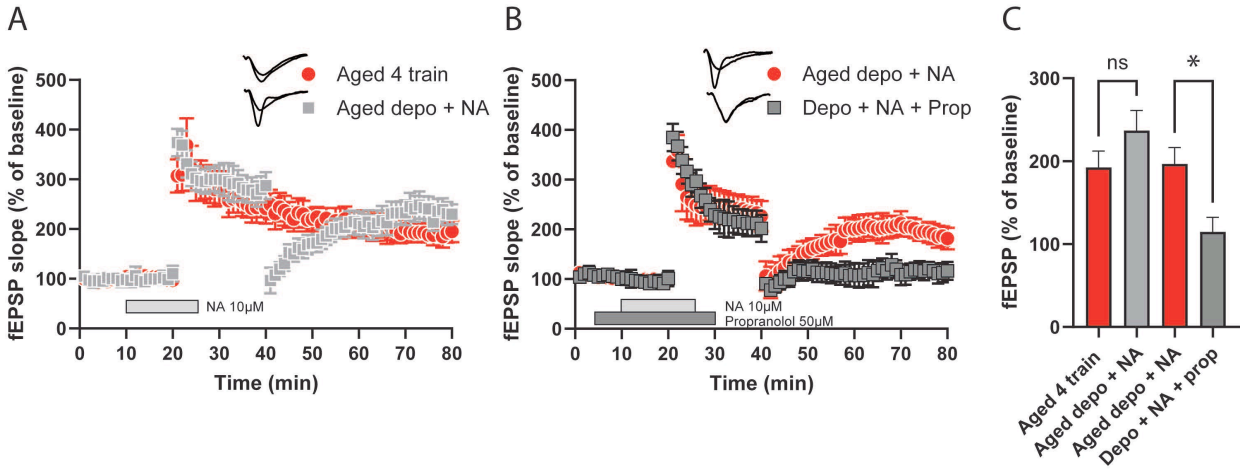

Figure 3.4. Noradrenaline restores the temporal window for depotentiation in aged slices through activation of beta-adrenergic receptors

(**A**) Four train LTP was used as a positive control (n = 7, red circles). NA application prior to HFS produced LTP which was resistant to depotentiation induced through 5 Hz, 3 min stimulation applied after 20 min (n = 6, grey squares). (**B**) Pairing NA with propranolol prevented the rescue of potentiation by NA (n = 7; dark grey squares) which was reversed by Dpt (n =7; pink squares) applied 20 min after HFS. (**C**) Summary histogram for these experiments comparing levels of potentiation 50-60 min after the application of multiple trains of HFS (ANOVA with Tukey's posthoc test; $*p < 0.05$). ns = not significant. Induction stimulation not shown. All fEPSP traces (insets) were sampled at 10 min into baseline and 55 min post HFS. Calibration bars: 2 mV, 2 msec.

3.3.5 NA engages translation regulation to confer resistance to depotentiation in aged mouse CA1

β-AR stimulation facilitates the activation of signaling pathways that couple to translation regulation (Gelinas and Nguyen, 2005; Maity et al., 2020; O'Dell et al., 2010). Accordingly, we next tested if translation regulation is required for the Dpt blocking effects of NA in hippocampus slices from aged mice. Aged hippocampus slices were treated with the translation repressor emetine (20 μM) overlapping with NA prior to inducing LTP. Although LTP induction was unaffected, subsequent Dpt erased LTP in slices treated with NA + emetine (**Fig. 3.5A**). Compared to NA + HFS treated slices (mean fEPSP slopes were 168.5 ± 17.5%), the addition of emetine reduced the synaptic responses (mean fEPSP slopes were 116.4 ± 13.2%). Comparison of LTP 50-60 min after HFS revealed a significant decrease in synaptic potentiation in emetine treated slices ($t_{(10)} = 2.378$, $p < 0.05$, **Fig. 3.5B**). These results suggest that NA recruits translation-dependent mechanisms to re-establish resistance to Dpt in aged slices, supporting a model in which dysregulation of protein synthesis impairs synaptic plasticity in aged neural circuits.

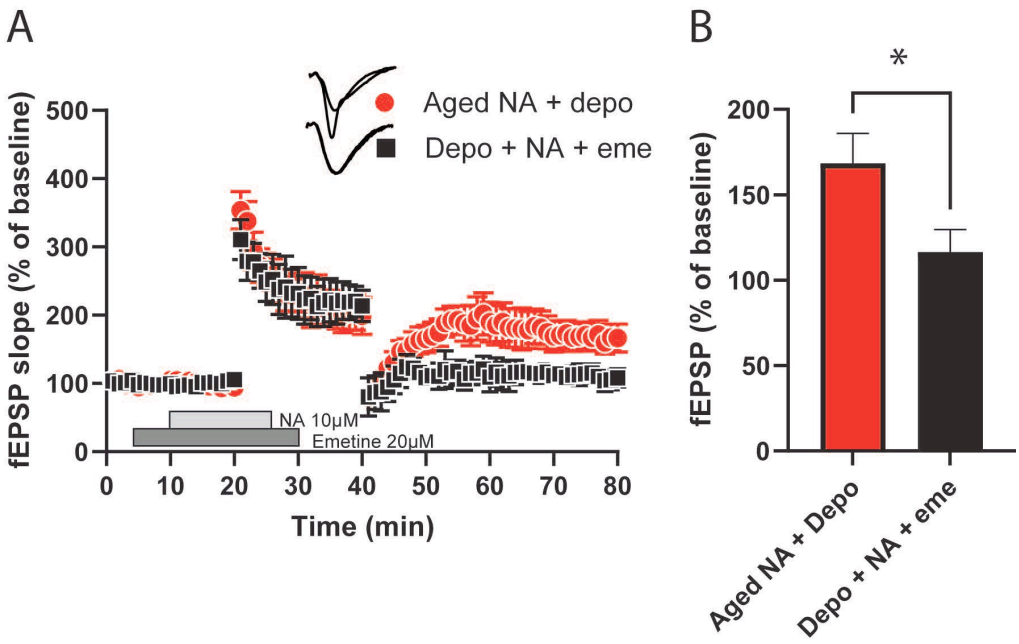

Figure 3.5. Noradrenaline recruits translation regulation to confer resistance to depotentiation in aged mouse CA1 (**A**) NA paired with HFS induces LTP that is resistant to Dpt (n = 6, red circles). Co-application of emetine and NA prior to HFS produced LTP which was depotentiated following 5 Hz, 3 min stimulation (n = 8, black squares). (**B**) Summary histogram for these experiments comparing levels of potentiation 50-60 min after the application of multiple trains of HFS (Student's t test with Welch correction; $*p < 0.05$). All fEPSP traces (insets) were sampled at 10 min into baseline and 55 min post HFS. Calibration bars: 2 mV, 2 msec.

3.4 Discussion

How aging alters synaptic plasticity in brain regions required for memory is a long-standing question that has met with conflicting results (Barnes, 2003; Costenla et al., 1999; Huang and Kandel, 2006; Kumar et al., 2007; Rex et al., 2005). To assess the status of LTP during aging, we first tested if LTP was compromised in the CA1 region of aged mouse hippocampus slices. We found that multiple forms of LTP were comparable between young and aged hippocampus. These results indicate that most forms of LTP demonstrate similar induction and expression profiles in both young and aged slices within the first 60 minutes following LTP induction. This relatively narrow sampling window (~1 hr) did not determine if later components of LTP expression were altered. It will be important to test time points extending beyond 1 hr going forward to further establish if the maintenance of LTP is preserved in aged neural circuits. A primary study linking LTP and memory performance during aging indicated that LTP decay rate was correlated with memory loss (Barnes and McNaughton, 1985). However, alternative studies testing LTP at aged synapses have reported null effects, impairments or enhancements of LTP during aging (Costenla et al., 1999; Huang and Kandel, 2006; Kumar et al., 2007; Rex et al., 2005). Our data is in line with null findings, which suggests that alternative synaptic mechanisms may underlie cognitive decline associated with advanced age.

Increased susceptibility to depotentiation offers an alternative synaptic model predicting the onset of aged-base neural circuit deficiencies. In contrast to LTD (Dudek and Bear, 1992; Mulkey and Malenka, 1992), depotentiation is readily induced in aged rodent hippocampus (Norris et al., 1996). Initially, LTP was comparably susceptible to Dpt independent of age, provided the depotentiating stimulus was applied rapidly after LTP induction. However, previous findings

indicate that depotentiation is easier to induce in aged rats (Norris et al., 1996). Consistent with this finding, our results revealed changes in susceptibility to Dpt that emerge during aging in mouse CA1. Thus, although canonical forms of LTP appear intact during aging, there is a notable increase in the temporal window (~20 min) following LTP induction during which Dpt can be elicited in aged mouse hippocampus.

A gradual yet global decline in neuromodulatory inputs has been reported within the brain during normal aging (Dahl et al., 2023; Karrer et al., 2017; Liu et al., 2020). Neuromodulators, including dopamine and noradrenaline, lower the threshold for inducing long lasting synaptic modifications through downstream engagement of translation regulatory pathways (Connor et al., 2011; Gelinas and Nguyen, 2005; Huang and Kandel, 1995; Maity et al., 2020; Navakkode et al., 2007). Within the noradrenergic system, activation of β-ARs facilitates the induction of enduring forms of LTP through engagement of ERK, mTOR and the cAMP-dependent Epac signaling cascades (Connor et al., 2012, 2011; Gelinas and Nguyen, 2005; Maity et al., 2020; Winder et al., 1999). Conversely, α-ARs play more modest roles in hippocampal synaptic plasticity (Nguyen and Connor, 2019; O'Dell et al., 2010). Our data revealed that NA acts primarily through β-ARs to restore resistance to Dpt within aged synapses, as the rescuing effects of NA were prevented by antagonism of β- but not α1/α2-adrenergic receptors. Collectively, our findings indicate that NA acting primarily through β-ARs reverses the expanded temporal window for Dpt observed in aged slices to time frames consistent with synapses in young hippocampal slices.

Interestingly, neuromodulation originating from the locus coeruleus noradrenergic outputs that project throughout the brain (Liu et al., 2020; Sara, 2009), and processes governing translation regulation (Fando et al., 1980; Schimanski and Barnes, 2010) become compromised

during aging. Accordingly, reduced neuromodulator tone with advanced aging may decrease synaptic consolidation by limiting the necessary recruitment of protein synthesis that would stabilize molecular and structural changes at synapses that support potentiation. Resetting of Dpt thresholds within the aged CA1 by NA required protein synthesis, as demonstrated by the failure to reset resistance to Dpt in the presence of the translation repressor, emetine. Notably, ERK and mTOR pathways activated downstream from β-ARs converge on eIF4E-mediated elongation to upregulate protein synthesis required for enduring forms of LTP (Gelinas et al., 2007; Gelinas and Nguyen, 2005). Additionally, NA specifically drives the synthesis of GluA1, indicating that a potential mechanism for regulating susceptibility to Dpt may be through the generation of GluA1-containing AMPARs (Maity et al., 2015), which are rapidly trafficked into synapses in response to LTP-inducing stimuli to bolster postsynaptic currents. It will be important to test if there is a shift in the overall expression or synaptic content of AMPARs that limits susceptibility to Dpt in aged synapses and whether upregulation of AMPARs following β-AR stimulation is required for synaptic consolidation and subsequent suppression of depotentiation.

Canonical forms of synaptic weakening in the hippocampus include long-term depression and depotentiation. These forms of synaptic plasticity diverge mechanistically, as some evidence suggests that LTD requires GluN2B-containing NMDARs whereas DPT requires GluN2A (Zhang et al., 2009). Aging is associated with a gradual reduction in the expression of GluN2B, which would bias aged synapses toward GluN2A-mediated depotentiation (Brim et al., 2013). Additionally, adenosine receptors coupling to Gi/o proteins that mediate suppression of cAMP have been specifically linked to Dpt (Huang et al., 1999). These findings suggest reduced GluN2B function in tandem with suppression of cAMP and downstream signaling cascades such as

Epac/PKA by adenosine may promote the functional redirection of activity-dependent synaptic changes away from potentiation towards depotentiation.

Consistent with this model, NA-mediated activation of β-ARs would bias metabotropic signaling towards Ga/s proteins that increase cAMP levels and downstream engagement of PKA/Epac to bias potentiation towards synaptic consolidation (Maity et al., 2020). Moreover, β-AR stimulation increases phosphorylation of Ser1166 on GluN2B which enhances NMDAR function and Ca^{2+} signaling in spines (Murphy et al., 2014), which would bolster synaptic consolidation and constrain the DPT time window.

Functionally, Dpt stimulation increases activation of Rap2 and c-Jun NH2-terminal kinase (JNK), which selectively regulate the surface expression of AMPA receptor subunits with long cytoplasmic tails (GluA1 and GluA2L)(Zhu et al., 2005). Activation of Rap2 and JNK promote internalization of GluA1 and GluA2L to downregulate glutamatergic transmission (Zhu et al., 2005). β-AR stimulation increases the synthesis and lateral insertion of GluA1 during NA-LTP through enhanced phosphorylation of S845 on GluA1 (Maity et al., 2015; Tenorio et al., 2010). When considered in the context of impaired LTP and dysregulated translation during aging, this suggests NA may converge on both the generation and/or trafficking of AMPAR subunits to re-establish synaptic consolidation in aged neural circuits. In our study, the translation repressor emetine blocked the restorative effects of NA on susceptibility to Dpt. Although further evidence is required, our results are consistent with an upregulation of AMPA receptor translation and/or trafficking that constrains DPT windows and allows synaptic consolidation to occur.

Collectively, our data suggests that aging significantly expands time windows during which recently potentiated synapses are subject to activity-dependent erasure. Furthermore, they

indicate that although the downregulation of neuromodulatory inputs associated with aging may not compromise the induction of LTP, this reduction may alter synaptic consolidation that renders synapses immune to depotentiation. Taken together, we propose that NA-induced stimulation of β-ARs plays an *in vivo* role in maintaining synaptic plasticity, through translation-dependent processes that are compromised with aging. Both human and animal genetic studies have linked synaptic pathology associated with aging to altered neuromodulation, downregulated translation and compromised synaptic plasticity(Barnes, 2003; Burke and Barnes, 2006; Karrer et al., 2017; Liu et al., 2020; Schimanski and Barnes, 2010; Small et al., 2011). Our findings indicate that age-related cognitive disorders could be associated with dysfunction of neuromodulatory systems and dysregulation of translation. Given that the application of NA was sufficient for preventing Dpt in aged hippocampus slices, this suggests that re-establishing memory capacity for those of advanced age may be possible through preventing excessive synaptic depotentiation.

In conclusion, we uncovered unique *ex vivo* roles of the endogenous neuromodulator, noradrenaline in resetting the parameters of a rarely studied synaptic phenomenon, depotentiation. Given that depotentiation is readily induced in the adult and aged brain, the physiological relevance of this finding may bare directly on subtle yet key cellular changes that increase the likelihood of pathological forgetting with aging. Given established deficits in global neuromodulation and associated diminished translational capacity with advancing age, we propose excessive depotentiation as a novel underlying pathology contributing to aging associated memory impairments that could be amenable to therapeutics targeting the noradrenergic system.

Supplementary figure

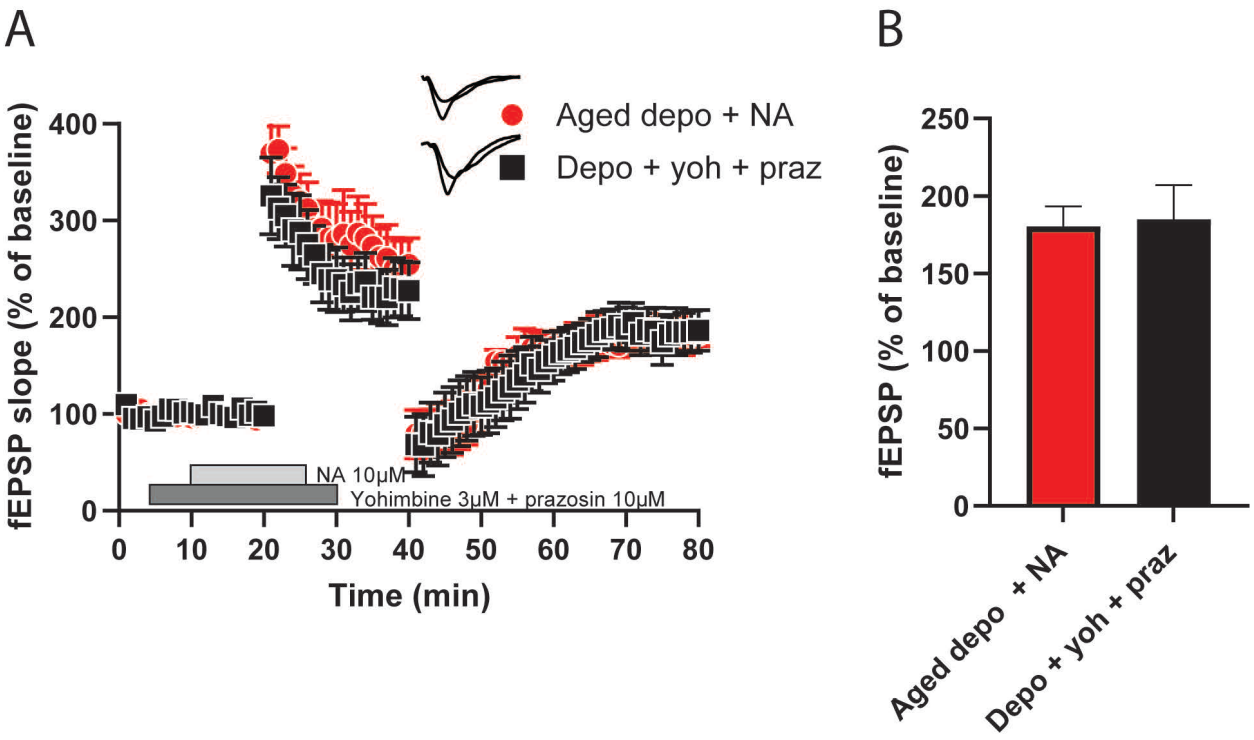

Figure 3.6. Alpha-adrenergic receptors are not required for NA-mediated resetting of resistance to depotentiation in aged CA1 (**A**) NA paired with HFS induces LTP that is resistant to Dpt (n = 5, red circles). Co-application of yohimbine and prazosin which inhibit α1- and α2-ARs, respectively overlapping with NA paired with HFS (n = 6, black squares) produced LTP which was similar in amplitude slices treated with NA alone. (**B**) Summary histogram for these experiments comparing levels of potentiation 50-60 min after the application of multiple trains of HFS (Student's t test with Welch correction; $p > 0.05$; not significant). Induction stimulation not shown. All fEPSP traces (insets) were sampled at 10 min into baseline and 55 min post HFS. Calibration bars: 2 mV, 2 msec.

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CHAPTER 4: LTP DECAY

Extrasynaptic NMDARs repress LTP through Rac1-mediated suppression of protein synthesis*

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Abstract

An underappreciated yet crucial aspect of memory optimization is the removal of unnecessary memories from brain circuits, or forgetting. A growing body of evidence indicates that erasure of neuronally stored information is an active process, dependent upon dedicated, intrinsic cellular mechanisms. The activity-dependent increase in synaptic strength known as long-term potentiation (LTP) is widely considered a cellular analog for memory formation. Accordingly, exploring processes underlying the decay of LTP should provide insight into the molecular mechanisms required for forgetting at the cellular level. To explore how LTP decays, we first tested the activity-dependence of this phenomenon. Halting basal stimulation after inducing LTP resulted in a substantial retention of synaptic potentiation that extended the duration of LTP. We next explored the relative contribution of different NMDA receptor subunits to potentiation decay. We found that GluN2B containing NMDA receptors were required for LTP decay, and that these receptors were localized to extrasynaptic sites. Next, we observed that inhibition of an established forgetting molecule, Rac1, also reduced extrasynaptic LTP decay. We next sought to determine if blocking LTP decay converts LTP into a translation-dependent form. Consistent with links between stable long-lasting forms of LTP, blocking Rac1 converted decaying LTP into a long-lasting,

translation-dependent LTP. Finally, we provide evidence that Rac1 represses pro-LTP pathways, as blocking Rac1 increased ERK and eIF4E phosphorylation, indicating upregulated translation. Thus, decay of LTP recruits extrasynaptic GluN2B-containing NMDA receptors and Rac1, which may promote active forgetting through suppression of parallel pro-LTP signaling cascades coupled to translation regulation. Our data suggest that protecting synaptic potentiation from LTP decay may unmask memory promoting signaling cascades within the hippocampus capable of maintaining synaptic strength required for memory genesis.

4.1 Introduction

An underappreciated yet crucial aspect of memory function is the removal of unnecessary memories from neuronal circuits, or forgetting. Traditional views on forgetting postulated that this was a mostly passive process (Ebbinghaus, 2013), However, an emerging theory (Davis and Zhong, 2017; Medina, 2018; Moreno, 2021) supported by recent findings states that erasure of information at the neuronal level is an active process, dependent upon intrinsic cellular mechanisms (Berry et al., 2012; Cervantes-Sandoval et al., 2020, 2016; Hardt et al., 2014; Sachser et al., 2016). In mammals, both ongoing neurogenesis (Akers et al., 2014; Epp et al., 2016; Richards and Frankland, 2017) and synaptic potentiation decay (Barnes and McNaughton, 1985; Dong et al., 2015; Hardt et al., 2014; Villarreal et al., 2002; Xiao et al., 1996) have been proposed as intrinsic drivers of forgetting. Synaptic potentiation decay can be effectively studied using a well-established cellular analog of memory known as long-term potentiation (LTP) (Abraham et al., 2024; Bliss and Collingridge, 1993; Bliss and Lomo, 1973). LTP models cellular processes engaged during learning and memory, and genetic or pharmacological manipulations that promote LTP decay enhance forgetting (Awasthi et al., 2019; Hardt et al., 2014; Sachser et al., 2017, 2016; Villarreal et al., 2002). LTP decay refers to the active reduction of this synaptic potentiation, returning synapses toward their baseline efficacy.

NMDARs, critical for LTP induction (Liu et al. 2004; Bliss and Collingridge 1993), also play a pivotal role in its decay. Calcium influx through NMDARs triggers signaling pathways that promote synaptic weakening and memory loss, indicating that ongoing NMDAR activation serves as a priming event for cellular forgetting. Accordingly, early results showed that NMDAR antagonism prevents LTP decay *in vitro* and extends the duration of spatial memories (Villarreal

et al., 2002). *In vivo* inhibition of NMDARs within the dorsal hippocampus following water maze training prolonged the duration of associated spatial memories from days to weeks (Shinohara and Hata, 2014). Similarly, both object location and recognition memories were extended by peripheral administration of memantine and MK-801, non-competitive antagonists of NMDARs (Sachser et al., 2016).

The relative contribution of the different NMDAR subunits to LTP decay and active forgetting may depend on the potentiation protocol or type of memory that is being probed for erasure. Decay of spatial memory in the Morris water maze was accelerated in the presence of the GluN2B antagonist, Ro 25-6981 but prevented by the GluN2A antagonist, NVP (Shinohara and Hata, 2018). Others found that blocking GluN2B-containing NMDARs with ifenprodil prevents the decay of LTP within area CA1 *in vivo*, suggesting that these receptors initiate depotentiation or related processes that could weaken memories (Sachser et al., 2016). Consistent with this, infusion of the Ro25-6981 in the dorsal hippocampus (dHPC) prevented forgetting of object location long-term memories (Migues et al., 2019). Taken together, these finding indicate that ongoing basal neuronal activity recruits NMDARs to promote the active decay of synapse potentiation and memories.

The removal of GluA2-containing AMPARs from the postsynaptic membrane has also previously been linked to LTP decay (Hardt et al., 2014). Activity-dependent endocytosis reduces the number of functional AMPARs, thereby weakening synaptic transmission. Preventing GluA2-dependent endocytosis with the peptide GluA23Y prevents the decay of a transient form of LTP (Early-LTP; E-LTP), converting it into a more enduring form akin to late-LTP (Dong et al., 2015). E-LTP decay occurs rapidly, often within hours, driven by the endocytosis of AMPARs particularly

those containing the GluA2 subunit, from the postsynaptic membrane (Hardt et al., 2014; Migues et al., 2016). LTP decay is thus an active, energy-dependent process, distinct from passive rundown, and is mediated by molecular machinery that reverses synaptic strengthening established during LTP induction.

Both LTP decay and memory erasure are subject to neuromodulation which engages dedicated intracellular signals to drive potentiation rundown. Within drosophila, ongoing dopaminergic neuron activity following a learning event promotes rapid forgetting of olfactory memories (Berry et al., 2012). Dopamine engages distinct receptors and cell types that supply forgetting signals (Berry et al., 2018; Himmelreich et al., 2017), including the small GTPase Rac1, which is linked to accelerated memory decay (Cervantes-Sandoval et al., 2020; Lv et al., 2019).

Rac1 has conserved functions in regulating forgetting, as blocking Rac1 extends memory duration, whereas rendering Rac1 active erases memory in rodents (Jiang et al., 2016; Liu et al., 2018a, 2016). These studies support the existence of an evolutionarily conserved biomolecular system, dedicated to forgetting, predicated on Rac1 regulation. Notably, NMDAR-mediated Ca^{2+} influx is sufficient for activating Rac1, possibly through phosphatidylinositol-3-kinase (PI3K) and PKCι/λ (Cui et al., 2021). Whereas Rac1-PI3K-induced signaling mediates the induction of LTP, Rac1 inhibits PKMζ via LIMK (Cui et al., 2021). However, although both NMDARs and Rac1 have been heavily implicated in the neuronal mechanisms of memory erasure, there is a general paucity of evidence regarding the select roles for the NMDAR subunits nor their location, or the downstream effectors for Rac1-induced forgetting in rodents, leaving a major gap in our understanding of active forgetting mechanisms in mammalian neural circuits.

Accordingly, we sought to understand the cellular basis for memory erasure through

probing NMDAR-mediated mechanisms driving LTP decay. Using hippocampal slices derived from mice, we sought to identify mechanisms that drive the reversal of synapse potentiation in a major memory circuit (hippocampus SC-CA1 pathway) as a proxy for cellular forgetting within the mammalian CNS. In young adult mouse hippocampus slices, we found that LTP decay is mediated by extrasynaptic NMDARs containing GluN2B subunits. Activation of these receptors appears to recruit Rac1 as inhibition of Rac1, similar to blocking GluN2B, prevented LTP decay.

Consistent with this, Western blotting revealed that induction of GluN2B-mediated decaying LTP increased the phosphorylation of Rac1. To probe how Rac1 activity mediates LTP decay, we tested the effects of Rac1 inhibition on LTP properties. Stimulation with a transient LTP protocol in the presence of a Rac1 inhibitor facilitated the conversion of weak LTP into a stable, protein synthesis-dependent form of synaptic potentiation. In accord, LTP was disrupted by blocking translation which prevented the expression of enduring LTP following Rac1 inhibition. Taken together, our study highlights a novel mechanism through which synaptic potentiation is actively reversed through extrasynaptic NMDAR-mediated recruitment of Rac1 and downstream suppression of translation.

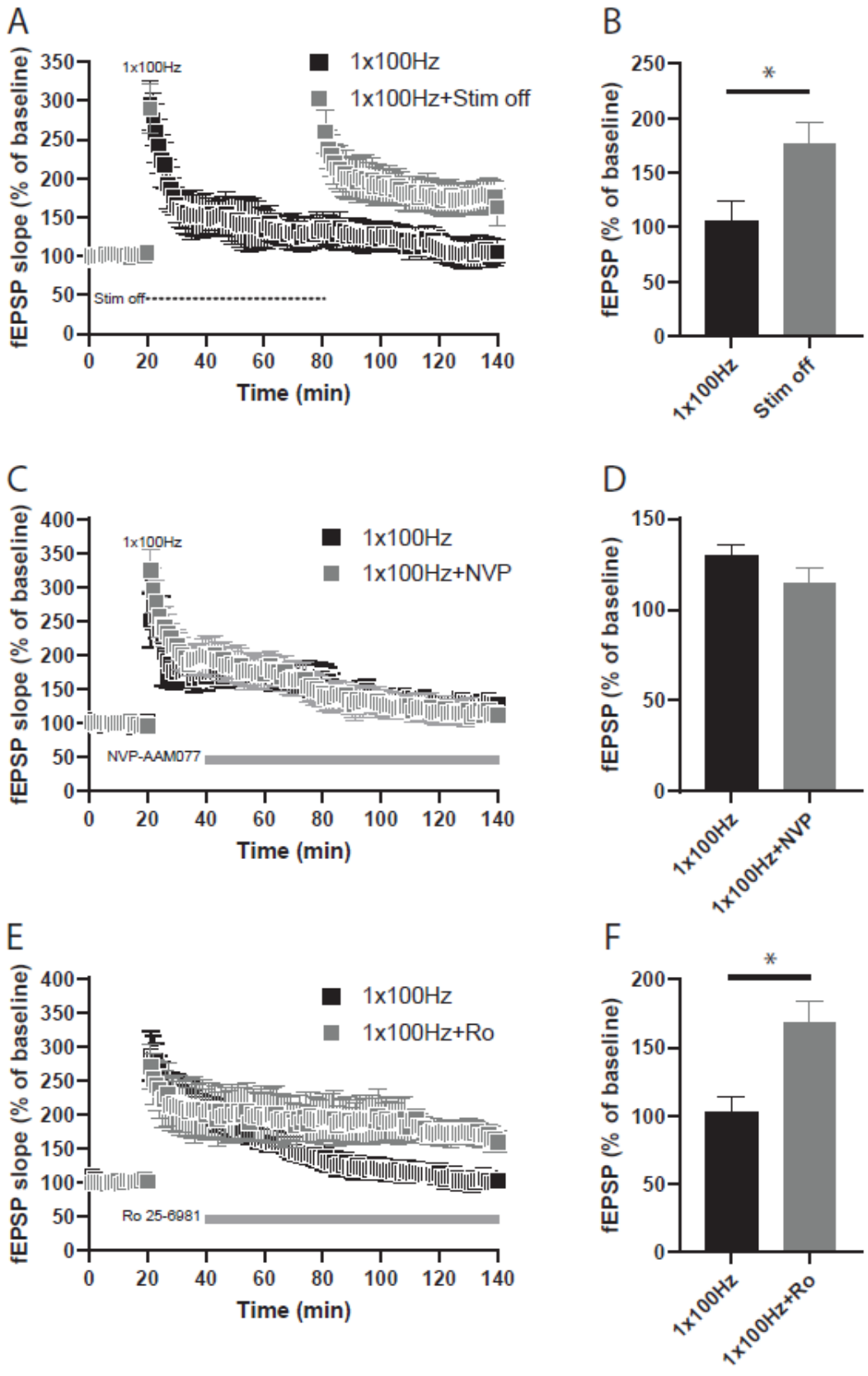
4.2 Results

4.2.1 Single train LTP decays in an activity-dependent manner

To confirm whether ongoing synaptic activity promotes LTP decay, we performed field excitatory postsynaptic potential recordings (fEPSPs) at the Schaffer collateral-CA1 pathway within mouse hippocampus slices (**Fig. 4.1A**). Stimulating and recording electrodes were placed with stratum radiatum of area CA1, a region characterized by an elevated concentration of

glutamatergic synapses forming on pyramidal cell apical dendrites. To test the activity-dependence of LTP decay, high frequency stimulation (1x100Hz, 1 sec) LTP was induced, a single pulse was applied to confirm LTP induction, and then baseline stimulation was halted for 1 hr. Controls consisted of the same LTP induction protocol followed with immediate resumption of baseline (1 pulse/min) stimulation.

HFS induced potentiation which decayed within ~90 min following application of the induction stimuli (mean fEPSP slopes were 105.71±18.1% 110-120 min post HFS) (**Fig. 4.1A**). In contrast, in slices that underwent LTP induction followed immediately by cessation of baseline stimulation, potentiation was maintained at an elevated level (**Fig. 4.1A,B**), supportive of a diminished rate of decay (mean fEPSP slopes were 175.53±20.8% 110-120 min post HFS). Comparisons of LTP amplitude during the last 10 min of each recording indicated that HFS-induced LTP decayed more rapidly when baseline stimulation was maintained for the duration of the recordings ($p < 0.05$; **Fig. 4.1B**). These data indicate that ongoing synaptic activity promotes potentiation decay within area CA1 of the mouse hippocampus.


A
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fEPSP slope (% of baseline)
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Stim off
C
1x100Hz
1x100Hz+NVP
NVP-AAM077
fEPSP slope (% of baseline)
Time (min)
D
fEPSP (% of baseline)
1x100Hz
1x100Hz+NVP
E
1x100Hz
1x100Hz+Ro
Ro 25-6981
fEPSP slope (% of baseline)
Time (min)
F
fEPSP (% of baseline)
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1x100Hz+Ro

Figure 4.1. The decay of LTP requires ongoing synaptic activity and is mediated by GluN2B-containing NMDARs (A) 1 train LTP (1x100Hz, 1s) stimulation induces a decaying LTP that returns to baseline in ~90 min (n = 8, black squares). Halting baseline stimulation (1 stimulation per minute as test pulse intensity) immediately after HFS produced LTP which was sustained for 1 hr (n = 6, grey squares). (B) Summary histogram for these experiments comparing levels of potentiation during the last 10 min of recordings (t-test with Welch correction; $t(10.93) = 2.533$, $^{*}p < 0.05$). Stopping stimulation maintained elevated potentiation relative to slices undergoing normal basal stimulation (C) Comparison of single train stimulation (n = 8, black squares) with slices undergoing single train stimulation paired with the GluN2A receptors antagonist, NVP-AAM077 (.1 μM, application time represented by grey bar; n = 8, grey squares) applied 20 min after HFS. Comparison of potentiation during the last 10 min of recording revealed no detectable differences between groups, $p > 0.05$, as shown in the summary histogram (D). (E) Application of the GluN2B antagonist, Ro25-6981, (grey squares, n = 8), relative to drug-free slices (black squares, n = 8) revealed that blocking GluN2B prevented the decay of LTP (t-test with Welch correction; $t(12.62) = 3.400$, $^{*}p < 0.001$). n = # of slices. Data reported with error bars representing the S.E.M..

4.2.2 LTP decay requires GluN2B-containing NMDAR subunits

Given the relatively high expression of GluN2A and GluN2B in the rodent hippocampus (Kirson and Yaari, 1996; Monyer et al., 1994), and their prominent roles in canonical forms of synaptic plasticity (Bliss and Collingridge, 1993; Connor and Wang, 2016), our initial analysis focused on these subunits. We first tested whether GluN2A subunit inhibition altered the decay rates of HFS-induced LTP. The selective GluN2A antagonist, NVP-AAM077 (.1 μM), was bath applied to hippocampus slices 20 minutes after inducing LTP with HFS. The decay of LTP was still evident in the presence of NVP which appeared to approach baseline over the course of the recording. (**Fig. 4.1C**). Upon quantifying the fEPSP amplitudes between NVP treated and non-treated slices, we failed to detect a change in the levels of potentiation during the last 10 minutes of the recordings (**Fig. 4.1D**) (mean fEPSPs were 129.41±6.6% and 114.70±7.9% for slices exposed to HFS or HFS paired with NVP, respectively).

Next, we tested whether inhibition of GluN2B subunits alters the rate of potentiation decay. To probe the effects of GluN2B inhibition on LTP decay, we applied Ro25-6981 (1 μM) 20 min after applying HFS. LTP appeared to be maintained in the presence of Ro. Comparisons conducted during the last 10 min of recordings revealed a significant increase in potentiation in slices treated with Ro relative to drug-free controls (**Fig. 4.1E,F**; mean fEPSPs were 102.62±11.3% and 168.68±15.9% for slices exposed to HFS or HFS paired with Ro, respectively; $p < 0.05$). Taken together, these data suggest whereas GluN2A was dispensable for LTP decay, ongoing synaptic activity recruits GluN2B to drive the decay of LTP, indicating that NMDARs containing GluN2B constrain LTP potentiation within mouse SC-CA1 glutamatergic synapses.

4.2.3 Extrasynaptic NMDA receptors containing GluN2B subunits mediate LTP decay

NMDA receptors demonstrate distinct localization, with GluN2B subunits primarily found at extrasynaptic sites (Hardingham et al., 2002; Stocca and Vicini, 1998). These differential distribution patterns contribute to alternative forms of plasticity as glutamate spillover associated with excessive synaptic stimulation preferentially acts on extrasynaptic NMDARs, which can depress synaptic strength (Hardingham et al., 2002; Liu et al., 2013). To further determine the relative contribution of synaptic and extrasynaptic NMDARs to LTP decay, we combined activity dependent blockade of synaptic NMDARs with pharmacological dissection of extrasynaptic NMDAR subunits during LTP decay.

To isolate extrasynaptic NMDA receptors, we bath applied an activity-dependent NMDAR inhibitor, MK-801 (10 μM), which enters and occludes the ion pore of activated NMDARs. This was followed by a train of stimulation (5Hz, 16 sec) designed to activate primarily synaptic receptors, thereby facilitating activity-dependent blockage by MK-801 (Yang et al., 2017). To ensure activation of extrasynaptic NMDARs, two trains of HFS (2x100Hz, 20 sec intertrain interval) were subsequently applied which induced LTP that decayed in ~90 min (**Fig. 4.2A**). Mean fEPSPs were 100.78±8.0% 100-110 min after HFS alone.

In order to determine the relative contribution of individual subunits to extrasynaptic LTP decay, extrasynaptic NMDARs were again isolated and LTP was induced while blocking GluN2A with NVP. LTP decay that was equivalent to control conditions was observed (**Fig. 4.2B**), as fEPSPs returned to baseline (mean fEPSPs were 102.48±9.4% 100-110 min after HFS). Next, we ran the same protocol while blocking GluN2B-containing NMDARs with Ro (**Fig. 4.2C**) which resulted in maintained LTP (mean fEPSPs were 138.11±11.9% 100-110 min after HFS).

An ANOVA comparing fEPSP amplitudes across control, NVP and Ro treated slices

revealed that potentiation was significantly enhanced only when GluN2B was inhibited when compared 100-110 min post-HFS ($F_{(2, 30)} = 4.487$, $p < 0.01$). Subsequent Tukey's posthoc comparisons found that treatment with Ro prevented the decay of LTP, relative to both NVP and control treatment slices ($p < 0.05$), **(Fig. 4.2D)**. These data indicate that the inhibiting extrasynaptic GluN2B but not GluN2A prevented the gradual decay of LTP, consistent with a role for extrasynaptic GluN2B-containing NMDARs as primary drivers of activity-dependent potentiation decay. This finding is consistent with a glutamate spillover effect during post-HFS basal stimulation that engages extrasynaptic GluN2B receptors and associated molecular events to constrain synaptic potentiation.

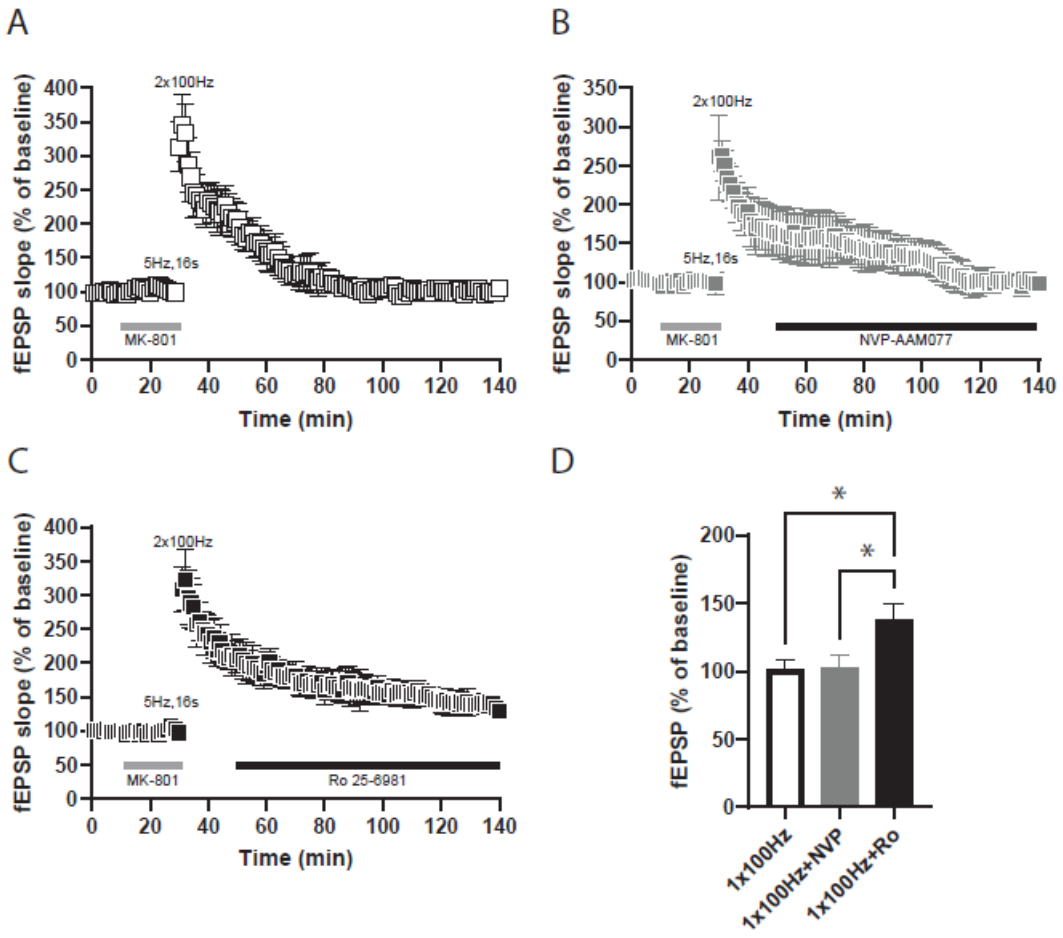


Figure 4.2. Extrasynaptic GluN2B-containing NMDA receptors are required for LTP decay

(A) Application of the activity-dependent channel blocker, MK-801 for 20 minutes, followed by theta frequency stimulation (5Hz, 16 s) allows for inhibition of synaptic NMDARs. Slices were subsequently stimulated with 2x100Hz stimulation with an intertrain interval of 20 sec to induce extrasynaptic NMDA receptor-dependent LTP (n = 12, white squares). (B) Following the isolation of extrasynaptic NMDA receptors with MK-801 paired with theta stimulation, 2x100Hz stimulation was applied, followed 20 min later by the application of NVP-AAM077 (.1μM) to determine if extrasynaptic GluN2A receptors modulate LTP decay (n = 11, grey squares). (C) The same protocol as outlined in (B) was used to test if inhibition of extrasynaptic GluN2B receptors with Ro25-6981 alters LTP decay (n = 10, black squares). (D) Summary histogram for this series of experiments comparing levels of potentiation during the last 10 min of recordings An ANOVA revealed significant differences between groups ($F_{(2, 30)} = 4.487, p < 0.01$), and subsequent Tukey's posthoc comparisons found that treatment with Ro prevented the decay of LTP, relative to both NVP and control treatment slices (*$p < 0.05$), indicating that extrasynaptic GluN2B receptors are required for the onset of LTP decay. n = # of slices. Data reported with error bars representing the S.E.M..

4.2.4 Rac1 is engaged during extrasynaptic GluN2B-mediated LTP decay

Previous studies in drosophila and rodents have identified activated Rac1 as a critical regulator of forgetting, demonstrating that Rac1 inhibition prevents forgetting in various memory-dependent tasks (Cui et al., 2021; Jiang et al., 2016; Liu et al., 2018b, 2016). To probe the molecular effectors acting downstream of extrasynaptic NMDARs that mediate LTP decay, we

next carried out molecular assessment of Rac1's role in acute brain slice LTP decay experiments. To test this, we isolated extrasynaptic NMDARs and induced LTP in the presence of NSC 23766, a selective Rac1 inhibitor.

Similar to blocking GluN2B-containing NMDARs, LTP induced in the presence of NSC revealed that blocking Rac1 maintained synaptic potentiation (**Fig. 4.3A**; mean fEPSPs were 138.24±8.7% and 106.32±4.9% 100-110 min after HFS in Rac1 inhibitor treated and control slices, respectively). Comparisons to drug free controls revealed that inhibition of Rac1 prevented LTP decay ($p < 0.01$; **Fig. 4.3B**). Given the prominent role of Rac1 in forgetting and the comparable decay kinetics observed under both Rac1 activation and GluN2B stimulation protocols, (**Fig. 4.2D**), these results suggest that GluN2B activation recruits Rac1 to promote LTP decay at CA1 glutamatergic synapses.

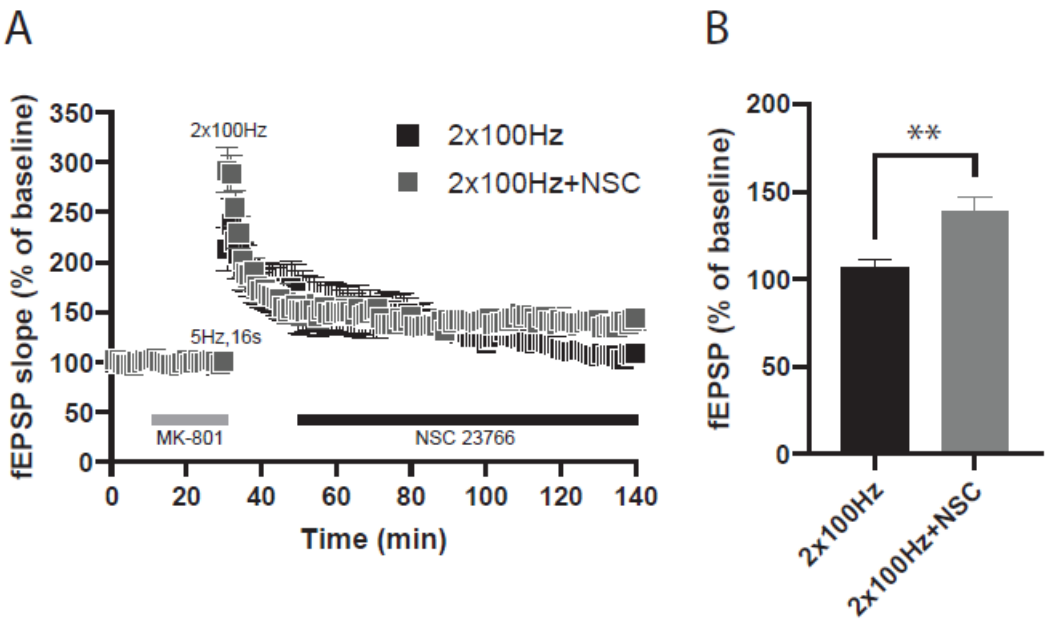


Figure 4.3. Rac1 inhibition prevents the decay of extrasynaptic NMDAR-LTP (A) Induction of extrasynaptic NMDAR-dependent LTP results in a decaying form of LTP (n = 8, black squares). Bath application of a selective Rac1 inhibitor, NSC 23766 (50 μM), 20 min after HFS reduced LTP decay (n = 8, grey squares). (B) Summary histogram for these experiments comparing levels of potentiation during the last 10 min of recordings (t-test with Welch correction; t(11.07) = 3.194, *$p < 0.05$). Blocking Rac1 activity significantly enhanced the magnitude of extrasynaptic NMDAR-LTP, consistent with an inhibition of LTP decay. n = # of slices. Data reported with error bars representing the S.E.M..

4.2.5 Blocking Rac1 converts decaying LTP to a translation-dependent, enduring form of LTP

Rac1 regulates multiple signaling cascades that regulate bidirectional forms of synaptic plasticity (Cui et al., 2021), including those coupling to extracellular signal regulated kinase, ERK. Notably, Rac1 crosstalks with and inhibits ERK signaling (Cui et al., 2021; Jiang et al., 2016; Liu et al., 2018b, 2016) which is canonically linked to pro-LTP pathways, including those coupling to synaptically localized translation regulation (Schafe et al., 2008; Winder et al., 1999; Zhai et al., 2013). Accordingly, we next sought to determine if inhibiting Rac1 during LTP decay converts decaying LTP into a translation-dependent, enduring form. To test this, we isolated extrasynaptic NMDARs and induced LTP while inhibiting Rac1. This was followed by application of the translation repressor, emetine. Relative to slices in which protein synthesis was intact, treatment with emetine reinstated LTP decay (**Fig. 4.4A**; mean fEPSPs were 142.48±5.8% and 105.92±11.0% ~100-110 min after HFS in emetine treated and control slices, respectively). These data suggest that inhibiting Rac1 following HFS de-represses pro-LTP pathways that recruit protein synthesis to stabilize synaptic potentiation.

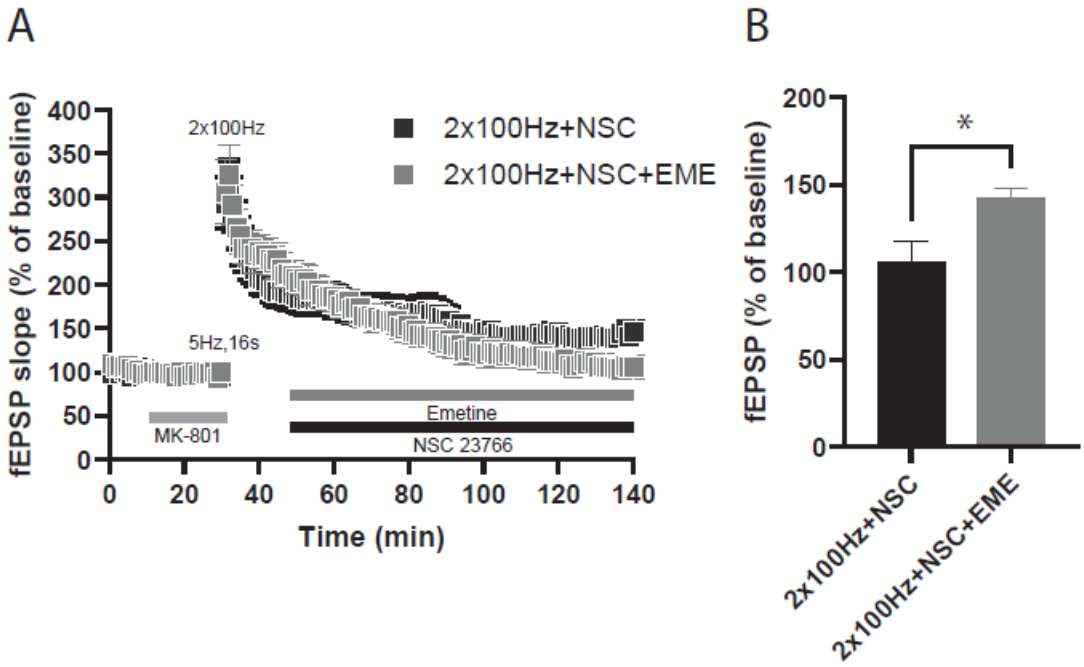


Figure 4.4. Blocking LTP decay with Rac1 inhibition unmasks a translation-dependent form of LTP (A) Induction of extrasynaptic NMDAR-dependent LTP paired with a Rac1 inhibitor, NSC (50 μM; n = 8, black squares), prevents LTP decay. Pairing Rac1 inhibition with the translation repressor emetine (20 μM) results in decaying LTP (n = 10, grey squares). (B) Summary histogram for these experiments comparing levels of potentiation during the last 10 min of recordings (t-test with Welch correction; $t(10.22) = 2.753$, $^{*}p < 0.05$). Inhibiting translation with emetine indicates that Rac1 inhibition converts LTP into an enduring form that requires protein synthesis. n = # of slices. Data reported with error bars representing the S.E.M..

4.2.6 Rac1 suppresses ERK-dependent translation regulation during LTP decay

To further confirm the recruitment of Rac1 and downstream engagement of translation regulation during LTP decay, we first quantified total and activated (phosphorylated) Rac1 in microdissected CA1 slices that had been either stimulated with HFS or undergone HFS paired with Rac1 inhibition. Western blot analysis revealed that although total Rac1 remained unchanged across conditions, induction of HFS-induced LTP upregulated phosphoRac1, indicating that stimulation protocols that induce decaying LTP are sufficient for recruiting Rac1 (**Fig. 4.5A, B**; $p < 0.05$).

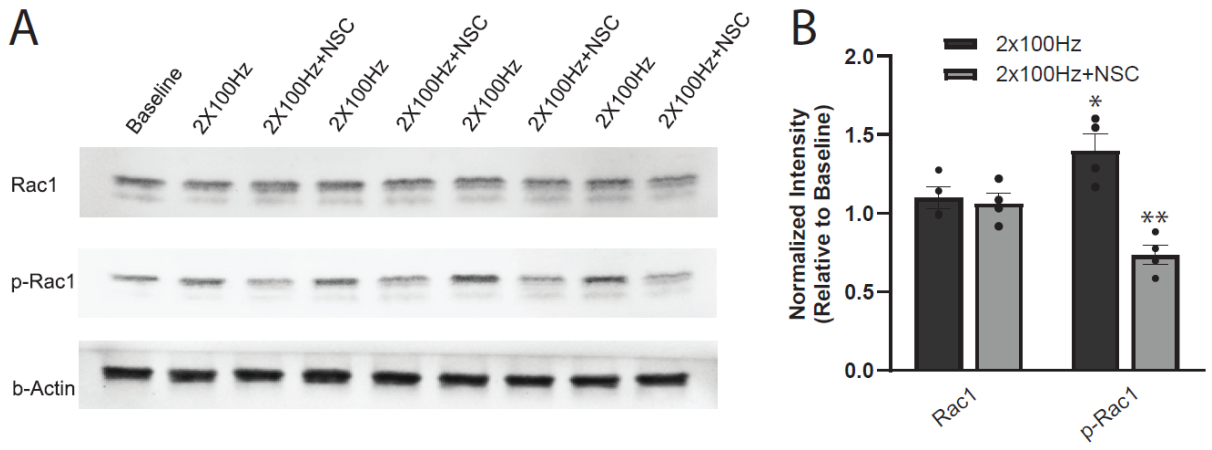
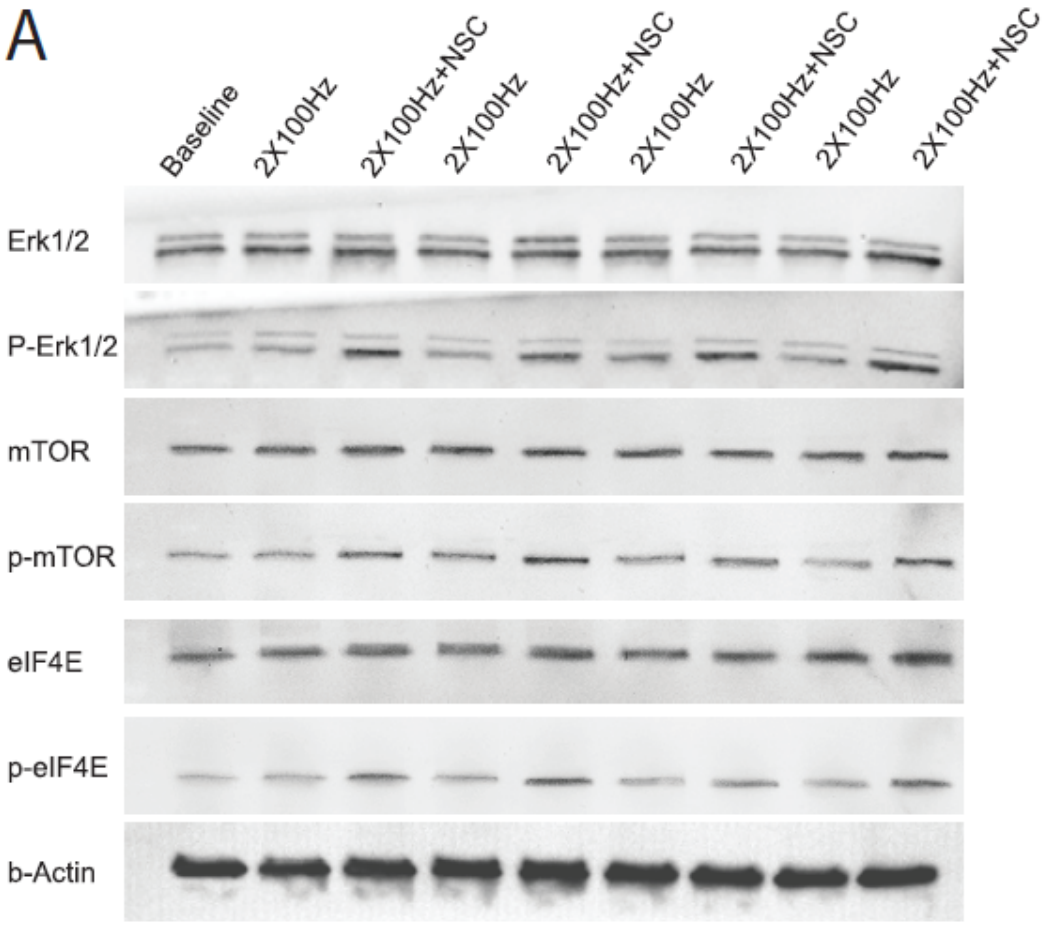


Figure 4.5. Extrasynaptic NMDAR activation through tetanic stimulation induces sustained Rac1 activation that reversed by Rac1 inhibition (A) Representative Western blot analysis of Rac1 and phosphorylated Rac1 (p-Rac1) protein levels in microdissected CA1 hippocampal slices. Slices were subjected to baseline condition (sliced and recovered for 1.5hrs) or high-frequency stimulation (HFS) at 2x100 Hz with or without NSC 23766 (NSC, a Rac1 inhibitor, 50 μM) applied 20 min after HFS. After ~100-110 min post HFS, CA1 was microdissected and processed for Western blot analysis. Lanes include: Baseline (control), 2x100 Hz, 2x100 Hz + NSC, and additional replicates. β-Actin was used as a loading control. Rac1 and p-Rac1 bands indicate total

Rac1 protein and its active phosphorylated form, respectively. (B) Summary of Western blot data, showing normalized relative intensity of Rac1 and p-Rac1 levels. A two-way ANOVA revealed significant differences between groups ($F_{(4, 26)} = 15.61$, $p < 0.001$), and subsequent Šidák's multiple posthoc tests found that HFS at 2x100 Hz significantly increased p-Rac1 levels relative to baseline levels (*$p < 0.05$), indicating that HFS was sufficient for inducing Rac1 activation. NSC 23766 treatment reduced this HFS-induced increase (**$p < 0.01$), confirming Rac1 activity reduction following inhibition by NSC. A Shapiro-Wilk test indicated normal within group data distributions. Total Rac1 levels remained unchanged across conditions. Data are presented as mean ± S.E.M.

Canonical signaling cascades converging on translation initiation at the synapse include those that couple to activation of ERK and mTOR (Kelleher et al, 2004). ERK and mTOR converge to regulate the translation initiation factor, eIF4E (Gelinas et al., 2007). Additionally, Rac1 has established roles in repressing ERK activity, suggesting that blocking Rac1 activation may have downstream effects on ERK signaling. Accordingly, we next probed for altered levels or activity of these translation regulators. To do this, we compared microdissected CA1 slices treated with HFS to those in which HFS was paired with a Rac1 inhibitor. Similar to Rac1, basal levels of ERK, mTOR, or eIF4E remained constant across conditions (**Fig. 4.6A**). However, inhibition of Rac1 during HFS resulted in a significant increase of both ERK and eIF4E phosphorylation, with a trend towards increased phospho-mTOR, indicating upregulation of this translation regulation pathway **Fig. 4.6B**; $p < 0.05$). These findings indicate that preventing LTP decay through Rac1 inhibition is sufficient for de-repressing protein synthesis normally required for enduring forms of LTP.


A
Baseline
2X100Hz
2X100Hz+NSC
2X100Hz
2X100Hz+NSC
2X100Hz
2X100Hz+NSC
2X100Hz
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Erk1/2
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mTOR
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eIF4E
p-eIF4E
b-Actin

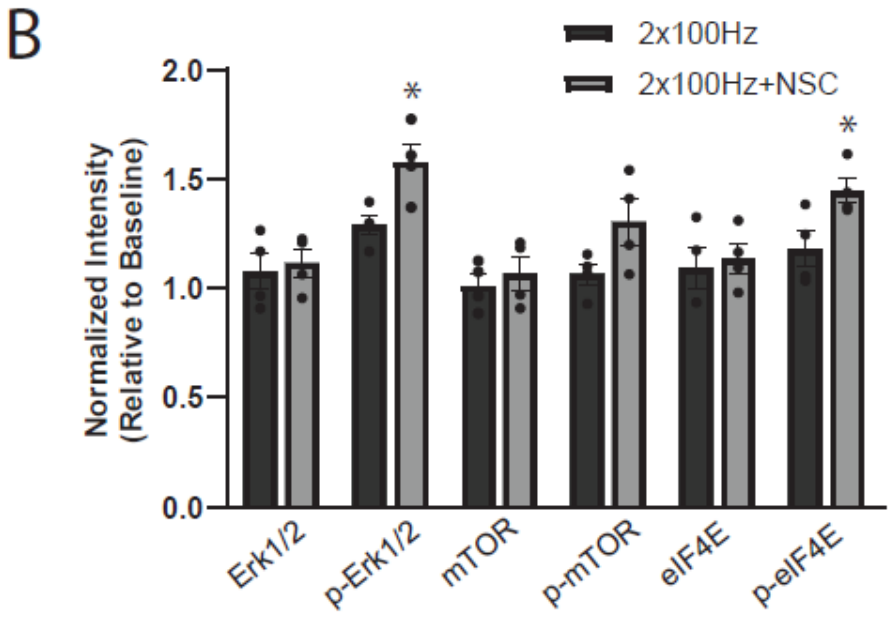
B
2x100Hz
2x100Hz+NSC
Normalized Intensity
(Relative to Baseline)
2.0
1.5
1.0
0.5
0.0
*
*
Erk1/2
p-Erk1/2
mTOR
p-mTOR
eIF4E
p-eIF4E

Figure 4.6. Blocking LTP decay with Rac1 inhibition unmasks a translation-dependent form of LTP (A) Representative Western blot analysis of translation regulation pathway protein expression and phosphorylation in microdissected hippocampal slices subjected to HFS or HFS paired with that Rac1 inhibitor, NSC. After ~100-110 minutes post HFS, CA1 was microdissected and processed for Western blot analysis. Lanes include: Baseline (control), 2x100 Hz, 2x100 Hz + NSC. β-Actin was used as a loading control. (B) Quantification of Western blot data, showing normalized relative intensity of total ERK and phosphorylated ERK (p-Erk1/2), total mTOR and phosphorylated mTOR (p-mTOR), and total eIF4E and phosphorylated eIF4E (p-eIF4E), as ERK, mTOR and eIF4E constitute a translation initiation pathway within hippocampal glutamatergic synapses. Although a two-way ANOVA ($F_{(11, 81)} = 6.151$, $p < 0.001$), and subsequent Šidák's multiple posthoc tests found that whereas HFS at 2x100 Hz modestly increased p-Erk1/2, NSC treatment paired with HFS significantly enhanced p-Erk1/2 ($^{*}p < 0.05$), indicating that Rac1 inhibition de-represses ERK signaling. Similarly, although p-eIF4E levels were modestly increased by HFS alone, pairing HFS with NSC significantly increased p-eIF4E levels relative to HFS ($^{*}p < 0.05$), indicating that Rac1 activation increases translation initiation within CA1 synapses. Total ERK, mTOR and eIF4E levels remained unchanged across treatments. A Shapiro-Wilk test indicated normal within group data distributions. Data are presented as mean ± S.E.M.

Finally, Given the prominent roles for AMPARs in bidirectional forms of synaptic plasticity, we next probed if GluA1 or GluA2 levels or phosphorylation were altered by Rac1 inhibition during HFS. Although no differences were detected in overall GluA1 or GluA2 protein levels, phosphoGluA2 was significantly upregulated in slices undergoing Rac1 inhibition during HFS (**Fig. 4.7B**). This finding indicates that preventing LTP decay, in addition to upregulating protein synthesis, increases the posttranslational modifications of GluA2 associated with increased trafficking and synaptic insertion of GluA2-containing AMPARs.

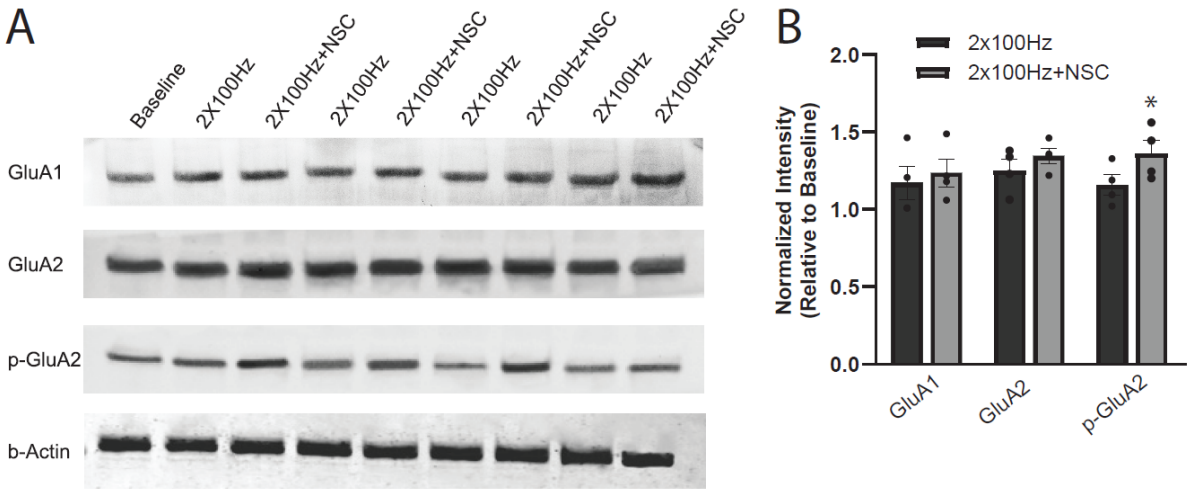

Figure 4.7. Inhibition of LTP decay through Rac1 inhibition increases synaptic GluA2 phosphorylation (A) Representative Western blot analysis of GluA1, GluA2 and phosphorylated GluA2 (p-GluA2) protein levels in microdissected CA1 hippocampal slices. Slices were subjected to baseline condition (sliced and recovered for 1.5hrs) or high-frequency stimulation (HFS) at 2x100 Hz with or without NSC 23766 applied 20 min after HFS. After ~100-110 min post HFS, CA1 was microdissected and processed for Western blot analysis. Lanes include: Baseline (control), 2x100 Hz, 2x100 Hz + NSC, and additional replicates. β-Actin was used as a loading

control. GluA1, GluA2 and p-GluA2 bands indicate total GluA proteins and active phosphorylated GluA2, respectively. (B) Summary of Western blot data, showing normalized relative intensity of GluA1, GluA2 and p-GluA2 levels. A two-way ANOVA failed to detect differences between groups ($F_{(4, 26)} = 15.61$, $p < 0.001$), however, subsequent Šidák's multiple posthoc tests found that HFS at 2x100 Hz paired with NSC increased p-GluA2 levels relative to HFS alone ($^{*}p < 0.05$), indicating that phosphorylation of GluA2 associated with synaptic retention of GluA2 is increased when LTP decay is blocked. Total GluA1 and GluA2 levels remained unchanged across conditions. A Shapiro-Wilk test indicated normal data distributions. Data are presented as mean ± S.E.M.

4.3 Discussion

In this study, we further advanced our understanding of how induction of a transient, decaying form of LTP simultaneously engages signaling pathways that both promote and constrain LTP. While previous studies have demonstrated that ongoing synaptic activity following LTP induction recruits NMDARs that promote the decay of LTP, major questions remained surrounding the location (synaptic/extrasynaptic), and the relative contribution of subunits to this phenomenon. Our study demonstrated that extrasynaptic GluN2B is required for the decaying component of LTP. This result is in line with previous studies showing that GluN2B inhibition extends the maintenance of LTP (Sachser et al., 2016), while demonstrating for the first time that these receptors localize to extrasynaptic regions.

Mechanistically, it appears that GluN2B activation engages Rac1 signaling to promote LTP decay. Previous studies established that Rac1 likely serves as a "forgetting molecule" as pharmacological or genetic manipulations that reduced Rac1 activity extend both memory duration

and LTP maintenance (Cui et al., 2021; Jiang et al., 2016; Liu et al., 2018b, 2016). How does HFS simultaneously induce pro-LTP and LTP decay pathways? Single train HFS, similar to the protocol used here, is sufficient to activate ERK in hippocampal slices, particularly in the CA1 region. Notably, ERK phosphorylation (p-ERK) is rapidly induced within minutes post-HFS, correlating with LTP induction (Schafe et al., 2008), as synaptic NMDAR opening facilitates calcium influx, which stimulates Ras-Raf-MEK-ERK. This ERK signaling cascade is well-established as an essential mediator of synaptic potentiation and long-term memory within the hippocampus (Kelleher et al., 2004; Schafe et al., 2008; Tsokas et al., 2007; Winder et al., 1999)

In parallel, extrasynaptic GluN2B-containing NMDARs preferentially engage signaling pathways that oppose potentiation (Hardingham et al., 2002). Accordingly, GluN2B activation upregulates Rac1 activity directly through phosphorylation of Rac1 GEFs. For example, Tiam1 a Rac1-specific GEF highly expressed in the hippocampus, is phosphorylated at specific serine/threonine residues following GluN2B stimulation, enhancing its GEF activity and promoting Rac1 activation (Tolias et al., 2005). Tiam1-induced activation of Rac1 alters dendritic spine morphology and AMPA receptor trafficking to suppress synaptic strength (Benoist et al., 2013; Blanco et al., 2025; Cui et al., 2021). GluN2B-mediated recruitment of another GEF, Kalirin-7, also upregulates Rac1. Interestingly, Kalirin-7 knockout mice exhibit impaired LTD, suggesting that Kalirin-7-mediated Rac1 activation regulates actin dynamics necessary for LTD-associated spine remodeling (Lemtiri-Chlieh et al., 2011).

Although GluN2B-NMDARs are often activated in pathological conditions (e.g., high glutamate levels), which can induce LTD-like mechanisms such as spine retraction, our data suggests that Rac1 signaling may link extrasynaptic GluN2B activation to potentiation suppression

through an alternative mechanism. Specifically, inhibition of Rac1 de-repressed the onset of a translation dependent form of LTP, indicating that absent Rac1-mediated LTP decay, single train stimulation may be able to recruit protein synthesis. As mentioned above, single HFS is sufficient to recruit ERK (Tsokas et al., 2007) which has well-established roles in synaptic protein synthesis (Kelleher et al., 2004; Winder et al., 1999). Moreover, a single HFS train also activates the mammalian target of rapamycin (mTOR) pathway in hippocampal slices, though evidence is slightly less extensive than for ERK. mTOR phosphorylation (p-mTOR) and downstream targets like p70S6K or 4E-BP1 are upregulated following single trains of HFS (Tsokas et al., 2007). ERK and mTOR converge on translation through p70S6K or 4E-BP1 which regulate cap-dependent local (synaptic) protein synthesis mediated by the translation initiation complex, eIF4E (Gelinas et al., 2007; Kelleher et al., 2004; Santini et al., 2014).

Our results confirm that in addition to changes in ERK activity, downstream recruitment of eIF4E is observed following the induction of LTP paired with inhibition of Rac1. This complements our data showing that preventing translation during Rac1 inhibition re-establishes LTP decay. Taken together, these data provide the basis for a model which explains how competing intracellular signaling pathways can both promote and constrain LTP induced with HFS, with one pathway coupling to ERK-mediated translation regulation to enhance LTP, whereas the second pathway activates Rac1 to promote the delayed onset of LTP decay.

What is the purpose of these parallel pathways? The ability to constrain or set thresholds for synapse potentiation endows neural networks with molecular thresholds that prevent encoding of innocuous information within synapses, thereby maintaining a readily available pool of synapses for encoding of more salient information. Simultaneously, the delayed onset of LTP decay

affords time-limited opportunities for surpassing consolidation thresholds which could be bolstered through subsequent neuromodulation or further experience driven neuronal activity (Moreno, 2021; Davis and Zhong, 2017).

It is important to note that use of HFS may not be as physiologically relevant as use of a theta burst stimulation (TBS) paradigm, which better approximates endogenous firing rates of pyramidal neurons under conditions of cognitive load, including spatial exploration (Larson and Munkácsy, 2015). It will be important to determine if TBS engages mechanisms similar to those identified for HFS, or whether this stimulation protocol acts through alternative means to engage LTP decay mechanisms.

Rac1-dependent forgetting was initially demonstrated in *Drosophila*, in which selectively increasing or decreasing Rac1 activity hastened the erasure or enhanced the duration of aversive olfactory memories, respectively (Shuai et al., 2010). A similar mechanism has been observed in the mammalian CNS, as various types (spatial, contextual, social) of hippocampus-dependent memories, were subject to Rac1-mediated decay (Jiang et al., 2016; Liu et al., 2016, 2018; Wu et al., 2019). These findings suggest the existence of a phylogenetically conserved mechanism for promoting forgetting predicated on Rac1 activity within specific time windows relative to memory acquisition (Lv et al., 2019).

Mechanistically, our findings indicate that the molecular pathway for triggering the cellular correlates of forgetting begins with extrasynaptic GluN2B-containing NMDARs. This is consistent with reports implicating GluN2B subunits in LTD, as GluN2B knockout or GluN2B loss-of-function knock-in transgenic models show impaired LTD (Brigman et al., 2010; Shin et al., 2020). Evidence implicating extrasynaptic NMDARs in synapse depression was shown through initial comparisons of synaptic versus extrasynaptic NMDARs, with activation of the latter

generating LTD (Lu et al., 2001). Moreover, LTP can be effectively blocked through prolonged theta-frequency stimulation which induces glutamate spillover and activation of extrasynaptic GluN2B receptors (Delgado et al., 2018). Given that the prominent role for GluN2B subunits in synapse weakening and the predominant extrasynaptic expression of GluN2B in adult neurons (Stocca and Vicini, 1998), these findings indicate a critical role for extrasynaptic GluN2B in tightly regulating and constraining synaptic strength enhancements.

What are the putative roles for LTP decay induced through extrasynaptic-NMDARs? As this pool of NMDARs are activated by glutamate spillover, extrasynaptic NMDARs are well positioned to serve modulatory roles in synaptic transmission through countervailing forms of synaptic plasticity that weaken synaptic strength. Extrasynaptic NMDARs containing GluN2B subunits respond to ambient glutamate levels to fine-tune synaptic responses, modify plasticity thresholds and provide dendritic signal amplification, and facilitate integration of synaptic inputs. Our data shows that activation of GluN2B containing extrasynaptic receptors biases signaling cascades towards synaptic weakening, enabling neurons to restrain cellular information encoding by preventing excessive synaptic potentiation.

From a cognitive perspective, the outcomes of this type of regulation are likely to be twofold, with extrasynaptic NMDA receptor activation supporting cognitive flexibility by sustaining a reserve pool of readily available synapses, while in parallel supporting neural network stability by limiting potentiation. This has implications for designing targeted therapies that modulate extrasynaptic NMDARs without disrupting synaptic function, particularly for cognitive disorders. For example, memantine, a low-affinity NMDAR antagonist, preferentially blocks extrasynaptic NMDARs at low doses, preserving cognitive functions while mitigating excitotoxicity in conditions like Alzheimer's disease (Karimi Tari et al., 2024).

Many human memory disorders and animal genetic studies have linked synaptic pathology causally with neuropsychiatric conditions characterized by excessive or exaggerated forgetting (Berry et al., 2024). Rac1 is genetically linked with several different types of neuropsychiatric disorders including Alzheimer's, Parkinson's and dementia (Berry et al., 2024). These disorders could be the result of elevated Rac1 expression or activity, which would amplify cellular forgetting. Indeed, excessive synapse loss and activation of Rac1 are hallmark features in Alzheimer's models (Wu et al., 2019). In accord, administration of Rac1 inhibitors was shown to have a prophylactic effect against neurodegenerative insults and in some mouse models for Alzheimer's disease has restored synaptic function (Wu et al., 2024). This suggests that targeting molecular events associated with LTP decay may have neuroprotective effects.

Our identification of extrasynaptic GluN2B-containing NMDA receptors, upregulated Rac1 activity, and changes in phosphorylation of canonical protein synthesis pathway regulators, further support our conclusion that recruitment of Rac1 shunts synapse potentiation through crosstalk and repression of pro-LTP signals. Thus, the neurodegenerative phenotypes observed in Alzheimer's disease models that demonstrate compromised plasticity may be likely due to elevated susceptibility to LTP decay, which contribute to the synaptic pathologies described above.

Future studies are needed to follow-up on some aspects of the current study. First, although our fEPSP and Western blot data suggest that extrasynaptic GluN2B recruits Rac1, it remains possible that this might also result from the activation of GluN2C or 2D. To address this possibility, it is important to conduct further electrophysiological analysis of LTP decay either in the presence of selective GluN2C/2D antagonists or in GluN2 knockout models (Upmanyu *et al*, 2022). Additionally, whether these results will be ubiquitously observed throughout the hippocampus axis or in other subregions (DG, CA2, CA3) remains to be determined, as this study

used slices randomly sampled throughout the hippocampal dorsal/ventral axis, which has differential roles in mediating behaviorally disparate types of memories. Finally, changes in the complement of the remaining synapse proteome associated with LTP decay remain unknown, leaving a substantive gap in our understanding of alterations in other synaptic players likely to causatively alter synaptic and behavioral phenotypes associated with forgetting.

In conclusion, we uncovered a unique, endogenous mechanism through which synapse weakening during LTP decay functions. Activation of GluN2B following HFS is sufficient for inducing parallel potentiation and decay pathways, endowing synapses with an additional means for calibrating synaptic strength. Rac1 operating downstream of these receptors mediates this capacity for bidirectional modifications which depends on the timing of changes in Rac1 status relative to the stimulating or memory-inducing event. Meanwhile, LTP promoting pathways initiated by HFS, simultaneously or after a delay, engage potentiation decay pathways that limit potentiation or reverse it altogether. Given links between bidirectional forms of plasticity with disorders of memory (PTSD, AD), and other neurological conditions associated with pathologized forgetting (Berry et al., 2024), we propose impaired (PTSD) or accentuated (AD) LTP decay as an underlying mechanism, and suggest that associated molecular processes offer valuable models for understanding the nature and pathologies of forgetting.

4.4 Materials and methods

4.4.1 Animal and ethics statement

All animal experiments were carried out in accordance with Canadian Council on Animal Care guidelines and were approved by the York University Animal Care Committee Mice were group-

housed (two to five per cage) and maintained on a 12-h light/dark cycle. For all experiments and analyses, the experimenters were blind to genotype. Male adult mice (8-12wks) from the C57BL/6J genetic background (Charles River) were used for all experiments.

4.4.2 Synaptoneurosomes preparation

Following the application of LTP decay stimulation protocols, synaptoneurosomes were isolated from mouse hippocampus area CA1 microdissected tissue (C57BL/6, 8–12 weeks old), using the differential filtration method described below. Briefly, brain regions of interest (CA1 hippocampus) were dissected on ice in ice-cold oxygenated artificial cerebrospinal fluid supplemented with protease inhibitor cocktail (Roche, 1 tablet per 10 mL buffer). Microdissected slices were homogenized in 1 mL glass homogenizing tube containing 200 µL of chilled modified Krebs-Henseleit buffer (mKRBS) [containing (in mM): 118.50 NaCl, 4.70 KCl, 1.18 MgCl2·6H2O, 2.50 CaCl2·2H2O, 1.18 KH2PO4, 24.90 NaHCO3, 10.00 glucose, pH adjusted to ~7.40 using 1.0 N HCl]. A protease inhibitor cocktail (104 mM AEBSF, 80 µM aprotinin, 4 mM bestatin, 1.4 mM E-64, 2 mM leupeptin and 1.5 mM pepstatin) was included in the buffer to limit proteolysis activity. Slices were then homogenized with 20 manual pestle strokes over ice, and the homogenate placed in a 1.5 mL microtube for 10 min on ice to allow for gravity sedimentation. Synaptoneurosomes were prepared by drawing the supernatant into a 1 cc Luer lock syringe and then passing it through a 13 mm filter holder (Millipore Sigma, Ontario, Canada) containing 3 layers of pre-wetted (mKRBS) 100 µm pore nylon filters (Millipore Sigma). Filtrate was then collected in a small Petri dish on ice and air was passed through the filter holder to ensure all filtrate was collected. The filtrate was than drawn into a new 1 cc Luer lock syringe and passed through a 13 mm filter holder containing a single pre-wetted (mKRBS) 5 µm nitrocellulose

Durapore membrane filter (Millipore Sigma) into a 1.5 mL microtube; after this, 1 mL of air was passed through the filter holder to ensure all the filtrate was collected. Microtubes were spun at 1000 × g for 15 min at 4°C, supernatants were discarded, and the pellet was re-suspended in 100 µL non- ionizing lysis buffer (10 mM Tris, 25 mM EDTA, 100 mM NaCl, 1 % [v/v] Triton X-100, and 1 % [v/v] NP-40, pH 7.4) containing protease and phosphatase inhibitors and stored at -80°C.

4.4.3 Protein Quantification

Protein concentrations were determined using the Pierce Rapid Gold BCA Protein Assay Kit (Thermo Fisher Scientific) according to the manufacturer's recommended protocol. A bovine serum albumin (BSA) standard curve was prepared by diluting the provided 2 mg/mL BSA standard in ultrapure water to generate a series of standards ranging from 0 to 2,000 µg/mL (0, 20, 40, 125, 250, 500, 750, 1,000, 1,500, and 2,000 µg/mL). For each sample and standard, 10 µL was added in duplicate to a 96-well microplate (Thermo Fisher Scientific). The working reagent was prepared by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B (50:1 ratio). A total of 200 µL of the working reagent was added to each well containing samples or standards. The microplate was covered, gently mixed on a plate shaker for 30 seconds, and incubated at room temperature (25°C) for 5 minutes. Absorbance was measured at 480 nm using a microplate reader within 10 minutes of incubation to ensure stability of the colorimetric reaction. A standard curve was generated by plotting the absorbance values of the BSA standards (corrected for the blank) against their known concentrations using GraphPad Prism (version 9.0). A second-order polynomial (quadratic) fit was applied to the standard curve to account for the non-linear response of the Pierce Rapid Gold BCA assay. Protein concentrations of unknown samples were calculated by interpolating their absorbance values against the standard curve. Each sample was measured in

duplicate, and the average concentration was used.

4.4.4 Immunoblotting

All samples were thawed on ice and the synaptoneurosome samples were then denatured in Laemmli sample buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue) at 95°C for 5 min. Protein samples were then loaded into either 10, 12, or 4-20 % SDS-polyacrylamide gels. After this, proteins were electrophoretically separated at 100 V for 1.5 h in 1× Tris-Glycine-SDS running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3) using a Bio-Rad Mini-PROTEAN Tetra system. Proteins were transferred from the gel onto PVDF membranes via dry transfer using the Trans-Blot Turbo system (Bio-Rad). Membranes were blocked using either 5 % (w/v) bovine serum albumin (BSA, BioShop, Ontario, Canada), or 5 % (w/v) non-fat milk in Tris-buffered saline with Tween-20 (TBST) for 1 h at room temperature and incubated with relevant antibodies overnight at 4°C with gentle agitation. All primary antibodies were diluted in blocking buffer specific to the application. The following antibodies were used: anti- GluA1 (Cell Signaling Technology, #13185S), anti-GluA2 (Cell Signaling Technology,#13607), anti-phospho (Y869, Y873, Y876)-GluA2 (Cell Signaling Technology, #3921), anti- Rac1/Cdc42 (New England BioLabs, #4651S), anti-phospho-Rac1/Cdc42 (New England BioLabs, # 2461S), anti- p44/42 MAPK (Erk1/2) (New England BioLabs, # 9102S), anti- phosphor-p44/42 MAPK (Erk1/2) (New England BioLabs, # 9106S), anti-eIF4E Antibody (Cell Signaling Technology, #9742), anti-phospho-eIF4E (Ser209) (Cell Signaling Technology, #9741), anti-mTOR Antibody (Cell Signaling Technology, #2972), anti-phospho-mTOR (Ser2448) (Cell Signaling Technology, #2971), β-Actin (Cell Signaling Technology, #3700). On the following day, membranes were incubated in mouse or rabbit (Cell

Signaling Technology, #7074, #7076) antibody conjugated to horseradish peroxidase and immunoblotted using Luminata Classico (Millipore Sigma), or Crescendo (Millipore Sigma) ECL substrate for 2 min. Membranes were imaged using a ChemiDoc Imaging System (Bio-Rad). Exposure times were optimized (typically 10–60 seconds) to avoid signal saturation while ensuring detection of faint bands.

All synaptoneurosome preparations and Western blot experiments were performed in duplicates to ensure reproducibility. Antibody dilutions and incubation conditions were optimized to minimize non-specific binding. When reprobing was necessary, membranes were stripped using a mild stripping buffer (200 mM glycine, 0.1% SDS, 1% Tween-20, pH 2.2) for 10 minutes, followed by extensive washing in TBST.

Band intensities were quantified using ImageJ software (NIH). The intensity of each target protein band was normalized to the intensity of a loading control (β-actin) to account for variations in protein loading across synaptoneurosome samples. Relative protein expression or phosphorylation levels were calculated by comparing normalized band intensities to a control condition (baseline).

4.4.5 Electrophysiology

Mice underwent cervical dislocation after which whole brains were rapidly removed and placed in ice-cold cutting solution composed of (in mM): 124 NaCl, 10 D-glucose, 26 $NaHCO_3$, 2.5 KCl, 1.25 NaH_2PO_4, 2 $CaCl_2$ and 1 $MgSO_4$ at pH of 7.3–7.4 and by 95% oxygen and 5% carbon dioxide and the osmolarity adjusted to 290–300 mOsm/L (unless stated, all chemicals and drugs were purchased from Sigma-Aldrich, Canada). Acute 400 μm hippocampus slices were obtained using a Mcilwain chopper and then transferred to an incubation chamber (BSK12,

Scientific Systems Design, Mississauga, ON, Canada) containing a standard artificial cerebral spinal fluid (ACSF) comprised of (in mM): 124 NaCl, 10 D-glucose, 26 $NaHCO_3$, 2.5 KCl, 1.25 NaH_2PO_4, 2 $CaCl_2$ and 1 $MgSO_4$ at pH of 7.3–7.4 and osmolarity of 290–300 mOsm/L. ACSF was bubbled continuously with carbogen (95%O_2/5%CO_2). Slices recovered in the chamber at 30 ºC for 1hr prior to experiments. Extracellular field excitatory postsynaptic potentials (fEPSPs) were recorded using an interface-chamber (Scientific Systems Design, Georgetown, Canada) and a MultiClamp 700B amplifier. fEPSPs were generated by stimulating the Schaffer collateral pathway using two bipolar nickel-chromium electrodes placed in the stratum radiatum area of the CA1 and recorded with a glass microelectrode filled with ACSF (resistance 2-3 MΩ). Input/output curves were generated to establish baseline stimulation values (10-100 μA). Extracellular recordings were acquired using Clampex 11.2. Analysis was done through a combination of Clampfit 11.2 and WinLTP 3.00.

Drugs (source in brackets): NVP-AAM077 (NVP; Hellobio) was dissolved in dimethyl sulfoxide (DMSO) to create a stock solution, which was then diluted in aCSF to achieve a final DMSO concentration of less than 0.1% to minimize solvent effects, and applied at a concentration of 0.1 μM. Ro 25-6981 (Ro; Sigma), used at a concentration of 1 μM and dissolved in DMSO. NSC 23766 (NSC; Sigma) was dissolved in DDH_2O and applied at a concentration of 50 μM. Emetine (Sigma) was dissolved in DDH_2O used at a concentration of 20 μM.

4.4.6 Plasticity protocols

Baseline fEPSP responses were set at 40% of maximum evoked responses. Stable baselines were run at this baseline for 20 min and slices were rejective if responses varied by > 10% during this period. LTP was induced with either single train (HFS: 1x100Hz, 1 sec at test

pulse intensity) or 2 train of HFS at 20 sec intertrain intervals. For isolation of extrasynaptic receptors, MK-801 (100μM) was applied 20 min prior to applying brief theta frequency stimulation comprised of 5Hz for 16 sec (Yang et al., 2017). Potentiation of fEPSP responses was determined by plotting post HFS responses as a function of the baseline stimulation mean.

4.4.7 Statistical testing

Analysis was performed by using GraphPad Prism 10 (GraphPad Software).Statistical comparisons were made with two-tailed Student's unpaired t-tests, one sample t-tests, one-way ANOVA with post hoc Tukey's multiple comparison tests, two-way repeated measures ANOVAs with Tukey's post hoc tests, as indicated in the figure legends. All data are reported as the mean ± standard error of the mean (SEM). No statistical significance (ns) was defined as $p > 0.05$, while statistical significance was defined as $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$. For Western blot analysis, data were expressed as mean ± standard error (SEM) from four independent biological replicates and two technical replicates. Statistical analysis was performed using GraphPad Prism (version 9.0). Significant differences were assessed using one-way ANOVA with post-hoc Šidák's test for multiple comparisons, with a p-value < 0.05 considered statistically significant.

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CHAPTER 5: DISCUSSION AND FUTURE DIRECTIONS

5.1 Introduction

The vast majority of research investigating the biology of memory has focused primarily on cellular and molecular events required for encoding (learning) and storage (memory). However, a growing body of research supports the notion that forgetting is an essential feature of memory regulation and cognitive flexibility, which warrants further exploration. My thesis explores these mechanisms through two projects focused on different forms of synaptic weakening as models for molecular processes that may erase memories. The first project explored how aging enhances susceptibility to depotentiation, revealing an ageing-related protracted time window in which synapses are susceptible to activity-dependent potentiation reversal.

The second project probed novel mechanisms through which NMDA receptors engage Rac1-mediated decay of LTP through suppression of signaling cascades that stabilize potentiation in synapses. Broadly, these separate yet complementary projects align with recent advances in synaptic plasticity research, which emphasize the interplay between synaptic weakening and memory erasure (Davis and Zhong, 2017; de Snoo and Frankland, 2025; Hardt et al., 2013; Richards and Frankland, 2017; Sachser et al., 2017). Below, I discuss my findings in the light of recent literature, critique my approaches, and discuss future directions for this research to better contextualize my discoveries within the field of memory research.

5.2 Depotentiation

Depotentiation, the activity-dependent reversal of LTP, has emerged as a potential neuronal model for forgetting, as it weakens previously strengthened synaptic connections (Bashir and Collingridge, 1994; Fujii et al., 2020, 1991; Sanderson, 2012). My research was designed to

determine if exaggerated depotentiation could serve as a model for age-related memory impairments, and if so, provide mechanistic insights into why aged synapses are susceptible to this type of synaptic strength reversal.

Depotentiation is a form of synaptic plasticity characterized by the reversal of LTP, typically induced by low-frequency stimulation (LFS, typically 1–5 Hz) following stimulation protocols that initiate LTP (Fujii et al., 1991; Sanderson, 2012). In contrast to LTD which depresses naïve (unstimulated) synapses (Collingridge et al., 2010; Connor and Wang, 2016), depotentiation reduces synaptic strength gains to baseline levels (Fujii et al., 1991; Sanderson, 2012), effectively erasing the potentiation often associated with memory encoding. As memories are thought to be encoded by strengthened synapses, depotentiation's ability to specifically weaken these connections positions it as a cellular mechanism for forgetting. Indeed, studies suggest that depotentiation during REM sleep, particularly at the troughs of theta oscillations, may facilitate the erasure of irrelevant information, supporting the hypothesis that sleep serves a restorative, housekeeping function for memory circuits (Poe, 2017; Poe et al., 2000).

In the hippocampus, depotentiation is mediated by NMDA receptor activation and protein phosphatase activity (Latif-Hernandez et al., 2016; Qi et al., 2013). Accordingly, depotentiation involves dephosphorylation of synaptic proteins, such as AMPA receptors, which promotes endocytosis and weakens synaptic transmission (Zhu et al., 2005). This process is similar in nature to active forgetting, where synapses are actively weakened to promote erasure of irrelevant memories to maintain cognitive flexibility (Anderson and Hanslmayr, 2014; Awasthi et al., 2019; Davis and Zhong, 2017).

My data identified altered translation as a key factor that renders aged synapses more susceptible to depotentiation. Similar to enduring memories (Schimanski and Barnes, 2010; Yang et al., 2019), L-LTP requires *de novo* protein synthesis which is impaired in aged neural circuits (Ryan et al., 2015; Schimanski and Barnes, 2010) . Increased susceptibility to in aged slices is consistent with a general downregulation or alternatively a protracted onset of translation-dependent synaptic consolidation. This supports a model (**Figure 4**) in which the capacity for *de novo* translation is impaired or alternatively, takes longer to reach a protein production level at which depotentiation would be ineffective, thus increasing the timeframe in which potentiation at these synapses can be reversed.

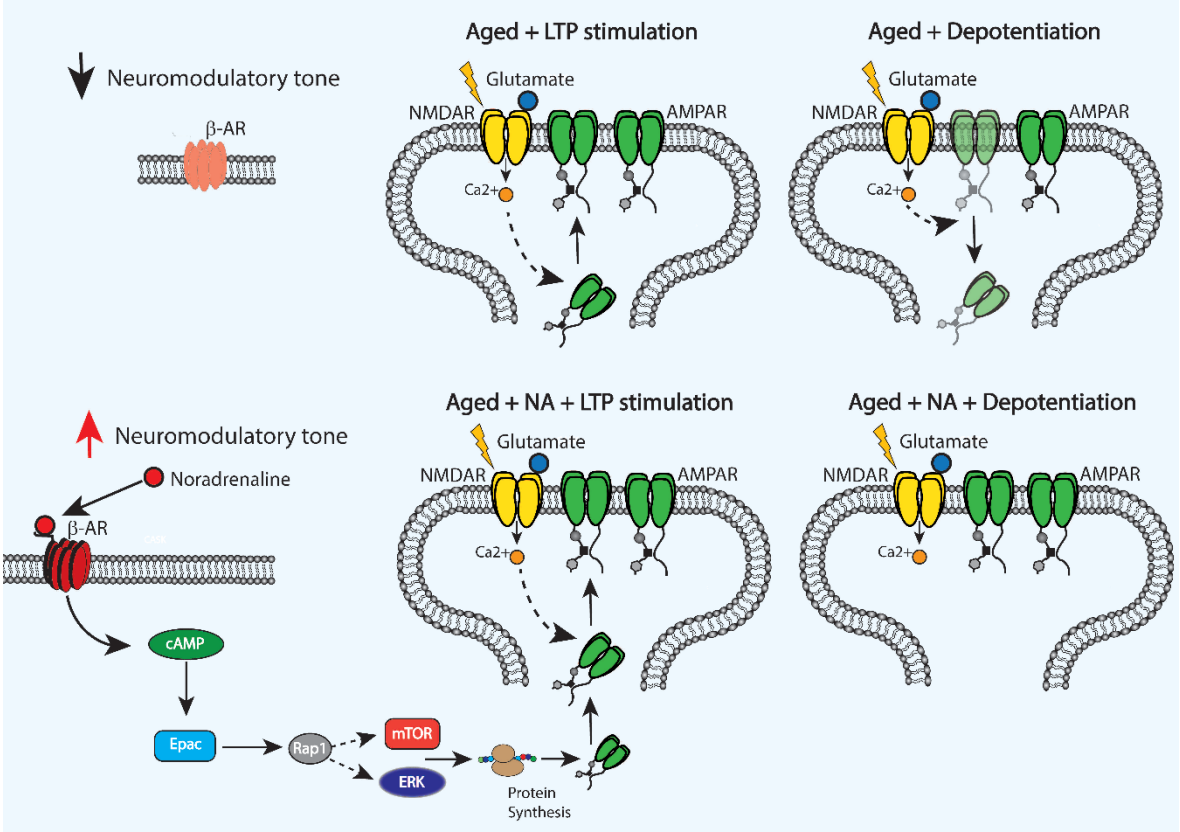

Figure 5.1. Synaptic mechanisms of increased susceptibility to depotentiation in aged mouse hippocampus and the rescue of depotentiation immunity through noradrenaline.

This figure illustrates the synaptic changes associated with long-term potentiation and depotentiation in the aged mouse hippocampus, with and without neuromodulation by NA. **Top right**: Aged synapses more readily undergo depotentiation which may be a consequence of reduced synaptic translation which would normally stabilize LTP through mechanisms that retain LTP-induced AMPAR sequestration within synapses. **Bottom left**: Enhancing neuromodulatory tone through activation of β-adrenergic receptors upregulates translation-dependent mechanisms that facilitate LTP consolidation. Specifically, noradrenaline enhances LTP through β-AR activation, increasing cAMP, which activates Epac, mTOR, ERK, and protein synthesis, amplifying AMPAR production, trafficking or insertion. **Bottom right**: Resistance to depotentiation is re-established through noradrenaline-mediated potentiation stabilization. Arrows indicate signaling pathways; dashed lines represent modulation or downstream effects. Generated with Adobe Illustrator.

Collectively, these findings indicate that LTP impairments may not stem from compromised induction but rather reflect changes in downstream regulatory processes that govern the ability of potentiated synapses to fully "consolidate" that is, engage key molecular mechanisms that stabilize synaptic strength gains and render synapses immune to depotentiation. My initial findings support this model, as the normally temporally limited window for depotentiation is increased at aged SC-CA1 synapses.

Questions remain surrounding how this deficit would manifest at a molecular level. Does translational machinery take longer to access or engage in response to synaptic activity? Are additional factors that modulate LTP consolidation mechanisms compromised with ageing? My experiments with noradrenaline support the last postulate, as they focused on mechanisms that could be modified by changes in neuromodulation, as discussed below.

5.3 Neuromodulation with ageing

Neuromodulation, mediated by neurotransmitters like dopamine, acetylcholine, and norepinephrine, enhances LTP and cognitive function (Fuchsberger et al., 2025; Galvez et al., 2016; Nguyen and Connor, 2019). In aged brains, neuromodulatory systems are compromised (Bakhtiarzadeh et al., 2023; Krohn et al., 2023), exacerbating LTP deficits (Dahl et al., 2023).

A critical neuromodulatory system demonstrating altered activity patterns (Krohn et al., 2023; Ludwig et al., 2024) and downregulation (Gargano et al., 2020; Liu et al., 2020) during ageing is the noradrenergic system. Notably, locus coeruleus activity and noradrenaline levels are reduced with advancing age which impairs noradrenergic receptor-mediated enhancement of LTP (Luo et al., 2015). These findings suggest that loss of noradrenergic tone is a contributing factor to age related decrements in neuronal and cognitive function.

5.4 Translation regulation with ageing

Advanced aged is associated with a general decline in translation regulation which has been observed in the hippocampus (Ryan et al., 2015; Schimanski and Barnes, 2010; Yang et al., 2019), which is in line with my results showing a protracted susceptibility to depotentiation. This decline in translational capacity may affect the production of plasticity-related proteins including glutamatergic receptors (AMPA and NMDA receptors) (Brim et al., 2013; Luo et al., 2015), which are essential for memory consolidation and synaptic remodeling. Studies in aged rodents demonstrate that protein synthesis is required for a longer period following training-induced learning to establish long-term memory, indicating reduced efficiency in translation regulation (Schimanski and Barnes, 2010). Aged rats show impaired LTP maintenance, correlating with decreased synaptic protein levels and altered dendritic morphology (Schimanski and Barnes, 2010).

The molecular basis of impaired translation regulation in aging includes altered activity of signaling pathways, such as the mTOR pathway, which regulates protein synthesis, and altered expression of translation initiation factors such as eIF4E (Yang et al., 2019), which have also been implicated in the neuropathology of Alzheimer's disease (Ghosh et al., 2020). These deficits contribute to a loss of synaptic stability, reducing the brain's capacity to encode and maintain memories. Taken together, these phenomena indicate that memory deficits associated with advancing age likely represent the synergistic impact of altered translation regulation coupled with a general downregulation of neuromodulatory inputs throughout the brain.

5.5 Rescuing LTP with noradrenaline

Beta-adrenergic receptors, activated by noradrenaline, play a significant role in modulating synaptic plasticity. These G-protein-coupled receptors enhance LTP by activating the cAMP-dependent (PKA, Epac) signaling pathways, which promote translation necessary for late-phase LTP (L-LTP) (Connor et al., 2011; Maity et al., 2020; O'Dell et al., 2015). In young animals, pairing the application of an E-LTP stimulation protocols with β-adrenergic receptor agonists, such as isoproterenol, facilitates L-LTP induction in hippocampal slices through recruitment of ERK, mTOR and local (synaptic) protein synthesis (Connor et al., 2011; Gelinas et al., 2007; Gelinas and Nguyen, 2005). During aging, the noradrenergic system undergoes significant changes, including a 20% loss of locus coeruleus (LC) neurons, the primary source of noradrenaline, and reduced β-adrenergic receptor expression (Liu et al., 2020). These alterations may contribute to impaired LTP and memory deficits through reducing the production or phosphorylation of plasticity-related proteins that would normally be maintained through noradrenergic tone.

The depotentiation deficits I observed here could, therefore, be the direct result of a downregulation of noradrenergic modulation throughout the brain or reflect a more generalized downregulation of global translational capacity. Although both of these possibilities remain, it is important to note that my "aged" model was 12-14 months which represents approximately the mouse equivalent of middle age, an age where neuromodulation is likely intact. It will be important to dissociate the relative contribution of downregulated neuromodulatory tone, adrenergic receptor levels and/or signaling pathways downstream of these receptors are altered in middle age and if so, to identify the precipitating cellular events.

5.6 Future directions: Depotentiation

Importantly, studying aged mice required maintaining our mice in grouped house conditions for extended periods of time. While not aversive *per se*, this type of housing fails to recapitulate the mental stimulation associated with diverse and physically challenging conditions wild mice would be subjected to under naturalistic conditions. Interestingly, studies in which mice are exposed to enriched physical environments, which better approximate naturalistic conditions have shown restorative effects on aged cognition. For example, when eighteen-month-old mice were housed in either standard or enriched environments (larger cages with running wheels and novel toys) under group or isolated conditions for two months, behavioral tests revealed that isolated mice exhibited significant declines in spatial and social memory compared to group-housed controls. These deficits were mitigated in isolated mice housed in enriched environments which correlated with preserved synaptic protein levels, myelination and elevated brain-derived neurotrophic factor levels, while also reducing glial cell activation and NOD-like receptor protein 3 inflammasome activity in the hippocampus (Wang et al., 2018).

Another study found that environmental enrichment rescued the epigenome within hippocampal dentate gyrus of aged mice. Genome-wide DNA methylation sequencing revealed that exposure to an enriched environment counteracted age-related CpG hypomethylation at target sites of the methyl-CpG-binding protein Mecp2 (Zocher et al., 2021), a key regulator of neuronal function, plasticity and neurogenesis. Collectively, these findings demonstrate that a stimulus-rich environment can mitigate aging-induced plasticity, epigenetic and memory deficits and indicate that the prolonged unnatural housing I necessarily had to use for my study may induce covert mental deficiencies that while going behaviorally undetected, nevertheless impact synaptic function.

Finally, questions remain regarding whether alternative LTP induction protocols may result in potentiation that is similarly less resistant to depotentiation in aged synapses. For my experiments, a "condensed" LTP protocols consisting of 4 trains with intertrain intervals of 20 seconds was used to induce robust LTP. Spaced protocols (5 or 10 min intertrain intervals) may yield LTP that is more resistant to LTP decay as the prolonged intertrain intervals allow more time for synaptic consolidation processes to be engaged. It will be important to test different protocols (spaced 4 train, TBS) going forward to further establish the nature of depotentiation susceptibility in aged synapses. An additional caveat is that many studies have used hippocampal slices, which may not fully capture the physiological conditions of aging brains. Incorporating *in vivo* models would partially remedy this shortcoming, as recordings in neural circuits within the intact brain would better approximate normal physiological responses.

5.7 Conclusion: Depotentiation

Depotentiation of recently potentiated synapses offers a compelling neuronal model for forgetting by weakening synaptic connections that directly encode memories. My findings indicate that ageing alters the consolidation time frame for LTP, predicated on a protracted time frame for translation related mechanisms, providing a cellular framework for understanding memory deficits in old age. In light of established memory deficits with ageing (Burke and Barnes, 2006), these findings indicate that newly formed memories, under conditions of reduced neuromodulation, remain labile and subject to erasure through further, ongoing neuronal activity. Conversely, these findings offer hope through 2 avenues: 1) β-adrenergic receptor activation holds promise for restoring synaptic plasticity and 2) Although synaptic consolidation takes longer, the observed

deficits may represent a delayed mechanism and not a complete loss of the capacity for maintaining synaptic strength through protein synthesis.

5.8 LTP decay

While the induction and maintenance of LTP have been extensively studied, the mechanisms governing active weakening of synapses (LTP decay) remain less understood yet are critical for understanding the dynamics of memory processes, including forgetting. Importantly, LTP decay differs from depotentiation in that depotentiation is induced using low frequency stimulation (Bashir and Collingridge, 1994), whereas LTP decay is initiated by the same tetanic stimulation that induces LTP (Dong et al., 2015; Villarreal et al., 2002).

NMDA receptors play a pivotal role LTP induction (France et al., 2022; Volianskis et al., 2013) and depotentiation (Latif-Hernandez et al., 2016; Zhu et al., 2005), with emerging evidence suggesting their involvement in LTP decay. As ionotropic glutamate receptors essential for synaptic plasticity, NMDA receptors serve as key regulators of activity-dependent changes in neural connectivity. It is well established that individual NMDA receptor subunits confer distinct functional properties (France et al., 2022; Ingram et al., 2024; Volianskis et al., 2013) which was the rationale for comparing the effects of GluN2A and GluN2B antagonists on LTP decay in my study.

Typically composed of two GluN1 subunits and two GluN2 (A-D) or GluN3 (A-B) subunits, there are multiple configurations in which these receptors can present (Dore et al., 2016). This is an important limitation of the present work as even though the relative specificity of the antagonists used has been validated, the effects of these pharmacological tools on receptors

composed of diverse subunits (e.g. diheteromeric, triheteromeric receptors) cannot be determined using this approach.

Additionally, emerging evidence suggests that beyond typical ionotropic NMDA receptor signaling, NMDA receptors are capable of metabotropic effects that can also alter synaptic transmission and plasticity (Dore et al., 2016; Nabavi et al., 2013). Thus, although NMDA receptors may contribute to LTP decay through calcium influx and subsequent activation of canonical intracellular cascades, they may also engage metabotropic signals that act primarily or in tandem with ionotropic mechanisms to destabilize synaptic enhancements.

Nevertheless, the subunit composition and subcellular location of NMDA receptors clearly influence their kinetics, calcium permeability, and downstream signaling, all of which have the potential to impact LTP decay. Within the hippocampus, the major GluN2 subunits in the hippocampus—GluN2A and GluN2B—have been most studied in this context.

GluN2A-containing NMDA receptors are associated with faster channel kinetics and are predominantly expressed at mature synapses (Collingridge and Monaghan, 2022; Hansen et al., 2017; Massey et al., 2004). Studies suggest that GluN2A receptors contribute to LTP induction but have a limited role in LTP decay. However, although my data indicate that NVP, a GluN2A selective antagonist, failed to alter LTP decay kinetics, this needs to be qualified by the understanding that current NMDA receptor antagonists lack specificity and effects on heteromeric NMDA receptors are limited. Accordingly, validation of this finding will require genetic approaches (e.g. GluN2A or 2B knockouts) to more effectively test the role of the individual subunits in LTP decay.

Alternatively, GluN2B-containing NMDA receptors exhibit slower deactivation kinetics, higher calcium permeability and are highly expressed extrasynaptically in adult hippocampus

(Hansen et al., 2017), making them prime candidates for modulating LTP decay. These receptors are enriched at extrasynaptic sites and in developing synapses but persist in adulthood. Others have shown that pharmacological inhibition of GluN2B with ifenprodil or Ro 25-6981 prevents LTP decay in CA1 hippocampal neurons, indicating that GluN2B activation is necessary for synaptic weakening (Sachser et al., 2016). However, this study did not further resolve the distinct role that subcellular localization may have on physiological outcomes.

Interestingly, excessive GluN2B activation, particularly at extrasynaptic sites, may trigger signaling pathways that promote synapse weakening (Delgado et al., 2018; Hardingham et al., 2002). My data showed that application of Ro 25-6981 20 minutes after inducing HFS prevented decay of LTP and selective stimulation of GluN2B-containing NMDA receptors at extrasynaptic sites induced a rapidly decaying form of LTP. Collectively, this research identifies extrasynaptic GluN2B as a critical regulator of activity-dependent synaptic weakening. It is important to acknowledge that the relative contribution of GluN2C and/or GluN2D subunits remains to be determined. These subunits are less abundant in the hippocampus and are expressed in specific neuronal populations, including interneurons (Hansen et al., 2017). It will be important to test the relative contribution of these subunits in other neuron types as LTP decay processes will likely differ as a function of cell type or NMDA receptors they express.

5.9 GluN2B receptors and LTP decay

GluN2B-containing NMDA receptors are particularly well suited to modulate LTP decay due to the differential activation kinetics and coupling to distinct signaling pathways. For example, GluN2B receptors bind calcium/calmodulin-dependent kinase II (CaMKII), a key mediator of LTP maintenance (Rumian et al., 2024). However, prolonged GluN2B activation may lead to CaMKII

inactivation or redistribution within the synapse, potentially destabilizing LTP (Huang et al., 2014; Mayford et al., 1995). Additional studies are required to further probe alternative signaling pathways that are engaged during LTP decay. Although speculative, the interesting possibility remains that the type of memory or firing patterns of neurons involved in engram formation may recruit distinct LTP decay pathways.

How are GluN2B receptors preferentially stimulated to promote LTP decay? Following LTP induction, ongoing low-frequency synaptic activity typically driven by baseline stimulation may preferentially activate extrasynaptic GluN2B receptors. This is supported by studies showing that prolonged low-frequency stimulation (1 Hz) reverses LTP in a GluN2B-dependent manner (Fujii et al., 2020). Moreover, my data as well as others (Villarreal et al., 2002) demonstrate the halting basal stimulation following LTP induction preserves LTP until stimulation resumes, consistent with a requirement for modest synaptic activity to drive the decay of LTP. These data indicate that high-frequency stimulation promotes either direct glutamate spillover or works in tandem with an indirect but tonic upregulation of glutamate release primed by the LTP induction stimulus which activates extrasynaptic GluN2B receptors, initiating signaling that counteracts LTP maintenance. This phenomenon is observed under pathological conditions, such as ischemia, where excessive glutamate release accelerates LTP decay (Berry et al., 2024).

5.10 RAC1 recruitment during LTP decay

Rac1, a small GTPase, is a key downstream effector of NMDA receptor signaling, regulating cytoskeletal dynamics and synaptic plasticity (Shuai and Zhong, 2010). NMDA receptor activation, particularly via GluN2B, stimulates Rac1 through calcium-dependent pathways. NMDA receptor-mediated calcium influx activates guanine nucleotide exchange factors, such as

Tiam1 and Kalirin-7, which catalyze Rac1 activation. Tiam1 is enriched at synapses where it promotes Rac1-dependent actin remodeling (Tolias et al., 2005). Accordingly, activated Rac1 reorganizes the actin cytoskeleton which in turn alters dendritic spine morphology. In the context of LTP decay, excessive Rac1 activation may destabilize spines, reducing synaptic efficacy (Benoist et al., 2013). For example, Rac1 inhibition with NSC 23766 enhances LTP stability in hippocampal neurons (Liu et al., 2016). Thus, preventing Rac1-mediated remodeling of dendritic spines should stabilize spines such that they are more resistant to activity-dependent breakdown which would depress synaptic transmission.

Beyond directly initiating signaling cascades coupling to LTD and depotentiation pathways (Benoist et al., 2013; Cui et al., 2021), Rac1 may also crosstalk with pro-LTP pathways. ERK, a member of the mitogen-activated protein kinase (MAPK) family, is essential for the consolidation of LTP, particularly in the hippocampus (Schafe et al., 2008; Zhai et al., 2013). ERK couples to signaling cascade that initiate new protein synthesis to sustain synaptic changes beyond the early phase (E-LTP) (Gelinas et al., 2007; Kelleher et al., 2004; Santini et al., 2014). In dendrites, ERK activates signals that converge on eIFE4E, which regulates translation initiation (Hoeffer et al., 2013). This enhances synthesis of plasticity related synaptic proteins, such as PSD-95 and AMPA receptor subunits (Kelleher et al., 2004) including GluA1 which is rapidly inserted into the synaptic membrane during LTP (Maity et al., 2015). These cascades ensure that ERK coordinates downstream translational responses, enabling the structural and functional changes required for L-LTP.

In the context of LTP decay, Rac1 activation has previously been associated with ERK Inhibition. Thus, Rac1 activation, triggered by LTP stimulation or enhanced neuronal activity associated with learning and memory formation, serves as a countervailing mechanism that limits

LTP. Specifically, Rac1 activates p21-activated kinase (PAK), which phosphorylates Raf-1 at inhibitory sites (Ser259), reducing Raf-MEK-ERK signaling (Benoist et al., 2013). Therefore, through inhibiting ERK, Rac1 reduces downstream recruitment of translational machinery, potentially restricting L-LTP maintenance. Consistent with this notion, I observed that inhibition of Rac1 enhanced ERK and eIF4E phosphorylation and prevented LTP decay.

This interplay between ERK and Rac1 highlights a sophisticated regulatory network that my data suggests may serve as a molecular "brake" on LTP expression. Whereas ERK drives the molecular changes necessary for long-lasting synaptic potentiation by coupling synaptic activity to protein synthesis, Rac1 may restrict ERK activity to prevent excessive potentiation or act as a molecular gate that only permits potentiation expression under optimized conditions (**Figure 5.1**).

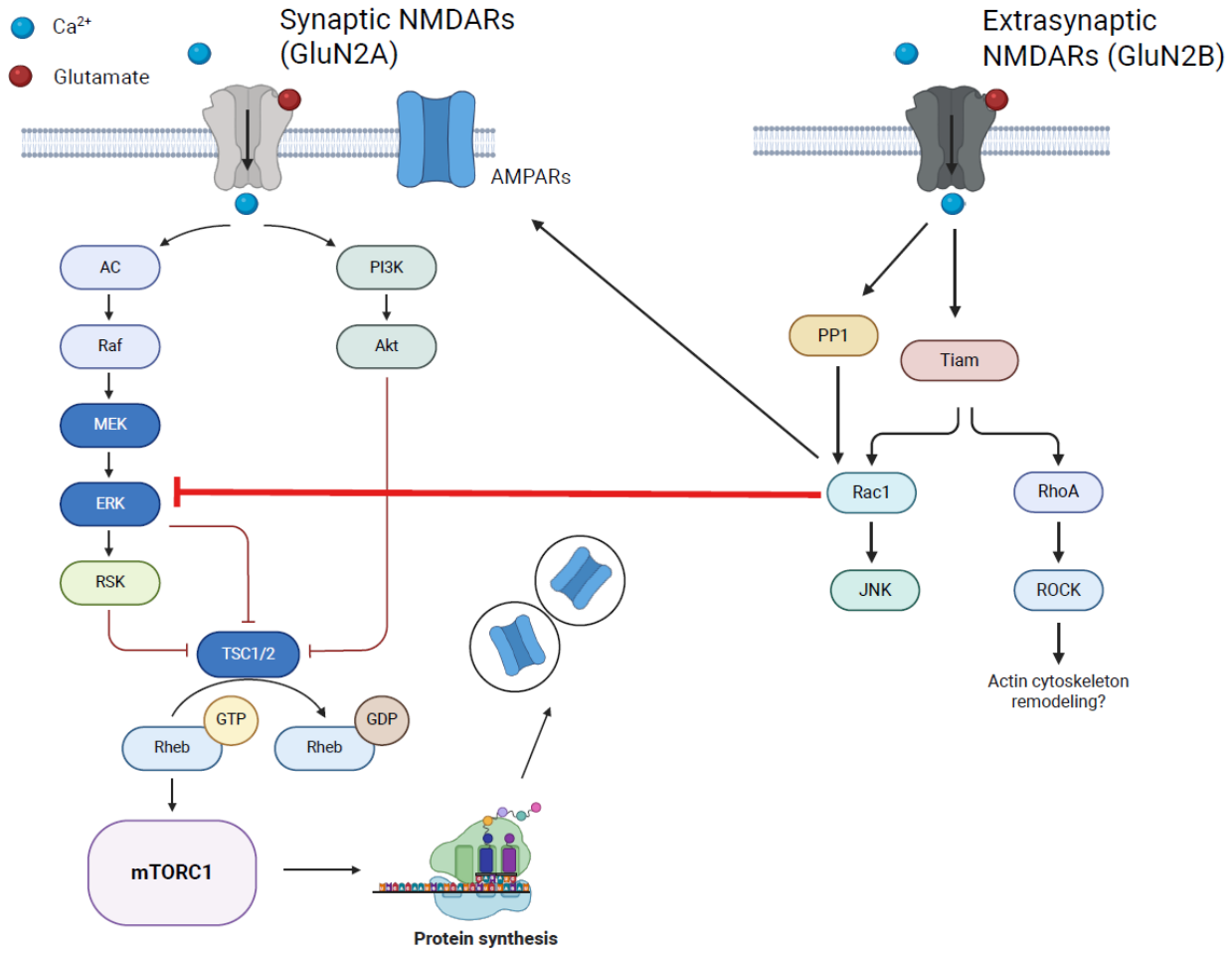

Figure 5.2. Mechanism of Rac1-mediated LTP decay via crosstalk and inhibition of ERK signaling cascades coupled to translation regulation. Schematic diagram illustrating the differential signaling pathways activated by synaptic and extrasynaptic NMDA receptors (NMDARs) in hippocampal neurons, focusing on Rac1's role in promoting LTP decay. **Left side**: Synaptic GluN2A-containing NMDARs are activated by glutamate released by HFS, triggering Ca^{2+} influx and activating the ERK signaling cascade. This pathway inhibits TSC1/2, activating Rheb and mTORC1, leading to protein synthesis that supports LTP maintenance. **Right side**: Extrasynaptic GluN2B-containing NMDARs activate Rac1 through PP1 and Tiam1, which

inhibits the ERK pathway (indicated by the red inhibitory line). This crosstalk suppresses ERK signaling, reducing protein synthesis and promoting LTP decay. Created with BioRender.com

Collectively, and in line with my findings, these findings suggest that the negative regulation of ERK by Rac1 following the induction of transient forms of LTP ensures precise control of synaptic plasticity through setting the conditions for potentiation erasure. Understanding these mechanisms provides insights into the molecular basis of forgetting and offers the potential therapeutic targets for neurocognitive disorders characterize by excessive or impaired forgetting.

5.11 Discussion: LTP decay

Similar to many studies which rely on antagonists like Ro or ifenprodil, which lacks absolute NMDA receptors subunit specificity, this approach limits the ability to completely isolate GluN2B-specific effects from those of other subunits. Moreover, these methods fail to recapitulate the temporal resolution of many forms of forgetting as LTP decay may occur over hours to days *in vivo*, whereas use of acute slices is restricted to the minutes-hours time frame which may not capture long-term processes. *In vivo* studies, particularly in GluN2 subunit specific knockout mice are needed to further test and validate my findings.

Beyond, these critical issues, it will be important to determine the universality of so-called forgetting molecules like Rac1. Neurons and neural circuits are subject to cellular, synaptic and molecular heterogeneity that influences properties of LTP. Therefore, it is reasonable to assume that this diversity will similarly be observed mechanisms of LTP decay that will likely vary as a function of cell type, synapse type and across brain regions, developmental stages, and stimulation

protocols (Hanson et al., 2017). Moreover, the simplistic notion that pre- and postsynaptic regions are the chief participants in synaptic plasticity is antiquated and now requires a more wholistic analysis that incorporates additional players including glial cells, neuromodulation, and changes in intrinsic properties of the cell beyond the synapse.

5.12 Conclusions: LTP decay

Dysregulated LTP decay is implicated in disorders like Alzheimer's disease, where GluN2B and Rac1 dysfunction are prominent (Wu et al., 2019). Interestingly, excessive Rac1 activation has been identified in an AD model, which also demonstrates impaired LTP (Wu et al., 2019). It will be critical to test if Rac1 activation or the downstream effects (e.g. ERK suppression) linked to LTP decay (thus promoting forgetting) could provide new therapeutic avenues for erasing excessively encoded memories associated with PTSD.

In conclusion, my project uncovered a unique, endogenous mechanism through which synapse weakening during LTP decay functions. Activation of GluN2B following HFS is sufficient for inducing parallel potentiation and decay pathways, endowing synapses with an additional means for calibrating synaptic strength. Rac1 operating downstream of these receptors mediates this capacity for bidirectional modifications which depends on the timing of changes in Rac1 activation status relative to the stimulating or memory-inducing event. Meanwhile, LTP promoting pathways initiated by HFS, simultaneously or after a delay, engage potentiation decay pathways that limit potentiation or reverse it altogether. Given links between bidirectional forms of plasticity with disorders of memory (PTSD, AD), and other neurological conditions associated with pathologized forgetting (Berry et al., 2024), we propose impaired (PTSD) or accentuated

(AD) LTP decay as an underlying mechanism and suggest that associated molecular processes offer valuable models for understanding the nature and pathologies of forgetting.

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