# University of Alberta

Neuromodulation of Heterosynaptic Plasticity in Mouse Hippocampus

by

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A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Centre for Neuroscience



Fall, 2011
Edmonton, Alberta

Dedicated to my family for the love and support they have always given me

ABSTRACT

The ability of synapses to undergo lasting changes in synaptic strength is considered the primary cellular mechanism for associative memory formation in the mammalian brain. Synapses in the hippocampus, a brain structure required for new memory genesis, exhibit a remarkable capacity for activity-dependent modification known as "synaptic plasticity". Our understanding of the neurobiological correlates of learning and memory has been significantly advanced through the study of one particular form of hippocampal synaptic plasticity known as long-term potentiation (LTP). As LTP has been linked to long-term memory formation, characterizing the mechanisms through which synaptic plasticity is regulated is crucial for advancing our understanding of the brains ability to encode and store information.

Hippocampal synaptic plasticity and long-term memory can both be enhanced through activation of neuromodulatory receptors. Noradrenaline is an endogenous neuromodulatory transmitter which is secreted in response to novelty and arousing experiences. Neuromodulatory receptor stimulation can influence synaptic plasticity in a cell-wide manner known as heterosynaptic plasticity. Heterosynaptic plasticity requires the generation and sequestration of “plasticity proteins”. Once generated, these plasticity proteins are available for capture at additional synapses, provided that synaptic activity sufficient for setting a molecular "tag" has taken place. Beta-adrenergic receptors (β-ARs) are activated by noradrenaline, resulting in the engagement of downstream signaling cascades capable of augmenting synaptic function. The present thesis identifies mechanisms through which β-ARs enhance heterosynaptic plasticity using electrophysiological recording methods in mouse hippocampal slices.

I found that inducing homosynaptic LTP by pairing high-frequency stimulation with ISO application facilitated the induction of LTP by subthreshold stimulation applied heterosynaptically. This form of plasticity requires ERK and mTOR activation, as inhibition of these kinases prevents LTP at either synaptic pathway. Inhibition of translation during ISO application (but not during heterosynaptic tetanization) similarly blocked LTP expression. Additionally, I found that inhibiting GluA2 endocytosis expanded the temporal window for heterosynaptic plasticity to 1 hour.

For my second project, I sought to determine if β-AR-dependent heterosynaptic plasticity was altered in a mouse model of Fragile X Syndrome (FXS). Interestingly, when beta-adrenergic receptor-dependent LTP was induced in hippocampal slices from Fragile X mental retardation knockout mice (Fmr1 KO), heterosynaptic LTP was enhanced relative to wild-type littermate controls. This enhancement appeared to result from increased basal activation of mTOR, as inhibition of mTOR failed to block homosynaptic LTP.

My third and final project investigated the mechanisms through which β-ARs and muscarinic receptors synergistically regulate synaptic plasticity. When beta-adrenergic and muscarinic receptor agonists were co-applied, stimulation with a low-frequency stimulation protocol (5 Hz, 5 s) which is normally subthreshold for LTP, resulted in enduring LTP. This form of LTP requires extracellular signal-regulated kinase and protein synthesis. Thus, I was able to further characterize how different neuromodulatory receptors can co-facilitate the induction of enduring synaptic plasticity which is considered the cellular basis for formation of new memories

# ACKNOWLEDGEMENTS

When reading through commentaries on the "state of science" published over the last several years, one quickly gets the impression that the underlying theme is one emphasizing collaboration. This is never more true than in the completion of a doctoral degree. Acquiring a Ph.D. is a truly collaborative process requiring tremendous efforts from many individuals, all of which I am truly thankful for.

I would like to extend my sincerest gratitude to Dr. Peter Nguyen as my supervisor. Pete's steadfast support and guidance have been invaluable in my development as a scientist. I will always be thankful for all Pete has taught me both in and out of the lab, across a range of disciplines including science, history, art and football. Under Pete's supervision, my doctoral studies were a challenging and rewarding experience, the lessons from which, will serve me well in both my academic career and life in general.

Special thanks also go to all past and present members of the Nguyen Laboratory whom I've had the pleasure of working with. I would like to thank Gustavo Tenorio and Sabya Maity for creating an intellectually stimulating environment and providing the occasional helping hand, sympathetic ear, and pint of beer.

I would also like to extend my appreciation to the members of my committee: Dr. Greg Funk and Dr. Declan Ali for providing their expertise and helpful suggestions concerning my research. Special thanks to the external examiner, Dr. Jaideep Bains, for generously donating his time and effort and providing insights which will no doubt, improve my science.

Additionally, I would like to thank my collaborators including Dr. Eric Klann and Dr. Charles Hoeffer (NYU) for generously providing me with Fmr1 transgenic mice. Thanks also to Dr. Yu Tian Wang (UBC) for providing GluR2 trafficking inhibitory peptides.

I wish to thank all the members of both the Centre for Neuroscience and the Department of Physiology, including the professors, fellow graduate students and the helpful secretaries for their support and creating a facilitative atmosphere.

Finally, I would like to give my sincerest thanks and love to my family for providing unfailing support. My parents have been wellsprings of inspiration. Their incredible work ethic and unique perspectives on life have served as excellent models which will continue to guide me throughout my future endeavors. Without their advice, encouragement and monk-like ability to put up with my scientific prattling, I would not have realized my true potential.

Finally, I would like to thank the Natural Sciences and Engineering Research Council of Canada (NSERC), the Alberta Heritage Foundation for Medical Research (AHFMR), the Canadian Institute of Health Research (CIHR), the Faculty of Graduate Studies and Research at the University of Alberta and The Centre for Neuroscience for generously providing financial support during my doctoral studies.

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LIST OF ABBREVIATIONS, SYMBOLS, AND NOMENCLATURE

| | |
|---|---|
| 4E-BP | 4E-binding protein |
| AC | adenylyl cyclase |
| ACSF | artificial cerebrospinal fluid |
| AMPAR | L-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor |
| ANOVA | analysis of variance between groups |
| β-AR | beta-adrenergic receptor |
| Ca+2 | calcium |
| CA | cornu ammonis |
| cAMP | adenosine 3', 5'-cyclic monophosphate |
| CaM | calmodulin |
| CaMKII | calcium/calmodulin-dependent protein kinase II |
| CCAC | Canadian council on animal care guidelines |
| CREB | cyclic AMP response element binding protein |
| DAG | Diacyglcerol |
| DEPO | depotentiation |
| DG | dentate gyrus |
| DMSO | dimethylsulfoxide |
| eIF4E | eukaryotic initiation factor 4E |
| EME | emetine |
| EPSP | excitatory postsynaptic potential |
| ERK | extracellular-signal regulated kinase |
| fEPSP | field excitatory postsynaptic potential |
| Fmr1 | Fragile X mental retardation 1 |
| FXS | Fragile X syndrome |
| G-protein | guanine nucleotide-binding regulatory protein |
| GluR | glutamate receptor |
| HFS | high-frequency stimulation |
| ISO | isoproterenol |
| IACUC | Institutional Animal Care and Use Committee |
| IEG | immediate early gene |
| LFS | low-frequency stimulation |
| LTP | long-term potentiation |
| LTD | long-term depression |
| LTM | long-term memory |
| MAPK | mitogen-activated protein kinase |
| MEK | mitogen activated protein kinase kinase |
| MF | mossy fibre |
| Mg+2 | magnesium |
| mGluR | metabotropic receptor |
| mRNA | messenger RNA |
| mTOR | mammalian target of rapamycin; |
| M | muscarinic receptor |
| NMDAR | N-methyl-D-aspartic acid receptor |
| NSF | N-ethylmaleimide sensitive factor |

| | |
|---|---|
| PICK1 | protein interacting with C-kinase 1 |
| PKA | cAMP-dependent protein kinase |
| PKC | protein kinase C |
| PKM | protein kinase M |
| PPF | paired pulse facilitation |
| PSD | postsynaptic density |
| RAP | rapamycin |
| RNA | ribonucleic acid |
| S | stimulating electrode |
| SC | Schaeffer collateral |
| SEM | standard error of the mean |
| WT | wildtype |

# CHAPTER I:

## General Introduction

## INTRODUCTION

A neurobiological correlate of memory requires a highly plastic mechanism which can be modified by experience. Specialized communication zones between neurons known as synapses fulfill the requirement for modifiability in memory formation. Both behavioral tasks that require new memory and simulation of learning-related cellular activity engage long-term changes in synaptic function known as synaptic plasticity. Synaptic plasticity is the leading cellular candidate for memory formation in the mammalian brain. Studies dating back to the 1970s have characterized a form of synaptic plasticity known as long-term potentiation (LTP). LTP is an activity-dependent enhancement of synaptic strength which has been correlated with the genesis of new memories in several different brain structures including the hippocampus. The hippocampus is critically involved in the formation of mammalian declarative, episodic and spatial memories.

Hippocampal synaptic plasticity and long-term memory can both be bolstered through activation of neuromodulatory receptors. Neuromodulatory transmitters, including noradrenaline, stimulate metabotropic receptors which initiate intracellular signaling cascades that modify the ability of synapses to undergo long-term changes required for memory. Moreover, neuromodulatory receptor stimulation can influence synaptic plasticity in a cell wide manner known as heterosynaptic plasticity. Heterosynaptic plasticity requires the generation of plasticity proteins that can be subsequently sequestered at other synapses converging on the same post-synaptic cells provided synaptic activity suprathreshold for setting a molecular "tag" has taken place. This process of synaptic tagging provides a cellular mechanism for associative memory formation as it would allow for different components of an experience to be bound together through heterosynaptic integration.

The endogenous neuromodulator, noradrenaline is secreted in response to experiences that require attention, elicit arousal, or during exposure to new environments or novelty. Noradrenaline acts through stimulation of alpha- and beta-adrenergic receptors (β-ARs). The present thesis identifies mechanisms through which β-ARs enhance heterosynaptic plasticity using electrophysiological recording methods in mouse hippocampal slices. To determine if β-

ARs can initiate heterosynaptic plasticity, I used a two pathway protocol in which two separate synaptic pathways converging on the same post-synaptic cells are monitored. I found that inducing homosynaptic LTP through β-AR activation at one synaptic pathway (S1) could facilitate the induction of LTP at a second synaptic pathway (S2) when low-frequency stimulation (5 Hz, 10 s) normally subthreshold for LTP, was applied 30 minutes later. This form of heterosynaptic plasticity required mammalian target of rapamycin (mTOR), extracellular signal-regulated kinase (ERK) and translation as inhibitors of all three applied during beta-adrenergic receptor stimulation prevented the expression of LTP at both synaptic pathways.

Interestingly, this form of heterosynaptic plasticity was augmented in a mouse model of Fragile X Syndrome (FXS) which was the basis for my second project. When I induced beta-adrenergic receptor-dependent LTP in the fragile X mental retardation 1 (Fmr1) knockout mouse hippocampus, heterosynaptic LTP was enhanced relative to wild-type littermate controls. This enhancement appeared to be the result of increased basal activation of mTOR as application of rapamycin (an mTOR antagonist) failed to block homosynaptic LTP.

Finally, I sought to determine if β-ARs could work synergistically with another important neuromodulatory receptor, muscarinic receptors, to augment hippocampal synaptic plasticity. I found that pairing of beta-adrenergic and muscarinic receptor agonists lowered the threshold for the induction of LTP by a low-frequency stimulation protocol (5 Hz, 5 s). This form of LTP required protein kinase C, and protein synthesis. Thus, I was able to characterize how different neuromodulatory receptors can co-operate to facilitate the induction of long-term synaptic changes that constitute a cellular correlate of memory. In a broader context, my research has provided further insight into how changes in the modifiability of synaptic transmission confer the brain with the remarkable capacity to encode and store information.

## LEARNING AND MEMORY: INITIAL INSIGHTS LINKING MEMORY TO THE BRAIN

The first documented reference to the brain is found in the Edwin Smith surgical papyrus, with the original version (Smith's was copied in the 17th century) dating to approximately the 13th century (Minagar et al., 2003; Rose, 2009). In what appears to be a battlefield surgeon's account, the papyrus describes the protrusion of cortical tissue following a skull fracture. In over 40 accounts, the author goes on to record what could be considered the first documented attempt at correlating symptoms with damage to the body, spinal cord, and brain. Following Galen's contention that mental faculties reside in the cerebrum (Green, 2003), the Byzantine Poseidonus was the first to report that damage to the posterior brain impaired memory (Manzoni, 1998). Poseidonus' observations were not lost on Thomas Willis (1621-1675), perhaps the first scientist to attribute memory processes to the cerebral cortex (Gross, 1999).

## PRELIMINARY INVESTIGATIONS OF HIPPOCAMPAL FUNCTION

Early anatomists at the Alexandrian School for Medicine noticed that when viewed together the two hippocampi resembled the horns of a ram. Thus, the nomenclature "cornus ammonis" (horn of the ram) which still persists to this day in the subdivisions of the hippocampus, CA1-CA3 (Andersen et al., 2007). The term "hippocampus" itself was originally coined in 1564 by the Bolognese anatomist, Giulio Cesare Aranzi due to the striking similarities in shape between the hippocampus and the seahorse (Andersen et al., 2007). Although originally prescribed a role in olfaction (Ferrier, 1876; Penfield and Erickson, 1941) and emotion (Kluver and Bucy; 1937; Papez, 1995), studies suggesting a mnemonic function were accumulating (Wernicke, 1881; Korsakov, 1889; Bekhterev, 1900) (see Brown and Scahfer (1888) for an early example of memory dysfunction following bilateral hippocampal lesions in a primate). It would

take more than half a century and the characterization of side effects of a life-saving neurosurgical procedure to provide more definitive evidence for the hippocampus in memory function.

## MEMORY SYSTEMS

The notion that memory is not expressed as a unitary mental process extends back into the 19th century (for review, see Squire, 2004). Maine de Biran (1804) drew the distinction between mechanical, sensitive, and representative memory (Anderson et al., 2007). In *Principles of Psychology*, James (1890) considered memory and habit separate mental processes. In his classic work, Tolman (1948) argued for the existence of multiple types of memory whereas Winograd (1975) suggested a dichotomy composed of procedural and declarative knowledge. A distinction between multiple types of human memory systems was proposed by Endel Tulving (1972) who further defined memories as declarative (having a contextual component) or semantic (purely factual, not requiring context).

Initial evidence for divergent memory processes in humans did not emerge until the characterization of (Scoville and Milner, 1957) and initial experimentation (Milner, 2005) using patient Henry Molaison (HM). In 1957 HM whom suffered from intractable epilepsy, elected to undergo a bilateral hippocampectomy to provide relief from his seizures. The procedure reduced the seizures, however, it left HM with a profound anterograde memory deficit. Strikingly, he was no longer able to form new declarative, episodic or spatial memories (Scoville and Milner, 1957; Corkin, 2002). Over the next 50 years, studies of HM conducted by Dr. Brenda Milner and others (Scoville and Milner, 1957; Corkin et al., 1997; see Corkin, 2002 and Squire 2009 for review) characterized the nature and degree of HM's memory impairments. Several key observations from this case study revolutionized our understanding of memory function: 1) The

medial temporal lobe (MTL) is required for distinct types of memory including episodic and declarative memories 2) There are multiple memory systems capable of functioning independent of the MTL as demonstrated by the sparing of working and implicit memory following MTL bisection 3) The MTL is required for storage and consolidation of new memories demonstrated by a temporally graded retrograde memory deficit (Squire, 2009). These concepts were extended and applied during studies of amnesic patients which revealed a correlation between hippocampal lesions and formation of memories with episodic and semantic (declarative) components relative to procedural and implicit (nondeclarative) memory deficits (Cohen and Squire, 1980; 1981; Squire, 1986, 2004). Based on this distinction, Squire proposed a model in which the "medial temporal lobe system " (composed of the hippocampal formation, perirhinal cortex, and parahippocampal cortex) is responsible for encoding declarative memories distinct from other memory systems (striatum: stimulus-response habits; neocortex: percepts and priming; amygdala: emotional and social learning and cerebellum: stimulus-response timing) (Squire and Zola-Morgan, 1988; 1991; 1997; Squire, 2004 ).

## HIPPOCAMPAL FUNCTION

What does the hippocampus do? Initial theories of hippocampal function suggested roles in olfaction, sensation, perception and higher-order cognitive functions (reviewed by Andersen et al., 2007). During the 1980s the medial-temporal lobe memory system theory was put forth by Larry Squire which suggested a specialized role for the hippocampus proper along with the perirhinal and parahippocampal cortices in declarative memory formation (Cohen and Squire, 1980). Case studies of patients with MTL lesions provided the bulk of data supporting the MTL theory (Squire and Zola-Morgan, 1991). Lacking definitive tests for declarative memory in animal models, investigations of the MTL theory were stalled considerably until the 1990s.

However, prior to the proposal of the MTL theory, data from intrahippocampal single unit recordings from awake, mobile rats suggested an alternative function for the hippocampus: encoding information about an animal's location in environments (O'Keefe and Dostrovsky, 1971). The cognitive map theory states that individual neurons within the hippocampus fire when an animal is in a particular space and are involved in forming spatial maps of new environments (O'Keefe and Dostrovsky, 1971). This was demonstrated by increased action potential frequency of individual hippocampal neurons during spatial exploration. The subsequent development of primate models of MTL dysfunction provided new insights into hippocampal function.

## A ROLE IN LEARNING AND MEMORY: HUMAN STUDIES AND ANIMAL MODELS

Early case studies provided crucial insights into the cellular basis for learning and memory. Post-mortem dissections of human cortical tissue revealed abnormal hypotrophy of MTL structures in patients demonstrating early-onset dementia and memory dysfunction as a result of alcoholism (Korsakoff's Syndrome)(Kopelman et al., 2009). Based on studies of amnesic patients, Cohen and Squire (1980) drew a distinction between "declarative" and "procedural" memory deficits. They proposed that amnesics with damage restricted to the MTL (Scoville and Milner, 1957) are impaired in forming memories for facts and events (declarative) with spared motor learning and cognitive skills (procedural) (Squire et al., 1984; Squire and Zola-Morgan, 1991; Squire et al., 2004).

Data from imaging studies, which indirectly measure region-specific brain activity (PET scan; 2-deoxyglucose utilization), provided evidence of increased activity in the hippocampus following learning, consistent with a role in processing new memories (Schacter et al., 1996; Bontempi et al., 1999). PET studies also demonstrated increased hippocampal activation during tasks requiring comparing and contrasting of nonspatial information (Henke et al., 1997; 1999)

consistent with data demonstrating a correlation between relational processing and increased activity in the hippocampus (Cohen et al., 1997).

Consistent with animal models, the human hippocampus is capable of processing spatial information, as demonstrated by depth electrode recordings which identified "place cells" (Ekstrom et al, 2003). Episodic memories are mental representations of temporally and contextually defined experiences (Tulving, 1972). From a behavioral perspective, episodic memories consist of the "what, when and where" content encoded during experiences (Tulving, 2002). Episodic memory requires "binding" of multi-modal information into coherent percepts representing location, time and context (events). Recordings taken from hundreds of neurons in the hippocampus and entorhinal cortex using electrodes implanted in epileptic patients provided the first direct evidence for reactivation of hippocampal neurons and conscious recall of a past experience (Gelbard-Sagiv et al., 2008).

Hippocampal integrity is required for encoding contextual and temporal components of experience (episodic memory) (Viskontas et al., 2000; Cipolotti et al., 2001; Viskontas et al., 2009; Paz et al., 2010). Human episodic memory confers the ability to transcend temporal limitations, through mental time travel (Tulving, 2004). Recent research suggests that when hippocampal function is compromised, the ability to imagine future scenarios is impaired (Addis and Schacter, 2008; Addis et al., 2010; but see Squire et al., 2010). Hence, studies using human subjects demonstrate that cellular activity within the hippocampus is involved in cognitive processes requiring access to stored information.

PRIMATES STUDIES

Early attempts to model amnesia patients with MTL damage, failed to replicate the observed memory deficits (Iverson, 1976). However, this incongruency between human and

primate data was resolved following the refinement of behavioral tests, capable of testing memory functions specifically mediated by the MTL. In a landmark study, recognition memory was tested using a delayed non-matching to sample (DNMTS) task. DNMTS requires the ability to discriminate between recently presented and novel objects-a form of recognition memory. Rewards (sugar puffs) were given for correctly displacing novel objects which is indicative of rule learning (displace new item = reward) (Gaffan, 1974).

Fornix lesions impaired recognition memory at long (minutes) but not short (seconds) delays between stimuli presentations (Gaffan, 1974). Combined hippocampal and amygdala lesions similar to those observed in HM, similarly impaired recognition memory performance (Mishkin, 1978; Zola-Morgan and Squire, 1985; Squire et al., 2004). Consistent with data from studies of human amnesiacs, MTL lesions do not affect motor, perceptual or cognitive skills as demonstrated by normal learning rates in tasks testing these domains (Zola-Morgan and Squire, 1984; Alvarez-Royo et al., 1992). Collectively, these studies confirmed the notion that MTL damage is responsible for human anterograde amnesia by showing a selective deficit in new, long-term memory formation with a relative sparing of short-term memory, and memory acquired prior to MTL excision.

The hippocampus has been implicated in the transfer of short-term memory to long-term memory, known as consolidation. To probe the role of the MTL system in memory consolidation Zola-Morgan and Squire (1990) trained monkeys on a series of object discrimination problems that were divided over several weeks (2, 4, 8, 12, 16) prior to hippocampal and parahippocampal lesions. Lesioned monkeys demonstrated impaired recall for recently learned objects discrimination problems relative to problems learned 12 or 16 weeks prior to surgery. Consistent with studies of HM (Squire, 2009), these results suggested a

temporally graded retrograde amnesia indicative of a time-dependent memory consolidation process that requires the hippocampus.

Spatial correlates of hippocampal processing have been observed in monkeys operating motorized cabs through an environment. Single cell recordings taken from the hippocampus while "driving" revealed place cells (Matsumura et al., 1999), similar to those observed in rodents (O'Keefe, 1976; O'Keefe and Speakman, 1987). In addition to the profound anterograde memory deficit observe in HM, he also exhibited retrograde memory loss (Corkin, 2002). Consistent with this, selective lesions of either the hippocampus (Zola-Morgan et al., 1992; Alvarez et al., 1995; Zola et al., 2000) or the perirhinal and parahippocampal cortices (Murray and Mishkin, 1986; Zola-Morgan et al., 1989b; Suzuki et al., 1993; Meunier et al., 1993) in monkeys produced severe impairment of explicit long-term memory. These results suggest that in addition to encoding new memories, the hippocampus may also be required for long-term consolidation or memory retrieval.

RODENTS

Single cell recordings from hippocampal cells of freely moving rats provided the basis for the “cognitive map” theory of hippocampal information processing (O’Keefe and Dostrovsky, 1971; O’Keefe and Burgess, 1996). This theory was supported by the observation that select principal neurons, termed "place cells" (O’Keefe, 1976), fire when rats traverse a specific location within an environment (O’Keefe and Dostrovsky, 1971; O’Keefe and Burgess, 1996). Polygamous male meadow voles which have extended territorial ranges, have larger hippocampi and demonstrate superior spatial learning relative to monogamous males and females (Gaulin and Fitzgerald, 1989; Jacobs et al., 1990). Additionally, spatial learning and novelty exploration increase levels of c-fos and zif268 in rat hippocampus (Guzowski et al.,

2001; Jenkins et al., 2004) indicative of neural plasticity associated with memory formation. Further evidence that the hippocampus is required for spatial memory came with the introduction of the Morris Water Maze (WMW)(Morris, 1981). Lesions restricted to the hippocampus prevent free recall in probe tests of memory for hidden platform location (Morris et al., 1982; Sutherland et al., 1983). More selective lesions revealed that damage restricted to dorsal (but not ventral) hippocampus prevented normal spatial learning (Moser et al., 1993, 1995).

Additionally, a form of learning in which rats socially transfer food preference to naive conspecifics was found to be sensitive to lesions targeting the hippocampus (Winocur, 1990) consistent with the relational processing theory (Bunsey and Eichenbaum, 1995).

In contextual fear conditioning, an animal learns to associate a shock (UCS) with the distinctive apparatus (CS; context) in which it is administered (Winocur et al,. 1987; Kim and Fanselow, 1992). A single training session generates long-term (weeks or longer), hippocampus-dependent memories (Kim and Fanselow, 1992; Phillips and LeDoux, 1992). Additionally, rodents have demonstrated the capacity for "episodic-like" (Griffiths et al., 1999) memory characterized by behavioral demonstrations of retained knowledge relating to the what, where and when components of an experience (Tulving, 1972). Using food caching experiments, researchers demonstrated that scrub jays are capable of deciding which food stuffs (what) to pursue based on the length of time that had elapsed (when), and location (where) (Pravosudov and Clayton, 2002) consistent with episodic-like memory.

## NEUROANATOMY: THE TRI-SYNAPTIC CIRCUIT

Among the multitude of significant contributions to the field of neuroscience made by the Spanish neuroanatomist, Santiago Ramon y Cajal, is his work on hippocampal neuroanatomy in which he diagrammed the hippocampal circuit and predicted the direction of information flow

within this circuit (Cajal, 1894) .The hippocampus can be divided into anatomically distinct subregions including the dentate gyrus (DG), Ammon's horn (CA1-CA4) and the subiculum (Lorente de No, 1934).

Electrophysiological (Andersen et al., 1971) and anatomical (Blakstad et al., 1970) studies revealed a high degree of lamellar organization within the subdivisions of the hippocampus (Andersen et al., 1971). Development of the in vitro CNS slice preparation (Li and MCIlwaing, 1957; Collingridge, 1995), and its refinement for hippocampal slices (Bliss and Richards, 1971; Skrede and Westgaard. 1971) provided the basis for characterization of cellular connections throughout this circuit (Andersen et al., 2007). The hippocampus consists of a tri-synaptic circuit in which cortical information from the entorhinal and perirhinal cortices is shuttled to the dentate gyrus (DG) via the perforant pathway. Axons of granule cells of the DG form the mossy fiber pathway which synapses onto CA3 pyramidal cell dendrites. CA3 cells form connections both reciprocally within CA3 and outside of the CA3 region via the Schaeffer collateral pathway which terminates in area CA1. Pyramidal cells in CA1 project to the subiculum which along with CA1 cells, complete the hippocampal processing loop via connections to deep layers of the entorhinal cortex (Andersen et al., 2007)(see Fig. 1.1).

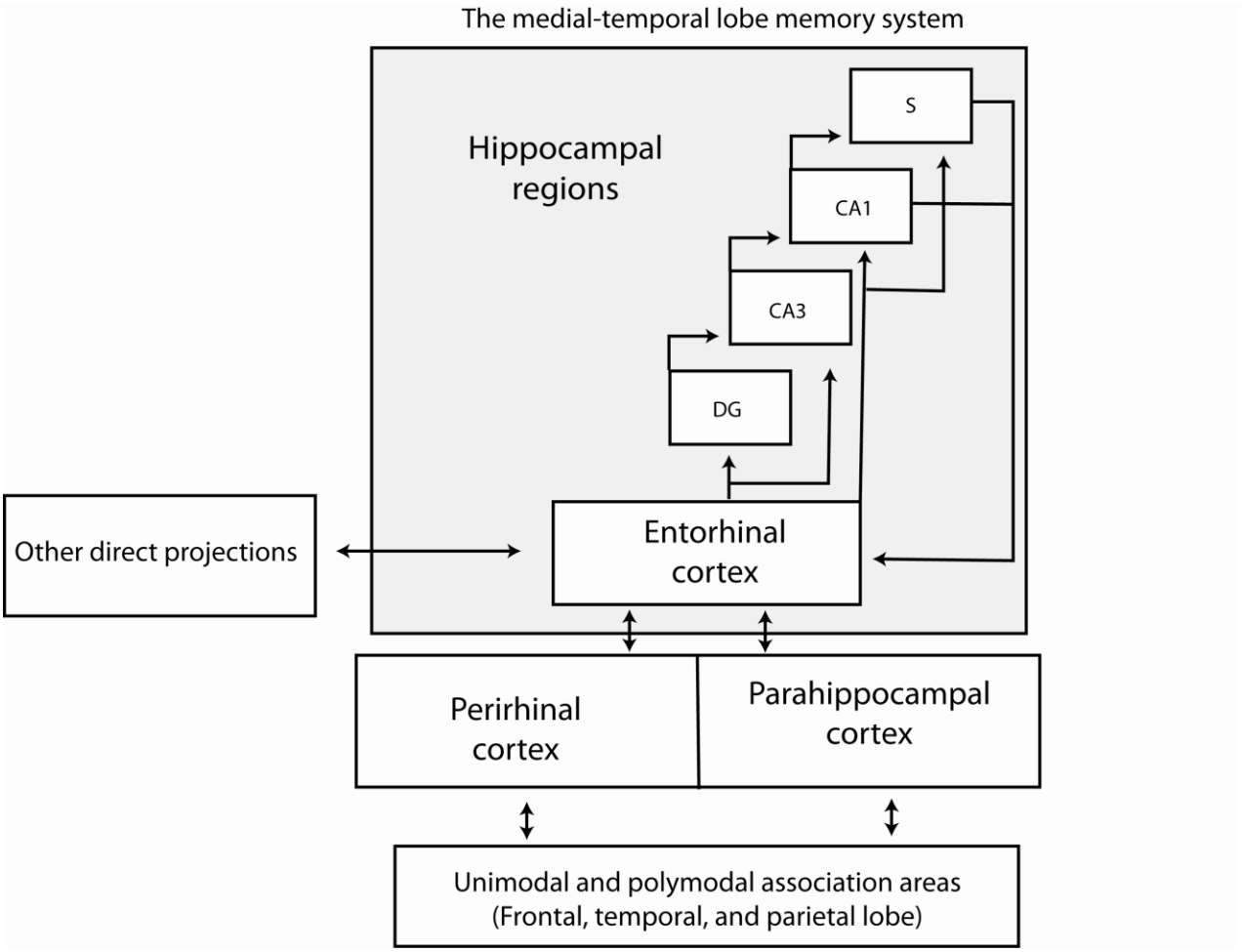


Figure 1.1. The tri-synaptic loop and cortical connections. Information from unimodal and polymodal association areas is projected to perirhinal and parahippocampal cortices which in turn project to the entorhinal cortex. The entorhinal cortex projects to the dentate gyrus (DG) through the perforant pathway. Connections from the DG form the mossy fiber pathway which form synapses on CA3 pyramidal cells. Schaeffer collateral fibers consisting of CA3 axons project to CA1. CA1 axons terminate in the subiculum (S) and entorhinal cortex which completes the cellular circuit and carries information out of the hippocampus, back to cortical association areas. Adapted from Andersen et al., 2007.

**SYNAPTIC PLASTICITY**

Researchers had determined that the hippocampus was required for memory formation but the location of the molecular and cellular "traces" of memory, the engram (Semon, 1908), remained elusive. Nobel Prize winning (1906; shared with Camillo Golgi) anatomist Santiago Ramon Y Cajal, postulated that intercellular transmission may change in an experience dependent manner (García-López et al., 2007), and that altered synaptic function (synaptic

plasticity) may be the cellular basis for memory (Cajal, 1894). Shortly thereafter (1897), the term "synapse" was introduced by the Nobelist (awarded 1932) Charles Sherrington , used to describe the signal transmission zone between "neurons" (Molnar and Brown, 2010). The notion that changes in synaptic efficacy might constitute the cellular equivalent of memory was formalized by the Canadian psychologist Donald Hebb (1949). Hebb postulated that associative mechanisms during neural activity would generate new neural networks capable of supporting memory formation (Cooper, 2005).

Although several forms of synaptic plasticity have been proposed as cellular correlates of memory including post-tetanic potentiation in the sympathetic ganglia (Larabee and Bronk, 1939) and spinal cord (Lloyd, 1949; Spencer et al., 1966), or enhanced magnitude of synaptic response during high-frequency stimulation in hippocampus (Cragg and Hamyln, 1955; Kandel and Spencer, 1961), none surpassed the 30 minute mark in terms of duration. Definitive evidence for Hebbian plasticity was provided through work conducted in Per Andersen's lab which revealed that repeated, high-frequency tetanization induced long-lasting increases in synaptic responses (Lømo, 1966; Lømo, 2003) that could last hours (Bliss and Lomo, 1970, 1973). This activity-dependent enhancement of synaptic strength would later be dubbed long-term potentiation (LTP) (Douglas and Goddard, 1975).

LONG-TERM POTENTIATION

Synaptic plasticity, an activity-dependent change in the strength of neuronal connections, is thought to underlie our ability to encode memories which can last a lifetime (Bliss and Collingride, 1993; Kandel, 2001). Investigations into the mechanisms supporting synaptic plasticity revealed a great deal about how memory circuits work at the cellular and molecular level. One form of plasticity, called long-term potentiation (LTP), has been linked to hippocampus-dependent memory formation (Whitlock et al., 2006). LTP is a stimulation-

dependent increase in synaptic efficacy which can last from hours to years, in vitro and in vivo respectively (Lomo, 1966; Bliss and Lomo, 1973; Abraham et al., 2002).

Studies aimed at determining the signaling cascades required for LTP have provided further insight into the neural basis of memory formation (Kandel, 2001; Malenka and Bear, 2004). The primary trigger for the induction of LTP is a rise in post-synaptic Ca2+ levels. This was demonstrated by loading postsynaptic cells with EGTA, a spatial Ca2+ buffer (Lynch et al., 1983). EGTA prevented the induction of LTP. Richard Morris' group (1986) determined that NMDA receptor activation is required for the induction of LTP, as blocking NMDARs using the competitive antagonist APV, prevented the induction of LTP. If LTP is required for memory formation, APV should similarly prevent learning. Consistent with this theory, intrahippocampal perfusion with APV prevented long-term spatial memory formation (Morris et al., 1986). Rats treated with APV spent equal amounts of time in each quadrant of the MWM during probe trials, whereas rats trained with NMDAR function intact spent more time swimming in the quadrant where the hidden platform had been located (Morris et al., 1986). Interestingly, recent data has called into question Morris' original contention that NMDARs were required for a LTP-like cellular mechanism. Using an antagonist that specifically blocks glutamate binding to NR2B isoforms of the NMDAR Ge et al. (2010) were able to show that non-selective antagonist APV may have prevented spatial learning through inhibition of LTD (which similarly requires NMDAR activity) as well as LTP.

Despite the wealth of data investigating the mechanisms involved in LTP, there is a relative paucity of data demonstrating LTP-like synaptic changes associated with learning in vivo. One study measuring changes in fEPSPs in vivo demonstrated an enhancement of synaptic strength which coincided with new learning (Whitlock et al., 2006). Conversely, exploration of a

novel environment was correlated with a β-AR-dependent long-term decrease in synaptic responses which correlated with spatial memory formation (Lemon et al., 2009). These results are consistent with a synaptic plasticity model of learning and memory formation.

## SYNAPTIC TAGGING AND CAPTURE

Experience is a contextually rich, temporally fluid phenomenon. Thus, memories must represent multiple components of experience aggregated into a unified percept. For memory formation to take place, synaptic plasticity should demonstrate several features including associativity, cooperativity and specificity (Lynch, 2004). One of the main strengths of the synaptic model of memory formation is the concept of specificity. The requirement of specificity dictates that only those synapses that are active during memory formation should be incorporated into the synaptic network responsible for encoding that experience. A cellular mechanism for recording temporally separate, yet related events requires the ability to associate synaptic changes over time. Given the sheer numbers of synapses (100 trillion) in the CNS, this greatly increases the computational power of memory circuits. However, this leads to an important question; How are ubiquitously expressed mRNA and proteins appropriately targeted to activated synapses?

Initial evidence attempting to elucidate the mechanisms through which mRNA and proteins are appropriately targeted during synaptic plasticity was provided by Frey and Morris (1997). They found that multiple trains of high-frequency stimulation applied to one synaptic pathway generated LTP that could be captured following a single train of HFS applied heterosynaptically (Frey and Morris, 1997, 1998). A theory for maintaining synaptic specificity was originally put forth by Frey and Morris (1997) in which they described the phenomenon of synaptic tagging and capture (STC). The basic tenets of STC are as follows: during the induction

of LTP, a local (synaptic) molecular event is triggered which sets an activity-dependent "tag". This tag serves as a local index of synaptic activity and is capable of sequestering plasticity related proteins (PRPs) that are simultaneously being generated (either at the dendritic spine and/or the soma). Once generated, these PRPs can be captured at other synapses converging on the same post-synaptic cell provided those synapses produce an activity-dependent synaptic tag (Frey and Morris, 1997; Barco et al., 2002; see Morris 2006; Redondo and Morris, 2011 for review). There is a finite time widow for the capture of plasticity proteins that depends on the half-lives of the synaptic tags and the PRPs (Frey and Morris, 1998). Additionally, synaptic capture can still take place when the synaptic tags are set prior to the generation of PRPs (Frey and Morris, 1998).

Studies in *Aplysia* have similarly provided evidence for heterosynaptic processes at the cellular level (Martin, 2002). Normally, a single dose of serotonin applied to *Aplysia* sensory-motor-neuron synapses induces short-term facilitation (STF), which relies on covalent modification of preexisting proteins. However, if STF is preceded by long-term facilitation (LTF) induced at another synaptic input converging on the same neuron, the STF is transformed to LTF (Casadio et al., 1999; Martin, 2002). This process of heterosynaptic transfer of LTF requires PKA, CREB and protein synthesis. These data suggest that induction of STC may be an evolutionarily conserved mechanism for facilitating heterosynaptic plasticity that could play a role in associative memory formation.

## SYNAPTIC TAGS

What is the nature of the synaptic tag? Originally, it was postulated that the synaptic engram (tag) may result from activation of a local (synaptic) molecule capable of interacting with plasticity proteins (Martin and Kosic, 2002)(Fig.1.1). This local molecular (activation of

regulatory proteins including kinases and phosphatases) event would have to be transient and reversible to prevent network saturation and to maintain synaptic specificity (Martin and Kosik, 2002). Protein kinases and phosphatases demonstrate properties consistent with a capacity to function as synaptic tags (Young and Nguyen, 2005; Young et al., 2006). Indeed, genetic reduction of cAMP-dependent protein kinase A (PKA) activity impairs synaptic capture (Young et al., 2006). Additionally, pharmacologically preventing interactions between PKA and A-Kinase anchoring proteins which recruit PKA to specific subcellular compartments, prevented synaptic tagging and capture (Huang et al,. 2006). However, recent data suggest that additional mechanisms beyond kinase or phosphatase interactions are involved in synaptic tagging by demonstrating that structural modifications to synaptic spines, which requires a multitude of molecular events, plays a role in synaptic tagging (Redondo et al., 2010). Recent evidence suggests that structural (spine) changes are initiated during synaptic plasticity (Engert and Bonhoeffer, 1999; Ramachandran and Frey, 2009). Moreover, this structural plasticity is required for synaptic tagging (Ramachandran and Frey, 2009) and is dynamically regulated by molecular activity (Redondo et al., 2010).

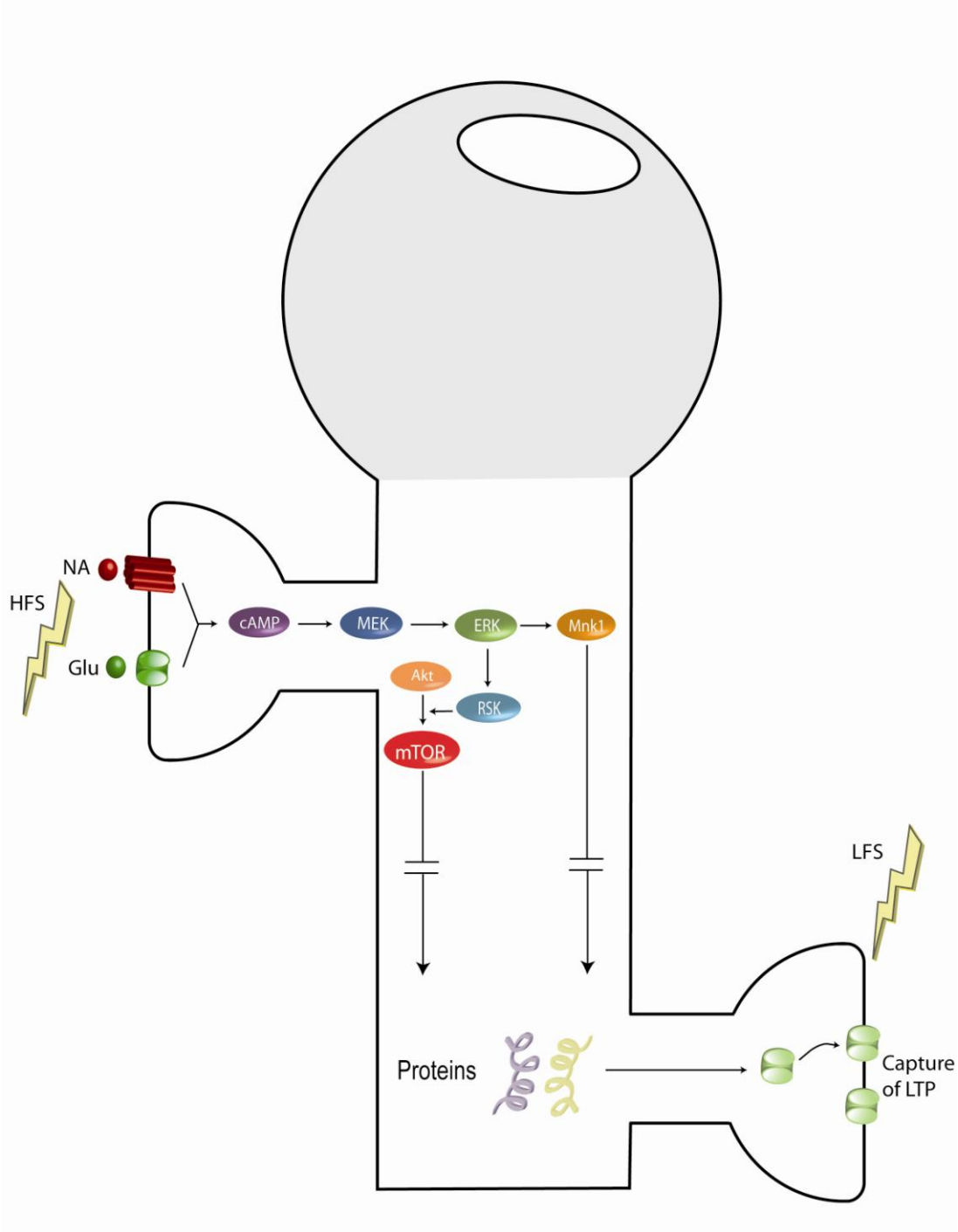


Figure 1.2. Synaptic tagging and capture induced by beta-adrenergic receptor activation. High-frequency stimulation paired with beta-adrenergic receptor activation engages translation. The resulting plasticity proteins can be captured heterosynaptically, provided a local (synaptic), activity-dependent synaptic tag is set. Once set, these synaptic tags can capture plasticity proteins resulting in expression of long-lasting LTP heterosynaptically.

## PLASTICITY-RELATED PROTEINS

Proteins synthesized during LTP serve to stabilize synaptic changes thereby facilitating the expression of enhanced synaptic strength. Although the proteins synthesized remain to be determined there is evidence which suggests that the proteins may either be directly involved in enhanced synaptic transmission (GluR subunits)(Ju et al., 2004) or may play a more regulatory role (translation of the protein synthesis machinery itself) (Tsokas et al., 2005). In addition, recent data suggests that there may be both process-dependent (LTP, LTD) tags and plasticity proteins (Sajikumar et al., 2005; Sajikumar et al., 2007; Sajikumar and Korte, 2011). A protein kinase C isoform, protein kinase M zeta (PKM ζ), has been identified as a plasticity protein specific to LTP processes (Sacktor et al., 1993; Osten et al., 1996; Serrano et al., 2005). Strikingly, injection of the myristoylated Zeta inhibitory peptide (ZIP) which blocks PKM ζ, can prevent the maintenance of memories weeks after initial memory formation (Shema et al., 2007; 2009; 2011; see Sacktor, 2011 for review). PKM ζ seems to exhibit prion-like properties in that once activated, PKM ζ maintains activity in a constitutive manner (Sacktor et al., 1993). Synaptic activity drives the cleaving of PKM to PKM ζ which makes this isoform an ideal candidate for a plasticity protein as it is both synaptically localized (specific) and requires synaptic activity (an associative process) to be generated.

## EVIDENCE FOR SYNAPTIC TAGGING-LIKE MECHANISMS: BEHAVIOURAL STUDIES

Despite the wealth of data supporting STC in vitro, behavioral studies have yet to provide definitive evidence of the STC phenomenon in vivo. Behavioral data supporting the STC model showed that the duration of LTP (synaptic activity capable of setting a tag) can be enhanced by exposure to a novel environment (representing a "strong" stimulus capable of inducing plasticity protein generation) (Kemp and Manahan-Vaughan, 2004). Similarly, providing water

(representative of a strong, plasticity-inducing stimulus) to water-deprived rats enhances the duration of LTP (Seidenbecher et al., 1997). More direct tests on memory per se, were conducted using rats trained in an inhibitory avoidance task; when rats were exposed to a novel environment before or after "weak" inhibitory avoidance training (which produces a mild form of LTM), the duration of LTM was significantly increased (Moncada and Viola, 2007). The facilitation of memory endurance was not observed following intrahippocampal injections of D1/D5 receptor antagonists or translation inhibitors. Complementary data showing that the duration of food caching memory could be extended by exploration of a novel environment and that the effects of novelty were prevented by inhibition of D1/D5 receptors and protein synthesis has recently been shown (Wang et al., 2010). Importantly, novelty exploration can upregulate immediate early genes (IEGs) thereby increasing levels of plasticity proteins (Guzowski et al., 1999). Collectively, these studies demonstrate that experiences that can upregulate plasticity proteins can facilitate long-term changes in synaptic plasticity and boost the duration of memory.

From a broader perspective, STC could provide a cellular correlate for associative memory formation as it would provide a synaptic basis for binding together distinct components of an experience into a unified memory. Additionally, this is a process which is bolstered through activation of neuromodulatory receptors (Wang et al., 2010)., which suggests that an understanding of how metabotropic receptor activation facilitates synaptic plasticity is critically important for elucidating the cellular mechanisms of memory formation.

## NOARDRENERGIC RECEPTORS: A ROLE IN SYNAPTC PLASTICITY

Activation of the noradrenergic system and subsequent noradrenaline (NA) release, promotes plasticity in brain structures that mediate enduring behavioral changes indicative of learning and memory (Harley et al., 1989; Devauges and Sara, 1991; Sara, 2009). The primary

source of hippocampal NA is the locus coeruleus , a brainstem nuclear group located bilaterally in the caudal pons (Moore and Bloom, 1979). Noradrenergic fibers project throughout the brain (Segal et al., 1973; Foote et al., 1983) and are capable of modulating global CNS functions including arousal, attention, sleep and memory (Kety, 1972; Ashton Jones et al., 2000; Berridge and Waterhouse, 2003; Carter et al., 2010; Takahashi et al., 2010). Neurons in the locus coeruleus demonstrate altered firing rates in response to novelty, stress, emotive experiences, and attentional shifts (Rasmussen and Jacobs, 1986; Abercrombie and Jacobs, 1987; Sara et al., 1994; Ashton-Jones et al., 1999) which modulates neural plasticity in the hippocampus (Segal and Bloom, 1976; Walling and Harley, 2004; Lemon et al., 2009).

## NORADRENERGIC RECEPTORS

Activation of locus coeruleus neurons results in increased secretion of noradrenaline. Noradrenaline binds to metabotropic receptors resulting in the activation of G proteins and subsequent regulation of intracellular signals. Noradrenergic receptors are divided into two classes; alpha (α) and beta (β), with several subtypes per class exhibiting unique expression profiles throughout the brain (McCune et al., 1993). NA enhances encoding, storage and retrieval of information (Hu et al., 2008) suggesting a selective role in long-term memory (LTM) (Izquierdo et al., 1998; Ji et al., 2003a; Ji et al., 2003b; Murchison et al., 2004)

## ALPHA-ADRENERGIC RECEPTORS

The cellular effects of NA are dependent on the concentration of NA, receptor localization and receptor subtype (Daly et al., 1981). Alpha-1 adrenergic receptors are expressed throughout the CA subfields where they are found on pyramidal and glial cells, in addition to astrocytes (Lerea and McCarthy, 1990; Duffy and MacVicar; 1995; Papay et al., 2006) although

significant interspecies variations exists in receptor localization (Zilles et al., 1993). Alpha-ARs couple to G(q) proteins which initiate phospholipase C and subsequent hydrolysis of phosphatidyl inositol diphosphate (PIP2) to inositol triphosphate (IP3) and diacyglcerol (DAG). IP3 liberates sequestered Ca2+, whereas DAG promotes kinase-substrate interactions through trafficking of protein kinase C (PKC) to the plasma membrane (Sirviö and MacDonald, 1999).

Noradrenaline can elicit a dose-dependent, bi-phasic response in cell excitability characterized by an initial depression followed by potentiation of population spikes (Curet and de Montigny, 1988). Results from Mynlieff and Dunwiddie (1988) showed that the initial depression was prevented by antagonizing a1-ARs, a result they confirmed using the a1-AR agonist phenylephrine, which decreased population spike amplitude in CA1. High levels of NA can also suppress synaptic potentials. In area CA1 induction of NA-dependent LTD requires a1-ARs (Scheiderer et al., 2004) which enhance LTD through an ERK-dependent mechanism (Vanhoose et al., 2002; Scheiderer et al., 2008). Both beta and a1-ARs are also found on astrocytes. Activation of astrocytic a1-ARs may serve to regulate calcium oscillations and glutamate release following metabotropic glutamate receptor stimulation (Muyderman et al., 2001) and may also play a role in gliogenesis (Papay et al., 2006). Finally, a1-AR-mediated depolarization of inhibitory interneurons has been reported (Hillman et al., 2009) which provides an alternative mechanism for suppressing synaptic transmission and cellular excitability in the hippocampus.

Recent examinations of the role of a1-ARs in hippocampal memory processing demonstrated that application of the selective a1-AR agonist, methoxamine, facilitated the consolidation of short-term to intermediate memory and intermediate to long-term memory using a discriminative avoidance task in chicks (Gibbs and Bowser, 2010). The selective antagonist

prazosin prevented memory consolidation by methoxamine. These effects appeared to rely on astrocytic a1-ARs as application of inhibitors of metabolic processes mediated by astrocytes (glycogenolysis or oxidation) prevented memory facilitation. These results provide evidence that a1-AR stimulation alone is sufficient for augmenting memory in contrast with previous results which suggested a co-facilitory role in spatial learning which required muscarinic receptors (Puumala et al., 1998) consistent with in vitro experiments (Scheiderer et al., 2007).

The a2-AR is located predominantly at the presynaptic terminal (Milner et al., 1998) where it performs a heteroinhibitory function, capable of regulating the release of non-noradrenergic transmitters including glutamate, (Boehm, 1999) and serotonin (Frankhuyzen and Mulder, 1982) which accounts for its efficacy as an antidepressant target (de Boer, 1995). In addition, a2-ARs also limit NA release through downregulation of presynaptic cAMP, which suggests an autoinhibitory function (Frankhuyzen and Mulder, 1982; Pittaluga and Raiteri, 1987). Consistent with a role in autoinhibition, local application of clonidine (an a2-agonist) to area CA1 decreased firing rates of pyramidal cells in response to LC stimulation (Curet and Montigny, 1988).

BETA-ADRENERGIC RECEPTORS

Beta-adrenergic receptor stimulation is the primary mechanism through which the noradrenergic system engages physiologic mechanisms underlying encoding and storage of new information. The noradrenergic system can boost LTP through activation of beta-adrenergic receptors which initiate cellular and molecular events that mediate synaptic plasticity (Thomas et al., 1996; Gelinas and Nguyen, 2005; see Gelinas and Nguyen, 2007 and O'Dell et al., 2010 for reviews). Beta-adrenergic receptors are found throughout the mammalian brain including the hippocampus (Wanaka et al., 1989; Nicholas et al., 1993). The primary subtypes expressed are

the β-1 and β-2 with β-3 showing limited expression (reviewed in Gelinas and Nguyen, 2007). beta-adrenergic receptors engage downstream signaling cascades through initiation of Gαs and subsequent AC-dependent cAMP generation (Minocherhomjee and Roufogalis, 1982; Raymond, 1995).

Activation of beta-adrenergic receptors can enhance depolarization through regulation of calcium-dependent potassium channels and voltage-dependent calcium channels (Madison and Nicoll, 1986; Hoogland and Saggau, 2004). Additionally, beta-adrenergic receptors boost synaptic and cellular excitability through initiation of signaling cascades coupling to downstream regulation of kinases that couple to translation (Straube et al., 2003; Gelinas and Nguyen, 2005; Gelinas et al., 2007; Tenorio et al., 2010

Beta-adrenergic receptors can boost synaptic plasticity throughout the hippocampus in a subregion specific manner. Tetanization of perforant path fibers which project to the DG generates LTP which requires β-adrenergic receptors (Bramham et al., 1997; Munro et al., 2001). Additionally, depletion of NA in the dentate gyrus decreases LTP (Stanton and Sarvey, 1985). LTP at mossy fiber-CA3 synapses requires beta-adrenergic stimulation as inhibition of β-ARs with propranolol prevents the expression of long-lasting LTP induced by multiple trains of high-frequency stimulation (Huang et al, 1994; Huang and Kandel, 1996). Conversely, in areas CA3, CA1 and the subiculum, activation of β-ARs decreases the threshold for LTP induction and maintenance (Huang and Kandel, 1996; Thomas et al., 1996; Katsuki et al, 1997; Winder et al., 1999; Gelinas and Nguyen, 2005; Huang and Kandel, 2005, Tenorio et al., 2010). Similar to memory formation (Davis and Squire, 1984; Deadwyler et al., 1987; Huang et al., 1996; see Abraham and Williams, 2008; Costa-Mattioli et al., 2009 for review), beta-adrenergic receptor-

dependent LTP requires translation (Gelinas and Nguyen, 2005, Gelinas et al., 2007; Tenorio et al., 2010).

Consistent with a role in long-term memory formation, inhibition of β-ARs impairs the formation of both spatial and contextual fear memory (Sara et al., 1999; Cahill et al, 2000; Ji et al., 2003a, 2003b). Conversely, activation of beta-adrenergic receptors facilitates hippocampal long-term plasticity necessary for memory formation (Kemp and Manahan-Vaughan, 2008; Lemon et al., 2009). β-ARs have also been implicated in memory retrieval (Cahill et al., 1994; Barros et al., 2001; Murchison, 2004). Restoring NA levels in mutant mice engineered to lack noradrenaline, improved memory retrieval through a mechanism apparently requiring beta-1 ARs (Muchison et al., 2011). Injection of NA into the hippocampus preferentially facilitated long-term memory (Izquierdo et al., 1998) suggestive of a role in consolidation (Izquierdo and Medina, 1997; Sara et al., 1999). Finally, β-ARs have also been implicated in "extinction", a form of memory requiring formation of new associations to altered stimulus contingencies (Ouyang and Thomas, 2005). Thus, β-ARs are capable of mediating several components of memory, which provides further impetus for understanding the mechanisms through which they alter hippocampal function.

**ACETYLCHOLINE: CHOLINERGIC RECEPTORS**

Studies of the motor endplate in the 1950s determined that the primary neurotransmitter responsible for end-plate potentials is acetylcholine (Fatt and Katz, 1950). In addition, cholinergic terminals are found throughout the brain, with the primary projections to the hippocampus originating in the medial septum and the diagonal band of Broca (Dutar et al., 1995; Cobb et al., 1999). Acetylcholine critically modulates numerous facets of cognitive functioning through activation of both nicotinic and muscarinic receptors (Hasselmo, 1999).

Acetylcholine release from cholinergic projections triggers the theta rhythm in the hippocampus (Dudar, 1977) which has been correlated with learning and memory (Huerta and Lisman, 1995; Hoffman et al., 2002). Cholinergic agonists applied to hippocampal tissue facilitates synaptic and cellular plasticity (Shinoe et al,. 2005; Dringenberg et al., 2008; Scheiderer et al., 2008; Buchanan et al., 2010) and can enhance memory function (Huerta and Lisman, 1995; Hoffman et al., 2002; Hasselmo, 2006; Herrera-Morales et al., 2007).

NICOTINIC RECEPTORS

Nicotinic receptors (nAChRs) exhibit a pentameric structure consisting of combinations of alpha (α1-α10) and beta (β2-β4) subunits which determine receptor structure and kinetics (Fenster et al., 1997; Nai et al., 2003). The primary neuronal nAChR subtype is α4β2 which conducts primarily monovalent ions (Na+, K+), however, the Ca2+-permeable α7 subunit is highly expressed in the hippocampus where it localizes both pre- and post-synaptically (Seguela et al., 1993; Fabian-Fine et al., 2001). Nicotine can induce hippocampal LTP (Fujii and Sumikawa, 2000) and improve cognitive functions, including memory (Davis et al., 2007; See Kenny and Gould, 2008 for review).

MUSCARINIC RECEPTORS

Similar to nAChRs, muscarinic receptors (mAChRs) are found throughout the CNS. Muscarinic receptors couple to G-proteins which once activated, can augment post-synaptic responses on temporal and spatial scales far exceeding nACRs. There are 5 known muscarinic receptor subtypes (M1-M5) which can be further subdivided based on the signal cascades and cellular responses they mediate. M1, M3 and M5 receptors couple to Gq/11 which triggers the hydrolysis of PIP2 to IP3 and DAG (Brown, 2010). IP3 liberates intracellular Ca2+ from the

smooth endoplasmic reticulum (SER) through activation of IP3 and ryanodine receptors whereas DAG mobilizes protein kinase C (PKC) to the plasma membrane thereby facilitating kinase-substrate interactions (Brown, 2010). M2 and M4 are located predominantly at presynaptic terminals where they decrease neurotransmitter secretion through negative regulation of adenyl cyclase. Additionally, there is some evidence that stimulation of both M1 and M4 results in increased cAMP synthesis (Migeon and Nathanson, 1994). The primary muscarinic receptor expressed in rodent hippocampus is the M1 subtype which is found on pyramidal cells, with higher expression levels being observed in CA1 relative to CA3 and dentate gyrus (Levey et al., 1995). M2 and M4 demonstrate lower expression levels restricted largely to inhibitory interneurons. M3 and M5 are expressed at low levels relative to other receptor subtypes.

Although the M3 muscarinic receptor has recently been implicated in the memory enhancing effects of muscarinic receptor stimulation (Poulin et al., 2010), previous research has demonstrated that the M1 muscarinic receptor is primarily responsible for enhancing hippocampal synaptic plasticity (Anagnostaras et al., 2003; Scheiderer et la., 2008; Giessel and Sabatini, 2010). M1 receptor stimulation potentiates NMDAR responses which facilitates post-synaptic depolarization and Ca2+ influx (Sur et al., 2003; Shinoe et al., 2005). M1 muscarinic receptors also enhance synaptic potentials through inhibition of small conductance potassium channels (Giessel and Sabatini, 2010).

Previous research has shown that muscarinic receptors facilitate the induction of LTP (Auerbach and Segal, 1994; Shinoe et al., 2005) through M1 receptor activation (Anagnostaras et al., 2003; Shinoe et al., 2005; Buchanan et al, 2010; Giessel and Sabatini, 2010). Similarly, in vivo experiments have also implicated the M1 receptors in enhanced LTP following weak stimulation of afferents converging on CA1 pyramidal cell basal dendrites (Ovsepian et al.,

2004). Thus, muscarinic receptor activation is capable of augmenting synaptic efficacy in the hippocampus which suggests a role in learning and memory.

## MUSCARINIC EFFECTS ON LEARNING AND MEMORY

Muscarinic receptors modulate memory formation as demonstrated by studies using scopolamine (a muscarinic receptor antagonist) which impaired the retention of contextual fear memory when administered around the time of training (Wiener and Messer, 1973; Anagnostaras et al., 1995; Wallenstein and Vago, 2001). Long-term spatial memory formation was similarly impaired when scopolamine was given prior to training in the Morris water maze (Herrera-Morales et al., 2007). Infusion of a muscarinic antagonist into the ventral hippocampus also impaired social transmission of food preference (Carballo-Márquez et al., 2009). Collectively, these studies support the view that muscarinic receptors are particularly involved in the genesis of hippocampus-dependent, long-term memories.

## NEUROMODULATION OF TRANSLATION REGULATION

The establishment of long-lasting synaptic plasticity requires protein synthesis (Flexner et la., 1963; Stanton and Sarvey, 1984; Deadwyler et al., 1987; Frey et al., 1988; Huang et al., 1996; Kandel, 2001; Tanaka et al, 2008) which can be localized to dendrites (Feig and Lipton, 1993; Kang and Schumann, 1996; Huber et al., 2001; Gelinas and Nguyen, 2005). Translation during long-term potentiation (LTP) and long-term depression (LTD) are importantly linked to memory function in the mammalian brain (Whitlock et al., 2006; Lemon et al., 2009). Although the function(s) of newly synthesized proteins in maintaining long-term synaptic changes has yet to be fully elucidated (but see Woo and Nguyen, 2003), there is strong evidence that neuromodulatory receptors including β-adrenergic and muscarinic receptors, can couple to

protein synthesis regulatory signaling cascades (Feig and Lipton, 1993; Gelinas and Nguyen, 2005; Gelinas et al., 2007; Scheiderer et al, 2008). β-ARs engage translation via ERK- and mTOR-dependent translation initiation (Gelinas et al. 2007). ERK and mTOR signaling cascades are implicated in the translational regulation of various forms of synaptic plasticity (Tang et al., 2002; Kelleher et al., 2004a; Kelleher et al., 2004b; Banko et al., 2006; Gelinas et al., 2007). Initiation of these signaling cascades provides a molecular mechanism for the enhanced maintenance of LTP generated by activating β-adrenergic receptors during LTP induction (Gelinas and Nguyen, 2005). An important question in the neuroscience of learning and memory is: How are innocuous events separated from significant events that should be learned and remembered? Neuromodulators such as NA and ACh act through initiation of translation, thereby providing a means for marking stimuli as worthy of encoding and storage. This could provide a neural mechanism for enhancing the saliency of stimuli which in the absence of neuromodulators would be sub-threshold for associative learning and memory.

**NEUROLOGICAL DISORDERS: FRAGILE X SYNDROME**

Neurological disorders have been linked to dysregulated neuromodulatory system function for decades (Adolfsson et al., 1979; Auld et al., 2002). Fragile X syndrome (FXS) is a genetic disorder characterized by cognitive deficits including mental retardation (Schneider et al., 2009). Recent evidence suggests that the primary neuropathology linked to the cognitive deficits observed in Fragile X syndrome is decreased levels of fragile X mental retardation protein (FMRP) (Nosyreva and Huber, 2006; Pfeiffer and Huber, 2009; see Fatemi and Folsom for review) . FMRP is an RNA binding protein which has been implicated in the trafficking and repression of transcripts (Laggerbauer et al., 2001; Li et al., 2001). In the absence of FMRP basal translation rates increase (see Waung and Huber, 2009), with several proteins linked to synaptic

plasticity (Muddashetty et al., 2007; Waung et al., 2009; Schütt et al., 2009) demonstrating elevated expression levels. Pioneering work conducted by Mark Bear and Kim Huber (2004) has demonstrated that metabotropic glutamate receptor (mGluR) signaling is altered in FXS. Specifically, they were able to show that long-term depression induced by pairing chemical activation of mGluRs with low-frequency stimulation is enhanced in the FXS mouse model (Fmr1 knockout) (Huber et al, 2002; Bear et al, 2004). Enhanced mGluR-LTD appears to be the result of dysregulated translation as application of a translation inhibitor does not block LTD in Fmr1 knockout mouse hippocampus (Nosyreva and Huber, 2006). Long-term depression induced by mGluR agonists initiates downstream signaling cascades (including ERK and mTOR) which couple to translation regulation at synapses (Huber et al., 2000; Gallagher et al., 2004; Hou et al., 2006) and are similarly engaged by beta-adrenergic (Gelinas et al., 2007) and muscarinic receptors (Watabe et al., 2000; Volk et al., 2007; Deguil et al., 2008)(Fig. 1.3). As protein synthesis is critically involved in cellular processes linked to memory, understanding how synaptically localized protein synthesis is dysregulated in Fmr1 mice should provide insights into memory deficits observed in FXS.

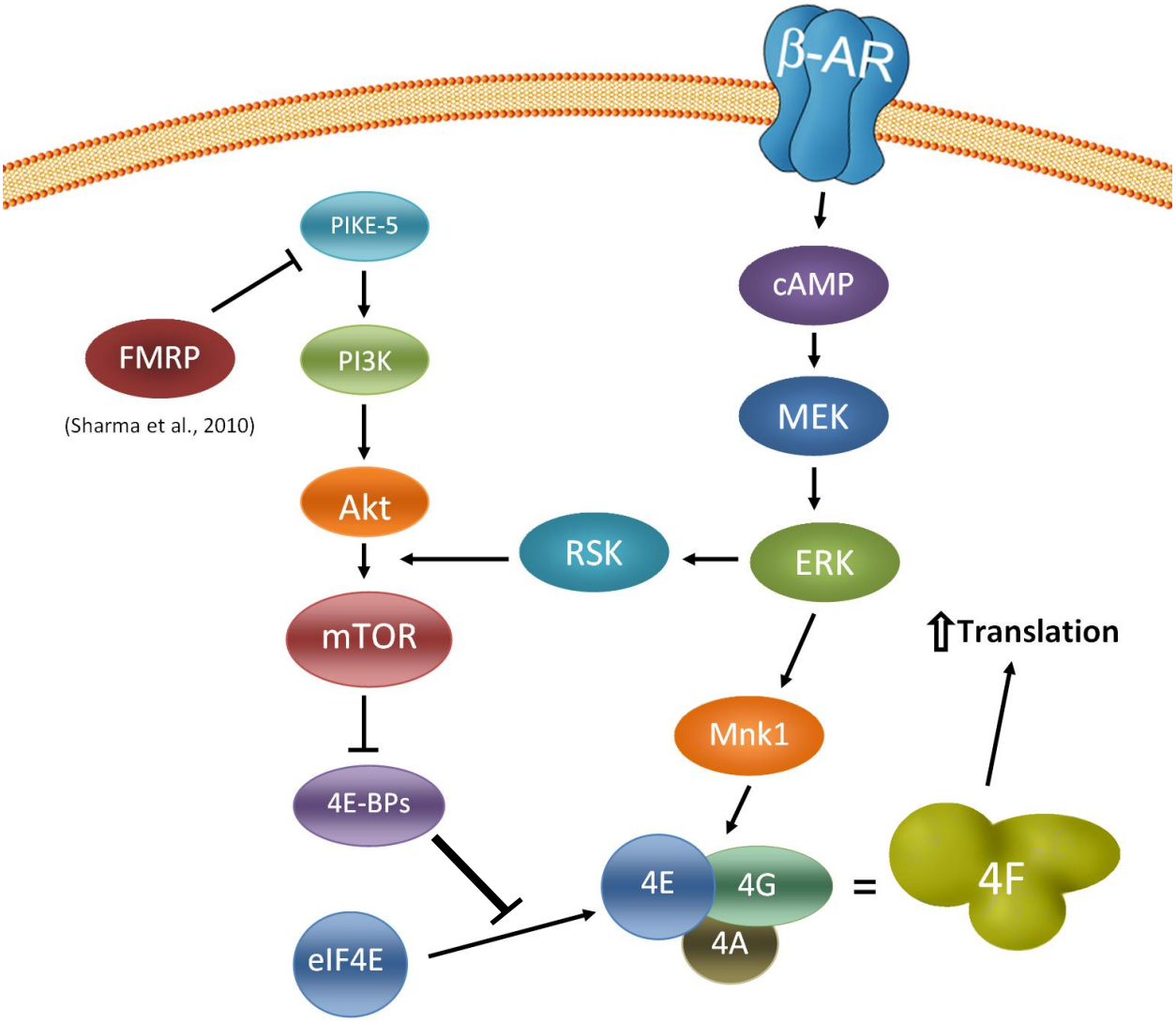


Figure 1.3. FMRP acts as an inhibitory constraint on translation. Through inhibition of PIKE-S, FMRP can prevent downstream mTOR activation which prevents phosphorylation of the eIF4E binding protein (4E-BP), thus maintaining 4E in a bound and inactive state. Beta-adrenergic receptors similarly converge on mTOR to boost translation through activation of mTOR .

## THESIS OBJECTIVES

Understanding how unified percepts are encoded on extended time scales has recently emerged as central to determining how new memories are formed. Neuromodulators can initiate protein synthesis capable of boosting synaptic plasticity required for long-term synaptic changes underlying LTP and long-term memory. Determining the mechanisms through which neuromodulators mediate their effects on synaptic plasticity and protein synthesis, should advance our understanding of the cellular and molecular basis of learning and memory. Consequently, the primary objective of my research is to characterize the intracellular mechanisms engaged by neuromodulators during the induction and expression of homo- and heterosynaptic plasticity.

The first objective of my thesis was to determine how beta-adrenergic receptors influence synaptic plasticity in a cell-wide manner. My second objective was to determine if beta-adrenergic receptor-dependent heterosynaptic plasticity is augmented in a mouse model of Fragile X syndrome and if so, the mechanistic differences responsible for the aberration(s). Finally, the third objective was to determine how two different neuromodulatory receptors (beta-adrenergic and muscarinic) implicated in learning and memory processes, interact to regulate protein synthesis capable of enhancing synaptic plasticity. I have attempted to address the following questions in my thesis:

1. Can beta-adrenergic receptors facilitate heterosynaptic plasticity?
2. What are the cellular mechanisms required for heterosynaptic plasticity?
3. Is heterosynaptic plasticity altered in a mouse model for Fragile X syndrome?
4. Can co-activation of beta- and muscarinic receptors alter hippocampal plasticity?

The primary goal of this thesis is to elucidate some of the cellular mechanisms initiated by neuromodulatory receptors and determine how these mechanisms influence synaptic plasticity required for memory formation in mammalian brains. Characterization of these mechanisms will advance our understanding of the cellular basis of memory and may provide the basis for novel neurocognitive therapeutics in the future.

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# CHAPTER II:

General Methodology

## Animals

### *C57BL/6 Mice*

C57BL/6 is a widely used inbred strain for neuroscience research. It is frequently used as a background strain for generating congenic and transgenic mice. Male C57BL/6 mice, aged 7-13 weeks, obtained from Charles River, Canada (unless otherwise stated) were used for all experiments. Animals were housed at the University of Alberta using guidelines approved by the Canadian Council on Animal Care (CCAC). Mice were maintained at New York University and at the University of Alberta consistent with Institutional Animal Care and Use Committee (IACUC) and CCAC guidelines. Controls were wildtype littermates of mutant mice. I was blind to genotypes.

## Slice Preparation and Electrophysiology

Following cervical dislocation and decapitation, the brain was removed from the skull and transferred to a beaker of ice-cold artificial cerebrospinal fluid (ACSF; composition in mM: 124 NaCl, 4.4 KCl, 1.3 $MgSO_4$, 1.0 $NaH_2PO_4$, 26.2 $NaHCO_3$, 2.5 $CaCl_2$, 10 Glucose). Following a brief recovery period, the hippocampus was dissected from the cortical tissue through gentle separation using surgical spatulas. The hippocampus was sectioned (transverse plane; 400µm thickness/slice) using a manual tissue chopper (Stoelting). Slices were transferred to a glass petri dish containing ice-cold ACSF which was continually oxygenated. Transversely sectioned hippocampal slices were subsequently transferred to an interface recording chamber. This interface chamber maintained the slice environment at 28$^{o}$C and allowed continuous perfusion with ACSF (1-2mL/min) aerated with carbogen (95% $CO_2$ and 5% $O_2$) .

Following a minimum 2 hr recovery period, extracellular field excitatory postsynaptic potentials (fEPSPs) were recorded with a glass microelectrode (1-3 MΩ) which contained ACSF. Both the recording and stimulating electrodes were placed in stratum radiatum of area CA1. Electrical stimulation of the Schaeffer collateral fibres using an extracellular bipolar nickel-chromium electrode was used to evoke fEPSPs. The stimulation intensity (0.08 ms pulse width) was adjusted to generate fEPSPs with amplitudes of approximately 40% of maximal fEPSP sizes. The resulting fEPSPs were used to establish baseline by stimulating 1/min for at least 20 min. Slices with maximal fEPSP sizes of less than 3 mV were not used.

For two-pathway experiments (Fig. 2.1), a dual stimulating electrode protocol was used in which two stimulating electrodes were placed in the stratum radiatum on opposite sides of the recording electrode. The independence of the two pathways was verified by the absence of paired-pulse facilitation when two sets of stimuli were delivered to the two pathways at 50, 75, 100, 150 and 200 ms interpulse intervals (Fig. 2.2).

All evoked fEPSPs were low pass filtered (1-3 KHz) and amplified by an intracellular amplifier (AxoClamp 2B). Evoked responses were digitized using the Digidata 1440A acquisition system (Axon Instruments), at a rate of 20 KHz and were recorded with pCLAMP 10.0 software (Axon Instruments). Data analysis was performed offline using Clampex 10.0 software (Axon Instruments) by measuring the initial slope of the evoked fEPSPs (Johnston and Wu, 1995).

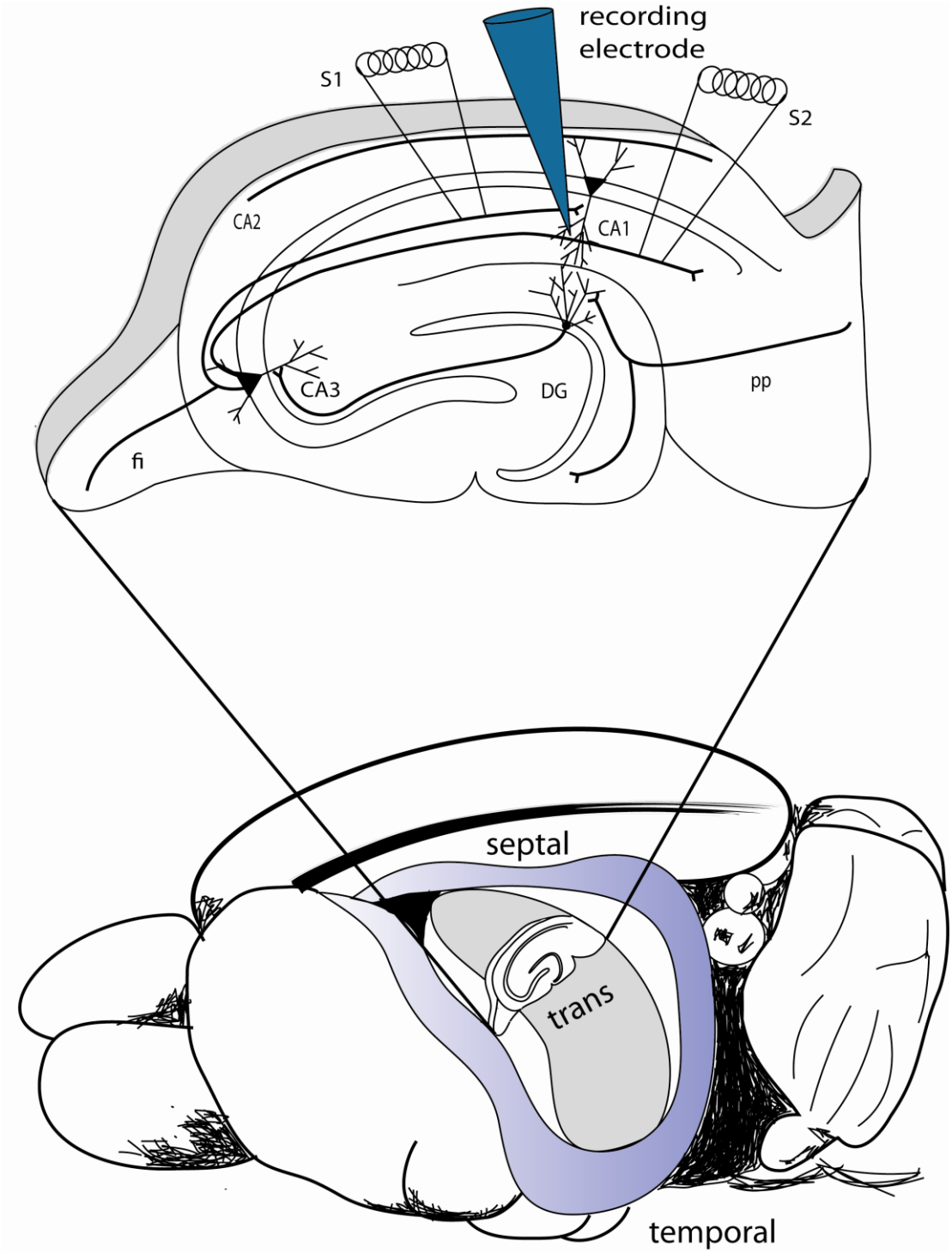



Figure 2.1. Experimental configuration for conducting two-pathway recordings. Two separate stimulating electrodes (S1 and S2) flank a recording electrode located in stratum radiatum or area CA1. Stimulating electrodes are staggered relative to each other to ensure independence of synaptic pathways. Adapted from Andersen et al., 2007.

Electrodes

Bipolar stimulating electrodes were created by twisting two nickel chromium wires (final diameter, approximately 130μm; A-M Systems) together and inserting them into a glass electrode. One end of the wires was flamed to remove the formvar coating which allowed for better electrical contact to the dual channel stimulator (Grass S88). Glass recording electrodes were fabricated in house using a P-47 flaming brown micropipette puller (Sutter Instruments). The recording electrodes (resistance of approximately 1 – 3 MΩ) were filled with ACSF prior to commencing experiments.

Stimulation Protocols

LTP was induced by applying either a single one-second train (100 Hz, at test strength), 100 Hz paired with isoproterenol (1μM, applied for 15 min.). Theta-pulse stimulation (5 Hz, 5 s or 5 Hz, 10 s) was used as a form of subthreshold, low-frequency stimulation. Depotentiation was induced by applying low-frequency stimulation (LFS) after LTP induction (5 Hz, 3 min).

Drugs

Several drugs were used to characterize the cellular mechanisms involved in changes in synaptic function (see Methods sections of individual chapters). Concentrated stock solutions were made by dissolving drugs in the appropriate solvents. In preparation for experiments, the stock solutions were diluted with ACSF to the desired final concentration. Drug solutions were then bath-applied to hippocampal slices (1-2ml/min). Solutions in which DMSO was used as the solvent were prepared with DMSO levels of less than 0.1%. At this concentration, DMSO did not affect basal synaptic transmission or LTP (data not shown). All experiments using

photosensitive drugs were performed under dimmed-light conditions. Drug experiments were interleaved with drug-free controls.

Data and Statistical Analysis

The initial slope of the fEPSP was used as an index of synaptic strength (Johnston and Wu, 1995). Baseline slope values were established by applying 1 pulse per min at 40% of maximal evoked fEPSPs over a period of 20 min prior to experimental manipulations. For each experiment, fEPSP slopes were expressed as a percentage of baseline slope values. Statistical comparisons were conducted using a Student's t-test for 2 groups or an ANOVA with a Tukey-Kramer post-test for 3+ groups using Instat software (GraphPad). Results were considered significant at a level of $p < 0.05$. All values shown are mean ± standard error (SEM), with n = number of slices.

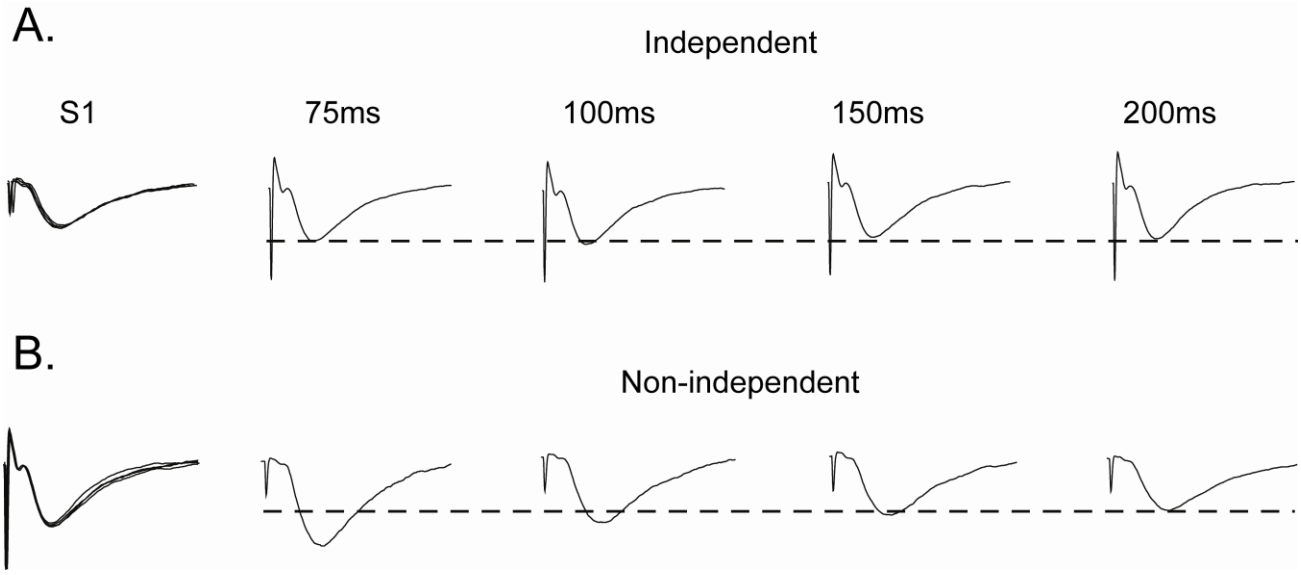



Fig. 2.2. Testing for independence of synaptic pathways. A. Progressive reduction in the interpulse interval between synaptic pathways demonstrates no significant interactions between stimuli applied at S1 (overlapping traces, far left) and S2 (individual pulses starting at far right 200ms and continuing to 75ms at left), suggesting that stimuli are activating non-overlapping (independent) groups of synapses. B. When the synaptic pathways are non-independent, progressively decreasing the interpulse interval results in an increase in the amplitude of the fEPSP as the time between stimuli at S1 and S2 is decreased. This is due to residual presynaptic calcium from the first pulse which would enhance neurotransmitter release and evoked responses if both stimuli were activating the same population of synapses.

## CHAPTER III:

# Activation of beta-adrenergic receptors enhances heterosynaptic plasticity in mouse hippocampus

Portions or this chapter has previously been published (Journal of Physiology, Copyright 2011; Wiley Publishing). I conducted all experiments and co-authored this manuscript. Yu Tian Wang provided the TAT-GluR2 peptide. Peter Nguyen co-authored the manuscript.

**Abstract**

Noradrenaline critically modulates the ability of synapses to undergo long-term plasticity on time scales extending well beyond fast synaptic transmission. Noradrenergic signaling through beta-adrenergic receptors (β-ARs) enhances memory consolidation and can boost the longevity of LTP. Previous research has shown that stimulation of one synaptic pathway with a protocol that induces persistent, translation-dependent LTP can enable the induction of LTP by subthreshold stimulation at a second, independent synaptic pathway. This heterosynaptic facilitation depends on the regulation and synthesis of proteins. Recordings taken from area CA1 in mouse hippocampal slices showed that induction of β-AR-dependent LTP at one synaptic pathway (S1) can facilitate LTP at a second, independent pathway (S2) when low-frequency, subthreshold stimulation is applied after a 30 minute delay. β-AR-dependent heterosynaptic facilitation requires protein synthesis as inhibition of mammalian target of rapamycin (mTOR), extracellular signal-regulated kinase (ERK), or translation, prevented homo- and heterosynaptic LTP. Shifting application of a translational repressor, emetine, to coincide with S2 stimulation did not block LTP. Heterosynaptic LTP was prevented in the presence of the cell-permeable cAMP-dependent protein kinase A inhibitor, PKI. Conversely, the time window for inter-pathway transfer of heterosynaptic LTP was extended through inhibition of GluR2 endocytosis. My data show that activation of β-ARs boosts the heterosynaptic expression of translation-dependent LTP. These results suggest that engagement of the noradrenergic system may extend the associative capacity of hippocampal synapses through facilitation of intersynaptic crosstalk.

Introduction

Noradrenaline (NA) is a neuromodulatory transmitter secreted in response to arousing or novel stimuli (Aston-Jones and Bloom, 1981; Sara and Segal, 1991; see Berridge and Waterhouse, 2003 for review). Activation of beta-adrenergic receptors by NA engages signaling mechanisms which facilitate neuroplasticity (Lacaille and Harley, 1985; Harley et al., 1996; Gelinas and Nguyen, 2005) and memory genesis (Izquierdo et al., 1998; Straube et al., 2003; Lemon et al., 2009; reviewed in O'Dell et al., 2010). Stimulation of beta-adrenergic receptors in the hippocampus, a brain structure required for memory formation (Scoville and Milner, 1957; Zola-Morgan et al., 1986; Eichenbaum, 2000), facilitates activity-dependent increases in synaptic strength (Thomas et al., 1996; Gelinas and Nguyen, 2005) known as long-term potentiation (LTP) (Bliss and Lomo, 1973; Bliss and Collingridge, 1993; Neves et al., 2008).

Previous research has shown that β-adrenergic receptors enhance LTP through regulation of protein synthesis (Walling and Harley, 2004; Gelinas and Nguyen, 2005; Gelinas et al., 2007). Translation regulation can serve as a priming mechanism for long-term synaptic changes in a cell-wide manner (Frey and Morris, 1997), including heterosynaptic metaplasticity (Abraham et al, 2001; Abraham et al., 2007). Similarly, heterosynaptic facilitation, a form of synaptic plasticity in which synaptic activity at one group of synapses initiates cellular mechanisms capable of facilitating synaptic strength at another group of synapses converging on the same postsynaptic cells, requires protein synthesis (Frey and Morris, 1997; 1998). As beta-adrenergic receptors couple to signaling cascades implicated in translation regulation (Gelinas et al., 2007), I sought to determine if the beta-adrenergic receptor agonist, isoproterenol (ISO), could enhance heterosynaptic facilitation in mouse hippocampus, CA1.

I characterized the effects of beta-adrenergic receptor activation on heterosynaptic facilitation of LTP using an in vitro, dual synaptic pathway protocol. In mouse hippocampus area CA1, two independent populations of synapses contacting the same postsynaptic cells were monitored to determine the effects of prior induction of homosynaptic β-AR-dependent LTP at one pathway (S1) on the subsequent induction of heterosynaptic long-term potentiation at a second pathway (S2). Herein, I have characterized the mechanisms through which β-ARs mediate heterosynaptic long-term potentiation.

Methods

*Ethical Approval*: The experiments and methods of this paper were approved by the University Animal Policy and Welfare Committee (UAPWC) at the University of Alberta using guidelines approved by the Canadian Council on Animal Care (CCAC). Approximately 150 C57BL/6 mice (aged 7-12 wks) were used for these experiments. Hippocampal tissue slices were harvested following cervical dislocation and decapitation in accordance with UAPWC and CCAC guidelines.

*Electrophysiology*. Transverse hippocampal slices (400 μm thickness) were prepared as described by Nguyen and Kandel (1997). Briefly, following cervical dislocation and decapitation, the hippocampus was removed and sliced using a manual tissue chopper (Stoelting, Wood Dale, IL). Slices were maintained in an interface chamber at 28 °C and perfused 1-2 ml/min with artificial CSF (ACSF) composed of the following (in mM): 124 NaCl, 4.4 KCl, 1.3 MgSO4, 1.0 NaH2PO4, 26.2 NaHCO3, 2.5 CaCl2 and 10 glucose, aerated with 95% O2 and 5% CO2. Slices were allowed to recover for 120 min prior to experiments. Extracellular field EPSPs (fEPSPs) were recorded with a glass microelectrode filled with ACSF (resistances, 2–3 MΩ) and

positioned in the stratum radiatum of area CA1. fEPSPs were elicited by using two bipolar nickel-chromium electrodes placed in stratum radiatum to stimulate two separate sets of inputs converging onto the same postsynaptic population of neurons. Interpathway paired-pulse facilitation elicited by successive stimulation through the two electrodes at 75-, 100-, 150- and 200-ms intervals was used to test for independence of synaptic pathways during baseline acquisition and at the conclusion of experiments. Pathways were considered independent when no facilitation was observed following pairs of pulses. Stimulation intensity (0.08 ms pulse duration) was adjusted to evoke fEPSP amplitudes that were 40% of maximal size (Gelinas and Nguyen, 2007; Woo and Nguyen, 2003). Subsequent fEPSPs were elicited at the rate of once per minute at this "test" stimulation intensity, with S1 stimulation preceding S2 stimulation by 200 ms.

After establishing a 20 min baseline recording, β-AR-dependent LTP was induced by applying one train of high-frequency stimulation (HFS; 100 Hz, 1 s duration at test strength) following a 10 min application of the β-AR agonist, isoproterenol (ISO; 1μM). ISO was applied for an additional 5 mins following HFS. 30 min after HFS at S1, a LFS (5 Hz, 10 s duration) was applied to S2. Depotentiation was induced using a previously established protocol consisting of 5 Hz, 3 min stimulation applied 15 min after LFS (Young and Nguyen, 2005). To assess the duration of activity-dependent synaptic changes, homosynaptic LFS (5 Hz 10 s) was applied followed by a 30 min or 1 h delay prior to heterosynaptic (1x100 Hz, 1 s paired with ISO) stimulation.

*Drugs*: The β-AR agonist [*R*(-)-isoproterenol(+)-bitartrate; Sigma, St. Louis, MO] was prepared daily as concentrated stock solutions at 1 mM, in distilled water and applied at a final concentration of (1 μM; Sigma). The translation inhibitor, emetine (EME; Sigma) was dissolved

in DMSO to a stock concentration of 20 mM in distilled water. At lower concentrations than those used here (20 µM), EME blocked protein synthesis by >80% in hippocampal slices (Stanton and Sarvey, 1984). EME was perfused for 20 min prior to commencing experiments. The β-AR antagonist (±)-propranolol hydrochloride (PROP; Sigma) was prepared daily in distilled water as a 50 mM stock solution and was applied at a final concentration of 50 µM. A MEK inhibitor, 2-(2-amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one (PD 98059) (50 µM; Sigma), was prepared in DMSO at a stock concentration of 10 mM and was applied 30 min prior to experiments. Rapamycin (Sigma), an mTOR inhibitor was dissolved in DMSO to make stock solutions at 1 mM, diluted to 1 µM and was applied 30 min prior to experiments. The cell membrane permeant PKA inhibitor 14-22 Amide (Calbiochem, La Jolla, CA) was dissolved in ACSF (1 mM stock) and applied at 1 µM final concentration. An inhibitor of $GluR_2$ endocytosis, the peptide Tat-$GluR2_{3Y}$ and a scrambled control peptide, Tat-$GluR2_{3S,}$ were applied at 4 µM, 30 minutes prior to LFS and during the delay between weak and strong stimulation. Due to photosensitivity of the drugs, experiments were performed under dimmed light conditions. Drug experiments were interleaved with drug-free controls.

*Data analysis.* Axon Clampex (10.2) (Molecular Devices) was used for fEPSP analysis. To quantify changes in synaptic strength, the initial slope of the fEPSP was measured (Johnston and Wu, 1995) . The average "baseline" slope values were acquired over a period of 20 min before experimental protocols were applied. fEPSP slopes were measured at either 120 or 90 min after HFS or LFS for comparisons of LTP. Student's *t* test was used for statistical comparisons of mean fEPSP slopes between two groups, with a significance level of $p < 0.05$. For comparison of more than two groups, one-way ANOVAs were conducted followed by Tukey–Kramer tests for *post hoc* comparisons. The Welch correction was applied in cases in which the SDs of groups

being compared were significantly different. All values shown are means ± SEM, with *n* = number of slices.

Results

**β-Adrenergic receptor activation facilitates heterosynaptic LTP in mouse CA1**

In the first set of experiments, I tested the idea that β-adrenergic receptor activation primes heterosynaptic LTP in mouse CA1. Initially I assessed whether application of high-frequency stimulation (HFS; 100 Hz, 1 s) to one synaptic pathway (S1) could facilitate heterosynaptic LTP induced by low-frequency stimulation (LFS; 5 Hz, 10 s) applied 30 min later to a second, independent set of synapses. High-frequency stimulation generated potentiation which decayed to baseline in < 2 h (Fig. 3.1*A*; fEPSPs 120 min after HFS were 111±10%, n=8). Low-frequency stimulation applied 30 min after HFS similarly induced decremental potentiation (fEPSPs 90 min after LFS were 103±8%, n=8). These results suggest that neither 100 Hz nor 5 Hz stimulation alone is sufficient for initiating long-lasting (> 2 h) LTP.

Next, I asked whether β-AR application alone would facilitate the induction of LTP by subthreshold stimulation applied heterosynaptically. Application of isoproterenol (ISO; 1μM) for 15 min, thirty minutes before low-frequency stimulation (5 Hz, 10 s) at S2, failed to facilitate LTP at either S1 (fEPSPs were 102±8% 120 min post ISO application) or S2 (fEPSPs at S2 were 99±9% 90 min after LFS, n=10) (Fig. 3.1*B*). Thus, activation of β-ARs in the absence of HFS does not initiate heterosynaptic facilitation.

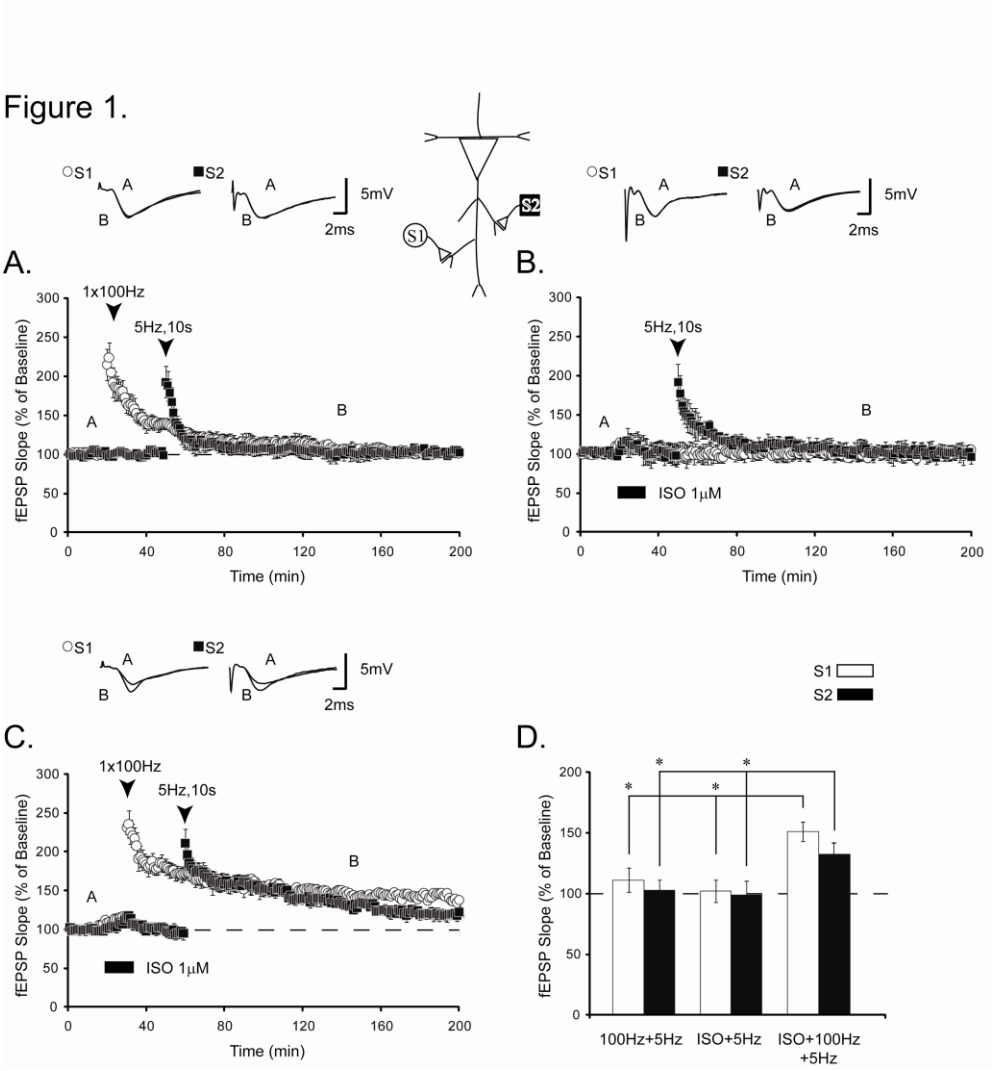



Figure 3.1 Beta-adrenergic receptor activation primes heterosynaptic facilitation. *A.* 100 Hz stimulation alone (open circles) induces transient (< 2 h) LTP which does not facilitate heterosynaptic LTP following low frequency stimulation (5 Hz, 10 s; filled squares) at a second synaptic pathway (n=8). LFS does not induce persistent changes in synaptic strength as fEPSPs at S2 returned to baseline in < 1 h. *B.* Low frequency stimulation 25 min after application of ISO alone has no long-lasting effects on synaptic transmission (n=8), which shows that ISO alone is insufficient for facilitating heterosynaptic LTP. *C.* Application of 1x100 Hz stimulation to S1 (filled circles) paired with isoproterenol (ISO) facilitates the induction of LTP (open squares) that can subsequently be captured by low-frequency stimulation at S2 (n=10). *D.* Summary histogram comparing fEPSP slopes obtained 120 min after HFS at S1 (white bars) and 90 min after LFS at S2 (black bars). "*" indicates significant differences between treatment groups. Sample traces were taken 10 min after commencement of baseline recordings, 120 min after stimulation at S1 and 90 min after stimulation of S2. Results in C represent means ± SEM, $*p < 0.05$.

Previous research has demonstrated that pairing β-AR activation (ISO, 1μM) with HFS (100 Hz, 1 s) induces long-lasting LTP which requires translation (Gelinas and Nguyen, 2005; Gelinas et al., 2007). Can prior induction of β-AR-dependent LTP facilitate heterosynaptic long-term potentiation? Pairing ISO (1 μM) with HFS homosynaptically (S1) induced LTP that was potentiated to 151±8% (n=10) of baseline 2 hrs post stimulation (Fig. 3.1*C*). LFS applied 30 min later to S2 generated enhanced LTP (fEPSPs were 131±7% of baseline 90 min after LFS; Fig. 1*C*). An ANOVA that compared fEPSPs 120 min after either 100 Hz alone, ISO alone or ISO+100 Hz at S1 demonstrated significant differences between groups ($F_{(2,25)} = 9.77$; $p < 0.01$). Subsequent Tukey-Kramer *post hoc* tests revealed that ISO+HFS generated homosynaptic LTP that was significantly greater than either 100 Hz or ISO alone ($p < 0.05$). When differences between heterosynaptic pathways stimulated with LFS following 100 Hz alone, ISO alone or ISO+100 Hz applied homosynaptically were compared, a significant difference between groups was detected ($F_{(2,25)} = 4.86$; $p < 0.02$). Tukey-Kramer *post hoc* tests showed that subthreshold LFS at S2 induced long-lasting LTP only when it was preceded by ISO+100 Hz stimulation at S1 ($p < 0.05$; Fig. 3.1*D*). As heterosynaptic LTP was not facilitated by either ISO alone or 100 Hz HFS, these results show that pairing β-adrenergic receptor activation with 100 Hz HFS elicits stable LTP that can be transferred heterosynaptically in an activity-dependent manner.

**Heterosynaptic facilitation elicited by ISO application requires β-adrenergic receptors**

To determine if β-ARs are necessary for ISO-induced heterosynaptic facilitation, I co-applied a β-AR antagonist, propranolol (50 μM), overlapping with ISO and high-frequency stimulation. Induction of homosynaptic LTP was completely blocked in the presence of propranolol (Fig. 3.2*A*). fEPSPs in slices treated with propranolol were 109±6% 120 min post stimulation (n=9) which was significantly less ($p < 0.001$) than control slices not exposed to

propranolol (fEPSPs were 153±8 120 min post HFS, n=8) (Fig. 3.2*B*). Comparison of fEPSPs 120 min after stimulation at S1 revealed that propranolol applied overlapping with S1 stimulation also blocked the expression of heterosynaptic LTP (propranolol treated slices = 102±4%, controls =122 ± 6%, $p < 0.05$; Fig. 3.2*C*).

Figure 2.

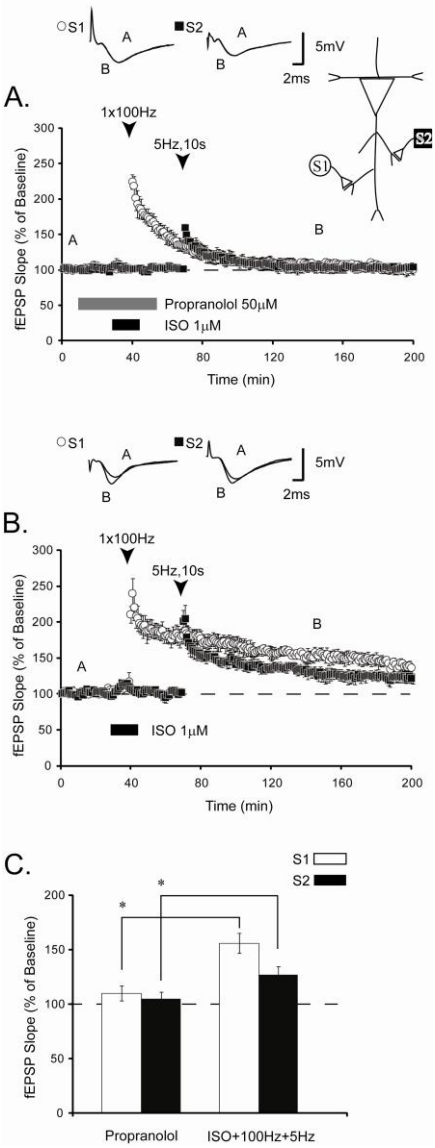


Figure 3.2. β-ARs are required for heterosynaptic enhancement of LTP. *A*. Application of the β-AR antagonist, propranolol, overlapping with ISO blocked the induction of LTP at S1 (open circles) and prevented heterosynaptic LTP at S2 (filled squares)(n=9). *B*. Both homosynaptic β-AR-dependent LTP (open circles) and heterosynaptic LTP were significantly enhanced relative to slices treated with propranolol (n=8). *C*. Summary histogram comparing fEPSP slopes obtained 120 min after HFS at S1 (white bars) and 90 min after LFS (black bars) at S2. Sample traces were taken 10 min after commencement of baseline recordings and 120 and 90 min after stimulation after HFS at S1 and LFS at S2 respectively. Results in C represent means ± SEM, * $p < 0.05$.

**Protein synthesis is required for the β-AR-dependent heterosynaptic facilitation**

As β-ARs gate LTP through translation regulation (Gelinas and Nguyen, 2005; Gelinas et al., 2007), I investigated whether heterosynaptic facilitation induced by activating β-adrenergic receptors requires protein synthesis. To test this, I applied a protein synthesis inhibitor, emetine (EME, 20 μM) 20 min prior to and overlapping with homosynaptic β-AR-dependent LTP. At this concentration, EME inhibits protein synthesis by > 80% in hippocampal slices (Stanton and Sarvey, 1984). When HFS was paired with ISO in the presence of EME, homosynaptic LTP was significantly reduced (fEPSPs were 104± 9% of baseline 120 min post HFS, n=11)(Fig. 3.3*A*). Heterosynaptic LTP was similarly inhibited when emetine was applied overlapping with S1 (fEPSPs were 101± 5% 90 min after LFS, Fig. 3.3*A*). This suggests that translation during β-AR-dependent LTP facilitates the subsequent induction of heterosynaptic LTP.

Heterosynaptic transfer of LTP may be a process that is independent of protein synthesis as it is mediated by activation of specific kinases (Redondo et al., 2010). To determine if heterosynaptic transfer of LTP induction is independent of translation, I shifted the application of EME to coincide with S2 stimulation. If protein synthesis during homosynaptic LTP induction is sufficient for facilitating heterosynaptic LTP 30 min later, shifting application of EME to

coincide with LFS at S2 should not prevent the expression of heterosynaptic LTP. Consistent with this hypothesis, bath application of EME during S2 stimulation failed to block either homosynaptic (fEPSPs were 141± 8% 120 min after HFS) or heterosynaptic LTP (fEPSPs were 128±9% 90 min after LFS, n=9) (Fig. 3.3*B*). An ANOVA that compared fEPSPs at S1 120 min after conjoint ISO application and HFS in the presence of emetine, during shifting of EME to overlap with LFS, or with no EME (Fig. 3.3*C*), demonstrated significant differences between groups ($F_{(2,27)} = 8.15$; $p < 0.01$). Subsequent Tukey-Kramer *post hoc* tests revealed that emetine significantly inhibited LTP only when applied during HFS at S1 ($p < 0.05$). Comparisons of LTP at S2 (ANOVA; ($F_{(2,27)} = 5.97$; $p < 0.02$) revealed similar results in which heterosynaptic LTP was significantly reduced ($p < 0.05$) only when EME was applied during ISO+HFS at S1 (Fig. 3.3*D*). Comparisons between control and EME shift experiments revealed no difference between groups ($p < 0.05$). Thus, shifting the application of EME to coincide with S2 LFS did not block the expression of LTP at either synaptic pathway. Thus, similar to heterosynaptic mechanisms engaged by multiple trains of HFS, protein synthesis is required for the generation, but not the heterosynaptic transfer, of LTP. Our second pathway data also show that inhibition of protein synthesis does not affect basal synaptic transmission in hippocampal slices, consistent with previous reports (Krug et al., 1984; Frey et al., 1988; Nguyen et al., 1994; Scharf et al., 2002; Gelinas and Nguyen, 2005).

Figure 3.

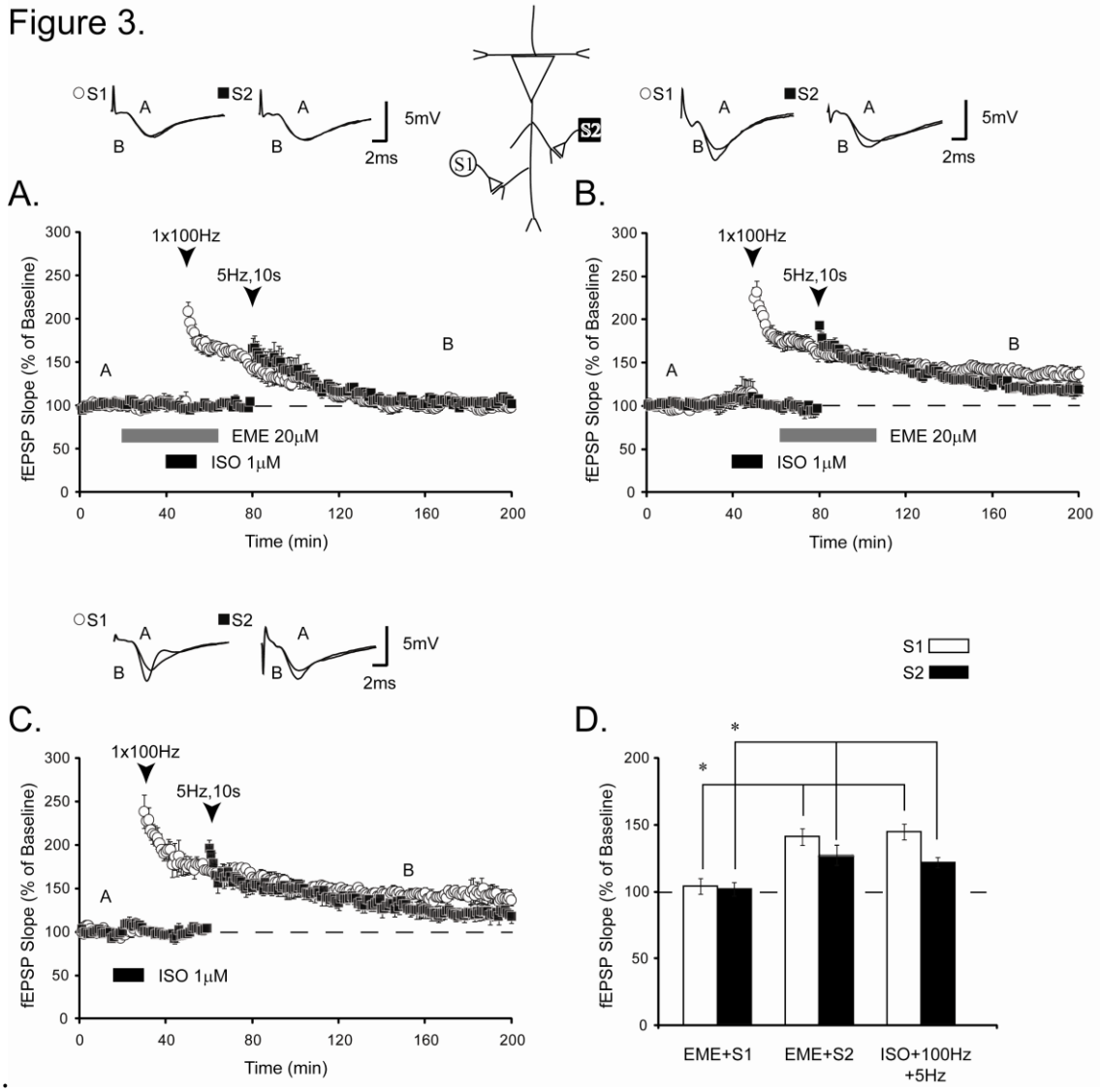



Figure 3.3. Protein synthesis is necessary for heterosynaptic facilitation induced by β-ARs. *A.* Slices treated with the translation inhibitor, emetine (EME), during β-AR activation paired with high-frequency stimulation did not express LTP at S1 (filled circles) or S2 (open squares; n=11). *B.* Shifting emetine to coincide with low-frequency stimulation at S2 had no significant effect on LTP at either synaptic pathway, as LTP was still expressed (n=9). *C.* LTP in the presence of emetine at S2 was similar in magnitude to translation inhibitor-free controls (n=8). *D.* Summary histogram comparing fEPSP slopes obtained 120 min after HFS (white bars) at S1 and 90 min after LFS at S2 (black bars). "*" indicates significant differences between treatment groups. Sample traces were taken 10 min after commencement of baseline recordings and 120 after HFS. Results in C represent means ± SEM, $*p < 0.05$.

**β-adrenergic receptors engage cap-dependent translation to facilitate heterosynaptic long-term potentiation**

Stimulation of β-ARs with isoproterenol regulates protein synthesis at synapses through initiation of extracellular signal regulated kinase (ERK) and mammalian target of rapamycin (mTOR) signaling pathways (Gelinas et al., 2007). I sought to determine if ERK and mTOR pathways, which are coupled to cap-dependent translation, are involved in the heterosynaptic facilitation initiated by β-adrenergic receptor activation.

I examined the effects of a MEK inhibitor, PD 98059 (50 μM), on β-adrenergic receptor-mediated heterosynaptic LTP. Pairing 100 Hz stimulation with ISO in the presence of PD 98059 inhibited LTP at both S1 (fEPSPs were 113 ± 9% 120 min after HFS) and S2 (fEPSPs were 98 ± 8% 90 min after LFS, n=8) (Fig. 3.4*A*). Additionally, when the mTOR inhibitor, rapamycin (1 μM), was applied prior to and overlapping with induction of homosynaptic β-AR-LTP, expression of both homo- and heterosynaptic LTP was impaired (fEPSPs were reduced to 110 ± 12% 120 min after a HFS stimulation at S1 and 101±6% 90 min after LFS at S2, n=6) (Fig. 3.4*B*). An ANOVA that compared fEPSPs 120 min after conjoint ISO application and HFS in the presence of PD 98059, rapamycin, or no drug (Fig. 3.4*C*) showed significant differences between groups ($F_{(2,21)} = 6.79$; $p < 0.01$). Subsequent Tukey-Kramer *post hoc* tests revealed that both PD 98059 and rapamycin significantly inhibited LTP at S1 ($p < 0.05$; Fig. 3.4*D*). Furthermore, the PD 98059 and rapamycin groups did not significantly differ from each other in their impairment of LTP ($p > 0.05$). An ANOVA comparing responses at my second synaptic pathway revealed differences between groups at S2 90 min following LFS ($F_{(2,21)} = 5.61$; $p < 0.02$). When applied with S1, both PD 98059 and rapamycin inhibited expression of heterosynaptic LTP at S2 ($p < 0.05$). Thus, ISO activates β-adrenergic receptors to establish LTP that requires downstream

activation of both ERK and mTOR. Coupled with my emetine experiments, my data suggest that β-adrenergic receptor activation recruits cap-dependent translation to engage translation regulation capable of stabilizing LTP heterosynaptically.

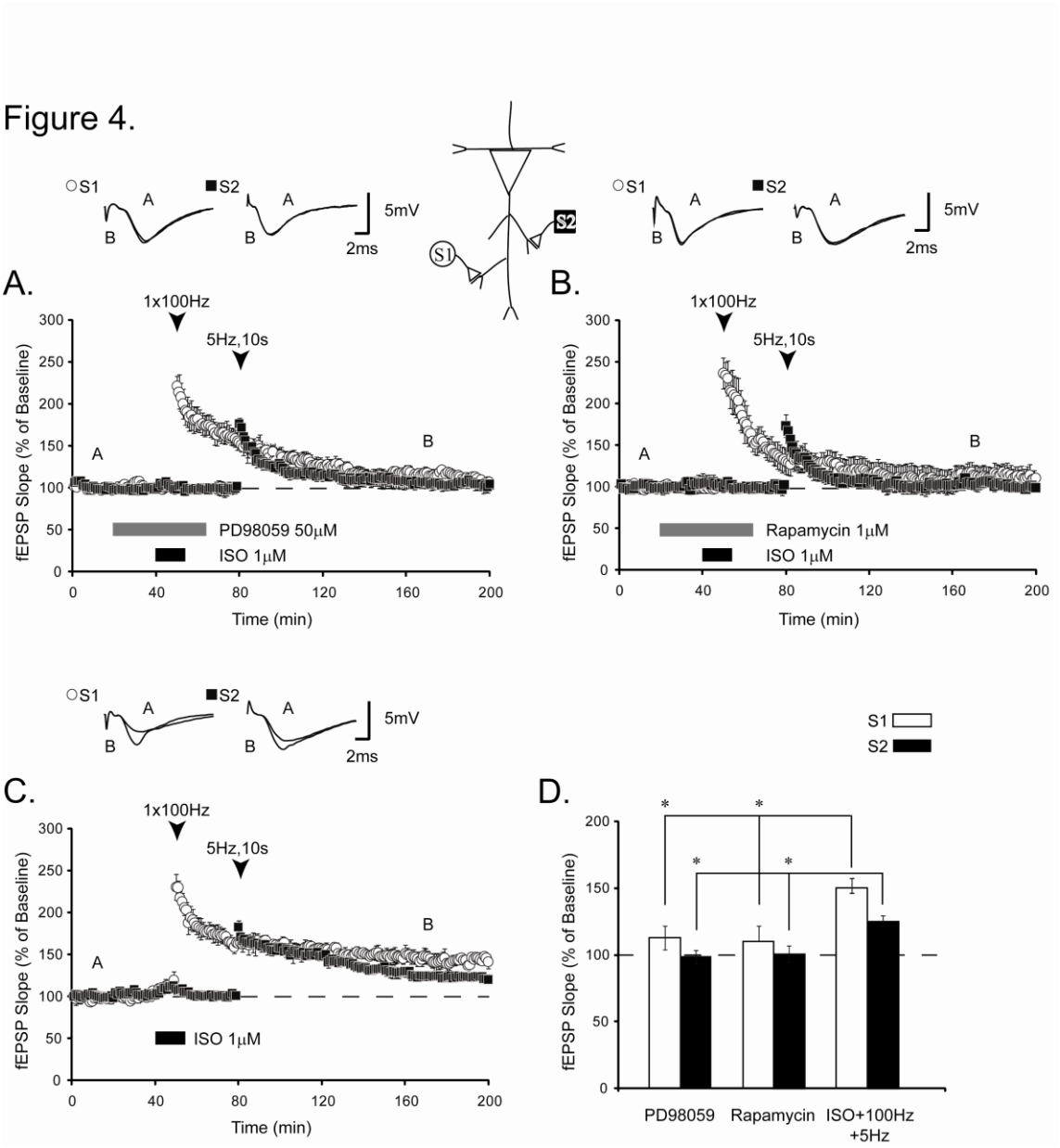



Figure 3.4. β-AR-dependent heterosynaptic facilitation requires ERK and mTOR. *A.* Application of the MEK inhibitor, PD98059, overlapping with ISO and HFS at S1 prevented the induction of homosynaptic LTP (S1; open circles) as well as the subsequent expression of heterosynaptic LTP (S2; filled squares)(n=8). *B.* Slices treated with rapamycin (mTOR inhibitor) similarly exhibited decremental LTP at both S1 (open circles) and S2 (filled squares; n=6) . *C.* Relative to drug free controls (n=8), fEPSPs in slices treated with PD98059 and mTOR were significantly less at both homo- and heterosynaptic inputs. D. Summary histogram comparing fEPSP slopes obtained 120 min after HFS at S1 (white bars) and 90 min after LFS at S2 (black bars). Sample traces were taken 10 min after commencement of baseline recordings and 120 min after HFS. Results in D represent means ± SEM, $*p < 0.05$.

**Heterosynaptically captured LTP is immune to depotentiation**

Depotentiating (DPT) stimuli can lead to the reversal of LTP during a restricted time interval soon after LTP induction (Young and Nguyen, 2005). Importantly, LTP stabilized through translation dependent mechanisms cannot be reversed by a DPT stimulus (O'Dell and Kandel, 1994; Woo and Nguyen, 2003; Young and Nguyen, 2005). If translation products generated during ISO+100 Hz, are captured at S2 following LFS, I hypothesized that this form of LTP should be immune to DPT. To test this, I paired ISO application with 100 Hz HFS, waited 30 min and applied 5 Hz for 10 s to a second independent synaptic pathway. Following a 15 min delay, LFS (5 Hz for 3 min) was given to S2 in an attempt to depotentiate established LTP. I found that LTP could not be persistently erased; following a reversal to below baseline, fEPSPs recovered to the previously potentiated values (Fig. 3.5*A*). fEPSPs were 129 ± 6% 90 min after LFS (n=6) which was not significantly different from control slices not given a DPT stimulus (control fEPSPs were 124 ± 9% 90 min after S2 stimulation, n=6, $p > 0.05$) (Fig. 3.5*B*,*C*). To confirm that the depotentiating stimulus is sufficient for reversing previously potentiated LTP I used a stimulation protocol which induces translation independent LTP (1x100 Hz stimulation) (Duffy et al., 2001). Application of 5 Hz 3 min stimulation 15 min after brief HFS reversed LTP (Fig. 3.5*D*) which resulted in fEPSPs that were significantly reduced (108± 3% of baseline, n=6) relative to slices tetanised with 100 Hz stimulation only (Fig. 5E; 132± 2%, n=6; $p < 0.05$).

Figure 5.

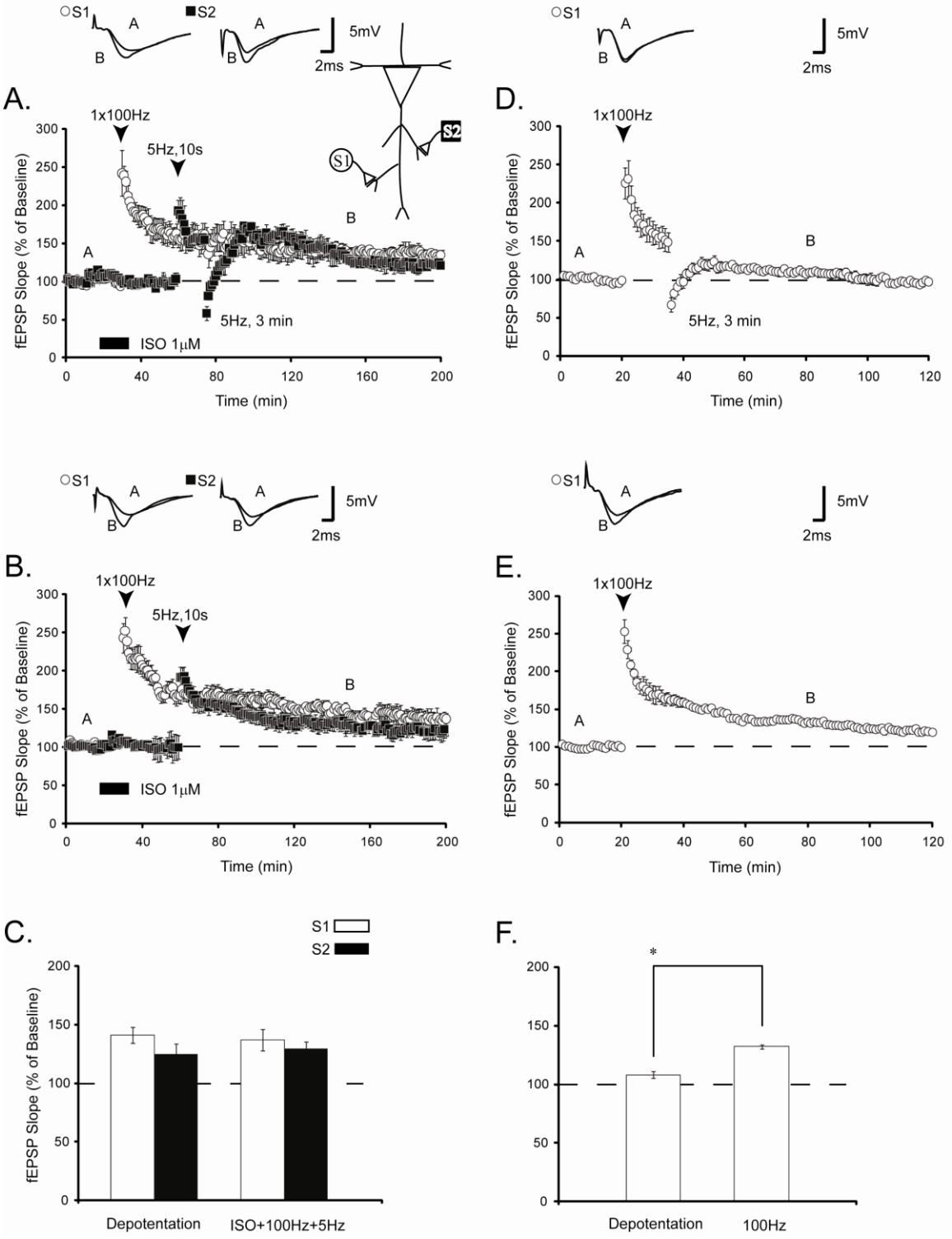

A.
S1
S2
A
B
5mV
2ms
1x100Hz
5Hz,10s
5Hz, 3 min
ISO 1μM
fEPSP Slope (% of Baseline)
Time (min)
D.
B.
E.
C.
S1
S2
Depotentation
ISO+100Hz+5Hz
F.
*
100Hz

Figure 3.5 Heterosynaptic LTP is immune to depotentiation. *A*. To determine if heterosynaptic LTP is immune to depotentiation (DPT), an activity dependent reversal of established LTP, I applied a depotentiating stimulus (5 Hz, 3 min). Following induction of homosynaptic β-AR-dependent LTP (open circles; n=6) at S1, LFS (5 Hz, 10 s) was applied to S2 (filled squares) thirty minutes later. Fifteen min after LFS at S2, a depotentiating stimulus (5 Hz, 3 min) was applied. Following an initial depression, LTP at S2 returned to previous potentiated levels which were not significantly different from slices not given a DPT stimulus (*B*). Homosynaptic LTP in which HFS was paired with ISO similarly was not significantly different when compared 2 h post stimulation. *C*. Summary histogram comparing fEPSP slopes obtained 120 min after HFS (white bars) and 90 min after LFS at S2 (black bars). *D*. Depotentiation induced with 5 Hz, 3 min is sufficient for reversing previously potentiated (100 Hz, 1 s) stimulation. 100 Hz stimulation was followed by DPT stimuli which resulted in a reversal of LTP which was significantly reduced relative to LTP induced with 100 Hz alone (*E*, *F*) Sample traces were taken 10 min after commencement of baseline recordings and either 60 or 120 min after stimulation. Results in C and F represent means ± SEM.

**Heterosynaptic transfer of β-adrenergic receptor-dependent LTP requires PKA**

cAMP-dependent protein kinase (PKA) possesses properties required of a local synaptic activity indicator as it is localized at synapses, is activity-dependent, and inactivates with time (Huang et al., 2006;Young et al., 2006). Previous research has identified PKA as a mediator of heterosynaptic facilitation induced by repetitive HFS (Navakkode et al., 2004; Young et al., 2006). Does heterosynaptic transfer of LTP generated by pairing β-adrenergic receptor activation with HFS require PKA? To address this question, I paired ISO with HFS in the presence of a membrane permeant inhibitor of PKA, PKI (1 μM). I found that LTP was intact following PKA inhibition, with S1 displaying potentiation of 152 ± 11% 120 min after HFS. Similarly, 5 Hz stimulation at S2 was also potentiated following PKA inhibition at S1; fEPSPs were potentiated to 126± 8% of baseline 90 min after stimulation (n=8; Fig. 3.6*A*). Next, PKI was shifted to coincide with S2 stimulation. Homosynaptic LTP was not blocked when PKI was paired with S2 (fEPSPs were 148±8% of baseline 120 min post HFS; Fig. 3.6*B*). However, LTP

was not expressed heterosynaptically (fEPSPs decayed to 102± 5% 90 min after LFS; n=10) (Fig. 6*B*). An ANOVA comparing homosynaptic LTP induced in the presence of PK1 with S1, paired with S2, or no drug revealed (Fig. 3.6*C*) no significant differences between groups ($p > 0.05$; Fig. 6*D*). An ANOVA comparing heterosynaptic LTP demonstrated that bath application of PKI only inhibited heterosynaptic LTP when it was present during LFS at S2 ($F_{(2,23)} = 6.19$; $p < 0.01$). Subsequent Tukey-Kramer *post hoc* tests showed that LFS paired with PKI induces decaying LTP which is significantly less ($p < 0.05$) than either PKI with S1 or ISO+HFS controls (Fig. 3.6*D*). Slices treated with PKI homosynaptically were similar to ISO+HFS controls ($p > 0.05$) These data suggest that PKA is not required for plasticity protein generation, but is necessary for heterosynaptic transfer of LTP.

Figure 6.

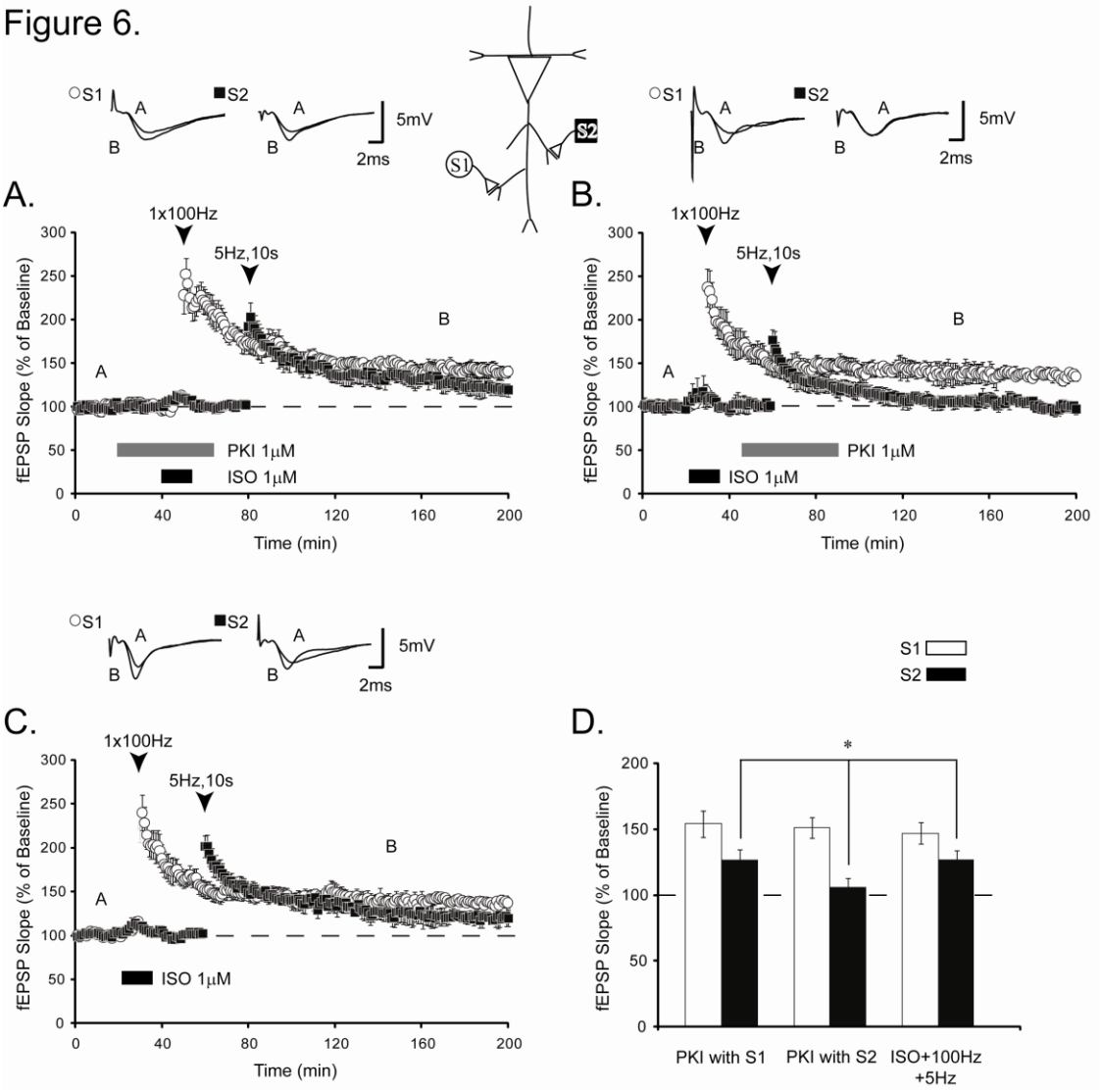



Figure 3.6. PKA is required for heterosynaptic transfer of LTP. *A.* Application of the membrane-permeant PKA inhibitor, PKI, overlapping with 1x100 Hz simulation paired with ISO (open circles), did not prevent the induction of homosynaptic LTP or the transfer of LTP to a second synaptic pathway (filled squares) (5 Hz, 10 s; n=8). *B.* Shifting PKI application to overlap with low-frequency stimulation prevented the heterosynaptic transfer of LTP to S2 (filled squares; n=10). Heterosynaptic LTP was significantly reduced relative to slices treated with PKI during HFS or PKI-free controls. LTP at S1 was unaffected (open circles) as determined by comparisons with PKA inhibitor free controls (*C.*) (n=6). *D.* Summary histogram comparing fEPSP slopes obtained 120 min after HFS (white bars) at S1 and 90 min after LFS at S2. Sample traces were taken 10 min after commencement of baseline recordings and 120 min after S1 stimulation. Results in C represent means ± SEM, $*p < 0.05$.

**LTP can be transferred heterosynaptically when LFS precedes HFS**

Previous research has demonstrated that weak, subthreshold stimulation applied within a limited time window before strong LTP-inducing stimulation, can still be facilitated in a heterosynaptic manner (Frey and Morris, 1998). These experiments allow for determination of the temporal limitations for heterosynaptic facilitation. In the light of these data, I investigated whether my form of heterosynaptic facilitation can similarly be expressed in a "retrograde" manner, with LFS (S1) preceding ISO paired with 100 Hz stimulation (S2). I observed that prior application of 5 Hz stimulation, which normally only induces a transient (< 1 h) form of LTP, produced LTP lasting > 2 h, provided that β-AR-dependent LTP was induced 30 min later (Fig. 3.7*A*) (fEPSPs were 124±6% 120 min after 5 Hz, n=6). However, when the duration between LFS and ISO+HFS was extended to 1 h, no transfer of LTP was observed (Fig. 3.7*B*, fEPSPs were 103 ± 4% 120 min after 5 Hz, n=8). Comparisons between LFS applied 30 min or 1 h before heterosynaptic HFS showed that prior application of a weak stimulus could capture subsequently induced LTP, only at the 30 min interval. Mean fEPSP slopes from the 30 min delay group were substantially higher than the 1 h group (Fig. 3.7*C*; $p < 0.02$). β-AR-dependent homosynaptic LTP induced 30 min and 1 hr after LFS were similar in magnitude ($p > 0.05$).

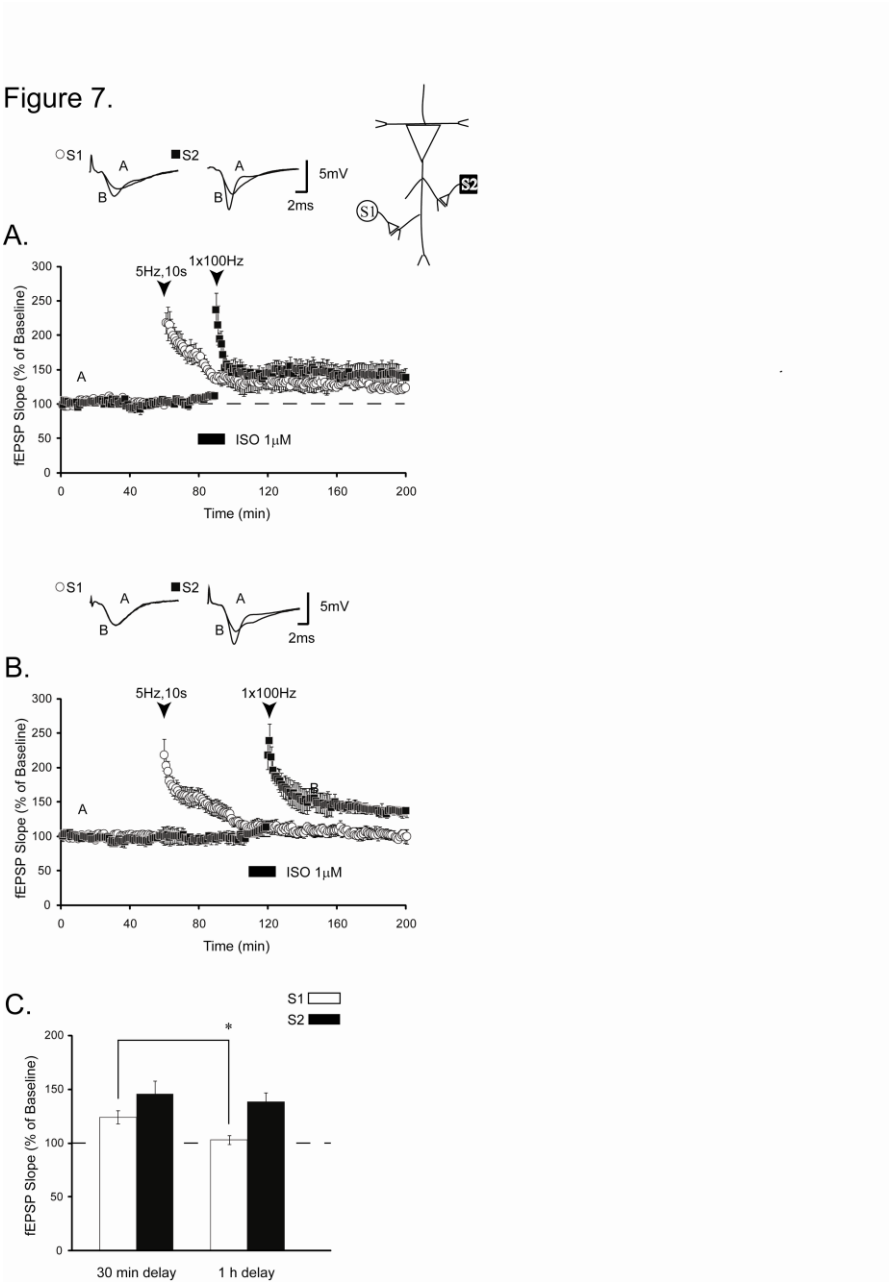


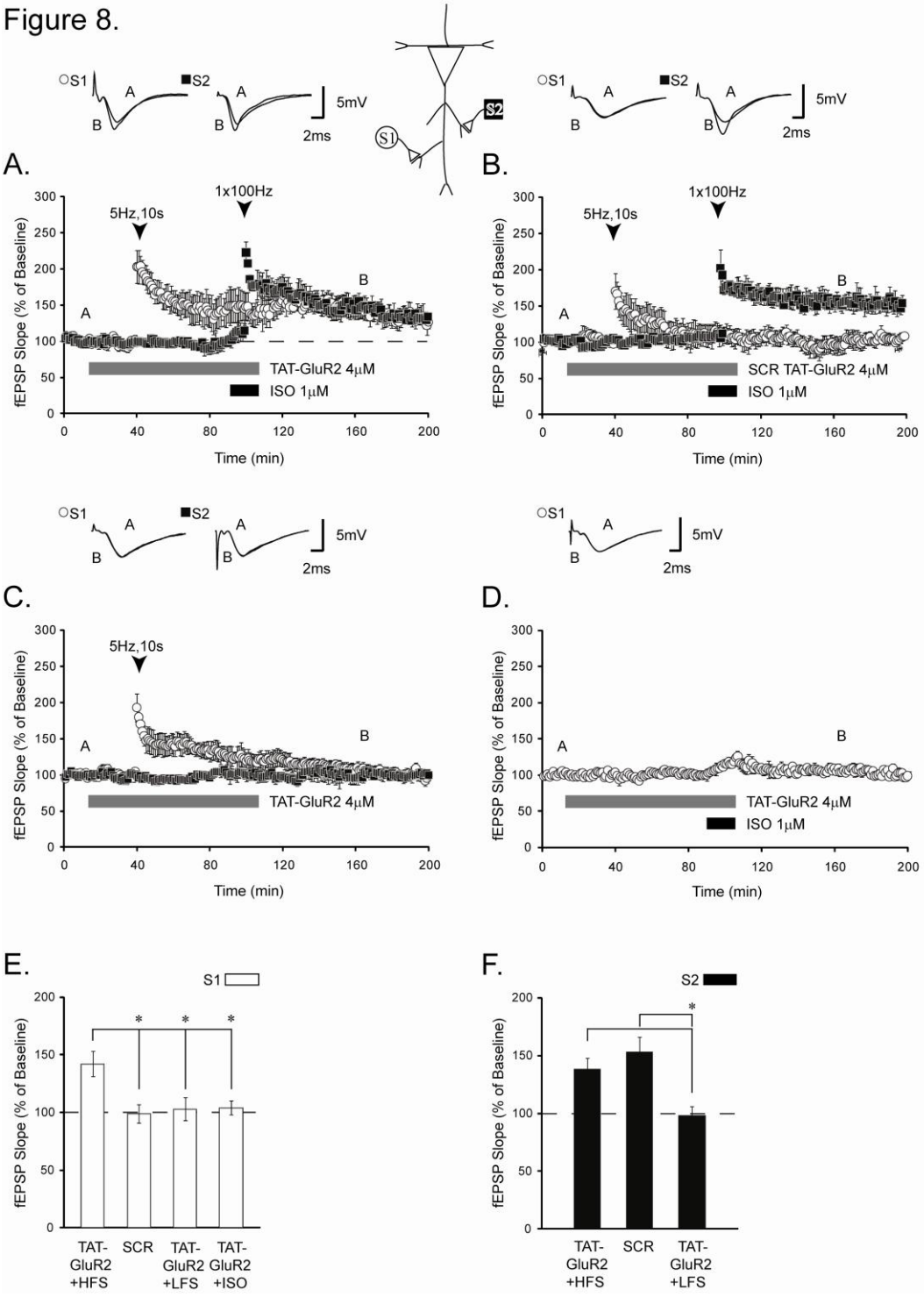

Figure 3.7. Prior LFS facilitates heterosynaptic transfer of LTP. *A.* Prior application of LFS (5 Hz, 10 s: S1; open circles), which normally induces transient LTP, enables long-lasting potentiation when β-AR LTP is induced heterosynaptically (S2; filled squares) 30 min later (n=10). *B.* Increasing the delay between LFS at S1(open circles; n=8) and HFS + ISO at S2 (S2; filled squares) to 1 h prevents the heterosynaptic transfer of long-lasting LTP. *C.* Summary histogram comparing fEPSP slopes obtained 120 min after HFS (white bars) at S1 and LFS at S2 (black bars). Sample traces were taken 10 min after commencement of baseline recordings and 120 min after stimulation. "*" indicates a significant difference between treatment groups. Results in C represent means ± SEM , $*p < 0.05$.

**Preventing GluR2 endocytosis extends the duration of the temporal window for heterosynaptic facilitation of LTP.**

Identification of mechanisms that could extend the time window for intersynaptic crosstalk is crucial for understanding the mechanisms required for heterosynaptic LTP. AMPA receptor trafficking and insertion have been identified as mechanisms involved in facilitating heterosynaptic long-term potentiation (Yao et al., 2008). To determine if GluR trafficking is involved in β-AR-dependent heterosynaptic facilitation, I asked whether preventing GluR internalization following LFS could increase the lifetime of the molecular activity trace. Using a membrane-permeant Tat-GluR2$_{3Y}$ peptide which prevents endocytosis of GluR2, I tested the hypothesis that the duration of the time window for effective heterosynaptic transfer of LTP could be extended by blocking endocytosis of GluR2-containing receptors (thereby maintaining these receptors at the synapse). Tat-GluR2$_{3Y}$ (4μM) was applied 30 min prior to and 1 h after LFS (5 Hz, 10 s). Following a 1 h delay, LTP at a second pathway was induced by pairing ISO with HFS (100 Hz, 1 s). I found that fEPSPs were potentiated to 142±11% (n=4; Fig. 3.8*A*) 120 min after LFS. To determine if the effects of preventing GluR2 endocytosis facilitated the heterosynaptic expression of LTP, I conducted several control protocols. First, I applied the inert Tat-GluR2$_{3s}$ scrambled control peptide to assess the effects of this peptide application. The Tat-GluR2$_{3s}$ scrambled peptide failed to enhance the expression of homosynaptic LTP when induced prior to heterosynaptic β-AR-dependent LTP (fEPSPs were 99±8% of baseline 120 min after LFS (n=4; Fig. 3.8*B*). Next I determined if preventing GluR2 endocytosis with the active peptide is sufficient for enhancing the duration of LTP independent of β-AR stimulation. I applied active Tat-GluR2$_{3Y}$ paired with 5 Hz, 10 s alone. Tat-GluR2$_{3Y}$ coupled with 5 Hz stimulation induced transient LTP which returned to baseline in less than 2 h (fEPSPs were 103±10% 120 min after

LFS; n=4) (Fig. 3.8*C*). Finally, I applied Tat-GluR2$_{3Y}$ with ISO alone to test whether prevention of GluR2 endocytosis paired with beta-adrenergic receptor stimulation affected baseline transmission. Application of Tat-GluR2$_{3Y}$ with ISO in the absence of stimulation induced a transient potentiation which returned to baseline in less than 30 min (fEPSPs were 103±6% 90 min after the termination of drug application; Fig. 3.8*D*, n=6). An ANOVA comparing either Tat-GluR2$_{3Y}$ with LFS, Tat-GluR2$_{3Y}$ paired with ISO and high-frequency stimulation, scrambled peptide Tat-GluR2$_{3s}$ paired with heterosynaptic ISO+HFS or Tat-GluR2$_{3Y}$ paired with ISO revealed a significant effect ($F_{(3,17)} = 6.76$; $p < 0.05$) (Fig. 3.8*E*). Tukey-Kramer *post hoc* tests demonstrated that only when active Tat-GluR2$_{3Y}$ was paired with the induction of β-AR-LTP was the temporal window for heterosynaptic transfer of LTP extended to 1 hr. There was no significant difference between the synaptic pathways receiving the strong stimulation protocol (fEPSPs were potentiated to 138±10% 90 min after HFS in Tat-GluR2$_{3Y}$ treated slices and 153±13% in slices exposed to the control scrambled peptide). However, both pathways receiving ISO+100 Hz stimulation were significantly more potentiated than the non-stimulated control pathway in the LFS paired with Tat-GluR2$_{3Y}$ experiment ($p < 0.05$). These findings show that blocking endocytosis of GluR2-containing receptors allows heterosynaptic transfer of persistent LTP to occur later than would normally be observed. The data also underscore the possibility that GluR2-containing receptors may serve as possible "tags" to enable heterosynaptic capture of translation-dependent LTP.

Figure 8.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B.
C.
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S1
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B
5mV
2ms
1x100Hz
5Hz,10s
fEPSP Slope (% of Baseline)
Time (min)
TAT-GluR2 4μM
SCR TAT-GluR2 4μM
ISO 1μM
TAT-GluR2 +HFS
SCR
TAT-GluR2 +LFS
TAT-GluR2 +ISO

Figure 3.8. Preventing GluR2 endocytosis extends the temporal window for transfer of heterosynaptic LTP. *A.* When GluR2 endocytosis is prevented through application of the Tat-GluR2$_{3Y}$ peptide, LFS (5 Hz, 10 s: S1; open circles) still expresses LTP when HFS+ISO is applied 1 h later (n=4). *B.* Application of a scrambled, inert version of the peptide (Tat-GluR2$_{3s}$) failed to extend the time window for heterosynaptic transfer of LTP when ISO+HFS was delayed to 1 hr post LFS (n=4). ISO+HFS generated homosynaptic, long-lasting LTP in the presence of both peptides, indicative of intact synaptic plasticity in both groups. *C.* Application of Tat-GluR2$_{3Y}$ peptide with 5 Hz stimulation fails to induce long-lasting LTP. When Tat-GluR2$_{3Y}$ was applied prior to and overlapping with 5 Hz, 10 s stimulation transient LTP was induced which returned to baseline in < 2 h. *D.* Pairing of ISO with Tat-GluR2$_{3Y}$ does not have any significant on baseline synaptic responses. To test for effects on basal synaptic transmission, Tat-GluR2$_{3Y}$ was paired with ISO application and basal synaptic responses were monitored. A transient, small ( < 20%) increase in synaptic potentiation was observed during ISO application. fEPSPs subsequently returned to baseline levels. *E, F.* Summary histogram comparing fEPSP slopes obtained 120 min after LFS at S1 (*E*; white bars) and 90 min after HFS at S2 (*F*; black bars). "*" indicates significant differences between groups. Sample traces were taken 10 min after commencement of baseline recordings and 120 min after stimulation. Results in C represent means ± SEM, *$p < 0.05$.

## Discussion

My results indicate that β-adrenergic receptor activation can facilitate heterosynaptic long-term potentiation in the adult mouse hippocampus. Neuromodulators gate both local protein synthesis (Huber et al., 2000; Gelinas and Nguyen, 2005; Navakkode et al., 2007) and activation of second messengers that can support heterosynaptic facilitation. Previous research showed that application of a cAMP phosphodiesterase inhibitor, rolipram, enabled the transformation of heterosynaptic, transient LTP into translation-dependent long-lasting LTP, suggestive of a cAMP-mediated increase in plasticity proteins (Navakkode et al., 2004). As noradrenergic receptors can facilitate protein synthesis-dependent LTP (Straube et al., 2003; Gelinas and Nguyen, 2005) and long-term memory (Cahill et al., 1994; Walling and Harley, 2004; Hu et al., 2007**;** Kemp and Manahan-Vaughan, 2008), translation regulation is critically involved in long-

term changes in synaptic strength required for memory formation. My findings suggest that cap-dependent translation is required for this form of heterosynaptic facilitation as inhibition of protein synthesis, ERK, and mTOR prevented the expression of homo- and heterosynaptic LTP. Here, upregulation of plasticity proteins through cap-dependent translation is sufficient for prolonging the duration of heterosynaptic LTP beyond 2 h.

The current study characterizes a novel heterosynaptic facilitation protocol which relies upon activation of β-ARs for its induction. I found that pairing β-adrenergic receptor activation with HFS induced LTP that could be transferred to a second synaptic pathway provided stimulation sufficient for sequestering LTP was applied within a finite time window (30 min). Additionally once captured, heterosynaptic LTP could not be depotentiated. Depotentiation is an activity-dependent reversal of previously established LTP (O'Dell and Kandel, 1994; Woo and Nguyen, 2003; Young and Nguyen, 2005). However, if a depotentiating stimulus is applied after a finite time window during which translation-dependent mechanisms have been engaged to stabilize recently induced synaptic potentiation, LTP can no longer be depotentiated (O'Dell and Kandel, 1994; Woo and Nguyen, 2003). When I applied a depotentiating tetanus 15 minutes after LFS applied to my second synaptic pathway, LTP was not reversed. This suggests that once plasticity-related proteins generated by prior β-AR activation have been captured, heterosynaptic LTP is stabilized thus conferring immunity to depotentiation.

How does β-adrenergic receptor activation facilitate heterosynaptic LTP? β-ARs regulate translation postsynaptically through initiation of ERK and mTOR pathways (Gelinas et al., 2007; Fig. 9). ERK and mTOR co-regulate cap-dependent translation through 4E-binding proteins (4E-BPs), which sequester and repress eukaryotic initiation factor 4E (eIF4E), which in turn forms

part of the eIF4F initiation complex (Kelleher et al., 2004; Richter and Sonenberg, 2005; see Klann et al., 2004, Banko and Klann, 2008 and Costa-Mattioli et al., 2009 for reviews). In the present study, application of emetine overlapping with LTP induction generated decremental LTP at both synaptic pathways, consistent with a necessity for translation regulation in the generation of plasticity proteins. Importantly, shifting application of emetine to coincide with S2 stimulation did not affect the expression of heterosynaptic LTP. These results are consistent with previous work which showed that proteins synthesized during strong S1 stimulation enable the induction of long-lasting LTP at S2 in a protein synthesis-independent manner (Frey and Morris, 1998).

Recent evidence from in vivo experiments demonstrated that prior exposure of rats to novel experiences promoted the transformation of short-term memory into protein synthesis-dependent long term memory in a form of "behavioural tagging", provided that novelty exposure took place within a limited time frame (Moncada and Viola, 2007). Both noradrenaline and dopamine are released in the hippocampus in response to novelty, where it has been postulated that they serve as learning signals (Kitchigina et al., 1997; Harley, 2004). Given that both beta-adrenergic (Walling and Harley, 2004; Lemon et al., 2009) and dopaminergic (Li et al., 2003) system activation induces cellular plasticity and boosts memory formation (Kemp and Manahan-Vaughn, 2008; Lemon and Manahan-Vaughn, 2006), these systems are well equipped for promoting the incorporation of new information into existing cellular networks representing previously encoded experiences.

In agreement with previous research, PKA was required for transfer of heterosynaptic LTP (Alarcon et al., 2006; Huang et al., 2006; Young et al., 2006). Inhibition of PKA failed to

prevent homosynaptic LTP, consistent with previous experiments which demonstrated that isoproterenol paired with HFS induces PKA-independent homosynaptic LTP (Gelinas et al., 2008). My present results suggest that translation regulation and gating of heterosynaptic facilitation occur through different mechanisms, with heterosynaptic transfer of LTP requiring PKA. What function does PKA serve in synaptic capture of LTP? PKA phosphorylates Ser845 of GluR1 which increases GluR insertion in response to synaptic activity (Esteban et al., 2003; Oh et al., 2006; Tenorio et al., 2010). Increased GluR trafficking could enhance synaptic capture both through boosting postsynaptic responses and providing additional slots for AMPAR insertion (Malinow, 2003). Previous data have established the dynamics of AMPAR subunit turnover at the synapse following the induction of LTP. Makino and Malinow (2009) have shown that GluR1 insertion is elevated immediately following the induction of LTP. These GluR1 receptors are subsequently replaced by GluR2-containing receptors during the expression of LTP which serves to maintain previously potentiated synaptic responses (Makino and Malinow, 2009). These data suggest that boosting the initial incorporation of GluR1 through Ser845 phosphorylation by PKA could increase the amount of postsynaptic AMPAR slots available for subsequent incorporation of GluR2, resulting in enhanced capture of LTP. Taken together, these results suggest that PKA activation may serve as a local synaptic indicator of previous synaptic activity following low-frequency stimulation.

Interestingly, increased synthesis of a constitutively active isoform of PKC, PKMζ, can prime heterosynaptic facilitation through upregulation of GluR2 (rat analogue for GluA2)-dependent AMPA receptor trafficking (Yao et al., 2008). My data indicate that disrupting AMPAR internalization extends the temporal window for heterosynaptic transfer of LTP from 30 min to 1 h. Recent data have implicated increased regulation of GluR2 trafficking in the

maintenance of both LTP and memory (Serrano et al,. 2005; Migues et al., 2010). AMPARs form heterodimers which exist primarily in GluR1-GluR2 and GluR2-GluR3 configurations (Wenthold et al., 1996). Preventing the constitutive endocytosis of GluR2-containing AMPARs may extend the duration of synaptic tags by maintaining recently incorporated GluR1-GluR2 heterodimers at the synapse. I observed that maintaining GluR2 at the synapse extended the duration of the synaptic tag to 1 hr. This result suggests that glutamatergic receptor trafficking determines the duration of synaptic tags. From a broader perspective, these data suggest that the temporal limits for associative memory formation are contingent upon the dynamics of experience-driven glutamate receptor trafficking. Synapses potentiated in response to environmental stimuli would be available for incorporation into cellular networks provided that AMPARs were trafficked to and maintained at the synapse (tagging), and stabilized through translation-dependent mechanisms initiated by neuromodulatory receptor stimulation (capture). Thus, GluR subunits may interact to facilitate synaptic plasticity, with GluR2 exocytosis acting to increase GluR levels to promote maintenance of LTP, and GluR1 acting to facilitate delivery of the receptors through phosphorylation, which has been correlated with reduced thresholds for LTP induction (Makino et al., 2011) and enhanced learning and memory (Lee et al., 2003; Hu et al., 2007).

My results with the Tat-GluR2$_{3Y}$ peptide, which inhibits GluR2 internalization, suggest that maintaining GluR2-containing AMPARs at the synapse is sufficient for prolonging the temporal window for intersynaptic crosstalk. The extension of the time window from 30 min to 1 h in the presence of Tat-GluR2$_{3Y}$ peptide is in line with previously established time frames for heterosynaptic LTP (Frey and Morris, 1998) and LTD (Sajikumar and Frey, 2004). As preventing GluR2 endocytosis facilitated heterosynaptic LTP at 1 h, this suggests that activity-

dependent GluR2 internalization may serve to define the effective temporal window for synaptic associativity across distinct synaptic pathways.

Figure 9.

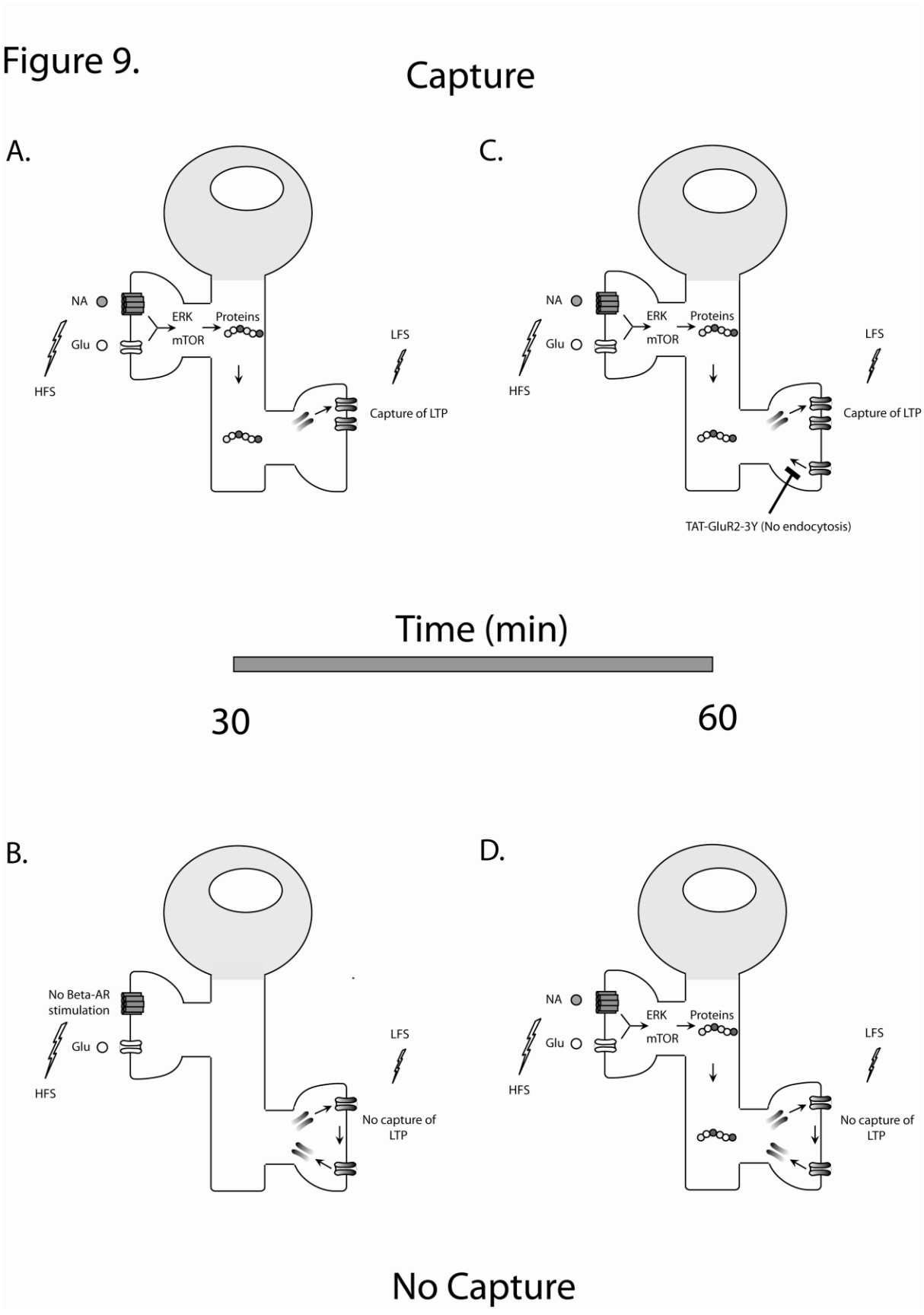
Capture
A.
NA
Glu
HFS
ERK
mTOR
Proteins
LFS
Capture of LTP
C.
NA
Glu
HFS
ERK
mTOR
Proteins
LFS
Capture of LTP
TAT-GluR2-3Y (No endocytosis)
Time (min)
30
60
B.
No Beta-AR stimulation
Glu
HFS
LFS
No capture of LTP
D.
NA
Glu
HFS
ERK
mTOR
Proteins
LFS
No capture of LTP
No Capture

Figure 3.9. Model for enhanced heterosynaptic long-term potentiation induced by β-AR-dependent LTP. *A*. Activation of β-ARs (shown here with the endogenous ligand noradrenaline; NA) paired with high-frequency stimulation (HFS) initiates intracellular signaling cascades that regulate protein synthesis (ERK, mTOR). ERK and mTOR converge on translational machinery to increase plasticity-related protein synthesis. These plasticity proteins can enhance heterosynaptic long-term potentiation by stabilizing AMPAR insertion induced by low-frequency stimulation. *B*. When β-AR-dependent LTP is not induced, LFS applied heterosynaptically induces a transient increase in AMPAR trafficking and insertion which, in the absence of protein synthesis upregulation is subsequently reversed through an endocytotic mechanism. Similarly, low-frequency stimulation applied prior to homosynaptic LTP induced with β-ARs does not capture LTP if the delay between stimuli is increased to 1 h (*D*.). *C*. Preventing GluR2 endocytosis following LFS extends the temporal window for heterosynaptic transfer of LTP. By stabilizing GluR2 in the membrane (with the Tat-GluR2$_{3Y}$ peptide), subsequently generated plasticity proteins can promote increased synaptic strength during a more extended time window, thereby overcoming homeostatic mechanisms that would drive removal of synaptically localized AMPARs and weaken synaptic strength.

The role of homosynaptically-induced translation products may be to stabilize primed AMPAR insertion following heterosynaptic stimulation (Fig. 3.9). Inhibiting GluR2 endocytosis appeared to extend the lifetime of the tag by maintaining synaptic incorporation of AMPARs which would normally be removed in the absence of plasticity proteins. Activation of beta-adrenergic receptors could facilitate heterosynaptic LTP through phosphorylation of AMPARs by PKA, in addition to increasing the generation of plasticity proteins which would enhance heterosynaptic facilitation. As heterosynaptic LTP maintenance continued beyond 1 h, Tat-GluR2$_{3Y}$-dependent enhancement of AMPAR surface expression appears to maintain potentiation until β-AR-dependent mechanisms are engaged to provide plasticity products capable of stabilizing long-term synaptic augmentation. Interestingly, application of the Tat-GluR2$_{3Y}$ appeared to increase the initial magnitude of synaptic potentiation following LFS relative to control peptide experiments (Fig. 3.8*A* and 3.9*B*). This could result from prevention of constitutive AMPA receptor endocytosis. Prevention of GluR2 endocytosis appears to maintain

GluR2-containing AMPARs with no observed increase in basal synaptic transmission when the Tat-GluR2$_{3Y}$ was applied alone (Fig. 3.8*D*). Increasing the pool of available AMPARs may prime these receptors for activity-dependent insertion into the postsynaptic membrane. These receptors could increase the capacity for AMPAR trafficking in response to synaptic stimulation (Hayashi et al., 2000; Ju et al., 2004). This would generate enhanced synaptic responses immediately following tetanisation. The elevated magnitude of the initial fEPSP potentiation observed in the presence of Tat-GluR2$_{3Y}$ (Fig. 3.8*A*) is consistent with this hypothesis.

What would be expected if GluR1 receptor endocytosis was prevented? Most forms of LTP require the rapid insertion of GluR1 during induction (Makino and Malinow, 2009). Blocking GluR1 endocytosis could result in either increased basal synaptic responses or the saturation of synaptic weights . Interestingly, recent data using phosphomimetic knock-in mice suggest that increasing GluR1 phosphorylation lowers the threshold for inducing LTP without affecting membrane insertion of GluR1 (Makino et al., 2011). Although not observed in the Makino et al. (2011) study, increasing GluR1 phosphorylation has previously been reported to enhance GluR1 expression which facilitated the induction of LTP (Oh et al., 2006). Importantly, most native receptors are GluR1/GluR2 heterodimers, and therefore, they are often trafficked together. However, the regulated endocytosis of this heterodimer is determined by sequences in the GluR2-tail, and therefore is GluR2-dependent, and not GluR1-dependent. As such, the endocytosis of heterodimers (both GluR1 and GluR2) will be inhibited by GluR2 tail peptides. Sequences in the GluR1 tail are only involved in constitutive endocytosis, but lack of signals for regulated endocytosis and thus, GluR1-derived peptide, may affect constitutive endocytosis, but not regulated endocytosis, and therefore, will have little effect on LTP maintenance.

My data suggest that preventing GluR2 endocytosis in conjunction with increased PKA activity, results in the synergistic facilitation of heterosynaptic LTP. Recent evidence has implicated GluR2 trafficking in the maintenance of LTP and memory (Migues et al., 2010). This process is mediated through mechanisms requiring the constitutively active PKC isoform, PKM-ζ (Sacktor, 2011). Activation of PKM-zeta increases NSF activity which interferes with PICK1-mediated sequestration of GluR2 (Araki et al., 2010). Once activated, NSF liberates GluR2 from PICK1-mediated extrasynaptic pools, which allows for synaptic delivery of GluR2, resulting in potentiation (Yao et al., 2008). My data are consistent with synaptic processes observed following the synthesis and phosphorylation of PKM-zeta. Preventing GluR2 endocytosis enhances levels of free GluR2 which can subsequently be driven into postsynaptic locations. An important mechanistic distinction is that in my protocol, PKA could substitute for PKM-zeta-dependent GluR2 trafficking by increasing the phosphorylation and synaptic delivery of GluR1 which is consistent with previous studies demonstrating a requirement for PKA in synaptic tagging (Young et al., 2006). It appears that endocytosis of GluR2-containing AMPARs may reset synapses which have not undergone translation-dependent potentiation back to basal states which could be considered a form of tag degradation. Both PKM-zeta-mediated GluR2 regulation and PKA activation could prevent this tag degradation through the synaptic maintenance of GluR2 and priming of GluR1 insertion respectively.

My main finding was an enhancement of heterosynaptic long-term potentiation in CA1 following the induction of homosynaptic β-AR-dependent LTP. My results suggest that increased noradrenergic signaling can prime heteroassociative processes requiring translation in a cell-wide manner. Synaptic protein synthesis may provide a mechanism for behavioral tagging, a process in which memory consolidation is enhanced by prior exposure to an open field and that

requires translation (Moncada and Viola, 2007). Open field exposure is a mildly aversive stimulus which engages the neuromodulatory systems capable of upregulating translation (Thomas et al., 1996; Winder et al., 1999; Straube et al., 2003; Gelinas and Nguyen, 2005). Taken together, my results suggest that activation of noradrenergic receptors enhances heterosynaptic facilitation which in turn, could provide a cellular mechanism for the remembrance of temporally spaced events during associative memory formation. My results add beta-adrenergic receptors to a growing list of neuromodulatory receptors that can engage cellular mechanisms capable of enhancing heterosynaptic facilitation.

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# CHAPTER IV:

## Fragile X mental retardation protein regulates heterosynaptic plasticity in the mouse hippocampus

Portions of this chapter has previously been published (Learning and Memory, Copyright 2011; Cold Springs Harbor). I conducted all experiments and co-authored the manuscript. Charles Hoeffer and Eric Klann provided fmr1 knockout mice. Peter Nguyen co-authored this manuscript.

**Abstract**

Silencing of a single gene, FMR1, is linked to a highly prevalent form of mental retardation, characterized by social and cognitive impairments, known as fragile X syndrome (FXS). The FMR1 gene encodes fragile X mental retardation protein (FMRP) which negatively regulates translation. Knockout of Fmr1 in mice results in enhanced long-term depression (LTD) induced by metabotropic glutamate receptor (mGluR) activation. Despite the evidence implicating FMRP in LTD, the role of FMRP in long-term potentiation (LTP) is less clear. Synaptic strength can be augmented heterosynaptically through the generation and sequestration of plasticity-related proteins, in a cell-wide manner. If heterosynaptic plasticity is altered in Fmr1 knockout (KO) mice, this may explain the cognitive deficits associated with FXS. I induced homosynaptic plasticity using the beta-adrenergic receptor (β-AR) agonist, isoproterenol (ISO), which facilitated heterosynaptic LTP that was enhanced in Fmr1 KO mice relative to WT controls. To determine if enhanced heterosynaptic LTP in Fmr1 KO mouse hippocampus requires protein synthesis, I applied a translation inhibitor, emetine (EME). EME blocked homo- and heterosynaptic LTP in both genotypes. I also probed the roles of mTOR and ERK in boosting heterosynaptic LTP in Fmr1 KO mice. Although heterosynaptic LTP was blocked in both WT and KOs by inhibitors of mTOR and ERK, homosynaptic LTP was still enhanced following mTOR inhibition in slices from Fmr1 KO mice. Because mTOR normally stimulates translation initiation, My results suggest that β-AR stimulation paired with derepression of translation results in enhanced heterosynaptic plasticity.

Introduction

Fragile X syndrome (FXS), a leading cause of mental retardation (Hagerman et al., 2009), is linked to silencing of the FMR1 gene (Verkerk et al., 1991; Eichler et al., 1994; Feng et al., 1995). FXS is characterized by mild to severe cognitive deficits including impaired learning and memory (Macleod et al., 2010) resulting from altered synaptic function (Huber et al., 2002; Bear et al., 2004, Hou et al., 2006; Zhang et al., 2009). The Fmr1 knockout (KO) mouse recapitulates the primary molecular pathology associated with FXS: a reduction in expression of the FMR1 protein product, fragile X mental retardation protein (FMRP) (Bakker et al., 1994). FMRP is an RNA binding protein that is implicated in processes critical for synaptic plasticity, including regulation of mRNA trafficking and translation (Corbin et al., 1997; Feng et al., 1997; Huber et al., 2002; Stefani et al., 2004, Bear et al., 2004; Weiler et al., 2004). Studies of the hippocampus, a brain structure involved in memory formation (Scoville and Milner, 1957; Zola-Morgan et al., 1986), revealed enhanced metabotropic glutamate receptor-dependent long-term depression (mGluR-LTD) in the absence of FMRP (Hou et al., 2006). mGluR-LTD is rendered insensitive to translation inhibitors in Fmr1 KO mouse hippocampus, suggestive of altered regulation of protein synthesis (Hou et al., 2006; Nosyreva and Huber, 2006).

Despite the evidence implicating FMRP in mGluR-LTD, it is less clear whether FMRP significantly impacts the expression of long-term potentiation (LTP), an activity-induced increase in synaptic strength believed to underlie specific types of learning and memory (Bliss and Collingridge, 1993; Godfraind et al., 1996; Paradee et al., 1999; Larson et al., 2005; Lauterborn et al., 2007).

Beta-adrenergic receptors (β-ARs) are G protein-coupled receptors which enhance hippocampal homosynaptic LTP and long-term memory formation through translation regulation

(Gelinas and Nguyen, 2005; Gelinas et al., 2007). As β-ARs and mGluRs both couple to signaling cascades which regulate translation (Banko et al., 2006; Gelinas et al., 2007), I asked whether β-AR-dependent heterosynaptic LTP was altered in Fmr1 KO mouse hippocampus. Frey and Morris (1997) have shown that the induction of protein synthesis-dependent LTP at one synaptic pathway (S1), can facilitate the generation of heterosynaptic LTP at a second set of synapses (S2). However, to date heterosynaptic processes have not been assessed in any mouse model of FXS. To test the hypothesis that β-AR-dependent heterosynaptic LTP is altered in Fmr1 KO mice, I induced heterosynaptic plasticity using a novel protocol consisting of β-AR activation paired with brief stimulation in one synaptic pathway followed later with milder stimulation at a neighbouring pathway.

I report that β-AR-dependent heterosynaptic LTP is altered in the hippocampus of Fmr1 KO mice. Although homosynaptic LTP was similar to WT controls, heterosynaptic LTP was enhanced in Fmr1 KO mouse hippocampus. This promotion of heterosynaptic plasticity may contribute to cognitive abnormalities in FXS, such as impaired learning during periods of heightened attention or arousal that recruit the noradrenergic neuromodulatory system and engage β-ARs.

## Materials and Methods

*Animals*: C57B*L*/6 congenic Fmr1 KO mice and their wild-type (WT) littermates (3-4 months) were used for all experiments. Animals were socially housed with food and water available *ad libitum* and were maintained on a 12 h on/off light cycle. Animals were housed at the University of Alberta using guidelines approved by the Canadian Council on Animal Care. Fmr1 KO mice and their wild-type littermates were generated in harem crosses using

hemizygous Fmr1 mutant or wild-type males crossed to heterozygote Fmr1 (+/-) females. Mice were weaned between 21-28 days and genotyped using standard methods using primer sequences and PCR instructions supplied by Jax Labs: (http://jaxmice.jax.org/protocolsdb).

*Electrophysiology*. After cervical dislocation and decapitation, transverse hippocampal slices (400-µm thickness) were prepared as described by Nguyen and Kandel (1997). Slices were maintained in an interface chamber at 28$^{o}$C which is a consistently used temperature for recording field responses on extended (hrs) time scales (Young et al., 2006; Gelinas and Nguyen, 2005, Gelinas et al., 2007). Slices were perfused at 1-2 mL/min with artificial cerebrospinal fluid (ACSF) composed of (in mM): 124 NaCl, 4.4 KCl, 1.3 $MgSO_4$, 1.0 $NaH_2PO_4$, 26.2 $NaHCO_3$, 2.5 $CaCl_2$, and 10 glucose, aerated with 95% $O_2$ and 5% $CO_2$. Extracellular field excitatory postsynaptic potentials (fEPSPs) were recorded with a glass microelectrode filled with ACSF (resistances, 2–3 MΩ) and positioned in the stratum radiatum of area CA1. fEPSPs were elicited by using two bipolar nickel-chromium electrodes placed on either side of the recording electrode in stratum radiatum to stimulate two separate sets of inputs converging onto the same postsynaptic population of neurons. Stimulating electrodes were staggered within stratum radiatum relative to each other to maximize independence of synaptic pathways. For simplicity, "S1" refers to the first or homosynaptic pathway and "S2" refers to the second or heterosynaptic pathway. The independence of the two pathways was confirmed by the absence of interpathway paired-pulse facilitation elicited by successive stimulation through the two electrodes at 75-, 100-, 150- and 200-ms intervals. Interpathway paired-pulse facilitation was assessed during baseline acquisition and at the conclusion of experiments. Stimulation intensity (0.08 ms pulse duration) was adjusted to evoke fEPSP amplitudes that were 40% of maximal size (Woo and Nguyen, 2003; Gelinas and Nguyen, 2007). Subsequent fEPSPs were elicited at the rate of once per

minute at this “test” stimulation intensity, with S1 stimulation preceding S2 stimulation by 200ms.

My first series of experiments were conducted using a standard "synaptic tagging and capture" protocol (Frey and Morris, 1997) in which repeated high-frequency stimulation (HFS) was induced by applying 4 trains (4x100 Hz, 1 s duration at test strength, 3 s interval) to S1. After 30 min, 1 train of HFS (1x100 Hz, 1 s) was applied to the second set of synaptic inputs (S2) (Woo et al., 2003; Young et al., 2006). β-AR-dependent LTP was induced by applying one train of HFS (100 Hz, 1 s duration at test strength) following a 10 min application of the β-AR agonist, isoproterenol (ISO; 1μM) (Gelinas and Nguyen, 2005). Post-HFS, ISO was applied for an additional 5 min. Thirty minutes after HFS at S1, low-frequency stimulation (LFS: 5 Hz, 10 s duration) was applied to S2. All experiments were performed blind to genotype.

*Drugs*: The β-AR agonist, *R* (-)-isoproterenol(+)-bitartrate (ISO, Sigma, St. Louis, MO) was prepared daily as concentrated stock solutions at 1 mM, in distilled water. The β-AR antagonist (±)-propranolol hydrochloride (PROP; 50 μM; Sigma) was also prepared daily in distilled water as a 50 mM stock solution. The GABA-A receptor antagonist, bicuculline (BICU; 10 μM; Sigma) was prepared as a 1mM stock solution and was used to assay for altered GABAergic inhibition. The mGluR5 antagonist, 6-methyl-2-(phenylethynyl) pyridine (MPEP, 10 μM; Sigma) was prepared as a 10 mM stock solution and perfused for 20 min prior to application of ISO. A translation inhibitor, emetine (EME; Sigma), was dissolved to a stock concentration of 20 mM in distilled water and perfused at 20 μM, 20 min prior to ISO application. At lower concentrations than 20 μM, EME blocked protein synthesis by >80% in hippocampal slices (Stanton and Sarvey, 1984). A MEK inhibitor, 2-(2-amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one (PD 98059) (50 μM; Sigma), was prepared in DMSO

at a concentration of 10 mM. An mTOR inhibitor, rapamycin (Rap; 1 μM; Sigma), was dissolved in DMSO to make stock solutions at 1 mM. PD98059 and Rap were perfused for 30 min prior to commencing experiments, overlapping with ISO application, and 10 min following ISO. All drug experiments were performed under dimmed light conditions due to photosensitivity of the drugs. Drug experiments were interleaved with drug-free controls.

*Data analysis.* The initial slope of the fEPSP was measured as an index of synaptic strength (Johnston and Wu, 1995). The average "baseline" slope values were acquired for 20 min before experimental protocols were applied. fEPSP slopes were measured at either 120 or 90 min after LFS or HFS for comparisons of LTP. Student's *t* test was used for statistical comparisons of mean fEPSP slopes between two groups, with a significance level of $p < 0.05$ (denoted as * on graphs). One-way ANOVA and Tukey–Kramer *post hoc* tests were done for comparison of more than two groups to determine which groups were significantly different from the others. The Welch correction was applied in cases in which the SDs of compared groups was significantly different. All values shown are means ± SEM, with $n$ = number of slices.

Results

Synaptic tagging induced by HFS alone is normal in Fmr1 knockout mouse hippocampus

Induction of translation-dependent LTP at one synaptic pathway can facilitate the induction of heterosynaptic LTP following the activity-dependent setting of "tags" which capture plasticity-related proteins (PRPs) necessary for expression of LTP (Frey and Morris, 1997; see Barco et al., 2008 and Frey and Frey, 2008 for reviews). Synaptic tagging and capture (STC) provides a mechanistic explanation for how translational and transcriptional products are appropriately targeted to previously active dendritic compartments, thereby maintaining synaptic specificity believed to be critical for cellular processes underlying memory formation (Morris, 2006; Barco et al., 2008; Frey and Frey, 2008). Regulated protein synthesis produces plasticity-related proteins (PRPs) necessary for heterosynaptic transfer of LTP (Frey and Morris, 1997). Knock out of Fmr1 reduces the expression of the translational repressor FMRP, which binds to several mRNAs that encode PRPs involved in synaptic plasticity (Hou et al., 2006; Muddashetty et al., 2007; Park et al., 2008; Schütt et al., 2009). Thus, I asked whether reduced expression of FMRP would alter synaptic tagging and capture of LTP, by using a stimulus protocol similar to one that is commonly implemented to probe tagging (Frey and Morris, 1997).

Two stimulating electrodes were placed in CA1 straddling a recording electrode positioned in stratum radiatum. Independence of synaptic pathways was confirmed using paired-pulse facilitation both before and at the conclusion of experiments. To determine if synaptic tagging was altered in FMR1 knockout mouse hippocampus I first established that synaptic tagging and capture could be induced in WT controls. A high-frequency stimulation protocol consisting of 4 trains of 100 Hz stimulation at 3 s intertrain intervals was applied to one synaptic pathway (S1; homosynaptic), in WT hippocampal slices which induced long-lasting (> 2hr) LTP

(Figure 4.1*A*). The magnitude of LTP in S1 as assessed 120 min after high-frequency stimulation was 154± 10% of baseline (n=9). When the same stimulation protocol was applied to Fmr1 KO mouse hippocampal slices, long-lasting LTP was induced (fEPSPs were potentiated to 158 ± 8% of baseline,120 min after HFS) which was not significantly different from WT controls ($p > 0.05$)(Figure 4.1*C*). Following a 30 min delay, one train of 100 Hz stimulation (1 s duration) was applied to a second synaptic pathway (S2) which converged on the same group of postsynaptic cells. One train of 100 Hz stimulation normally induces decremental LTP in mouse hippocampal slices which returns to baseline in < 2 h (Duffy et al., 2001; Gelinas and Nguyen, 2005). When preceded by multiple HFS trains of LTP homosynaptically, a single train of HFS applied heterosynaptically generated long-lasting LTP in both WT (mean fEPSP slope was 142± 8%) and Fmr1 KO (mean fEPSP slope was 144± 10%)(Figure 4.1*C*) slices as assessed 2 h post-stimulation. Although, no significant differences were detected between KO and WT slices at the second synaptic pathway ($p > 0.05$; Figure 1*C*), these results suggest that in both WT and Fmr1 KO mouse hippocampi, 4x100 Hz stimulation generates long-lasting LTP that can be captured at a second synaptic pathway in an activity-dependent manner.

To confirm that heterosynaptic facilitation of LTP had occurred, two control experiments were conducted in Fmr1 KO mouse hippocampal slices in which each pathway was stimulated alone. Either 4x100 Hz or 1x100 Hz alone was applied to S1 while monitoring heterosynaptic baseline responses at S2. Consistent with previous results (Frey and Morris, 1997), application of multiple high-frequency trains (4x100 Hz) induced LTP (fEPSPs were 156± 14% 120 min after HFS at S1, n=6, data not shown) that did not transfer to a second (S2) synaptic pathway receiving baseline stimulation (1 pulse per minute; fEPSPs were 101± 4% at 120 min). Next, I applied brief, HFS (1x100 Hz, 1 s) alone which failed to induce long-lasting synaptic changes at

either synaptic pathway (fEPSPs were 107± 9% of baseline at S1 and 98± 8% of baseline at S2 120 min post stimulation; n=4). My results show that HFS-dependent synaptic tagging and capture of LTP in Fmr1 KO mouse hippocampus is intact and similar to slices from wild-type mouse.

Figure 1.

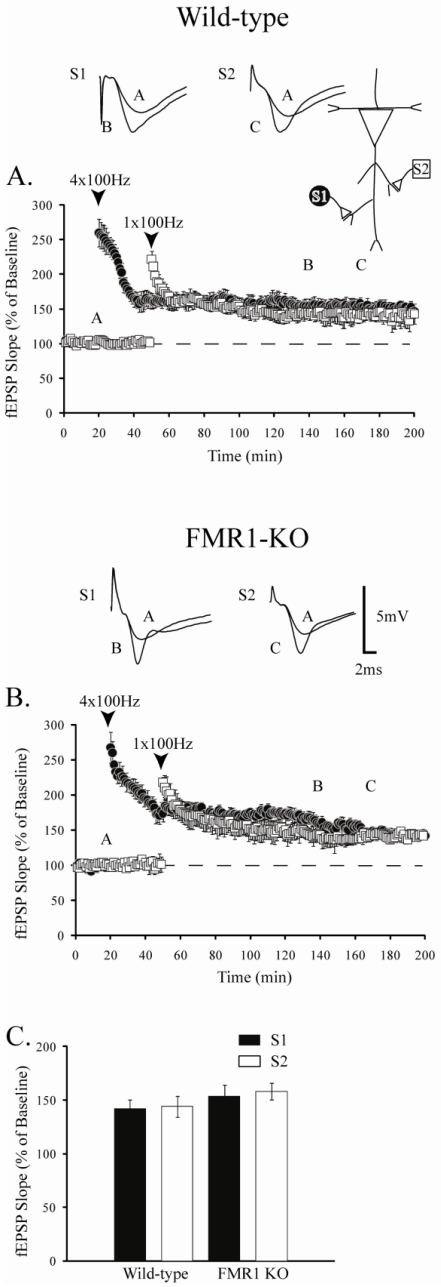


Figure 4.1. HFS-induced synaptic tagging and capture are not altered in Fmr1 knockout mice. *A.* Application of 4x100 Hz stimulation to S1 (filled circles) facilitates the induction of LTP by brief, HFS (1x100 Hz; open squares) at S2 (n=9). *B.* In Fmr1 KO slices, HFS-induced synaptic tagging and capture are similar to WT. Stimulation with repeated HFS at one synaptic pathway (4x100 Hz; filled circles) induces long-lasting LTP which can be captured heterosynaptically following a single train of HFS (1x100 Hz; open squares; n=8) at a second set of synapses converging on the same postsynaptic cells. *C.* Summary histogram comparing fEPSP slopes obtained in WT and KO slices 120 min after HFS at S1 and LFS at S2. No statistically significant differences were detected between WT and KO fEPSPs when homo- and heterosynaptic LTP were compared . Sample traces were taken 10 min after commencement of baseline recordings and 120 min after stimulation at S1 and S2. Results in C represent means ± SEM.

**Activation of β-ARs facilitates heterosynaptic transfer of LTP**

β-AR activation in area CA1 of mouse hippocampal slices facilitates induction of homosynaptic LTP by stimulation protocols normally subthreshold for inducing persistent, translation-dependent LTP (Thomas et al., 1996; Gelinas and Nguyen, 2005). As β-AR-dependent LTP requires *de novo* protein synthesis for its expression (Straube et al., 2003; Gelinas and Nguyen, 2005; Gelinas et al., 2007), I wanted to determine if inducing β-AR-dependent LTP at one synaptic pathway would enhance LTP at a second, independent set of synapses in WT slices. To do this, I repeated the two-pathway regimen previously described. Briefly, two stimulating electrodes were placed in stratum radiatum on either side of a recording electrode and staggered relative to each other to stimulate two independent groups of synapses. Using a β-AR-dependent LTP protocol previously established (Gelinas and Nguyen, 2005) consisting of 1x100 Hz for 1 s paired with ISO, LTP was induced at S1 (Figure 4.2*A*) which lasted > 2 h (mean fEPSP slope was 144±7% 120 min after HFS, Figure 4.2*E*). To test the idea that heterosynaptic LTP expression is facilitated by prior induction of β-AR-dependent LTP, I applied brief LFS (5 Hz, 10 s) to a second set of synapses. I found that subthreshold LFS in S2 induced long-lasting (> 2 h) LTP if it was preceded by the induction of β-AR dependent LTP in

S1 (mean fEPSP slopes were 119± 6% of baseline at S2, 2 h post stimulation; n=8, Figure 4.2*A*, *E*). To control for the effects of ISO application, I ran a control experiment consisting of ISO application (1 µM) for 15 min followed 25 min later with LFS (5Hz, 10 s). WT slices treated with ISO did not exhibit long-lasting potentiation in response to either ISO alone (fEPSPs were 98± 9% of baseline at S1 90 min after ISO application, n=6, data not shown) or LFS applied 25 min later to S2 (fEPSPs were 102± 7% of baseline 90 min after stimulation). Thus, ISO alone is insufficient for facilitating the subsequent induction of LTP by LFS in WT hippocampal slices. Next, I tested the hypothesis that 1x100 Hz alone is capable of enhancing the subsequent induction of heterosynaptic LTP by LFS in WT slices. Wild-type hippocampal slices tetanised with 100 Hz at S1 followed 30 min later with 5 Hz at S2 failed to generate LTP at either synaptic pathway. fEPSPs had returned to baseline at S1 (108± 6%) 120 min after HFS and at S2 (97± 9%) 120 min after LFS (n=6, data not shown). Based on these results, I wanted to determine if β-AR-dependent heterosynaptic plasticity is altered in Fmr1 KO mice.

Figure 2.

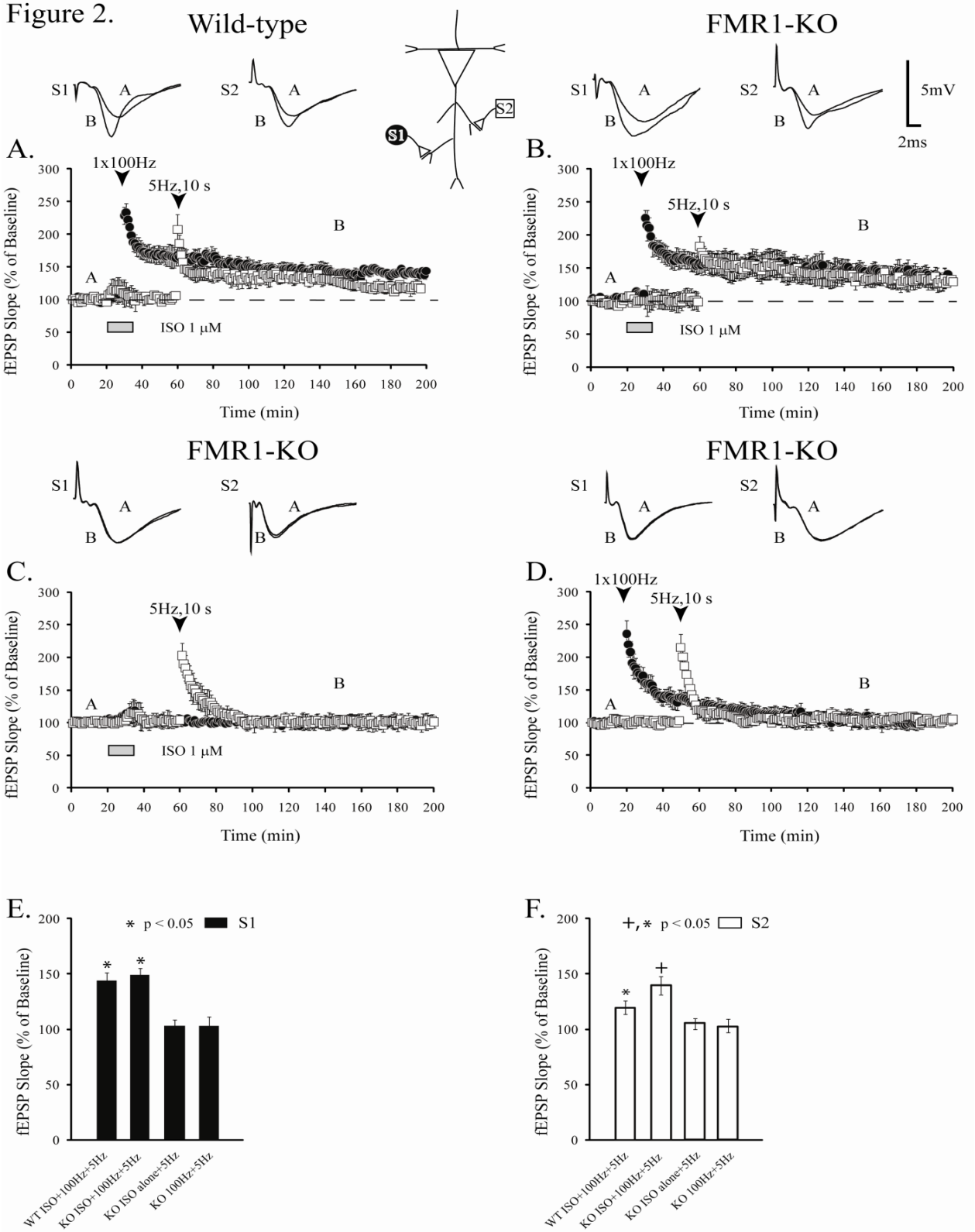

Wild-type
FMR1-KO
S1
S2
A
B
5mV
2ms
A.
B.
C.
D.
1x100Hz
5Hz,10 s
ISO 1 μM
fEPSP Slope (% of Baseline)
Time (min)
E.
F.
* p < 0.05
+,* p < 0.05
WT ISO+100Hz+5Hz
KO ISO+100Hz+5Hz
KO ISO alone+5Hz
KO 100Hz+5Hz

Figure 4.2. β-AR-dependent heterosynaptic plasticity is enhanced in Fmr1 KO mice. *A.* In WT slices, pairing one train of 100 Hz simulation with ISO application induces LTP (filled circles) which facilitates LTP at a second synaptic pathway (open squares) following LFS (5Hz, 10 s; n=8). LTP at S1 was significantly enhanced (denoted by *, Figure E) relative to S1 controls treated with ISO alone (C) or 100 Hz alone (D). Heterosynaptic LTP (open squares) was similarly enhanced (*, as shown in Figure F) relative to controls when preceded by ISO paired with 100 Hz homosynaptically. *B.* Similar to WT, homosynaptic long-lasting LTP was generated in Fmr1 KO hippocampal slices when 100 Hz was applied with ISO (open circles). However, heterosynaptic LTP was significantly potentiated (denoted as +) relative to both controls (C, D) and WT slices treated with ISO+100 Hz (A) 120 min after LFS at S2 (open squares; n=8). *C.* Application of ISO alone to Fmr1 KO slices prior to LFS induces a modest, short lasting increase in basal synaptic response which has no long-lasting effects on synaptic transmission (n=7). *D.* 100 Hz stimulation alone induces transient LTP which returns to baseline in < 2 hrs and does not facilitate heterosynaptic LTP following LFS at a second synaptic pathway in Fmr1 KO mouse slices (n=8). Importantly, LFS (5 Hz, 10 s) does not last more than 45 min. *E.* Summary histogram comparing fEPSP slopes obtained 120 min after HFS at S1. *F.* Summary histogram comparing fEPSP slopes 120 min after LFS at S2. Sample traces were taken 10 min after commencement of baseline recordings and 120 min after S1 stimulation. Results in panels E and F represent means ± SEM, $p < 0$.

**β-AR-dependent heterosynaptic LTP is enhanced in Fmr1 KO mice**

Metabotropic glutamate receptors (mGluRs) co-regulate translation by engaging signaling cascades including the extracellular-signal regulated kinase (ERK) and mammalian target of rapamycin (mTOR) pathways (Huber et al., 2000; Gallagher et al., 2004; Hou et al., 2004; Banko et al., 2006; Volk et al., 2007; Ronesi and Huber, 2008). In Fmr1 KO mice, deletion of FMRP decreases the requirement for protein synthesis in the expression of mGluR-LTD (Hou et al., 2006; Nosyreva and Huber, 2006). Similar to mGluRs, β-ARs couple to the ERK and mTOR signaling cascades to engage cap-dependent translation and promote persistent, translation-dependent homosynaptic LTP (Banko et al., 2006; Gelinas et al., 2007). Therefore, I wanted to determine whether β-AR-mediated LTP is altered homosynaptically and heterosynaptically in the absence of FMRP.

I applied the β-AR-dependent heterosynaptic plasticity protocol to Fmr1 KO mouse hippocampal slices. Briefly, following a 20 min baseline, ISO was applied for 10 min, then paired with 1x100 Hz stimulation at S1(homosynaptic) and maintained in ISO for an additional five minutes. When this protocol was applied to Fmr1 hippocampal slices, homosynaptic, long-lasting LTP was induced which was not significantly different from WT slices (mean fEPSP slopes were 152±6% in KO; n=8, and 147±7% in WT, 120 min after stimulation; n=8) (Figure 4.2*B*, *E*). Thirty minutes after HFS in S1, low-frequency stimulation (5 Hz, 10 s) was applied to a second, independent set of synapses converging on the same postsynaptic cells (S2). LFS applied heterosynaptically generated long-lasting LTP that was significantly enhanced in Fmr1 KO slices relative to WT controls (mean fEPSP slopes in KO = 139±8%, WT = 119±6%, as assessed 120 min post-LFS; $p < 0.05$) (Figure 4.2*F*). These results suggest that heterosynaptic plasticity is enhanced in response to β-AR stimulation in Fmr1 KO mouse hippocampus.

To determine if β-AR activation alone is sufficient for enhancing heterosynaptic plasticity, ISO (1 μM) was applied to in Fmr1 knockout hippocampal slices for 15 min. Application of ISO induced a modest, transient increase in fEPSPs at both synaptic pathways which returned to baseline, consistent with previously reported data (Gelinas and Nguyen, 2005). 60 min after ISO application, the mean fEPSP slopes at S1 were 103 ± 5% of baseline (n=7; Figure 4.2*C*). After a 25 min delay, low-frequency stimulation (5 Hz for 10 s) was applied to S2 which induced short-lasting LTP that decayed to baseline in < 1 h: mean fEPSP slopes were 105 ± 5% of baseline 90 min after 5 Hz stimulation (Figure 2*C*, *F*). Next, I sought to determine if a single tetanus of 100 Hz (1 s duration) to FMR1 KO mouse hippocampal slices could facilitate the expression of heterosynaptic LTP induced by LFS. Consistent with previous results, 1x100 Hz stimulation induced transient LTP that returned to baseline in < 2 h (mean fEPSP slopes were

104± 8% at S1 120 min after HFS, n=8; Figure 2D). Following a 30 min delay, low-frequency stimulation (5 Hz, 10 s) was applied to a second group of synapses converging on the same postsynaptic cells. Heterosynaptic low-frequency stimulation failed to induce long-lasting LTP (mean fEPSPs were 102± 6% at S2 when assessed 2 h after stimulation; n=8) (Figure 2*D*, *F*). Taken together, these data suggest that application of either ISO alone or 1x100 Hz stimulation without ISO, does not facilitate the future induction of heterosynaptic LTP in Fmr1 KO mouse hippocampal slices.

**Altered inhibition is not the primary cellular mechanism mediating β-AR-dependent heterosynaptic facilitation in Fmr1 KO hippocampus**

Alterations to GABAergic inhibition have previously been associated with neuropathologies observed in FXS rodent models (Burgard and Sarvy, 1991; El Idrissi et al., 2005; Curia et al., 2009). Hippocampal tissue from adult male Fmr1 KO mice exhibits reduced expression of the GABA-A receptor beta subunit, which is required for normal receptor function (El Idrissi et al., 2005). In addition, recent evidence suggests that ISO can reduce inhibitory drive within the hippocampus (Zsiros and Maccaferri, 2008). Therefore, I investigated whether altered inhibitory circuit function could account for the enhanced heterosynaptic LTP observed in Fmr1 KO hippocampus. To examine the effects of altered inhibitory function, I pre-applied the GABA-A receptor antagonist, bicuculline (BICU; 10 μM), for 20 min prior to, and overlapping with, ISO. If altered GABA-A receptor function is the mechanism responsible for enhanced heterosynaptic plasticity, then prior application of BICU should occlude the LTP enhancement generated by β-AR stimulation. Firstly, Fmr1 KO mouse hippocampal slices were exposed to a 20 min preincubation in BICU. Subsequently, a cocktail consisting of ISO and BICU was added to the bath for 15 min. Ten minutes into ISO+BICU application, 1x100 Hz, 1 s stimulation was

applied to S1. This protocol generated long-lasting LTP that was potentiated to 164± 12% (n=6) 2 h after stimulation in Fmr1 slices (Figure 4.3*B*). To test if heterosynaptic plasticity was altered by BICU, 25 min after homosynaptic 100 Hz stimulation, 5 Hz, 10 s stimulation was given to a second synaptic pathway. Heterosynaptic responses were potentiated to 171± 14%, of baseline 2 h after LFS (Figure 4.3*B*). It should be noted that Fmr1 slices treated with BICU exhibited epileptiform activity following LTP induction. This activity dissipated after 2 h and did not appear to affect the stability or consistency of my fEPSPs.

Next, wild type slices were treated with the same regimen. Preincubation with BICU followed by ISO paired with HFS induced long lasting LTP at S1 (fEPSPs were potentiated to 144± 10% 2 hr after stimulation; n=6, Figure 4.3*A*). Subsequent low-frequency stimulation (5 Hz) applied heterosynaptically generated long-lasting LTP which was potentiated to 154± 11% at 2 h post-tetanus (Figure 4.3*A*). When homosynaptic (S1) β-AR-dependent LTP in slices from WT (Fig. 2A) was compared to WT slices exposed to BICU (Fig. 4.3*A*) 2 h after stimulation, no significant differences were detected ($p > 0.05$; Figure 4.3*C*). Similarly, comparisons between homosynaptic LTP in Fmr1 KO slices in the presence or absence of BICU failed to reveal any significant differences ($p > 0.05$; Figure 3C). When heterosynaptic comparisons were conducted between fEPSPs 2 h after LFS in WT slices either exposed (Fig. 4.3*A*) or not exposed (Fig. 4.2*A*) to BICU, a significant enhancement of LTP in the presence of BICU was observed ($p < 0.01$; Figure 3D). Likewise, comparisons between heterosynaptic LTP in Fmr1 KO slices treated or not treated with BICU showed that BICU contributed to a significant increase in potentiation ($p < 0.05$; Figure 4.3*D*) 2 h after low-frequency stimulation. These results suggest that decreasing inhibition results in a further enhancement of heterosynaptic LTP in both genotypes and that

stimulation of β-ARs engages cellular plasticity mechanisms that are not primarily dependent on altered GABAergic receptor function.

Figure 3.

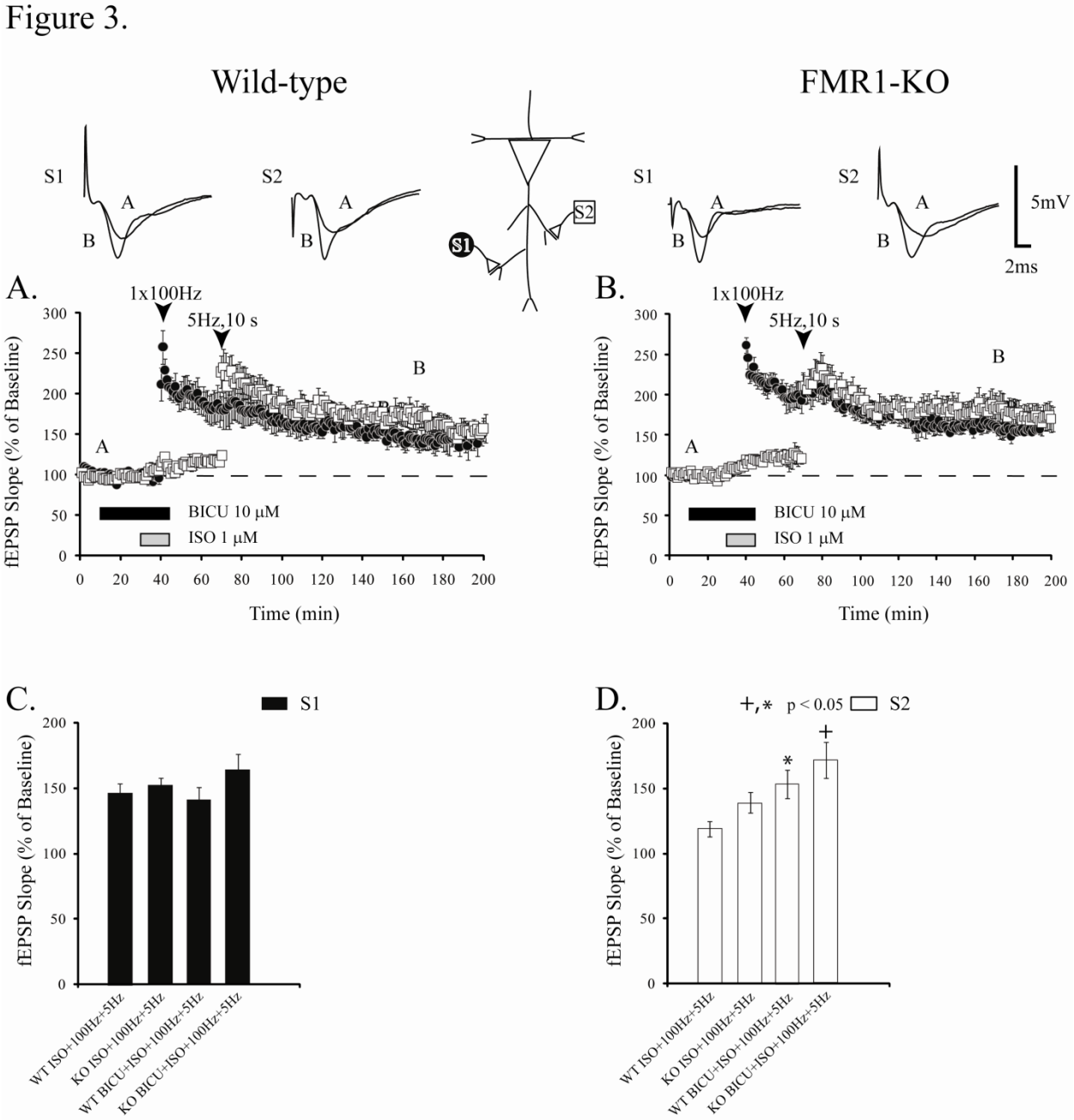


Figure 4.3. β-ARs facilitate heterosynaptic plasticity independent of altered GABA-A receptor function in Fmr1 KO hippocampus. *A*. To determine if enhanced heterosynaptic plasticity is the result of β-AR-mediated disinhibition, wild-type hippocampal slices were preincubated with the GABA-A receptor antagonist, bicuculline (BICU). Subsequent induction of homosynaptic β-AR-dependent LTP (filled circles) yielded long-lasting LTP that was not significantly different from WT slices not exposed to BICU (Figure C). Comparisons of heterosynaptic LTP in BICU-treated (open squares) and non-treated slices demonstrated a significant boosting of LTP in the presence of BICU (as denoted by *; Figure D). *B*. When BICU was added to the bath of Fmr1 KO slices, homosynaptic LTP (filled circles) was slightly, but not significantly, enhanced relative to BICU naive slices. When heterosynaptic LTP (open squares) was compared, prior application of BICU significantly enhanced the magnitude of subsequently induced LTP relative to Fmr1 slices not treated with BICU (denoted by +). *C*. Summary histogram comparing fEPSP slopes obtained 120 min after HFS at S1. *D*. Summary histogram comparing fEPSP slopes 120 min after LFS at S2. Sample traces were taken 10 min after commencement of baseline recordings and 120 min after S1 stimulation. Results in panels C and D represent means ± SEM, $p < 0.05$.

**β-AR activation is required for isoproterenol-induced heterosynaptic plasticity**

Genetic and pharmacological inhibition of β-ARs in area CA1 can interfere with expression of LTP and long-term memory formation (Winder et al., 1999; Ji et al., 2003). To test if β-ARs are required for heterosynaptic plasticity induced with isoproterenol, hippocampal slices from wild-type and Fmr1 knockout mice were pretreated with a β-AR antagonist, propranolol (PROP; 50μM). In wild-type mice, following a 10 min baseline, PROP was bath applied 20 min prior to, overlapping with and 10 min after ISO application. When high-frequency stimulation was applied homosynaptically, LTP was induced which decayed to baseline in < 2 h (Figure 4.4*A*; mean fEPSP slopes were 101 ± 8%, 120 min after HFS, n=9). When LFS (5 Hz, 10 s) was applied at a second set of synapses 30 min after homosynaptic high-frequency stimulation, I found that heterosynaptic plasticity was also blocked (mean fEPSPs were 98± 6% 90 min after LFS at S2)(Figure 4.4*A*). Similar to wild-type slices, prior application of propranolol prevented the induction of both homo- and heterosynaptic LTP (Figure 4.4*B*;

mean fEPSP slopes were 104± 3% 120 min after HFS at S1 and 101± 5% 90 min after LFS at S2, n =8)(Figure 4.4*C*). Thus, heterosynaptic plasticity produced by ISO application requires β-AR activation.

Figure 4.

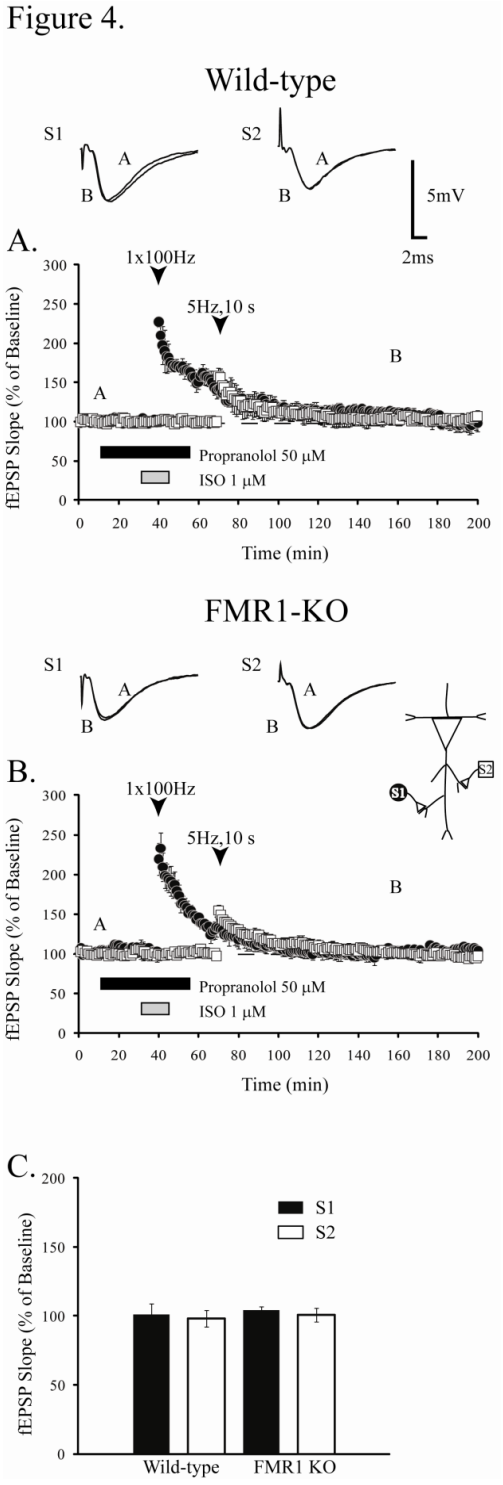


Figure 4.4. β-ARs are required for heterosynaptic facilitation of LTP. *A.* Application of the β-AR antagonist propranolol during β-AR activation inhibited both homosynaptic (filled circles) and heterosynaptic (open squares) LTP in WT slices (n=9). *B.* Similarly, In Fmr1 KO slices propranolol application overlapping with ISO impaired LTP at S1 (filled circles) and S2 (opens squares; n=8). *C.* A Summary histogram comparing fEPSP slopes obtained 120 min after HFS at S1 and 90 min after LFS at S2 demonstrates that propranolol blocked the maintenance of LTP both homo- (S1; black bars) and heterosynaptically (white bars) in both wild-type and Fmr1 KO genotypes.. Sample traces were taken 10 min after commencement of baseline recordings and 120 and 90 min after stimulation. Results in C represent means ± SEM.

**Prior low-frequency stimulation can express LTP when β-AR-dependent LTP is generated heterosynaptically**

The synaptic tagging hypothesis states that local molecular traces induced by synaptic activity serve as "tags" which can sequester plasticity-related proteins generated within a discrete time window before or after tags are set (Frey and Morris, 1997). In “weak before strong” stimulation experiments, the activity-dependent tag is set by mild stimulation prior to the generation of plasticity-related proteins by a later, stronger stimulus (Frey and Morris, 1998). To determine if prior synaptic potentiation can be facilitated by the future induction of β-AR-dependent LTP I first applied low-frequency stimulation (5 Hz, 10 s) to WT slices in pathway S1. Twenty-five minutes later, β-AR-dependent LTP was induced heterosynaptically (S2). Prior LFS induced long-lasting LTP (mean fEPSP slope was 122±9% 120 min post stimulation, n=8) provided that LTP was induced heterosynaptically through pairing of ISO with high-frequency stimulation (1x100 Hz). (Figure 4.5*A*; fEPSPs at S2 were 144±9% 90 min post HFS). Similar to WT slices, low-frequency stimulation applied 25 min prior to the induction of heterosynaptic β-AR-dependent LTP induced homosynaptic LTP that was still potentiated 2 h after LFS. Comparisons between slices from Fmr1 KO and WT mice revealed no significant differences in the magnitude of LTP either homosynaptically ($p > 0.05$; Figure 4.5*B*; mean fEPSP slope was

120±8% at S1 in Fmr1 KOs, n=8) or heterosynaptically (Figure 4.5*C* $p > 0.05$; mean fEPSPs were 147±9% 90 min after HFS in Fmr1 KOs). These data suggest that low-frequency stimulation initiates an activity-dependent molecular trace which lasts 25 min, that can interact with plasticity products generated by a strong stimulus applied heterosynaptically at a future time point.

Figure 5.

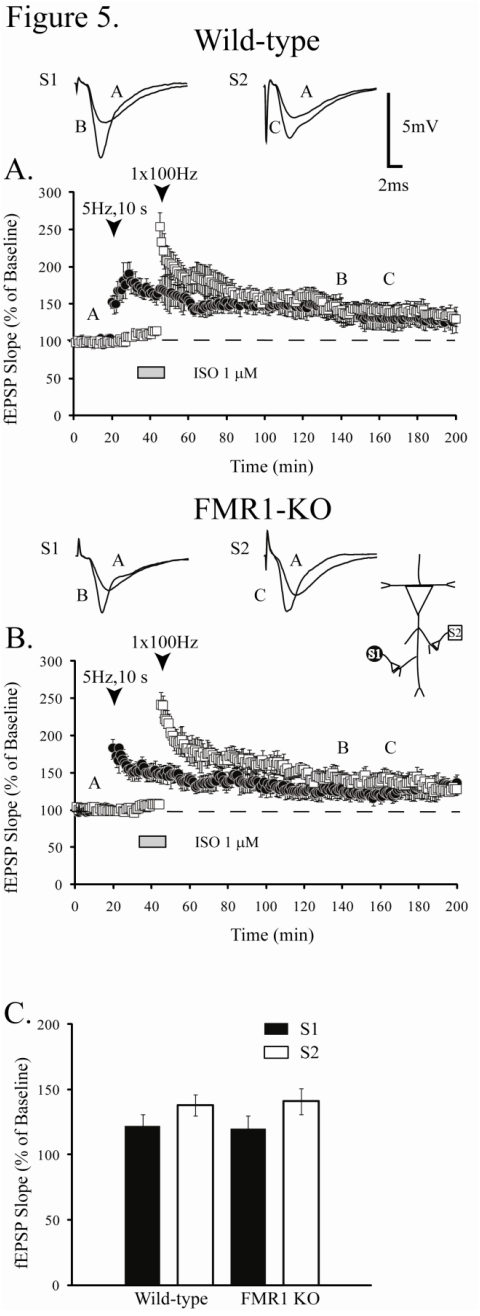


Figure 4.5. Heterosynaptic facilitation of LTP is still observed when LFS precedes ISO paired with HFS at a second synaptic pathway. *A.* Application of LFS (5 Hz, 10 s: S1; filled circles) 25 min prior to ISO application paired with HFS, still shows heterosynaptic facilitation (S2; open squares) (n=8). *B.* Similar to WT, prior LFS (S1; open circles) in Fmr1 KO hippocampal slices allows for the transfer of LTP induced by β-AR activation paired with HFS at a second synaptic pathway (S2; open squares; n=8). Comparisons between WT and Fmr1 KO mouse genotypes (C) 120 min after LFS at S1 or HFS paired with ISO at S2 failed to demonstrate significant differences in mean fEPSP slopes. These results suggest that application of the low-frequency "weak" stimulus homosynaptically can capture subsequently induced LTP heterosynaptically regardless of genotype. *C.* Summary histogram comparing fEPSP slopes obtained 120 min after HFS at S1 (black bars) and LFS at S2 (white bars). Sample traces were taken 10 min after commencement of baseline recordings and 120 min after stimulation. Results in C represent means ± SEM.

**mGluR activation is not required for enhanced heterosynaptic LTP in Fmr1 KO mouse hippocampus**

mGluR-dependent LTD is enhanced in Fmr1 KO mouse hippocampus. Normally, mGluR LTD requires protein synthesis for maintenance of GluR internalization (Nosyreva and Huber, 2006; Nakamoto et al., 2007). In Fmr1 KO mouse hippocampus, mGluR LTD does not require translation, suggestive of an increase in basal protein synthesis (Hou et al., 2006; Nosyreva and Huber, 2006). It is unclear whether heterosynaptic LTP in Fmr1 KO mouse hippocampus requires mGluR activation. To determine whether mGluRs are required for heterosynaptic facilitation of LTP by β-ARs in Fmr1 mouse hippocampal slices, I used the mGluR5 antagonist, MPEP (10 μM). Application of MPEP 20 min prior to and overlapping with β-AR stimulation paired with 100 Hz tetanus in S1, did not affect LTP expression in either synaptic pathway: mean fEPSP slopes in S1 were 147±8% 2 h after tetanisation in Fmr1 KO slices (n=6; Figure 4.6*A*). When compared to WT controls not exposed to MPEP (144±6%, n=8; Figure 4.6*B*) there was no significant difference in homosynaptic LTP ($p > 0.05$). However, hippocampal slices from Fmr1 KO mice, treated with MPEP, still displayed heterosynaptic LTP that was enhanced

relative to WT slices not exposed to MPEP: mean fEPSP slopes in KOs were 144±8% of baseline which is significantly elevated relative to wild-type slices (mean fEPSPs were 118±6% of baseline), when compared 120 min after stimulation ($p < 0.05$, Figure 4.6*C*). Thus, the enhancement of heterosynaptic LTP in slices from Fmr1 KO mice does not appear to require mGluR-5 activation.

Figure 6.

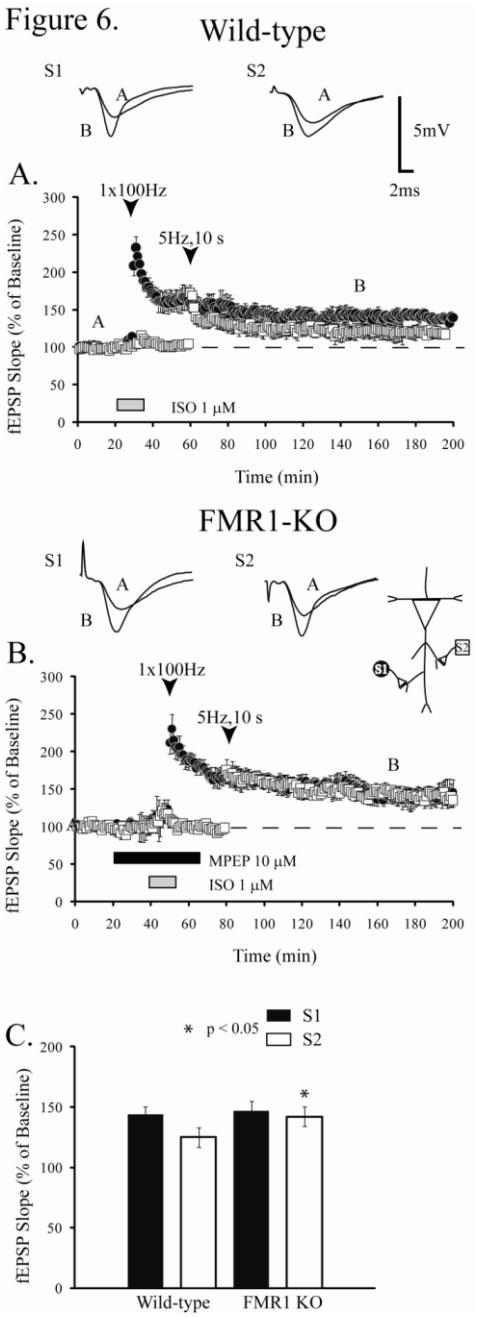


Figure 4.6. MPEP does not block the β-AR-dependent enhancement of heterosynaptic plasticity in Fmr1 KO mice. *A.* Induction of β-AR-dependent LTP at one synaptic pathway (S1; filled circles) facilitates the subsequent expression of heterosynaptic LTP (S2; open squares) in WT slices (n=8). *B.* The same protocol applied to Fmr1 KO mouse hippocampal slices in the presence of the mGluR5 antagonist MPEP, induces heterosynaptic plasticity (S2; open squares) that is still enhanced relative to WT controls (n=6). *C.* A Summary histogram comparing fEPSP slopes obtained 120 min after HFS at S1 (black bars) revealed no significant differences in homosynaptic LTP. Heterosynaptic LTP (S2; white bars) was significantly elevated (*) 120 min after LFS in Fmr1 KO slices treated with MPEP when compared to WT slices not treated with MPEP. Sample traces were taken 10 min after commencement of baseline recordings and 120 min after HFS. Results in C represent means ± SEM, $p < 0.05$.

**β-AR-dependent heterosynaptic LTP requires translation in Fmr1 KO mice**

Protein synthesis is critically involved in the generation of LTP and long-term memory formation. Results from Auerbach and Bear (2010) suggest that priming of LTP by prior activation of mGluRs, involves a reduced requirement for translation in Fmr1 KO mice, consistent with enhanced synthesis of PRPs. Previously, cap-dependent translation was shown to be a critical target for regulation during β-AR-induced synaptic plasticity (Gelinas et al., 2007). As β-ARs and mGluRs both engage cap-dependent translation, I asked whether β-AR activation in Fmr1 knock-out mice renders LTP immune to protein synthesis inhibition. To test this hypothesis, I preincubated slices in a translational inhibitor, emetine (EME). Homosynaptic LTP elicited by pairing β-AR activation with 1x100 Hz high-frequency stimulation in S1 decayed in the presence of EME. Mean fEPSP slopes were 115±10% in KO (n=8) and 107±13% in WT (n=7), $p > 0.05$, 120 min after HFS (Figure 4.7*A, B*). When compared to homosynaptic LTP induced with β-ARs in WT controls (mean fEPSP slopes were 144±9% 120min after HFS, n=6, data not shown), Tukey-Kramer *post hoc* tests revealed that emetine significantly inhibited LTP in both WTs and KOs ($p < 0.05$). Bath application of emetine overlapping with ISO likewise inhibited LTP heterosynaptically when 5 Hz, 10 s LFS was applied 30 min later in S2 relative to

EME-free control slices (Figure 4.7*A*, *B*) (mean fEPSP slopes in the presence of EME were 106 ± 6% in KO (n=8) and 104±10% in WT (n=7), $p > 0.05$, 90 min after LFS)(Figure 4.7*C*). When compared to heterosynaptic LTP induced with β-AR stimulation in WT controls (127±7%, 90 min after LFS, data not shown), Tukey-Kramer *post hoc* tests revealed that heterosynaptic LTP in both wild-type and Fmr1 knockout slices ($p < 0.05$) (Figure 4.7*D*) decayed in the presence of EME. Thus, when compared two hours post-stimulation, Fmr1 KO mouse slices exhibited significant decreases in potentiation at both S1 and S2 which was similar to WT slices exposed to EME.

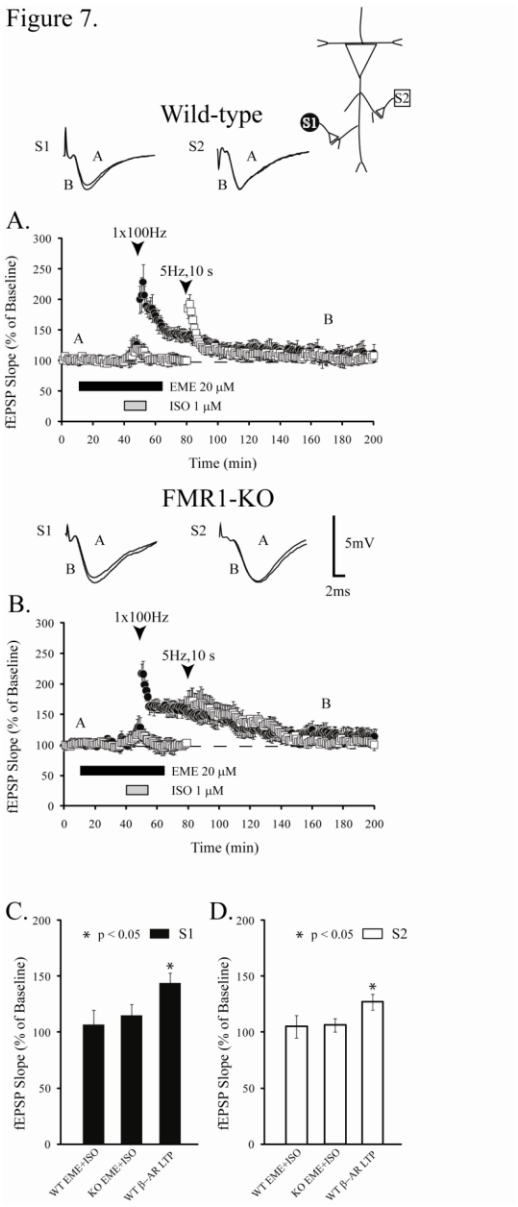



Figure 4.7. Knockout of Fmr1 does not confer immunity to translation inhibition. *A.* WT slices treated with EME during β-AR activation did not express LTP at S1 (filled circles) or S2 (open squares; n=7) when compared to β-AR-dependent LTP induced in WT slices without EME. *B.* Similar to WT, EME blocked both homo (filled circles) and heterosynaptic (open squares) plasticity in Fmr1 KO slices. Comparisons were made with β-AR-dependent heterosynaptic plasticity induced in WT slices (data not shown, n=6). *C.* Summary histogram comparing fEPSP slopes obtained 120 min after HFS at S1 (black bars) demonstrates a significant (*) enhancement of LTP in WT slices not exposed to EME relative to WT and Fmr1 slices treated with EME. Similarly, LTP in both WT and Fmr1 KO slices treated with EME was significantly (*) less 90 min after LFS at S2 (white bars) than compared to WT slices without EME. Sample traces were taken 10 min after commencement of baseline recordings and 120 after HFS. Results in C represent means ± SEM, $p < 0.05$.

**Knockout of Fmr1 confers immunity to inhibition of mTOR but not ERK**

β-ARs recruit the mammalian target of rapamycin (mTOR) signaling pathway to facilitate LTP maintenance through translation initiation (Gelinas et al., 2007). I assessed whether heterosynaptic LTP was affected by an mTOR inhibitor, rapamycin, in Fmr1 knockout mouse hippocampus. Wild-type slices treated with rapamycin (RAP) demonstrated reduced homo- and heterosynaptic plasticity. Application of RAP 30 min prior to, overlapping with, and 10 min after ISO paired with high-frequency stimulation inhibited the expression of homosynaptic LTP as assessed two hours after HFS (mean fEPSP slopes in S1 were 108 ± 12%). Subsequent (30 min post-HFS) stimulation with low-frequency stimulation at S2 generated decremental LTP which was 108 ± 9% 90 min post LFS (Figure 4.8*A*; n=9). Interestingly, Fmr1 KO mouse slices treated with RAP demonstrated intact homosynaptic LTP. Homosynaptic LTP was significantly enhanced (mean fEPSP slope was 140±9% in S1, 120 min post stimulation) in rapamycin-treated slices (Figure 4.8*B*, *E*; n=6, $p < 0.02$) relative to wild-type slices exposed to RAP. However, inhibition of mTOR still attenuated LTP heterosynaptically in Fmr1 mouse slices. Mean fEPSP slope at S2 90 min after low-frequency stimulation was 115±9% ($p > 0.05$ relative to WT) (Figure 4.8*F*). To control for potential synaptic crosstalk generated by heterosynaptic LFS, the RAP experiments were repeated in Fmr1 KO slices in the absence of LFS in S2. Consistent with my previous results, RAP failed to inhibit homosynaptic LTP (fEPSPs were 137±12% in S1 120 min after HFS, $p < 0.05$ compared to WT slices exposed to RAP; data not shown). Stimulation of a second control pathway showed that RAP did not adversely affect baseline synaptic responses (fEPSPs were 96±10% 2 h after RAP washout at S2). These results support previous research (Sharma et al., 2010), which found that mTOR signaling was intact and enhanced in the absence of FMRP.

Because the ERK pathway cooperates with mTOR to regulate translation-dependent, β-AR-mediated LTP in the mouse hippocampus (Banko et al., 2006; Gelinas et al., 2007; Gelinas et al., 2008), I asked whether homo- and heterosynaptic LTP in Fmr1 KO mouse hippocampus were affected by inhibiting MEK, which my group has previously established as a pharmacological blocker of β-AR-dependent synaptic enhancement (Gelinas et al., 2005). In WT slices, induction of LTP was blocked homosynaptically (mean fEPSP slope was 116± 11%, 120 min after HFS) and heterosynaptically (mean fEPSP slope was 105± 7%, 90 min post LFS) (Figure 4.8*C*, *E*) by a MEK inhibitor, PD98059 (n=6) when it was applied 30 min before as well as overlapping with and 10 min after ISO+HFS at S1. In slices from Fmr1 KO mice, LTP was also blocked by PD98059: mean fEPSP slopes were 103± 8% at S1 and 98± 6% at S2 (Figure 4.8*D, E*; n=7) of baseline 120 and 90 min post stimulation respectively.

Figure 8.

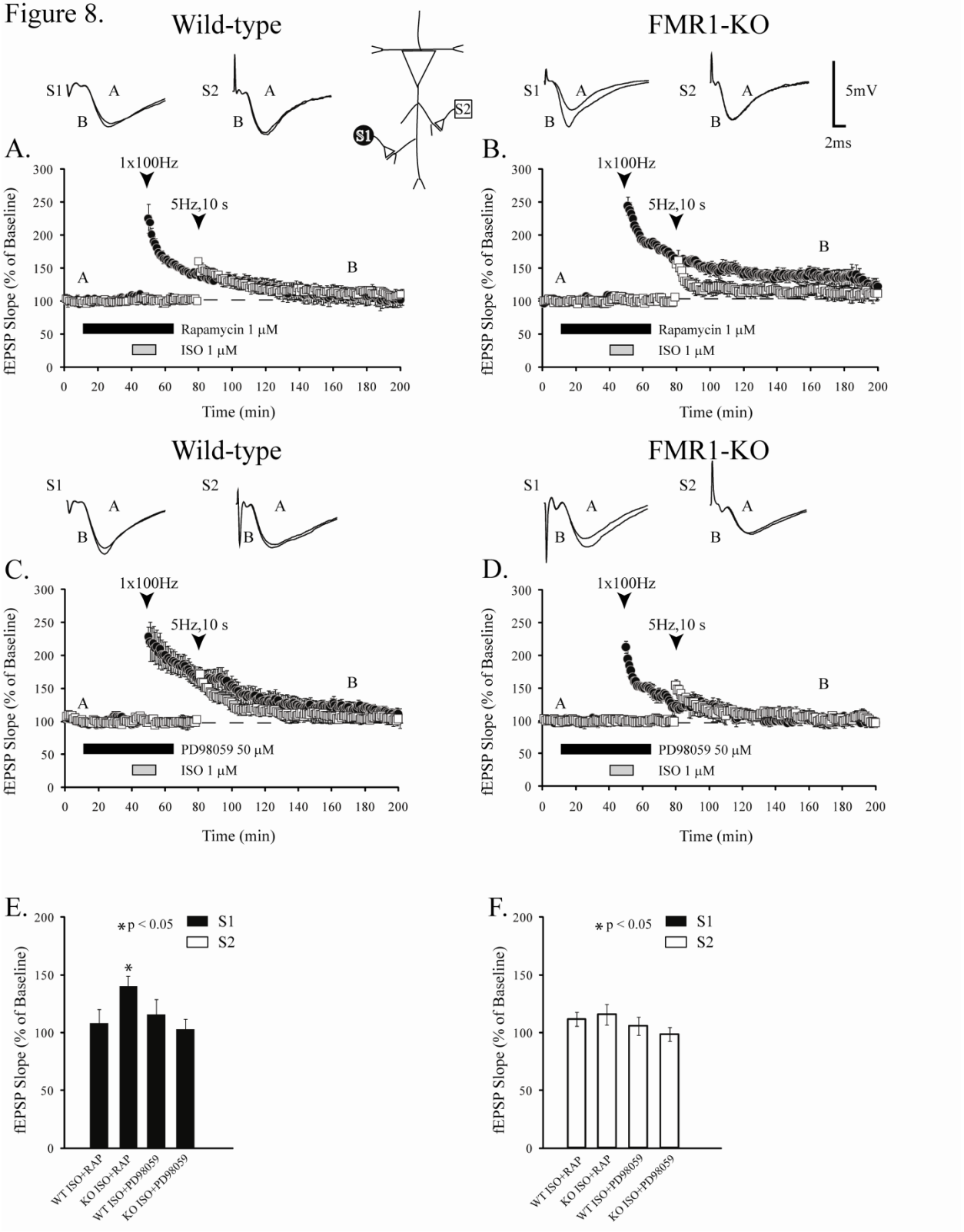

Wild-type
FMR1-KO
S1
S2
A
B
5mV
2ms
A.
B.
C.
D.
1x100Hz
5Hz,10 s
fEPSP Slope (% of Baseline)
Time (min)
Rapamycin 1 μM
ISO 1 μM
PD98059 50 μM
E.
F.
* p < 0.05
WT ISO+RAP
KO ISO+RAP
WT ISO+PD98059
KO ISO+PD98059

Figure 4.8. Inhibition of mTOR does not block β-AR-dependent homosynaptic LTP in Fmr1 KO mice. *A*. Application of rapamycin completely blocked homo- (filled circles) and heterosynaptic (open squares) plasticity in WT slices (n=9). *B.* In Fmr1 KO mice, co-application of rapamycin and ISO blocked the heterosynaptic facilitation of LTP at S2 (open squares). However, LTP at S1 (filled circles) was not blocked by mTOR inhibition and was significantly elevated (as denoted by the *) relative to WT slices exposed to RAP (n=6). *C.* In WT slices, application of the MEK inhibitor PD98059 prevented the expression of LTP at both S1 (filled circles) and S2 (open squares; n=6). D. Similar to WT, inhibition of MEK in Fmr1 hippocampal slices blocked LTP expression at both synaptic pathways (S1: filled circles, S2: open squares; n=7). *E*. Summary histogram comparing fEPSP slopes obtained 120 after HFS at S1 (black bars) shows that inhibition of mTOR failed to prevent homosynaptic LTP in Fmr1 KO hippocampal slices. *F*. Heterosynaptic LTP (white bars) was inhibited in both genotypes by treatment with either RAP or PD98059. Sample traces were taken 10 min after commencement of baseline recordings and 120 after stimulation. Results in E and F represent means ± SEM, $p < 0.05$.

## Discussion

Fragile X syndrome is characterized by behavioural (Hagerman and Hagerman, 2002; Spencer et al., 2005; Moon et al., 2006) and cognitive (Tsiouris et al., 2004; Macleod et al., 2010) abnormalities that have been linked to altered metabotropic receptor signaling and translation regulation (Huber et al., 2002; Bear et al., 2004; Kelleher et al., 2008; see O'Donnell and Warren, 2002 and Pfeiffer and Huber, 2009 for review). The noradrenergic system, acting through metabotropic adrenergic receptors, regulates multiple processes including sleep, attention, learning, memory, and arousal (Gelinas and Nguyen, 2007; Berridge and Waterhouse, 2008). β-ARs also mediate synaptic plasticity involved in encoding the association of disparate events over time (Shapiro et al., 2006). Heterosynaptic facilitation provides a mechanism for associating stimuli over time by integrating synaptic events following induction of persistent synaptic plasticity (Barco et al., 2008; Frey and Frey, 2008).

A previously defined heterosynaptic protocol known as "synaptic tagging and capture" (STC) is commonly initiated by applying HFS (4x100 Hz) at one pathway (S1) which generates

plasticity-related proteins (PRPs) that can be captured by activity-dependent "tags" set at a second synaptic pathway (S2) (Frey and Morris, 1997). Neuromodulators gate both protein synthesis and activation of second messengers that can support this type of heterosynaptic plasticity (Gelinas and Nguyen, 2005; Navakkode et al., 2007). Application of a cAMP phosphodiesterase inhibitor, rolipram, enabled the heterosynaptic transformation of translation-independent LTP induced by one 100 Hz train into translation-dependent LTP, suggestive of a cAMP-mediated increase in plasticity products (Navakkode et al., 2004). Noradrenergic receptors can facilitate the generation and regulation of plasticity proteins capable of prolonging LTP (Winder et al., 1999; Straube et al., 2003; Gelinas and Nguyen, 2005) and boosting long-term memory (Cahill et al., 1994; Hu et al., 2007; Kemp and Manahan-Vaughan, 2008). Interestingly, prior exposure of rats to novel experiences engages the noradrenergic system (Kitchigina et al., 1997) to promote the consolidation of short-term memory into protein synthesis-dependent long-term memory, that is likely to involve "behavioural tagging" (Moncada and Viola, 2007; Ballarini et al., 2009). Taken together, these results suggest that β-AR activation engages intracellular pathways critical for heterosynaptic facilitation of LTP. Herein, I describe a novel heterosynaptic plasticity protocol which relies upon activation of β-ARs for its induction. I found that pairing β-AR activation with high-frequency stimulation induces LTP that could be transferred to a second synaptic pathway provided that stimulation sufficient for generating a molecular activity trace was applied.

How does β-AR activation facilitate heterosynaptic plasticity in Fmr1 KO mouse hippocampus? One of the core phenotypes of FXS models is enhanced epileptogenesis as a result of dysregulated inhibitory circuit function (Burgard and Sarvy, 1991; El Idrissi et al., 2005; Curia et al., 2009). This fact, coupled with the ability of isoproterenol to depress inhibitory

interneurons, suggests that altered inhibitory circuit function may be responsible for enhanced heterosynaptic LTP in Fmr1 KO mice. However, I found that disinhibition through application of the GABA-A receptor antagonist bicuculline failed to occlude the effects of β-AR activation. Indeed, consistent with previous data (Burgard and Sarvy, 1991), co-application of ISO and BICU resulted in further enhancement of heterosynaptic LTP (additionally, there was a trend for enhanced homosynaptic LTP in Fmr1 KO slices but this failed to reach statistical significance). My results do not support the idea that altered GABAergic inhibition could account for enhanced heterosynaptic LTP in Fmr1 mice.

The mGluR theory of FXS states that stimulation of mGluR1/5 leads to enhanced synthesis of LTD-related plasticity proteins, which are normally repressed through FMRP-dependent mRNA regulation (Bear et al., 2004; Pfeiffer and Huber, 2007). The enhancement of heterosynaptic LTP in Fmr1 KO mice observed following my β-AR-dependent heterosynaptic protocol did not require mGluR activation, as application of the mGluR5 antagonist MPEP had no effect on the magnitude of LTP at either synaptic pathway in Fmr1 KO slices. The mGluR theory has been extended to include signaling molecules that affect cap-dependent translation, including ERK and mTOR (Hou et al., 2006; Volk et al., 2007; Sharma et al., 2010). Importantly, β-AR activation has been linked to regulation of cap-dependent translation through ERK and mTOR pathways, both of which are dysregulated in Fmr1 KO mouse hippocampus (Hou et al., 2006; Kim et al., 2008; Sharma et al., 2010). These pathways are critical for coupling mGluRs and β-ARs to protein synthesis (Banko et al., 2006; Gelinas et al., 2007) through interactions with eIF4E, a translation initiation factor required for eIF4F initiation complex formation and translation initiation (Costa-Mattioli et al., 2009; Richter and Klann, 2009). My data suggest that activation of the ERK pathway is required for homosynaptic LTP and for

heterosynaptic facilitation of LTP in both WT and Fmr1 KO mouse hippocampus. In contrast, activation of mTOR is required for heterosynaptic LTP, but not for homosynaptic LTP, in Fmr1 KO mouse hippocampus. Although the mTOR inhibitor rapamycin prevented the expression of heterosynaptic LTP, homosynaptic LTP was still intact. Upregulated mTOR signaling may lower the threshold for initiation of protein synthesis by elevating the levels of "free" eIF4E (Sharma et al., 2010), thereby boosting heterosynaptic plasticity.

As inhibition of protein synthesis, but not inhibition of mTOR, blocked heterosynaptic facilitation of LTP, decreased levels of FMRP appear to affect processes upstream of mTOR-mediated translation regulation. Previous results suggest that in Fmr1 KO mice, mTOR signaling is elevated, which may facilitate mGluR-dependent LTD (Sharma et al., 2010; Hoeffer and Klann, 2010). Hyperphosphorylation of eIF4E-binding proteins (4E-BPs) by mTOR regulates translation initiation by increasing levels of unbound eIF4E. Once dissociated from the 4E-BPs, eIF4E is able to complex with other initiation factors to engage translation (Klann et al., 2004; Richter and Klann, 2009). Dysregulation of the PI3K-mTOR pathway in the absence of FMRP has recently been reported (Sharma et al., 2010) and could provide a mechanism for enhanced β-AR-dependent synaptic facilitation (Figure 4.9*A*). As β-AR-dependent synaptic plasticity was partially immune to mTOR inhibition, my results suggest that, in a broader perspective, dysregulation of mTOR signaling may be an important cause of altered synaptic plasticity which both mGluRs and β-ARs critically modulate.

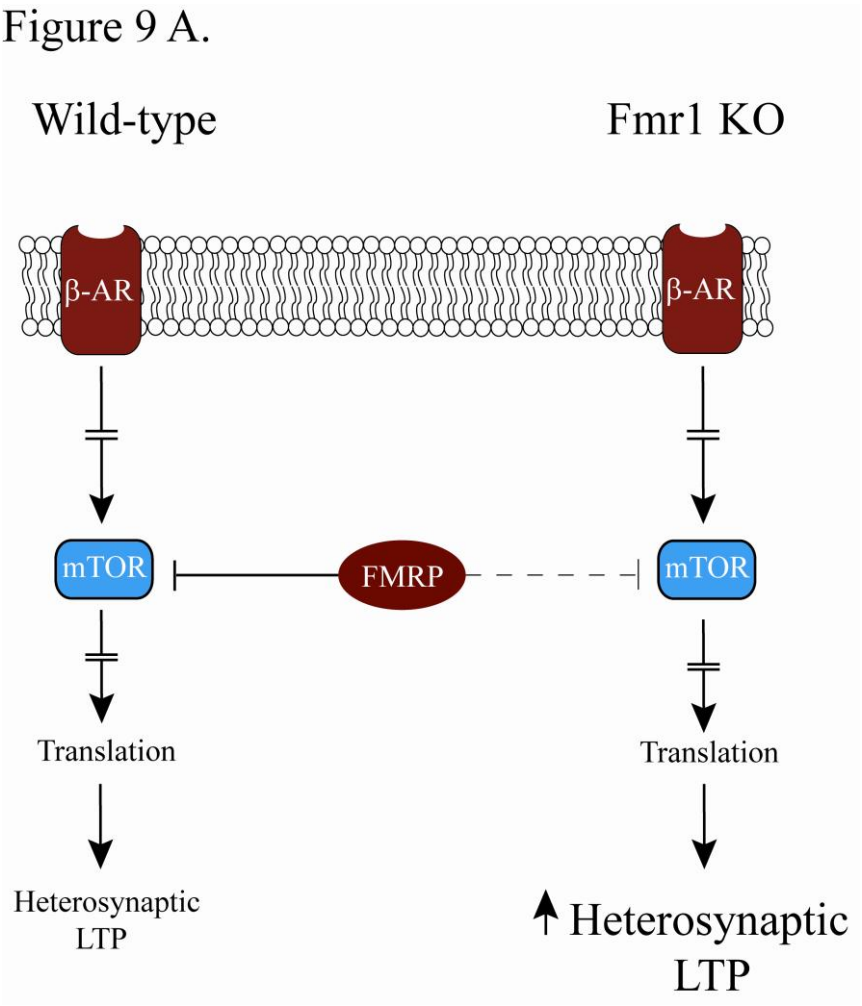


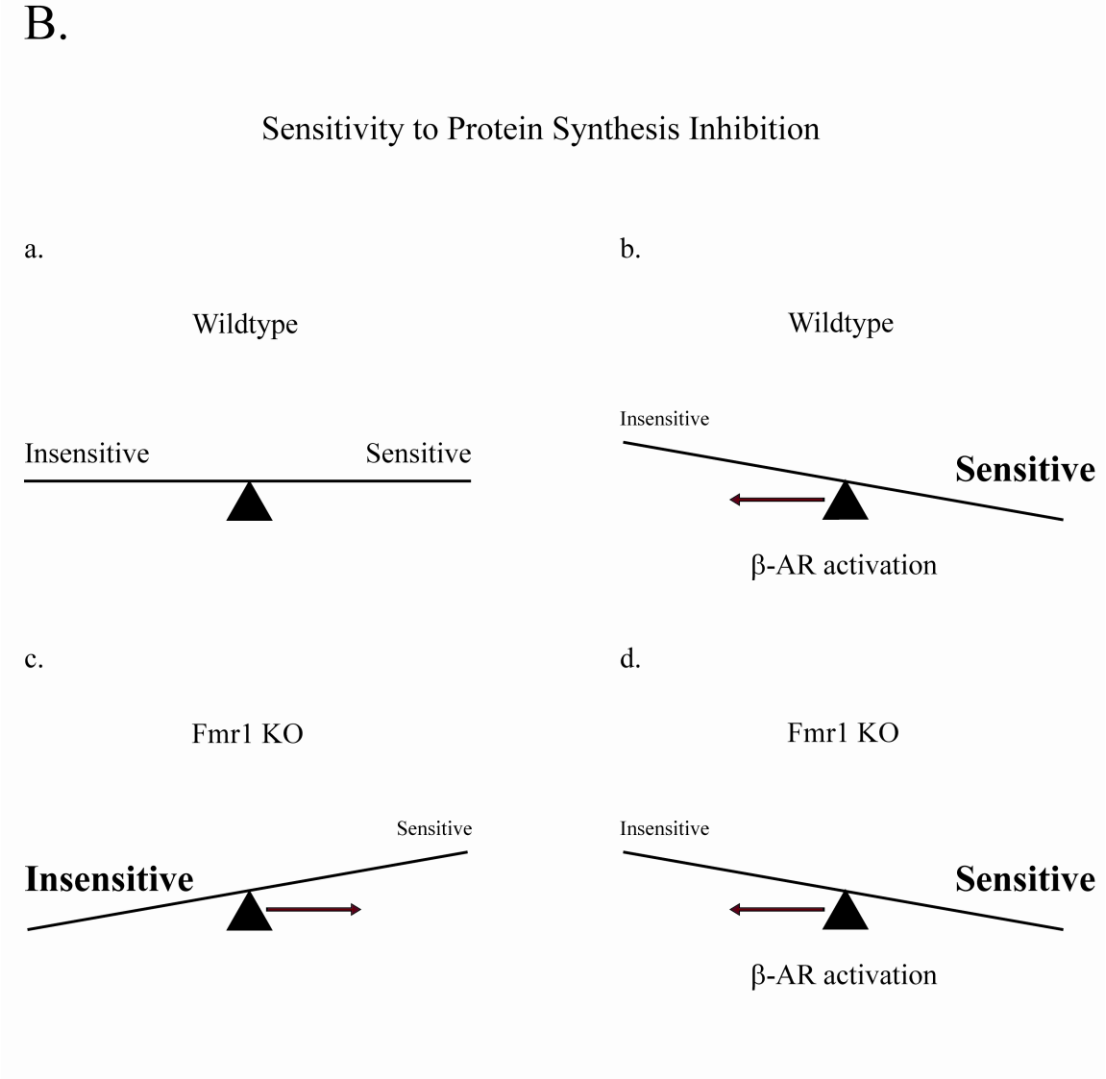

Figure 4.9. *A*. Increased mTOR signaling in the absence of FMRP enhances heterosynaptic plasticity. In wild-type mouse hippocampus, FMRP represses mTOR activity. The mTOR signaling cascade can regulate protein synthesis mechanisms necessary for heterosynaptic transfer of LTP. Without the repressive effects of FMRP (right side), mTOR activity is elevated resulting in dysregulated translation and exaggerated heterosynaptic plasticity.

Interestingly, β-AR-dependent heterosynaptic facilitation in Fmr1 mouse hippocampus is blocked by a protein synthesis inhibitor, in contrast to mGluR-LTD, which is immune to translational inhibition (Nosyreva and Huber, 2006). Evidence suggests that FMRP may act as a brake on cap-dependent translation, evident from the increased basal eIF4F initiation complex formation observed in Fmr1 KO mice (Sharma et al., 2010). FMRP suppresses translation

through interaction with cytoplasmic FMRP-interacting protein (CYFIP1) which binds to eIF4E, thereby preventing formation of the eIF4F initiation complex (Napoli et al., 2008). My data suggest that translation is still required for heterosynaptic plasticity mediated by β-ARs in both WT and Fmr1 KO mice. These results contrast with mGluR-LTD (Nosyreva and Huber, 2006) and mGluR-primed LTP (Auerbach and Bear, 2010) in Fmr1 knockout mice, which persist in the presence of translation inhibitors. The disparity in these data could be the result of a higher demand for plasticity proteins in heterosynaptic plasticity protocols relative to single pathway experiments. Enhanced basal protein synthesis may be sufficient for overcoming translational inhibition using protocols that do not exhaust the availability of PRPs. Indeed, previous research has described a form of inter-synaptic competition for plasticity proteins under conditions of reduced protein synthesis (Fonseca et al., 2004). When plasticity protein synthesis is limited, the maintenance of LTP at one synaptic pathway occurs at the expense of a second previously potentiated synaptic pathway, in a process known as "competitive maintenance" (Fonseca et al., 2004). Thus, even in circumstances of enhanced basal protein synthesis, the sharing of plasticity proteins required for heterosynaptic facilitation of synaptic changes may require additional translation to maintain LTP in two pathways.

Temporally and spatially restricted translation gates persistent synaptic plasticity by generating proteins necessary for long-term modifications of synaptic structure and function. Fmr1 knockout mice exhibit alterations in translation regulation (Nimchinsky et al., 2001). Enhanced protein synthesis has been demonstrated in the hippocampus of Fmr1 KO mice (Qin et al., 2005; Dolen and Bear, 2008) with several of the dysregulated mRNAs encoding proteins involved in LTP, LTD and long-term memory (GluR1, Arc, PSD-95, MAP1B) (Hou et al., 2006; Muddashetty et al., 2007; Park et al., 2008; Schütt et al., 2009). As β-ARs couple to both the

mTOR and ERK signaling cascades which can co-regulate translation, I propose that activation of β-ARs resets the sensitivity of long-term synaptic changes to translational inhibition (Figure 9B). Previous data have demonstrated an abnormally rapid ERK dephosphorylation in response to mGluR1/5 stimulation in synaptoneurosomes isolated from Fmr1 knockout mouse cortical tissue (Kim et al., 2008). Thus, β-AR activation may compensate for downregulated ERK activity observed in Fmr1 knockouts, thereby re-establishing the homeostasis between ERK and mTOR signaling necessary for regulated translation. As a result, the sensitivity of long-term synaptic modifications to translational control would be reset to levels similar to that observed in wild-type mice (Fig. 4.9*B*). The impact of β-AR-mediated tagging and enhanced heterosynaptic plasticity on cognition in Fmr1 knock-out mice remains to be determined.




*B.* Activation or β-ARs confers sensitivity to protein synthesis inhibition. *a*. This schematic diagram represents the sensitivity of mouse hippocampal synaptic plasticity to protein synthesis inhibition. In wild-type mice, the fulcrum is centered, indicative of situations in which the ability of synapses to undergo translation-dependent changes is modifiable. *b*. β-AR activation shifts the fulcrum to the left through upregulation of protein synthesis, thereby increasing the sensitivity of synaptic changes (LTP) to translation inhibition. *c*. In Fmr1 knockout mouse hippocampus, the fulcrum is shifted to the right under basal conditions, rendering certain forms of synaptic plasticity (mGluR-dependent LTD) insensitive to inhibition of protein synthesis. *d*. Activation of β-ARs enhances activation of signaling cascades that regulate translation, resetting the sensitivity to protein synthesis inhibition (shifting the fulcrum to the left) in Fmr1 knockout mouse hippocampus, thereby restoring the translation-dependence of synaptic plasticity.

Overall, my data show an enhancement of heterosynaptic plasticity in Fmr1 knock-out mouse hippocampus following the induction of homosynaptic β-AR-dependent LTP. This form of synaptic plasticity requires translation, suggestive of a β-AR-mediated re-establishment of sensitivity to protein synthesis inhibition. Given that β-AR antagonists are already being used clinically with positive results (Rosenberg, 2007), β-ARs and their downstream effectors are logical targets for the pharmacologic treatment of fragile X syndrome (Restivo et al., 2005; Kelley et al., 2008). Increased heterosynaptic plasticity in response to noradrenergic system activation may promote hyperconsolidation of synaptic changes across multiple neural networks that would normally be repressed when FMRP is present. Synaptic hyperconsolidation could stem from a lowered threshold for heterosynaptic plasticity, thereby compromising the network- and synapse-specific changes in synaptic weights that are believed to be necessary for normal learning to occur. This may lead to the cognitive impairments observed in fragile X syndrome.

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# CHAPTER V:

## Neuromodulation of the endurance of hippocampal LTP by co-activation of muscarinic and beta-adrenergic receptors

**Abstract**

Cellular processes that underlie information storage, learning, and memory involve long-term potentiation (LTP), an activity-dependent, persistent increase in synaptic strength. Neuromodulators such as noradrenaline (NA) and acetylcholine (ACh) can act synergistically to regulate LTP in the hippocampus. Previous research has shown that co-activation of beta-adrenergic and cholinergic receptors paired with theta frequency (5 Hz,5 sec) stimulation induces long-term potentiation in the hippocampal CA1 region, which is dependent upon extracellular signal-regulated kinase (ERK) activation. As ERK is involved in intracellular signaling cascades which initiate translation, I hypothesized that co-application of the beta-adrenergic agonist, isoproterenol (ISO), and the cholinergic agonist, carbachol (CAR) would induce LTP which is dependent upon protein synthesis. Herein, I characterize molecular cascades which couple β1-adrenergic and M1 muscarinic receptors to translation regulation. I found that ISO+CAR paired with 5 Hz, 5 sec stimulation induces LTP which requires ERK, mTOR and translation. Taken together, my results suggest that co-activation of beta-adrenergic and muscarinic receptors can modulate hippocampal synaptic plasticity by lowering the threshold for induction of translation-dependent LTP.

Introduction

The cellular basis of memory formation has been attributed to activity-dependent, enduring modifications of synaptic function known as long-term potentiation (LTP) (Bliss and Lomo, 1973; Bliss and Collingridge; Kandel, 2001). Synaptic plasticity including LTP has been demonstrated in the hippocampus following events which induce behavioural changes indicative of learning and memory (Whitlock et al., 2006). The hippocampus is a brain structure required for forming new memories (Scoville and Milner, 1957; Zola-Morgan et al., 1986; Neves et al., 2008). The ability of hippocampal synapses to undergo long-term potentiation can be altered through activation of neuromodulatory systems. The cholinergic and noradrenergic neuromodulatory systems are capable of enhancing synaptic plasticity (Hu et al., 2007; Fernández de Sevilla et al., 2008; Dringenberg et al., 2008) and long-term memory formation (Cahill et al., 1994; Harley et al, 2006; Kukolja et al., 2009). Conversely, blocking cholinergic or noradrenergic receptors impairs memory function (Wallenstein and Vago, 2001; Kemp and Manahan-Vaughan, 2008). Acetylcholine affects neuronal function through stimulation of both nicotinic and muscarinic receptors which can initiate bidirectional changes in synaptic strength (Leung et al., 2003; Scheiderer et al., 2008). Noradrenaline activates alpha- and beta-adrenergic receptors which can elicit opposing effects on cell excitability and synaptic plasticity (Gelinas et al., 2007; Lemon et al., 2009). Activation of muscarinic (Shinoe et al., 2005; Li et al., 2007; Poulin et al., 2010) or beta-adrenergic receptors (Thomas et al., 1996; Walling and Harley, 2004; Gelinas and Nguyen, 2005; Lemon et al., 2009) can boost LTP and memory formation.

Previous research has demonstrated that co-activation of alpha and muscarinic receptors facilitates the induction of long-term depression (Scheiderer et al., 2008). Long-term depression induced by alpha and M1 receptors is facilitated through a synergistic elevation of extracellular

signal regulated kinase (ERK) phosphorylation. A similar effect on ERK stimulation was observed when the non-selective beta-adrenergic receptor agonist isoproterenol (ISO) was paired with carbachol (CAR), a broad-spectrum muscarinic agonist (Watabe et al., 2000). However, pairing ISO with CAR lowered the threshold for LTP induction in contrast to the facilitative effects of LTD observed when alpha and muscarinic receptors were co-stimulated. Given that ERK activation initiates translation (Gelinas et al., 2007; Banko and Klann, 2008), the results of the Watabe et al.(2000) study suggest that beta- and muscarinic receptor agonists could facilitate the induction of long-term potentiation through a protein synthesis-dependent mechanism. Based on these observations, I sought to further characterize the downstream signaling cascades engaged by co-application of ISO and CAR.

I report that, in line with previous data (Watabe et al., 2000), co-activation of beta and muscarinic receptors lowers the threshold for the induction of LTP by brief low-frequency stimulation (5 Hz, 5 s). I identified the β1 and M1 receptor subtypes as the primary receptors responsible for enhancing synaptic plasticity, as application of pirenzepine (M1 muscarinic receptor antagonist) or CGP (β1-AR antagonist) prevented the induction of LTP. I also observed that inhibition of ERK and mTOR similarly prevented the maintenance of LTP induced by ISO paired with CAR. To probe the role of translation, I applied the protein synthesis inhibitor emetine overlapping with receptor stimulation. Emetine blocked the maintenance of LTP, indicating a requirement for translation. Thus, co-activation of beta and muscarinic receptors recruits local translation to enhance synaptic strength. As cholinergic and noradrenergic dysfunction has been associated with neurodegenerative diseases (Caccamo et al, 2006; Yu et al., 2011), my results suggest these neuromodulatory systems may be a viable therapeutic target for the restoration of long-term memory in these disorders..

Materials and Methods

*Animals*: Male C57B*L*/6 mice (7-14 weeks) were used for all experiments. Animals were housed at the University of Alberta according to Canadian Council on Animal Care (CCAC). All mice were maintained on a 12 h on/off light cycle in social housing, with food and water available *ad libitum*.

*Electrophysiology*: Following cervical dislocation and decapitation hippocampi were removed and placed in ice-cold ACSF. Each hippocampus was dissected out of the remaining tissue and sliced in the transverse plain (400-µm thickness) (as described in Nguyen and Kandel, 1997). Slices were then transferred to an interface chamber and maintained at 28$^{o}$C which is a consistently used temperature for recording field responses on extended (hrs) time scales (Young et al., 2006; Gelinas and Nguyen, 2005, Gelinas et al., 2007). Artificial cerebrospinal fluid (ACSF) composed of (in mM): 124 NaCl, 4.4 KCl, 1.3 $MgSO_4$, 1.0 $NaH_2PO_4$, 26.2 $NaHCO_3$, 2.5 $CaCl_2$, and 10 glucose, aerated with 95% $O_2$ and 5% $CO_2$, was applied at a flow rate of 1-2 mL/min. Extracellular field excitatory postsynaptic potentials (fEPSPs) were recorded with a glass microelectrode filled with ACSF (resistances, 2–3 MΩ). Both the recording electrode and a bipolar nickel-chromium stimulating electrode were positioned in the stratum radiatum of area CA1. fEPSPs were elicited by stimulating Schaeffer Collateral (SC) fibers projecting onto CA1 apical dendrites. Maximal evoked EPSPs were generated by increasing the stimulation intensity until no further increase in EPSP amplitude could be obtained. Subsequent test pulses (1/min; 0.08 pulse duration) were adjusted to elicit a fEPSP that was 40% of the maximum evoked value (Woo and Nguyen, 2003; Gelinas and Nguyen, 2007). fEPSPs elicited at this levels constituted our baseline responses.

LTP requiring co-activation of β-AR and muscarinic receptor-dependent LTP was induced by applying one train of low-frequency stimulation (5 Hz, 5 s) following a 10 min co-application of the β-AR agonist, isoproterenol (ISO; 200nM) and the muscarinic agonist, carbachol (CAR; 200 nM) (Watabe et al., 2000). Following LFS, ISO and CAR were applied for an additional 5 min.

*Drugs*: The β-AR agonist, *R* (-)-isoproterenol(+)-bitartrate (ISO, Sigma, St. Louis, MO) and the muscarinic receptor agonist carbachol (CAR, Sigma) were prepared daily as concentrated stock solutions at 1 mM, in distilled water and co-applied at a final concentration of 200nM each. The β-AR antagonist (±)-propranolol hydrochloride (PROP; 50 µM; Sigma) was also prepared daily in distilled water as a 50 mM stock solution. Both the specific β1-AR antagonist, (±)-2-Hydroxy-5-[2-[[2-hydroxy-3-[4-[1-methyl-4-(trifluoromethyl)-1H-imidazol-2-yl]phenoxy]propyl] amino]ethoxy]-benzamide methanesulfonate salt (CGP-20712A; CGP) and β2-AR antagonist, (±)-1-[2,3-(Dihydro-7-methyl-1H-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol hydrochloride (ICI 118,551; ICI) (Sigma) were prepared at stock concentrations of 1 mM and diluted to a final concentration of 1µM. The muscarinic receptor antagonist, atropine (Sigma) was dissolved in ethanol at 50 mM stock concentration and diluted to 50 µM prior to application. An M1 muscarinic receptor antagonist, pirenzepine dihydrochloride (PIR), was dissolved in distilled water (1mM stock) and applied at 250 nM. The M3/M5 muscarinic receptor antagonist, 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP; 1 µM; Sigma), was dissolved in DMSO yielding a stock concentration of 1 mM. Methoctramine, an M2/M4 antagonist, was prepared in distilled water at 1 mM stock concentration and diluted to 1µM.

To assay for downstream activation of kinases, a protein kinase C inhibitor, chelerythrine chloride (CHEL; 1 µM; Sigma), and cAMP-dependent protein kinase (PKA) inhibitor (PKA

Inhibitor 14-22 Amide, Cell -Permeable, Myristoylated; PKI; 1 µM; Calbiochem, La Jolla, CA) were dissolved in distilled water. Both CHEL and PKI were applied 20 min prior to and overlapping with ISO+CAR. An inhibitor of SK channels, apamin,(Sigma) was dissolved in acetic acid to produce a 1 mM stock solution and applied at a final concentration of 100 nM. A MEK inhibitor, 1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio] butadiene (U0126, 20 µM; Sigma), was prepared in DMSO at a concentration of 20 mM. An mTOR inhibitor, rapamycin (Rap; 1 µM; Sigma), was dissolved in DMSO to make stock solutions at 1 mM. U0126 and Rap were perfused for 20 min prior to commencing experiments, overlapping with ISO application, and 10 min following ISO. A translation inhibitor, emetine (EME; Sigma), was dissolved to a stock concentration of 20 mM in distilled water and perfused at 20 µM, 20 min prior to ISO application. At lower concentrations than 20 µM, EME was shown to block protein synthesis by >80% in hippocampal slices (Stanton and Sarvey, 1984). All drug experiments were performed under dimmed light conditions due to photosensitivity of the drugs. Drug experiments were interleaved with drug-free controls.

*Data analysis.* The initial slope of the fEPSP was measured as an index of synaptic strength (Johnston and Wu, 1995). The average "baseline" slope values were acquired for 20 min before experimental protocols were applied. fEPSP slopes were measured at either 60 or 90 min after LFS for comparisons of LTP. Student's *t* test was used for statistical comparisons of mean fEPSP slopes between two groups, with a significance level of $p < 0.05$ (denoted as * on graphs). One-way ANOVA and Tukey–Kramer *post hoc* tests were done for comparison of more than two groups to determine which groups were significantly different from the others. The Welch correction was applied in cases in which the SDs of compared groups was significantly different. All values shown are means ± SEM, with $n$ = number of slices.

Results

Given that both cholinergic and noradrenergic receptors are capable of augmenting synaptic strength (Gelinas and Nguyen, 2005; Dringenberg et al., 2008) and prior evidence suggesting interactions between these neuromodulatory systems (Watabe et al., 2000; Scheiderer et al., 2008), it is important to identify mechanisms mediating the potential synergistic effects of beta and muscarinic receptor activation. In line with previous research, I found that co-application of ISO and CAR facilitates the induction of LTP by a subthreshold, low-frequency stimulation protocol (fEPSPs were potentiated to 137±9% of baseline 60 min post LFS, n=10) (Fig. 1E). To determine if LFS alone is sufficient for inducing LTP, 5 Hz stimulation was applied in the absence of receptor agonists. LFS alone induced a transient potentiation of fEPSPs which returned to baseline in < 45 min (fEPSPs were 102±3% of baseline 60 min after stimulation, n=8) (Fig. 5.1*A*). Similarly, application of either ISO or CAR paired with LFS failed to induce LTP. Application of CAR paired with LFS induced transient LTP which returned to baseline in less than 45 min. fEPSPs were 100±4% of baseline 60 min after stimulation (n=10; Fig. 5.1*B*). Pairing ISO with LFS resulted in decremental LTP which was reduced to 98±4% of baseline 60 min post LFS (n=12; Fig. 5.1*C*). To test if co-application of ISO and CAR is sufficient for inducing synaptic plasticity, both drugs were co-applied while monitoring baseline fEPSPs. A 15 min application of ISO and CAR was insufficient for augmenting synaptic responses. When measured 60 min after termination of drug application, fEPSPs were 98±6% (n=10) of baseline (Fig. 5.1*D*). An ANOVA comparing all groups revealed a significant effect of treatment ($F_{(4,45)} = 8.85$; $p < 0.01$). Tukey-Kramer post-hoc test revealed that persistent LTP was seen only when ISO and CAR were co-applied and paired with low-frequency stimulation (5Hz, 5s). Comparisons of fEPSP magnitudes 60 min post-stimulation

showed that ISO+CAR+5Hz induced LTP which was significantly enhanced relative to all other groups.

Figure 1.

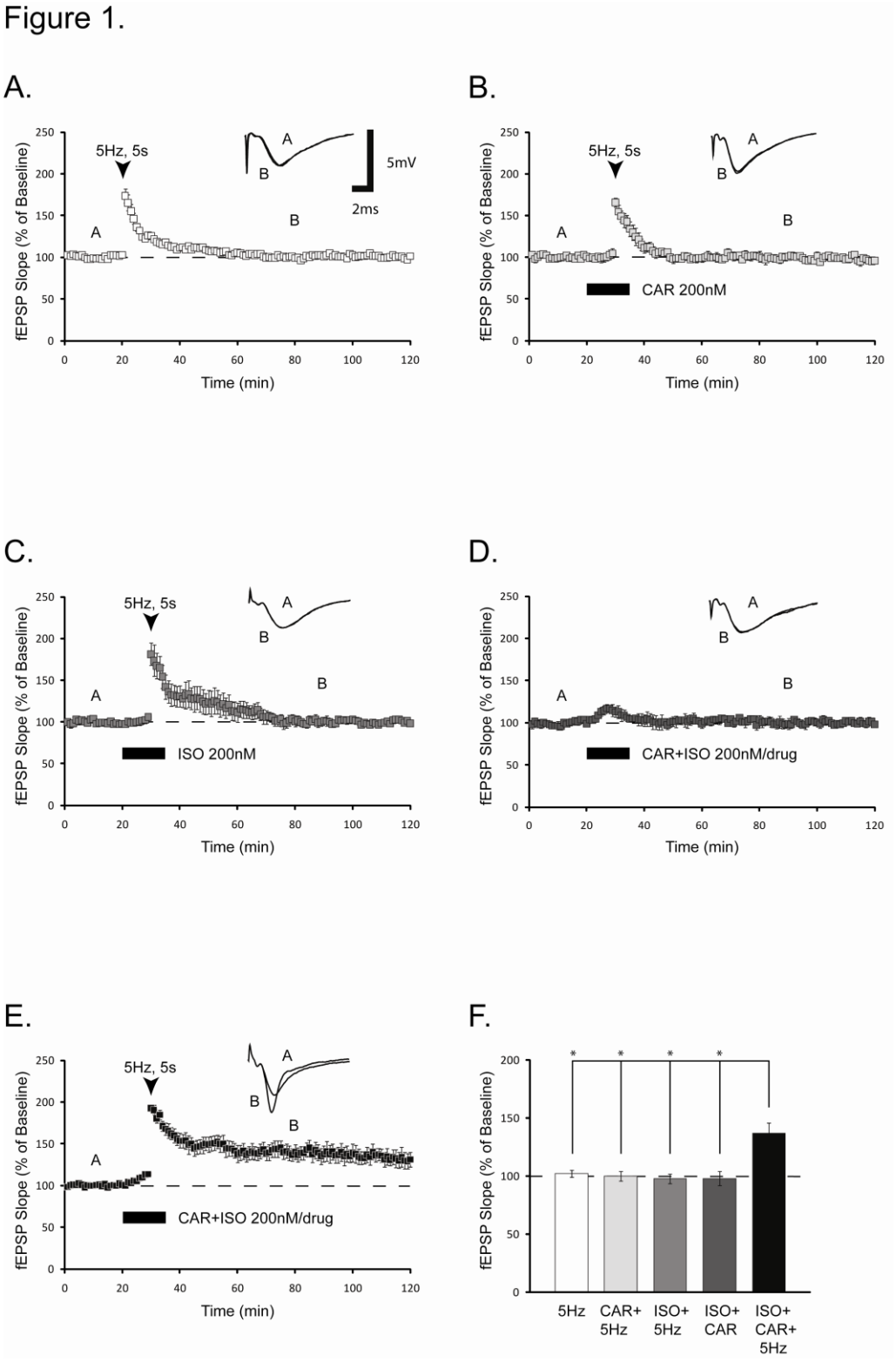

Figure 5.1. Co-activation of beta and muscarinic receptors facilitates the induction of LTP by low-frequency stimulation. *A*. Tetanization of Schaeffer collateral fibers with 5 Hz stimulation for 5 S induces transient (< 45 min) LTP (n=8). *B.* Activation of muscarinic receptors alone does not enhance the magnitude of duration of LTP in response to LFS (n=10). *C*. Application of ISO for 10 min followed by LFS similarly failed to induce LTP (n=12). *D*. Pairing of ISO with CAR elicits a modest enhancement of basal synaptic responses which return and maintain pre-drug application levels (10). *E*. When LFS is applied following a 10 min application of ISO+CAR, the duration of LTP is significantly enhanced relative to all other treatments (n=10). *F*. Summary histogram comparing fEPSP slopes obtained 60 min after LFS. "*" denotes significant differences between treatment groups. Sample traces were taken 10 min after commencement of baseline recording and 60 min after stimulation. Results in F represent means±SEM, * $p < 0.05$.

**β1- but not β2-ARs are required for LTP induced by ISO+CAR**

To determine if beta-adrenergic receptors are required for the induction of LTP by ISO and CAR, I tested the effects of a general beta-adrenergic receptor antagonist, propranolol (PROP; 50 μM) on LTP. Application of PROP blocked the expression of long-lasting LTP; fEPSPs were 98±4% of baseline (n=9) 60 min after tetanization (Fig. 5.2*A*). To further characterize the β-AR subtype responsible for the ISO induced effects on LTP, I used the β1-specific antagonist CGP (1 μM). Specific inhibition of β1 during ISO+CAR application blocked the induction of LTP (fEPSPs were 99±4% of baseline 60 min post stimulation, n=9) (Fig. 5.2*B*). In contrast, inhibition of β2-ARs with the specific beta-2 antagonist, ICI (1 μM), failed to block LTP (Fig. 5.2*C*). Sixty min after stimulation, fEPSPs were still potentiated to 128±5% (n=8) of baseline. An ANOVA comparing groups treated with antagonists demonstrated a significant effect of drug treatment on LTP ($F_{(3,30)} = 15.44$; $p < 0.01$) (Fig. 5.2*E*). Subsequent Tukey-Kramer post-hoc analysis revealed that both propranolol and CGP significantly reduced the persistence of LTP relative to slices treated with ISO, CAR and 5Hz stimulation in the absence of antagonists (Fig. 5.2*D, E*). Conversely, inhibition of β2 yielded LTP which was not significantly different from antagonist-free controls ($p > 0.05$).

Figure 2.

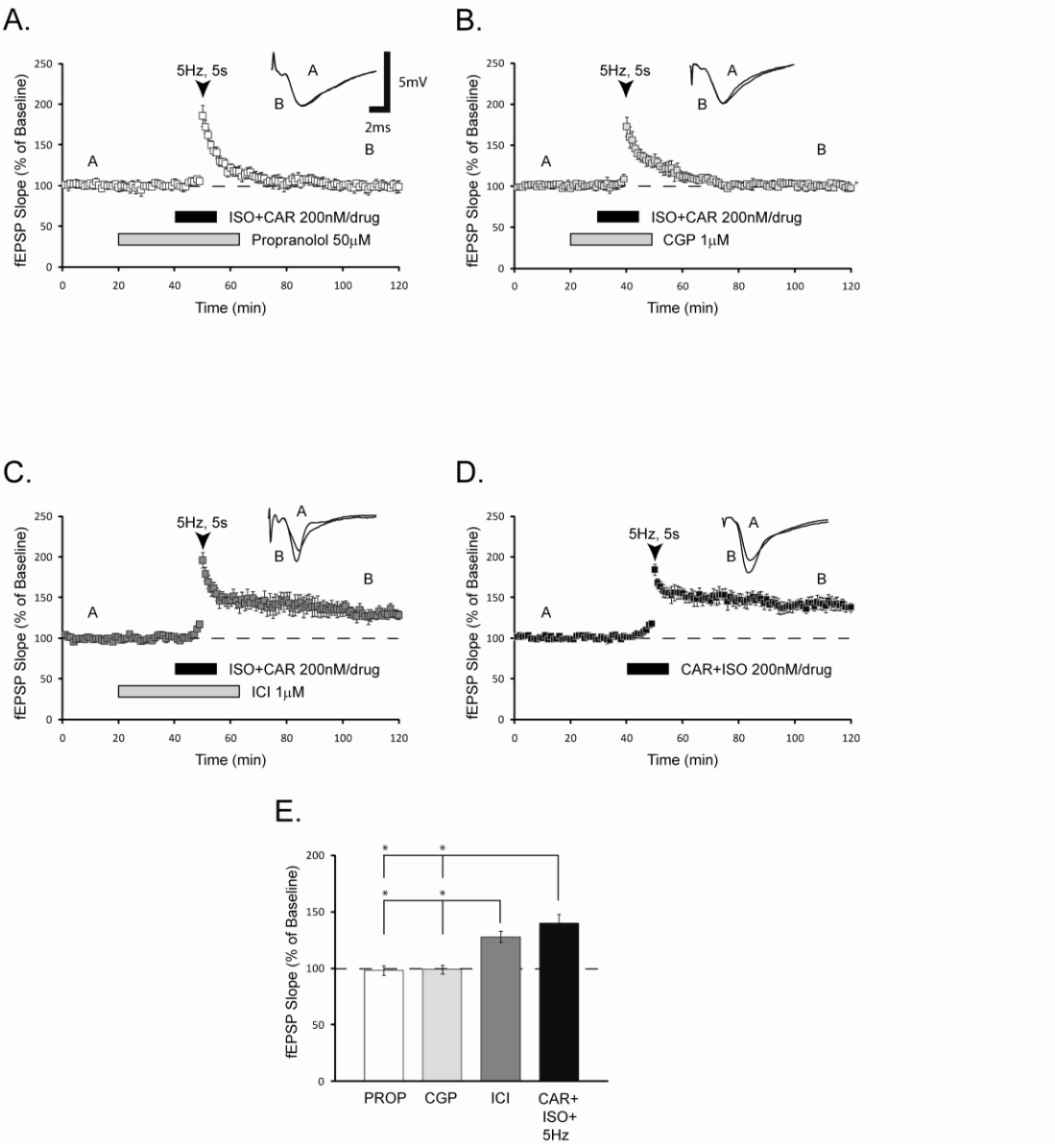


Figure 5.2. β-ARs are required for enhancement of LTP induced by ISO paired with CAR. *A.* Application of the β-AR antagonist, propranolol, overlapping with ISO+CAR prevented the induction of LTP (n=9). *B.* Specific inhibition of β1 through treatment with CGP completely abolished enhancing effects of ISO paired with CAR on LTP (n=9). *C.* Blocking β2-ARs with the specific antagonist ICI, failed to prevent the induction of LTP. *D.* Summary histogram comparing fEPSP slopes obtained 60 min after LFS. Sample traces were taken 10 min after commencement of baseline recordings and 60 min after stimulation. Results in D represent means ± SEM, * $p < 0.05$.

**Inhibition of the M1 muscarinic receptor subtype blocks ISO+CAR LTP**

As an initial step in the characterization of the muscarinic component of ISO+CAR LTP, I applied the broad-spectrum muscarinic receptor antagonist atropine (50 µM). Application of atropine overlapping with stimulation of beta- and muscarinic receptors blocked LTP. fEPSPs returned to baseline (97±5%) within 60 min of LFS (Fig. 5.3*A*; n=8). Next, I applied the selective M1 muscarinic receptor antagonist pirenzepine (PIR; 250 nM). Long-term potentiation was not observed in slices exposed to PIR; (fEPSPs were 103±4% of baseline 60 min after tetanization; n=12; Fig. 5.3*B*). To determine if M3 receptors are required for ISO+CAR-LTP, I used the M3 antagonist, 4-DAMP (1 µM). Blocking M3 receptors did not prevent the expression of LTP. fEPSPs were potentiated to 131±5% (n=8; Fig. 5.3*C*) of baseline when assessed 60 min after 5 Hz stimulation. Next, I sought to determine if M2/ M4 receptors are engaged during ISO and CAR-induced LTP. Induction of LTP in the presence of the M2/M4 antagonist, methoctramine (250 nM), yielded LTP which was potentiated to 135±6% of baseline 60 min after stimulation (n = 6; Fig. 5.3*D*). An ANOVA was conducted to compare all slices exposed to inhibitors and control slices treated with ISO+CAR+5 Hz. A significant effect of treatment was observed ($F_{(4,37)} = 11.27$; $p < 0.01$) (Fig. 5.3*E*). Tukey-Kramer post-hoc tests revealed that blocking muscarinic receptors non-selectively with atropine prevented the expression of LTP ($p < 0.01$). Similarly, inhibition of M1 but not M2/M4 or M3, prevented the expression of LTP ($p < 0.01$). Slices treated with 4-DAMP (M3) or methoctramine (M2/M4) were indistinguishable from ISO+CAR+5Hz controls ($p > 0.05$) (Fig. 5.3*E*).

Figure 3.

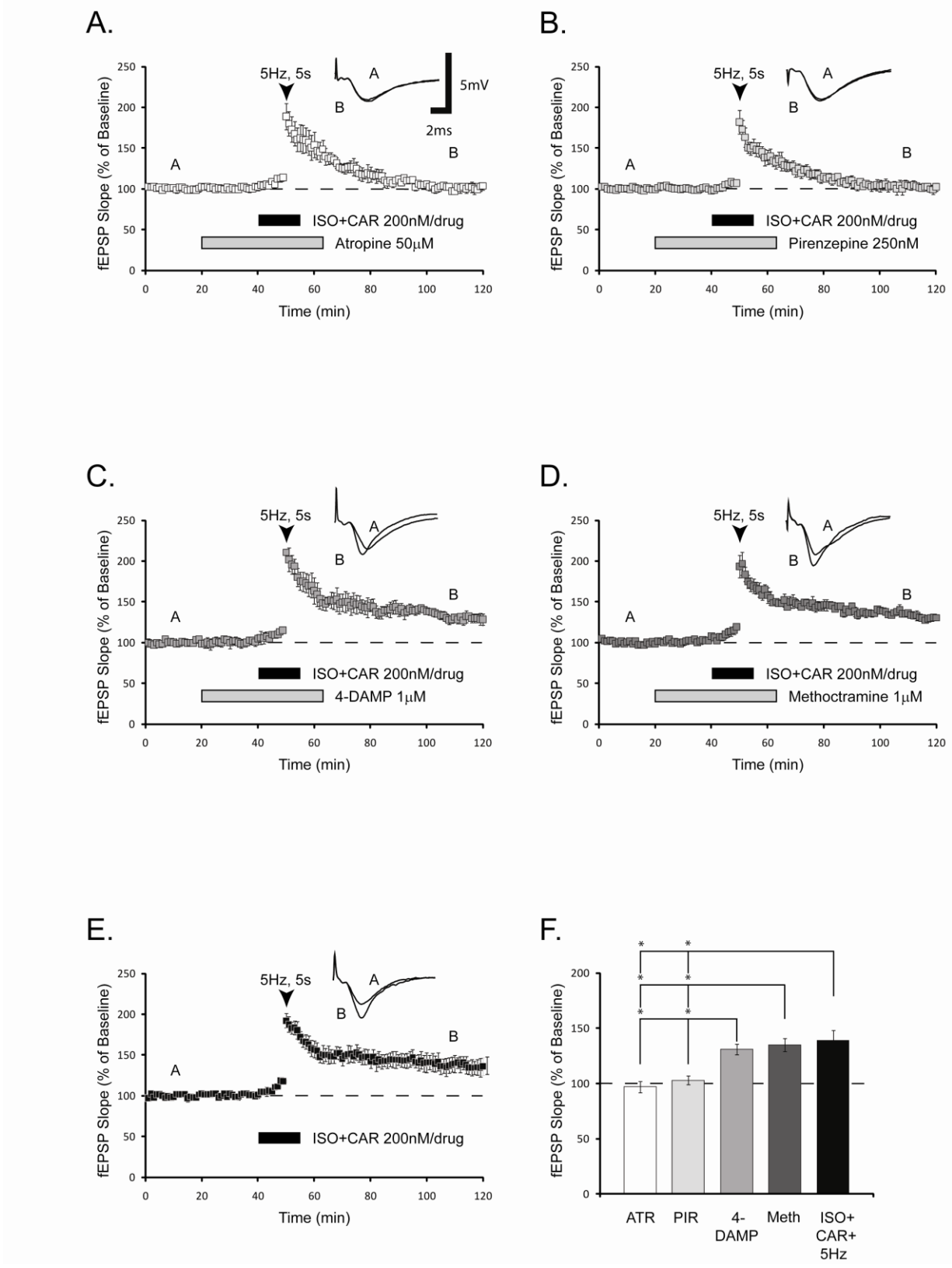
A.
B.
C.
D.
E.
F.
5Hz, 5s
5mV
2ms
A
B
fEPSP Slope (% of Baseline)
Time (min)
ISO+CAR 200nM/drug
Atropine 50μM
Pirenzepine 250nM
4-DAMP 1μM
Methoctramine 1μM
ATR
PIR
4-DAMP
Meth
ISO+CAR+5Hz

Figure 5.3. Muscarinic receptors are required for LTP induced by co-activation of beta and muscarinic receptors. *A.* Treating slices with the broad-spectrum muscarinic receptor antagonist atropine, resulted in decremental LTP which returned to baseline in < 45 min (n=8). *B.* The M1 muscarinic receptor antagonist pirenzepine similarly inhibited the induction of LTP when applied overlapping with ISO and CAR application (n=12). *C.* Conversely, application of the M3 muscarinic receptor antagonist failed to prevent LTP as potentiation was still observed 60 min post stimulation (n=8). *D.* Inhibition of M2/M4 receptors with methoctramine yielded LTP which was similar to non-methoctramine controls (n=6). *E.* Slices treated with ISO+CAR+5Hz demonstrated significantly enhanced LTP which was enhanced relative to atropine and pirenzepine treated slices (n=8). *F.* Summary histogram comparing fEPSP slopes obtained 60 min after LFS "*" indicates significant differences between treatment groups. Sample traces were taken 10 min after commencement of baseline recordings and 60 min after LFS. Results in F represent means ± SEM, $*p < 0.05$.

**Inhibition of PKC and PKA prevents the expression of LTP following co-activation of beta and muscarinic receptors.**

Both muscarinic and beta-adrenergic receptors can modulate synaptic function through downstream activation of kinases including PKC and PKA (Winder et al., 1999; Fernández de Sevilla and Buño, 2010). Recent evidence has implicated M1-driven activation of PKC in the facilitating effects of muscarinic receptor stimulation on synaptic plasticity (Buchannan et al., 2010). Thus, I tested whether PKC is required for LTP induced by activation of beta- and muscarinic receptors. Inhibition of PKC with chelerythrine (CHEL; 1µM) during ISO+CAR application resulted in short lasting LTP which was 106±4% of baseline 60 min after LFS (n=8; Fig. 5.4*A*). Next, I sought to determine if PKA is required for LTP induced by ISO and CAR. Application of PKI (1µM) overlapping with ISO+CAR prevented the expression of LTP (fEPSPs were 110±5% of baseline 60 min after stimulation; n=11) (Fig. 5.4*B*). An ANOVA comparing slices treated with CHEL, PKI, and control slices not exposed to inhibitors (134±7%; n=8)(Fig. 5.4*C*), showed a significant difference between groups ($F_{(2,24)} = 7.11$; $p < 0.01$). Post-hoc tests showed that application of either CHEL or PKI resulted in decremental LTP which was

significantly reduced relative to slices treated with ISO+CAR and low-frequency stimulation (Fig. 5.4*D*). There was no significant difference between CHEL and PKI exposed slices ($p > 0.05$).

Figure 4.

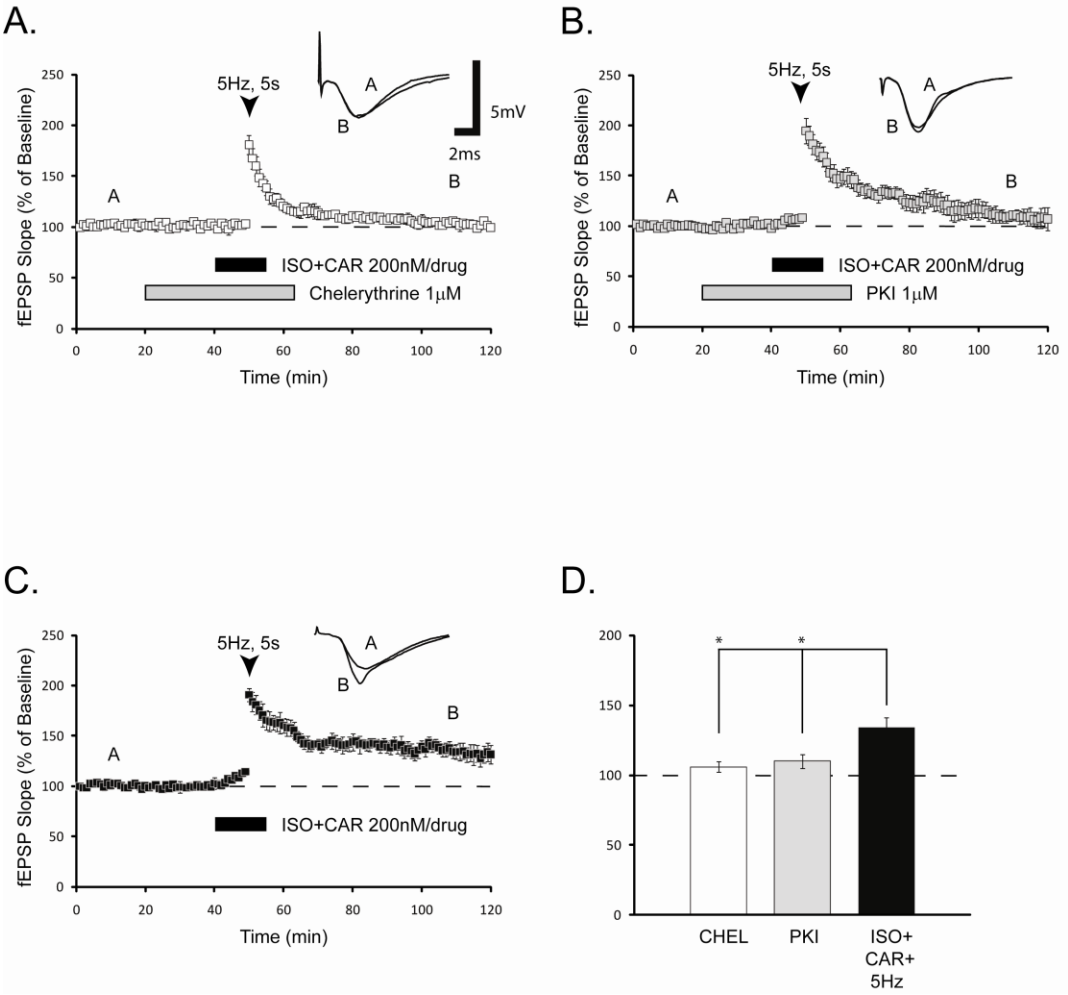


Figure 5.4. Both PKC and PKA are required for facilitated LTP observed during co-stimulation of beta and muscarinic receptors. *A.* To determine ISO+CAR LTP requires PKC, I applied a broad-spectrum PKC antagonist, chelerythrine. fEPSPs were significantly reduced following chelerythrine treatment (n=8). *B.* Pairing of the membrane permeant PKA inhibitor, PKI with co-stimulation of beta and muscarinic receptors similarly prevented the bolstering of LTP (n=11) observed in slices naive to kinase inhibitors (*C.*, n=8). *D.* Summary histogram comparing fEPSP slopes obtained 60 min after LFS. "*" indicates significant differences between treatment groups. Sample traces were taken 10 min after commencement of baseline recordings and 60 min after LFS. Results in D represent means ± SEM, $*p < 0.05$.

**Blocking SK channels does not further enhance LTP-induced with ISO and CAR**

Inhibition of SK (small conductance, $Ca^{2}$+-sensitive K+ channels) has recently been identified as a mechanism through which muscarinic receptors enhance neural excitability and synaptic strength (Buchannan et al., 2010; Giessel and Sabatini, 2010). Both muscarinic and beta-adrenergic receptors can enhance cellular excitability through inhibition of SK channels (Faber et al., 2008; Buchannan et al., 2010; Giessel and Sabatini, 2010 ). To test the notion that SK channels are inhibited during ISO+CAR application, I applied the bee venom extract apamin (100 nM), which blocks SK channels. If inhibition of SK channels is not involved in enhanced LTP observed following ISO+CAR treatment, I would expect a further increase in the magnitude of synaptic potentiation when ISO+CAR are applied in the presence of apamin. Application of apamin overlapping with ISO+CAR induced LTP which was potentiated to 136±6% of baseline (n=6) 90 min after LFS (Fig. 5.5*A*). A Students t-test comparing apamin treated slices with ISO+CAR controls (131±8% of baseline, n=6; Fig. 5.5*B*) 90 min after tetanization failed to detect any significant difference between groups ($p > 0.05$) (Fig. 5.5*C*). Thus, as application of apamin failed to enhance the magnitude of LTP, the effects of co-activation of beta- and muscarinic receptors appear to be at least in part, due to inhibition of SK channels.

Figure 5.

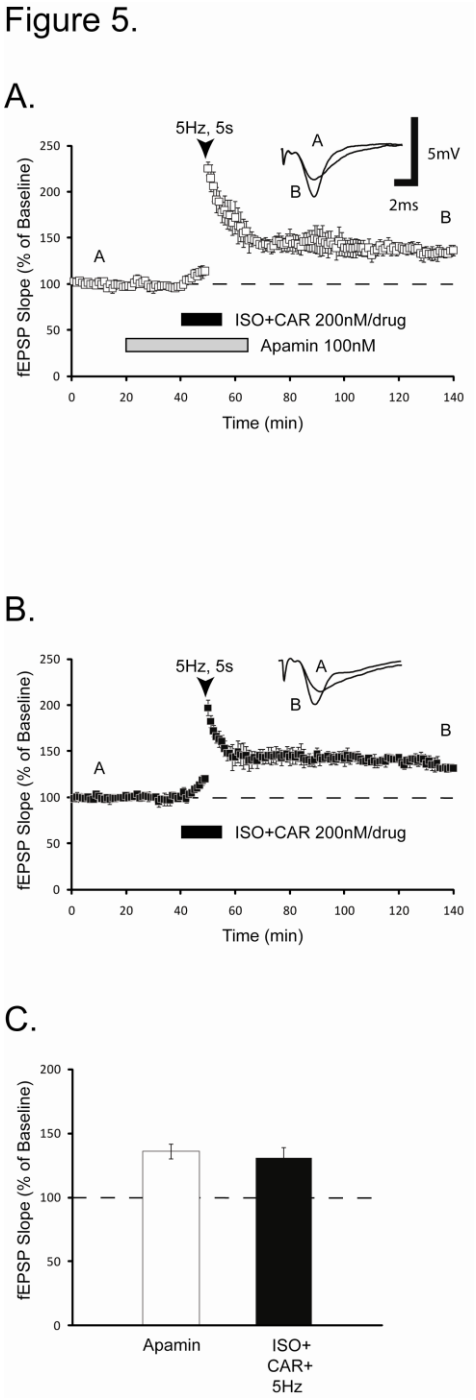


Figure 5.5. Inhibition of SK channels does not further bolster LTP. *A.* Application bee venom apamin which inhibits SK channels, overlapping with ISO and CAR treatment (n=6) results in LTP which is not significantly different from control slices (B., n=6) not treated with apamin. *C.* Summary histogram comparing fEPSP slopes obtained 90 min after LFS. Sample traces were taken 10 min after commencement of baseline recordings and 60 min after S1 stimulation. Results in C represent means ± SEM, $*p < 0.05$.

**ERK and mTOR are required for LTP induced by ISO+CAR**

Both beta-adrenergic (Gelinas and Nguyen, 2005) and muscarinic receptors (Feig and Lipton, 1993) can promote translation-dependent LTP. Stimulation of beta-adrenergic receptors upregulates translation though signaling cascades coupling to mammalian target of rapamycin (mTOR) and ERK (Gelinas et al., 2007). To test the hypothesis that co-activation of beta and muscarinic receptors can engage downstream mTOR activity to facilitate LTP, I used the mTOR inhibitor, rapamycin (RAP; 1µM). Slices treated with RAP during simultaneous activation of beta and muscarinic receptors demonstrated decremental LTP (fEPSPs had returned to 97±6% of baseline 90 min after 5 Hz stimulation; n=6)(Figure 5.6*A*). It is known that pairing ISO with CAR induced LTP mediated through extracellular signal-regulated kinase (ERK) (Watabe et al., 2000). To test this, I inhibited ERK using the MEK inhibitor (MEK is the only known kinase upstream of ERK), U0126 (20µM) during the co-activation of beta and muscarinic receptors. Consistent with previous findings, inhibition of MEK prevented the expression of LTP (90 min after LFS in the presence of U0126 fEPSPs had diminished to 98±7% of baseline, n=8) (Fig. 5.6*B*). As both ERK and mTOR couple to translation regulation (Gelinas et al., 2007; Banko et al., 2005), I next explored whether inhibition of protein synthesis impairs LTP induced by co-activation of beta and muscarinic receptors.

Figure 6.

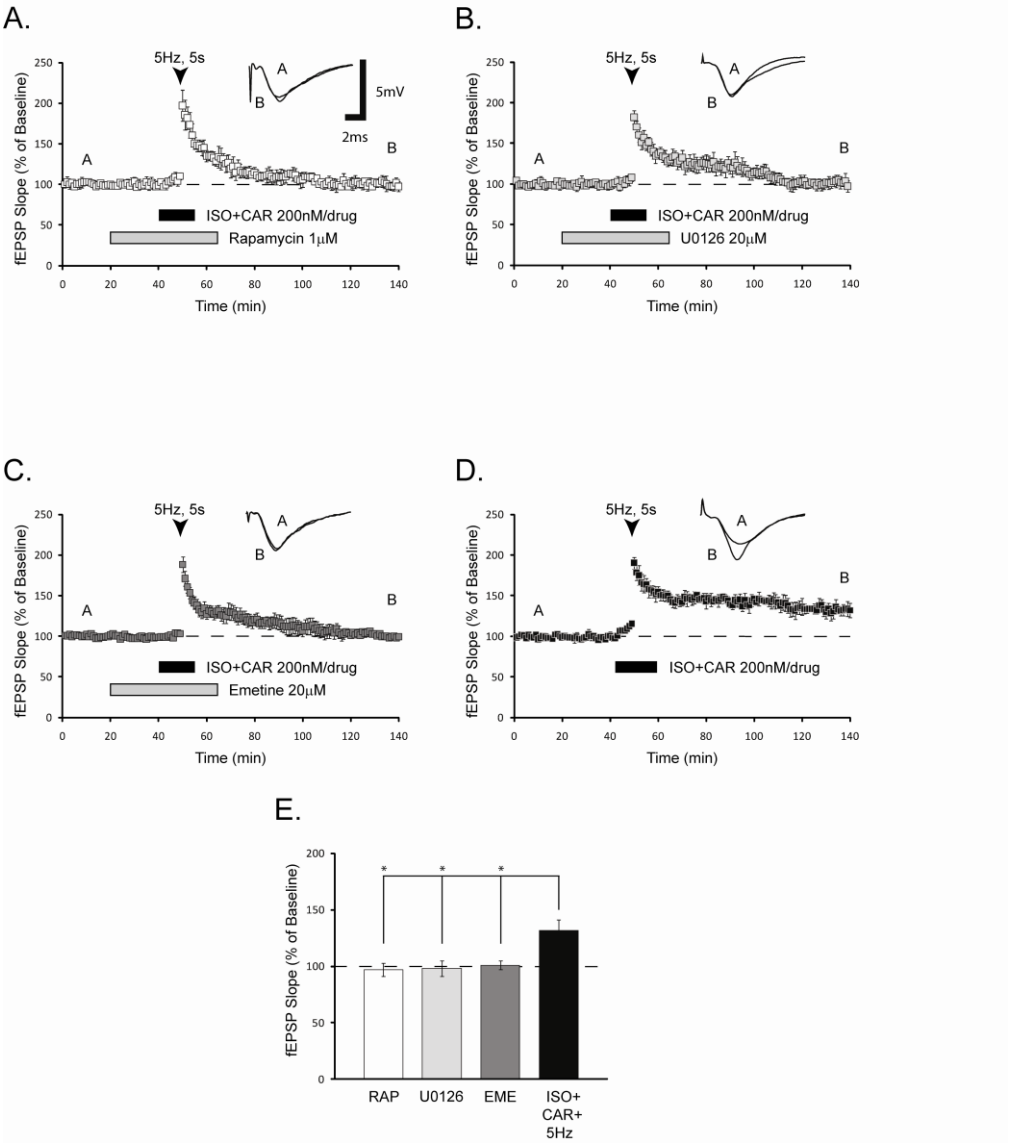


Figure 5.6. Translation is engaged to enhance LTP during co-application of ISO and CAR. *A.* Application of the mTOR inhibitor rapamycin overlapping with ISO and CAR prevented the induction of LTP (n=6). *B.* Slices treated with U0126 (MEK inhibitor) similarly exhibited decremental LTP (n=8). *C.* Relative to drug free controls (*D.* n=8), fEPSPs in slices treated with the translation repression emetine were significantly less when compared 90 min post stimulation. *E.* Summary histogram comparing fEPSP slopes obtained 90 min after LFS. Sample traces were taken 10 min after commencement of baseline recordings and 90 min after HFS. Results in E represent means ± SEM, *$p < 0.05$.

**ISO and CAR engage translation to facilitate LTP**

A requirement of protein synthesis has been observed in enduring forms of both synaptic plasticity and long-term memory (Abraham and Williams, 2008). Thus, I sought to determine if protein synthesis is necessary for expression of LTP induced by ISO paired with CAR. I found that application of a translational repressor, emetine (EME; 20μM), during ISO+CAR treatment generated LTP which reverted to baseline levels (101±4%) when measured 90 min after LFS. Subsequent comparisons (ANOVA) between slices treated with RAP, U0126, EME or no inhibitor demonstrated a significant effect of treatment on the magnitude of LTP as assessed 90 min post stimulation ($F_{(2,24)} = 7.11$; $p < 0.01$). Inhibition of mTOR, ERK, or translation resulted in LTP that was substantially reduced relative to ISO+CAR controls (Figure 5.6*C, D*). No significant difference between EME, RAP or U0126 treated groups was observed ($p > 0.05$)

Discussion

Neuromodulators including noradrenaline and acetylcholine can modify a diverse range of neural functions including synaptic plasticity (Gelinas and Nguyen, 2005; Scheiderer et al., 2008). Much research has focused on the effects of these neurotransmitters in isolation; however, few studies have addressed potential interactions between neuromodulators. Previous research has established that co-activation of beta and muscarinic receptors reduces the threshold for induction of LTP (Watabe et al., 2000). Also, noradrenergic and muscarinic signals converge to augment the ability of hippocampal synapses to undergo long-term depression (Scheiderer et al., 2008). Here, I have confirmed the initial results of Watabe et al. (2000) and extended the characterization of mechanisms by which beta and muscarinic receptors synergistically mediate long-lasting synaptic plasticity.

My data suggest that when paired together, sub-micromolar concentrations of isoproterenol and carbachol initiate intracellular signaling cascades capable of extending the duration of synaptic potentiation induced by a low-frequency stimulation protocol (5 Hz) which normally produces transient (< 30 min) LTP. Herein, I have shown that the effects of ISO are mediated through β1- but not β2-adrenergic receptors, as the facilitative effects of ISO+CAR were blocked by CGP (β1 antagonist) but were preserved in the presence of ICI (β2 antagonist). The β1-AR couples to Gαs which elicits cAMP generation and activates downstream signaling kinases including PKA and ERK (Winder et al., 1999; Vanhoose and Winder, 2003). Isoproterenol can activate β1-ARs (Fowler and O'Donnell, 1988) which have been specifically implicated in theta frequency LTP protocols (Winder et al., 1999).

I have also further clarified the muscarinic receptor component which appears to require M1, but not M2/M4 or M3/M5, muscarinic receptors. Application of pirenzepine inhibited LTP whereas methoctramine or 4-DAMP failed to block the enhancement of LTP observed in the presence of ISO and CAR. Similar to beta-adrenergic receptors (Gelinas et al., 2007), M1 muscarinic receptors are capable of inducing ERK activation (Berkely et al., 2001) which has been linked to translation-dependent long-term depression in rat visual cortex (McCoy and McMahon, 2007). Recently it has been demonstrated that M1 muscarinic receptor boosts long-term potentiation through inhibition of SK channels which results in amplified NMDA receptor activation (Buchannan et al., 2010; Giessel and Sabatini, 2010).

What cellular mechanisms are initiated by co-activation of beta and muscarinic receptors to enhance LTP induction? There is substantial evidence implicating SK channels, which are responsible for the apamin-sensitive AHP (after-hyperpolarization) in synaptic plasticity (Hammond et al., 2006; Buchanan et al., 2010). Inhibition of SK channels results in

depolarization, increased cell excitability and decreased spike-frequency accommodation (Stocker, 2004), all of which can increase synaptic augmentation and boost memory formation (Hammond et al,. 2006). Both PKC and PKA can mediate their cellular effects partially through inhibition of SK channels (Faber et al., 2008; Oh et al., 2009; Buchanan et al., 2010). I tested the hypothesis that SK channel inhibition is involved in LTP induced by co-activation of beta and muscarinic receptors through the application of apamin prior to and overlapping with ISO and CAR. Apamin inhibits SK channels which can modify the ability of synapses to undergo synaptic plasticity (Ngo-Anh et al., 2005; Hammond et al., 2006). If the effects of ISO and CAR are mediated through a mechanism *not* requiring SK channels, co-applying apamin with ISO and CAR should result in a further boosting of LTP. Interestingly, no significant differences were detected between slices exposed to ISO and CAR and slices also exposed to apamin. These data implicate SK channel inhibition as a mechanism through which ISO and CAR lower the threshold for the induction of LTP.

My results suggest that co-activation of beta and muscarinic receptors engages PKC and PKA to increase the duration of LTP. Long-term potentiation is associated with AMPAR trafficking and membrane insertion, which are highly dynamic processes subject to regulation through phosphorylation mediated by kinases (Makino and Malinow, 2009; Makino et al., 2011) Activation of beta-adrenergic receptors increases membrane insertion of GluR1 through PKA-dependent phosphorylation (Esteban et al., 2003; Hu et al., 2007; Tenorio et al., 2010). Similarly, GluR1 insertion was upregulated following in vitro treatment with ACh, an effect mediated through M1-dependent signaling (Fernández de Sevilla et al., 2008). Although not determined in the Fernández de Sevilla study, activation of PKC downstream of muscarinic receptors can increase phosphorylation of Serine-831 on GluR1, a key event implicated in AMPAR trafficking

and insertion (Boehm et al., 2006). Future research will be needed to determine the role of AMPA receptor trafficking in LTP induced through co-activation of beta and muscarinic receptors.

Inhibition of mTOR prevented the maintenance of LTP induced by ISO paired with CAR. Although evidence implicating muscarinic receptors in mTOR regulation is sparse, one report has implicated the M3 muscarinic receptor in mTOR regulation (Slack and Blusztajn, 2008). As my results have implicated the M1 muscarinic receptor but not M3, this suggests that the β1-AR may be primarily responsible for mTOR regulation (see Gelinas et al., 2007). Previous research has implicated ERK in both long-term memory formation and synaptic plasticity (Atkins et al., 1998; Banko et al., 2006; Gelinas et al., 2007). Initiation of translation is enhanced following ERK activation which has been linked to enduring changes in synaptic strength (Tang et al., 2002; Kelleher et al., 2004; Gelinas et al., 2007). Both β1-ARs (Roberson et al., 1999) and muscarinic receptors (Berkely et al., 2008) are capable of ERK activation. Additionally, both muscarinic and beta adrenergic receptors can engage PKC and PKA signaling cascades to increase phosphorylation of ERK (Roberson et al., 1999). Thus, I sought to determine if ERK activity is required for LTP induced by co-activation of muscarinic and beta-adrenergic receptors. U0126, which prevents activation of the only known upstream kinase for ERK (MEK1/2), was used to determine if ERK activation is required for LTP. Consistent with previous data (Watabe et al., 2000), inhibition of ERK prevented the maintenance of LTP induced by co-activation of beta and muscarinic receptors. Taken together, these results suggest that co-stimulation of beta-adrenergic and muscarinic receptors induces LTP which requires mTOR and ERK activation. As both mTOR and ERK are capable of inducing translation regulation which has been implicated in enduring changes in synaptic function (Banko et al.,

2006; Gelinas et al., 2007), these results provide evidence for a potential dual neuromodulatory receptor-initiated mechanism for augmenting long-term memory.

The synthesis of proteins has been implicated in many forms of synaptic plasticity and memory formation (see Klann et al., 2004, Richter and Sonenberg, 2005 and Costa-Mattioli et al., 2009 for reviews). Persistent LTP requires translation (Stanton and Sarvey, 1984; Frey et al., 1988; Kandel, 2001; Scharf et al, 2002; Gelinas and Nguyen, 2005. As both ERK and mTOR are capable of regulating translation through interactions with Mnk1 and 4E-binding proteins respectively (Gelinas et al., 2007; Costa-Mattioli et al., 2009), my results suggest that translation is required for enduring synaptic potentiation following co-stimulation of beta and muscarinic receptors. Consistent with this postulate, inhibition of translation with emetine prevented the maintenance of LTP. The effects of co-activating beta and muscarinic receptors on memory formation in vivo have yet to be determined.

Determination of how diverse neuromodulatory receptors interact to regulate synaptic plasticity should provide novel insights into memory consolidation processes during distinct behavioural states. Disruption of both cholinergic and noradrenergic neuromodulatory systems has been implicated in the pathology of neurodegenerative disorders(Caccamo et al., 2006; Yu et al., 2011). Further understanding of how neuromodulatory systems interact is critical for generation of precise therapeutic interventions in the treatment of memory disorders.

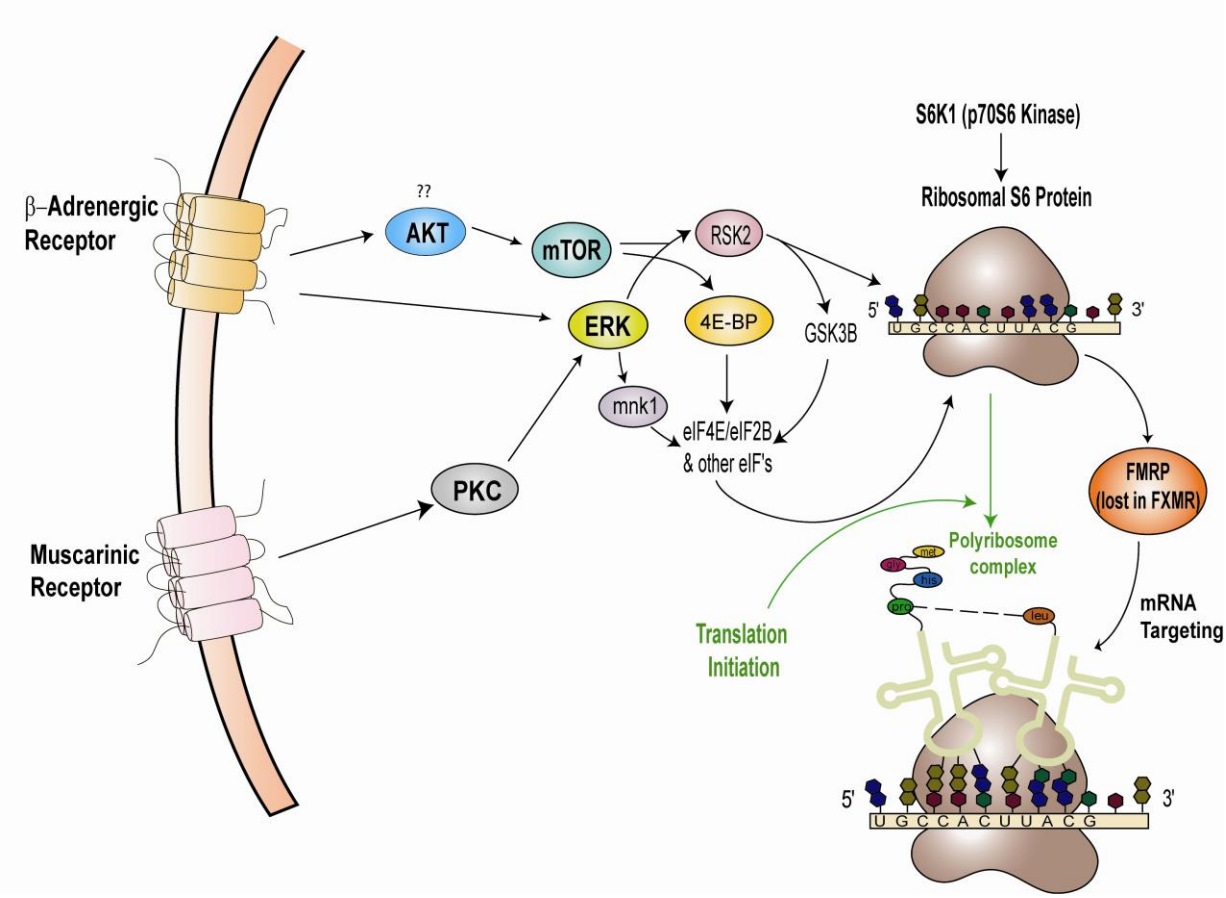


Figure 5.7. Co-activation of muscarinic and beta-adrenergic receptors boosts LTP through translation regulation. Signaling cascade downstream of beta and muscarinic receptor converge of ERK and mTOR which couple to translation regulation. mTOR increases phosphorylation on the eIF4E binding protein, 4E-BP, resulting in the activation of eIF4E. eIF4E is required for the formation of the pre-initiation complex and subsequent translation initiation. ERK-dependent activation of mnk1 also enhances translational capacity. Adapted from Sweatt, 2003.

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# CHAPTER VI:

General Discussion

## NEUROMODULATION OF HIPPOCAMPAL SYNAPTIC PLASTICITY: SUMMARY OF THESIS FINDINGS

How are experiences translated into neural circuit changes capable of supporting the genesis of new adaptive behavioral strategies? Clearly, experiences modify behavioral repertoires through synaptic modifications which influence circuit dynamics and ultimately brain function. A fundamental series of questions remains: How does the brain selectively initiate the encoding of relevant information? How are temporally separate components of an experience bound together into a coherent memory? What are the cellular mechanisms mediating long-term changes in cellular function underlying memory processes? My data suggest that neuromodulation provides answers to these questions.

Research initiated by Richard Morris, Julie Frey, Cliff Abraham and others has provided initial evidence that the ability of synapses to undergo modifications can be influenced through heterosynaptic mechanisms (Frey and Morris, 1997; 1998; Abraham et al., 2001; Le Ray et al, 2004; Abraham et al., 2007; Redondo et al., 2010; see Redondo and Morris, 2011 for review). The synaptic tagging and capture hypothesis provides an attractive model for investigating how populations of synapses interact to facilitate the association of information on extended time scales. The initial postulates that primary rounds of translation and transcription can support future heterosynaptic plasticity have been confirmed (Frey and Morris, 1998). These results provide a link between initial work conducted using *Aplysia* which demonstrated a requirement for translation and transcription in the expression of LTF (Martin et al., 1997; Martin, 2002) and memory by showing how local translation could support the initial encoding of memory which would later be reinforced through transcriptional mechanisms (Wang et al., 2009).

Neuromodulation of LTP has been proposed to be a mechanism for boosting long-term memory (LTM) in the mammalian brain because activation of metabotropic receptors initiates signaling cascades which couple to translation and transcription (Gelinas et al., 2007; Granado et al., 2008; Navakkode et al., 2010). As well, altered neuromodulatory function is correlated with impaired LTP, defective long-term memory function and neuropathologies underlying several human diseases associated with cognitive dysfunction (Flexner et al., 1985; Caccamo et al., 2006; Antonova et al., 2010; Kroes et al., 2010; Yu et al., 2011). Given the importance of neuromodulatory systems to brain function, LTP, and memory formation, my research has further enhanced our understanding of the mechanisms through which neuromodulatory receptors regulate long-term synaptic plasticity in the hippocampus, a brain structure critically involved in the genesis of enduring memories.

**Heterosynaptic Facilitation of LTP**

The first objective of my research was to determine if, similar to other neuromodulatory receptors (O'Carroll and Morris 2004; Sajikumar and Frey, 2004), beta-adrenergic receptors were capable of initiating heterosynaptic facilitation of LTP. I found that stimulation of β-ARs (as described in *Chapters 3 and 4*) facilitates heterosynaptic plasticity through the activation of ERK and mTOR downstream of translation initiation. Electrophysiological and pharmacological tools were used to show that the recruitment of ERK and mTOR is required during the induction of homosynaptic LTP, consistent with previous studies that used much stronger induction regimens (Kelleher et al., 2004; Banko et al., 2006; Gelinas et al., 2007). Furthermore, inhibiting either of these kinases during the induction of LTP at the first synaptic pathway prevented the subsequent induction of LTP at the second synaptic pathway. Consistent with the STC hypothesis, blocking

translation during "strong" (1X100 Hz paired with ISO) but not during "weak" (5 Hz, 10 s) stimulation prevented the expression of both homo- and heterosynaptic LTP. This result is consistent with a model in which stimulation protocols which are suprathreshold for generating translation-dependent LTP can supply plasticity proteins for later use at separate synaptic inputs which receive stimulation subthreshold for initiating protein synthesis (Frey and Morris, 1998). Importantly, heterosynaptic stimulation requires the genesis of a local molecular trace capable of sequestering plasticity products. My results provide evidence that PKA can serve as a molecular tag indicative of synaptic activity. When a membrane-permeant PKA inhibitor (PKI) was applied overlapping with low-frequency stimulation at S2, capture of LTP was impaired. Interestingly, shifting PKI application to coincide with S1 stimulation failed to prevent LTP at either synaptic pathway. These results are consistent with results showing that pairing of 100 Hz stimulation with ISO generates LTP which requires EPAC (exchange protein activated by cyclic-AMP) but not PKA (Gelinas et al., 2008a), and theta-like (5 Hz) patterns of stimulation may preferentially engage PKA to facilitate LTP (Gelinas et al., 2008b).

**Enhanced Heterosynaptic Facilitation in Mice Lacking FMRP**

In *Chapter 4*, genetic and pharmacological techniques were used to determine if Fmr1 knockout mice, a model for Fragile X mental retardation syndrome, display altered synaptic function in response to β-AR stimulation. Heterosynaptic facilitation elicited by beta-adrenergic receptor stimulation was enhanced in slices taken from Fmr1 knockout mouse hippocampus relative to wildtype littermate controls. Similar to WT, heterosynaptic plasticity in Fmr1 KOs required intact ERK signaling during induction of homosynaptic LTP. However, heterosynaptic plasticity in Fmr1 KOs demonstrated a resistance to inhibition of mTOR relative to WT controls, demonstrated by increased maintenance of homosynaptic LTP in the presence of an mTOR

inhibitor. These results are consistent with a recent study conducted in Eric Klann's lab at NYU (Sharma et al., 2010) showing elevated rates of basal mTOR activation in Fmr1 KO mouse hippocampus.

**Interactions Between Neuromodulatory Systems Facilitates Synaptic Plasticity**

Finally, a combination of electrophysiological and pharmacological techniques were used to determine how two key neuromodulatory systems interact (*Chapter 5*). Through the combination of a β-AR and muscarinic receptor agonists, I found that low-frequency stimulation (5 Hz, 5 s) which normally induces transient potentiation (< 1 hr) could now elicit a form of long-lasting LTP (> 2 hr) that required translation. The co-application of these agonists initiated a PKC-dependent signaling cascade to enhance the magnitude and duration of LTP. Importantly, application of either agonists alone failed to facilitate LTP on times scales extending beyond 1 hr. Further upstream characterization of receptor subtypes revealed a dependence on β1 and M1 receptors, consistent with a postsynaptic locus of expression. The functional importance of this form of LTP is demonstrated by a requirement for translation which links this form of LTP with evidence implicating translation in long-term memory formation (Straube et al., 2003; Walling and Harley, 2004; Herrera-Morales et al., 2007).

**Neuromodulation of Protein Synthesis**

It is widely accepted that synaptic activity suprathreshold for engaging translation is critical for establishing enduring synaptic modifications (Stanton and Sarvey, 1984; Deadwyler *et al.*, 1987; Frey *et al.*, 1988; Nguyen *et al.*, 1994; Gelinas and Nguyen, 2005). The ability of these synapses to undergo activity-dependent modifications can be bolstered through activation of neuromodulatory receptors (Straube et al., 2003; Walling and Harley, 2004; Herrera-Morales

et al., 2007). Importantly, neuromodulators can expand the duration of synaptic modifiability to the minutes-to-hours range, well beyond normal fast synaptic transmission (milliseconds). My data support this contention by demonstrating that LTP can be captured 30 min before or after the activation of beta-adrenergic receptors, and that this process requires protein synthesis. Recently it was shown that beta-adrenergic receptor activation can prime translation which supports the establishment of long-lasting LTP by high-frequency stimulation applied 1 h later (Tenorio et al., 2010). Taken together, these results suggest that β-ARs are capable of modifying synaptic plasticity through regulation of protein synthesis. Which specific proteins are being synthesized remains an open question, although strong evidence has emerged that a constitutively active isoform of PKC, PKM-zeta is sufficient for maintaining enduring synaptic changes and long-term memories (Yao et al., 2008). An interesting question that has yet to be addressed is whether beta-adrenergic receptor-driven heterosynaptic facilitation similarly requires PKM-zeta for its expression.

Plasticity-related proteins synthesized in response to strong synaptic activity are distributed cell-wide and are captured at tagged synapses (Frey and Morris, 1997, 1998). My research adds beta-adrenergic receptors to a growing list of neuromodulator transmitters capable of inducing synaptic tagging, where pairing of ISO with HFS induces "strong" LTP that once induced can subsequently be captured at synapses tagged in the future.

Only when emetine was applied during strong stimulation was LTP prevented at both synaptic pathways. This provides indirect evidence that proteins synthesized during ISO+100 Hz stimulation are necessary and sufficient for facilitating heterosynaptic LTP. In contrast, shifting emetine to coincide with S2 stimulation does not prevent LTP at either synaptic pathway. Thus, once translation of pre-existing mRNA has been initiated and captured, LTP is immune to

translational arrest. Future studies should investigate which proteins could serve as putative plasticity-related proteins.

## THEORETICAL IMPLICATIONS OF THESIS FINDINGS

Tightly regulated translation is essential for normal cognitive functioning. Fragile X mental retardation syndrome is characterized by moderate to severe mental retardation including memory deficits (Hagerman et al., 2005). Similarly, FXS model mice exhibit phenotypes consistent with moderate cognitive deficits (Mineur et al., 2002; MacLeod et al., 2010). Clearly, determining which signaling pathways contribute these cognitive deficits is crucial for identifying novel therapeutic targets in the treatment of FXS. Clinical trials have already begun to test mGluR antagonists with promising results (Berry-Kravis et al., 2011). These studies indicate that metabotropic glutamate receptor activation is tuned to optimize synaptic function through downstream regulation of translation (Auerbach and Bear, 2010; Osterweil et al., 2010). As beta-adrenergic receptors similarly couple to these signaling cascades, augmenting noradrenergic function should prove to be therapeutically viable.

I have demonstrated that beta-adrenergic receptors are capable of enhancing heterosynaptic plasticity in two important ways; by lowering the threshold for the induction of homosynaptic LTP, and secondly, by upregulating translation. Thus, by initiating translation-dependent forms of LTP representative of a strong or highly salient environmental event, the mammalian brain could update this new memory through the incorporation of normally innocuous events within a finite time window relative to the strong memory-forming stimulus. The strong memory-inducing event would cause the release of noradrenaline and the engagement of protein synthesis which would allow the immediate encoding of attended experience. Once

these proteins are generated, they can be sequestered by synaptic activity induced by subthreshold or innocuous experiences, thereby encoding or incorporating these events into the initial strong memory. This would lead to qualitatively richer memories which would now be updated in both detail and temporal components. It should be noted that despite initial forays into behavioral tests of the synaptic tagging and capture hypothesis (Moncada and Viola, 2007), more direct evidence of this phenomenon is sorely needed and will likely require the combination of *in vivo* electrophysiological and real-time molecular event visualization experiments capable of monitoring discrete synaptic events at the dendritic level (ambitious, I know).

## CONCLUSIONS AND FUTURE DIRECTIONS

In conclusion, my data have further characterized the mechanisms through which neuromodulatory receptors regulate homo- and heterosynaptic LTP. I found that beta-adrenergic receptor stimulation gates the induction of heterosynaptic LTP, through upregulation of translation. Next, I identified altered heterosynaptic plasticity in a mouse model of Fragile X syndrome which appears to be due to an imbalance between ERK and mTOR signaling. Finally, I found that two different neuromodulatory receptor systems can interact to facilitate the induction of translation-dependent LTP.

In the preceding paragraphs of this discussion, a number of important issues for future exploration were identified and these include:

1) Identifying which proteins are synthesized following neuromodulatory receptor stimulation.

2) Determining how local synaptic changes interact with transcription to maintain synaptic alterations.

3) The identification of in vivo correlates of synaptic tagging and capture

To further clarify the roles of neuromodulators in memory function, multiple approaches will need to combined. Integrating electrophysiological and biochemical techniques with new optogenetic, and imaging technologies will provide the basis for further elucidation of neuromodulatory mechanisms engaged during memory formation. In vivo evidence for STC is needed and will require in vivo identification and recording of converging synaptic events during memory formation. To investigate interactions between neuromodulatory systems will require an additional level of sophistication. Noradrenergic and muscarinic fibers project throughout the brain and thus are able to engage distinct and interconnected brain structures to modify behaviour. A critically important question is how diverse structures coalesce to influence memory formation and behavioural adaptations. This will require simultaneous in vivo recordings of changes in network activity across several brain structures including the hippocampus, cortical storage sites and the neuromodulatory nuclear groups. These experiments would be bolstered through the use of optogenetic techniques which, with further refinement, could allow non-deleterious activation/deactivation of selective brain structures and cell types with a high degree of temporal flexibility (Fiala et al., 2010).

Overall, much remains to be determined regarding neuromodulation of memory formation. However, further elucidation of the cellular mechanisms of memory should be bolstered due to the implication of altered synaptic function in a broad range of neurobiological processes including pain, addiction, and human diseases associated with altered cognitive functioning.

Neuromodulatory receptors continue to gain prominence in the neuroscience of learning and memory. The severity of memory impairments associated with aging and neuropathological diseases including Alzheimer's and Parkinson's disease is strongly correlated with dysfunction of

neuromodulatory systems in the brain (Perez-Lloret and Rascol, 2010; Thathiah and De Strooper, 2011). The fact that neuromodulators can regulate synaptic function, learning and memory provides further impetus for understanding the cellular and molecular mechanisms through which they impact neural plasticity. Neuromodulation of LTP provides a cellular solution to the problem of how to integrate information over time. My data provide evidence that beta-adrenergic receptors are capable of expanding the temporal window for heterosynaptic transfer of LTP well beyond the limits of normal synaptic transmission. Beta-adrenergic receptors facilitate memory encoding by lowering the threshold for long-term synaptic modifications, including LTP. As LTP is the leading cellular candidate for neural encoding of new memories, my data further define the mechanisms through which changes in synaptic function are regulated in the mammalian hippocampus.

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Title of Publication: Neuromodulation of Heterosynaptic Plasticity in Mouse Hippocampus
Authors/Editors: Author: Steven A. Connor Date of Publication:
Publisher: Canadian National Library
Title of CSHLP Journal/Book: Learning and Memory Title of Article/Chapter: Fragile X mental retardation protein regulates heterosynaptic plasticity in the hippocampus CSHL Authors/Editors: Steven A. Connor, Charles A. Hoeffer, Eric Klann, Peter V. Nguyen.

Page Numbers: 208-220
Figure Numbers: 1-9
Figure Page Numbers: 208, 210, 211, 212, 213, 214, 215, 216, 217
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# Thesis Approval/ Program Completion

**Part 1: Completed by student and provided to examining committee at defense.**

UofA ID Number: 1149867 Name of Student: Steven Connor

Department: Centre for Neuroscience Degree Program: PhD Specialization (if applicable): n/a

Exact Title of Thesis: Neuromodulation of Heterosynaptic Plasticity in Mouse Hippocampus

**Part 2: Completed by examining committee at the end of the in camera discussion.**

Pass: Committee members sign below immediately. If one of the examiners fails the student but the student passes, that examiner does not have to sign. The committee chair initials on behalf of the external reader if the reader does not attend the doctoral exam.

Pass subject to revisions: Members wishing to do so may sign immediately. The committee chair or supervisor withholds signature until the thesis is amended satisfactorily and all other committee members have signed.

Adjourned or Failed: No member of the committee signs. The committee chair should consult Section 8 of the Graduate Program Manual for instructions.

**We, the members of the examining committee, certify that we have examined this thesis and recommend acceptance of this thesis in partial fulfillment of the requirements for the above noted degree.**

| | | |
|---|---|---|
| X Supervisor's Signature | PETER V. NGUYEN Name (please print) | August 20, 2011 Date |
| X Committee Member's Signature | Kathryn Todd Name (please print) | Aug 15/2011 Date |
| X Committee Member's Signature | DECLAN ALI Name (please print) | Aug 15, 2011 Date |
| X Committee Member's Signature | GREG FUNK Name (please print) | Aug 15, 2011 Date |
| X Committee Member's Signature | JAIDEEP BAINS Name (please print) | Aug 15, 2011 Date |
| X Committee Member's Signature | Name (please print) | Date |
| X Committee Member's Signature | Name (please print) | Date |
| X Committee Member's Signature | Name (please print) | Date |

**Part 3: Completed and submitted to FGSR by Graduate Coordinator/Department Chair following examination.**

Examination Date: 15 August 2011

Committee Recommendation: ☐ Pass ☐ Pass subject to revisions ☐ Adjourned ☐ Failed

I certify that: ☒ the student has met all degree requirements

☐ the Academic Integrity and Ethics requirement is:

- ☐ complete (mandatory if admitted as of September 2004; optional for others)
- ☐ not required (student began program before September 2004)
- ☐ not required (ethics completed in a previous degree)

☐ all grades, excluding thesis, have been entered

Graduate Coordinator/Department Chair (please print) Dave Collins Signature Aug 31, 2011 Date

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Steven Connor
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