Université de Montréal

Mechanisms of translation regulation in long-term synaptic plasticity

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Mémoire présenté à la Faculté des études supérieures en vue de l'obtention du grade de Maître en sciences en sciences neurologiques

Décembre 2014

This thesis entitled:

Mechanisms of translation regulation in long-term synaptic plasticity

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RÉSUMÉ ET MOTS CLÉS

Les souvenirs sont encodés dans le cerveau grâce aux configurations uniques de vastes réseaux neuronaux. Chaque connexion dans ces circuits est apte à être modifiée. Ces changements durables s'opèrent au niveau des synapses grâce à une synthèse de protéines *de novo* et génèrent ce qu'on nomme des traces mnésiques. Plusieurs preuves indiquent que, dans certaines formes de plasticité synaptique à long terme, cette synthèse a lieu dans les dendrites près des synapses activées plutôt que dans le corps cellulaire. Cependant, les mécanismes qui régulent cette traduction de protéines demeurent encore nébuleux. La phase d'initiation de la traduction est une étape limitante et hautement régulée qui, selon plusieurs chercheurs, constitue la cible principale des mécanismes de régulation de la traduction dans la plasticité synaptique à long terme. Le présent projet de recherche infirme cette hypothèse dans une certaine forme de plasticité synaptique, la dépression à long terme dépendante des récepteurs métabotropiques du glutamate (mGluR-LTD). À l'aide d'enregistrements électrophysiologiques de neurones hippocampiques en culture couplés à des inhibiteurs pharmacologiques, nous montrons que la régulation de la traduction implique les étapes de l'élongation et de la terminaison et non celle de l'initiation. De plus, nous démontrons grâce à des stratégies de knockdown d'expression d'ARN que la protéine de liaison d'ARNm Staufen 2 joue un rôle déterminant dans la mGluR-LTD induite en cultures. Dans leur ensemble, les résultats de la présente étude viennent appuyer un modèle de régulation de la traduction locale de protéines qui est indépendante de l'initiation.

Mots clés : dépression à long terme dépendante des récepteurs métabotropiques du glutamate (mGluR-LTD), régulation de la traduction locale de protéines, protéines de liaison d'ARNm, répression de la traduction, Staufen 2 (Stau2).

ABSTRACT AND KEY WORDS

Memories are encoded in the unique configurations of the vast neuronal networks of the brain. Each of these connections possesses the ability to be modified. Such long-lasting changes at the synapse often require the synthesis of new proteins that create what we call memory traces. Evidence suggests that the signal-induced activation of translation in some forms of synaptic plasticity occurs locally, at the activated synapses, rather than in the soma. However, the mechanisms regulating local and rapid *de novo* protein synthesis are poorly understood. The initiation step of translation is a highly regulated step and is believed to be the main target of control. The present research project challenges this view for a certain form of long-term synaptic plasticity, metabotropic glutamate receptor-dependent long-term depression (mGluR-LTD). We show, using electrophysiological recordings of dissociated hippocampal neurons in cultures coupled to pharmacological inhibitors, that translation regulation depends on elongation and termination, rather than initiation. Moreover, by exploiting RNA knockdown strategies, we demonstrate that the RNA-binding protein Staufen 2 plays a crucial role in mGluR-LTD induced in cultures. Altogether, the findings of the present study support a model of translation regulation that is downstream of initiation.

Key words: metabotropic glutamate receptor-dependent long-term depression (mGluR-LTD), local translation regulation, RNA-binding proteins (RBPs), translation repression, Staufen 2 (Stau2).

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

4E-BP	Eukaryotic initiation factor 4E-binding protein
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptor
AP2	Adaptor protein 2
Arc/Arg3.1	Activity regulated cytoskeletal-associated protein
BDNF	Brain-derived neurotrophic factor
Btz	Barentsz
CA1	Cornu ammonis 1
CA2	Cornu ammonis 2
CA3	Cornu ammonis 3
CaMKII	Calcium/calmodulin-dependent protein kinase II
CBP80	Nuclear cap-binding protein 80
c-Fos	FBJ osteosarcoma oncogene
CPEB1	Cytoplasmic polyadenylation element binding protein-1
CREB	Cyclic adenosine monophosphate (cAMP) response element binding protein
DAG	Diacylglycerol
DG	Dentate gyrus
DHPG	Dihydroxyphenylglycine
EC	Entorhinal cortex
eEF2K	Eukaryotic elongation factor 2 kinase
EF1A	Elongation factor 1A

eIF1A	Eukaryotic initiation factor 1 A
eIF2α	Eukaryotic initiation factor 2 α
eIF3	Eukaryotic initiation factor 3
eIF4F	Eukaryotic initiation factor 4F complex
eIF4G	Eukaryotic initiation factor 4G
EJC	Exon junction complex
e-LTP	Early-LTP
EPSC	Excitatory post-synaptic current
ERK	Extracellular signal-regulated kinases
fEPSP	Field excitatory post-synaptic potential
FMRP	Fragile X mental retardation protein
FXS	Fragile X syndrome
GDP	Guanosine diphosphate
GFP	Green fluorescent protein
GRIP-ABP	AMPAR-binding protein–glutamate receptor interacting protein
GSK3β	Serine/threonine kinase glycogen synthase kinase-3β
GTP	Guanosine-5'-triphosphate
HHT	Homoharringtonine
hnRNP	Heterogeneous nuclear ribonucleoprotein
HPC	Protein hippocalcin
IEG	Immediate early gene
IP_3	Inositol-1,4,5-trisphosphate
JNK	Jun N-terminal kinase

KH	K-homology
l-LTP	Late-LTP
LTD	Long-term depression
LTP	Long-term potentiation
MAP1B	Microtubule associated protein 1B
MAP2	Microtubule-associated protein 2
MAPK	Mitogen associated protein kinases
mEPSC	Miniature excitatory post-synaptic current
Met-tRNAi^{Met}	Methionyl initiator transfer RNA
mGluR	Metabotropic glutamate receptor
mGluR-LTD	Metabotropic glutamate receptor-dependent long-term depression
miRNP	Micro RNA particles
MNK	Mitogen-Activated Protein Kinase-Interacting Kinase
MOI	Multiplicity of infection
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cell
NSF	N-ethylmaleimide-sensitive factor
NO	Nitric oxide
NOS	Nitric oxide synthase
NMD	Non-sense mediated decay
NMDAR-LTD	NMDAR-dependent long-term depression
NMDAR-LTP	NMDAR-dependent long-term potentiation

NMDAR	N-methyl-D-aspartate receptor
Ophn1	Oligophrenin 1
P-bodies	Processing bodies
PDK1	Phosphoinositide-dependent kinase 1
PERK	Protein kinase-like ER kinase
PI3K	Phosphoinositide 3-kinase
PIKE	Phosphoinositide 3-kinase -enhancer
PIC	43S pre-initiation complex
PICK1	Protein interacting with C-kinase 1
PKA	Protein kinase A
PKC	Protein kinase C
PKMζ	Truncated form of protein kinase C, zeta
PLC	Phospholipase C
PP1	Protein phosphatase 1
PP2B	Protein phosphatase 2B
PPF	Paired-pulse facilitation
PP-LFS	Paired pulse low frequency stimulation
PPR	Paired pulse ratio
Pr	Probability of transmitter release
PRP	Plasticity-related protein
PTK	Protein tyrosine phosphatase
Rap1	Repressor activator protein 1
RBP	RNA-binding protein

RNP	Ribonucleoprotein particle
RPM	Ribopuromycilation
RSK1	Ribosomal S6 kinase-1
SC	Schaffer collateral
shRNA	Short hairpin RNA
SMD	Staufen-mediated decay
SRF	Serum response factor
Stau1	Staufen 1
Stau2	Staufen 2
siRNA	Small interfering RNA
STEP	Striatal-enriched protein tyrosine phosphatase
STP	Short-term potentiation
Upf1	Helicase up-frameshift 1
ZBP1	Zipcode-binding protein 1
Zif268	Early growth response 1

ACKNOWLEDGMENTS

I am deeply indebted to several people who have helped in bringing the best in me while writing this thesis. To all, I wish to convey my heartfelt gratitude, appreciation and acknowledgment. During the past two incredibly formative years, I have grown not only as a student and young scientist, but also as a more mature individual.

I want to thank both of my supervisors, Jean-Claude Lacaille and Wayne Sossin, for their guidance and advice. I benefited tremendously from their direction, technical support and above all encouragement. I am very appreciative of their many valuable suggestions and comments on this thesis. This work has been such a wonderful learning process for me and they have been major contributors to my development.

I am deeply grateful to have met fantastic colleagues, both in my lab and in the Department, without whom, this journey would not have been the same. I thank them for all their support and friendship. I will remember, most of all, the endless discussions, the laughter and the joy I felt of working surrounded by such remarkable individuals. I have learned from their depth of experience in science, but also in different aspects of life, and I hope to carry that with me as I begin a new adventure.

Finally, I would like to thank my family, especially my parents, for their unconditional support without which I would not be where I am now. Their faith in me has always empowered me to achieve bigger and greater things, to be ambitious and strive for excellence. I cannot express in words how thankful I am for everything they have given me.

CHAPTER I. INTRODUCTION

1.1 HIPPOCAMPAL-DEPENDENT LEARNING AND MEMORY

Although, today, the link between the hippocampus and memory is undisputed, neuroscientists were still searching for a clear hippocampal function until the mid-twentieth century (Andersen et al 2006). This impressive bulging brain structure of the temporal lobe attracted interest from the very start of brain investigations. It was implicated in a variety of functions ranging from olfaction (Brodal 1947) to harboring "the central emotive process of cortical origin" (Papez 1937) until the seminal and detailed report on the amnesic patient H.M. (Scoville & Milner 1957) provided direct evidence for a mnemonic function.

1.1.1 Role of hippocampus in learning and memory

H.M., who died in 2008, had been suffering from intractable epilepsy resistant to antiepileptic drug treatment for many years before he underwent bilateral resections of the medial portions of the temporal lobe. His seizures were successfully relieved, but he was left with a profound memory loss. Following the operation, H.M. was unable to retain any information about recent events or episodes of his life, people he had met, places he had visited or objects he had encountered (anterograde amnesia). His memories dating from some time prior to the intervention were also impaired (retrograde amnesia), although memories from early life appeared to be intact. In contrast, his general intellect, perceptual ability, working memory and some forms of long-term memory were unscathed (Milner et al 1968, Scoville & Milner 1957).

Since then numerous studies in both humans and animals have tried to elucidate how the hippocampus mediates memory processing. Learning and memory are extremely complex concepts involving a dynamic interplay between various brain regions that make different contributions to memory formation (Schacter & Tulving 1994, Squire 2004). To puzzle out the hippocampus' role amidst the organization of memory systems in the brain is not a simple task (Kandel 2001). What does the hippocampus do? Does it perform tasks that other brain regions cannot accomplish? In which phase of memory processing is the hippocampus involved? The main processes include the encoding of the

information into memory, the consolidation process for stability over time and the retrieval and reactivation of the memory during recall (Morris 2006). To add a layer of difficulty in answering these questions, "memory traces" are encoded at different levels. They can be stored in synaptic weights as specific synapses modify their connection strength by undergoing biochemical modifications (Martin et al 2000), but such changes are embedded within a larger neural ensemble to which the memory has been allocated (Guzowski et al 1999, Hall et al 2001, Hebb 1949, Reijmers et al 2007, Sakaguchi & Hayashi 2012).

Although much is yet to be understood, great strides have been made and some conclusions have been reached about the function of the hippocampus in humans: (1) Amongst the different types of memory (Bruner 1969, Ryle 1949, Winograd 1975), the hippocampus is involved in one particular type termed declarative memory that refers to the recollection of facts and events (Tulving 1983) and can be associative, abstract and context-dependent (Manns & Eichenbaum 2006). Located at the confluence of highly processed multimodal sensory inputs, the hippocampus can bind information from the neocortex to form memories that are representational and model the external world (Squire & Alvarez 1995). (2) Its involvement in the storage and recovery of memories diminishes, or in any case, changes with time as consolidation proceeds and memory traces are reorganized. However, whether the hippocampus is a temporary memory system (Squire 1992, Squire & Alvarez 1995) or is engaged to some extent in the retrieval and storage of certain remote memories is still a subject of debate (Moscovitch et al 2006, Sutherland & Lehmann 2011). (3) The hippocampal region and the adjacent cortex are not involved in working memory in addition to a wide range of implicit or non-declarative tasks (e.g. motor skill memory) (Squire & Zola 1996). (4) The hippocampal region is not involved in non-mnemonic aspects of cognition (Craig 2006).

1.1.2 Animal models of human amnesia

Lastly, it is important to note that although there is great structural similarity and strong evolutionary conservation of circuitry of the hippocampus (in contrast to the diversity of the neocortex), there are some functional differences across the mammalian taxon (Manns & Eichenbaum 2006, Squire et al 2004). While it is unequivocally involved in

the initial acquisition and temporary storage of declarative memory, the rat hippocampus appears to be heavily involved in the formation of cognitive maps and their navigation through space compared to its human counterpart that is most engaged by episodic memory. Such differences could be accounted for by the different kinds of information the animal hippocampus is receiving in comparison to the humans. Just like the hippocampi of the two human hemispheres may be performing slightly different functions due to lateralization even though they are structurally symmetrical (Morris 2006), the rat hippocampus provides an output that is appropriate to the animal, not to humans. As Manns and Eichenbaum (2006) illustrate it ever so well, "it is perhaps not surprising that an animal such as the rat, whose survival normally depends on nighttime foraging, would place a premium of remembering spatial locations of important items. It is also not surprising that the human hippocampus, spoiled by the wonderful elaborative ability of its complex neocortex, would more likely reflect the paradoxes of episodic memory, being abstract yet structured, detailed yet imperfect."

1.2 NEUROANATOMY OF THE HIPPOCAMPAL FORMATION

1.2.1 Anatomical organization

The hippocampal formation is a discernible C-shaped tridimensional structure of the medial temporal lobe found in both hemispheres. In monkeys and humans, it lies horizontally on the floor of the temporal horn of the fourth ventricle. In rats, the structure curves (more vertically oriented than in primates) from the medial aspects, or septal pole (dorsal hippocampal), to the bottom tip of the temporal lobe, or the temporal pole (ventral hippocampus) (Amaral & Lavenex 2006). The course the hippocampal formation follows is termed the septotemporal axis and much of its internal structure remains relatively the same throughout its length. Orthogonal (perpendicular) to this axis is the transverse plane that clearly reveals the cytoarchitectonically distinct adjoining structures that make up the hippocampal formation: the hippocampus proper, dentate gyrus, subicular complex (subiculum, presubiculum, parasubiculum) and entorhinal cortex (Amaral & Lavenex 2006). The hippocampus proper is further subdivided into three subfields that bear the latin name *cornu ammonis* (CA) for Ammon's Horn: CA1, CA2 and CA3. The general

term hippocampus is often used to refer to the hippocampus proper and dentate gyrus and will be used as such in this text. Moreover, we will focus on the rodent hippocampus since it is the animal model used in this study.

1.2.2 Cytoarchitectonic organization

The highly ordered organization of cells and terminating projections is one of the hallmarks of the hippocampus that has made it a model of choice for the study of the neurobiology of memory. The hippocampus consists of only one densely packed layer of principal neurons – it is an allocortical[1] region unlike the surrounding neocortex – that form two interlocking C's, reversed relative to each other, one of pyramidal cells of the hippocampus proper, and the other of granule cells of the dentate gyrus. Fibers originating from different cortical regions (including the hippocampus itself) make synaptic contact with distinct dendritic segments of their target principal neuron (Förster et al 2006). Thus, afferent fibers terminate in sharply segregated hippocampal layers to form a laminated network.

The principal neurons and zones of synaptic connections of the hippocampus proper are disposed in superimposed layers (Amaral & Lavenex 2006) (Fig. 1A):

(1) the lacunosum-moleculare layer is the deepest layer containing projections from the entorhinal cortex (perforant path) and extra-hippocampal inputs;
(2) the radiatum layer is the layer in which apical dendrites from the pyramidal cells extend and CA3-CA1 connections occur via the Schaffer collaterals from CA3 as well as associational connections[2];
(3) the pyramidal layer contains the cell bodies of pyramidal cells;
(4) the oriens layer contains the basal dendrites of the pyramidal cells that make contact with some of the Schaffer collaterals and associational fibers;
(5) the alveus layer, on the other hand, contains the axons of the pyramidal cell.

Much of these intrahippocampal projections travel along the transverse or oblique axis and exhibit a clear intrinsic connectivity (Andersen et al 1971) that we will discuss next.

[1] Allocortical: a term applied to cortical regions having fewer than six layers.
[2] Associational connections are ipsilateral CA3-CA3 connections.

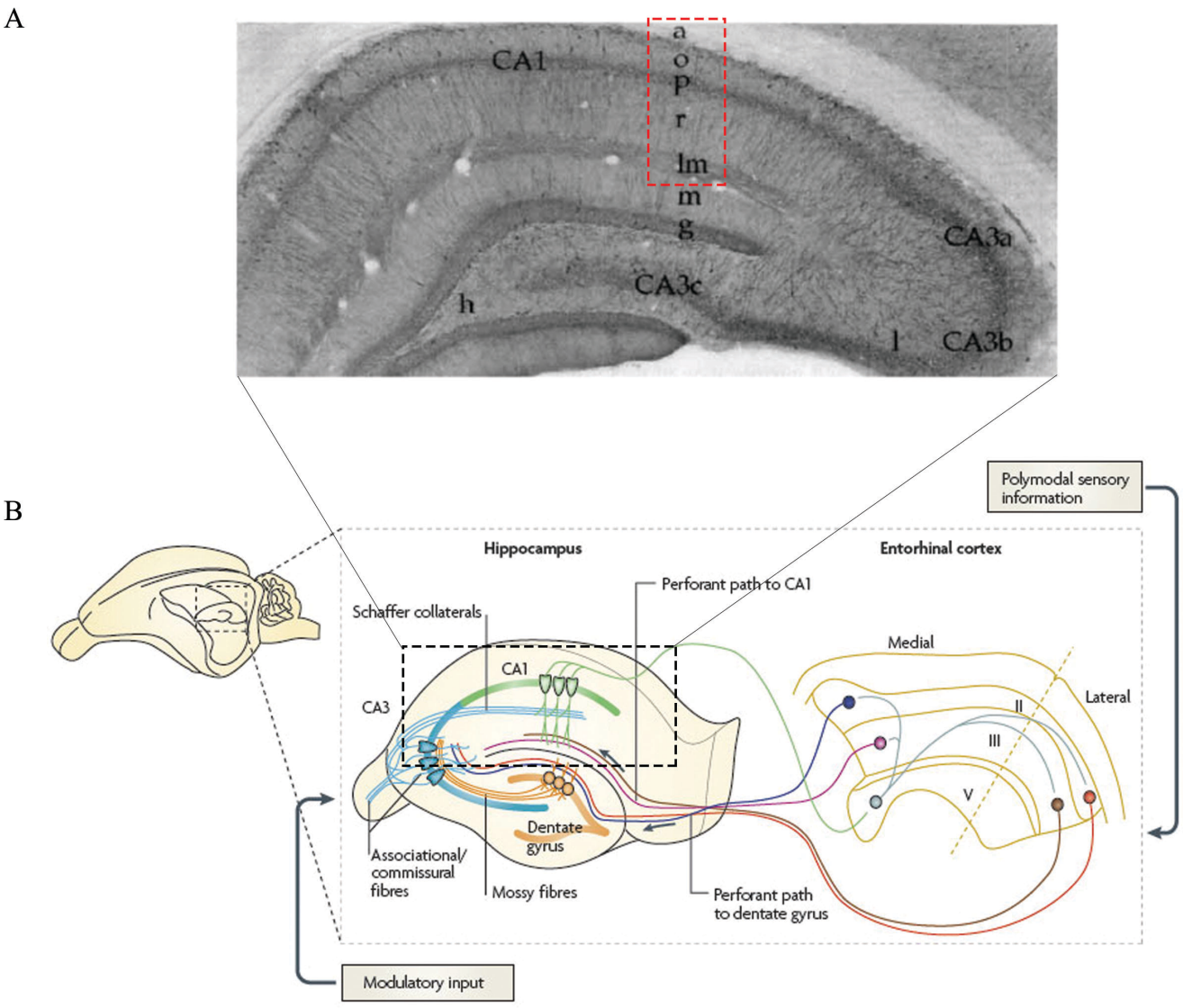

Figure 1. Basic anatomy and connectivity of the hippocampus

(A) Layers of the hippocampus proper. Alveus layer (a), oriens layer (o), pyramidal cell layer (p), radiatum layer (r), lacunosum-moleculare layer (lm). Figure adapted from Freund and Buzsaki (1996). (B) Diagram of the trisynaptic circuit of the hippocampus. Figure adapted from Neves et al (2008). See text for details.

1.2.3 The trisynaptic circuit

The highly convergent-divergent internal connections of the hippocampus are more complex than the trisynaptic circuit, which was described early on (Ramón y Cajal 1911), might suggest. Although serial elements of the circuit lie within a transverse plane, axonal projections also diverge along the longitudinal axis (Amaral & Witter 1989). Moreover, it is now clear that it is rather a portion of the functional circuitry of the hippocampal formation than its major contributor (Amaral & Lavenex 2006). However, this unidirectional synaptic flow through the three important excitatory synapses depicted below remain of great significance for hippocampal research.

Enthorinal cortex (EC) ➔ Dentate gyrus (DG) • DG ➔ CA3 • CA3 ➔ CA1

Synapse 1. The entorhinal cortex, via the perirhinal and parahippocampal cortices, receives a host of highly processed multimodal sensory inputs from various neocortical sources (Suzuki & Amaral 1994). This information is then relayed to the granule cells of the dentate gyrus via the **perforant path**. These entorhinal fibers perforate the subiculum before terminating into the outer molecular layer of the dentate gyrus (Fig. 1B). Of note, the entorhinal cortex also projects, to a lesser extent, to the hippocampus proper via the **temporammonic pathway**.

Synapse 2. Granule cells give rise to axons called **mossy fibers** (Fig. 1B) with unusually large boutons that form en passant synapses onto the CA3 pyramidal cells. This innervation stops at the border of CA3 with CA2, which is the main feature distinguishing these two regions. No other hippocampal projections are known to innervate CA3 (apart from CA3-CA3 commissural[3] and associational connections).

Synapse 3. The CA1 subfield represents the last stage of this intrahippocampal loop and is densely innervated by CA3 pyramidal axons, the **Schaffer collaterals** (Fig. 1B). These fibers extend through both the stratum radiatum and stratum oriens layers. CA1 pyramidal cells send axons that travel parallel to the alveus in stratum oriens and mainly project back to the entorhinal cortex and subiculum (Amaral & Lavenex 2006).

[3] Commissural connections refer to contralateral CA3-CA3 connections

Although pyramidal neurons largely outnumber any other cell type in the hippocampus, there is a great diversity of interneurons found in various layers of the hippocampus that play a crucial role in regulating and fine-tuning the activity of the network (Freund & Buzsaki 1996), ultimately modulating its output, thus adding to the complexity of the system.

1.2.4 Field excitatory post-synaptic potentials

A consequence of the laminar organization and connectivity of the hippocampus is the ability to generate large extracellular currents while stimulating Schaffer collaterals with an electrode in transverse slices. Such stimulation of parallel fibers causes the simultaneous synaptic activation of a population of cells and allows the observation of synaptic currents that would normally be too small to be detected in single unit recordings. When the recording electrode is placed parallel to the stimulating electrode, a negative change in potential occurs at the recording electrode relative to the reference electrode (ground) as positive depolarizing currents rush toward the *current sink* into the dendrites of activated cells. Synaptically generated current flows inside the cells and exit at the *current source* in the region of the soma where membrane area is greatest. A current loop is created and gives rise to field excitatory post-synaptic potentials (fEPSPs). With stronger stimulation, a population spike superimposed onto the rising phase of the fEPSP is observed and reflects the synchronous discharge of cells. The magnitude of the fEPSP is used as a measure of the efficacy of post-synaptic activation (Andersen et al 2006).

Hippocampal field potential studies were instrumental in further understanding synaptic function.

1.3 LONG-TERM SYNAPTIC PLASTICITY

The idea that changes in the strength of the synapse serve as elementary components of memory storage was first brought forward by Ramón y Cajal (1894). However, tangible evidence was only provided years later. The first direct evidence to support the notion that neural circuits are modified by learning came from studies of simple forms of learning in invertebrate systems, including the gill-withdrawal reflex of *Aplysia*

(reviewed in Mayford et al 2012). During this same period, Bliss and Lomo (1973) found *in vivo* synaptic responses in the dentate gyrus of the hippocampus to display plasticity in the rabbit following stimulation of the perforant path. Since then, long-term synaptic plasticity, and particularly long-term potentiation (LTP), has been the subject of intense study and found to be present at a large number of excitatory synapses in the brain. Moreover, not only can neurons undergo bidirectional changes, such that synapses can be potentiated or depressed, but they can also express multiple forms of LTP and long-term depression (LTD) that differ in their molecular mechanisms and time domains thus, conferring several computational advantages (Malenka & Bear 2004); hence the widely held belief that modulation of synaptic transmission constitutes the physical substrate of information storage in the brain (Martin et al 2000). Given the wide variety and flavors of synaptic plasticity found in different brain regions, the following section will describe forms of plasticity limited to one of the better characterized synapses in the hippocampus: the Schaffer collateral (SC) synapse, a monosynaptic connection between CA3 and CA1 pyramidal neurons (CA3-CA1).

1.3.1 Synaptic transmission at the Schaffer pathway

Communication at chemical synapses involves the exocytotic release of the content of synaptic vesicles from the presynaptic terminal, diffusion across the synaptic cleft, and binding to postsynaptic receptors. At the SC CA3-CA1 synapse, synaptic transmission is excitatory and glutamate is released onto tiny protrusions called dendritic spines. Glutamate receptors located at this synapse are: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptors (AMPARs), *N*-methyl-D-aspartate receptors (NMDARs), kainate receptors and metabotropic glutamate receptors (mGluRs).

AMPARs are ligand-gated ionotropic receptors that respond rapidly to neurotransmitters released in the cleft. Their activation leads to large influx of sodium and smaller efflux of potassium, such that the postsynaptic membrane is depolarized and excitatory post-synaptic currents (EPSCs) are generated. AMPARs are composed of four subunits, which can be a homomeric or heteromeric mixture of GluA1 to GluA4 subunits. Most AMPARs contain at least one GluA2 subunit which renders them calcium-impermeable while GluA2-lacking AMPARs are permeable to calcium (Luscher &

Malenka 2012). Hippocampal principal cells mainly express GluA1 and GluA2 (Keinanen et al 1990).

NMDARs are also ligand-gated ionotropic receptors, but they are also voltage-dependent since magnesium blocks them at resting membrane potential and depolarization is needed to drive the divalent cation from the channel (Nowak et al 1984). NMDARs, when opened, are permeable to the monovalent cations sodium and potassium, but, unlike most AMPARs, they have a high permeability to calcium (Jahr & Stevens 1987). They also have slower kinetics, longer open time and higher affinity for glutamate (Dingledine et al 1999). Of note, NMDARs can possess subunits that contain a binding site for glycine and D-serine.

Kainate receptors are also ligand-gated ionotropic receptors that mediate fast excitatory neurotransmission. Although they contribute little to EPSCs, they supplement glutamate transmission by enhancing and extending the postsynaptic depolarization. They can also modulate transmission presynaptically (Pavel et al 2006).

mGluRs are G-protein coupled receptors that, when bound to glutamate, trigger various signaling cascades. There are eight receptor subtypes categorized in three groups based on their pharmacological and functional properties: Group I (mGluR1, mGluR5), Group II (mGluR2, mGluR3) and Group III (mGluR4, mGluR6, mGluR7, mGluR8) (Shigemoto et al 1997). mGluRs are generally located peri-synaptically, thus they are thought to require strong synaptic activation and glutamate spillover for the receptors to be activated. Group I mGluRs have a somatodendritic distribution and their activation leads to increased excitability of the neuron via the modulation of potassium, calcium, and nonselective cations channels as well as increased intracellular calcium postsynaptically. In contrast, group III mGluRs found near the pre-synaptic terminal act to inhibit excitatory transmission at the SC CA3-CA1 synapse (Pavel et al 2006). On the postsynaptic pyramidal neuron, there are high levels of mGluR5 and lower levels of mGluR1, while mGluR7 is abundant presynaptically (Bliss et al 2006).

EPSCs evoked at low-rates are mediated in great part by activation of AMPARs (Davies & Collingridge 1989). NMDARs contribute little to the postsynaptic response during basal synaptic activity but are critical for synaptic plasticity since they require the temporal coincidence of ligand release and depolarization for current to pass through.

1.3.2 NMDA receptor-dependent LTP

NMDAR-dependent LTP (NMDAR-LTP) is the predominant form of synaptic plasticity in the brain (Bliss & Collingridge 1993). As explained above, the properties of NMDA receptors are such that the coincidence of glutamate release and post-synaptic depolarization is required to open the channel and cause maximal post-synaptic influx of Ca^{2+}. Calcium entry is an absolutely necessary trigger for NMDAR-LTP (Lynch et al 1983, Malenka et al 1992). Experimentally, activation of NMDARs is usually achieved by applying a high-frequency tetanic stimulation to the synapses or by directly depolarizing the postsynaptic neuron while applying a low-frequency synaptic stimulation (Citri & Malenka 2008). NMDAR-LTP exhibits a number of basic properties that relate to the properties of NMDARs. First, it is cooperative because a weak input, even if delivered at high frequency, does not induce LTP; a critical number of synapses must therefore be activated to reach threshold intensity. Second, it is associative because activity at one input can influence the ability of another active input to undergo plasticity. In other words, a weak input can be potentiated only if it coincides with a strong input. Finally, NMDAR-LTP is input specific since potentiation only occurs at synapses at which it is induced (Bliss et al 2006, Nicoll et al 1988).

As with other forms of synaptic plasticity, NMDAR-LTP involves phases of induction and expression. Following the appropriate pattern of stimulation of the Schaffer collaterals to activate NMDARs, an initial strong potentiation of the response is observed which decays over a period of 10 minutes to a stable, but persistently increased level compared to baseline when synaptic transmission is probed with a low stimulation rate (every 30 seconds). This initial phase is referred to as short-term potentiation (STP) and involves different mechanisms than those recruited in the long-term phase. The long-term phase, which can last from hours (in vitro) to days (in vivo), is in turn believed to be divided in two phases, early-LTP (e-LTP) and late-LTP (l-LTP), according to their respective sensitivity to protein synthesis inhibitors (Frey et al 1993), although this model has evolved (Reymann & Frey 2007). Different protocols of stimulation can also be used to isolate the early phase from the late phase (Frey & Morris 1997). It is important to keep in mind that LTP is not a unitary phenomenon and involves different molecular mechanisms that overlap in time. The multi-stage model of LTP is widely accepted and

these processes do seem to influence each other, but whether they consist of separate, parallel phases of expression or occur in series is still under investigation (Johnstone & Clark 2011, Park et al 2013, Reymann & Frey 2007).

1.3.2.1 From induction to expression

The expression of LTP can be achieved in several ways. Modifications to either pre- or postsynaptic terminals can lead to an enhanced synaptic transmission. Presynaptically, an increase in release probability would cause more glutamate to be released in the cleft and a larger postsynaptic response would be the consequence. On the other hand, this same observation can be made if there is an increase in the postsynaptic sensitivity to glutamate. This can be accomplished either through the modification of the receptor itself to enhance AMPAR conductance or the insertion of additional AMPARs into the postsynaptic density. The idea of 'unsilencing synapses' has also been suggested as a postsynaptic mechanism in which synapses previously lacking AMPARs are converted to functional synapses following LTP induction (Kerchner & Nicoll 2008). Extensive work has been done to clearly determine the locus of expression of NMDAR-LTP at the SC CA3-CA1 synapse (Bliss & Collingridge 2013). Although the controversy seems to have been resolved due to a large body of evidence pointing in the direction of new recruitment of AMPARs to silent synapses or to synapses already possessing some functional AMPARs (Kerchner & Nicoll 2008, Lynch 2004, Nicoll & Roche 2013), the subject is still a matter of debate (Bliss et al 2014, Johnstone & Clark 2011). These mechanisms of expression, however, are not mutually exclusive; the existence of one does not preclude the existence of another, even if their relative contribution is unequal. Moreover, the locus of expression may change overtime as STP progresses into the different stages of LTP (Johnstone & Clark 2011).

The induction of NMDAR-LTP is, by general consent, postsynaptic, but what are the biochemical cascades triggered by NMDAR activation that lead to the expression of LTP? Although there have been many proteins implicated in mediating LTP, it is generally agreed that calcium/calmodulin-dependent protein kinase II (CaMKII) activity is required for induction (Nicoll & Roche 2013). Calcium that entered through NMDARs binds to calmodulin, which then activates CaMKII. During this activation, CaMKII

undergoes autophosphorylation rendering it constitutively active and calcium independent. Calcium activated CaMKII translocates to the postsynaptic density where it can potentiate postsynaptic AMPA receptors in early phases of LTP. CaMKII enhances single channel conductance by phosphorylating specific sites on AMPAR subunits. Evidence also suggests that it is involved in the capture of AMPARs to the post-synaptic density, but its exact method of action remains unclear (Lisman et al 2012). Furthermore, the CaMKII/NMDAR complex is proposed to be a promising candidate for the maintenance of LTP and therefore the persistence of memory (Sanhueza & Lisman 2013).

Several other kinase cascades have also been found to be involved, some of them occurring in parallel. None of them seem to be obligatory since, depending on the conditions, LTP is not affected by inhibitors targeting their activity (Pavel et al 2006). Various factors are potentially at stake, but the induction protocol does appear to hold a determining role, which raises the possibility that there is more than one form of NMDAR-LTP at the SC CA3-CA1 synapse. Protein kinase A (PKA), protein kinase C (PKC), tyrosine kinases, the ERK/MAP kinase pathway (extracellular regulated kinases/mitogen associated protein kinases), phosphoinositide 3-kinase (PI3K), as well as the mammalian target of rapamycin (mTOR) and its downstream effectors, have all been implicated (Lynch 2004, Pavel et al 2006). Nitric oxide (NO) and brain-derived neurotrophic factor (BDNF) are just two substances amongst potential retrograde messengers to mediate the effects on presynaptic function (Regehr et al 2009). In the case of NO, calcium influx activates NO synthase (NOS) causing production of NO and diffusion from the postsynaptic to the presynaptic neuron to initiate presynaptic enhancement (Johnstone & Clark 2011).

1.3.2.2 Maintenance of late phase of LTP

We have seen that a plasticity-inducing event such as NMDAR-LTP activates distinct pathways that lead to post-translational modifications of pre-existing proteins and structural changes within the synapse. Specific alterations in protein synthesis are one of the consequences of the activation of such pathways and this is important for the induction of LTP. Protein synthesis is required for the persistence of synaptic change

during late phase LTP, whereas early phase LTP are unaffected by protein translation inhibitors (Frey et al 1988, Kelleher III et al 2004, Stanton & Sarvey 1984). When key signaling molecules and regulators of translation are disrupted, LTP is found to be abnormal in several of these instances (e.g. Alarcon et al 2004, Banko et al 2005, Costa-Mattioli et al 2005, reviewed in Costa-Mattioli et al 2009). But these studies do not provide information about whether protein synthesis initially derives in part from pre-existing mRNAs localized near the synapse and is independent of transcription or somatic translation or is general and somatic. In other words, where is protein translation occurring? Dendritic compartments are translationally competent since they contain all the necessary components of the protein synthesis apparatus (Tiedge & Brosius 1996). Moreover, some studies show that late phase LTP is maintained in synapses isolated from the soma by microsurgical cut (Vickers et al 2005) and destabilized microtubule networks (Vickers & Wyllie 2007). CaMKII protein levels are increased after tetanic stimulation (Ouyang et al 1999) and disrupting the dendritic localization of CaMKII mRNA transcript diminishes late-phase LTP (Miller et al 2002). Local application of protein synthesis inhibitors also impaired but did not completely block late phase LTP (Bradshaw et al 2003). While mRNA transcripts such as CaMKII, microtubule-associated protein 2 (MAP2), Shank and β-actin have been visualized in dendrites (Holt & Schuman 2013) and BDNF appears to mediate local protein synthesis that contributes to NMDAR-LTP (Leal et al 2014), it is still not clear how much protein is synthesized in the soma versus dendrites during LTP. Mechanisms of mRNA transport and regulation of local protein synthesis will be discussed in greater detail in section 1.4.

Following the induction of LTP, signaling pathways such as the ERK/MAPK and mTOR cascades upregulate the translation of 'plasticity-related proteins' (PRPs) (Costa-Mattioli et al 2009). However, the changes that are occasioned by the coordinated synthesis of PRPs are not inherently stable – there must be mechanisms in place to maintain them for periods that can last up to several hours *in vivo* (Klann & Sweatt 2008). One protein that has attracted a lot of interest in the last years is an atypical PKC isoform, PKMζ, that becomes, following its rapid synthesis and phosphorylation by phosphoinositide-dependent kinase 1 (PDK1), persistently active. It is thought to play a role in AMPAR trafficking to maintain enhancement of synaptic strength (Sacktor 2012).

Transcription can also become necessary in later phases to maintain the potentiation. A number of studies have shown the activation of specific patterns of gene expression following behavioral training (Guzowski 2002). Activity that induces LTP is linked to the nucleus by the activation of PKA, CaMKIV and MAPK and leads to the phosphorylation of the transcription factor CREB [cyclic adenosine monophosphate (cAMP) response element binding protein], which plays a central role in gating activity-regulated gene expression and the immediate early genes (IEGs) *c-Fos* (FBJ osteosarcoma oncogene) and *Zif268* (early growth response 1). *Arc/Arg3.1* (activity regulated cytoskeletal-associated protein) production, whilst it can depend on CREB activity, is also modulated by the transcription factors MEF2, SRF/Elk1 (serum response factor) and Zeste-like factor (Bramham et al 2010). IEGs subserve various cellular functions that are compatible with the structural and functional modifications that underlie synaptic plasticity (Abraham & Williams 2003, Guzowski 2002, Lynch 2004), thus they are believed to play a critical role in memory formation.

1.3.3 NMDA receptor-dependent LTD

Potentiation or depression of synaptic transmission can occur as a consequence of NMDAR activation, so what determines the direction of change? The degree and timing of calcium entry appears to be the determining factor in recruiting the intracellular molecules for the appropriate change in polarity (Artola & Singer 1993, Bliss et al 2006, Franks & Sejnowski 2002, Lisman 1989). In contrast to the induction of LTP, NMDAR-dependent LTD (NMDAR-LTD) is optimally induced by the repeated activation of the presynaptic neuron at low frequencies (Dudek & Bear 1992, Mulkey & Malenka 1992) to allow for a modest postsynaptic calcium entry (Malenka 1994). This suboptimal increase in calcium establishes a requirement for intracellular calcium store release, unlike during LTP induction (Nakano et al 2004).

1.3.3.1 From induction to expression

Mechanisms of expression in NMDAR-LTD appear to be largely postsynaptic with the removal of AMPARs from the synapse (Collingridge et al 2004), although there is

evidence that presynaptic alterations do occur and depend on several factors, notably the developmental stage (Collingridge et al 2010).

If LTP involves protein kinases, NMDAR-LTD activates protein phosphatases. The rise in postsynaptic calcium triggers the serine-threonine protein phosphatase cascade; calcium entering through NMDARs binds to calmodulin to activate calcineurin (protein phosphatase 2B, PP2B), which leads to the activation of protein phosphatase 1 (PP1) via dephosphorylation of inhibitor-1. PP1 acts to dephosphorylate several targets such as serine sites on AMPAR subunits. However, CaMKII, a protein kinase critical for potentiation of the synapse, is also a downstream target of calmodulin. It has been proposed that the different calcium dynamics are critical in determining which signaling pathway is activated (Lisman 1989).

NMDAR-LTD involves regulation of AMPAR subunit cycling between the synaptic membrane and the cytoplasm. Upon NMDAR-LTD induction, the N-ethylmaleimide-sensitive factor (NSF) dissociates from GluA2-containing AMPARs and there is increased binding of the protein interacting with C-kinase 1 (PICK1) to the GluA2 subunit. This PICK1-GluA2 interaction is required for hippocampal NMDAR-LTD (reviewed in Anggono & Huganir 2012). The neuronal calcium sensor protein hippocalcin (HPC) is a high-affinity Ca^{2+} sensor that promotes the exchange of NSF with the adaptor protein 2 (AP2) to destabilize AMPARs and initiate clathrin-mediated endocytosis of AMPARs. Another mechanism involves the scaffolding proteins AMPAR-binding protein–glutamate receptor interacting protein (GRIP-ABP). Like NSF, GRIP-ABP dissociates from AMPARs when PICK1 is activated (PICK1 changes conformation when bound to GluA2). NMDAR-LTD is also associated with phosphorylation by protein tyrosine kinases of tyr876 of GluA2, which may also aid the exchange of PICK1 for ABP–GRIP, although this is more associated with PKC phosphorylation of GluA2. Another target of PP1 is the multifunctional serine/threonine kinase glycogen synthase kinase-3β (GSK3β) and is required for NMDAR-LTD. Dephosphorylation of a serine residue leads to further activation of GSK3β (reviewed in Anggono & Huganir 2012, Bliss et al 2006, Collingridge et al 2010, Kemp & Bashir 2001) (Fig. 2).

1.3.3.2 Maintenance of late phase NMDAR-LTD

NMDAR-LTD is also accompanied by a protein synthesis-dependent phase that becomes evident 4 hours after low frequency stimulation in freely moving rats (Manahan-Vaughan et al 2000). Protein synthesis dependence was also found in organotypic slices as translation inhibitors caused a rapid recovery to baseline levels of transmission after induction of LTD (Kauderer & Kandel 2000). However, the same translation inhibitor did not affect NMDAR-LTD in acute slices (Huber et al 2000), but the plasticity was only monitored 60 minutes after induction. In contrast, a study in acute slices by Sajikumar and Frey (2003) shows a protein synthesis-dependent phases appearing 3-4 hours after induction, thus NMDAR-LTD does appear to involve protein synthesis for the maintenance of its late phase.

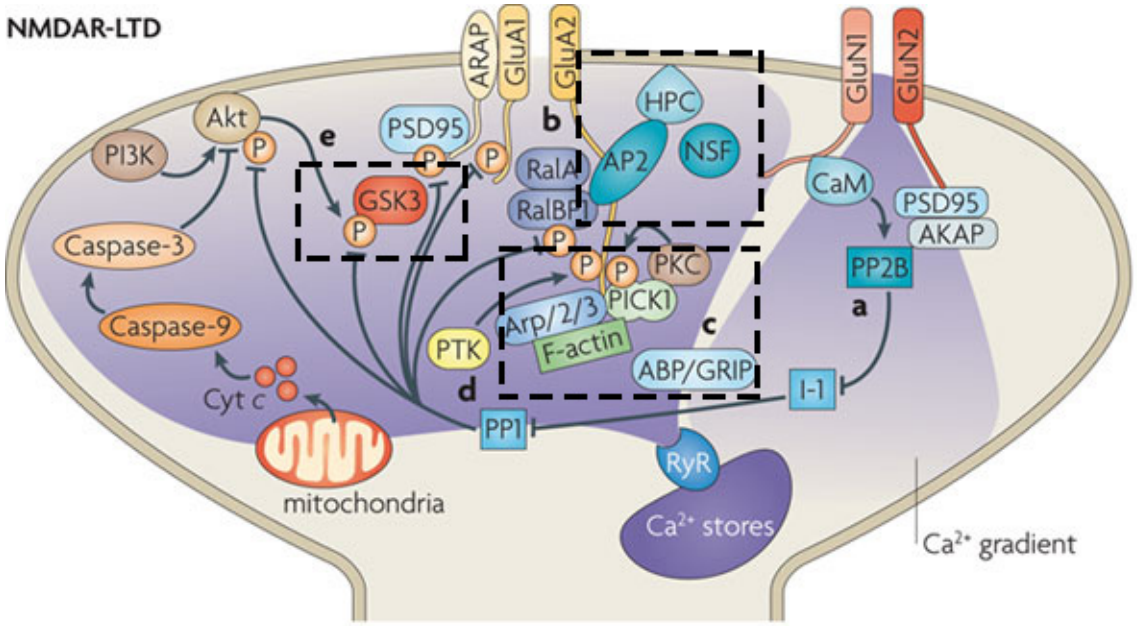

Figure 2. Signaling pathways involved in NMDAR-LTD.

Calcium enters through NMDA receptors (consisting of GluN1 and GluN2 subunits) causing the upregulation of PP1 and the release of calcium from intracellular stores. AMPA receptor (GluA1 and GluA2 subunits) anchoring is destabilized and receptors are internalized. Figure adapted from Collingridge et al (2010).

1.3.4 mGlu receptor-dependent LTD

At least two different forms of LTD exist at the SC CA3-CA1 synapse. Depression can be elicited by the sole activation of metabotropic glutamate receptors (mGluRs) (Huber et al 2001, Palmer et al 1997), in contrast to the forms of plasticity we have discussed previously. NMDAR-LTD and mGluR-LTD are believe to be mechanistically independent since they do not occlude each other (Oliet et al 1997, Palmer et al 1997); in other words, when mGluR-LTD is saturated, NMDAR-LTD can achieve further depression. Interestingly, while evidence suggests that NMDAR-LTD reverses and erases LTP (also called depotentiation), mGluR-LTD is instead superimposed on LTP (Oliet et al 1997). Moreover, NMDAR-LTD appears to be more prominent in neonatal and juvenile rats, but a developmental shift favors mGluR-LTD in adults (Kemp et al 2000). mGluR-LTD can be induced synaptically or chemically. The application of a paired pulse low frequency stimulation (PP-LFS) to the presynaptic neuron generates an LTD that was found to be dependent upon the activation of group I mGluRs (mGluR1 and mGluR5) (Huber et al 2000). Chemical induction is often used in *in vitro* preparations. The group I mGluR agonist, dihydroxyphenylglycine (DHPG), is thought to elicit an LTD that relies preferentially on mGluR5 (Faas et al 2002). Although both induction protocols are thought to involve similar mechanisms because they occlude each other (Huber et al 2001), some differences have been found (Gladding et al 2009b). While DHPG induced-LTD is completely calcium independent (Fitzjohn et al 2001), synaptically induced-LTD at the SC CA3-CA1 synapse is sensitive to intracellular calcium chelators (Bolshakov & Siegelbaum 1994, Oliet et al 1997). In these two studies, the calcium influx was identified to occur via T-type and L-type voltage gate calcium channels. However, a more recent study provided evidence that calcium is not an absolute requirement for synaptic induction of mGluR-LTD (small adjustments were made to the conventional PP-LFS protocol) (Kasten et al 2012).

1.3.4.1 From induction to expression

Whether the locus of expression is both presynaptic and postsynaptic or uniquely postsynaptic in mGluR-LTD is not clear, but it is evident that internalization of surface ionotropic GluRs occurs in response to mGluR activation (Gladding et al 2009a, Huang

et al 2004, Snyder et al 2001, Waung et al 2008, Xiao et al 2001, Zhang et al 2008). There is however conflicting evidence showing a lack of decreased postsynaptic sensitivity to uncaged glutamate (Rammes et al 2003) and enhanced responses to ionophoretic application of AMPA, kainic acid and NMDA (Tan et al 2003) following DHPG application. A presynaptic mechanism of expression is supported by findings indicating lasting increase in paired-pulse ratio, a reduction in miniature excitatory postsynaptic currents (mEPSCs) frequency and a decrease in neurotransmitter release following induction (Gladding et al 2009b).

Signaling pathways mediating mGluR-LTD are clearly distinct from those implicated in NMDAR-LTD. mGluRs function as G-protein-coupled receptors; receptor activity leads to G-protein activation promoting the exchange of GTP to GDP (guanosine-5'-triphosphate to guanosine diphosphate), which results in the modulation of protein-protein interactions and activation of second messenger cascades. Group I mGluR activation, is normally coupled to the activation of phospholipase C (PLC), the generation of diacylglycerol (DAG), inositol-1,4,5-trisphosphate (IP_3) and the release of calcium from intracellular stores, but, although this pathway is upregulated during mGluR-LTD (Mao et al 2005), it is not necessary (Fitzjohn et al 2001, Gallagher et al 2004, Schnabel et al 1999). The mitogen-activated protein kinase (MAPK) signaling cascades are activated as all three MAPK subclasses are involved: p38 MAPK, Jun N-terminal kinase (JNK), and ERK (Gallagher et al 2004, Moult et al 2008, Schmit et al 2013). MAPK cascades typically involve the sequential activation of a small GTPase (Ras), a MAPK kinase kinase (Raf), and a MAPK kinase (MEK). P38 MAPK is activated via the GTPase Rap1 (repressor activator protein 1), a pathway that is coupled to the endocytotic machineries and AMPAR internalization. ERK is also activated in this way and leads to the downstream activation of ribosomal S6 kinase-1 (RSK1), a key regulator of activity-dependent protein synthesis. Another important cascade coupled to translation regulation is the phosphoinositide 3-kinase-Akt-mammalian target of the rapamycin (PI3K-Akt-mTOR) pathway thought to mediate cap-dependent translation during mGluR-LTD in parallel with the MEK-ERK pathway. The mTOR pathway is activated through the coupling of mGluR5 with the postsynaptic-density scaffolding protein Homer that recruits the small GTPase that binds PI3K, PI3K-enhancer (PIKE), forming an mGluR-

Homer-PIKE complex. The formation of this complex turns on PI3K (Ronesi & Huber 2008) (Fig. 3). Protein tyrosine phosphatases (PTPs) are also implicated (reviewed in Anwyl 2006, Bliss et al 2006, Collingridge et al 2010, Gladding et al 2009b). An additional pathway activated by group I mGluR signaling alters the activity of the elongation factor 2 kinase (eEF2K) (Park et al 2008, Taha et al 2013, Verpelli et al 2010). Increased phosphorylation of eEF2 by eEF2K causes the arrest of general translation, whilst protein synthesis of specific transcripts such as Arc/Arg3.1 and the microtubule associated protein 1B (MAP1B) is increased (Davidkova & Carroll 2007, Park et al 2008).

1.3.4.2 Maintenance of late phase mGluR-LTD

mGluR-LTD exhibits a dependence on local protein synthesis within the first 10 minutes after induction (Huber et al 2000, Huber et al 2001, Park et al 2008, Waung et al 2008, Zhang et al 2008), unlike NMDAR-LTP/D. mGluR activation mediates the translation of "LTD proteins" such as striatal-enriched protein tyrosine phosphatase (STEP) (Zhang et al 2008), MAP1B (Davidkova & Carroll 2007), oligophrenin 1 (Ophn1) (Di Prisco et al 2014) and Arc/Arg3.1 (Park et al 2008, Waung et al 2008), which are involved in AMPAR endocytosis in some way. Other proteins synthesized during mGluR-LTD are the fragile X mental retardation protein (FMRP) (Weiler et al 1997), the ribosomal protein S6 and the elongation factor 1A (EF1A) (Antion et al 2008), which are proteins that themselves regulate translation.

mGluR-LTD is generally thought to involve translation rather than transcription, but there is evidence of the requirement for transcription factors 2 or 3 hours after induction. The modulation of factors such as NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), ETS domain-containing protein and CREB is dependent on the ERK and PI3K pathways (Gladding et al 2009b).

Interestingly, when mGluR antagonists are applied after the induction, LTD is reversed, but is reestablished without any further induction when the drugs are washed out (Palmer et al 1997). It is therefore tempting to suggest that mGluR activation must be maintained for mGluR-LTD to persist.

1.4 LOCAL ACTIVITY-DEPENDENT PROTEIN SYNTHESIS

A key mechanism for the persistence of synaptic changes at individual synapses is the requirement for local protein synthesis (Costa-Mattioli et al 2009, Pfeiffer & Huber 2006). Just as stress and growth signals can change the rate of synthesis of specific transcripts and bulk mRNAs, neurotransmission can control translation by regulating its machinery (Gal-Ben-Ari et al 2012).

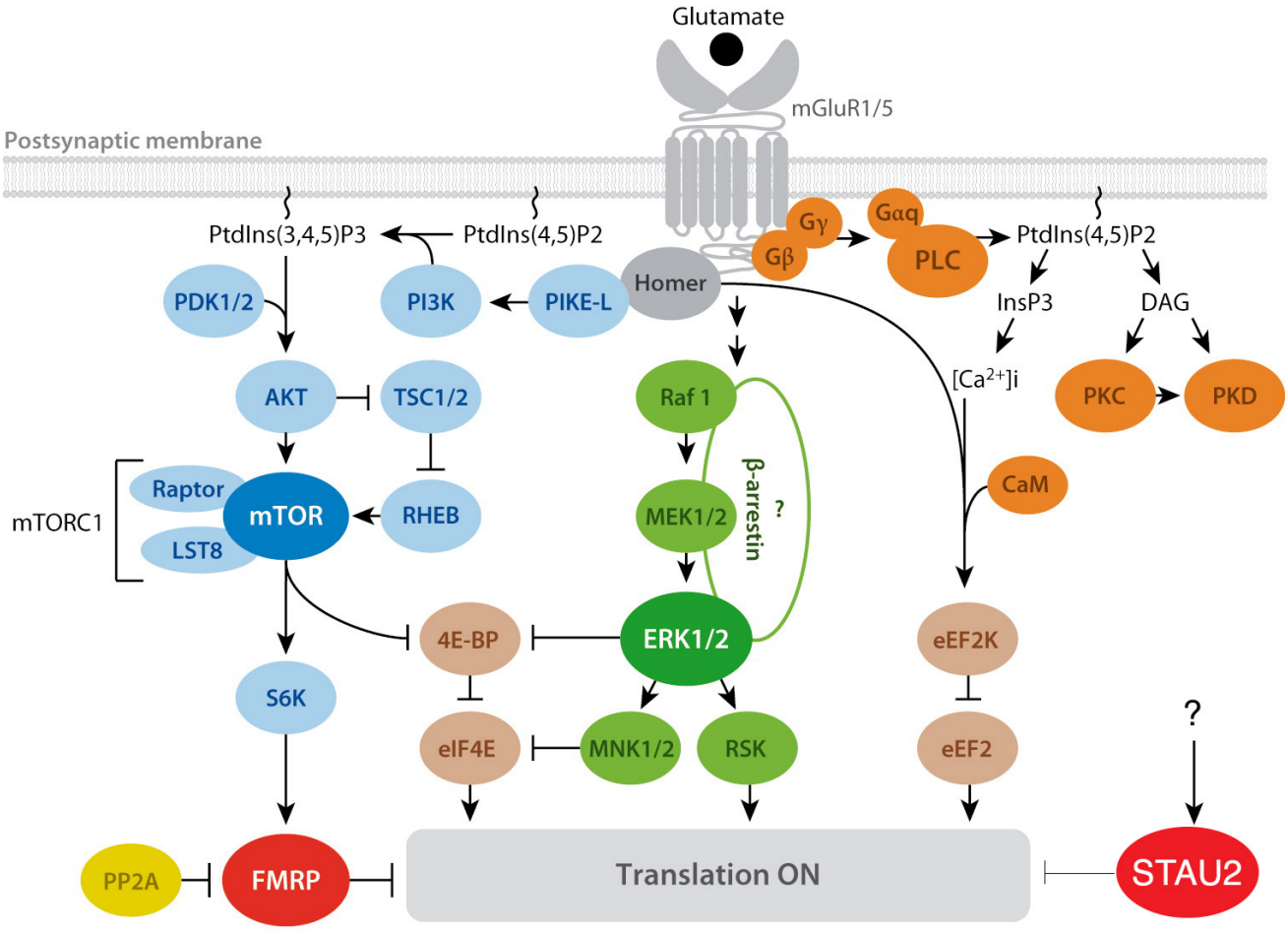

Figure 3. Signaling pathways involved in translation regulation during mGluR-LTD.

PLC/calcium-calmodulin pathway (orange), the mTOR pathway (blue), and the ERK pathway (green). FMRP and Stau2 are both known RNA-binding proteins that normally repress translation, but upon mGluR activation, promote de-repression. Question marks indicate undetermined associations. Arrows indicate a positive consequence on downstream components; perpendicular lines indicate an inhibitory consequence.

Abbreviations: [Ca^{2+}]i, calcium release from intracellular stores; CaM, calmodulin; ERK, extracellular signal–regulated kinase; FMRP, fragile X mental retardation protein; Gαq, Gβ, Gγ, heterotrimeric G proteins; InsP3, inositol-1,4,5-triphosphate; mGluR, metabotropic glutamate receptor; mTOR, mammalian target of rapamycin; PtdIns, phosphoinositides; PLC, phospholipase C; PP2A, protein phosphatase 2A; Raptor, regulatory-associated protein of mTOR. Figure adapted from Bhakar et al 2012.

1.4.1 Translation regulation

Translation occurs in three steps: initiation, elongation and termination. Synthesis of most proteins is driven by cap-dependent translation; in other words, translation initiation is most often the rate-limiting step and target for regulation (Costa-Mattioli et al 2009).

For initiation, the ribosome must be recruited to the mRNA. This is achieved through the interaction of the eukaryotic initiation factor (eIF) 4F complex with the 5'-m^7G-cap (Fig. 4A), which then recruits the 43S pre-initiation complex (PIC). PIC is comprised of the 40S ribosomal subunit, eIF1, eIF1A, eIF3 and ternary complex (Jackson et al 2010). The ternary complex, for its part, brings together eIF2, GTP and the specific initiator methionyl initiator transfer RNA (Met-tRNAi^{Met}) to form (eIF2)–GTP–Met-tRNAi^{Met} (Sonenberg & Hinnebusch 2009). PIC begins scanning the 5'-UTR for the AUG start codon (Fig. 4B). eIFs are released and the 60S subunit joins the 40S subunit to form the 80S complex (Fig. 4C) (Sonenberg & Hinnebusch 2009). Elongation factors are recruited to regulate elongation. Upon recognition of the stop codon, termination factors promote the release of the polypeptide chain from the mRNA and ribosome.

Inhibition of the formation of the ternary complex and the eIF4F complex are the two major ways of regulating translation initiation (Gkogkas et al 2010). Phosphorylation of eIF2α, a component of the ternary complex, prevents the functional reconstitution of the complex. On the other hand, eIF4F complex assembly requires the interaction of the cap-binding protein eIF4E with eIF4G. This binding interaction is disrupted and translation initiation is inhibited in the presence of the eIF4E-binding protein (4E-BP). Phosphorylation of 4E-BP by mTOR relieves inhibition. eIF4E can itself be phosphorylated by MNKs, a small family of protein kinases, some of which are regulated by MAPK signaling (Shveygert et al 2010).

The control of the level of peptide chain elongation, on the other hand, is mediated by the eukaryotic elongation factor 2 (eEF2). eEF2 is a GTP-binding protein that mediates the translocation of peptidyl-tRNAs from the A-site to the P-site on the ribosome as amino acids are added to the peptide chain. Phosphorylation of eEF2 inhibits eEF2–ribosome binding and arrests elongation. While general translation is slowed, eEF2 phosphorylation causes, by a mechanism that is unclear, the increased synthesis of specific transcripts (Bramham & Wells 2007).

Remarkably, these pathways that regulate the translation machinery have been shown to be upregulated by both NMDA and mGluR signaling (Gal-Ben-Ari et al 2012). Do these signaling pathways mediate control of general translation or a subset of mRNAS (Costa-Mattioli et al 2009)? Considerable evidence suggests that the coordinated translation of selective subsets of mRNAs, or "regulons" (Keene 2007), occurs in response to different patterns of synaptic transmission inducing plasticity (Costa-Mattioli et al 2009). So how is the concerted synthesis of specific plasticity-induced proteins achieved? One attractive mechanism involves RNA-binding proteins.

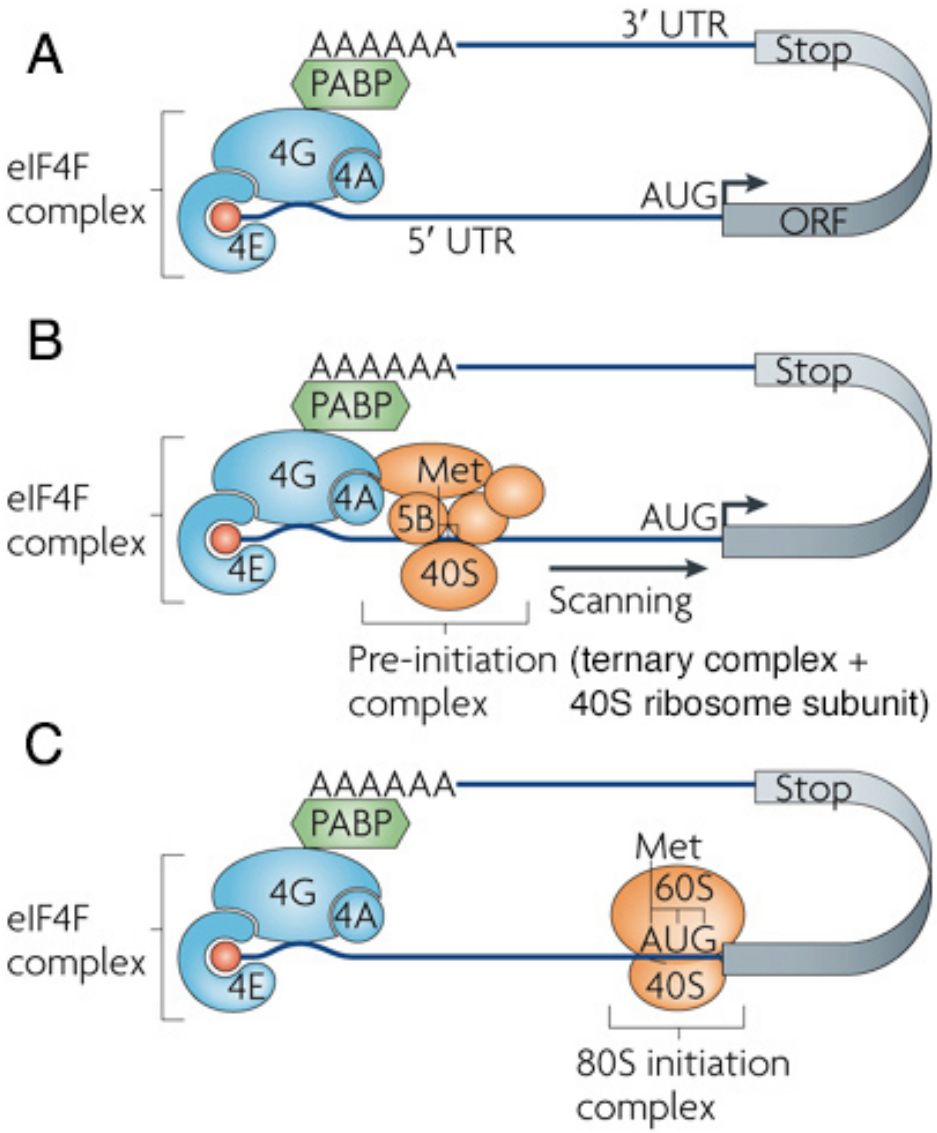

Figure 4. Steps of translation initiation

Figure adapted from Besse and Ephrussi (2008).

1.4.2 mRNA localization

For the local and rapid translation of specific proteins to occur on demand, mRNAs must first be transported to their final site of function. These subsets of transcripts are packaged into transport complexes called RNA granules. These higher-order assemblies contain important components of the translation machinery as well as RNA-binding proteins and their mRNA partners (Fritzsche et al 2013b, Sossin & DesGroseillers 2006) that render mRNAs translationally dormant (Besse & Ephrussi 2008). Synaptic activation

provokes their unmasking and release from their repressed state (Buxbaum et al 2014, Graber et al 2013). This dual function of mRNA localization and translation regulation confers several advantages (Martin & Ephrussi 2009). In addition to the ability to alter the synaptic input to one of its many dendrites without changing others, the neuron is provided with the opportunity to achieve this spatially restricted gene expression with a high temporal resolution.

RNA-binding proteins selectively bind transcripts for transport, hence their consideration as key candidates for differential regulation of plasticity-induced protein translation. Indeed, evidence suggests that different dendritic trafficking pathways exist which could allow for independent localization and distinct regulation of mRNAs involved in different forms of synaptic plasticity (Doyle & Kiebler 2011, Lebeau et al 2011, Mikl et al 2011).

1.4.3 RNA-binding proteins

Information about the destination of mRNA transcripts is encoded by cis-acting elements in the RNA most frequently found in the 3' UTR, which are recognized by trans-acting RNA-binding proteins (RBPs) (Besse & Ephrussi 2008). RNA-binding proteins are not only incorporated into RNA granules but are also components of a variety of cytoplasmic RNA structures: translating polysomes, processing bodies (P bodies), stress granules, micro RNA particles (miRNPs) or the RNA interfering silencing complex, and RNA transport particles. Transport particles contain, similarly to RNA granules, mRNAs, RNA-binding proteins, adaptors that couple to the motor complex and motors, but they are devoid of ribosomes (Sossin & DesGroseillers 2006). Emerging evidence is suggesting that the composition and the pool of RNA granules are more heterogeneous then we previously thought (Bramham & Wells 2007, Fritzsche et al 2013b, Graber et al 2013). Here, we describe a few RBPs that have been identified to play an important role in localization and translation regulation of mRNA transcripts during synaptic plasticity.

1.4.1.1 CPEB1

Cytoplasmic polyadenylation element (CPE) binding protein-1 (CPEB1) is found in the postsynaptic density and controls mRNA translation by regulating the length of the

poly(A) tail. It forms a dual activity complex through its association with various factors that can both activate and repress translation of its target mRNAs by adding or removing the poly(A) tail. CPEB1 at the 3'UTR anchors a complex of proteins that includes an eIF4F binding protein. When bound together, CPEB1 competes with eIF4G for eIF4E binding, thus represses initiation. CPEB1 phosphorylation induces polyadenation and translation of CaMKIIα through NMDA receptor signaling. Deficits in LTP and LTD have been observed in CPEB1 KO mice (Richter 2007).

1.4.1.2 ZBP1

The mRNA of β-actin contains a localization element in the 3'UTR called zipcode that is specifically recognized by zipcode-binding protein 1 (ZBP1) and is involved in its localization (Tiruchinapalli et al 2003) and translation repression (Hüttelmaier et al 2005). Abolition of the function of the zipcode by mutation of the element itself, treatment with specific antisense oligonucleotides, or knockdown/out of ZBP1 protein leads to the mislocalization of β-actin mRNA and subsequent alterations of cell morphology, motility, and adhesion as well as failures in synaptic growth and deficiencies in dendritic spine number, maturation, and arborization (Eliscovich et al 2013). Buxbaum et al (2014), by using single-molecule *in situ* hybridization approaches, showed that β-actin transcripts are in a masked state, unavailable to probes for binding, but upon depolarization, become unmasked. ZBP1 has also been found to bind and regulate at least 116 other mRNAs (Eliscovich et al 2013).

1.4.1.3 FMRP

The fragile X mental retardation protein (FMRP) has generated a lot of interest due to its pathophysiology. Fragile X syndrome results from the complete absence of this protein due to transcriptional silencing of the gene FMR1. Loss of FMRP is caused by a trinucleotide (CGG) repeat expansion that leads to hypermethylation and transcriptional silencing. It is a protein that is normally highly expressed in neurons, thus its absence causes moderate to severe intellectual disability and autistic features. FMRP is also expressed in other tissues of the body causing a wide spectrum of abnormalities.

FMRP possesses at least three RNA-binding domains. Two of these domains are hnRNP K-homology (KH) domains and a point mutation in the second one leads to a severe clinical presentation of the disease. FMRP is found in RNA granules and is therefore associated with mRNA transport, but it does not appear to be necessary, since, even in its absence, mRNA targets are correctly localized. It does however play a very important role in translation repression of "LTD proteins". Indeed, mGluR-LTD is exaggerated and protein synthesis independent in the absence of FMRP, suggesting that the necessary proteins had already been translated before mGluR activation. In other words, without FMRP, translation of these transcripts is unchecked. Interestingly, mGluR signaling is still required for LTD to occur in this pathological state; otherwise, the opposite effect would be expected. However, translation regulation of "LTD proteins" is not only achieved through FMRP. An additional mechanism is most likely recruited, since inhibiting mGluR5 can rescue some of the phenotypes. Were mGluR5 stimulation completely uncoupled to protein synthesis regulation due to the loss of FMRP, this rescue would not occur (Bhakar et al 2012).

1.4.1.4 Staufens

The Staufen family consists of proteins that contain double-stranded RNA-binding domains. Mammals possess two Staufen homologs, Stau1 and Stau2. Both of these RBPs are involved in controlling localization, translation and stability of their mRNA targets. They are found both in transport particles (Kiebler et al 1999) and in RNA granules (Krichevsky & Kosik 2001).

Stau1 and Stau2 share similar functions, but they are part of different complexes and differentially bind RNA (Duchaîne et al 2002, Monshausen et al 2001). Neurons lacking Stau2 displayed fewer, extended dendritic spines and this was associated with a reduction in the number of synapses (Goetze et al 2006). In addition, Stau2 is required for mGluR-LTD (Lebeau et al 2011). Similarly to Stau2 knockdown neurons, Stau1-deficient mice exhibit a decrease in dendritic protrusions, while those remaining shifted towards a more elongated shape. In contrast to Stau2, knockdown of Stau1 by small interfering RNAs (siRNAs) in hippocampal slice cultures impairs the late phase of the chemically induced NMDAR-LTP, but not mGluR-LTD (Lebeau et al 2008).

Both Staufens bind and transport mRNAs encoding proteins with known roles in synaptic plasticity. For example, Stau1 binds the CaMKIIα mRNA, while Stau2 links with the microtubule associated protein 1B (MAP1B), and β-actin mRNAs (Heraud-Farlow & Kiebler 2014). Some of the proteins that interact with Stau2 in RNA granules include FMRP, ZBP1, helicase up-frameshift 1 (Upf1), the nuclear cap-binding protein 80 (CBP80) and 7 ribosomal proteins associated with the large subunit. These provide strong evidence for translation repression during transport. Moreover, the EJC complex was absent in Stau2 granules suggesting translation has already undergone initiation (Fritzsche et al 2013b). In addition to their role in localization and translation regulation, Stau1 and Stau2 are implicated in mRNA degradation processes termed Staufen-mediated decay. The recruitment of Upf1 is crucial for this decay to occur (Heraud-Farlow & Kiebler 2014).

1.5 OBJECTIVES OF THIS MASTER'S THESIS

To quote my co-supervisor, Wayne Sossin, during one of our journal clubs, "if we want to understand memories, we must understand the molecular traces [that underlie them]." The consequence of synaptic changes can be very similar, in other words, it is hard to differentiate NMDAR-LTD from mGluR-LTD solely from the "output" engendered (they both cause depression), but the mechanisms that are responsible for the expression can be thoroughly different (Malenka & Bear 2004). With this perspective in mind, memories should be defined by their molecular traces, not by the stimulus used or the length of time during which plasticity is maintained. Extensive evidence demonstrates that some forms of synaptic plasticity rely on local synthesis of specific proteins for their expression (Pfeiffer & Huber 2006). Thus, in protein synthesis dependent synaptic plasticity, a set of necessary mRNA transcripts must be transported near the synapse to be available for translation following a memory-encoding stimulus. However, we have yet to understand how distinct learning paradigms lead to the production of such distinct proteins and molecular traces.

The goal of this thesis is therefore to better understand the translation control pathways and the molecular traces underlying one form of long-term synaptic plasticity, mGluR-LTD. We propose that one of the major mechanisms responsible for the specificity of proteins translated and their concerted increase in synthesis during mGluR-LTD is of polyribosomes stalled at the elongation step by the RNA-binding protein Staufen 2. In order words, translation of proteins is initiated in the soma, but paused at the elongation step, thus proteins are partially completed until they are transported to the synapse where mGluR signaling triggers the reactivation of translation and proteins are quickly made available. Staufen 2 is known to target the appropriate mRNAs to dendritic compartments, while repressing translation of these transcripts during transport in complexes called RNA granules (Sossin & DesGroseillers 2006). It is also required for mGluR-LTD (Lebeau et al 2011). Recently, we demonstrated that polyribosomes are indeed stalled and undergo reactivation during mGluR activation (Graber et al 2013). But what we have yet to elucidate is how the stalling complex is formed and what upstream signaling pathways trigger its disassembly to allow for translation to resume.

To achieve this, we needed a model of mGluR-LTD at the single cell level that allowed us to manipulate and dissect the pathways of translation regulation both using molecular and pharmacological approaches. We therefore used dissociated hippocampal neurons in culture and whole-cell patch recording to demonstrate that we could reliably induce mGluR-LTD with a group I mGluR agonist consistently with the work of several groups. Next, we investigated whether translation during mGluR-LTD was regulated at the initiation or elongation step in this preparation using specific inhibitors of protein synthesis. Finally, we investigated the role of Staufen 2 in mGluR-LTD using lentiviral transduction methods to knock it down.

Altogether, this thesis has shed light on a fundamental mechanism of translation control underlying the induction of mGluR-LTD. The concept of stalled polyribosomes is an innovative way of thinking about translation, since translation regulation is mostly believed to occur at the initiation step in neurons. This work provides a platform to further investigate the mechanisms implicated in a form of synaptic plasticity that is crucial for learning and memory.

CHAPTER 2. RESULTS

2.1 CONTRIBUTION OF THE AUTHOR

The first author, Sarah Hébert-Seropian, performed all electrophysiological experiments and their analysis. Tyson Graber provided the different lentivirus constructs and vectors. Julie Pépin made the cultured dissociated hippocampal neurons and transduced the neurons. Manuscript was written by Sarah Hebert-Seropian and revised by co-supervisors, Jean-Claude Lacaille and Wayne Sossin.

Translation control downstream of initiation during mGluR-LTD in cultured hippocampal neurons

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Manuscript prepared for submission

2.2 ABSTRACT

Some forms of synaptic plasticity require rapid, local activation of protein synthesis. It is widely believed that mRNAs are translationally repressed during transport to synaptic compartments, but the molecular mechanisms underlying this translational silencing, and its de-repression during plasticity, remain largely unknown. In many synaptic plasticity models, the initiation of translation is presumed to be the rate-limiting step and the main target of control. However, we recently showed, using field potential recording in acute hippocampal slices, that translational regulation in metabotropic glutamate receptor-mediated long-term depression (mGluR-LTD) occurs downstream of initiation, likely via de-repression of stalled polyribosomes. Here, we use a cell culture model to study mGluR-LTD at the single synapse/cell level and further investigate these mechanisms. Using whole cell recording from hippocampal neurons in dissociated cell culture, we show that chemically induced mGluR-LTD is expressed as a decrease in the frequency of miniature excitatory postsynaptic currents (mEPSCs) and is impaired by the translation elongation blocker, emetine. In contrast, homoharringtonine, a translation initiation blocker, does not affect mGluR-LTD. Moreover, we found that Staufen 2, an RNA-binding protein important for mRNA localization and translation regulation, plays an essential role in mGluR-LTD. Having established a cell culture model of mGluR-LTD, gene knockdown and rescue strategies may be used to determine the molecular mechanisms of translational control implicated in long-term synaptic plasticity.

2.3 INTRODUCTION

The remarkable ability of neurons to modify the efficacy of specific synapses in an activity-dependent manner is thought to be the physical substrate of long-term memory and information storage (Martin et al 2000). A key mechanism for the persistence of synaptic changes at individual synapses is the requirement for local protein synthesis. Metabotropic glutamate receptor-mediated long-term depression (mGluR-LTD) is a form of plasticity that critically depends upon local and rapid translation (Huber et al 2000). In patients with Fragile X Syndrome, the most common form of inherited mental retardation and autism, translation dysregulation that results from the absence of an RNA-binding protein, FMRP, causes an abnormal mGluR-LTD (reviewed in Bhakar et al 2012). Understanding mechanisms of translation control in mGluR-LTD is therefore crucial for understanding deficits in neuronal function and their implication for disease.

Hippocampal mGluR-LTD is induced by activation of group I metabotropic glutamate receptors (mGluRs) either by pharmacological or synaptic stimulation. Group I mGluR activation triggers signaling pathways that are coupled to the translation of specific mRNA transcripts (reviewed in Gladding et al 2009b). The mechanisms by which this signaling controls protein synthesis remain however elusive. Since translation initiation is very tightly regulated, it is commonly believed to be the rate-limiting step of translation (Herbert & Proud 2007). Indeed, some groups have suggested that cap-dependent translation is the main target of control for *de novo* protein synthesis during mGluR-LTD (Banko et al 2006, Di Prisco et al 2014, Huber et al 2000). On the other hand, evidence establishing an important role for regulation at the elongation step challenges this view (Davidkova & Carroll 2007, Graber et al 2013, Park et al 2008). Recently, we demonstrated that, in a type of RNA transport complex, polyribosomes are stalled at the elongation step and are reactivated following group I mGluR stimulation (Graber et al 2013).

Staufen1 (Stau1) and Staufen 2 (Stau2) are RNA-binding proteins (RBP) that have been implicated in the regulation of transcripts important for plasticity (Lebeau et al 2008, Lebeau et al 2011). They hold dissociable roles in two opposing forms of plasticity.

While the lack of Stau1 impairs long-term potentiation and leaves mGluR-LTD intact, Stau2 is similarly required for mGluR-LTD, but not for LTP (Lebeau et al 2008, Lebeau et al 2011). Staufens are a major component of mRNA transport complexes called RNA granules that contain all the necessary machinery for translation (Sossin & DesGroseillers 2006). Translation is widely believed to be repressed during transport (Besse & Ephrussi 2008), thus RBPs could provide a mechanism of translation regulation during plasticity as proteins are synthesized on demand. But even as we start to better understand the molecular composition of RNA granules and mechanisms of mRNA targeting, it remains unclear how RNA-binding proteins achieve translation repression and signal-induced translational activation at the synapse. Since Stau2 is implicated in translational control of mRNAs important for mGluR-LTD, our goal is to understand the mechanism by which it is able to selectively bind mRNAs that are distinct from those targeted by Stau1 and form the stalling complex.

In the present study, we set out to develop a culture model that will allow us to study mGluR-LTD electrophysiologically at the single cell level and manipulate the molecular pathways of RNA regulation using pharmacological and molecular biology approaches. With this culture model, we aim to understand the mechanisms underlying the stalling of polyribosomes and their reactivation during synaptic plasticity.

2.4 METHODS

All experiments were approved by the Animal Ethics Committee at Université de Montréal and according to the guidelines of the Canadian Council on Animal Care.

2.4.1 Dissociated hippocampal neurons

Rat hippocampi were dissected from embryonic day 18 Sprague-Dawley embryos (Charles River Laboratories). Neurons were dissociated and cultured as previously described (Lebeau et al 2011). Neurons were plated at a density of approximately 750 cells/mm^2 on 12-mm diameter pre-coated coverslips (Mandell) and grown on 12-well plastic tissue culture plates. Cultures were fed at 4DIV and replaced with fresh Neurobasal medium (NBM, Life Tech) supplemented with B27, N2, Pen/Strep, L-Glutamine once every 5-6 days. Experiments were performed at 18-25 DIV.

2.4.2 Lentivirus-mediated delivery of short hairpin RNA

To efficiently knockdown Staufen 2 in rat hippocampal neurons, lentiviruses were generated that express short hairpin RNA (shRNA) driven by a CMV promoter as previously described (Thomas et al 2009). A non-targeting, control shRNA sequence (AATTCTCCGAACGTGTCACGT), a sequence targeting all of the rat isoforms of Staufen 2 (ACTAGTGGACGCTTTATAGCC), or a sequence targeting rodent Upf1 isoforms (AGCAGCTTGTGGTAAATATAC) was incorporated into a shRNA cassette and subsequently cloned into a vector downstream of an emGFP ORF (pcDNA6.2.emGFP). The entire emGFP.shRNA cassette was then PCR-amplified and sub-cloned into a lentiviral expression vector (pRRL.emGFP.shRNA). VSV G pseudotyped virus was packaged by transient transfection of HEK293T helper cells with Lipofectamine 2000 and pMD2.g, pRSV-Rev, and pMDLg/pRRE packaging plasmids together with the pRRL.emGFP.shRNA. Virus was collected over 48 hours, cell debris removed by microfiltration, and virus was concentrated by centrifugation, resuspended in

PBS, pH7.3 and stored at -80. Virus was titered in HEK293T cells and hippocampal neurons were transduced at 7DIV with a multiplicity of infection (MOI) of 10. Transduction efficiency was assessed in each experiment by expression of emGFP. Percent knockdown efficiency was obtained by dividing the mean knockdown expression in shStau2-transduced neurons by that in the shControl-transduced neurons. Standard errors for individual neurons were added to obtain the percent error (Graber & Sossin 2014).

2.4.3 Electrophysiology

Coverslips with cultured hippocampal cells were placed in a recording chamber mounted on an inverted microscope equipped with epifluorescence (Nikon Eclipse T*i*-S). Cultures were superfused at 1ml/min at room temperature in Tyrode's solution consisting of the following (in mM): 140 NaCl, 4 KCl, 2 $MgCl_2$, 10 glucose, 10 HEPES, 2 $CaCl_2$, 0.1 picrotoxin, 0.001 TTX, adjusted to pH 7.4 (with KOH) and 310 mOsm. Whole cell recordings were obtained from GFP-expressing neurons with patch pipettes (3-6 mΩ) filled with the following (in mM): 125 K-gluconate, 2.6 KCl, 1.3 NaCl, 10 HEPES, 0.1 EGTA, 4 ATP-Mg, 0.3 GTP-Na, 14 phosphocreatine-Tris, adjusted to pH 7.2 and 285 mOsm. Cells were voltage clamped at -60 mV holding potential. Recordings with a holding current of <100 pA and with series resistance <35 MΩ that remained stable (<25% change) throughout the experiment were kept for analysis. For all LTD experiments, the group I metabotropic glutamate receptor (mGluR) agonist (S)-DHPG (100 μM, 5 min, Abcam) was added to Tyrode's solution. Emetine (40μM, Sigma) and HHT (20 μM, Sigma) were applied 10 min before, during, and 20 min after DHPG application. Recordings were made in the voltage-clamp mode using a Multiclamp 700A amplifier (Molecular Devices). Data acquisition was performed using the 1440A Digidata acquisition board and pClamp software (Molecular Devices). Data were digitized at 20 kHz. Detection and measurements of mEPSCs, which were collected over two 5-min time windows, just before DHPG application and 30 min after application, using MiniAnalysis program (Synaptosoft) after filtering traces at 1 kHz. Detection threshold was set at 5 pA and all detected events were verified by visual inspection. Paired-t-tests

were used for statistical evaluation of DHPG effects (pre vs post-DHPG) on mEPSC frequency and amplitude, and cell input resistance using GraphPad Prism 6 software. Statistical significance was set at $p < 0.05$.

2.5 RESULTS

2.5.1 mGluR-LTD in dissociated hippocampal neurons

Dissociated hippocampal cultures allow easy access to individual neurons for electrophysiology while also permitting both pharmacological and gene expression manipulations. It is therefore a very useful tool to study molecular mechanisms of plasticity at the single cell level. We firstly determined if we could reliably observe mGluR-LTD in cultured hippocampal neurons using voltage clamp recordings of miniature excitatory postsynaptic currents (mEPSCs). Application of the group I mGluR agonist DHPG (100 μM, 5 min) caused a significant depression in the frequency of mEPSCs (Fig. 1*B*, *C*). The frequency 30 min following treatment was significantly reduced to 84 ± 4% of baseline (n = 8, P = 0.04). No significant difference was observed in the amplitude of mEPSCs after DHPG application (Fig. 1*B, C*). The mean amplitude 30 min post DHPG was 101 ± 3% of baseline (P = 0.75).

In control experiments, untreated neurons (n = 4) showed no significant changes in mean frequency (100 ± 10% of baseline; P = 2.8) or amplitude (96 ± 4% of baseline; P = 0.48) of mEPSCs over the same time period. These data indicate that mGluR-LTD is present in hippocampal neuron cultures and is expressed as a decrease in mEPSC frequency. Having established a reliable paradigm to induce mGluR-LTD in our cultures that is consistent with previous findings (Snyder et al 2001), we next examined the molecular mechanisms that underlie this form of plasticity.

2.5.2 mGluR-LTD is blocked by inhibitors of translation elongation but not of translation initiation

We recently showed that mGluR-LTD at Schaffer collateral synapses is insensitive to homoharringtonine (HHT), an inhibitor of translation initiation or, more precisely, of the first round of elongation (Graber et al 2013). We also found that mGluR-LTD is prevented by emetine, an inhibitor of translation elongation (Graber et al 2013). Since these previous experiments measured changes in the synaptic transmission of a

population of cells using field potential recordings, we examined if mGluR-LTD was similarly independent of translation initiation at the single cell level using dissociated cultured neurons (Fig. 1*B, C*). Application of the elongation inhibitor emetine (40 μM) blocked the decrease in mEPSC frequency induced by DHPG, but similar application of the initiation inhibitor HHT (20 μM) did not. The mEPSC frequency was 105 ± 8% of baseline at 30 min following application of DHPG in the presence of emetine ($n = 6$, $P = 0.27$), but was significantly decreased to 77 ± 10% of baseline after DHPG in the presence of HHT ($n = 7$, $P = 0.04$). Amplitude of mEPSCs was not significantly changed by DHPG in these experiments. The mEPSC amplitude was 95 ± 5% of baseline at 30 min post-DHPG in emetine ($P = 0.28$) and 105 % ± 5% of baseline post-DHPG in HHT ($P = 0.47$).

These results showing a block of mGluR-LTD by the translation elongation inhibitor emetine indicate that such plasticity in cultured hippocampal neurons is dependent on *de novo* protein synthesis. Moreover, the lack of inhibition of mGluR-LTD by the translation initiation inhibitor HHT suggest that this synaptic plasticity is dependent on regulation of translation downstream of initiation, at the elongation step.

Activation of group I mGluRs by DHPG causes a short-term increase in cell input resistance likely due to the inhibition of a leak potassium conductance (Mannaioni et al 2001). To verify that the absence of mGluR-LTD in the presence of emetine was not due to a lack of effect of DHPG, we monitored cell input resistance during DHPG application. DHPG application caused a transient significant increase in cell input resistance that was similar in all treated conditions, including in the presence of emetine (132 ± 8 % of baseline for DHPG alone; 125 ± 5% of baseline for DHPG + Emetine; 140 ± 10 % of baseline for DHPG + HHT; Figure 1*E*, *F*). In the untreated condition, cell input resistance was unchanged at the same time period (103 ± 2 % of baseline; Figure 1*E*, *F*). Thus, in all experimental conditions, group I mGluRs were activated, indicating that the block of mGluR-LTD by emetine was not due to the lack of effect of DHPG.

To verify that the lack of mGluR-LTD in presence of emetine was not due to other nonspecific variations in basal experimental conditions, we compared basal membrane and synaptic properties in the different treatment conditions. We did not find any significant difference in baseline mEPSC frequency or amplitude. Neither were series

resistance, holding current and input resistance different between untreated and treated groups (DHPG, DHPG + emetine, or DHPG + HHT) (Table 1).

Overall, these results suggest that mGluR-LTD monitored at the single cell level using whole-cell patch clamp recordings in cultured hippocampal neurons is independent of translation initiation and dependent on translation elongation.

2.5.3 Knockdown of the RNA-binding protein Staufen 2 prevents mGluR-LTD in cultured neurons

Having established that mGluR-LTD in cultured neurons show similar hallmark properties to mGluR-LTD in acute slices, we next combined this chemical induction paradigm with molecular biological knockdown strategies to examine the mechanism of RNA regulation. Staufen 2 (Stau2) has been implicated in mGluR-LTD using knockdown strategies and field recordings in organotypic slices (Lebeau et al 2011). To investigate the role of Stau2 in mGluR-LTD at the single cell level, we utilized a lentivirus expressing short-hairpin (sh) RNA to knockdown Stau2 RNA and protein in our cultures of dissociated hippocampal neurons (shStau2) (Fig. 2*B*).

First, we verified the knockdown efficacy of the lentivirus by immunocytochemistry. Three days after transduction, protein expression of Stau2 assessed by immunocytochemistry in the soma of shStau2 neurons was reduced to 83.9 ± 14.7% (n = 9) of the protein expression detected in neurons transduced with a lentivirus expressing a control shRNA (shControl) (Graber & Sossin 2014). The efficacy of transduction and lentiviral expression was, on the other hand, estimated by the expression of green fluorescent protein (GFP). Therefore, Stau2 was effectively knocked down in neurons expressing GFP.

Next, we studied the effects of Stau2 knockdown on mGluR-LTD at the single cell level in cultured neurons. As expected, neurons transduced with shControl showed normal mGluR-LTD expressed as decrease in mEPCS frequency, but no change in amplitude. In contrast, Stau2 knockdown precluded the depression in frequency. Thirty minutes following bath application of DHPG, mEPSC frequency in neurons transduced with shControl was decreased to 78 ± 4% of baseline (n = 9, P = 0.0019) (Fig. 2*C, D*). In

neurons transduced with shStau2, mEPSC frequency was similar to baseline levels (103 ± 9% of baseline; $n = 8$, $P = 0.35$). Surprisingly, while shControl neurons did not show any change in the amplitude of mEPSCs after DHPG application, mEPSC amplitude was significantly decreased in shStau2-transduced neurons. Amplitude post-DHPG in shControl neurons was at 107 ± 5% of baseline ($P = 0.18$), whereas the amplitude in shStau2 neurons decreased to 91 ± 2% of baseline ($P = 0.015$). Our findings demonstrate that Stau2 is required for mGluR-LTD, which is expressed as a decrease in mEPSC frequency.

To make certain the block of mGluR-LTD observed in neurons with Stau2 knockdown is not due to experimental conditions or nonspecific changes in basal transmission as a result of the lentiviral transduction, we compared basal membrane and synaptic properties in the different experimental conditions. There were no significant difference in baseline mEPSC amplitude, series resistance or holding current between the shControl and shStau2 transduced neurons (Table 2). Thus, the knockdown of Stau2 most likely caused the impairment in mGluR-LTD.

To verify shStau2 block was not due to a lack of, or an interference with, mGluR activation due to lentiviral expression, we monitored passive membrane properties during DHPG application. We observed transient changes in cell input resistance in all DHPG treated groups (123 ± 4 % of baseline for shControl; 136 ± 9% of baseline for shStau2; Figure 2*E*, *F*), effects that are consistent with group I mGluR activation. Thus, virus expression did not impede activation of group I mGluRs (Fig. 2*E, F*).

Overall, these results indicate that the RNA-binding protein Staufen 2 is necessary to induce mGluR-LTD, and therefore plays a crucial role in the underlying mechanism.

2.6 DISCUSSION

Our results show that the application of the group I mGluR agonist DHPG in dissociated hippocampal neuron cultures elicits a long-term depression of excitatory synaptic transmission that is observed as a decrease in mEPSC frequency with no change in mEPSC amplitude. mGluR-LTD in cultured neurons is prevented by emetine, an inhibitor of translation elongation, but not by homoharringtonine, an inhibitor of translation initiation. Moreover, our findings indicate that the RNA-binding protein Staufen 2 is required for mGluR-LTD in cultured neurons. Taken together, these results indicate that DHPG application in dissociated hippocampal neurons coupled with lentiviral expression to manipulate proteins involved in RNA regulation is an effective approach to investigate the mechanisms underlying translation regulation during long-term synaptic plasticity. Moreover, our results provide further support for a model of translational control in mGluR-LTD that occurs downstream of initiation, likely through the reactivation of polyribosomes stalled at the elongation or termination stage (Graber et al 2013).

2.6.1 Expression mechanisms of mGluR-LTD

Our data show that the application of DHPG to dissociated hippocampal neuron cultures elicits a persistent decrease in the frequency of mEPSCs, but no change in their amplitude. These results are in agreement with previous findings both in hippocampal cultures and in hippocampal slices (Fitzjohn et al 2001, Jakkamsetti et al 2013, Moult et al 2006, Niere et al 2012, Sanderson et al 2011, Snyder et al 2001, Verpelli et al 2011, Waung et al 2008).

The site of expression of this depression in synaptic transmission is, on the other hand, not as clear. A change in mEPSC frequency but not in amplitude is classically believed to reflect a change in the probability of transmitter release (Pr) from the presynaptic neuron. DHPG application has also been reported to cause an increase in paired-pulse facilitation (PPF) (Fitzjohn et al 2001, Moult et al 2006, Rouach & Nicoll 2003) coupled with an increase in the coefficient of variation of EPSCs (Fitzjohn et al 2001, Moult et al 2006) and a decrease in the success rate of EPSCs (Fitzjohn et al 2001),

consistent with a presynaptic locus of expression. However, there is strong evidence that AMPA receptors at the postsynaptic membrane are internalized following mGluR-LTD induction (Gladding et al 2009a, Huang et al 2004, Jakkamsetti et al 2013, Snyder et al 2001, Waung et al 2008, Xiao et al 2001, Zhang et al 2008) and this activity-dependent endocytosis requires the postsynaptic synthesis of proteins (Snyder et al 2001) such as Arc/Arg1.3 (Waung et al 2008). In addition, mGluR-LTD induction pathways are clearly postsynaptic (Gladding et al 2009b). To reconcile these findings, the concept of 'silent synapses' has been proposed (Snyder et al 2001) and postulates that mGluR-LTD induction results in the internalization, in an all or none fashion, of postsynaptic AMPA receptors at certain synapses, rendering them functionally-silent. This postsynaptic model could explain all the electrophysiological findings discussed above, including PPF changes, if high probability synapses were to be selectively silenced (Fitzjohn et al 2001, Sanderson et al 2011).

Conflicting evidence does exist however in regards to PPF during mGluR-LTD. Huang and Hsu (2006) reported that DHPG caused a transient change in PPF during the acute phase, but it did not persist. On the other hand, Nosyreva and Huber (2005) showed that the site of mGluR-LTD expression appears developmentally regulated since PPF was more pronounced in neonatal rats (8-15 days postnatal) compared to adolescents (21-35 days postnatal). Nonetheless, changes in PPF (Moult et al 2006) as well as decreased transmitter release measured by fluorescence detection of vesicular zinc release (Qian & Noebels 2006) have been reported during mGluR-LTD across all age ranges. It is interesting to point out that the concentrations of DHPG used and its period of application vary considerably between these studies, although they are all able to induce synaptic depression at similar levels. In slices, the treatment protocol ranges from 50 μM for 5 minutes (Huang & Hsu 2006) to 100 μM for 20 minutes (Moult et al 2006). It is possible then that synapses experiencing stronger mGluR activation recruit additional signaling pathways that could lead to a greater presynaptic contribution.

Interestingly, although we found that Stau2 abolished mGluR-LTD as expressed by a decrease in mEPSC frequency, DHPG caused a significant decrease in amplitude in these transduced neurons. This has been reported in wild type neurons under some conditions in both cultures and acute slices (Xiao et al 2001). A decrease in amplitude,

but not in frequency can be interpreted as either a decrease in quantal size (less neurotransmitter in each vesicle) or a decrease in the sensitivity of the post-synaptic membrane to the neurotransmitter. We speculate that the lack of Stau2 prevents the induction of mechanisms underlying synapse silencing, hence the lack of change in frequency, while allowing Stau2-independent pathways to cause the internalization of some but not all AMPA receptors. These mechanisms would require further investigation.

Thus, although the effects of DHPG are rather consistent, the mechanisms of expression of mGluR-LTD are still not clearly understood. They likely involve both pre- and postsynaptic sites of expression whose relative contributions differ depending on the developmental stage and experimental conditions during induction. More work is needed to understand which pathways are responsible for these different expression mechanisms.

2.6.2 Translation regulation at the elongation step during mGluR-LTD

Local protein synthesis is crucial for mGluR-LTD (Huber et al 2000). The upregulated translation, following induction, of specific transcripts such as MAP1B, STEP and Arc/Arg3.1 that are directly linked to AMPA receptor endocytosis has been documented (Gladding et al 2009b). However, the mechanisms that regulate activity-dependent translation and allow such specificity during long-term synaptic plasticity remain largely elusive. Our findings shed light on one of these mechanisms.

RNA-binding proteins (RBPs) represent an attractive mechanism of translation regulation. They are proteins involved in correctly targeting mRNAs to their appropriate dendritic compartment (Besse & Ephrussi 2008). During transport, translation of these mRNAs is repressed, but this repression can be relieved upon plasticity inducing synaptic activity (Buxbaum et al 2014, Graber et al 2013). Thus, this dual function of localization and translation regulation of RBPs provides the neuron with a dynamic mechanism of translation control that can quickly respond to plasticity signals. Several RBPs exist that differentially bind mRNAs (Doyle & Kiebler 2011, Lebeau et al 2011) and these properties could very well give them the ability to define the translation “regulons” (Keene 2007), the set of mRNAs that are upregulated in a coordinated fashion during a specific form of plasticity.

Staufen 2 (Stau2) is an RBP that is both involved in the localization and translation regulation of transcripts (Krichevsky & Kosik 2001), notably the microtubule-associated protein 1B (MAP1B), a protein involved in mGluR-mediated AMPA receptor endocytosis and whose synthesis is promoted during mGluR-LTD (Lebeau et al 2011). In the absence of Stau2, basal MAP1B levels are decreased in dendrites and the increase in synthesis seen following mGluR-LTD induction is blocked. Recently, we were able to visualize ribosome-bound nascent chains by immunofluorescence, a technique called ribopuromycilation (RPM) (Graber et al 2013). This staining technique revealed intense punctas, indicative of polyribosomes, that colocalized with Stau2 and FMRP, markers for RNA granules. RNA granules are large mRNA transport complexes that contain RBPs, ribosomes and other components of the translation machinery in contrast to ribonucleoprotein particles (RNPs) that do not possess ribosomes (Sossin & DesGroseillers 2006). When cultured hippocampal neurons were pre-incubated with an initiation inhibitor to allow active ribosomes to naturally run-off, almost all the RPM stained punctas remained. These results suggest that ribosomes loaded on transcripts in RNA granules are stalled and this stalling occurs downstream of the initiation step. Thus, proteins are partially translated before they reach their dendritic compartment and await the appropriate synaptic signal to complete their synthesis. FMRP is also implicated in reversibly stalling polyribosomes (Darnell et al 2011). Consistent with these results, we found that mGluR-LTD in primary cultures is impaired by an inhibitor of elongation, but not of initiation. Furthermore, we demonstrated that knockdown of Stau2 blocked mGluR-LTD. These data suggest that translation of transcripts regulated downstream of initiation is required for mGluR-LTD.

Stalled polyribosomes are reactivated following mGluR activation and RNA granules disassemble, which are observed as a decrease in RPM puncta (Graber et al 2013). The upstream pathways that lead to this reactivation have yet to be elucidated, but an interesting candidate is the eukaryotic elongation factor 2 kinase (eEF2K), whose activity is upregulated during mGluR-LTD, causing eEF2 to be phosphorylated (Park et al 2008, Verpelli et al 2010). In this context, eEF2 phosphorylation slows general translation but the translation rate of certain proteins like MAP1B (Davidkova & Carroll 2007) and Arc/Arg1.3 (Park et al 2008) is actually increased. Further investigation is

needed to understand how phosphorylation of eEF2 could lead to the reactivation of stalled polyribosomes.

2.6.3 Translation regulation at the initiation step during mGluR-LTD

Multiple lines of evidence suggest that translational control during mGluR-LTD is cap-dependent, and therefore also depends on regulation at the initiation step. A very recent study demonstrated that regulation of the ternary complex through the phosphorylation of the eukaryotic initiation factor 2 alpha (eIF2α) is important for mGluR-LTD (Di Prisco et al 2014). DHPG application caused an increase in eIF2α phosphorylation (Di Prisco et al 2014, Trinh et al 2014) mediated through the RNA-activated protein kinase (PKR)-like ER kinase (PERK) (Trinh et al 2014). mGluR-LTD induced in slices from mice with deficient eIF2α phosphorylation was abolished (Di Prisco et al 2014). This result contrasts with a previous report that knocking out PERK, thus abolishing DHPG-induced increases in eIF2α phosphorylation, enhanced mGluR-LTD (Trinh et al 2014). One major difference between the PERK knockout and eIF2α knock-in mice in these two studies is that the eIF2α knock-in has a large basal effect on eIF2α while the PERK knockout does not. Possibly, it is the reduction in basal levels of eIF2α caused by the eIF2α kock-in that affects the neuron's ability to induce mGluR-LTD rather than the lack of signal-induced increase in phosphorylation.

Oligophrenin-1 (Ophn1) translation was increased in response to eIF2α phosphorylation, but not Arc/Arg1.3 (Di Prisco et al 2014), a protein regularly observed to be upregulated in mGluR-LTD (Huber & Lüscher 2010). It is possible that the eIF2α knock-in effects on basal eIF2α phosphorylation decreased Ophn1 basal levels to the point that mGluR-LTD is blocked. In addition, the Ophn1 increase in synthesis largely depends on the activation of mGluR1, rather than mGluR5 and is independent of FMRP regulation (Kasri et al 2011). DHPG-induced mGluR-LTD relies more heavily on sustained activation of mGluR5 and its downstream signaling (Huang & Hsu 2006, Palmer et al 1997) and FMRP is a major regulator of mGluR-LTD mRNA targets (Darnell et al 2011). Moreover, our findings suggest that the acute Ophn1 synthesis that would presumably occur through increased translation initiation is not required for mGluR-LTD.

4E-binding proteins (4E-BPs) have also been shown to play a role in mGluR-mediated translation regulation (Banko et al 2006). 4E-BPs compete with eIF4G for eIF4E binding. This binding interaction with eIF4E inhibits translation since the eIF4F complex cannot be formed, thus inhibits initiation. In 4E-BP knockout mice, mGluR-LTD was enhanced. Other conditions such as the pre-incubation with initiation inhibitors (HHT or the cap analog m^7GpppG) for at least an hour prior to induction shift mGluR-LTD (Graber et al 2013, Huber et al 2000) towards a more stringent requirement for initiation regulation as certain factors may be depleted.

While studies such as Banko et al (2006), Di Prisco et al (2014)'s provide evidence for upregulated translation initiation during mGluR-LTD, our findings suggest that these mechanisms are not important for this form of plasticity. Increased synthesis of Ophn1, but not Arc/Arg1.3, a protein shown in numerous reports to be crucial for mGluR-LTD (Jakkamsetti et al 2013, Niere et al 2012, Park et al 2008, Snyder et al 2001, Waung et al 2008), following eIF2α phosphorylation strongly implies that this is not a major pathway of translation regulation. On the other hand, enhanced mGluR-LTD in 4E-BP knockout mice is merely an indication that increasing the rate of initiation can support mechanisms of expression in mGluR-LTD, but does not suggest that it is required.

In conclusion, translation regulation of important mRNA targets during mGluR-LTD occurs downstream of initiation. Uncovering the details of this mechanism is crucial to understanding how neurons achieve temporal and spatial control of local protein synthesis during long-term synaptic plasticity. Moreover, it will shed light on how dysregulated translation leads to neuronal dysfunction. Finally, our findings suggest that the mGluR-LTD model in cultured neurons is an adequate tool to investigate the mechanisms of stalling and reactivation of ribosomes by Staufen 2, a crucial mechanism of translation regulation in neurons during mGluR-LTD.

2.7 REFERENCES

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2.8 TABLES

Table 1. Electrophysiological properties in neurons of each treatment group during baseline

	Untreated	DHPG	Emetine + DHPG	HHT + DHPG
n	4	8	6	7
DIV (days)	19.0 ± 0.6	20 ± 2	21 ± 2	18.6 ± 0.6
Amp. (pA)	11 ± 1	8.8 ± 0.8	10.8 ± 0.9	9.3 ± 0.8
Freq. (Hz)	2.7 ± 0.6	3 ± 2	5 ± 2	2.3 ± 0.6
Rise time (ms)	1.5 ± 0.2	1.3 ± 0.1	1.0 ± 0.1	1.3 ± 0.1
Decay time[a] (ms)	5.5 ± 0.4	5.9 ± 0.5	4.5 ± 0.3	4.4 ± 0.4
I_{hold} (pA)	-70 ± 40	-33 ± 4	-42 ± 7	-25 ± 4
R_{input} (mΩ)	210 ± 40	260 ± 30	270 ± 50	160 ± 20
R_s (mΩ)	22 ± 3	23 ± 2	21 ± 3	18 ± 2
V_r[b] (mV)	-54 ± 1	-54 ± 1	-56 ± 1	-61 ± 2

DIV, days in vitro; R_s, series resistance; V_r, resting potential, I_{hold}, holding current; R_{input}, input resistance
[a]Decay time calculated from 10 to 67% of peak
[b]Not corrected for junction potential

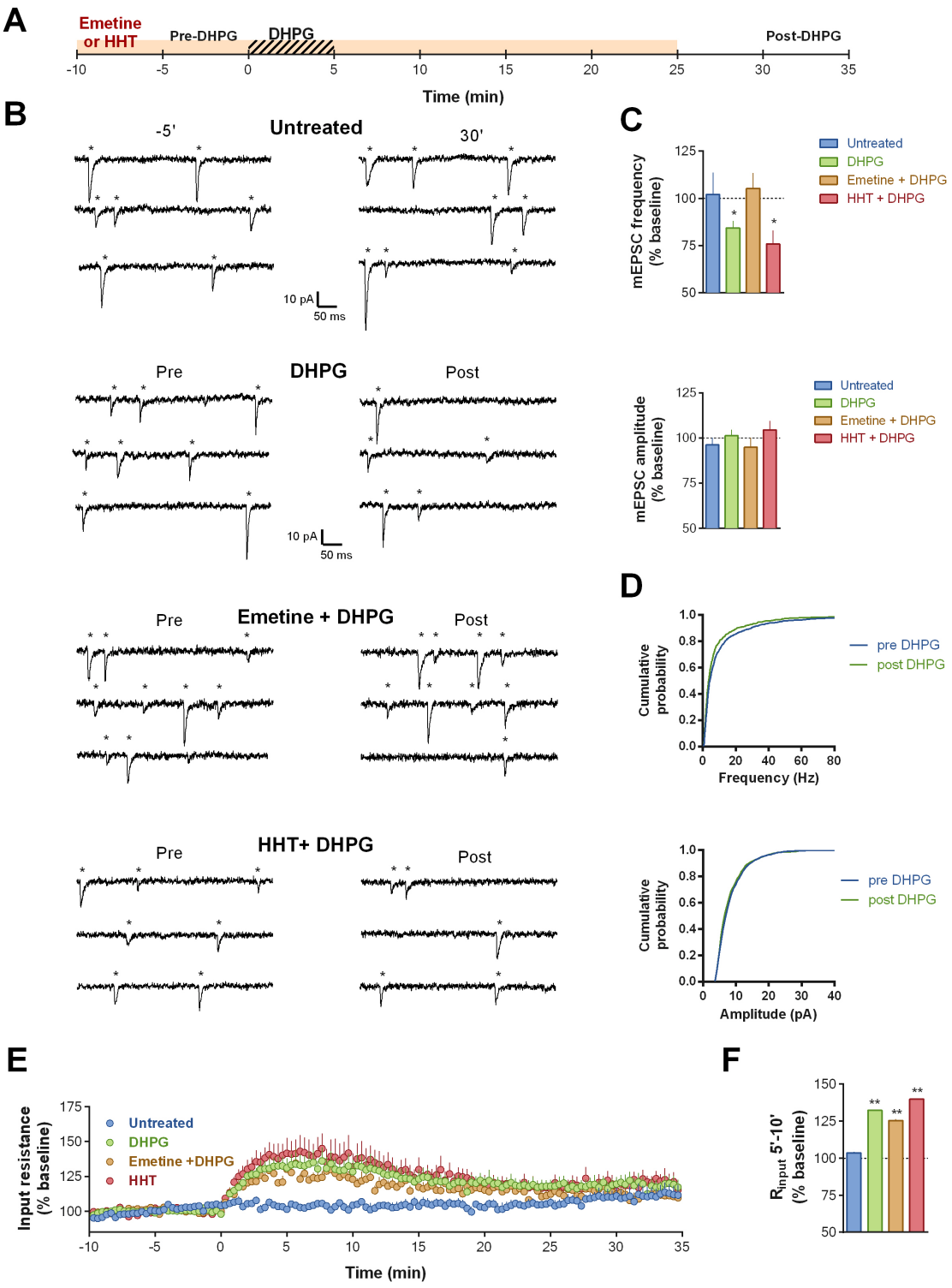
Table 2. Electrophysiological properties in transduced neurons during baseline

	shControl	shStau2
n	10	9
DIV (days)	22.2 ± 0.7	21 ± 1
Amp. (pA)	10.9 ± 0.8	13 ± 2
Freq. (Hz)	2.4 ± 0.2	4.3 ± 0.7[§]
Rise time (ms)	0.87 ± 0.07	0.85 ± 0.08
Decay time[a] (ms)	4.6 ± 0.5	4.2 ± 0.3
I_{hold} (pA)	-46 ± 10	-38 ± 7
R_{input} (mΩ)	170 ± 20	220 ± 20
R_s (mΩ)	16 ± 1	15 ± 2
V_r[b] (mV)	-57 ± 1	-57 ±1

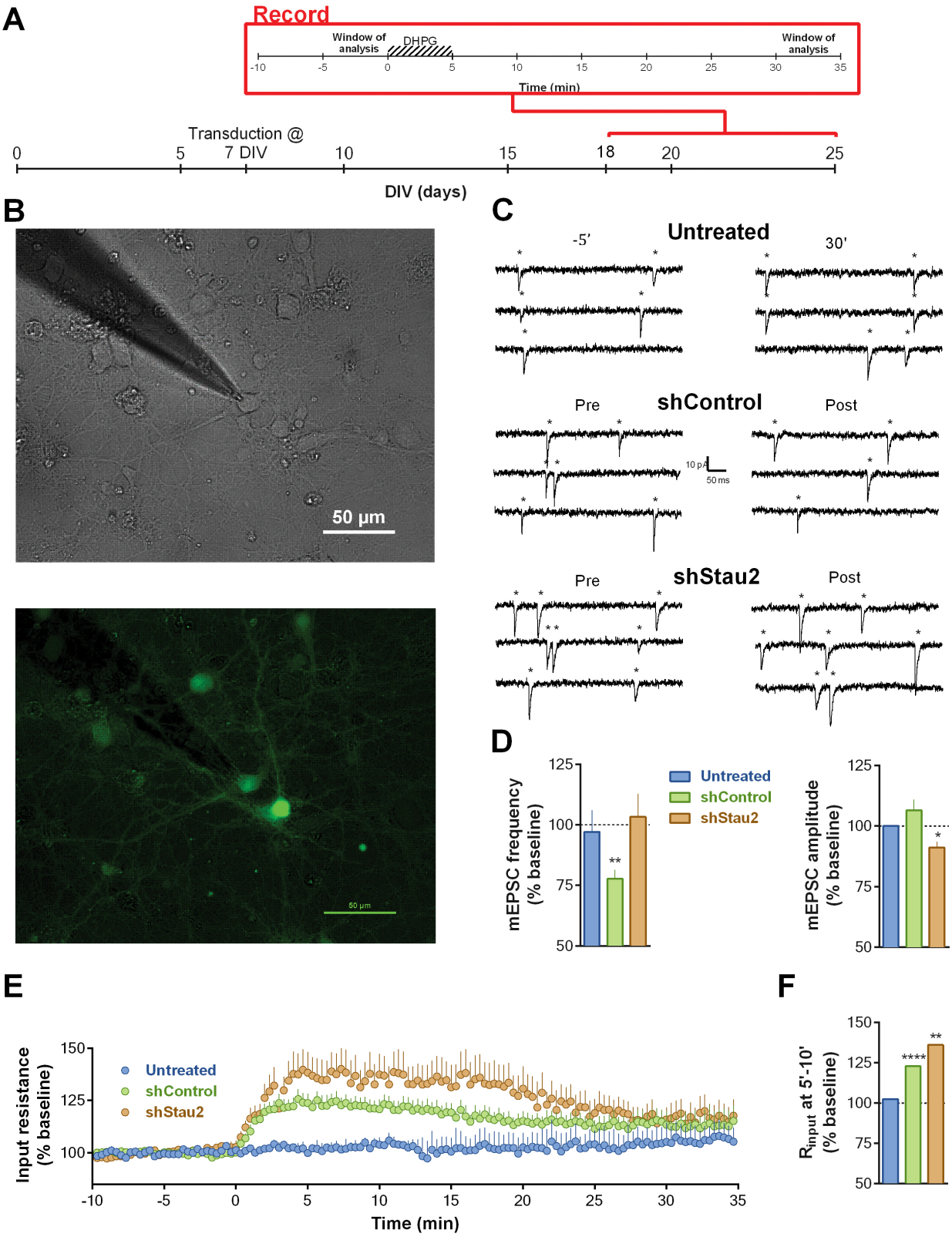
DIV, days in vitro; R_s, series resistance; V_r, resting potential, I_{hold}, holding current; R_{input}, input resistance
[a]Decay time calculated from 10 to 67% of peak
[b]Not corrected for junction potential
[§]Statistically different compared to the shControl group

2.9 FIGURES

2.9.1 Figure 5



2.9.2 Figure 6


A
Record
Window of analysis
DHPG
Window of analysis
-10 -5 0 5 10 15 20 25 30 35
Time (min)
Transduction @
7 DIV
0 5 10 15 18 20 25
DIV (days)
B
50 µm
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C
Untreated
-5'
30'
Pre
shControl
Post
10 pA
50 ms
Pre
shStau2
Post
D
mEPSC frequency (% baseline)
125
100
75
50
**
Untreated
shControl
shStau2
mEPSC amplitude (% baseline)
125
100
75
50
*
E
Input resistance (% baseline)
150
125
100
Untreated
shControl
shStau2
-10 -5 0 5 10 15 20 25 30 35
Time (min)
F
R input at 5'-10' (% baseline)
150
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2.10 LEGENDS

Figure 5. mGluR-LTD in cultured neurons and block by translation elongation inhibitor. **(A)** Diagram of timeline of drug application during the whole-cell recording experiments and time windows used for analysis of mEPSCs. **(B)** mEPSCs from representative cells at two time points (-5' and 30') in untreated neurons (top) or pre- and post-DHPG application in the treated groups (DHPG, Emetine + DHPG, HHT + DHPG). **(C)** Summary histograms of mEPSC frequency and amplitude for all cells. **(D)** Cumulative probability histograms of DHPG effects on mEPSC frequency and amplitude for the DHPG-treated cell depicted in **(B)** showing a significant change in the distribution of the instantaneous frequency of events (K-S test, $P = 0.0031$), but no change in the distribution of mEPSC amplitude ($P = 0.70$) post-DHPG. **(E)** Time plot for all cells of cell input resistance measured from hyperpolarizing pulses and normalized to baseline (-5 to 0 minutes pre-DHPG application) showing in all groups a transient increase in cell input resistance induced by DHPG. **(F)** Summary histogram showing a significant increase in R_{input} in cells exposed to DHPG compared to untreated cells at 5-10 min regardless of exposure to translation inhibitors. Untreated, n=4; DHPG, n = 8; Emetine + DHPG, n = 6; HHT + DHPG, n = 7. * $p<0.05$, ** $p<0.01$

Figure 6. Knockdown of the RNA binding protein Staufen 2 impairs mGluR-LTD. **(A)** Diagram of timeline of lentivirus transduction and whole-cell recording experiments. **(B)** Representative images of dissociated hippocampal neurons in cultures 21 DIV transduced with a lentiviral vector expressing a short hairpin RNA (shStau2) and emGFP. **(C)** mEPSCs from representative cells at two time points (-5' and 30') in untreated transduced neurons or pre- and post-DHPG in neurons transduced with a control shRNA sequence or shStau2. **(D)** Summary histograms of mEPSC frequency and amplitude for all cells. **(E)** Time plot for all cells of cell input resistance measured from hyperpolarizing pulses and normalized to baseline (-5 to 0 minutes pre-DHPG application) showing in all groups a transient increase in cell input resistance induced by DHPG. **(F)** Summary histogram showing an increase in R_{input} in cells exposed to DHPG compared to untreated cells at 5-10 min regardless of the lentiviral vector used. Untreated, n=2 (1 shScramble, 1 shStau2); shScramble, n = 9; shStau2, n = 8. * $p<0.05$, ** $p<0.01$, **** $p<0.0001$.

CHAPTER III. GENERAL DISCUSSION

3.1 REVIEW OF MAIN RESULTS

The objectives of this thesis were as follow:

1. To establish if we could reliably induce mGluR-LTD in cultures of dissociated hippocampal neurons with the group I mGluR agonist DHPG as previously reported.
2. To demonstrate that mGluR-LTD is independent of the initiation step of translation in cultured hippocampal neurons.
3. To determine if the RNA-binding protein Staufen 2 plays a crucial role in mGluR-LTD in cultured hippocampal neurons.

The objectives aimed at better understanding the molecular memory trace of mGluR-LTD using a single cell culture model amenable to both pharmacological and RNA knockdown studies.

With these objectives in mind, our results indicate that:

1. Following the application of DHPG, mGluR-LTD is expressed as a decreased mEPSC frequency, but no change in mEPSC amplitude.
2. Translation initiation is not necessary for mGluR-LTD.
3. Staufen 2 is required for mGluR-LTD.

These results strongly suggest that mGluR-LTD implicates a mechanism of translation regulation that is downstream of initiation. We propose this is achieved through the stalling of polyribosomes by the RNA-binding protein Staufen 2, which, upon the activation of mGluR signaling pathways, dissociates to relieve the translational repression and allow for local, rapid and coordinated synthesis of its targets (Fig. 7).

3.2 MECHANISMS OF LOCAL TRANSLATION REGULATION

The notion that learning and memory requires protein synthesis was proposed as early as 1948 by Monné and evidence to support this claim was first published in the late 1950s (see Sutton & Schuman 2006). Today, the importance of protein synthesis for memory is widely

recognized and several studies have also demonstrated its necessity for the maintenance of forms of plasticity such as NMDAR-dependent late-LTP and mGluR-LTD (Kelleher III et al 2004). What isn't as clear is whether this synthesis needs to be local (i.e. near the synapse).

Indeed, what makes the study of mGluR-LTD so interesting is its clear reliance on local protein synthesis (Pfeiffer & Huber 2006). In contrast, while there is evidence for local protein synthesis during NMDAR-dependent late-LTP (Sutton & Schuman 2006), whether it is necessary is not apparent. To make the distinction even more conspicuous, protein synthesis inhibitors block mGluR-LTD within 10 minutes following induction (Huber et al 2000) whereas NMDA-dependent late-LTP, while initially impaired, is blocked after more than an hour (Kelleher III et al 2004).

Thus, our work to uncover the molecular mechanisms underlying mGluR-LTD will give further insight into how local translation is accomplished and why this may be important for learning and memory.

3.2.1 Evidence for stalled polyribosomes

Local protein synthesis occurs on demand in response to plasticity-inducing synaptic activity. Translation regulators must therefore be able to wield a temporal and spatial control of protein synthesis. A number of RNA-binding proteins (RBPs) that can perform this very task have been described (Besse & Ephrussi 2008). One RBP that has attracted a lot of interest in the last 20 years for its direct relevance to Fragile X Syndrome (FXS), the most common cause of inherited autism, is FMRP. FXS is caused by the functional absence of FMRP due to a CGG triplet expansion repeat in the 5' UTR of the *FMR1* gene. FMRP is a negative regulator of translation (reviewed in Bhakar et al 2012, Darnell & Klann 2013), but the mechanism by which it represses translation has not been elucidated. FMRP is found in RNA granules of distinct composition (Fritzsche et al 2013a), but also in processing bodies and stress granules (Barbee et al 2006, Kim et al 2006), thus could play more than one role and have more than one mode of translation regulation, thereby making the understanding of its mechanism of action more challenging.

Nonetheless, several lines of evidence suggest that FMRP exerts a regulatory role after the initiation step. First, FMRP associates with polyribosome complexes (Ceman et al 2003,

Darnell et al 2011, Graber et al 2013, Khandjian et al 2004, Stefani et al 2004, Tamanini et al 1996, Weiler et al 1997). Second, phosphorylated FMRP associates with apparently stalled polyribosomes, while dephosphorylated FMRP is associated with actively translating polyribosomes (Ceman et al 2003). Stefani et al (2004) questioned the results reported by Ceman et al (2003) because of their use of the metabolic poison sodium azide. Even so, the results of Darnell and coworkers (Stefani et al 2004) indicate that a sizeable number of polyribosomes complexed with FMRP were retained on mRNAs despite the application of puromycin, an amino-acyl tRNA analog, that disrupts actively translating ribosomes, prematurely ending translation.

More convincingly, a brain polyribosome-programmed *in vitro* translation system quantitatively revealed that ribosome stalling occurs on FMRP target transcripts (Darnell et al 2011). The authors induced run-off of actively translating ribosomes with the use of puromycin or hippuristanol. Puromycin covalently binds to the nascent peptide preventing the addition of more amino acid, thus causes early peptide termination. Hippuristanol is an initiation inhibitor, thus allows natural run-off of actively translating ribosomes while preventing any new polyribosome formation by blocking initiation. Following this treatment, FMRP was still present in the heavy sucrose gradients suggesting that the polyribosomes associated with FMRP are not active, but rather stalled, hence their insensitivity to the translation inhibitors. In the absence of FMRP or when FMRP was present but bore a 130N mutation, FMRP mRNA targets were distributed to lighter fractions than when FMRP was present and functional. Moreover, by combining high-throughput sequencing (HITS) and crosslinking-immunoprecipitation (CLIP), a technique that consists of coimmunoprecipitating FMRP cross-linked by UV light to its mRNA targets, FMRP was revealed to mostly bind mRNA in various regions of the coding sequence (Darnell et al 2011). This is consistent with FMRP binding to polysomes rather than blocking initiation.

This combined evidence strongly suggests that FMRP is implicated in stalling ribosomes at the elongation step. However, it adds little to our understanding of the mechanism involved. Is FMRP directly responsible for the stalling? FMRP's binding domains (KH domains and RGG box) are thought to only recognize RNA binding motifs such as the 'kissing complexes' and G-quadruplexes (Wang et al 2012), but Darnell and colleagues (2011) did not find a consensus sequence amongst the RNA targets – FMRP showed a rather broad binding pattern,

so how is specificity achieved? A possible explanation is that FMRP is a component of a larger complex, but it is the combination and interaction of different elements that is responsible for specifically recognizing and differentially sorting mRNA targets (Änkö & Neugebauer 2012). Indeed, FMRP are part of at least two types of RNA granules: Staufen 2 (Stau2) and Barentsz (Btz)-containing granules (Fritzsche et al 2013a). Despite the existence of a few shared protein interactors (not necessarily direct protein-protein interactors) like FMRP, Stau2 and Btz RNA granules have different molecular compositions (e.g. translation factors and RBPs), evidence that RNA granules are heterogeneous structures. More importantly, there is mounting evidence that distinct RNA granules differentially bind mRNA transcripts (Heraud-Farlow et al 2013, Lebeau et al 2011, Mikl et al 2011). This raises the interesting possibility that discrete RNA granules regulate functionally related sets of RNAs (Hogan et al 2008, Keene 2007).

3.2.2 Role of Staufen 2 in mGluR-LTD

Stau2 is a member of a family of proteins that are implicated in RNA transport in neurons (Kohrmann et al 1999, Tang et al 2001), associate with ribosomes (Duchaine et al 2002, Marion et al 1999), and are linked with translation regulation (Krichevsky & Kosik 2001) and synaptic plasticity (Lebeau et al 2008, Lebeau et al 2011).

In the present study, we have shown that Stau2 is crucial for mGluR-LTD and therefore is likely involved in the regulation mRNAs that are necessary for this form of plasticity. Consistent with this finding, Stau2 modulates the expression of a subset of mRNAs that encode synaptic proteins (Heraud-Farlow et al 2013). Furthermore, we have recently demonstrated that Stau2 and FMRP markers colocalized with stalled polyribosomes that were directly visualized in neurons using a novel technique (Graber et al 2013). These stalled polyribosomes visualized as intense punctas of fluorescence were decreased in number following the application of DHPG to induce mGluR-LTD. This suggests that rather than stimulating initiation and causing an increased loading of ribosomes onto mRNA transcripts, mGluR activation causes stalled polyribosomes to be reanimated, thus quickly dissociating as they complete the translation of proteins (Fig. 7).

In agreement with these findings, we established in the present study that initiation is not important for mGluR-LTD in dissociated neuronal cultures. Therefore, ribosomes have already gone through initiation and proteins are partially completed before they are stalled. Only reactivation of elongation is then necessary. To support this data, initiation blockers do not impair the increase in MAP1B synthesis during mGluR-LTD (Graber et al 2013). MAP1B is an important protein linked to AMPAR endocytosis and known to be upregulated during mGluR-LTD (Davidkova & Carroll 2007). It is a target of both FMRP and Stau2 (Darnell et al 2001, Lebeau et al 2011). In addition, characterization of Stau2 RNA granules revealed that this high-order structure lacks components of the exon junction complex (EJC), an indicator that the mRNAs they contain have already undergone translation initiation (Fritzsche et al 2013a).

3.2.3 FMRP and Staufen 2 – an indispensable partnership

Stau2 and FMRP are both linked to translation regulation of mRNA targets important for mGluR-LTD and they have been found to genetically interact[4] (Bolduc et al 2008). However, they do not appear to be redundant elements, but rather play different roles within the same RNA granule. While the absence of Stau2 causes mGluR-LTD to be impaired as we have shown here, lack of FMRP is characterized by exaggerated mGluR-LTD (Huber et al 2002). Interestingly, without FMRP, mGluR-LTD becomes quite clearly independent of new protein synthesis (Park et al 2008, Weiler et al 2004). These observations made in the absence of functional FMRP have been explained as a deficiency in translation repression causing excessive protein synthesis that does not depend on plasticity signaling events. Moreover, evidence suggests the excessive basal protein synthesis in Fmr1 KO mice is not due to hyperactive mGluR signaling, but it is rather the translation machinery that is hypersensitive to normal mGluR signaling (Osterweil et al 2010).

In the absence of Stau2, mRNA targets important for mGluR-LTD are simply not transported to the synapse (Lebeau et al 2011). In contrast, FMRP appears to be a passive passenger of transport complexes, since in its absence mRNA localization is not affected

[4] Genetic interaction is the phenomenon observed when "mutations in two genes produce a phenotype that is surprising in light of each mutation's individual effects" - Mani R, St.Onge RP, Hartman JL, Giaever G, Roth FP. 2008. Defining genetic interaction. *Proceedings of the National Academy of Sciences* 105: 3461-66

(Steward et al 1998). Therefore, we can envision that in the presence of Stau2 and absence of FMRP, Stau2 correctly localizes mRNAs, but it is unable to maintain the repression of translation at the synapse because the interaction with FMRP is needed. Conceivably, FMRP is required to stabilize the Stau2 mediated stalling complex. On the other hand, FMRP cannot sustain transport of necessary mRNAs without Stau2.

3.3 FUTURE PERSPECTIVES

What is the mechanism of stalling? Which protein-protein interactions are important to decide the fate of a select population of mRNAs? How do these mechanisms differ for translation regulation in NMDA-dependent late-LTP? These are just some of the interesting questions in need of answers. We hope to address these outstanding questions by making use of the mGluR-LTD induction protocol we described in dissociated hippocampal neuron cultures in combination with various tools of molecular biology.

3.3.1 Mechanism of stalling elongation

Although our knowledge of the molecular composition of RNA granules, such as Stau2-containing granules, is gaining ground (Fritzsche et al 2013a, Heraud-Farlow et al 2013, Kanai et al 2004), we have little understanding of how Stau2 influences granule assembly and translation control. In addition to FMRP, Stau2 RNA granules contain Pura and DEAD box helicase 6 (DDX6), two translational repressors, as well as the nuclear proteins, nuclear polyadenylate binding protein 1 (PABPN1) and cap-binding protein 80 (CBP80) (Fritzsche et al 2013a). These findings provide further evidence that mRNAs in Stau2 granules are repressed. Moreover, they suggest that granule assembly begins in the nucleus (Heraud-Farlow & Kiebler 2014).

Another important constituent is the helicase up-frameshift 1 (Upf1), which is crucial to a process called non-sense mediated decay (NMD). The family of Staufens, Stau1 and Stau2 are themselves involved in the degradation of their mRNA targets via Staufen-mediated decay (SMD) and directly bind to Upf1 (Park & Maquat 2013). NMD and SMD elicit mRNA decay, a normal process important for cellular homeostasis in various tissues of the body. However, Staufens seem to engage to a greater extent in stabilizing rather than destabilizing

target mRNAs in neurons (Heraud-Farlow & Kiebler 2014). In fact, a recent study showed that higher Stau1 levels lead to an increased association with ribosomes onto certain mRNAs instead of SMD-driven mRNA degradation (Ricci et al 2014). Moreover, preliminary data obtained in our lab indicates that down-regulating Upf1 decreases the number of visualized stalled polyribosomes (Graber & Sossin 2014). Taken together, these data hint at a possible role for Upf1 in stabilizing the Stau-mediated stalling complex.

Thus, it would be interesting to determine if Upf1-Stau2 interactions are important for the formation of RNA granules activated during mGluR-LTD independently of SMD. A possible strategy would be to use neurons with knocked down Stau2, which we have characterized in the present study, and test the effects of overexpressing a mutated Stau2 that does not bind Upf1 using the lentiviral transduction approach. We could examine if, in the context of a disrupted Upf1-Stau2 interaction, but otherwise normal Stau2 function, dissociated hippocampal neurons can express mGluR-LTD induced with DHPG. This would further our understanding of mechanism underlying Stau2-mediated polyribosome stalling.

3.3.2 Distinct pathways of local translation regulation

Stau1 and Stau2 are paralogues that share a number of conserved features. However, they are found in distinct particles in dendrites of hippocampal neurons (Duchaine et al 2002). Moreover, they bind different sets of mRNAs, sharing but 30% of transcripts (Furic et al 2008).

Interestingly, down-regulation of Stau1 by small interfering (siRNAs) in organotypic hippocampal slices causes deficits in the late phase of LTP induced by forskolin treatment, but leaves the early form of LTP and mGluR-LTD intact (Lebeau et al 2008). On the other hand, knockdown of Stau2 in a similar fashion blocks mGluR-LTD without affecting forskolin-induced LTP (Lebeau et al 2011). These findings are supportive of a model of translation control in which different RBPs such as Stau1 and Stau2 coordinate distinct 'regulons' or pools of mRNAs implicated in translation-dependent forms of synaptic plasticity. In agreement with this line of thought, knocking down Stau2 lowers the levels of its mRNA target Map1b, without perturbing CaMKIIα (Lebeau et al 2011), a target of Stau1 (Kanai et al 2004, Kohrmann et al 1999). Considering the fact that NMDA-dependent late-LTP does not

rely on local protein synthesis in the same way that mGluR-LTD does, one might wonder if the mechanisms of repression mediated by Stau1 and Stau2 are similar. For one, initiation blockers impair NMDA-dependent late-LTP induced by theta burst stimulation (TBS) but not mGluR-LTD (Graber et al 2013). Whether this reflects the stalling of ribosomes at the initiation step for transcripts important for NMDA-dependent late-phase, or the requirement, in addition to local protein synthesis, for more general protein translation is not clear. Interestingly, a recent study found that Stau1 interacts mainly with the coding sequence (CDS) and 3' UTR of transcripts with a higher CDS guanine-cytosine base pair content (Ricci et al 2014).

A good starting point to understand the dissociable roles of Stau1 and Stau2 would be to discern which domains are important in defining their respective regulons. By replacing each of the RNA binding domains in Stau2 with the equivalent Stau1 region, we could generate Stau1/Stau2 protein chimeras. By evaluating the chimera's ability to rescue mGluR-LTD in neurons with down-regulated expression of Stau2, it could be possible to identify the Stau2 domains necessary for its role in mGluR-LTD. Next, we could insert this Stau2 domain in Stau1 in place of its equivalent binding domain and test whether this construct would be sufficient to rescue mGluR-LTD in Stau2 deficient neurons. This would help us gain knowledge of the critical domains that differentiate the Stau1 and Stau2 regulons.

3.3.3 Functional roles of mGluR-LTD

What is the relationship between mGluR-LTD in the hippocampus and behavior? Although many studies have investigated the role of LTP in behavior, much less is known about LTD. Protecting the network from saturating is an important role of LTD (Hu et al 2010) but some studies also suggest a role of hippocampal LTD in the processing of information (Collingridge et al 2010, Kemp & Manahan-Vaughan 2007).

Rodents have an innate preference for exploring new objects, a measurable behavior useful to assess whether the animal recognizes the object or not. Object recognition (OR) is an episodic-like form of memory that appears to be processed by the hippocampus. Low frequency stimulation of Schaffer collaterals normally generates a short-term depression in the CA1 region of freely moving rats, but after exploration of a new spatial environment, this

depression is facilitated (Kemp & Manahan-Vaughan 2004). In addition, a long-term depression of synaptic transmission is elicited by spatial object recognition learning (Goh & Manahan-Vaughan 2013b). Indeed, exposure to OR training (a pair of new objects in a familiar environment) and testing (one object is replaced with a new one in the same location) decreased synaptic strength. Further investigation revealed that, rather than object novelty per se, it is the novelty of the object position that is responsible for this synaptic change since a depression similar to the one elicited during novelty exploration was observed when the same objects were moved to a novel configuration (Goh & Manahan-Vaughan 2013b).

Second, interfering with NMDAR-LTD yields deficits in behavioral flexibility in both the Morris water maze and a delayed non-match to place T-maze task (Nicholls et al 2008). Transgenic mice with inhibited protein phosphatase 2A (PP2A) activity, which impairs NMDAR-mediated LTD, show no deficit in acquisition and memory in the Morris water maze, but are impaired in spatial reversal learning, which is the learning of a new platform location. Likewise, in the delayed non-match to place T-maze task, in which the animal needs to choose, after varying delays, the unvisited arm, the transgenic mice performed normally, but showed deficits when the location of the reward was modified. Finally, the administration of D-serine which potentiates NMDAR-LTD improved performances on spatial reversal tasks (Duffy et al 2007).

Thus, current evidence seems to assign at least two roles to LTD. First, spatial learning itself "endogenously" induces LTD of synaptic transmission in CA1 hippocampus (Goh & Manahan-Vaughan 2013b); and, second, LTD weakens previously encoded memory traces when new information is learned, providing behavioral flexibility in tasks that engage the hippocampus (Nicholls et al 2008).

Nevertheless, there are still gaps remaining in our understanding of the role of long-term depression in hippocampus-dependent behaviors. Despite our comprehension of NMDAR-LTD and mGluR-LTD as two very different processes *in vitro*, how they respectively contribute to behavior has not been elucidated. In fact, the exploration of a novel environment engages both receptors to generate LTD (Goh & Manahan-Vaughan 2013a). It would be interesting to investigate further the role of local protein synthesis and the implication of Staufen 2 in this type of learning.

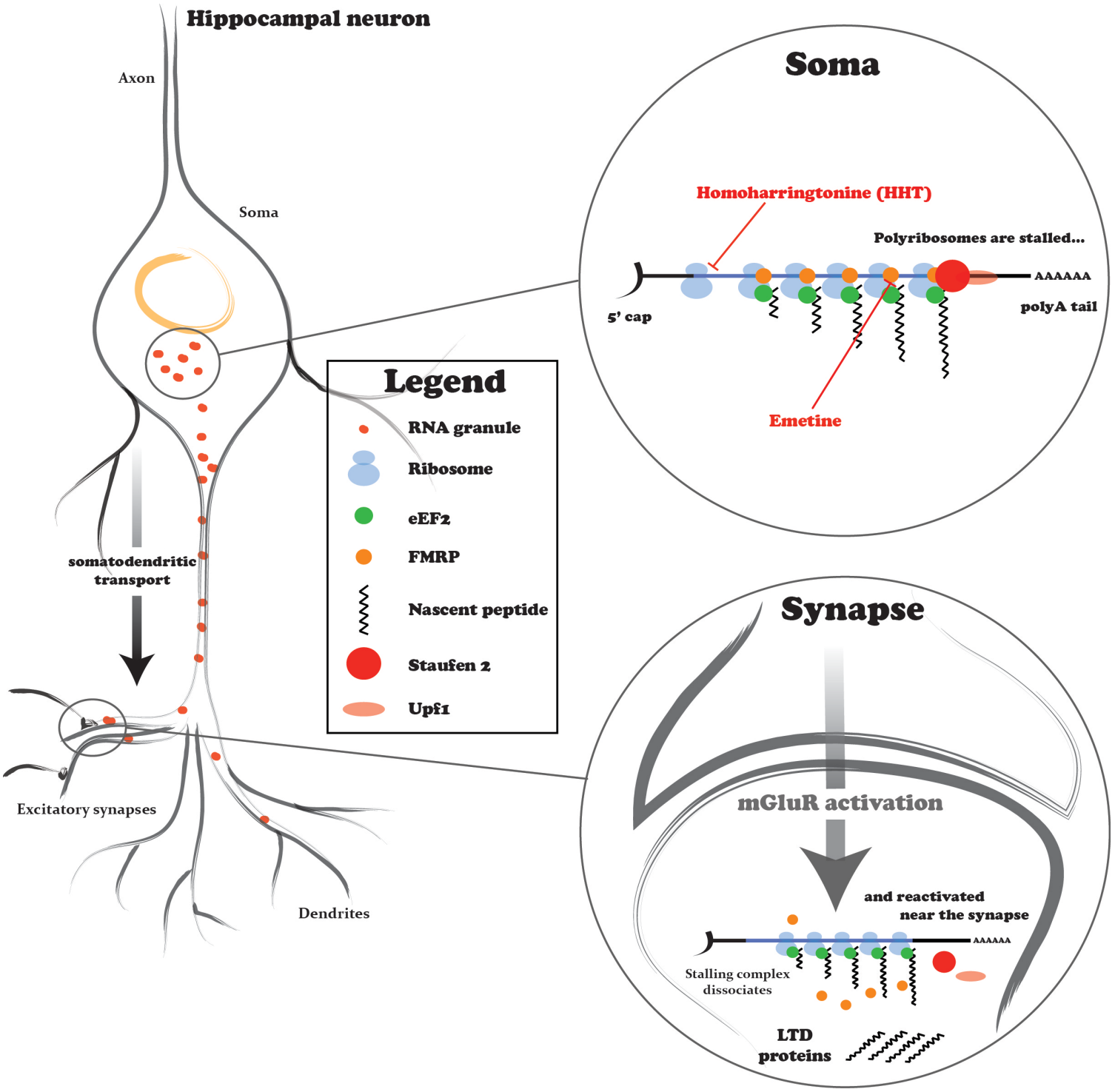

Figure 7. Model of translation regulation in mGluR-LTD.

Transcripts important for mGluR-LTD such as MAP1B are initiated in the soma. Proteins are partially completed when ribosomes are paused at the elongation step and the RNA granule is

formed. Staufen-mediated repression/stalling is stabilized by FMRP and requires Upf1 binding. Whether RNA granules pack more than one mRNA or mRNAs travel singly is a current area of investigation (Mikl et al 2011). During transport, translation is in a state of repression, which is maintained at the synapse. When group I mGluRs of hippocampal pyramidal cells are activated, signaling cascades cause the stalling complex to dissociate and polyribosomes become once again active to rapidly complete synthesis locally. The specific pathways responsible for de-repressing translation are yet to be elucidated. The proteins produced work together to internalize AMPA receptors and decrease synaptic transmission.

CHAPTER IV. GENERAL BIBLIOGRAPHY

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