THE ROLE OF LMO4 IN THE REGULATION OF HIPPOCAMPAL AND AMYGDALAR SYNAPTIC FUNCTION

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ABSTRACT

Synaptic activity can encode and store information in the brain through changes in synaptic strength as well as by control of gene expression. One corollary challenge becomes identifying these activity-dependent regulatory proteins and the underlying mechanisms associated with neuronal functions. By using biochemical, electrophysiological and behavioral approaches in combination with genetic and pharmacological manipulation, I report that LIM domain only 4 (LMO4) is a key regulator of calcium induced calcium release (CICR) and protein tyrosine phosphatase 1B (PTP1B) in the hippocampus and amygdala, respectively. Neuronal ablation of LMO4 in the glutamatergic neurons (LMO4KO) was associated with reduced promoter activity, mRNA, and protein expression of ryanodine receptor 2 (RyR2), suggesting the involvement of LMO4 in the transcriptional regulation. CICR function in LMO4KO mice was severely compromised, reflected by inefficient CICR-mediated electrophysiological responses including afterhyperpolarization, calcium rise from internal stores and glutamate release probability. These changes were accompanied with impaired hippocampal long term potentiation (LTP) and hippocampal-dependent spatial learning ability. LMO4 was also shown to exert a cytoplasmic regulation as an endogenous inhibitor for PTP1B that accounts for tyrosine dephosphorylation of mGluR5 in the amygdala. LMO4KO mice had elevated PTP1B activity and decreased mGluR endocannabinoid signaling, resulting in a profound anxiety phenotype. The potential clinical value of PTP1B/LMO4 is promising, given that intra-amygdala injection of the PTP1B inhibitor Trodusquemine or a PTP1B shRNA alleviated anxiety by restoring eCB signal in LMO4KO mice. Thus this study identified PTP1B as a potential therapeutic target for anxiety, besides the previous findings of its association with obesity and diabetes. Moreover, this PTP1B-

mediated anxiety may be a general mechanism during chronic stress. Collectively, these findings identify that LMO4 plays an essential role for non-genomic and genomic regulation in central neurons, providing a mechanism for LMO4 to modulate a wide range of neuronal functions and behavior.

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LIST OF ABBREVIATIONS

Abbreviations	Full Name
2-AG	2-arachidonoylglycerol
AC	adenylyl cyclase
Acb	accumbens
ACSF	artificial cerebrospinal fluid
ACTH	adrenocorticotropic hormone
AEA	anandamide
AHP	after-hyperpolarization
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid-type glutamate receptor
APP	amyloid precursor protein
APP	anteroposterior
APV	(2R)- amino-5-phosphonopentanoate
AR	adrenergic receptor
Arc	activity-regulated cytoskeletal associated protein
Bhlhb5	basic helix-loop-helix protein 5
BLA	basal lateral amygdala
BMP7	morphogenic protein 7
BNST	bed nucleus of the stria terminalis
BRCA1	breast-cancer susceptibility protein 1
CA	catecholamines
CaMKIV	calmodulin-dependent protein kinase IV
CB1R/CB2R	the cannabinoid receptor
CBP	CREB-binding protein
CDTA	calcium-dependent transacylase
CeA	central amygdala
CHPG	2-chloro-5-hydroxyphenylglycine
CICR	calcium-induced calcium release
CLIM1	cofactor of LIM homeodomain protein 1
CLIM2	cofactor of LIM homeodomain protein 2
CNS	central nervous system
CORT	corticosterone
COX-2	cyclooxygenase -2
CREB	cAMP response element-binding protein
CRH	corticotrophin releasing hormone
CtBP	C-terminal binding protein
CtIP1	C-terminal binding protein-interacting protein 1

DAGL	diacylglycerol lipase
DEAF1	deformed epidermal autoregulatory factor 1
DG	dentate gyrus
DG	diacylglycerol
DHPG	(S)-3, 5-dihydroxyphenylglycine
DSI	depolarization-induced suppression of inhibition
DV	dorsoventral
EC	entorhinal cortex
eCB	endocannabinoid
eEPSC	evoked excitatory post-synaptic current
ephrin B	erythropoietin-producing hepatoma interactor B
EPSC	excitatory postsynaptic current
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
ERα	estrogen receptor alpha
FAAH	fatty acid amide hydrolase
FAK	focal adhesion kinase
FMRP	fragile X mental retardation protein
GABA	γ-Aminobutyric acid
GPCR	G-protein-coupled membrane receptor
GR	glucocorticoid receptor
HDAC2	histone deacetylase 2
HFS	high-frequency stimulation
HPA	hypothalamic pituitary adrenal
HVA	high voltage activated channel
I mGluR	group I metabotropic glutamate receptor
IL-6	interleukin
InsP3R	Inositol 1,4,5-tris-phosphate receptor
IP3	inositol 1,4,5-trisphosphate
IPSC	inhibitory postsynaptic current
IRS1	insulin receptor substrate 1
IRS2	insulin receptor substrate 2
ISI	interstimulus interval
JAK	Janus kinase
LA	lateral amygdala
LIMK1	LIM kinase 1
LMO	LIM domain only
LMO4	LIM domain only 4
LPP	lateral perforant pathway
LTD	long term depression
LTDi	long-term depression of inhibitory postsynaptic current

LTP	long term potentiation
LVA	low-voltage activated channel
MAGL	monoacylglycerol lipase
MAPK	mitogen-activated protein kinase
mEPSC	miniature excitatory post-synaptic current
mGluR	metabotropic glutamate receptor
mGluR-LTD	mGluR-dependent long term depression
ML	mediolateral
MPEP	2, 2-methyl-6-(phenylethynyl) pyridine
MPP	medial perforant pathway
mTOR	mammalian target of rapamycin
NBQX	3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione
NES	nuclear export signal
NFT	neurofibrillary tangles
NGN2	neurogenin 2
NMDAR	N-methyl-D-aspartate receptor
pAEA	phosphoanandamide
PI	phosphatidylinositol
PI3K	phosphoinositide 3-kinase
PKA	protein kinase A
PLC	phospholipase C
PP	perforant pathway
PPARγ	peroxisome proliferator-activated receptor-γ
PPF	paired-pulse facilitation
PPR	paired-pulse ratio
PSD-95	postsynaptic density-95
PTP1B	protein tyrosine phosphatase 1B
PTSD	post-traumatic stress disorder
PVN	paraventricular nucleus of the hypothalamus
RER	receptor-driven endocannabinoid release
RGM A	repulsive guidance molecule A
ROI	regions of interest
RTK	receptor tyrosine kinase
RY	ryanodine
RYR	ryanodine receptor
RyR2	ryanodine receptor 2
SH2	src-homology-2
SH3	src-homology-3
shRNA	small hairpin RNA
SOCS3	suppressor of cytokine signaling 3
SPSS	statistical Package for the Social Sciences

SR	sarcoplasmic reticulum
SSRI	selective serotonin re-uptake inhibitor
STAT3	signal transducer and activator of transcription 3
STEP	striatal-enriched protein tyrosine phosphatase
TA	temporoammonic pathway
TG	thapsigargin
TGFβ	transforming growth factor beta
TM	transmembrane
Trodu	Trodusquemine
TRP	transient receptor potential
TRPV1	transient receptor potential vanilloid 1
TTX	tetrodotoxin
VGCC	voltage gated calcium channel

CHAPTER 1

GENERAL INTRODUCTION

1.1 LMO4 and its Functions

1.1.1 LMO4 and LIM Proteins

First discovered 25 years ago, the LIM domain is recognized as a cysteine- and histidine-rich tandem zinc-finger structure that functions as a modular protein-binding interface (Freyd et al., 1990; Karlsson et al., 1990; Schmeichel and Beckerle, 1994; Way and Chalfie, 1988). LIM domains are present in many proteins that have diverse cellular roles as regulators of gene expression, cytoskeleton organization, cell adhesion, cell motility and signal transduction. LIM proteins have been identified in both the nucleus and the cytoplasm, raising the possibility that LIM proteins might function as biosensors that mediate communication between the cytosolic and the nuclear compartments. LIM proteins can consist exclusively of LIM domains (LIM only, LMOs), or may also contain other domains such as homeodomains, catalytic domains (kinase or monooxygenase), cytoskeletal-binding domains or other protein-binding modules such as Src-homology-2 (SH2), Src-homology-3 (SH3) or postsynaptic density-95, discs large, zona occludens-1 domains. These features highlight the modular nature of the LIM domain, as well as the functional diversity of LIM proteins (Kadrmas and Beckerle, 2004; Zheng and Zhao, 2007).

LIM domain only 4 (LMO4) contains 165 amino acids. Together with short amino-terminal (22 residues) and carboxyl-terminal stretches (25 residues), LMO4 is composed of two tandem LIM domains which are separated by two amino acids (Grutz et al., 1998; Kadrmas and Beckerle, 2004; Sanchez-Garcia and Rabbitts, 1994; Zheng and Zhao, 2007).

Though it has a typical DNA-binding structure, zinc-finger motifs, LMO4 has not yet been shown to demonstrate the DNA-binding activity. In fact, LMO4 plays a role in the transcriptional regulation as a protein binding interface by forming complexes with selective transcription factors. Other members with LIM domain only (LMO) structure are found, named as LMO1, LMO2 and LMO3. Although LMO4 shares less than 50% homology with other LMO proteins (Kenny et al., 1998; Matthews et al., 2013), homology remains relatively higher in the LIM domains other than in the NH2- and C-terminal stretches between the four proteins. LMO family sequency alignment and homology are illustrated in Fig.1.1.

1.1.2 The Role of LMO4 in Tumorigenesis and Development

Previous studies of LMO4 have been focused on its function in tumorigenesis and development (Heberlein et al., 2009; Inan and Crair, 2007; Polleux et al., 2007). LMO4 as an autoantigen in human breast cancer was first described by Racevskis (Racevskis et al., 1999). The overexpression property in breast cancer has been later extensively reported (Montanez-Wiscovich et al., 2009; Sum et al., 2005; Visvader et al., 2001). Upregulation of LMO4 which promotes proliferation and inhibits differentiation of mammary epithelial cells contributes to breast cancer. LMO4 forms a complex with breast-cancer susceptibility protein 1 (BRCA1) and CtBP (C-terminal binding protein)-interacting protein 1 (CtIP1) and negatively regulates BRCA1-mediated transcription activation in breast cancer (Stokes et al., 2013; Sum et al., 2002). LMO4 also binds to estrogen receptor alpha (ERα) and suppresses ERα transactivation functions in the breast cancer progression (Singh et al., 2005). LMO4 and Deaf1 act in common to regulate normal development and breast cancer (Barker et al., 2008; Hahm et al., 2004; Hermanson et al., 1999; Kenny et al., 1998; Wang et al., 2004). In fact, by yeast two

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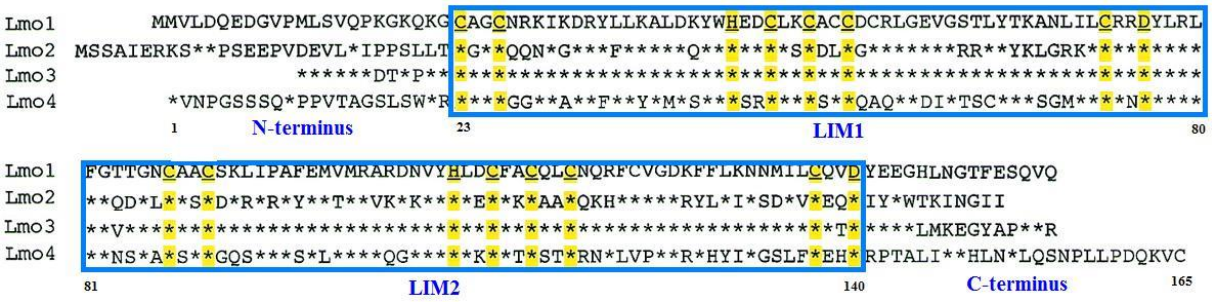

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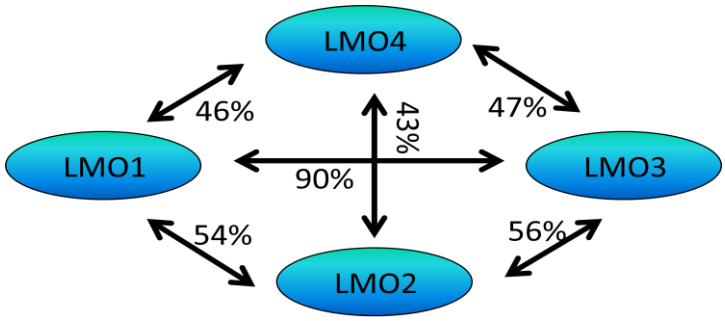

Figure1.1 LIM domain only family sequency alignment and homology

A, The four known members of the mouse LMO proteins are aligned with respect to LMO1. Sequence identity to LMO1 is indicated by a star (*). The sequences are broken into the four regions of the proteins. They all have two LIM domains and each LIM domain has two zinc-finger motifs.The zinc-binding residues are yellow-highlighted with underlines. Homology in the LIM domains is high between the four proteins but minimal in the N- and C-terminal stretches.

B, The pairwise sequence identity between mouse LMO proteins has been determined using CLUSTALW .

hybrid assay, LMO4 has been identified to interact with transcription factor $DEAF1_{404-438}$ (Cubeddu et al., 2012; Joseph et al., 2013; Sugihara et al., 1998). LMO4 seems to regulate the subcellular localization of the DEAF1 by blocking its export leading to its nuclear accumulation. Moreover, LMO4 interacts with SMAD proteins and functions as a co-activator on transforming growth factor beta (TGFβ) signaling in the epithelial oncogenesis (Lu et al., 2006b). In addition, LMO4 is an essential cofactor for SNAIL-mediated cadherin repression in neuroblastoma tumor formation (Ferronha et al., 2013). Another study shows that LMO4 elevation can disrupt the transcriptional complex and prevent histone deacetylase 2 (HDAC2) from binding to chromatin, leading to elevation of bone morphogenic protein 7 (BMP7) promoter activity (Wang et al., 2007). The dysregulated expression of LMO4 is also found in some other human tumors, including prostate cancer (Mousses et al., 2002); small cell lung cancer (Karachaliou et al., 2013; Taniwaki et al., 2006); pancreatic cancer (Murphy et al., 2008; Yu et al., 2008) ; rhabdomyosarcoma (Armeanu-Ebinger et al., 2011).

Beyond its role in oncogenesis, accumulating evidence implicates a role for LMO4 in the regulation of proliferation and differentiation of developing neurons. Germ line ablation of LMO4 in mouse causes embryonic lethality and gives rise to exencephaly (Lee et al., 2005; Ochoa et al., 2012; Tse et al., 2004). During retina development, LMO4 is required for the expression of basic helix-loop-helix protein 5 (Bhlhb5) by inhibitory interneurons (Duquette et al., 2010). Bhlhb5 is a transcription factor that has been previously demonstrated as a postmitotic regulator of area identity in layer II-V of the developing neocortex, e.g. somatosensory and caudal motor cortex (Joshi et al., 2008). LMO4 also targets the transcription factor GATA3 and associates with the formation of vestibular structure (Alvarado et al., 2009). Mice with conditional ablation of LMO4 in the cerebral cortex show

defects in thalamocortical connections during development (Kashani et al., 2006). The role of LMO4 in cortical area formation is supported by other studies showing that LMO4 is involved in the organization of the somatosensory barrel field (Huang et al., 2009) as well as the diversity of motor cortex projection neuron subpopulations (Cederquist et al., 2013). In addition, LMO4 forms a complex with neurogenin 2 (NGN2) and facilitates NGN2-mediated radial migration of cortical neurons and neurogenesis in the developing cerebral cortex (Asprer et al., 2011). There is also evidence that LMO4 governs a balance between generation of inhibitory and excitatory neurons in the ventral spinal cord (Joshi et al., 2009). All these functions of LMO4 in development and oncogenesis, either transactivation or transrepression, are associated with modulation of multiple transcription factors and cofactors in the form of transcription complexes.

1.1.3 The Role of LMO4 in Adult Brain

Recently, the function of LMO4 in the adult brain has drawn the attention of researchers. A previous study in our laboratory revealed a role of LMO4 in the adult mouse cortex as an essential hypoxia-inducible cofactor required for activation of peroxisome proliferator-activated receptor-γ (PPARγ) signaling after ischemic injury (Schock et al., 2008a). We also provided evidence that LMO4 mediates leptin function in selective hypothalamic nuclei through Janus kinase/Signal transducer and activator of transcription 3 (JAK/STAT3) signaling for regulation of fat metabolism (Zhou et al., 2012). Recently, we further demonstrated that LMO4, in the hypothalumus, is an endogenous inhibitor for protein tyrosine phosphatase 1B (PTP1B) (Pandey et al., 2013). PTP1B is localized in the endoplasmic reticulum (ER) and is considered as a therapeutic target for obesity and diabetes

due to its inhibition of insulin and leptin signaling (Bourdeau et al., 2005; Dube et al., 2005; Zabolotny et al., 2002). Our studies showed that LMO4 palmitoylation at its C-terminal cysteine residue is critical for inhibition in PTP1B activity through increase of inactive oxidized forms of PTP1B. Importantly, LMO4 palmitoylation is sensitive to metabolic stress. Mice, challenged with acute exposure to saturated fat, had less palmitoylated LMO4, less oxidized PTP1B, and increased PTP1B activity, resulting in rapid loss of central leptin signaling. In addition, Heberlein's group also discovered an adult- and amygdala-specific role for the transcriptional regulator LMO4 in fear learning (Maiya et al., 2012). Mice heterozygous for a genetrap insertion in the LMO4 locus with 50% reduction of LMO4 expression display enhanced freezing to both the context and the cue. Knockdown of LMO4 by lentiviral small-hairpin RNA in the basolateral complex of the amygdala recapitulated this enhanced conditioning phenotype.

LMO4 is expressed in a variety of adult brain regions that also show most intense hybridization staining during embryonic stages. These regions include the hippocampus (e.g., the CA3 layer), the amygdala, the cerebral cortex, the hypothalamus, the cerebellum, the dentate gyrus, the basal ganglia, olfactory structures, intralaminar and reticular nuclei of the ventral thalamus and medullary motor structures (Hermanson et al., 1999; Kenny et al., 1998). However, the high levels of LMO4 expression in the entorhinal cortex and CA1 hippocampus were dramatically and consistently decreased in vulnerable brain regions of Alzheimer's disease cases, indicating that LMO4 might be a marker for complexity of the disease phenotype (Leuba et al., 2004). We reason that the widespread but subregion-specific distribution of LMO4 suggests its role on various functions, such as synaptic, autonomic, motor and neuroendocrine regulation.

1.1.4 LMO4 as a Bimodal Sensor in both Nucleus and Cytoplasm

LMO4 was initially identified as a cofactor of many transcription factors and is found only in nucleus (Kashani et al., 2006; Manetopoulos et al., 2003; Schock et al., 2008a). Recent studies revealed its novel functions in the cytoplasm. Our work showed that LMO4 is present both in the cytoplasm and nucleus. LMO4 translocates from the cytoplasm to the nucleus in response to extracellular stimuli in F11 cells that mouse neuroblastoma N18TG-2 cells are fused with embryonic rat dorsal-root ganglion (DRG) neurons (Chen et al., 2007b). This is consistent with another study showing that LMO4 is localized throughout the cytoplasm and growth cones in embryonic rat cortical neurons, human SH-SY5Y cells, human Ntera2 neurons (Schaffar et al., 2008). In fact, several LIM-containing proteins, such as LIM kinase, the actin binding LIM protein and Paxillin, have been shown to function both in the cytoplasm and the nucleus (Kadrmas and Beckerle, 2004). In addition to nuclear proteins, including transcription factors and cofactors, LMO4 has been shown to interact with transmembrane receptors such as the interleukin (IL)-6-type cytokines, erythropoietin-producing hepatoma interactor B (ephrin B), and neogenin to modulate their signaling (Bong et al., 2007; Novotny-Diermayr et al., 2005; Schaffar et al., 2008). LMO4 has a positive regulatory role in IL-6 signaling. LMO4 possibly functions as a scaffold for stabilization of the glycoprotein 130 complex. In this complex, LMO4 not only interacts with JAK to promote STAT3 and its target gene expression, it also interacts with protein tyrosine phosphatase 2 and suppressor of cytokine signaling 3 (SOCS3) to attenuate IL-6 signaling by inhibiting JAK and degrading proteins (Novotny-Diermayr et al., 2005). Moreover, LMO4 was identified by yeast two-hybrid screen to associate with ephrin B1 (transmembrane receptor tyrosine kinase) which can signal from the cell surface to the nucleus via recruitment

of Stat3 (Bong et al., 2007). In addition, LMO4 interacts with the cytoplasmic domain of transmembrane neogenin receptor. LMO4 is released upon activation of neogenin repulsive guidance molecule A (RGM A) and promotes Rho A signaling which has been shown to inhibit neuroregeneration (Schaffar et al., 2008). It is also known that the Rho family of GTPases including RhoA and Cdc42 play important roles in the spine morphogenesis and structure plasticity by regulating the actin cytoskeleton organization. By using two-photon uncaging of glutamate to activate N-methyl-D-aspartate receptors (NMDARs), RhoA and Cdc42 can relay transient CaMKII activation to increase the spine volume while also inducing LTP (Murakoshi et al., 2011). Since LMO4 is a transactivator induced by calcium influx via voltage gated calcium channel (VGCC) or NMDAR activation in a similar time frame (Aizawa et al., 2004; Kashani et al., 2006), it is interesting to examine whether LMO4 is an indispensable messenger for the Rho signaling responsible for this type of plasticity as well as whether and how LMO4 acts as a messenger to translate the various external stimuli into changes in gene expression. Taken together, these observations provide ample support for the hypothesis that LMO4 couples signals from membrane receptors to changes in gene expression.

1.2 Learning, Memory and Hippocampus

1.2.1 Learning and Memory

Learning is the process of acquiring new information while memory is retention of that information for future use. The neuronal mechanisms of these processes have intrigued neurobiologists for centuries and clinicians have been motivated to seek treatments for their failures or disruptions.

There are many types of memory. Tulving (Tulving, 2006; Tulving, 2000) in his books listed as many as 256 different types of memory by counting the number of combination of the adjectives plus memory sorts. Based on the time that it persists, memory can be categorized into short-term and long-term memory. Visual and auditory memories are examples of short-term memory while olfactory, visual-spatial, verbal and skill/motor memories are examples of long-term memory. Memory can also be classified into declarative, procedural and emotional memory by information type (Byrne, 2008).

Information is believed to be represented in various brain regions that are involved in the formation and storage of different forms of memories for different amounts of time. Short-term memory depends on transient adaptations of neuronal connections in the frontal lobe and the parietal lobe, whereas long-term memories may involve more persistent changes throughout the brain. Specifically, declarative memories are processed, encoded and consolidated by the hippocampus then moved to cortex for storage. Procedural memories are thought to be processed mainly in the motor cortex, cerebellum and basal ganglia. Emotional memories are encoded primarily in the amygdala then relayed to hypothalamus (Fig 1.2.1).

1.2.2 Hippocampus

Since Scoville and Milner (Scoville and Milner, 1957) described profound memory loss following bilateral medial temporal lobe excision in the landmark patient H.M., the hippocampus has been recognized as the critical structure for learning and memory, including consolidation of information acquired in learning and the storage of long-term memory.

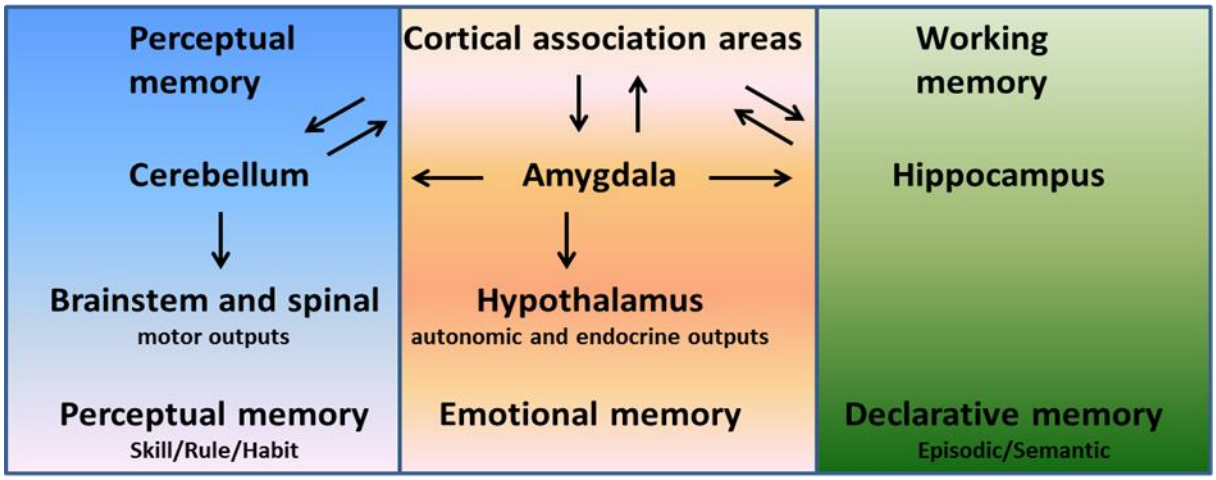

Fig 1.2.1 Anatomic pathway of main memory systems

Systems for perceptual and working memory involve networks between the cortical association areas and other memory systems. The perceptual memory system involves pathways through the cerebellum that connect directly with motor outputs. The emotional memory system involves pathways through the amygdala that connect directly with the hypothalamus and subsequent autonomic motor and endocrine outputs, and amygdala influences the strength of memories in other systems. The declarative memory system involves bidirectional interactions between the hippocampus and cortical association areas.

Hippocampus is part of the medial pallium which arises from the dorsal medial part of the neural tube. The hippocampal formation including dentate gyrus, CA3, CA1, is a three-layered structure characterized by largely unidirectional connections, whereas the surrounding region (parahippocampal region) is cortical in nature and six-layered (Byrne, 2008; Deng et al., 2010; Moser, 2011). The dentate gyrus (DG) contains modified pyramidal cells called granule cells that have only apical dendrites. The CA fields contain fairly typical pyramidal cells with both basal and apical dendrites covered with spines. The hippocampus gets most of its input from the neocortex via the entorhinal cortex. In addition, the hippocampus receives input from the septum and the hypothalamus via the fornix/fimbria. The schematic drawing (Fig 1.2.2) illustrates the main hippocampal circuits (Deng et al., 2010). The EC is the source of the temporoammonic pathway (TA) as well as perforant pathway (PP) which includes the lateral perforant pathway (LPP) and medial perforant pathway (MPP). Entorhinal layer II projects to DG and CA3 through PP, whereas layer III projects to CA1 through TA. The DG granule cells give rise to the mossy fibre pathway, which targets the proximal apical dendritic spines of CA3 pyramidal neurons. The CA3 pyramidal neurons project to CA1 apical dendritic spines through Schaffer collaterals pathway and eventually CA1 pyramidal neurons send projections out of the hippocampus back to the deep layer neurons of the EC. CA3 pyramidal cells also project down to the hypothalamus via the fornix, which include both input and output pathways (van Strien et al., 2009). The cortical areas surrounding the hippocampal formation and the nearby parahippocampal region are also necessary for human declarative memory.

Based on a clear stereotypical organization of neuronal layers, well-defined synaptic components and neural pathways as well as the essential roles in learning and memory,

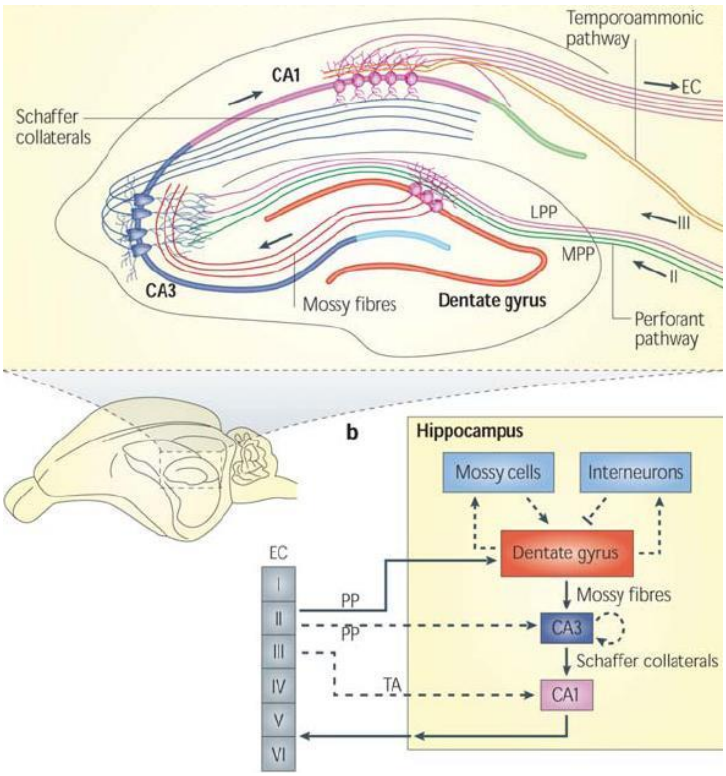

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Figure 1.2.2 The main hippocampal circuitry

A, An illustration of the hippocampal circuitry

B, Diagram of the hippocampal neural network. The traditional excitatory trisynaptic loop, composed of synapses between principal cells in three major subregions: the dentate gyrus, CA3 and CA1, describes a unidirectional route of incoming signals into the hippocampus. The entorhinal cortex (EC) is the source of the temporoammonic pathway (TA) as well as perforant pathway (PP) which includes the lateral perforant pathway (LPP) and medial perforant pathway (MPP). Entorhinal layer II projects to the dentate gyrus (DG) and CA3 through PP, whereas layer III projects to CA1 through TA. The DG granule cells give rise to the mossy fibre pathway, which targets CA3 pyramidal neurons. The CA3 projects to CA1 through Schaffer collaterals pathway and eventually CA1 pyramidal neurons send back projections to the deep layer neurons of the EC.

hippocampus has drawn intensive studies. Importantly, the cellular and molecular mechanisms of neuronal functions revealed in the hippocampus have been shown to be widely employed by other neural circuits in the brain.

1.3 Anxiety, Fear and Amygdala

1.3.1 Anxiety and Fear

Anxiety is the motivation associated with behaviors that occur to potential, signaled, or ambiguous threat while fear is the motivation associated with behaviors that occur to clearly threatening stimuli (Blanchard et al., 2008). Since characterizations are based on measurement of the intensity or persistence of the behaviors with which they are associated, anxiety and fear may integrate or overlap. Anxiety disorders are often conceptualized as the pathological counterpart of normal fear. They are amongst the most common psychiatric disorders. Statistics shows that anxiety disorders affect approximately 40 million adults (18%) age 18 years and older in the United States (Kessler et al., 2005) and about 12% of the Canadian population (Offord et al., 1996) in a given year. Individuals with an anxiety disorder are filled with fearfulness and uncertainty. They have excessive and unrealistic feelings that interfere with their lives. Psychological symptoms can range from discrete episodes of intense fear, such as in panic disorder, in post traumatic stress disorder, in social phobia, in specific phobias, to anxiety not attributable to specific factors but lasting for prolonged periods of time, such as in generalized anxiety disorder. Often these psychological symptoms are accompanied by autonomic (cardiovascular, gastrointestinal, etc) symptoms, as well as activation of the hypothalamic pituitary adrenal (HPA) axis which mediates the stress response. Anxiety disorders are usually treated with benzodiazepines or antidepressants,

especially selective serotonin re-uptake inhibitors (SSRIs). Benzodiazepines have a fast onset of action but they can induce some side effects, such as sedation, psychomotor retardation, memory impairment, withdrawal syndrome, and rebound anxiety. Antidepressants have a much better safety and tolerability profile but a slow onset of action. They can induce other side effects, such as various types of sexual dysfunction and suicidal behaviour. Development of novel therapeutic approaches other than the clinically widely used benzodiazepines and antidepressants could be a promising direction for researchers.

1.3.2 Amygdala Circuitry

The amygdala, first named by Burdach in early 19th century, is an almond-shaped mass of gray matter in the medial temporal lobe with a multinuclear complex comprised of 13 nuclei. These nuclei are divided into three major groups: the basolateral complex, the cortical nucleus, the centromedial nucleus (central and medial nucleus). The basolateral complex can be further subdivided into the basal nuclei, the lateral nuclei (LA), and the accessory basal nuclei (Amunts et al., 2005). A schematic illustration of the main amygdala input and output circuitry is shown in Figure 1.3 (Ciocchi et al., 2010; Ehrlich et al., 2009; Krasne et al., 2011). The lateral nuclei are considered to be the main target of afferents to the amygdala. It receives substantial inputs from sensory-related cortical areas, prefrontal cortex, hippocampus, hypothalamus and some midline and posterior thalamic nuclei. The LA then sends impulses to the rest of the basolateral complex including basal and the centromedial nuclei. The basal nuclei project to the cortex, hippocampus, nucleus accumbens and caudate-putamen, the amygdalo-hippocampal area, nuclei of the olfactory tract and lateral, anterior

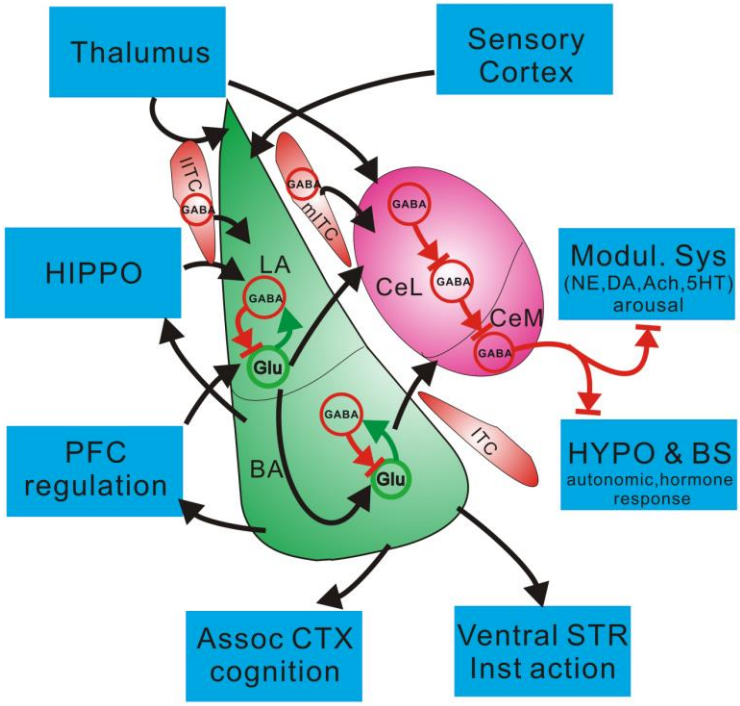

Figure 1.3 Anatomical organization of the amygdala circuitry

Black arrows represent overall flow of information in the amygdaloid complex. Excitatory neurons and inhibitory interneurons in amygdaloid nuclei are shown in green and red circles, respectively. In the LA and BA, local interneurons are part of feedforward and feedback circuits and control projection neuron output. mITCs and lITCs and relay feedforward inhibition to the CEA and BLA, respectively. CeM output is the major output from amygdala under inhibitory control receiving information from CeL. Intrinsic CeL inhibition may be also involved in regulation of CeL output.

LA, lateral amygdala; BA, basal amygdala; CeL, lateral subdivision of the central amygdala; CeM, medial subdivision of the central amygdala; mITC, medial intercalated cell cluster; lITC, lateral intercalated cell cluster; CEA, central amygdale; BLA, basolateral amygdala; HIPPO, hippocampus; HYPO, hypothalamus; BS, brainstem; PFC, prefrontal cortex; Assoc CTX, association cortex; Ventral STR, ventral striatum; Modul Sys, modulatory system; Inst action, instrumental action; Glu, glutamate; GABA, *gamma*-Aminobutyric acid; NE, norepinephrine; DA, dopamine; Ach, acetylcholine; 5-HT, 5-Hydroxytryptophan.

cortical and central nuclei as well as to the nucleus of the olfactory tract. The accessory basal nuclei receive sensory related information similar to the basal nuclei. The cortical nuclei which are known as olfactory amygdala, receives input from olfactory bulb and cortex for smell sensing. The centromedial nuclei are the main outputs for the basolateral complexes, and are involved in emotional arousal. The amygdala is the key brain region within the limbic system controlling anxiety and fear memory, a statement consistently supported by a large amount of studies using different experimental paradigms and measurement of fear responses (Ehrlich et al., 2009; Krasne et al., 2011; LeDoux, 2000; Maren, 2001, 2005; Maren and Quirk, 2004). In addition, the amygdala also regulates fear-related learning in other brain structures, such as the hippocampus and the cortex (McGaugh, 2004). By using optogenetics with two-photon microscopy, electrophysiology and anxiety assays in freely moving mice, an elegant study showed that increasing synaptic activity from BLA neurons onto the CeA was anxiolytic whereas reducing this activity was anxiogenic (Tye et al., 2011). MRI evidence indicates that the function of the amygdala is disrupted in anxiety disorders (Etkin et al., 2009). There is evidence that the amygdala is not limited as the interface of anxiety and fear, also contributions to social behavior, instrumental behavior and reward learning (Murray, 2007).

1.3.3 Stress-induced Anxiety Disorder

Stress is the real or interpreted threat to the physiological or psychological integrity of an individual or the disturbance to homeostasis. Selye (Selye, 1950) first made the distinction between stressors and stress. Stressors are defined as stimuli and stress is termed as the internal state experienced by the organism upon exposure to a stressor. Experimental stress

protocol can be differed by acute and chronic paradigms. An acute protocol is a brief one-time exposure to a single stressor. A chronic protocol can be either a prolonged one-time exposure to a single stressor or a repeated exposure to either the same (homotypic) or variable (heterotypic) stressor. A homotypic stressor typically involves habituation which dampens the detrimental effects of the stress response as well as increases the psychological tolerability. As part of the stress response two systems are activated: the sympatho-adrenomedullar system and the hypothalamic-pituitary-adrenal axis. Stress rapidly leads to the release of catecholamines (CAs) from the adrenal medulla, sympathetic nervous system and catecholaminergic neurons in the brain. CAs integrate the signals and activate corticotrophin releasing hormone (CRH) transcription in the hypothalamus. CRH-producing neurons in the paraventricular nucleus of the hypothalamus (PVN) then stimulate cells of the anterior pituitary to secrete adrenocorticotropic hormone (ACTH) which is then released into the blood system. At the adrenal cortex, ACTH stimulates glucocorticoid transcription and release (corticosterone in most rodents, cortisol in humans). Hormonal stress response prepare an organism for a threat by mobilizing energy stores, increasing arousal, focusing attention, and mediating cognition while dampening digestive, immune, and reproductive functions (Hill et al., 2010b).

The limbic system is the set of brain structures that forms the inner border of the cortex including hippocampus, amygdala, limbic cortex, fornix, hypothalamus, thalamus, etc. The limbic system is well-known to be involved in emotions. Particularly two limbic structures-hippocampus and amygdala have a pivotal role for memory formation and stress responses. Their unique features of stress-induced plasticity could have long-term impacts for cognitive performance and pathological anxiety. The mechanism is more complex than the simple

traditionally-proposed neural–endocrine model with involvement of catecholamines (CAs) and hypothalamic pituitary axis (HPA). It is likely to recruit other neural neuromodulators and the limbic structures (e.g. thalamus, prefrontal cortex, striatum, etc)(Kim and Diamond, 2002).

During a stressful experience, neurons have been suggested to receive a enhanced noradrenergic input from the locus coeruleus (Sara, 2009). Noradrenaline (one of CAs) first reaches limbic cells and exerts its rapid actions within seconds upon activation of G-protein-coupled membrane receptors (GPCRs). However, the wave only lasts less than 30 minutes (McIntyre et al., 2002). Meanwhile, the same neurons receive high levels of the corticosterones with a slower kinetics and reach the peak round 20 minutes later than stress onset. Normalization usually takes place after 1–2 hrs. Corticosterone can associate with other monoamines (dopamine, serotonin) or neuropeptides (CRH, vasopressin) and quickly change neuronal function in a nongenomic manner. There is a time window when limbic cells are exposed to elevated levels of both noradrenaline and corticosteroid hormones (Joels and Baram, 2009; Joels et al., 2011). Nevertheless, corticosterone may also alter neuronal activity through genomic mechanisms. Corticosteroid hormones easily pass the blood-brain barrier and activate all the receptive cells in the brain. Corticosteroid release is pulsatile, with inter-pulse intervals of one or two hours. The peak of the pulses follows an overall circadian rhythm and varies with low amplitudes around the start of the inactive period and high amplitudes towards the active period onset of the day. Exposure to stress causes a large surge of corticosteroids. Acute glucocorticoid secretion during stress may produce intense, long lasting memories of the events themselves. However, chronic over-exposure to circulating glucocorticoids may affect neuronal structure and survival and impair subsequent attention

and memory, leading to dendritic atrophy in hippocampal principal neurons and dendritic hypertrophy in amygdala principal neurons (Kim and Diamond, 2002; Sapolsky, 2000, 2003; Vyas et al., 2002) . Extreme stress experience may even induce profound amnesia, phobias, post-traumatic stress disorder (PTSD), which belong to the category of anxiety disorders. One of the key features of successful adaptation is remembering the important aspects of the stressful situation and reducing interference by unrelated material for future use. Limbic areas, in particular the amygdala, play an important role in establishing such memories.

1.4 Neurotransmission and Synaptic Plasticity

1.4.1 The Synapse

The term synapse comes from "synaptein", coined from the Greek "syn-" (together) and "haptein" (to clasp) by Sir Charles Scott Sherrington and colleagues. The synapse is a highly specialized structure where neurons communicate with each other by exchanging electrical or chemical signals. It is the fundamental ‘unit’ where memory and information is encoded, stored and retrieved. A typical neuron consists of a cell body (soma), one axon and multiple dendrites. The axon originates from the cell body and may extend for meters long, whereas dendrites are confined to the proximity of the neuron. At a synapse, the presynaptic neuron comes into close apposition with the postsynaptic neuron by plasma membrane. The majority of presynaptic part is located on an axon, others are located on a dendrite or soma. Both the pre- and post- synaptic sites contain extensive arrays of molecular machinery and carry out the signaling process.

There are mainly two different types of synapses (Kandel, 2013). In an electrical synapse, special channels called gap junctions, pass voltage changes and rapidly transfer the

signals from the presynaptic cell to the postsynaptic cell. In a chemical synapse, an endogenous chemical called a neurotransmitter is released from the presynaptic neuron, and binds to receptors (e.g. ligand-gated ion channels or ligand-binding G-protein coupled receptors) located in the plasma membrane of the postsynaptic cell. A postsynaptic cell may be either depolarized or hyperpolarized based on the property of the neurotransmitter and the receptors. Summation of numerous synaptic neurotransmissions on the same postsynaptic neuron determines its membrane potential and firing patterns, causing either an excitatory/ inhibitory response or activation of a biochemical intracellular signaling pathway. The residual neurotransmitter may be either enzymatically degraded by scavengers, or reuptaken back by transporters into the presynaptic site for reuse. Astrocytes also communicate with the synaptic neurons in response to synaptic activity and, in turn, regulate neurotransmission (Perea et al., 2009).

1.4.2 Major Neurotransmitters and their Receptors in Hippocampus and Amygdala

1.4.2.1 Glutamate and Glutamate Receptors

Glutamate is the most abundant excitatory neurotransmitter found in the mammalian brain. It binds to two distinct classes of receptors: ionotropic glutamate receptors (AMPA receptors, NMDA receptors and kainate receptors) and metabotropic glutamate receptors (mGluRs).

AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid)-type glutamate receptors (AMPARs) are the main transducers of rapid excitatory transmission in the mammalian brains (Anwyl, 2009; Collingridge et al., 2004; Derkach et al., 2007; Jiang et al., 2006). AMPAR functional properties are coupled to their trafficking, cytoskeletal dynamics and local protein synthesis (Bredt and Nicoll, 2003; Esteban, 2003; Malenka, 2003;

Minichiello, 2009; Shepherd and Huganir, 2007). AMPARs can form tetramers composed of four glutamate receptor subunits, GluR1 – GluR4. However, most AMPARs are heterotetramers comprised of GluR2 plus GluR1, GluR2 or GluR4 subunits. The most divergent regions of these subunits are their carboxyl (C) termini, which contain regulatory domains targeted by multiple intracellular signaling pathways. The C termini also interact with scaffold proteins that bind signaling proteins (e.g. kinases, phosphatases) as well as cytoskeletal proteins (e.g. actin) (Collingridge et al., 2004; Humeau et al., 2007; Malinow and Malenka, 2002; Roche et al., 1996; Tomita et al., 2003). These protein complexes may influence several aspects of AMPAR function including gating, trafficking, and stabilization at synapses. It is widely believed that the regulated AMPAR trafficking to or away from the synapse mediates the long-lasting activity-dependent changes in synaptic strength called long-term potentiation (LTP) or long-term depression (LTD) (Bliss and Lomo, 1973; Malinow and Malenka, 2002). In dendritic regions, AMPARs can be found in four non-synaptic compartments: (1) on the spine surface; (2) intracellularly in the spine; (3) on the dendritic surface; (4) intracellularly in the dendrite. Two routes for synaptic delivery of AMPARs are observed in neurons: (1) lateral diffusion from the surface (Borgdorff and Choquet, 2002; Heine et al., 2008; Petralia and Wenthold, 1992; Triller and Choquet, 2005); (2) exocytosis from intracellular compartments (Passafaro et al., 2001; Wang et al., 2008; Yang et al., 2008a, b). Makino and Malinow (Makino and Malinow, 2009) used recombinant surface-fluorescent receptors in organotypic rat hippocampal slices to distinguish surface or intracellular receptors. They demonstrated that following synaptic potentiation, both lateral movement and exocytosis of receptors occur in a time- and space-dependent manner with varied functions. Lateral movement of receptors from nonsynaptic sites enhances the number of receptors at the

synapse whereas exocytosis of receptors occurs onto the dendrite likely to replenish the local extrasynaptic pool of receptors available for subsequent synaptic potentiation.

N-methyl-D-aspartate receptors (NMDARs) are located either intra-synaptically or extra-synaptically. NMDARs are also often heterotetrameric complexes containing a 'dimer of dimers' conformation from the subunits NR1, NR2A-NR2D, and NR3A-NR3B (Madden, 2002). The NR1 subunit that binds the co-agonist glycine is widely expressed and obligatory for NMDAR-channel activity. The NR2 subunits (A-D) that bind to glutamate, convey different pharmacological characteristics and decay kinetics on functional NMDARs. The NR2A and NR2B are the two most abundant subunits in the brain. NR2A-containing NMDARs have more rapid kinetics than NR2B-containing NMDARs. Their expression levels often change during postnatal development. NR2B subunits are expressed abundantly at early developmental stages and subsequently migrate to extrasynaptic sites, whereas NR2A subunits progressively increase with development. NR3 A and B subunits that bind to glycine, have an inhibitory effect on receptor activity (Liu et al., 2004; Massey et al., 2004). NMDARs have the three fundamental domains: an extracellular domain that contains a modulatory and a ligand-binding residue; a membrane domain that contributes to the receptor's conductance, calcium permeability, and voltage-dependent magnesium block; and an extensive cytoplasmic domain that consists of residues that can be directly modified by a series of protein kinases and protein phosphatases, as well as residues that interact with a large number of adaptor, structural, and scaffolding proteins (Paoletti and Neyton, 2007). At resting membrane potential, the NMDA receptor is blocked by magnesium. The AMPA receptor is first activated by binding to glutamate and it then depolarizes the post-synaptic cell. This leads to NMDA receptor activation with associated calcium influx to trigger four main mechanisms

contributing to synaptic plasticity in spines: 1) the regulation by kinases and phosphatases of channels; 2) alterations of synaptic AMPAR properties, subunit composition; 3) actin reorganization and modulation of spine morphology; and 4) initiation of local protein synthesis in spines and dendrites (Derkach et al., 2007). These mechanisms are associated through a complex network of signal transduction pathways. Depending on the pattern of synaptic activity and the previous history of the synapse, bidirectional synaptic plasticity (LTP or LTD) can occur. This can involve AMPAR lateral trafficking between extrasynaptic and synaptic sites and changes in scaffolding and cytoskeletal proteins. Questions about the relationship of AMPAR and NMDAR plasticity, mRNA translation and dynamic cytoskeletal regulation remains and more insightful investigations are needed.

In contrast to the ionotropic glutamate receptors which mediate the fast excitatory neurotransmission, the metabotropic glutamate receptors (mGluRs) have been shown to play a more modulatory role in neuronal excitability and synaptic transmission. The mGluRs are family of G-protein coupled receptors that contain heterotrimeric complex of α, β, γ subunits. The mGluRs are subdivided into three groups based on sequence homology, second messenger coupling and pharmacology. Group I (mGluR1 and mGluR5) are coupled to Gq/11 protein, phospholipase Cβ and intracellular calcium signaling as postsynaptic receptors involved in increasing neuronal excitability. In addition, activation of postsynaptic mGluRs may inhibit neurotransmission by generating the retrograde messengers (e.g. endocannabinoids) and exerting presynaptic effects on transmitter release. (S)-3, 5-dihydroxyphenylglycine [(S)-3, 5-DHPG], the first and most selective agonist at group I mGluRs, has similar potencies at mGluR1 and mGluR5. Though 2-chloro-5-hydroxyphenylglycine (CHPG) is a selective mGluR5 agonist, it has not been widely used

due to its relatively weak potency and efficacy. LY367385 is a commonly-used selective antagonist of mGluR1 while CPCCOEt is the first highly selective negative allosteric modulator of mGluR1. Both MTEP and MPEP remain highly useful selective mGluR5 antagonists because of the relatively high potency, selectivity and brain penetration. Group II (mGluR2 and mGluR3) and group III (mGluR4, mGluR6, mGluR7, mGluR8) are coupled to Gi/o proteins and inhibit adenylyl cyclase as presynaptic receptors involved in reducing neurotransmitter release. LY354740 is the highly selective agonist of group II mGluRs and L-AP4 is the one of group III mGluR (Niswender and Conn, 2010; Pinheiro and Mulle, 2008). These generalized physiological roles of mGluRs maintain in many instances, however there are also exceptions: activation of mGluR1 by synaptic stimulation or brief agonist application induces hyperpolarization rather than depolarization in midbrain dopamine neurons (Valenti et al., 2002); mGluR1 and mGluR5 have distinctive roles in the regulation of the excitability in CA1 pyramidal cells where mGluR1 activation leads to somatic calcium elevation and depolarization while mGluR5 activation inhibits Ca2+- activated potassium currents reflected by afterhyperpolarization potentials and potentiates NMDA receptor currents (Mannaioni et al., 2001).

The mGluR has a large extracellular domain to bind glutamate and transmit signals through the receptor protein to intracellular partners. The C-terminal tails of mGluR is critical for receptor activity regulated by protein- protein interactions. The widespread distribution of mGluRs throughout the central nervous system (CNS) suggests the ability to participate in many neuronal functions, and thus the mGluRs represent attractive drug targets for therapeutic intervention in a variety of neurological and psychiatric disorders such as anxiety,

depression, schizophrenia, pain, Alzheimer's disease and Parkinson's disease (Anwyl, 2009; Coutinho and Knopfel, 2002; Gasparini and Spooren, 2007; Niswender and Conn, 2010).

1.4.2.2 GABA and GABA Receptors

γ-Aminobutyric acid (GABA) is the main inhibitory neurotransmitter which plays a role in regulating neuronal excitability and network connection in the CNS. It binds to specific transmembrane receptors called GABA receptors in both pre- and postsynaptic neuronal processes. There are two classes of GABA receptors: $GABA_A$ receptors, which are ligand-gated ion channels (ionotropic receptors) and $GABA_B$ receptors, which are G protein-coupled receptors (metabotropic receptors). In the brain, most inhibitory effects of GABA are mediated by GABAA receptors, which are heteropentameric chloride channels formed by various combination of different types of subunits (e.g. α 1-6, β1-3, γ1-3, δ, ε, π, θ). Each subunit consists of four hydrophobic transmembrane (TM1–4) domains, where TM2 is believed to line the pore of the chloride channel. In addition, each receptor subunit consists of a large intracellular domain between TM3 and TM4, which is the site for various protein interactions as well as the site for various post-translational modifications that modulate receptor activity. At the large extracellular N-terminus, besides the active binding site for the GABA, $GABA_A$ receptor contains a number of different modulatory allosteric binding sites for other drugs (e.g. benzodiazepines, nonbenzodiazepines, barbiturates, general anaesthetics, neurosteroids, picrotoxin, etc). $GABA_A$Rs with different subunit composition have distinctive physiological and pharmacological properties, are differentially distributed in different subcellular regions throughout the brain. Benzodiazepine-sensitive receptors that are largely synaptically located and mediate the majority of phasic inhibition, are composed of α (1, 2, 3

or 5) together with β and γ subunits. On the contrary, benzodiazepine-insensitive receptors that are predominantly extrasynaptic situated and mediate tonic inhibition, contain a special population of α (4 or 6) together with β and δ subunits (Jacob et al., 2008). Mild inhibition neuron firing by enhancing $GABA_A$ activation causes a reduction of anxiety in the patient (an anxiolytic effect) while an overdose may induce general anesthesia.

Mature neurons maintain a low concentration of chloride due to the activity of K^+/Cl^- co-transporter and thus in most cases, activation of the $GABA_A$ receptor allows a net flow of chloride through its pore into the cell, resulting in hyperpolarization and an inhibitory effect on excitability. However, the gradient of chloride is reversed in immature neurons with a relative high concentration of chloride, activation of the $GABA_A$ receptor produces efflux of Cl^- ions from the cell, leading to depolarization and a primarily excitatory action in the developing brain (Ben-Ari et al., 2007; Ganguly et al., 2001).

1.4.2.3 Endocannabinoids and Cannabinoid Receptors

Endocannabinoids (eCBs) are a class of neuroactive lipids that can activate cannabinoid receptors and participate in multiple physiological processes ranging from memory, anxiety, addiction, appetite, pain to neuroprotection (Di Marzo, 2009, 2011; Kano et al., 2009). In particular, eCBs produce anxiolytic and mood elevating effects thus promoting relaxation and reducing stress (Green et al., 2003). The endocannabinoid system consists of the cannabinoid receptors (CB1R, CB2R), eCBs and the enzymes that synthesize and degrade endocannabinoids. CB1Rs, which are the most abundant GPCR in the central nervous system, are present at high levels in GABAergic interneurons as well as in lower amounts at glutamatergic terminals, proximal axons, dendrites and cell bodies. CB1Rs are also functionally expressed in CA1 astrocytes (Han et al., 2012). CB2 receptors were thought to be

restricted within the immune system, but their existence in neurons and microglial cells has been demonstrated (Nunez et al., 2004; Van Sickle et al., 2005). There is a consensus that both CB1Rs and CB2Rs can control both central and peripheral functions, including neuronal transmission and inflammation, hormone release, respiratory and reproductive functions, and energy metabolism (Di Marzo, 2009; Mackie, 2008). Anandamide (AEA) and 2-arachidonoylglycerol (2-AG) are well characterized natural ligands for cannabinoid receptors. Both AEA and 2-AG are produced postsynaptically in a Ca^{2+}-dependent manner. However, AEA has relatively lower endogenous quantities and displays lower intrinsic efficacy than 2-AG. AEA has also a distinct biochemical route for synthesis and catabolism in comparison with 2-AG. For example, AEA is synthesized from phospholipid precursors by a calcium-dependent transacylase (CDTA) whereas 2-AG is produced by phospholipase C (PLC) and diacylglycerol lipase (DAGL) respectively. AEA is degraded postsynaptically to arachidonic acid (AA) by fatty acid amide hydrolase (FAAH), whereas 2-AG is presynaptically hydrolyzed to AA by monoacylglycerol lipase (MAGL), though cyclooxygenase -2 (COX-2) may degrade both AEA and 2-AG via an oxidation process. Evidence suggests that AEA is primarily synthesized tonically under basal conditions and serves as a "gatekeeper" that must be lowered to maintain HPA-axis functioning, whereas 2-AG may be mainly produced phasically in an activity-dependent manner (Hill et al., 2009b). Importantly, AEA but not 2-AG is a ligand for transient receptor potential vanilloid 1 (TRPV1), which is a six-transmembrane (TM) domain integral membrane protein containing a nonselective cation-permeable pore region as well as cytosolic C- and N-terminal domains. The binding site is located on the intracellular face thus raising the possibility that when anandamide is biosynthesized in cells expressing TRPV1, it will activate this receptor before being released,

thereby regulating calcium homeostasis as a second messenger (Pertwee et al., 2010). A common model for eCB signaling is schematically illustrated in Figure 1.4.1 modified from Figure 2 (Ahn et al., 2008). Unlike traditional neurotransmitters, eCBs are usually synthesized on demand at postsynaptic neurons rather than stored in vesicles (Ahn et al., 2008; Rodriguez de Fonseca et al., 2005). eCBs then act retrogradely and bind to presynaptically-located CB1Rs. Once activated, CB1Rs couple through the G_i /G_o class of G-proteins mainly to inhibit adenylyl cyclase and to activate inwardly rectifying potassium channels (Di Marzo, 2009; Twitchell et al., 1997). These actions serve to reduce probability of neurotransmitter release and inhibit synaptic neurotransmission in excitatory and inhibitory circuits thus fine tuning the strength of a particular synapse (Freund et al., 2003).

1.4.3 Synaptic Plasticity

In general, the term 'synaptic plasticity' refers to the ability of the neuronal synapse to change transmission in strength in response to experience-driven external stimuli over synaptic pathways. It is also known as Hebbian theory, proposed by the Canadian psychologist Donald Olding Hebb in his book "The Organization of Behavior: A Neuropsychological Theory" (Hebb, 1949). The model is best expressed by this quote: "When an axon of cell A is near enough to excite cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased." It describes a mechanism of neuron adaptation how elevation in synaptic efficacy arising from the presynaptic cell repeatedly and persistently stimulates the postsynaptic cell. It is also paraphrased as 'Cells that fire together, wire together.' Synaptic plasticity is believed to be a fundamental

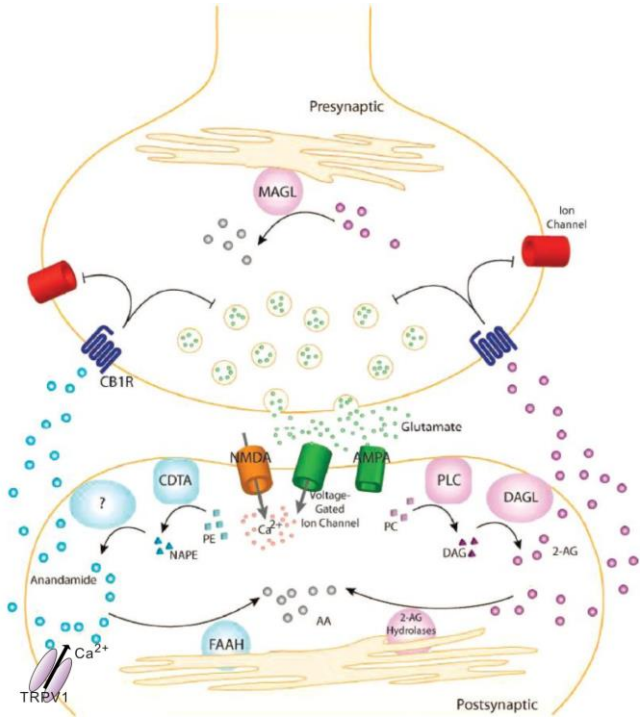

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Figure 1.4.1 A general model for endocannabinoid-based retrograde signaling

Anandamide is synthesized from phospholipid precursors by a calcium-dependent transacylase (CDTA) and one or more other still uncharacterized enzymes. 2-Arachidonoylglycerol (2-AG) is synthesized from phospholipid precursors by phospholipase C (PLC) and diacylglycerol lipase (DAGL). Endocannabinoids then migrate from postsynaptic neurons to CB1 receptors (CB1R) located on presynaptic neurons. Once activated, CB1Rs couple through the G_i/G_o class of G-proteins to regulate ion channels and to inhibit neurotransmitter release. The retrograde signaling of endocannabinoids is then terminated by degradative enzymes. Anandamide is hydrolyzed to arachidonic acid (AA) primarily by fatty acid amide hydrolase (FAAH), located in the postsynaptic neuron. 2-AG is hydrolyzed to AA primarily by monoacylglycerol lipase (MAGL) in the presynaptic neuron, though other 2-AG hydrolases may also participate in this process. AEA but not 2-AG can also activate transient receptor potential vanilloid 1 (TRPV1) and subsequently regulates calcium homeostasis as a second messenger.

mechanism employed by the nervous system for memory formation. It is also playing a role in many psychological diseases.

Synaptic plasticity is often classified by time scale as short term plasticity vs long term plasticity or distinguished by polarity as synaptic facilitation vs synaptic depression. Two typical experiment examples of how synaptic transmission can be reversibly enhanced or reduced are LTP and LTD, whose mechanisms have been the subject of considerable studies over the past few decades. LTP is a long-lasting enhancement in signal transmission derived from synchronization between two neurons whereas LTD is a prolonged weakening of transmission efficacy. LTP can be experimentally induced at all ages of animal models. On the contrary, LTD is developmentally regulated and only prevalent at young ages. Since the majority of excitatory synaptic transmission in the brain is AMPA-mediated, LTP and LTD are normally depicted by the enhancement or weakening of AMPAR-mediated currents. Although the expression mechanisms underlying synaptic plasticity vary amongst different brain regions, both LTP and LTD are initiated by depolarization or/and activation of NMDARs and/or mGluRs. The Ca^{2+} influx through NMDARs and/or mGluR further engages cascades of signaling molecules to modify synaptic transmission.

1.4.3.1 Calcium-dependent Synaptic Transmission and Plasticity

Calcium influx into the cytosol mediates a wide range of cellular responses, including neurotransmitter release, plasticity, excitability, gene transcription, cell division, survival, and differentiation (Berridge, 1998; Gomez-Ospina et al., 2006; Kisilevsky and Zamponi, 2008). Calcium might enter cells through ligand-gated receptors (e.g. NMDAR, TRPV1 described in previous sections) and VGCCs. VGCCs are composed of up to five subunits, including the main α1 subunit and α2δ, β and γ as auxiliary subunits. They can be divided into several

subfamilies, including the L-type (Cav1.1-1.4); the P/Q-type (Cav2.1); the N-type (Cav2.2); the R-type (Cav2.3); and the T-type (Cav3.1-3.3) channels, or can be broadly classified into high and low-voltage activated channels (HVA and LVA channels, respectively). Different types of VGCCs exert specific physiological functions as well as electrophysiological and pharmacological properties (Kisilevsky and Zamponi, 2008). T-type belongs to LVA calcium channel that activate in response to small membrane depolarizations with rapid gating kinetics and a small conductance. In contrast, HVA channel requires stronger membrane depolarizations for activation and can be further classified into N-, P-, Q-, R-, and L-types, based on their pharmacological profiles. N-type channels are selectively blocked by ω-conotoxins GVIA, MVIIA. P- and Q-type channels are sensitive to ω-agatoxin IVA. R-type channels are potently inhibited by the spider toxin SNX-482, and L-type channels are sensitive to dihydropyridines (e.g. nifedipine). VGCCs subtypes also exhibit specific subcellular distributions, e.g. L-type calcium channels are mainly expressed on cell bodies and facilitate calcium-dependent gene transcription (Dolmetsch et al., 2001; Weick et al., 2003), whereas both N-type and P/Q-type channels are expressed at presynaptic nerve terminals and govern evoked neurotransmitter release (Ishikawa et al., 2005).

There is mounting evidence suggesting that calcium-induced calcium release (CICR) from intracellular pools including endoplasmic reticulum (ER) or sarcoplasmic reticulum (SR) is a powerful modulator of neurotransmitter release, at least under some conditions (Emptage et al., 2001; Llano et al., 2000; Peng, 1996; Zhang et al., 2009). But the contributing role of CICR to setting basal release probability during low-frequency neurotransmission may be equivocal (Carter et al., 2002). CICR was first discovered in muscle cells crucial for excitation-contraction coupling in the late of 60s by the pioneering works of Weber and

Ebashi (Endo, 1977). Later, CICR is found to be a widely-occurring cellular signaling process present in many other cell types, such as neurons and pancreatic beta cells. Ryanodine (RYRs) and Inositol 1,4,5-tris-phosphate receptors (InsP3Rs) are the two major receptors that are located on the endoplasmic reticulum and are responsible for releasing Ca2+ from the internal store (Berridge, 1998). Like the plasma membrane, the ER, named a neuron within a neuron, has shown both integrative and regenerative properties that might play important roles in synaptic transmission and plasticity.

CICR can modulate neuronal excitability by altering membrane potential. Pyramidal neurons display prominent after-hyperpolarizations (AHPs) following either a single action potential or bursts of action potentials (Alger and Nicoll, 1980; Hotson and Prince, 1980; Schwartzkroin and Stafstrom, 1980). AHPs that are mediated by calcium-activated potassium channels, modify neuronal activity by suppressing firing patterns and contribute to spike frequency adaptation. Although the calcium gating these potassium conductances arises in large part from voltage-gated calcium channels (Marrion and Tavalin, 1998; Pineda et al., 1999; Pineda et al., 1998; Sah, 1996), CICR contributes a significant portion of the calcium responsible for the generation of the AHP. This is supported by the observations that the AHP is reduced by CICR inhibitors such as ryanodine or thapsigargin while facilitated by caffeine (Akita and Kuba, 2000; Kuba et al., 1983; Pineda et al., 1999; Sah, 1996; Sah and McLachlan, 1991; Torres et al., 1996).

It is well established that intracellular calcium concentration ($[Ca^{2+}]_i$) is the single most important factor in the regulation of both pre- and post- synaptic plasticity (Berridge, 1998; Zucker, 1999). In presynaptic terminals, extensive studies have shown that, in addition to calcium influx from VGCC and ligand-gated receptors, CICR contributes to

neurotransmitter release (Emptage et al., 2001; Llano et al., 2000; Peng, 1996; Zhang et al., 2009). The basic premise is that calcium release from internal stores through activation of InsP3Rs or RYRs is capable of raising and maintaining the high level of local calcium above the threshold necessary to trigger exocytosis and the dense-cored vesicles release. Three components are thought to be accountable for regulation of presynaptic calcium signaling including Ca^{2+} / Ca^{2+} sensing proteins, G_{i}/o βγ subunit of the G protein coupled receptors (GPCRs) and SNARE proteins (Catterall and Few, 2008). In the postsynaptic neurons, $[Ca^{2+}]_i$ can also trigger a variety of long-lasting synaptic modification including LTP and LTD. How can this factor mediate the bidirectional effects of potentiation and depression within the same synapse? Based on the fact that LTP induction requires a stronger stimulus than LTD induction, it is proposed that this bidirectional switch might rely on the magnitude and duration of calcium signal in the proper spatial and temporal organization. It is supported by the observations that brief tetanic stimulation normally inducing LTP could generate LTD when Ca^{2+} entry was limited either by applying low level of NMDAR antagonist APV or by voltage clamping cells at more negative membrane potentials, and that a large and brief calcium elevation was accompanied with LTP while a modest and prolonged calcium influx occurred during LTD (Cummings et al., 1996; Lisman, 1989; Malenka et al., 1992). The molecular targets of calcium actions in synaptic transmission and plasticity require more investigation in the different synapses of the brain regions by advanced experimental tools and techniques.

1.4.3.2 Endocannabinoid-mediated Synaptic Transmission

Activation of cannabinoid receptors by eCBs causes various effects through multiple signaling pathways within different brain regions. Since the earlier reports (Kreitzer and

Regehr, 2001; Maejima et al., 2001; Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001), it has been established that eCBs mediate retrograde signaling in the CNS and contribute to several forms of short-term and long-term synaptic plasticity. eCBs are postsynaptically produced and act either phasically in an activity-dependent manner or tonically in a basal tone. eCBs presynaptically bind to cannabinoid receptors and suppress release of neurotransmitter either transiently or persistently. So far, various forms of eCB-mediated short term plasticity (eCB-STD) and eCB-mediated long term plasticity (eCB-LTD) in the hippocampus and amygdala have been reported. The retrograde signaling can be modulated by either the functionality of cannabinoid receptors or enzymatic pathways in the generation and degradation of endocannabinoids (Chevaleyre et al., 2006; Di Marzo, 2009, 2011; Freund et al., 2003; Kano et al., 2009; Mechoulam and Parker, 2013).

Kano *et al.* (Kano et al., 2009) summarized three modes for eCB-based retrograde signaling for eCB-STD: A), Ca^{2+}-driven endocannabinoid release mode in the case of depolarization induced suppression of inhibition (DSI) or depolarization induced suppression of excitation (DSE) : when a large Ca^{2+} elevation (micromolar range) is caused by strong depolarization through VGCCs, by activation of NMDA receptors (Ohno-Shosaku et al., 2007; Pitler and Alger, 1992) or by Ca^{2+} release from internal stores (Isokawa and Alger, 2006), 2-AG is produced from diacylglycerol (DG) by diacylglycerol lipase (DGL) through a pathway independent to phospholipase C (PLC) but presumably dependent on DGL; B), basal receptor-driven endocannabinoid release (RER) mode : at basal Ca^{2+} levels, strong activation of Gq/11-coupled receptors (e.g.,metabotropic glutamate receptor group I/V mGluR1/5) stimulates phospholipase C (PLC) which hydrolyzes phosphatidylinositol (PI) into DG and inositol 1,4,5-trisphosphate (IP3) (Hashimotodani et al., 2007; Neu et al., 2007);

C), Ca^{2+}-assisted RER: it is similar to A mode and it requires calcium influx (Melis et al., 2004; Ohno-Shosaku et al., 2002). However, in C mode, only small Ca^{2+} elevation (submicromolar range), which is subthreshold for basal RER, is coincided with weak activation of G_q/11- coupled receptors through either VGCCs or NMDA receptors, PLC activation is enhanced and 2-AG is produced. In A or C modes, calcium rise might also origin from the ER, e.g., by the PLC product, IP3. In any of the three modes, the released 2-AG binds to presynaptic CB1R and suppresses neurotransmitter release. In addition to eCB-STD, eCB-LTD has also been demonstrated in both the hippocampus and amygdala. The most common initial steps of eCB-LTD induction is the activation of postsynaptic group I metabotropic glutamate (Glu) receptors (I-mGluR) following repetitive synaptic stimulation or by application of I-mGluR agonist. These receptors couple to PLC via Gq/11 subunits and promote DAG formation followed by conversion into 2-AG by DGL. 2-AG is then released from the postsynaptic neuron and binds presynaptic type 1 CB1Rs. Postsynaptic Ca^{2+} can regulate eCB mobilization by either a PLC-dependent or a PLC-independent manner. This Ca^{2+} elevation arises from VGCCs or through NMDARs. Ca^{2+} released from the ER by the PLC product IP3, can also make contribution. At the presynaptic terminal, the CB1R activation inhibits adenylyl cyclase (AC) via $G_{i/o}$, reducing protein kinase A (PKA) activity. eCB-LTD induction may also require a presynaptic Ca^{2+} rise through presynaptically located VGCCs, NMDARs or from Ca^{2+} induced release from internal stores. Activation of the Ca^{2+}-sensitive phosphatase calcineurin and the decrease of PKA activity, shifts the kinase/phosphatase activity balance, thus promoting dephosphorylation of an unidentified presynaptic target that mediates a long-lasting reduction of transmitter release (Heifets and Castillo, 2009). The active zone protein RIM1α and the vesicle-associated protein Rab3B are

also necessary for LTDi (Castillo et al., 2012; Tsetsenis et al., 2011).

Tasker laboratory (Di et al., 2003) defined a mechanism in hypothalamus that eCB synthesis induced by postsynaptic activation of membrane-bound glucocorticoid receptors via G-protein signaling cascade, was the workhorse for glucocorticoids to inhibit local neuronal transmission and subsequently hypothalamic hormone secretion. This pioneer work provides a possible interaction between the actions of endocannabinoids and glucocorticoids in the hypothalamus that regulates stress response. This growing evidence reiterates the notion that the eCB system is a rapid mediator in response to stress and stress hormones in many other brain regions including hippocampus and amygdala (Campolongo et al., 2009; Hill et al., 2006; Hill et al., 2010a; Hill et al., 2010c; Karst et al., 2010). Potentially, glucocorticoid and endocannabinoid cross-talk has been occurring throughout evolutionary phylogeny to regulate rapid responses to stress (Hill and McEwen, 2009; Tasker, 2006; Tasker and Herman, 2011). eCB signaling pathway interaction with the stress axis is summarized in Figure 1.4.2. It needs to be mentioned that suppression of amygdalar eCB signaling by stress contributes to the activation of the hypothalamic−pituitary−adrenal axis to elevate production of the stress hormone glucocorticoid (Hill et al., 2009a). However, chronic elevation of glucocorticoid as occurs in chronic stress further suppresses eCB signaling in the limbic system including the amygdala and hippocampus and this feed-forward mechanism exacerbates anxiety disorders (Bowles et al., 2012; Hill et al., 2012; Hill et al., 2010b). The potential clinical value of cannabinoid modulation is highlighted by the dense expression of the CB1 receptor in regions known to be important for anxiety and emotional memory (Katona et al, 1999, 2000, 2001; Freund et al, 2003; van der Stelt and Di Marzo, 2003). For example, CB1 immunoreactivity is

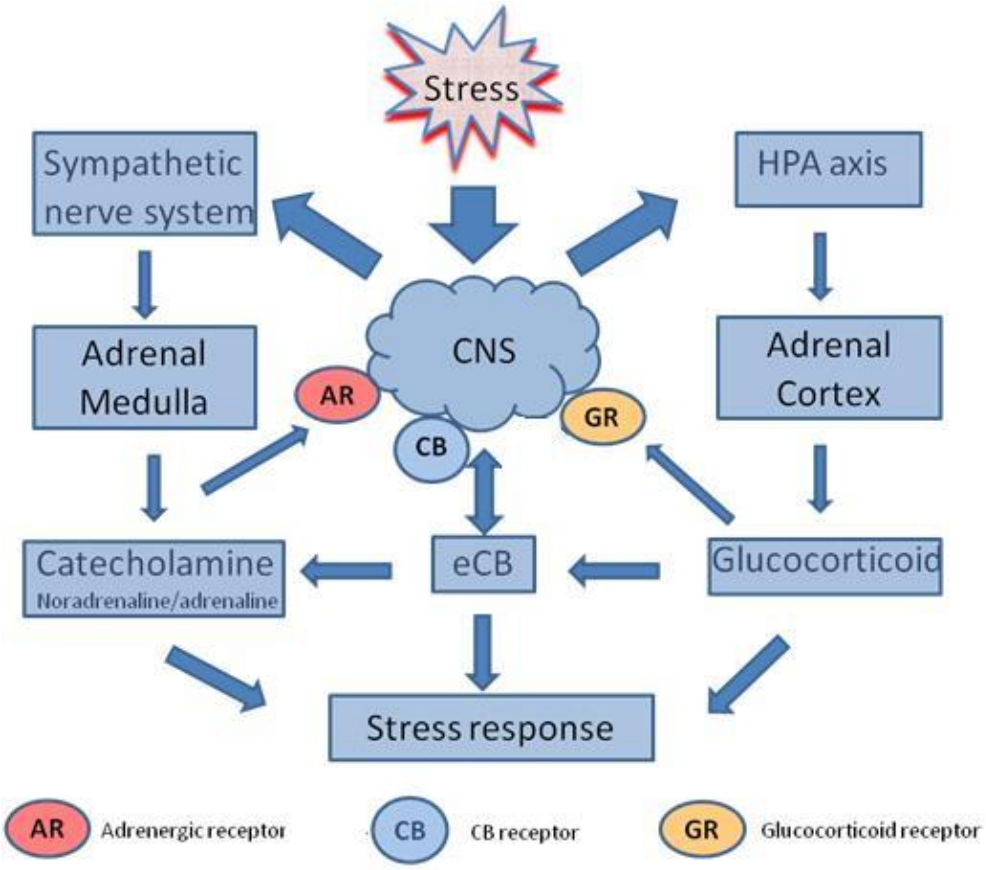

Figure 1.4.2 A proposed endocannabinoid (eCB) signaling pathway and points of interaction with the stress axis

Stressful stimuli simultaneously activate the sympathetic nervous system and the hypothalamic–pituitary–adrenal (HPA) axis, leading to elevated circulating levels of catecholamines and glucocorticoids that have impact on the stress response. A, Stressors change expression levels of adrenergic receptor (AR), cannabinoid receptor (CB), glucocorticoid receptor (GR); B, eCBs reduce HPA activity (activity enhanced by CB1 receptor blockade); C, eCBs disinhibit catecholamine release; D, glucocorticoids increase eCBs synthesis; E, eCBs bind CB1; F, glucocorticoids bind glucocorticoid receptors; G, noradrenaline binds β-adrenergic receptors.

expressed in a subset of GABA-containing interneurons in the basal lateral amygdala (BLA) (Katona et al., 2001), where aversive memories might be formed and stored (Medina et al., 2002). eCB signaling in BLA has been implicated in extinction of aversive memories (Marsicano et al., 2002). Moreover, the critical involvement of eCB-mediated transmission in extinction potentially has important clinical implications for anxiety disorders such as phobias, PTSD in humans and any other diseases associated with the expression of classically conditioned fear in animals. Investigation of the detailed mechanisms of eCB would help improve our understanding of the molecular underpinnings of eCB roles in emotional memory and anxiety.

1.4.3.3 mGluR-dependent Synaptic Plasticity

mGluR-dependent long-term synaptic depression (mGluR-LTD) is the most important form of mGluR-dependent synaptic plasticity associated with many brain diseases such as mental retardation, autism, Alzheimer's disease, Parkinson's disease and drug addiction (Luscher and Huber, 2010) mGluR-LTD was first described at the granule cell parallel fiber synapses onto Purkinje cells in the cerebellum (Ito et al., 1982) and this form was found widely-employed in many brain regions such as hippocampus, neocortex, dorsal and ventral striatum and spinal cord (Bear and Abraham, 1996; Bellone et al., 2008; Gladding et al., 2009b; Jorntell and Hansel, 2006; Kemp and Bashir, 2001; Luscher and Huber, 2010). Most studies have been focused on the postsynaptic Group I mGluRs (mGluR1/5). mGluR1 is in complementary distribution with mGluR5 in the brain regions (Ferraguti and Shigemoto, 2006) and both are mainly located in a perisynaptic zone surrounding the ionotropic receptors (Lujan et al., 1996). mGluR1 immunoreactivity is most intense in Purkinje cells of the

cerebellar cortex and neurons in the thalamus, lateral septum, the pallidum while mGluR5 staining is broadly distributed in the cerebral cortex, hippocampus, subiculum, striatum, nucleus accumbens and lateral septal nucleus (Ferraguti and Shigemoto, 2006). mGluR5 is the predominant subtype of I-mGluR expressed in the amygdala (Allen Brain atlas, http://www.brain-map.org). I-mGluR coupled to Gαq stimulates PLC to form DAG and IP3 to further initiate signaling processes. Ca^{2+} elevation, IP3 sensitive Ca^{2+} stores, PLC and PKC activity are required by cerebellar mGluR-LTD but they seem unlikely essential for hippocampal mGluR-LTD (Fitzjohn et al., 2001; Moult et al., 2006). However, recent sophisticated study provided evidence of local Ca^{2+} rise and Ca^{2+} release from internal stores during mGluR-LTD using two photon uncaging of glutamate onto individual spines of hippocampal CA1 neurons (Holbro et al., 2009). The spines with fluorescent ER protein expression were more susceptible to mGluR-LTD than the spines lacking of ER protein.

Although mGluR-LTD is mediated by distinct pre- or postsynaptic mechanisms that alters in the brain region, all these forms of synaptic plasticity rely on rapid synthesis of proteins, commonly-called 'LTD proteins'(Mameli et al., 2007; Waung and Huber, 2009; Yin et al., 2006). Amongst these LTD proteins, activity-regulated cytoskeletal associated protein (Arc), fragile X mental retardation protein (FMRP), amyloid precursor protein (APP), striatal-enriched protein tyrosine phosphatase (STEP) and Homer1 are best studies (Bassell and Warren, 2008; Carty et al., 2012; Goebel-Goody et al., 2012; Shankar et al., 2008; Szumlinski et al., 2006; Waung and Huber, 2009; Waung et al., 2008). There are also some exceptions implicating absence of protein synthesis for mGluR-LTD (Hou and Klann, 2004; Nosyreva and Huber, 2005). In these circumstances, postsynaptic mechanisms were possibly employed for mGluR-LTD through tyrosine dephosphorylation or AMPAR endocytosis

where existing 'LTD protein' levels are likely enough for on-going activity.

mGluRs-LTD appear to recruit two signaling pathways, the extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) and phosphoinositide 3-kinase/mammalian target of rapamycin (PI3K/mTOR) pathways. mGluR-LTD in hippocampus relies on stimulation of both ERK and the PI3K/mTOR pathway (Gallagher et al., 2004; Hou and Klann, 2004). Similarly, mGluR-LTD in the cerebellum (Ito-Ishida et al., 2006) and bed nucleus of the stria terminalis (BNST) also require ERK signaling (Grueter et al., 2006) while mGluR-LTD in the VTA depends on mTOR signaling (Mameli et al., 2007). In the near future, specific pharmacological and optogenetic tools as well as genetic manipulations in mice will aid to advance our understanding of how mGluRs-mediated synaptic plasticity contribute to complex learning behaviours and various neurological diseases, such as addiction, anxiety, depression, fragile X syndrome, Parkinson's and Alzheimer's disease.

1.5 Rationale, Hypotheses and Objectives

1.5.1 LMO4 and its Potential Role in Synaptic Function

The molecular mechanisms underlying activity-dependent modification of synaptic strength have been under intensive investigation because of their fundamental importance in brain function and dysfunction. Recent growing data point to LMO4 as a potential important player in synaptic regulation because of its role both in the nucleus as a transcription cofactor and in the cytoplasm as a protein-binding interface coupling with many signaling molecules (Heberlein et al., 2009; Inan and Crair, 2007; Polleux et al., 2007). LMO4 can serve as either an activator or a repressor in multiple transcription complexes and mediate various neuronal

functions. Through a transactivation trap assay, LMO4 was identified as a transcription activator in response to calcium influx (Aizawa et al., 2004). Kashani and his colleagues further demonstrated that calcium influx via VGCCs and NMDA receptors contributes to synaptically-induced LMO4-mediated transactivation (Kashani et al., 2006). LMO4 could mediate activity-dependent gene activation via calcium/calmodulin-dependent protein kinase IV (CaMKIV) and MAPK in rat cultured cortical neurons. Moreover, co-immunoprecipitation experiments in 293T cells showed that LMO4 interacts with the cAMP response element-binding protein (CREB) and forms a complex with the cofactor of LIM homeodomain protein 1 (CLIM1) and protein 2 (CLIM2) to regulate the thalamocortical morphogenesis during cortex development (Kashani et al., 2006). Notably, the CaMKIV-CREB pathway, which regulates the development and plasticity of dendrites, has been shown to influence synapse formation. The over-expression of a constitutively active form of CaMKIV or wild type CREB in the rat hippocampus increases LTP at the CA1 synapse and enhances the generation of new silent synapses which are considered to be potent cellular and molecular substrates for LTP and experience-dependent modification (Marie et al., 2005). Furthermore, LMO4 downregulation likely alters the function of neural circuits that enhance behavioural sensitivity to cocaine (Heberlein et al., 2009; Lasek et al., 2011; Lasek et al., 2010). Approximately 50% decrease in mRNA levels of the GluR1 subunit of the AMPA receptor has been observed in mouse Acb tissue expressing a small hairpin RNA targeting LMO4 (shLMO4). It is uncertain whether LMO4-dependent cocaine sensitivity is mediated by a direct effect on the transcription of AMPAR subunits, or by an indirect effect on a scaffolding protein or other downstream signaling molecules.

Another study showed that LIM kinase 1 (LIMK-1) knockout mice exhibit significant abnormalities in spine morphology and in synaptic function, including enhanced hippocampal LTP. The knockout mice also showed altered fear responses and spatial learning. These findings suggest that LIMK-1 plays a critical role in dendritic spine morphogenesis and brain function by regulation of actin filament dynamics through inhibition of ADF/cofilins. Interestingly, LIMKs share a high similarity with the LIM sequence of LMOs. Importantly, LIM kinases contain targeting sequences that can direct them to either the nucleus or the cytoplasm (Yang and Mizuno, 1999), and they carry out nuclear functions in cell-cycle progression as well as cytoskeletal organizing activities (Roovers et al., 2003). An intriguing possibility is that LMO4 might modulate LIMK activity and affect dendritic spine morphology and synaptic function.

As described in a previous section, LMO4 has been suggested to be involved in several non-nuclear signaling pathways such as IL-6, ephrin B, neogenin-Rho A by a direct binding or as a second messenger (Bong et al., 2007; Novotny-Diermayr et al., 2005; Schaffar et al., 2008), which are known to play a role in synaptic plasticity function (Balschun et al., 2004; Lim et al., 2008; Tancredi et al., 2000). LMO4 acts as a scaffold for the IL-6 glycoprotein 130 complex and positively regulates IL-6 signaling (Novotny-Diermayr et al., 2005). IL-6 expression has been shown to increase during high frequency stimulation (HFS) induced LTP either in rat hippocampus slices or in freely moving rats but IL-6 levels beyond the physiological range in turn inhibited LTP maintenance (Balschun et al., 2004; Tancredi et al., 2000). A yeast-two-hybrid screen found that LMO4 binds to ephrin B (Bong et al., 2007). Ephrin B and the EphB receptor tyrosine kinases (RTKs) play crucial roles in CNS development and plasticity (Klein, 2004, 2012; Sloniowski and Ethell, 2012). EphB-ephrinB

signaling can be bi-directionally activated through ephrin B (presynaptically-located, reverse signaling) or eph receptors (postsynaptically-located, forward signaling). Ephrin B reverse signaling is known to activate Jak2 (Pasquale, 2010). Activation of presynaptic ephrin-B signaling rapidly (within 30 minutes) enhances synaptic neurotransmitter release and activity-dependent synaptic efficacy (i.e. LTP) (Lim et al., 2008). EphB forward signaling typically involves activation of the intrinsic tyrosine kinase activity and tyrosine phosphorylation of intracellular effector proteins which in turn regulate the activity of Rho GTPases including RhoA to modulate cytoskeletal dynamics. Activation of postsynaptic EphB signaling in primary cortical neurons increases NMDAR-dependent calcium influx by promoting NMDAR phosphorylation (Takasu et al., 2002). There is also evidence that RhoA activation is downstream of an LMO4-mediated RGM A-neogenin signal transduction pathway. Other possible intrinsic tyrosine kinases involved include focal adhesion kinase (FAK), paxillin, the tyrosine phosphatase (SHP2), the tyrosine kinase MERTK and intersectin (ITSN) for Rho A activation (Schaffar et al., 2008). RhoA is known to transduce CaMKII activation into spine enlargement and LTP induction (Murakoshi et al., 2011). Besides the large eph family of receptor tyrosine kinase (RTK), LMO4 is also an essential mediator of another RTK family: epidermal growth factor receptor (EGFR, ErbB, Neu, Her2). LMO4 is downstream target of ErbB2-PI3K pathway in breast cancer cell cycle progression (Montanez-Wiscovich et al., 2009). Given the fact that LMO4 can form IL-6 gp 130 complex with both tyrosine kinase JAK2 and phosphatase SHP2 and SOCS3 in the IL-6 gp130 complex (Novotny-Diermayr et al., 2005), LMO4 is likely a protein binding interface for modulation of the effectors of tyrosine phosphorylation balance and thus plays its important role in post-translational modification. This suggestion is also supported by our unpublished data that LMO4 binds and

negatively controls ER-located protein tyrosine phosphatase 1B (PTP1B) activity (Pandey et al., 2013). In this study, we aimed at investigation of LMO4-mediated PTP1B-dependent signaling molecules that are playing the important role in synaptic function.

Taken together, the binding properties of LMO4 in nucleus and cytoplasm suggest that it might act as a bimodal regulator for signaling the various external stimuli into changes in gene expression at both a transient non-genomic level and a delayed genomic level.

1.5.2 Rationale

It is shown that LMO4 is an important transcriptional co-regulator. Before starting my studies, my lab had screened for genes whose expression levels are modulated by ablation of LMO4 by carrying out a non-biased high-throughput microarray analysis. Total RNA was isolated from primary cortical neuron culture derived from LMO4 germline ablated (Chen et al., 2007a) and littermate control embryos, amplified, and hybridized to oligonucleotide microarrays to identify differentially expressed transcripts. Although the expression of several genes was altered in neurons with LMO4 ablated (data not shown), we were intrigued by the gene ryanodine receptor 2 (RyR2). There are a few reasons: 1), RyR2 expression had a robust decrease of up to 40% between WT and LMO4 KO ($P < 0.05$); 2), this effect showed some specificity since the expression of the other two subtypes RyR1 and RyR3 was not significantly different (data not shown); 3), The expression pattern of RyR in neurons is widely overlapped with that of LMO4 and RyR2 is the predominant subtype distributed in the adult brain (Hermanson et al., 1999; Kenny et al., 1998; Mori et al., 2000; Padua et al., 1992; Sharp et al., 1993); 4), RyRs are calcium gated calcium channels that control the calcium release from the internal stores and thus contribute to calcium induced calcium release (CICR)

and synaptic plasticity (Fill and Copello, 2002; Peng, 1996). Together, RyR2 is likely a regulatory target of LMO4. Because germline ablation of LMO4 causes embryonic lethality, we used a conditional LMO4 KO mouse line (Camk2αCre/LMO4flox) for functional studies of LMO4 in the central nervous system, where LMO4 is ablated in all glutamatergic neurons upon Camk2α recombinase expression. The first part of my study had been focused to investigate the calcium-dependent neuronal function of LMO4 in the hippocampus where LMO4 is intensively expressed (Hermanson et al., 1999; Kenny et al., 1998). Electrophysiolgical and behavioural characterizations had been carried out on hippocampal-associated learning and memory.

LMO4 is a regulator for the activity of key transcription factors in the nucleus (de la Calle-Mustienes et al., 2003; Hahm et al., 2004; Joshi et al., 2009; Kashani et al., 2006; Lu et al., 2006a; Manetopoulos et al., 2003; Schock et al., 2008a; Sum et al., 2002; Wang et al., 2007). On the other hand, LMO4 modulates several tyrosine kinases in the cytoplasm (Bong et al., 2007; Gomez-Smith et al., 2010; Novotny-Diermayr et al., 2005; Schaffar et al., 2008; Zhou et al., 2012). Recently, we reported that LMO4 is an endogenous inhibitor for the tyrosine phosphatase PTP1B that controls hypothalamic leptin signaling (Pandey et al., 2013). Our preliminary data showed that ablation LMO4 in glutamatergic neurons (Camk2αCre/LMO4flox) had an increased activity for PTP1B phosphatase in the amygdala (Figure 3.5A). We also identified that mGluR5 is a substrate for PTP1B by co immunoprecipitation experiment (Figure 3.5C). Given the facts that mGluR modulation, presumably via mGluR-eCB signaling, is essential for emotion control in the amygdala where LMO4 is also highly expressed, we asked whether LMO4 expression in the amygdala affects anxiety behavior and whether PTP1B is a new therapeutic target for anxiety and other

psychiatry disorders. Biochemical, electrophysiological and behavioural assays were carried out to investigate amygdala-associated emotional state.

1.5.3 Hypotheses

- LMO4 regulates calcium-induced calcium release and synaptic plasticity in the hippocampus ;
- LMO4 modulates anxiety through PTP1B-mediated mGluR5-dependent eCB signaling in the amygdala.

1.5.4 Objectives

- To examine the role of LMO4 on regulation of calcium-induced calcium release and synaptic plasticity in the hippocampus ;
- To investigate the role of LMO4 in the amygdala on modulation of the PTP1B activity and the subsequent mGluR5 function as well as to elucidate the potential clinical values of PTP1B for anxiety relief and stress reduction.

CHAPTER 2

Manuscript #1

LIM Domain Only 4 (LMO4) Regulates Calcium-Induced Calcium Release and Synaptic Plasticity in the Hippocampus

Zhaohong Qin, Xun Zhou, Mariana Gomez-Smith, Nihar R. Pandey, Kevin F. H. Lee, Diane C. Lagace, Jean-Claude Béïque, and Hsiao-Huei Chen. LIM domain only 4 (LMO4) regulates calcium-induced calcium release and synaptic plasticity in the hippocampus. J Neurosci 2012; 32, 4271-4283.

2.1 Statement of Author Contribution

Z.Q., J.-C.B., and H.-H. C. designed research; Z.Q. did all the electrophysiology recordings, calcium imaging and organotypic slice culture, X.Z. did the biochemistry experiments, M.G.-S. performed Morris water maze test, N.R.P. and K.H.L. contributed to some unpublished preliminary data, and H.-H.C. performed research; H.-H.C. contributed unpublished reagents/analytic tools; Z.Q., X.Z., M.G.-S., D.C.L., J.-C.B., and H.-H.C. analyzed data; Z.Q., J.-C.B., and H.-H.C. wrote the paper.

2.2 Title Page

LMO4 regulates calcium-induced calcium release and synaptic plasticity in the hippocampus

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Any Conflict of Interest: None

2.3 Abstract

The LIM domain only 4 (LMO4) transcription factor activates gene expression in neurons and regulates key aspects of network formation, but the mechanisms are poorly understood. Here, we show that LMO4 positively regulates ryanodine receptor type 2 (RyR2) expression, thereby suggesting that LMO4 regulates calcium-induced calcium release in central neurons (CICR). We found that CICR modulation of the afterhyperpolarization of CA3 neurons in hippocampal slices from mice carrying a forebrain-specific deletion of LMO4 (LMO4 KO) was severely compromised, but could be restored by single-cell overexpression of LMO4. In line with these findings, two-photon calcium imaging experiments showed that the potentiation of RyR-mediated calcium release from internal stores by caffeine was absent in LMO4 KO neurons. As a last test, we found that the overall facilitatory effect of CICR on glutamate release induced during trains of action potentials was likewise defective in LMO4 KO, confirming that CICR machinery is severely compromised in these neurons. Moreover, the magnitude of CA3-CA1 long-term potentiation was reduced in LMO4 KO mice, which defect appears to be secondary to an overall reduced glutamate release probability in these mice. These cellular phenotypes in LMO4 KO mice were accompanied with deficits in hippocampus-dependent spatial learning as determined by the Morris water maze test. Thus, our results establish LMO4 as a key regulator of CICR in central neurons, providing a mechanism for LMO4 to modulate a wide range of neuronal functions and behavior.

2.4 Introduction

Neuronal and synaptic activity can encode and store information in the brain not only through changes in synaptic strength but also by long-lasting control of gene expression. Experience exerts this control in part by modulating the level and/or function of several calcium-dependent regulatory proteins involved in gene regulation (Flavell and Greenberg, 2008). A number of experimental approaches have been developed to screen for transcription factors regulating gene expression involved in many aspects of neuronal development, including dendritic branching, synapse maturation, and synapse elimination (see review in (Flavell and Greenberg, 2008)). One corollary challenge becomes identifying the genes downstream of these activity-dependent gene regulatory proteins and ultimately to link these genetic programs with defined cellular and behavioral measures.

Using a transactivator trap screen, the LIM domain only 4 (LMO4) protein was identified as a calcium-responsive transactivator (Aizawa et al., 2004) that activates gene expression in an activity-dependent manner (Kashani et al., 2006). LMO4 is a small protein (165 amino acids) that contains 2 protein-interacting LIM domains. LMO4 serves not only as a cofactor of many transcription factors (Kashani et al., 2006; Manetopoulos et al., 2003; Schock et al., 2008b), it also interacts with transmembrane receptors to modulate their signaling (Bong et al., 2007; Novotny-Diermayr et al., 2005). Whether LMO4 couples signals from membrane receptors to changes in gene expression is not known, although we showed that LMO4 is present in the cytoplasm and nucleus and translocates from the cytoplasm to the nucleus in response to extracellular stimuli (Chen et al., 2007b).

LMO4 is expressed early during central nervous system development (Chen et al., 2002b; Kenny et al., 1998). Mice with germline ablation of LMO4 die prior to birth with

exencephaly (Hahm et al., 2004; Lee et al., 2005; Tse et al., 2004), whereas mice with conditional ablation of LMO4 in the cerebral cortex show defects in thalamocortical connections (Kashani et al., 2006). However, the cellular processes that might be responsible for this defect are ill-defined, in part illustrating the relative paucity of experimental data on the functional roles played by this activity-regulated protein.

Here, following a lead from a microarray screen that identified downregulation of ryanodine receptor 2 (RyR2) in LMO4 null cortical neurons, we characterized the role of LMO4 as regulator of both RyR2 expression and function. To this end, and to circumvent the lethality of the germline knock-out, we generated mice with postnatal ablation of LMO4 by mating LMO4flox mice with mice expressing Cre-recombinase under the control of a CaMK2α minigene (Casanova et al., 2001). Electrophysiological recordings and calcium imaging showed that the calcium-induced calcium release (CICR) machinery was severely compromised in the hippocampus of LMO4 KO mice, which directly translated in altered excitability metrics of CA3 pyramidal neurons and synaptic plasticity in the hippocampus. These hippocampal cellular phenotypes were accompanied with learning deficits as determined in the Morris water maze test.

2.5 Materials and Methods

Camk2αCre/LMO4flox mice.

CamK2αCre/LMO4flox (LMO4 KO) and LMO4flox littermate mice were genotyped and maintained on a CD-1 background as described (Schock et al., 2008b). Camk2αCre/LMO4flox mice and age-matched Camk2αCre/(LMO4flox/-), (LMO4 Het) or LMO4flox/flox (WT) controls were used in all experiments. All procedures for animal use were approved by the University of Ottawa Animal Care and Veterinary Service and carried out according to institutional guidelines and in accordance with those of the Canadian Council on Animal Care.

Cell culture and transfection.

F11 cells, a hybridoma between rat E12 dorsal root ganglion neurons and a mouse neuroblastoma line were maintained as described (Chen et al., 2007a; Chen et al., 2007b). For quantitative RT-PCR, F11 cells were seeded in 6-well plates and transfected 24 hours later by using lipofectamine 2000 (Invitrogen).

RyR2 promoter construct and activity assay.

The *RyR2* promoter region spanning from −765 to −104 (+1 being the putative transcription start site) was amplified by PCR from mouse tail genomic DNA as described previously (Pfeffer et al., 2009) using the following primers: (F) 5-TTctcgagCGCATTCAGTGATCG-3, (R) 5-TTaagcttGACCTCAAGTCCAAGG- 3 (lower cases represent *XhoI* and *HindIII* liner sequences). The *RyR2 Xho*I/*HindIII* fragment was

cloned into the pGL4.10-Basic luciferase reporter vector (Promega) and verified by sequencing. For promoter activity assays, 1 μg of RyR2 luciferase promoter was co-transfected with 100 ng of CMV-beta galactosidase reporter in the presence of 400 ng of LMO4 expression vector or LMO4shRNA into F11 cells in 6-well plates. Appropriate empty vector or scrambled shRNA was used as a control. Cells were harvest 24 hours after transfection and RyR2 promoter-driven luciferase activity was normalized to beta galactosidase to control for transfection efficiency, as described previously (Chen et al., 2007a; Gomez-Smith et al., 2010).

RNA extraction and quantitative RT-PCR.

Total mouse hippocampal RNA was extracted and purified from 30 mg samples (wet weight) dissected from 1 month old mutant and control mice, while F11 cells were lysed 36 hours post-transfection using TRIzol Reagent (Invitrogen). RNA was reconstituted in sterile, nuclease-free water, and sample concentrations were determined spectrophotometrically at OD 260nm. After DNase treatment of 2 μg of total RNA (TURBO DNA-free kit, Ambion), dNTP and random decamers were used for cDNA synthesis in 20 μl reaction (Applied Biosystems). 0.1 μl of the resulting cDNA products was evaluated using real time PCR. Target genes were amplified and evaluated using the Rotor-Gene 3000 (Corbett Research) and SYBR green detection (DyNAmo SYBR Green qPCR kit, New England Biolabs). PCR Cycling conditions were as follows: 10 min at 95°C, and then 40 cycles at 95°C for 10 s, followed by 56°C for 15 s and 72°C for 20 s. Cyclophilin A was used as an endogenous control (housekeeping) gene. Specific ryanodine receptor primer sequences described previously (Chakroborty et al., 2009) were as follows: *RyR1*: (F) 5-

TCTTCCCTGCTGGAGACTGT-3, (R) 5-GTGGAGAAGGCACTTGAGG-3; *RyR2*: (F) 5-TCAAACCACGAACACATTGAGG-3, (R) 5-AGGCGGTAAAACATGATGTCAG-3; *RyR3*: (F) 5-CTGGCCATCATTCAAGGTCT-3, (R) 5-GTCTCCATGTCTTCCCGTA-3. *CycloA*: (F) 5-GGCCGATGACGAGCCC-3, (R) 5-GTCTTTGGAACTTTGTCTGCAAAT-3. LMO4: (F) 5-GGACCGCTTTCTGCTCTATG-3, (R) 5-AGCACCGCTATTCCCAAAT-3. Each sample was evaluated in triplicate. Amplification data were analyzed using the comparative cycle threshold (ΔΔCt) method after normalization to Cyclophilin A. A two-tailed *t* test was used to determine statistical significance at $p<0.05$.

In situ hybridization.

Tissues were processed for in situ hybridization with digoxigenin-labeled antisense or sense riboprobes, as previously described (Duquette et al., 2010). For more reliable comparisons of gene expression patterns, wild-type and mutant tissues were processed on the same slides.

Western blot analysis.

Hippocampal protein extracts from 1 month old mice were harvested and prepared for Western blot analysis as described previously (Chen et al., 2007a; Gomez-Smith et al., 2010). Primary mouse monoclonal antibodies to ryanodine receptor 2 (Affinity Bioreagents, Golden, CO) (Stutzmann et al., 2006) and to glyceraldehyde phosphate dehydrogenase (GAPDH, Abcam) were used.

Electrophysiology.

3 to 5 week-old transgenic mice (CamK2αCre/LMO4flox) and littermate controls (LMO4flox) were used in all experiments. As previously described (Béïque et al., 2006) mice were deeply anesthetized with isoflurane in an enclosed chamber and decapitated. The brain was quickly removed and immersed in ice-cold artificial cerebrospinal fluid (aCSF) containing (in mM): 119 CholineCl, 2.5 KCl, 4.3 $MgSO_4$, 1.0 NaH_2PO_4, 1.0 $CaCl_2$, 11 glucose, and 26.2 $NaHCO_3$, continuously bubbled with 95% O_2 and 5% CO_2, pH 7.4 and 300 μm-thick coronal slices of the hippocampus were obtained with a Vibratome. Brain sections were kept at 34°C for 30 min in oxygenated aCSF with the following composition (in mM): 119 NaCl, 2.5 KCl, 1.3 $MgSO_4$ 1.3, 1.0 NaH_2PO_4, 2.5 $CaCl_2$, 11 glucose, and 26.2 $NaHCO_3$, pH 7.4, and then at room temperature until recordings.

Visualized whole-cell recordings were performed using infrared differential interference contrast optics. Slices were placed in a perfusion chamber mounted on a movable stage assembly on a fixed upright microscope (Nikon FN, Nikon) and perfused with oxygenated aCSF (95% O_2 – 5% CO_2; 2ml/min) at room temperature. Recording pipettes (3 – 5MΩ) were pulled on a Narishige PC-10 (Narishige Co.) from borosilicate glass capillaries (World Precision Instruments) and filled with one of two intracellular solution containing (in mM): For voltage-clamp experiments: 115 $CsMeSO_4$, 2.8 NaCl, 0.4 EGTA, 5 TEA-Cl, 20 HEPES, 3.0 MgATP, 0.5 Na_2GTP, 10 Na-phosphocreatine, pH adjusted to 7.2 with CsOH. For current-clamp experiments: 115 K-gluconate, 20 KCl, 2 $MgCl_2$, 10 HEPES, 10 Na-phosphocreatine, 4 NaATP, and 0.3 NaGTP, pH adjusted to 7.2 with KOH. The $GABA_A$ receptor antagonist bicuculline methobromide (20 μM) was included unless otherwise indicated. Whole-cell recordings were obtained from CA1 and CA3 pyramidal neurons of the hippocampus. In some experiments, the stimulation bipolar electrode was inserted into

the stratum radiatum near the border of CA1 pyramidal layer under visual control to stimulate the Schaffer collateral fibers. Testing stimuli of 0.067 Hz, 10 μs duration were adjusted to evoke approximately 50 – 100 pA amplitude excitatory postsynaptic current (eEPSC). A Multiclamp 700B amplifier (Molecular Devices) was used for whole cell recordings and analyzed using pClamp 10.0 and OriginPro 8. Recordings were filtered at 3 kHz, digitized at 10 kHz, and stored on a PC. Access resistance was continuously monitored by applying a 75 ms, 5 mV hyperpolarizing pulses 250 ms before the eEPSCs, and cells were used for recording only if the access resistance was maintained at≤10 MΩ. Spontaneous mEPSCs were acquired at -70 mV in control ACSF solution supplemented with 1μM tetrodotoxin (TTX). Only events greater than 7.5 pA were included in the analysis which corresponds to the event detection limit of the noisiest of the recordings included in the analysis.

Probability of release was estimated by analyzing the variance of EPSC amplitudes (Malinow and Tsien, 1990; Martin and Buno, 2003). We estimated the effects of caffeine on release probability by plotting ($Mean^2$ / variance caffeine) / ($Mean^2$ / variance baseline) against_ (Peak amplitude caffeine /Peak amplitude baseline) and computed linear fits (Bekkers and Stevens, 1990; Faber and Korn, 1991; Manabe et al., 1993; Martin and Buno, 2003).

Organotypic slice culture.

Hippocampal slice cultures were prepared from 6- to 8-day-old mice as described previously (Béïque and Andrade, 2003; Béïque et al., 2007; Stoppini et al., 1991). Biolistic transfections were conducted 3–6 days later with the Helios Gene Gun (Bio-Rad), using 1.0-μm gold particles coated with DNA. Gold particles were coated with two cDNAs: dsRed and

LMO4-EGFP. We have previously shown that, neurons that are effectively transfected express both plasmids in >90% of cases (Béïque and Andrade, 2003).

Two-photon calcium imaging.

Simultaneous electrophysiological and optical recordings were carried out from CA3 pyramidal cells using similar conditions as those described above in the electrophysiology section (for current-clamp recordings), except that the intracellular pipette solution was supplemented with Alexa Fluor 594 (30 μM; to outline morphology) and with the calcium indicator Fluo-4FF (200 μM). We initially favored this low affinity (and low capacity) dye to avoid dye saturation following trains of action potentials in conditions of enhanced CICR. The imaging was obtained on an Olympus FV 1000 with a 40X/0.8 NA objective and excitation was obtained with a Mai Tai Deep See Ti:Sapphire laser (Spectra-Physics, Mountain View, Ca, USA) tuned at 810 nm. Analysis was carried out offline using Image J. $\Delta F/F_0$ were calculated by averaging fluorescence intensities in two or three selected regions of interest (ROIs), located on the soma but excluding the nucleus, and were expressed as follows: $\Delta F/F_o = (F-F_{baseline})/(F_{baseline}-F_{background})$. Image acquisition was on neuronal soma and no particular efforts were devoted to adjust acquisition to focal planes encompassing proximal dendrites.

Morris water maze test.

The Morris water maze test was performed in the Faculty of Medicine Behavior Core Laboratory at the University of Ottawa. All testing was performed between 08:00 and 12:00 AM and animals received 1hr of habituation to the testing room daily. Each mouse was

handled for 3-4 days before training. Mice at age 7-8 weeks old were trained for 9 days (4 trials per day, inter-trial interval pf 1hr, random start location in 1 of 4 positions) to find a submerged platform at a fixed position. Distal cues in the testing room, such as patterned cardboard on a white wall, were provided as spatial references. Each trial lasted 1 min or until the mouse found the platform and mice remained on the platform for 15 sec prior to being removed to the home cage. If the mice did not find the platform within 1 min on training the mice were guided to the platform by the experimenter. Latency to reach the platform, distance traveled to reach the platform, swim speed, % time in thimotaxsis region were measured using automated video tracking software from Noldus (Ethovision). On day 10, the platform was removed from the pole and the probe trial (trial length 1 min) was administered and percent time spent in the target quadrant and numbers of platform crossings were measured using Noldus system.

Statistical analysis

All data is presented as means ± SEM. *N* refers to the number of cells for voltage clamp or current clamp and in all other outcomes refers to number of mice. Differences between treatments were analysed using Student's t test for paired data or one-way repeated measures ANOVA followed by Bonferroni's post hoc test for comparisons. For water maze data a 2-way (genotype sex) ANOVA was completed followed by Bonferroni's post hoc test. Significance was accepted if $p<0.05$.

2.6 Results

LMO4 modulates ryanodine receptor 2 expression in neurons.

To begin examining the role of LMO4 in neuronal function, we first sought to screen for genes whose expression levels are modulated by ablation of LMO4. To this end, we carried out a non-biased, high throughput microarray analysis. Total RNA was isolated from 2 week old primary cultured cortical neurons derived from LMO4 germline-ablated (Chen et al., 2007a) and littermate control embryos, amplified and hybridized to oligonucleotide microarrays in order to identify differentially expressed transcripts. Although the expression of several genes was altered in neurons with LMO4 ablated (not shown), we were intrigued by the robust decrease (*ca.* 40%) in the expression of the ryanodine receptor type 2 (RyR2; $p < 0.05$). This effect showed some specificity since the expression of RyR1 and RyR3 were not significantly different between WT and LMO4 KO (not shown).

Because germline-ablation of LMO4 causes embryonic lethality, we generated a conditional LMO4 knock-out mouse line (Camk2αCre/LMO4flox, abbreviated hereafter LMO4 KO) for functional studies of LMO4 in the central nervous system. *In situ* hybridization and quantitative RT-PCR LMO4 expression in the hippocampus confirmed effective gene deletion in the mice after the first postnatal month (Fig. 2.1A, B). Direct quantitative PCR showed that the mRNA level of RyR2, but not of RyR1 or RyR3, was significantly decreased in the brain of LMO4 KO (Fig. 2.2A) compared to littermate control mice, thereby confirming the microarray finding obtained in germline KO. This finding was further confirmed by western blot analysis (Fig. 2.2B) that revealed reduced RyR2 protein levels in hippocampal protein extracts of LMO4 KO mice compared to littermate controls.

Figure 2.1 LMO4 expression in the hippocampus is ablated in Camk2αCre/LMO4flox mice

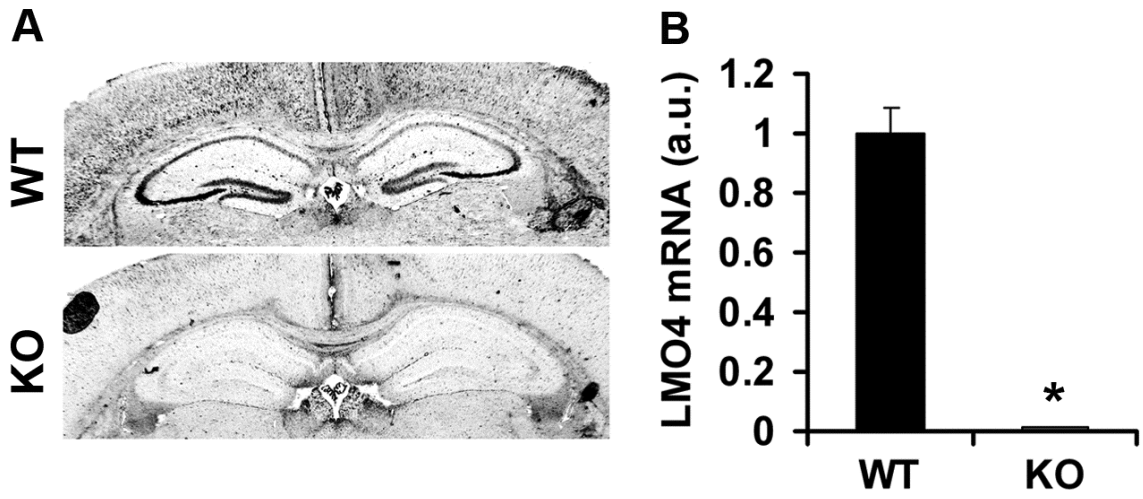

Figure 2.1 LMO4 expression in the hippocampus is ablated in Camk2αCre/LMO4flox mice

A, *In situ* hybridization revealed high levels of LMO4 mRNA in the hippocampal neurons of wild type littermate controls (WT) and is absent in Camk2αCre/LMO4flox mice (KO). Scale bar, 800 μm. ***B***, quantitative RT-PCR revealed 95% reduction of LMO4 mRNA in the hippocampus of KO compared to controls. n = 6 for WT and KO.

Figure 2.2 LMO4 regulates RyR2 expression

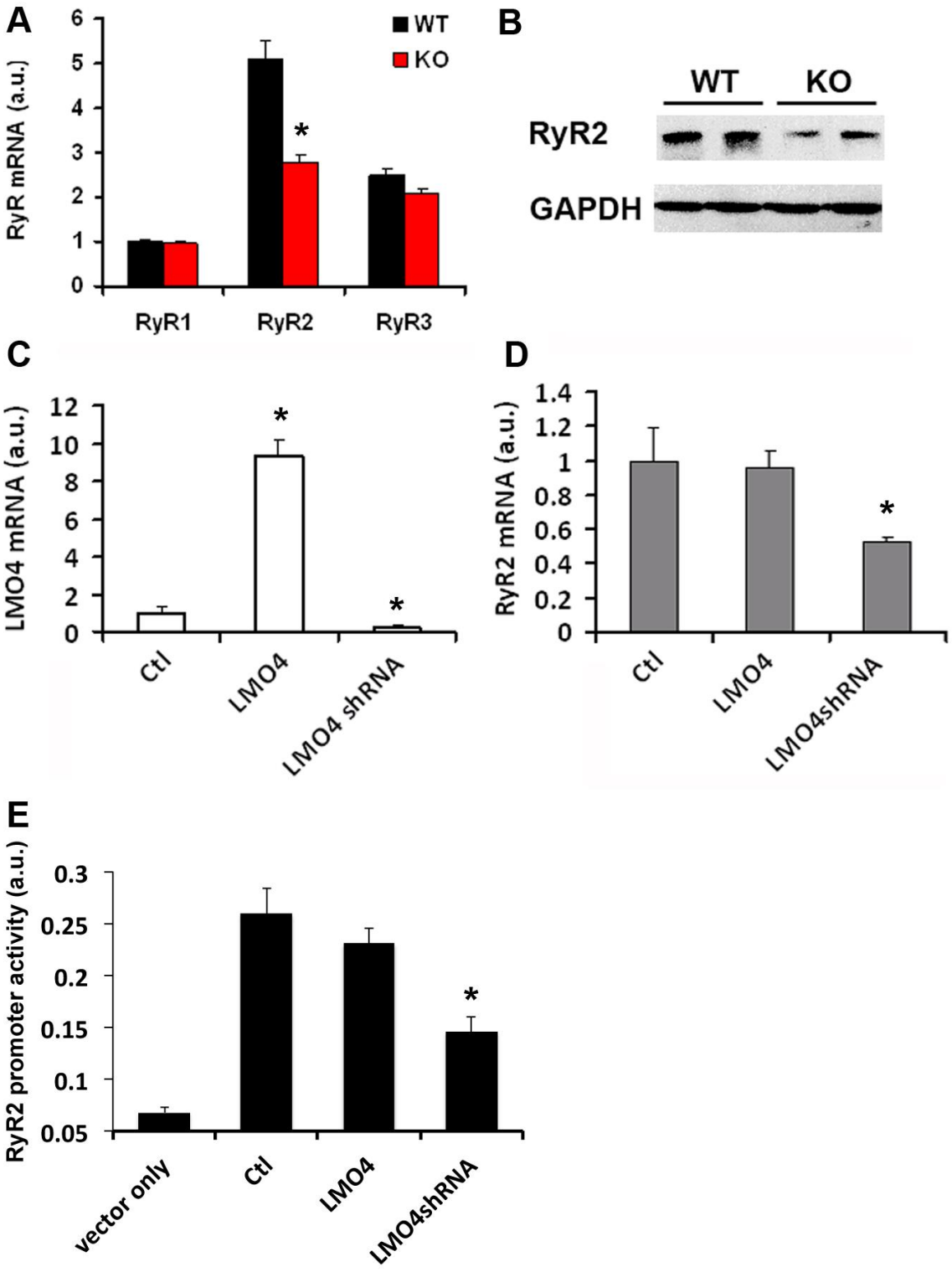

Figure 2.2 LMO4 regulates RyR2 expression

A, Quantitative PCR revealed a 50% reduction in RyR2 mRNA in the hippocampus of LMO4 KO mice (n=6 for each genotype). ***B***, Western blot of hippocampal protein revealed a reduction in RyR protein expression in LMO4 KO compared to littermate WT mice. ***C***, Quantitative PCR determined the levels of LMO4 mRNA in F11 neuronal cells transfected with an LMO4 expression vector (LMO4) or an LMO4shRNA expression vector. Following transfection of LMO4 expression or LMO4shRNA vector, LMO4 levels were elevated to 9 fold or reduced to 25 % relative to control, respectively. Ctl, control F11 cells transfected with empty expression vectors. ***D***, Quantitative PCR of RyR2 in F11 neuronal cells over-expressing LMO4 or with LMO4 knockdown by shRNA. In *C* and *D*, the same RNA extracts were used for quantification. n = 6 for each condition. ***E***, RyR2 promoter activity assay in F11 cells transfected with an LMO4 expression vector (LMO4) or an LMO4shRNA expression vector. n=6.

To further examine the role of LMO4 in modulating the expression of RyR2, we determined the effects of direct manipulations of LMO4 expression by transient transfection in F11 neuronal cells on the level of RyR2 mRNA. First, we found that overexpression of LMO4 (about 9 fold; Fig. 2.2C) did not elevate RyR2 mRNA levels, as determined by quantitative PCR (Fig. 2.2D). However, knock-down of endogenous LMO4 expression with shRNA (Fig. 2.2C) robustly reduced RyR2 mRNA levels in these cells (Fig. 2.2D). To further investigate the modulation of RyR2 by LMO4, we next determined whether LMO4 regulated the activity of a RyR2 promoter fragment driving luciferase expression by transient transfection in F11 cells. Over-expression of LMO4 led to only a modest increase in promoter activity, although knock-down of LMO4 with shRNA markedly reduced the RyR2 promoter activity (Fig. 2.2E). Together, these results show that LMO4 regulates RyR2 expression.

CICR contribution to the AHP is abolished in LMO4 KO

RyRs are calcium-gated calcium channels that control the release of calcium from internal stores and as such contribute to calcium-induced calcium release (CICR) (Fill and Copello, 2002; Iino, 1989; Peng, 1996). The involvement of LMO4 in regulating mRNA and protein expression of RyR2 suggest that LMO4 may be a key and central modulator of CICR in central neurons. To test this hypothesis, we compared several cellular processes known to be directly influenced by CICR in wild-type and LMO4 KO neurons.

Pyramidal cells of the hippocampus exhibit large afterhyperpolarizations (AHPs) following action potential discharges (Alger and Nicoll, 1980; Hotson and Prince, 1980; Schwartzkroin and Stafstrom, 1980) These AHPs are mediated by calcium-activated

potassium channels and contribute to spike frequency adaptation, a hallmark of this cell type. Although the calcium gating these potassium conductances originates in large part from membrane voltage-gated calcium channels (Marrion and Tavalin, 1998; Pineda et al., 1999; Pineda et al., 1998; Sah, 1996), CICR contributes a significant portion of the calcium responsible for the generation of the AHP (Akita and Kuba, 2000; Kuba et al., 1983; Pineda et al., 1999; Sah, 1996; Sah and McLachlan, 1991; Torres et al., 1996). We therefore reasoned that the AHP could serve as a reliable proxy to probe the function of CICR in wild-type and LMO4 KO neurons.

We carried out whole-cell electrophysiological recordings from CA3 pyramidal neurons of the hippocampus in current-clamp mode. A brief current injection of increasing amplitude (500 ms, 50-400 pA) induced reliable action potential firing that was followed, upon cessation of the depolarizing current, by an AHP (Fig. 2.3). As shown in Fig. 2.3, the amplitude of the AHP was dependent on the number of action potentials fired during the depolarizing step. Interestingly, the amplitude of the AHP was significantly smaller in CA3 neurons from LMO4 KO compared to WT mice (Fig. 2.3A, B). This is unlikely to result from differences in passive membrane properties since input resistance was indistinguishable between WT and KO neurons (WT: 109.7 ±5.7 MOhm (n=25); KO: 107.9 ± 6.9 MOhm (n=24); P=0.751). To determine whether compromised machinery of CICR in LMO4 KO neurons might be responsible for this effect, we analyzed the behavior of the AHP following manipulations of CICR. Blocking CICR with ryanodine (100 μM; > 1 hour treatment) significantly reduced the amplitude of the AHP in WT CA3 neurons (Fig. 2.3$A_{1,2}$), suggesting that, in keeping with previous reports (Akita and Kuba, 2000; Kuba et al., 1983; Pineda et al., 1999; Sah, 1996; Sah and McLachlan, 1991; Torres et al., 1996), internal stores contribute a

Figure 2.3 Afterhyperpolarization is reduced in LMO4 KO CA3 neurons

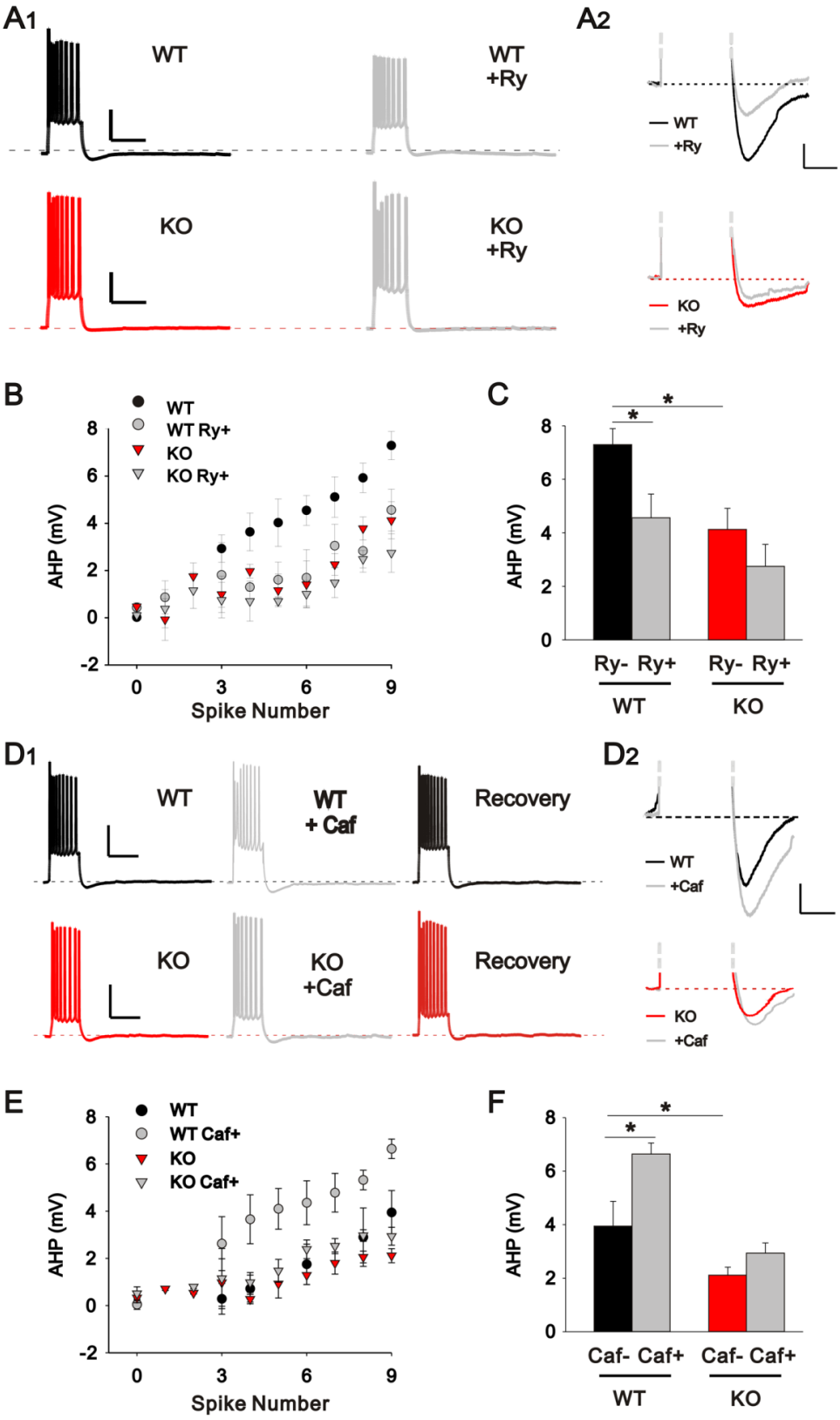

Figure 2.3 Afterhyperpolarization is reduced in LMO4 KO CA3 neurons

A_1, Superimposed current traces showing the afterhyperpolarization (AHP) measured in a WT and LMO4 KO CA3 neurons in control and following ryanodine treatment. Scale bar, 30mV, 500ms. ***A_2***, Enlarged traces of the AHP in ***A_1*** under control and ryanodine treatment. Scale bar, 2 mV, 250 ms. ***B***, the amplitude of AHP is expressed as a function of the spike number. ***C***, A bar graph comparing the amplitudes of the AHP (WT, n=9; LMO4, n=9). Ryanodine significantly reduced AHP in WT neurons (WT Ry+, n=11), but has no effect on LMO4 KO neurons (KO Ry+, n=12). *, P <0.05. ***D_1***, Superimposed traces of the AHP in control conditions , following caffeine administration and recovery from a WT and LMO4 KO CA3 neurons. Scale bar, 30mV, 500ms. ***D_2***, Enlarged traces of the AHP in ***D_1*** under control and caffeine treatment are combined. Scale bar, 2 mV, 250ms. ***E***, the amplitude of the AHP is plotted as a function of the number of action potentials. ***F***, bar graph comparing the amplitudes of the AHP. Littermate control neurons (WT caf-, n=7) had larger AHP compared to LMO4 KO neurons (KO caf-, n=10). Caffeine significantly increased the AHP in littermate control (WT caf+, n=7) but had little effect in LMO4 KO neurons (KO caf+, n=10). *, P <0.05. Mice age: 4-5 weeks.

significant portion of the calcium responsible for triggering the AHP in neurons. Interestingly, the same treatment of ryanodine to slices derived from LMO4 KO animals failed to reduce the amplitude of the AHP (compared to non-treated control sister slices; Fig. 2.3B,C). Because the amplitude of the AHP in KO slices closely matches that observed in WT slices treated with ryanodine, these results suggest that the reduction of the AHP seen in LMO4 KO reflects compromised CICR machinery in these cells.

To further test this idea, we next tested the effect on the AHP of facilitating the CICR process by caffeine. As previously reported in CA1 neurons (Torres et al., 1996), we found that the acute administration of caffeine (10 mM) significantly enhanced the amplitude of the AHP in wild type CA3 neurons (Fig. 2.3D_1,D_2, E, F). In addition to enhancing CICR, caffeine also blocks adenosine receptors and phosphodiesterase. However, these non-specific actions likely do not contribute to the effect reported here on the AHP since adenosine antagonists has no effect on the AHP (Torres et al., 1996) and phosphodiesterase inhibitors reduce, rather than increase, the AHP (Andrade and Nicoll, 1987; Madison and Nicoll, 1986). Interestingly, we found that the same treatment of caffeine was devoid of any effect on the AHP of CA3 neurons from LMO4 KO slices (Fig. 2.3D_1, D_2, E, F). Together, these results attest to the presence of compromised CICR in LMO4 KO neurons.

We next determined whether the AHP observed in CA1 pyramidal neurons (Madison and Nicoll, 1984) was also reduced in LMO4 KO slices. Intriguingly, we found that the amplitude of the AHP in CA1 cells was of similar amplitude in WT and LMO4 KO animals ($p= 0.368$; not shown). The reasons underlying this cellular subtype specificity are unclear at present. It may reflect, in principle, the differential contribution of KCNQ channel subtypes to the AHP in these two subregions of the hippocampus (Tzingounis et al., 2010) or simply stem

from the lower density of LMO4 levels in CA1 than in CA3 neurons (Fig. 2.1A). Regardless of these possibilities, our analysis of the contribution of CICR to the generation of the AHP in hippocampal CA3 pyramidal neurons shows that the CICR machinery is severely compromised in LMO4 KO neurons. These effects are fully consistent with the robust reduction of RyR2 in LMO4 knock-out animals.

Rescue of AHP with LMO4 over-expression in LMO4 KO slices.

We next sought to determine whether restoring the expression of LMO4 in LMO4 KO neurons would rescue the AHP deficit. To this end, we prepared organotypic slices from WT and LMO4 KO. Whole-cell recordings from CA3 neurons in this preparation revealed that these neurons expressed an AHP in response to trains of action potentials, although it was somewhat smaller than that recorded in acute slices. Nevertheless, we observed that the amplitude of the AHP was significantly smaller in slices prepared from LMO4 KO, compared to WT (Fig. 2.4), thereby recapitulating in organotypic slices the overall phenotype observed in acute slices. We then overexpressed LMO4 (along with dsRed) by means of biolistic transfection. Whole-cell recordings from fluorescently labeled transfected CA3 neurons 24-48 hours following transfection revealed that expression of LMO4 restored AHP in LMO4 neurons to a similar level as seen in WT neurons (Fig. 2.4), whereas KO cells transfected with dsRed alone showed no effect (data not shown). Together, these results show that the deficit of the AHP observed in LMO4 KO is rescued by single-cell overexpression of LMO4 and implies that this deficit reflects a cell-autonomous mechanism and does not reflect a broader anomaly in the KO.

Figure 2.4 Afterhyperpolarization is rescued in LMO4 KO CA3 neurons by restoring LMO4 expression

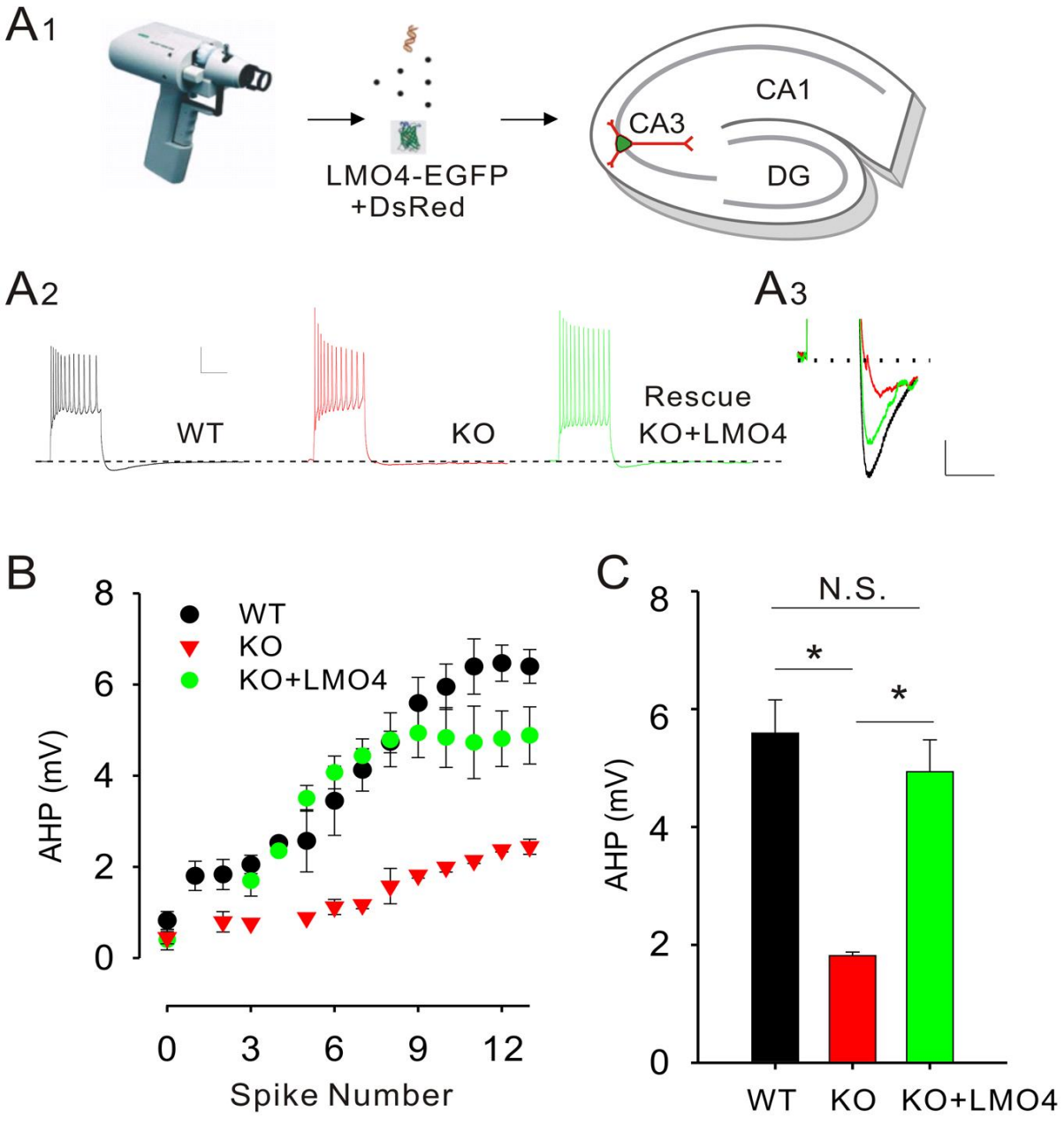

Figure 2.4 Afterhyperpolarization is rescued in LMO4 KO CA3 neurons by restoring LMO4 expression

A_1, Schematic representation of transgene delivery into neurons of organotypic slice culture by gene gun. ***A_2***, Superimposed current traces showing the afterhyperpolarization (AHP) measured in organotypic slice cultures of CA3 neurons from WT, KO and KO transfected with LMO4-EGFP (KO + LMO4). Scale bar, 20 mV, 250 ms. ***A_3***, Enlarged traces of the AHP in ***A_1*** under control and transfected neurons. Scale bar, 2 mV, 500 ms. ***B***, the amplitude of AHP is expressed as a function of the spike number (WT, n=12; KO, n=12; KO + LMO4, n=6). ***C***, A bar graph comparing the amplitudes of the AHP when 9 spikes were induced. AHP was significantly reduced in KO compared to WT neurons, but was rescued in LMO4-EGFP transfected KO neurons. *, $P < 0.05$.

Caffeine facilitation of CICR during a train of action potentials is abolished in LMO4 KO slices

Our results thus far show that the changes in the behavior of the AHP in CA3 pyramidal neuron induced by acute pharmacological challenges to CICR machinery are abolished in LMO4 KO slices. The most straightforward interpretation of these results is that the RyR2-mediated calcium release induced by trains of action potentials is reduced in LMO4 KO. To further support this interpretation, we carried out multiphoton calcium imaging experiments from CA3 neurons in WT and LMO4 KO slices. In current-clamp mode, action potential firing induced by direct current injection was accompanied by robust increases in intracellular calcium concentration (Fig. 2.5). This rise in intracellular calcium, and the amplitude of the AHP recorded from these same cells, was well correlated with the number of action potentials elicited during current injection (Fig. 2.5B, C, D). In WT neurons, bath administration of caffeine (10 mM; 10-15 minutes) lead to an increase in the amplitude of the AHP , in keeping with our previous observations in acute slices (Fig. 2.3), along with a robust enhancement of intracellular calcium triggered by trains of action potentials (Fig. 2.5). Remarkably, the potentiating effect of caffeine on both the AHP and intracellular calcium was abolished in CA3 neurons from LMO4 KO slices. Together, these results support the notion that CICR is severely compromised in LMO4 KO.

CICR-mediated facilitation of glutamatergic transmission in CA1 is impaired in LMO4 KO mice.

Despite some controversies (Carter et al., 2002), a number of studies have shown that, at least under certain conditions, calcium from CICR contributes to neurotransmitter release

Figure 2.5 Imaging spike-evoked calcium signals in CA3 neurons

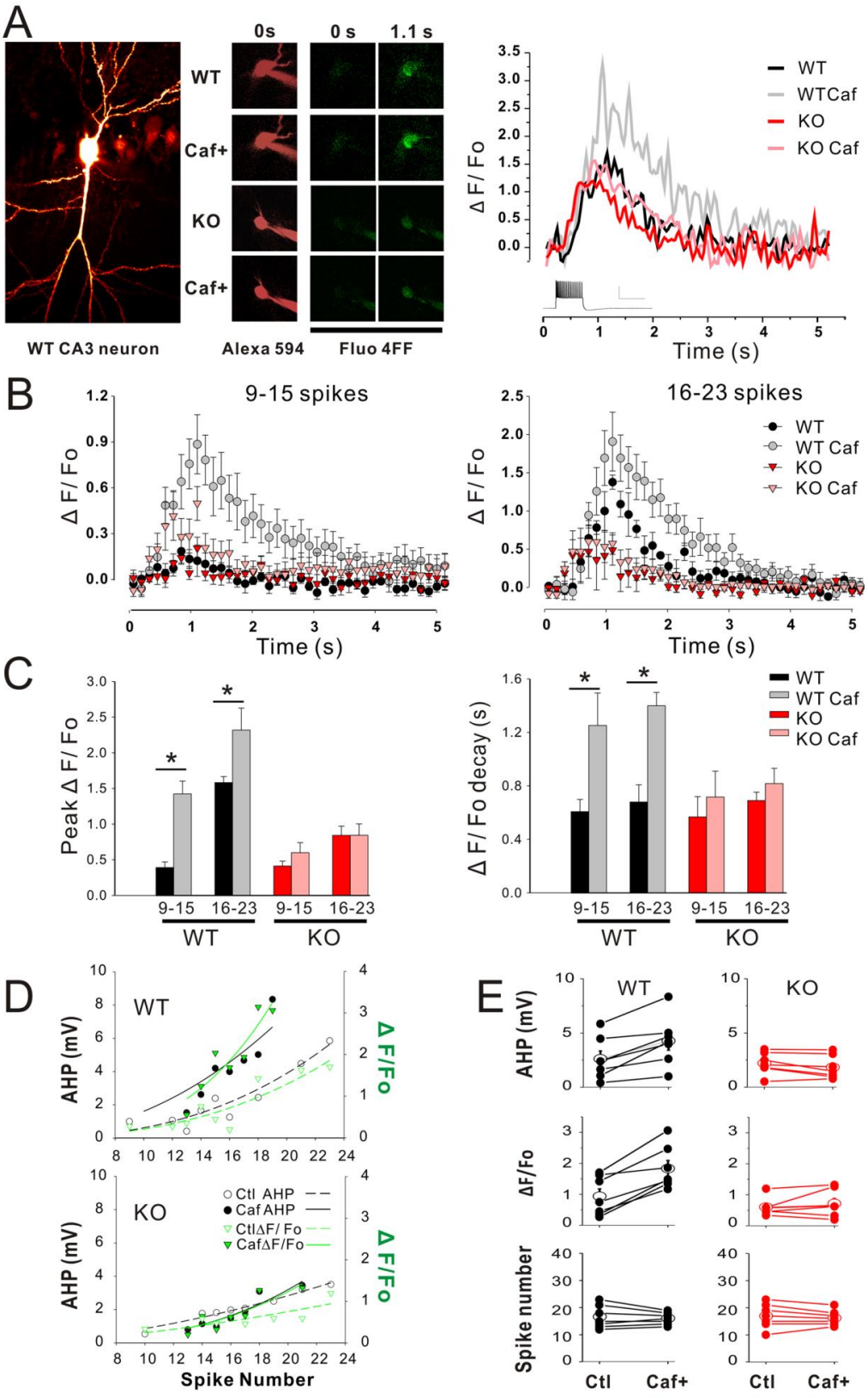

Figure 2.5 Imaging spike-evoked calcium signals in CA3 neurons

A, a representative CA3 neuron image with dendrites on the left. The right panel shows Alexa Fluor 594 hydrazide images (left, 0 s) and Fluo-4FF images of spike-evoked calcium signals from WT and KO CA3 neurons at resting levels (middle, 0 s) and at peak levels (right, 1.1 s) before and after caffeine treatment (10 mM 10 -15 min) treatment. Superimposed traces to the right show time course of increasing somatic fluorescence ratio signals (ΔF/Fo) evoked by 500 ms current injection of 400 pA before and after caffeine treatment. Calcium signals were measured from the soma (excluding the nucleus) on an average of 2-3 different areas with 80 video frames of time course traces obtained (65 ms per frame), respectively, in a representative WT neuron, a KO neuron before and after caffeine treatment. ***B***, average time course traces of somatic fluorescence calcium signals (ΔF/Fo) were binned according to the number of spikes. ***C***, peak values of ΔF/Fo (left) and the decay constant (tau, right) are shown from WT and KO CA3 neurons binned to the different number of spikes (9-15 and 16-23). Both of the peak ΔF/Fo and the decay time constant were significantly increased in WT neurons before and after caffeine treatments (n=7) but not KO neurons (n=9). $p<0.01$, paired Student's t test. ***D***, the amplitude of the AHP (black dots, values refer to the left Y axis) and ΔF/Fo (green triangles, values refer to the right Y axis) with or without caffeine are plotted as a function of the spike numbers. Data points are fitted for AHP (black) and ΔF/Fo (green) with non-linear regression power equation displayed by continued or dashed lines for caffeine treatment or control, respectively. ***E***, Values of paired AHP, ΔF/Fo and spike numbers (top to bottom) are shown and connected by lines before and after caffeine treatment from the same set of CA3 neurons. Mice age: 3 weeks.

(Emptage et al., 2001; Llano et al., 2000; Peng, 1996; Zhang et al., 2009). These studies prompted us to examine several determinants of glutamatergic synaptic transmission in LMO4 KO. As a first-pass test, we recorded AMPAR-mediated miniature excitatory post-synaptic currents (mEPSCs) from CA1 pyramidal neurons whose main glutamatergic input originates from the CA3 region. Interestingly, we found that frequency of mEPSCs was robustly reduced in LMO4 KO mice compared to littermate controls (Fig. 2.6A) whereas there was no difference in the amplitude of mEPSCs. Provisionally, we interpreted these results as indicating lower glutamate release probability in LMO4 KO mice.

We next determined release probability using a more direct experimental approach by comparing the rate of blockade of NMDAR-mediated currents by the irreversible and use-dependent NMDAR blocker MK-801. This method is a reliable and widely used approach to evaluate release probability (Hessler et al., 1993; Rosenmund et al., 1993). As expected, bath administration of MK-801 (30 μM; during AMPAR blockade) induced a use-dependent, progressive block of NMDAR-mediated evoked excitatory post-synaptic currents (eEPSCs) that could be fitted by a single exponential (Fig. 2.6B). We found that the rate of decline of NMDAR-mediated eEPSCs induced by MK-801 was close to twofold slower in LMO4 KO mice compared to WT littermate controls (τ = 44.97 versus 24.69; Fig. 2.6B), indicating a marked decrease in glutamatergic release probability in LMO4 KO mice. As a last test to monitor release probability, we next carried out paired-pulse ratio analysis (Dobrunz and Stevens, 1997). As expected for CA1 recordings, we found paired-pulse facilitation (PPF) of AMPAR-mediated eEPSCs in WT slices. Somewhat unexpectedly, we failed to observe any significant change in PPF in LMO4 KO slices (Fig. 2.6C). As such, our assessment of release probability in LMO4 KO yielding different approaches provides ambiguous results. Because

Figure 2.6 Release probability in LMO4 KO mice

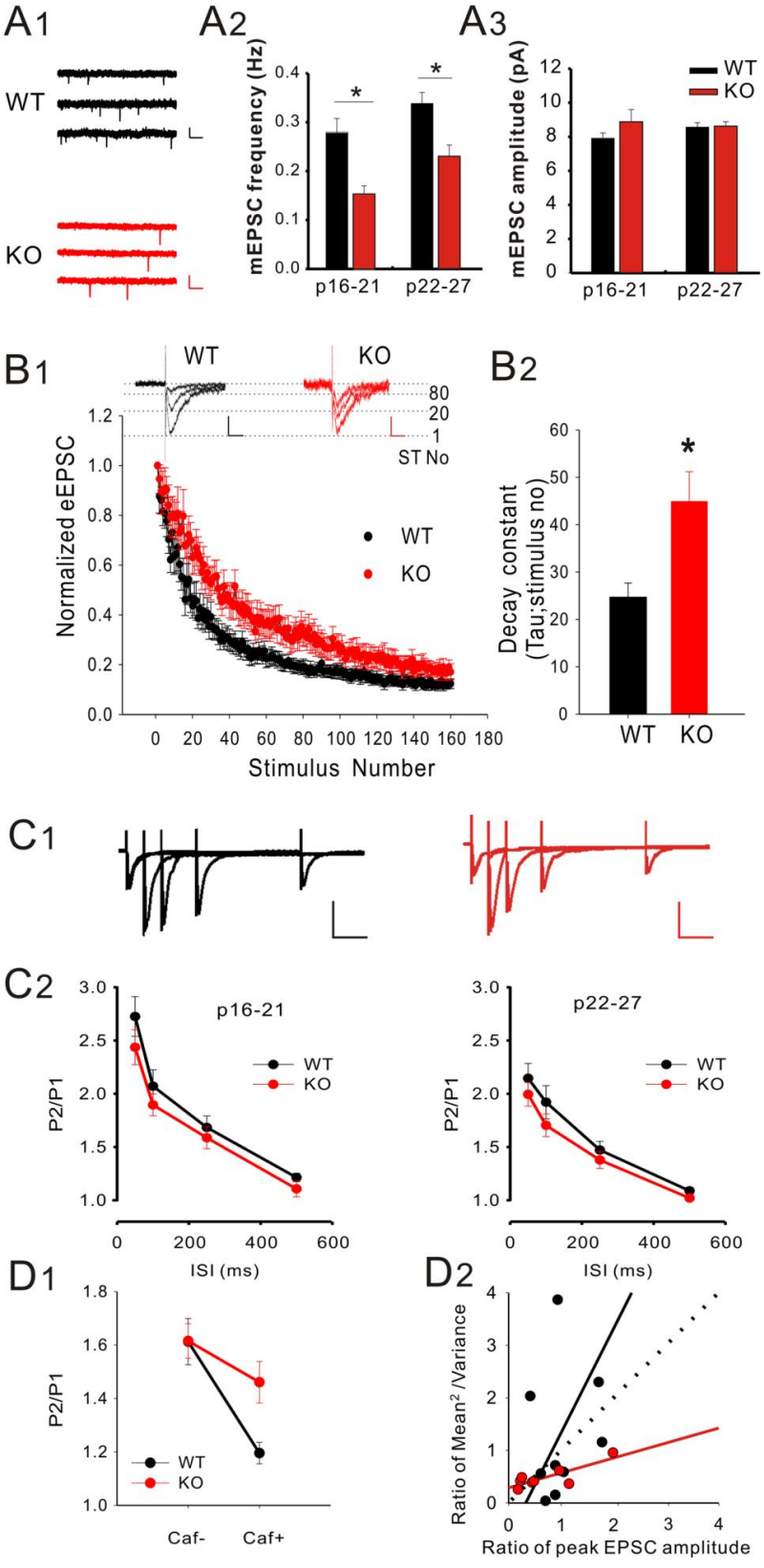

Figure 2.6 Release probability in LMO4 KO mice

A1, Current traces showing mEPSCs from WT and a KO CA1 pyramidal neurons. Scale bars: 10 pA, 2 s. Top: P16; middle: P22; bottom: P27. The frequency (***A2)*** and amplitude (***A3***) of mEPSCs were binned according to the age of the animals. n=7, WT; n=9, KO at P16-21; n= 23, WT; n=27, KO at P22-27. *, $p<0.05$. ***B1,*** the 1st, 20th and 80th NMDAR-mediated EPSC current traces in the presence of the irreversible NMDAR inhibitor MK-801 is shown for a WT (left) and KO (right) CA1 neuron. Scale bars, 50 pA / 50 ms. The progressive block of NMDAR-mediated EPSCs is plotted as a function of the pulse number and could be fitted by a mono-exponential decay. ***B2,*** The decay constant was significantly increased in KO (44.97 ms; n=7) compared to littermate control neurons (24.69 ms; n=9; $p<0.01$, unpaired Student's t test). ***C1,*** Current traces depicting AMPAR-mediated eEPSCs induced by paired pulses delivered at different interstimulus intervals (ISIs) obtained from a WT and a KO neuron are shown. Scale bar, 50 pA / 50 ms. ***C2,*** The average ratios (P2/P1) of AMPAR-mediated eEPSCs obtained with different ISIs in WT mice (n =13, P16-21; n=13, P22-27) were not significantly different at each age group from those obtained in LMO4 KO mice (n =12, P16-21; n=16, P22-27). ***D1***, Dot plots showing the average ratios (P2/P1) of AMPAR-mediated eEPSCs at 50 ms ISI before and after caffeine (10 mM) treatment. Caffeine significantly reduced the P2/P1 which reflects the increased transmitter release in littermate control (WT, n=10) but had little effect in LMO4 KO neurons (KO, n=8). *, $P<0.05$. ***D2***, Dot plots summarizing CV analyses of the effects of caffeine (10 mM) on eEPSC in brain slices. Dot plots show the ratio [($Mean^2$/variance caffeine)/($Mean^2$/variance baseline)] as a function of the ratio (Peak amplitude caffeine/Peak amplitude baseline).

the contribution of CICR to basal release probability is somewhat controversial, we did not further attempt to resolve this discrepancy. However, we did ask whether acutely increasing CICR by an acute caffeine treatment would enhance release probability, thereby providing another index to monitor CICR in LMO4 KO. As previously observed (Martin and Buno, 2003), bath administration of caffeine significantly enhanced release probability of glutamate onto WT CA1 neurons, as inferred by both paired-pulse ratio analysis (Fig. 2.6D_1) and coefficient of variation analysis (Fig. 2.6 D_2). Caffeine however did not modulate release probability in LMO4 KO (Fig. 2.6D). Altogether, these results indicate that the effectiveness of a pharmacological activation of RyR-mediated CICR was abolished in LMO4 KO slices.

CICR-mediated facilitation of glutamatergic transmission during trains of action potentials in CA1 is impaired in LMO4 KO mice.

The contribution of CICR to synaptic release elicited by prolonged trains of action potentials has been well demonstrated (Zhang et al., 2009). We therefore investigated synaptic responses in CA1 to repetitive stimuli of increasing frequency in WT and LMO4 KO mice (Fig. 2.7A). As expected, low frequency stimulation (1 Hz; 10 pulses) induced eEPSCs that showed neither facilitation nor depression in either littermate control or LMO4 KO neurons. At 10 Hz, eEPSCs exhibited facilitation in both WT and LMO4 KO mice. Interestingly, a robust reduction in the synaptic facilitation induced by a 20 Hz stimulation became manifest in LMO4 KO neurons and this impairment was also present at the highest frequency tested (50 Hz). That altered calcium-dependent signaling was involved in this compromised synaptic facilitation in LMO4 KO animals was suggested by the rescue of this defect by increasing extracellular concentrations of calcium (to 5 and 7.5 mM; Fig. 2.7B).

Figure 2.7 The facilitation of eEPSCs induced by high frequency stimulation is impaired in LMO4 KO mice

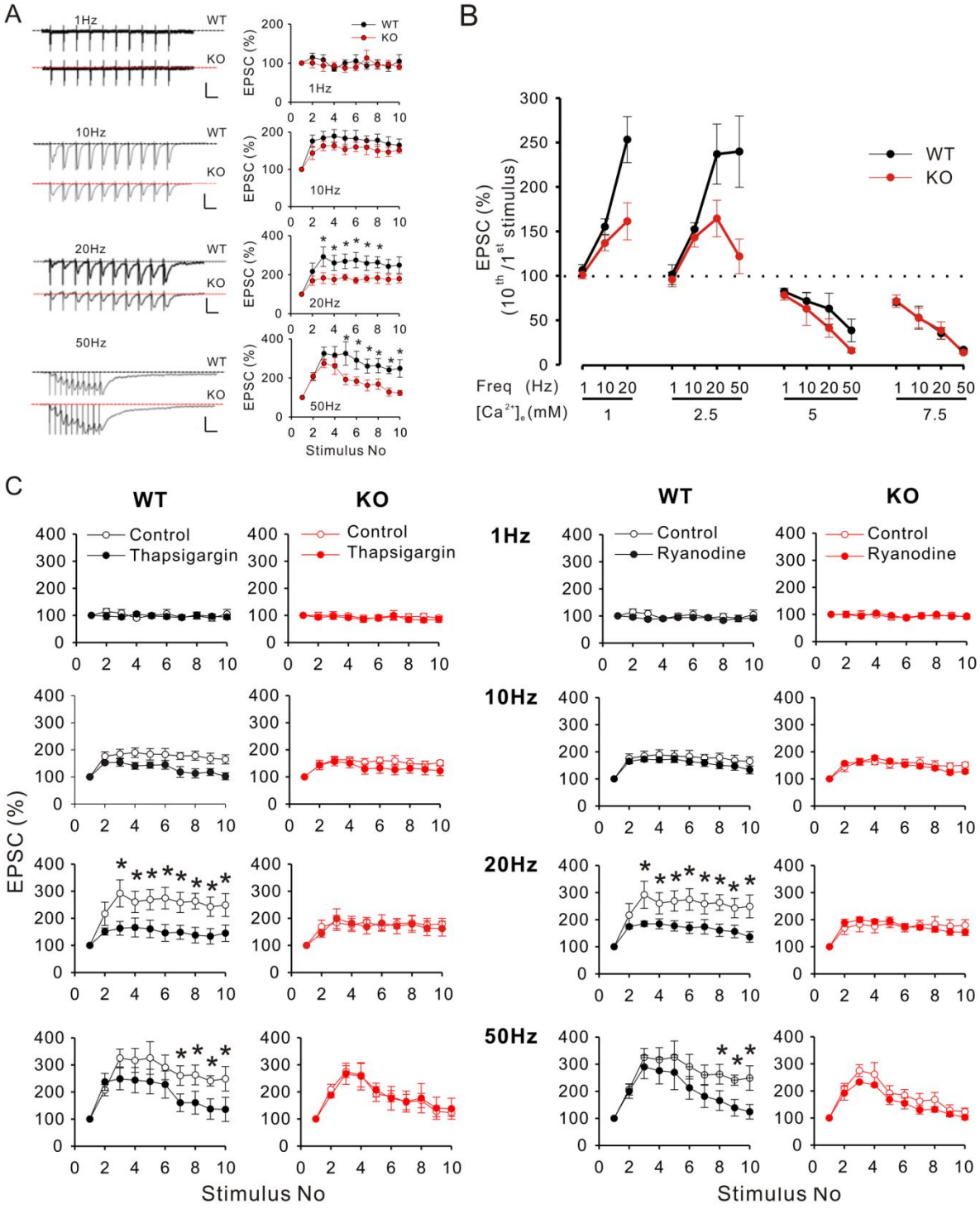

Figure 2.7 The facilitation of eEPSCs induced by high frequency stimulation is impaired in LMO4 KO mice

A, Left panel shows current traces from CA1 recordings depicting synaptic facilitation elicited by trains of increasing frequency (1, 10, 20 and 50 Hz; n=11 for both WT and KO). External calcium concentration = 2.5 mM. Scale bars for 1 Hz: 50 pA, 1s; 10 Hz: 50 pA, 100 ms; 20 & 50 Hz: 100 pA, 50 ms. ***B,*** Percentage change of EPSC elicited by the 10^{th} stimulus normalized to the response to the 1^{st} stimulus at indicated external calcium concentrations. Synaptic facilitation deficits in KO neurons were compensated by elevated external calcium concentration. n= 11 for each genotype at each calcium concentration. *, $P<0.05$. ***C,*** The effects of thapsigargin (TG) and ryanodine (RY) treatments on synaptic facilitation were compared in WT (Left) and LMO4 KO slices (right). The control trains are reploted here from ***A*** for clarity. All data points are presented as mean ± SEM n =11 for groups except n=6 for RY treated KO group.

We reasoned that the altered behavior in short-term plasticity outlined above in LMO4 KO slices could reflect the putative generalized impairment of CICR in these mice. We analyzed this short-term plasticity following depletion of intracellular calcium stores by treating slices with thapsigargin (Treiman et al., 1998). In keeping with a previous report (Zhang et al., 2009), we found that a significant proportion of the facilitation of eEPSC by high frequency trains was blocked by thapsigargin treatment in WT slices (Fig. 2.7C). Interestingly, thapsigargin had no effect on the behavior of trains of eEPSCs elicited in LMO4 KO slices. Similarly, treatment with ryanodine, at a concentration that blocks RyRs and prevents CICR, also suppressed synaptic facilitation at 20 Hz in WT neurons but had no effect in LMO4 KO neurons (Fig. 2.7D). Because the CICR-independent portion of facilitation observed in WT mice closely matched that observed in LMO4 KO, collectively, these results strongly support the notion that CICR contributes to the maintenance of facilitation during prolonged trains and that this process is severely compromised in LMO4 KO.

Hippocampal long-term potentiation is impaired in LMO4 KO mice

Having determined the effects of LMO4 deletion on short-term plasticity, we next asked whether ablation of LMO4 was accompanied with deficits of long-term potentiation (LTP). To address this possibility, we obtained whole-cell recordings from CA1 pyramidal neurons and applied a standard pairing LTP protocol (i.e., 100 pulses delivered at 1 Hz while holding the cell at 0 mV). This protocol induced a robust LTP in WT slices. Interestingly, the magnitude of the potentiation was markedly reduced in slices from littermate LMO4 KO mice (Fig. 2.8A). Although a plethora of mechanisms could in principle account for this defect in LTP, we wondered whether the reduction in release probability in LMO4 KO could underlie

this defect simply by reducing the number of successful synaptic release events during the LTP-inducing protocol. To address this possibility, we reasoned that increasing the number of pulses during the LTP protocol would rescue the defect in LTP if it stemmed solely from a presynaptic release probability deficiency. We therefore repeated the experiments using a higher stimulus number during the protocol. However, these experiments were soon complicated by the fact that the magnitude of the LTP in these conditions was so strong that it elicited in several recordings the appearance of unclamped action potentials despite efforts to begin the recordings with small baseline eEPSCs (*ca.* 20-50 pA; not shown). We thus repeated the entire series of LTP experiments with the intracellular recording solution supplemented with the cell-impermeant sodium channel blocker QX-314. Using a 100 pulse LTP protocol, inclusion of QX-314 did not induce any phenotypic changes since the magnitude of LTP was still severely compromised in LMO4 KO slices (compare Fig. 8*B1* to 8*A*). However, this defect was no longer present when 300 and 600 pulses (at 1 Hz) were applied during the LTP induction protocol (Fig. 2.8B2, B3). Altogether, these results are consistent with the observation that glutamate release probability is lower in LMO4 KO slices and suggest that this defect translates in a shift in the induction rules of LTP in the hippocampus.

LMO4 KO mice exhibit spatial learning and memory deficits in a Morris water maze test

To determine if the overall changes in release probability in the LMO4 KO animals was associated with a defect in a hippocampus-dependent spatial learning task we conducted a Morris water maze test. During 9 days of training the escape latency (time taken to get to

Figure 2.8 Impaired LTP in the CA1 neurons in LMO4 KO mice

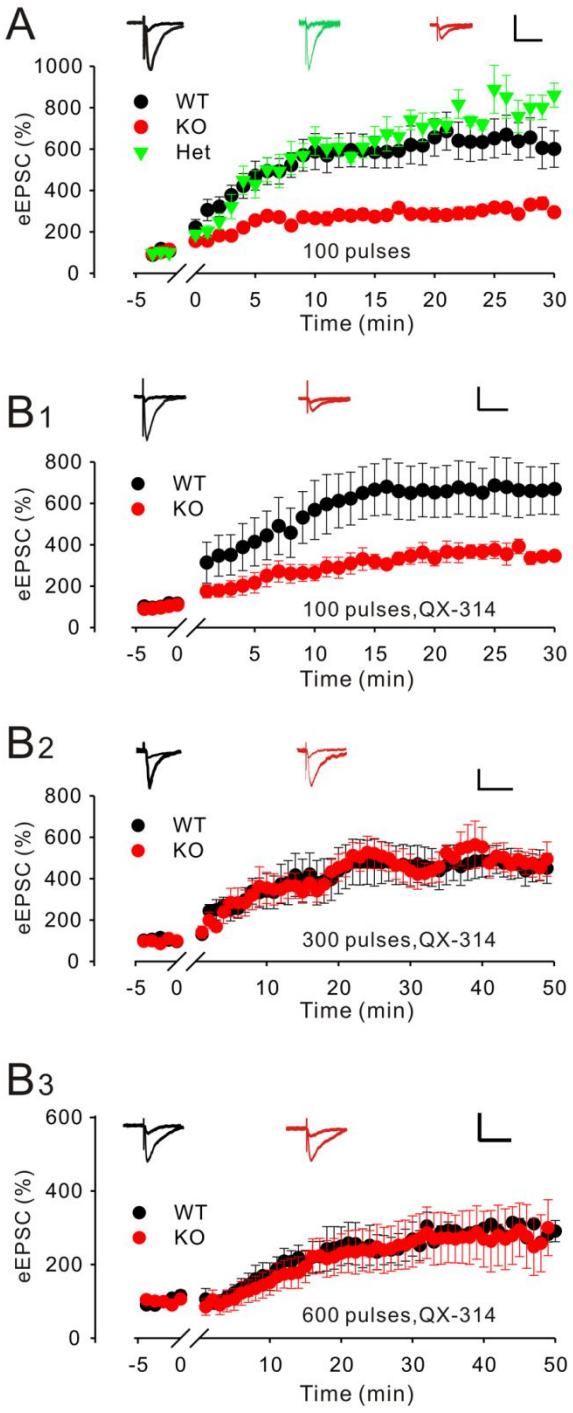

Figure 2.8 Impaired LTP in the CA1 neurons in LMO4 KO mice

A, Current traces of AMPAR-mediated eEPSCs from CA1 neurons at baseline and after LTP were induced by a pairing protocol (100 pulses delivered at 1 Hz at 0 mV). The magnitude of LTP was significantly reduced in LMO4 KO mice. n=10, WT; n=6, Het; n=9, KO. Scale bars: 100 pA, 50 ms. *In* ***B,*** the effects of increasing the number of pulses delivered during the pairing protocol (*i.e.,* 100, 300 and 600 pulses while holding the cell at 0 mV) were compared in WT and LMO4 KO slices. In (***B***), the intracellular recording solution was supplemented with QX-314. In these conditions, the magnitude of LTP was significantly reduced in LMO4 KO mice following the 100 pulse LTP-induction regimen (***B_1***; n=8, WT; n=7, KO. Scale bars: 100 pA, 50 ms.), but not following the 300 (***B_2***; n=6, WT; n=4, KO. Scale bars: 100 pA, 50 ms) and 600 (***B_3****;* n=11, WT; n=11, KO. Scale bars: 100 pA, 50 ms) pulse regimens.

hidden platform) was significantly longer for LMO4 KO mice compared to their littermate controls, indicating a spatial learning deficit (Fig. 2.9A). In agreement with this interpretation, during the probe trial the LMO4 KO mice also spent less time in the BR quadrant where the escape hidden platform had been located during training (Fig. 2.9B). On the day of the probe test, LMO4 KO mice also took 3 times longer than littermate controls to first cross over where the hidden platform was previously located during training (Fig. 2.9C). This deficit is unlikely to reflect a generalized alteration in motor function since WT and KO mice displayed indistinguishable swimming speeds (Fig. 2.9D). These results suggest that spatial learning and memory is impaired in LMO4 KO mice.

Figure 2.9 Camk2αCre/LMO4flox mice exhibit spatial learning and memory deficits in Morris water maze hidden platform test

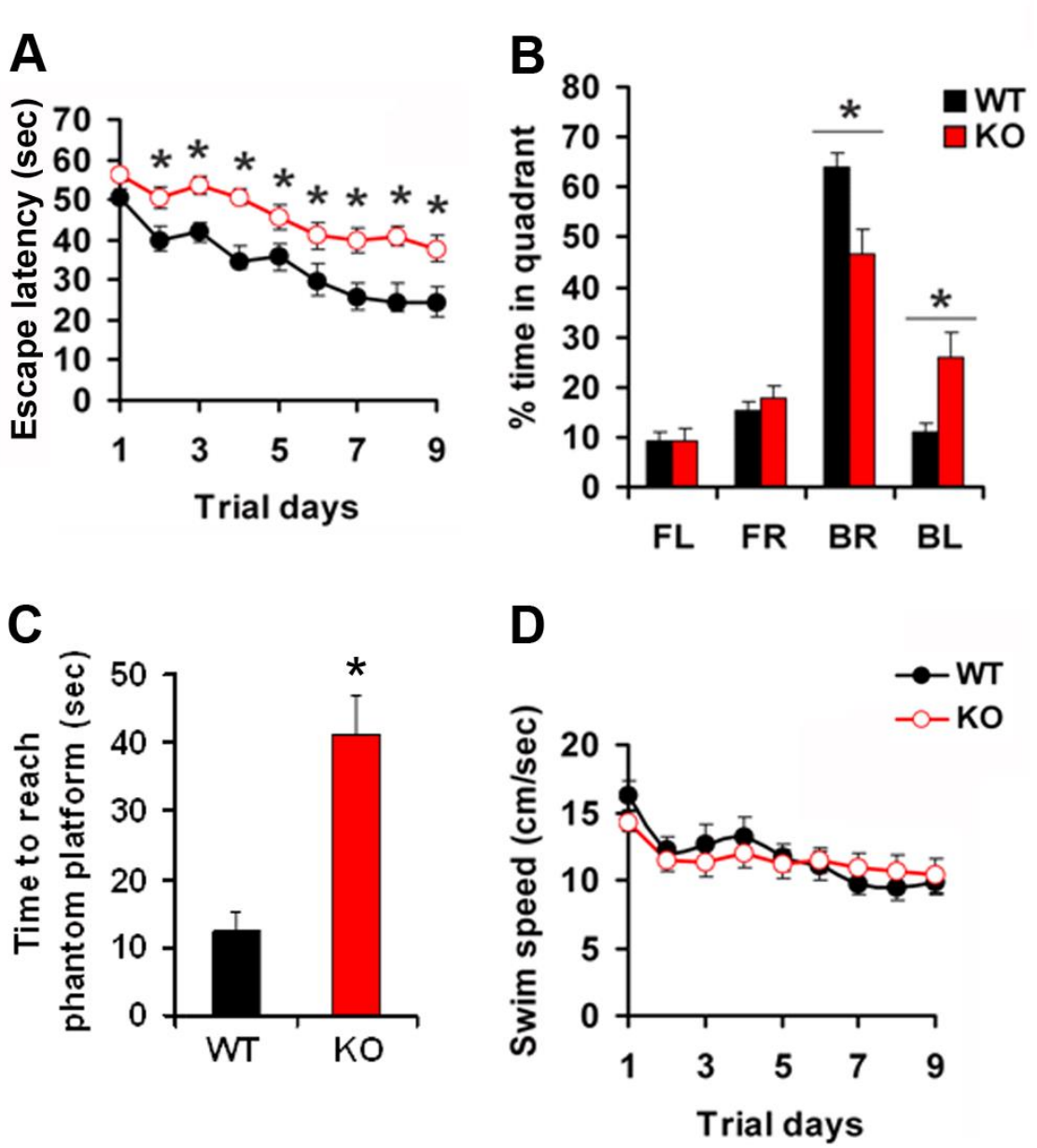

Figure 2.9 Camk2αCre/LMO4flox mice exhibit spatial learning and memory deficits in Morris water maze hidden platform test

A, During the 9 days of training LMO4 KO mice took a longer time to find the platform I the water maze task. Data represent means of 4 trials ± SEM. ***B***, On Probe trail (Day 10) LMO4 KO mice spent significantly less time in the quadrant (BR) where the platform was previously located during training. (quadrants: FL, front left; FR, front right; BL, back left (target quadrant); BR, back right. ***C***, On probe test LMO4 KO mice took 3 times longer to first cross over where the platform was previously located during training. ***D***, Swimming speeds were not different between WT and KO mice. *, $p<0.05$. n=12, WT, n=15, KO.

2.7 Discussion

In this study, we show that LMO4 is an important regulator of the molecular machinery controlling CICR in central neurons. First, we found that ablating LMO4 in neurons is associated with reduced RyR2 mRNA and protein expression. This reduction likely reflects a direct transcriptional function for LMO4 since we found that it positively regulates the RyR2 promoter in a luciferase assay. We next sought to functionally assess the role of LMO4 in controlling CICR in central neurons by monitoring in a forebrain-specific LMO4 KO a number of electrophysiological responses known to be modulated by CICR. Thus, we found that the CICR modulation of the AHP in hippocampus CA3 neurons was severely reduced in LMO4 KO and that this deficit likely reflected compromised release of calcium from internal stores, as determined in multiphoton calcium imaging experiments. As an additional test, we then found that the CICR-mediated modulation of glutamate release probability onto CA1 pyramidal neurons was likewise abolished in recordings from LMO4 KO slices. These changes were accompanied with impaired hippocampal LTP and hippocampal-dependent behavioral tasks. Together, these results identify a key role of LMO4 in controlling central synapse and neuronal function and that disruption of these functions results in important behavioral deficits.

The role of LMO4 in neuronal function is only beginning to be explored. A previous study described a key role of LMO4 in the patterning of thalamocortical connections, a function that was ascribed to its transcriptional role (Kashani et al., 2006). However, how this occurs is unclear, in part emphasizing our fragmentary understanding of the cellular roles played by this protein. To begin addressing this limitation, we carried out a non-biased, high throughput screen to identify potential targets modulated by LMO4. This effort led us to the

identification of RyR2 as a gene whose level of expression depends upon LMO4. Although direct DNA binding has not been demonstrated for LMO4, this protein interacts with the transcription factor CREB to regulate gene expression in response to synaptic activity and calcium influx through both L-type voltage sensitive calcium channels and NMDA receptors (Kashani et al., 2006). CREB is known to activate the RyR2 promoter (Ziviani et al., 2011) and LMO4 may work through CREB to regulate the expression of RyR2. Thus, LMO4 is strategically positioned to tune the gain of CICR, a biologically powerful mechanism that modulates neuronal function, in a manner dependent on overall neuronal excitability.

Because it is well established that a significant portion of the calcium that triggers AHP originates from internal stores (Akita and Kuba, 2000; Kuba et al., 1983; Pineda et al., 1999; Sah, 1996; Sah and McLachlan, 1991; Torres et al., 1996), we reasoned that studying the behavior of the AHP in response to manipulations aimed either at increasing or blocking CICR would provide a valid approach to probe the CICR machinery in LMO4 KO. That the amplitude of the AHP in CA3 pyramidal neurons from LMO4 KO was smaller than littermate controls provided an indication that LMO4 might regulate CICR. This idea was further confirmed by the observations that: 1) blocking CICR had no effect on the amplitude of the AHP in LMO4 KO neurons while this same manipulation significantly reduced that in WT neurons and; 2) the ability of caffeine to increase the AHP was severely curtailed in LMO4 KO neurons. Importantly, multiphoton calcium imaging experiments confirmed that the caffeine-induced potentiation of calcium release from internal stores during a train of action potentials was abolished in LMO4 KO slices. As such, these findings strongly argue that the CICR machinery is compromised in LMO4 KO mice and that this reduction likely stems from the reduced levels of RyR2.

There is mounting evidence suggesting that calcium from CICR is a powerful modulator of neurotransmitter release, at least under some conditions (Emptage et al., 2001; Llano et al., 2000; Peng, 1996; Zhang et al., 2009). These studies therefore prompted us to examine several determinants of synaptic release in LMO4 KO slices. As a first step, we assessed basal glutamate release probability in WT and KO slices. Intriguingly, the different, but complimentary, approaches used to estimate glutamate release probability yielded inconsistent outcomes. Indeed, whereas the frequency of mEPSCs and the rate of block of NMDAR-mediated current by the use-dependent blocker MK-801 both concurred in indicating that release probability was lower in CA1 synapses of LMO4 KO compared to WT slices, paired-pulse ratio of eEPSCs was indistinguishable between WT and KO. The reasons underlying this discrepancy are unclear at present. Of relevance here, we failed to observe any changes in paired-pulse ratio in CA1 neurons following blockade of CICR (Fig. 2.7), in keeping with a previous study (Carter et al., 2002). Although the contributing role of CICR to setting basal release probability during low frequency neurotransmission may be considered to be equivocal (Carter et al., 2002), it is clear that acute pharmacological challenges that enhance calcium release from internal stores can lead to a marked enhancement of release probability (Martin and Buno, 2003; and Fig. 2.6D). That this effect is severely abolished in LMO4 KO slices, irrespective of the inconsistencies between the different experimental methods used to probe basal release probability, provides yet another line of evidence supporting the role of LMO4 as a key modulator of CICR.

As a means to provide alternative evidence supporting the role of LMO4 in controlling CICR, we reasoned that the recently described ability of CICR to modulate the facilitation of glutamate release induced by a burst of action potentials (Zhang et al., 2009) would provide a

reliable index to further assess CICR function in LMO4 KO slices. As expected, a train of 10 pulses induced a robustly facilitating postsynaptic response when applied at frequencies higher than 10 Hz. Manipulations that altered CICR revealed that calcium from internal stores significantly contributed to maintain this synaptic facilitation during trains of action potentials (*i.e.*, the CICR contribution begins to be apparent after the third to fourth stimulus of a high stimulus train). In our hands, the maximal (and likely saturating) synaptic facilitation induced by high frequency bursts was attained during the 20 Hz stimulation regimen, since a 50 Hz stimulation did not further facilitate the postsynaptic response (*i.e.*, *ca*. a 3 fold potentiation). Altogether, we have recapitulated the findings of (Zhang et al., 2009) that show that CICR robustly and dynamically modulate release probability, in part in contributing to the maintenance of release fidelity during trains of action potentials.

Having established in our hands short-term plasticity regimens that are robustly modulated by CICR processes, we then analyzed the behavior of these trains in LMO4 KO slices. In keeping with LMO4's putative role in modulating CICR, the magnitude of the facilitation induced by high frequency trains in slices from LMO4 KO CA1 neurons was markedly smaller than that observed in WT slices and, second, closely matched that obtained in WT slices treated with either thapsigargin or ryanodine. Importantly, these same treatments were devoid of any effect on high frequency train responses induced in LMO4 KO slices. Altogether, these data add to and support those obtained on the behavior of the AHP in CA3 neurons and outline a robust deficit of CICR in neurons devoid of LMO4.

It is striking to note that synaptic release phenotypes highly analogous to those we show here in LMO4 KO mice have recently been reported to occur in presenilin 1 & 2 double knockout mice (Zhang et al., 2009). A GeoProfile report (GDS787 / 98122_at / Lmo4 / Mus

musculus) indicates that the expression of LMO4 is dramatically reduced in the forebrain of these double knockout mice (Beglopoulos et al., 2004) whereas our qPCR analysis, conversely, showed that presenilin 1 and 2 expression in hippocampus was not altered in LMO4 KO mice (data not shown). Although in need of formal support, these findings collectively raise the intriguing possibility that, at least in part, the deficiency associated with presenilin deletion might actually be secondary to reduced LMO4 expression and thereby mplicate LMO4 in the etiopathology of Alzheimer's disease. Along this line, decreased expression of LMO4 in the entorhinal cortex and in the hippocampal region was reported in non-familiar Alzheimer's disease brains, and this reduction was correlated with the amount of neurofibrillary tangles (NFT), degenerating neurons, the severity of senile plaque deposition and an increase in β amyloid (Leuba et al., 2004). Altogether, our study shows that important neuronal and synaptic mechanisms relying on CICR are tightly controlled by LMO4 as early as 1 month of age. Because dysregulated calcium homeostasis and compromised synaptic function precede histopathological manifestations of neurodegeneration (Oddo et al., 2003; Rowan et al., 2003), it will be important to determine whether dysregulated LMO4 function or expression might contribute to the progression of Alzheimer's disease.

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CHAPTER 3

Manuscript #2

Restoring mGluR5 and endocannabinoid signaling by inhibition of PTP1B relieves anxiety

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3.1 Statement of Author Contribution

Z.Qin, N.R.Pandey, X.Zhang, J.-C.Beique, A.F.Stewart and H.-H. Chen designed research; Z.Qin did all the behaviour and electrophysiology experiments; N.R.Pandey and X.Zhou performed all the biochemistry experiments; T.Zaman assisted in the intra-amygdala injection; D.C.Lagace, X.Zhang, J.M.Brunel, J.-C.Beique and H.-H.Chen contributed unpublished reagents/analytic tools; Z.Q, N.R.Pandey and H.-H.Chen analyzed data; Z.Qin, A.F.Stewart, and H.-H.Chen wrote the paper.

Title Page

Restoring mGluR5 and endocannabinoid signaling by inhibition of PTP1B relieves anxiety

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Running title: Anxiolytic effect of a PTP1B inhibitor

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Number of figures (7) and tables (0)

Keywords: metabotropic glutamate receptors, stress, glucocorticoids,

3.3 Abstract

Activation of metabotropic glutamate receptor (mGluR) produces endocannabinoids (eCB). Inadequate eCB signaling is thought to underlie stress-induced anxiety. Previously, we reported that LMO4 is an endogenous inhibitor of the protein tyrosine phosphatase PTP1B. Here, we show that mGluR5 is a substrate of PTP1B. Mice with LMO4 deleted in glutamatergic neurons are anxious. This phenotype is associated with increased PTP1B activity and impaired mGluR5-dependent eCB signaling in the basolateral amygdala. Blocking PTP1B activity by intra-amygdalar injection of the PTP1B inhibitor Trodusquemine or a PTP1B shRNA restored amygdalar mGluR5-dependent eCB signaling and relieved anxiety in LMO4 mutant mice. Elevated PTP1B activity was also observed in the amygdala of wild type mice exposed to stress and in cultured neurons exposed to corticosterone, suggesting a general mechanism underlying stress. Collectively, these findings indicate that blocking PTP1B activity to restore mGluR5 and eCB signaling may have a therapeutic application in the treatment of anxiety.

3.4 Introduction

Anxiety disorders affect approximately 40 million adults in the United States each year (Kessler et al., 2005). The amygdala is central to the processing of fear and anxiety and MRI evidence indicates that the function of the amygdala is disrupted in anxiety disorders (Etkin et al., 2009). The basolateral amygdala (BLA) consists of 90% glutamatergic neurons (Carlsen, 1988) that send projections to the central amygdala (CeA), which contains almost exclusively GABAergic inhibitory neurons (McDonald, 1982). Amygdala circuitry is important in the control of anxiety. Increasing synaptic activity from BLA neurons onto the CeA is anxiolytic whereas reducing this activity is anxiogenic (Tye et al., 2011).

Endogenous cannabinoids (eCBs) modulate synaptic strength and are involved in the regulation of emotional states. eCBs are synthesized and released from postsynaptic neurons upon stimulation and signal retrogradely by binding to CB1 receptors at the presynaptic terminals to inhibit neurotransmitter release. CB1 receptors are present at high levels in inhibitory GABAergic interneurons and at lower levels in excitatory glutamatergic terminals, proximal axons, dendrites and cell bodies (Heifets and Castillo, 2009; Katona and Freund, 2008; Wilson and Nicoll, 2001). Increasing eCB levels by blocking fatty acid amide hydrolase (FAAH), an enzyme that degrades anandamide, or administration of low doses of CB1 receptor agonists produces anxiolytic, whereas CB1 antagonist produces anxiogenic behaviors in rodents (Ganon-Elazar and Akirav, 2009; Mechoulam and Parker, 2013; Viveros et al., 2005). Notably, CB1 knockout mice are anxious (Haller et al., 2004).

Stress suppresses amygdalar eCB signaling and contributes to the activation of the hypothalamic–pituitary–adrenal axis to elevate production of the glucocorticoid stress hormones (Hill et al., 2009a). However, sustained elevation of glucocorticoids as occurs in

chronic stress (Hill et al., 2010b) further suppresses eCB signaling in the limbic system including the amygdala and hippocampus and this feed-forward mechanism is thought to exacerbate anxiety disorders (Bowles et al., 2012).

Activation of postsynaptic type I metabotropic glutamate receptors, namely mGluR1 and mGluR5, mobilizes the synthesis and release of 2-arachidonoylglycerol (2-AG) and anandamide (AEA) (Azad et al., 2004; Heifets and Castillo, 2009; Jung et al., 2005; Varma et al., 2001). AEA and 2-AG are the two major eCBs whose expression is altered by acute and chronic stress (Hill et al., 2010b). mGluR5 is highly expressed in all regions of the amygdala, including the BLA. mGluR5 knockout mice exhibit an abnormal response to stress (Brodkin et al., 2002). In addition, mGluR5 dysfunction contributes to the anxiety phenotype in a mouse model of fragile-X syndrome (Dolen et al., 2007; Ronesi et al., 2012), where coupling of mGluR5 to eCB signaling is abnormal (Maccarrone et al., 2010). mGluR activation induces eCB-mediated synaptic plasticity in the amygdala GABA synapses (Azad et al., 2004; Zhu and Lovinger, 2005). Tyrosine phosphorylation of mGluR5 maintains the receptors in a high signaling state (Orlando et al., 2002; Tozzi et al., 2001). Thus, mGluR5-dependent eCB production and its modulation of anxiety behavior are likely to be sensitive to altered tyrosine phosphatase activity.

The LIM domain only protein LMO4 is a small protein that modulates several signaling pathways through protein-protein interaction. In the nucleus, LMO4 regulates the activity of key transcription factors (de la Calle-Mustienes et al., 2003; Hahm et al., 2004; Joshi et al., 2009; Kashani et al., 2006; Lu et al., 2006a; Manetopoulos et al., 2003; Schock et al., 2008a; Sum et al., 2002; Wang et al., 2007). In the cytoplasm, LMO4 modulates several tyrosine kinases (Bong et al., 2007; Gomez-Smith et al., 2010; Novotny-Diermayr et al., 2005;

Schaffar et al., 2008; Zhou et al., 2012). We recently reported that LMO4 is an endogenous inhibitor of the tyrosine phosphatase PTP1B (Pandey et al., 2013). Mice with glutamatergic neuron-specific ablation of LMO4 (LMO4KO) have increased PTP1B phosphatase activity in the hypothalamus (Pandey et al., 2013). LMO4 is highly expressed in the BLA and we asked whether disrupted expression of LMO4 in the amygdala affects anxiety behavior. Here, we report that LMO4KO mice are anxious and have impaired mGluR5 function and reduced eCB signaling in the BLA. We found that PTP1B activity is markedly elevated in the amygdala, that PTP1B targets mGluR5 for tyrosine dephosphorylation and that blocking PTP1B activity with a PTP1B inhibitor or PTP1B-specific antisense shRNA restores mGluR5 function and alleviates anxiety in LMO4KO mice. Elevated PTP1B activity in the amygdala was also observed in wild type mice subject to chronic stress, indicating that PTP1B activity is sensitive to stress and that elevated PTP1B activity may be a general mechanism underlying anxiety.

3.5 Results

LMO4KO mice are anxious due to impaired eCB signaling. LMO4 is abundantly expressed in the BLA (Fig. 3.1A) and is completely ablated in the amygdala of LMO4KO (CamK2αCre/LMO4flox) mice (Fig. 3.1B). Given that the BLA is a key center controlling anxiety(Tye et al., 2011), we tested whether loss of LMO4 would influence anxiety. Three behavioral tests revealed heightened anxiety in LMO4KO mice: suppressed spontaneous tendency to explore/avoid certain zones of a given environment, i.e. reduced time spent and fewer entrances to the open arm in the elevated plus maze test (Fig. 3.1C), reduced transitions between dark and light chambers in the light preference test (Fig. 3.1D), and reduced time spent in the center arena in the open field test (Fig. 3.1E). Locomotor function cannot account

Figure 3.1 LMO4KO mice are anxious due to reduced eCB signaling

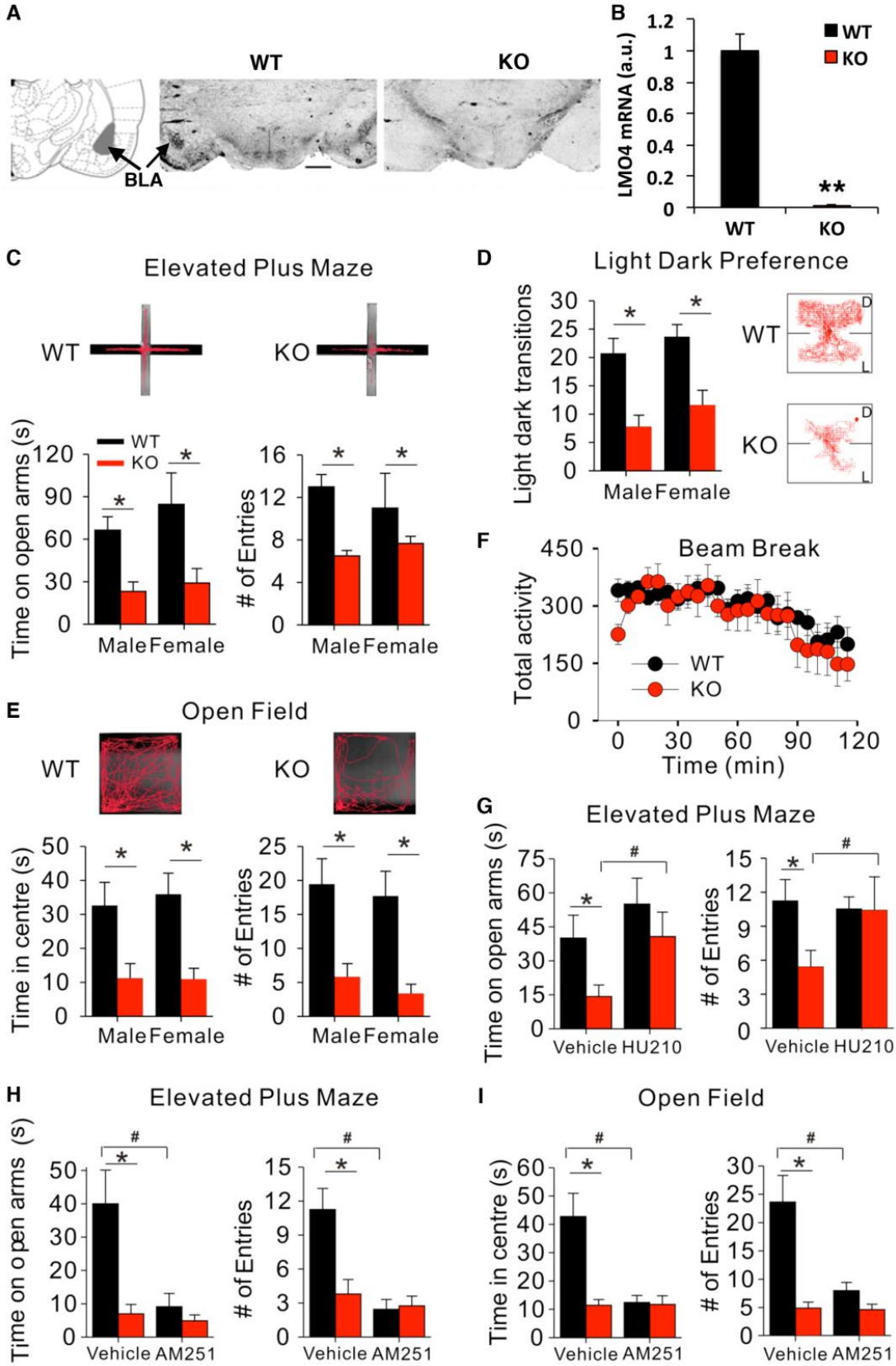

Figure 3.1 LMO4KO mice are anxious due to reduced eCB signaling

(A) *In situ* hybridization revealed LMO4 mRNA at high levels in the basal lateral amygdala (BLA) of wild type littermate controls (WT) and its absence in Camk2αCre/LMO4flox mice (KO). Scale bar, 800 μm.

(B) Quantitative RT-PCR confirmed ablation of LMO4 mRNA in the amygdala. Mean ± SEM, n=6 per group. **, $p<0.001$.

(C-E) Three behavioral tests: (C) elevated plus maze, (D) Light/dark preference test, and (E) open field test revealed a marked anxiety phenotype in LMO4KO mice. N=12 per gender for each genotype.

(F) Locomotor activity, measured by autonomic beam break over a 2 h period, was similar between littermate KO and WT mice.

(G) Intra-peritoneal treatment with the CB1 agonist HU210 (10 μg/kg) abolished the anxiety phenotype of LMO4KO mice.

(H-I) The anxiogenic effect of CB1 cannabinoid receptor antagonist AM251 was occluded in LMO4KO mice. Twenty minutes after intra-peritoneal injection of AM251 (3 mg/kg), mice were subjected to (H) elevated plus maze and (I) open field tests. Mean ± SEM, n = 8-12 mice per group. *, $p<0.05$.

for these behavior differences since locomotor activity was similar between KO and WT (Fig. 3.1F).

Anxiety in LMO4KO mice was extinguished by intra-peritoneal administration of a CB1 agonist HU210 (Fig. 3.1G). On the other hand, blocking CB1 receptors with the antagonist AM251 caused a marked increase in anxiety in littermate control mice. This effect was occluded in LMOKO mice that are already anxious (Fig. 3.1H & 3.1I). These results indicate that impaired eCB signaling underlies the anxiety phenotype in LMO4KO mice.

Elevated PTP1B activity in the amygdala of LMO4KO mice is associated with reduced tyrosine phosphorylation of mGluR5. Previously, we reported that LMO4 is an endogenous inhibitor of PTP1B phosphatase activity in the hypothalamus (Pandey et al., 2013) that promotes the inactivation of PTP1B through oxidation of a cysteine residue in the catalytic domain (Mahadev et al., 2001). Consistent with our previous study, an *in vitro* assay revealed a 3-fold elevation of PTP1B tyrosine phosphatase activity in amygdala lysates from LMO4KO mice (Fig. 3.2A) that was not due to elevated PTP1B protein levels (Fig. 2B). Instead, elevated PTP1B activity reflects lower levels of the oxidized, inactive form of PTP1B in LMO4KO amygdala compared to littermate controls (Fig. 3.2B).

Since eCB production is tied to the activity of metabotropic glutamate receptors (mGluR) (Azad et al., 2004; Heifets and Castillo, 2009; Jung et al., 2005; Varma et al., 2001) and tyrosine phosphorylation of mGluR5 is required for high levels of mGluR5 signaling (Orlando et al., 2002), we postulated that elevated PTP1B activity in LMO4KO amygdala might affect mGluR5 phosphorylation and eCB production. To test this, we used F11

Figure 3.2 Knockdown of LMO4 causes elevated PTP1B activity, reduced phosphorylation of mGluR5 at tyrosine residues, and impaired mGluR5 signaling.

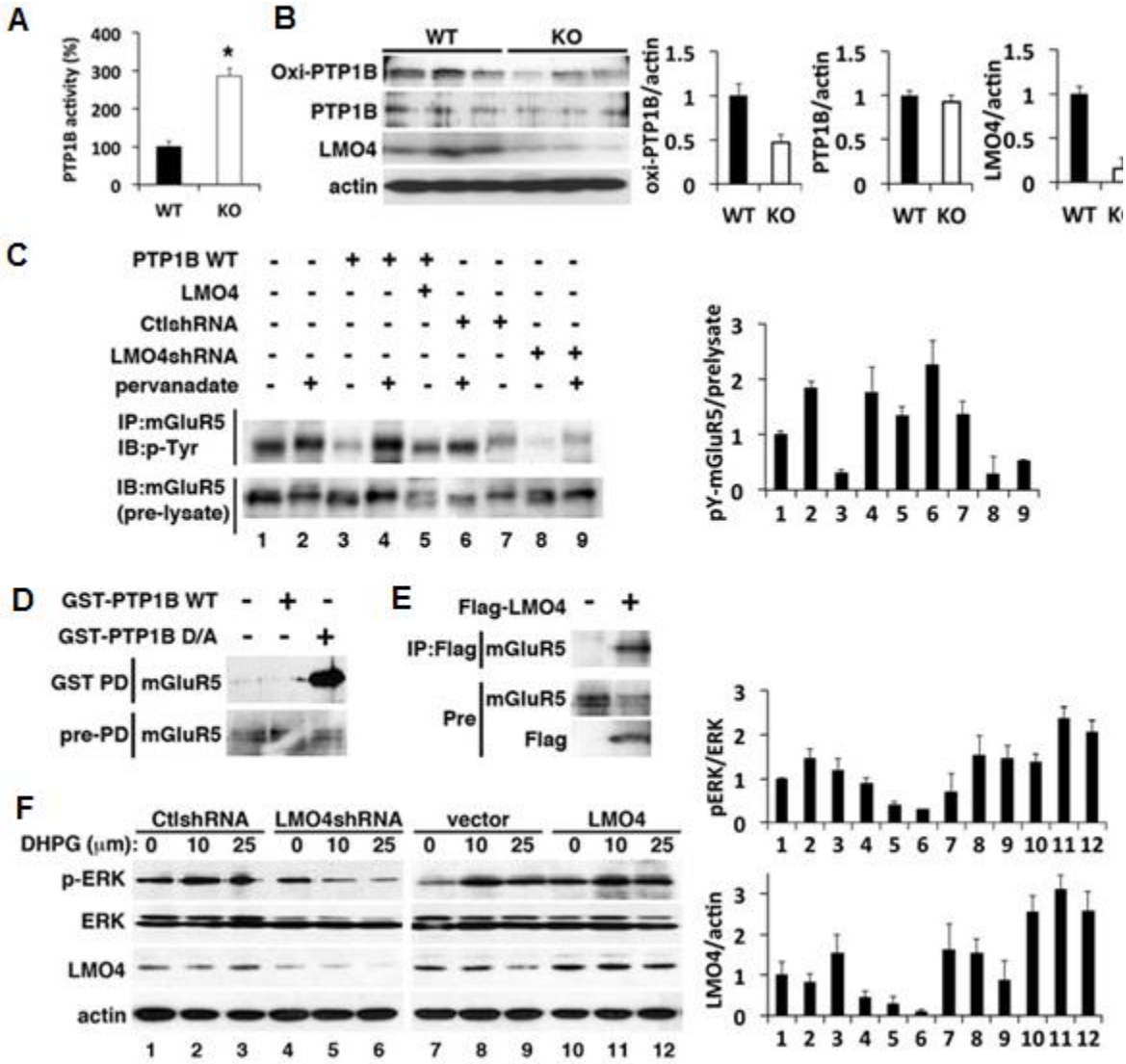

Figure 3.2 Knockdown of LMO4 causes elevated PTP1B activity, reduced phosphorylation of mGluR5 at tyrosine residues, and impaired mGluR5 signaling.

(A) PTP1B activity is elevated in LMO4KO amygdala. n= 8 mice per group. *p <0.05. Error bars represent s.e.m.

(B) PTP1B protein levels are not changed. Rather, there was less PTP1B oxidation (oxi-pTP1B) in the amygdala of LMO4KO compared to littermate controls. n= 3 per genotype.

(C) F11 neuronal cells transfected with PTP1B expression vector (lane 3) or LMO4shRNA (lane 8) caused reduced tyrosine phosphorylation of mGluR5 Representative blots are shown from 3 experiments.

(D) Endogenous mGluR5 in F11 neuronal cells was pulled down (PD) with a substrate-trapping mutant (D/A) of GST-tagged PTP1B protein.

(E) LMO4 co-immunoprecipitated with mGluR5 in F11 cells.

(F) Transient transfection of F11 neuronal cells with LMO4shRNA reduced (lanes 4-6) whereas LMO4 over-expression (lanes 10-12) increased DHPG-induced ERK phosphorylation. (B, C, F) blots are quantified in the right panels.

neuronal cells to confirm that mGluR5 is phosphorylated at tyrosine residues by immunoprecipitating endogenous mGluR5 and immunoblotting with a phosphotyrosine-specific antibody (Fig. 3.2C). Importantly, mGluR5 tyrosine phosphorylation was markedly reduced by PTP1B over-expression (lane 3) and this effect was reversed by the pan-tyrosine phosphatase inhibitor pervanadate (lane 4). Moreover, over-expression of LMO4 increased (lane 5) whereas knockdown of LMO4 reduced mGluR5 tyrosine phosphorylation (compare lane 8 to 7). Thus, modulation of LMO4 affects mGluR5 phosphorylation.

We next asked whether mGluR5 is a substrate of PTP1B. Using a catalytically inactivated mutant of PTP1B that binds but does not dephosphorylate or release its substrate (i.e., a substrate-trapping mutant) (Stuible et al., 2008), we observed a strong interaction of this mutant PTP1B with mGluR5 in a GST pulldown assay (Fig. 3.2D), indicating that mGluR5 is a *bone fide* substrate of PTP1B. Co-immunoprecipitation showed that mGluR5 also interacts with LMO4 (Fig. 3.2E), suggesting that LMO4 may regulate mGluR5 function in a complex with PTP1B.

We next examined the effect of LMO4 modulation on mGluR5-mediated signaling in F11 neuronal cells. Metabotropic glutamate receptors activate phospholipase C and promote extracellular signal-regulated kinase (ERK) signaling (Kim et al., 2008). The mGluR agonist DHPG caused a rapid elevation in phosphorylated ERK and this response was markedly attenuated with LMO4 knockdown by shRNA (Fig. 3.2F, compare lanes 5 and 6 to lanes 2 and 3) and augmented by over-expression of LMO4 (Fig. 3.2F, compare lanes 10-12 to lanes 7-9). Thus, altered LMO4 expression is sufficient to affect mGluR5 signaling in neurons.

mGluR5-dependent eCB production is impaired in LMO4KO amygdala. We next tested whether the impaired mGluR5-mediated ERK signaling that we observed with knockdown of LMO4 in F11 cells would be reflected in a deficit in mGluR5 function and mGluR5-dependent eCB production in the amygdala of LMOKO mice. To this end, we conducted electrophysiological assessments by whole cell recording in brain slices containing the amygdala (Fig. 3.3A). Activation of mGluR1/5 with the agonist DHPG results in depolarization by increasing inward currents, in part by the inhibition of K^+ channels and activation of a transient receptor potential (TRP)-like conductance (Desai et al., 1994; Gee et al., 2003; Mannaioni et al., 2001; Rae et al., 2000). mGluR1/5 functionality was monitored by a DHPG-induced inward current in WT BLA neurons that was markedly attenuated in LMO4KO BLA neurons (Fig. 3.3B). Pre-treatment with the mGluR5 antagonist MPEP blocked over 60% of the DHPG-induced change of holding current (Fig. 3.3B), indicating that mGluR5 is the predominant type I glutamate receptor in BLA neurons. These results showed that mGluR5 function is impaired in LMO4KO BLA neurons.

To determine the consequence of impaired mGluR signaling on eCB production in amygdala slices, we measured the effect of DHPG on the paired-pulse ratio (PPR) and long-term depression (LTD) of inhibitory postsynaptic currents (IPSC). Both responses reflect eCB-mediated synaptic suppression of GABA release (Azad et al., 2004; Maccarrone et al., 2008; Marsicano et al., 2002). In littermate control BLA neurons, DHPG increased the PPR and this effect was blocked by pretreatment with the CB1R antagonist AM251 (Fig. 3.3C). On the contrary, DHPG did not increase the PPR in LMOKO BLA neurons. In addition, DHPG induced LTDi in WT but not in LMO4KO BLA neurons (Fig. 3.3D). Together, these findings lead us to conclude that mGluR5-mediated eCB signaling is impaired in LMO4KO mice.

Figure 3.3 mGluR5 function and mGluR5-dependent eCB modulation of neurotransmission is impaired in LMO4KO amygdala.

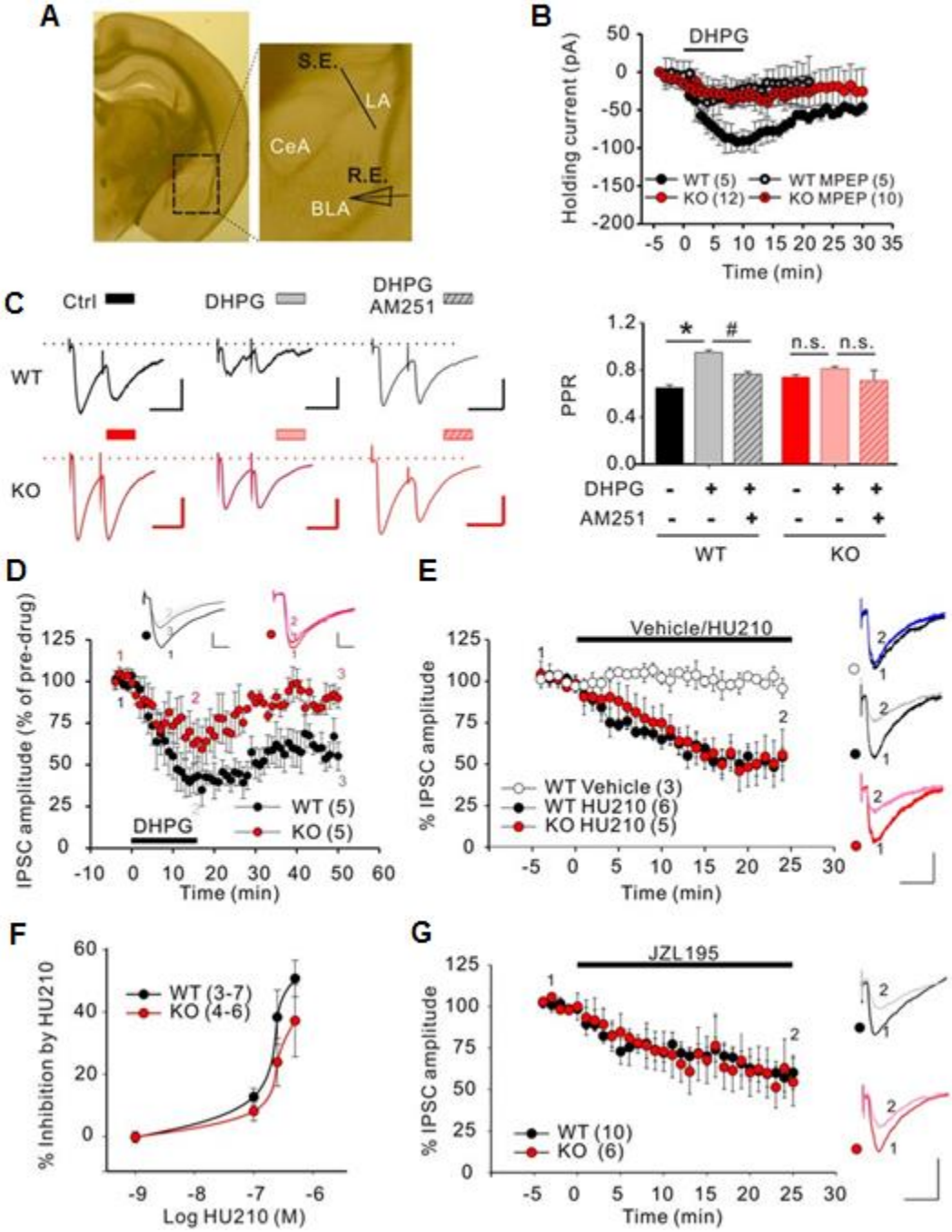

Figure 3.3 mGluR5 function and mGluR5-dependent eCB modulation of neurotransmission is impaired in LMO4KO amygdala.

(A) Positions of recording and stimulating electrodes for patch-clamp recording. S.E., stimulating electrode. R.E., recording electrode. CeA, central amygdala. LA, lateral amygdala. BLA, basolateral amygdala.

(B) Change of holding current following administration of the mGluR1/5 agonist DHPG was markedly attenuated in LMO4KO compared to control BLA neurons. The mGluR5 antagonist MPEP blocked the DHPG-induced change of holding current. Number of the recorded cells are in parentheses.

(C) DHPG increased the paired-pulse ratio (PPR, P2/P1) in WT but not in LMO4KO BLA neurons and this effect was blocked by pre-treatment with CB1R antagonist AM251. n=7-8. Current traces depicting GABA-mediated eIPSCs induced by paired pulses before, 15 minutes after DHPG application without or with AM251 from WT and LMO4KO BLA neurons are shown above the graph; scale bar, 200 pA/50 ms.

(D) DHPG-induced long-term depression of IPSC (LTDi) was markedly attenuated in LMO4KO compared to littermate control (WT) BLA neurons. Representative traces before (trace 1: black for WT and red for KO) and 15 min after DHPG treatment (trace 2:grey for WT and pink for KO) are shown above the graph, scale bar: 100 pA/10 ms.

(E) Treatment of BLA neurons with CB1 agonist HU210 (500 nM) reduced eIPSC amplitude to a similar degree.

(F) WT and LMO4KO BLA neurons have similar dose-dependent responses to HU210 at 1, 100, 250 and 500 nM.

(G) JZL 195 (1 μM) reduced eIPSC amplitude to a similar degree in LMO4KO and littermate control BLA neurons. * & #, $p<0.05$. n.s., not significant. Error bars represent s.e.m..

The deficits of eCB signaling in LMO4KO mice could be attributable to a decrease in CB1 receptor function or to lower eCB contents (Heifets and Castillo, 2009; Long et al., 2009; Wamsteeker et al., 2010). Thus, we assessed the functionality of CB1 receptors by administering an exogenous CB1 receptor agonist HU210 and found a similar reduction in the amplitude of eIPSCs (Fig. 3.3E) and a similar dose-response to this agonist (Fig. 3.3F) in LMO4KO and littermate control BLA neurons. In addition, administration of JZL195 (Long et al., 2009) that increases eCB contents by blocking fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL), the primary enzymes responsible for degrading the endocannabinoids AEA and 2-AG, respectively, restored eCB-mediated inhibition of eIPSCs in LMO4KO BLA neurons (Fig. 3.3G). Taken together, these results indicate that the reduced eCB signaling observed in LMO4KO mice is not due to a defective CB1 receptor, but rather is due to reduced eCB levels.

The PTP1B inhibitor Trodusquemine normalizes PTP1B activity, restores mGluR5 function and alleviates the anxiety phenotype of LMO4KO mice. Since ablation of LMO4 is associated with elevated PTP1B activity and reduced mGluR5 phosphorylation and function, we asked whether a small molecule PTP1B-specific inhibitor Trodusquemine (Krishnan et al., 2011; Lantz et al., 2010; Pandey et al., 2013) would restore mGluR5 phosphorylation and signaling in LMO4-deficient cells. Indeed, in F11 neuronal cells where LMO4 was knocked down by shRNA, Trodusquemine restored tyrosine phosphorylation of mGluR5 (Fig. 3.4A, compare lanes 7 & 8 to 5 & 6). Moreover, Trodusquemine also restored mGluR5-mediated ERK phosphorylation in these LMO4 knockdown cells (compare lane 8 to

Figure 3.4 The PTP1B inhibitor Trodusquemine normalizes PTP1B activity, restores mGluR function and alleviates the anxiety phenotype of LMO4KO mice.

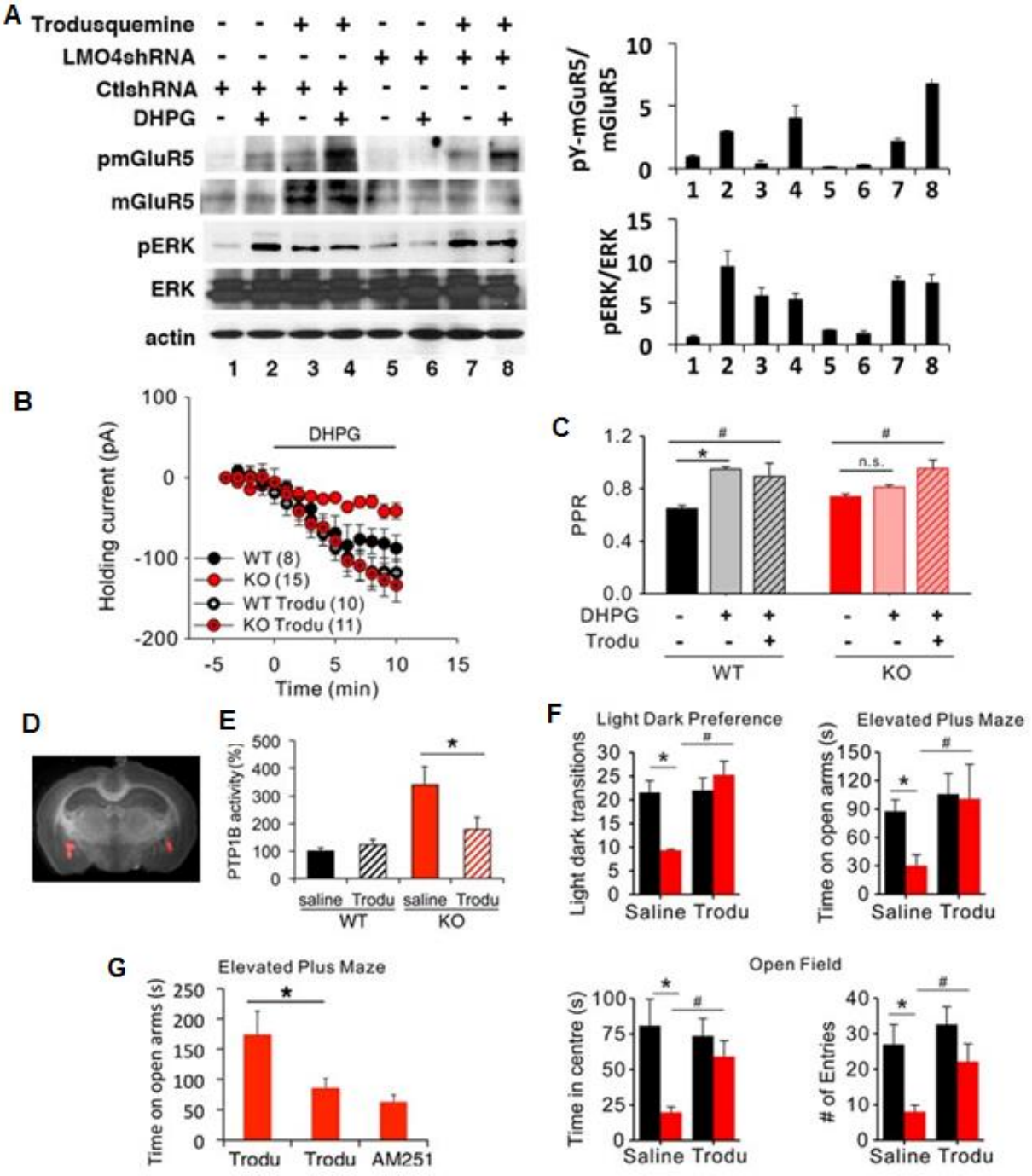

Figure 3.4 The PTP1B inhibitor Trodusquemine normalizes PTP1B activity, restores mGluR function and alleviates the anxiety phenotype of LMO4KO mice.

(A) Pre-treatment with Trodusquemine (10 μM) for 30 minutes restored ERK phosphorylation in response to mGluR1/5 agonist DHPG in F11 neuronal cells. Blots are quantified in the right panels.

(B) Addition of Trodusquemine in the intracellular recording electrode rapidly rescued DHPG-induced holding currents in LMO4KO BLA neurons.

(C) Trodusquemine restored the facilitation of paired pulse ratio (PPR) in response to 10 minutes of bath applied DHPG in LMO4KO BLA neurons.

(D) Co-injection of fluorescent dye confirmed the position of Trodusquemine administration within the amygdala.

(E) Trodusquemine treatment normalized PTP1B activity in LMO4 KO amygdala. 1 week after intra-amygdalar Trodusquemine or saline injection, amygdala protein extracts were harvested to measure PTP1B activity. n=12 per group.

(F) A single bilateral stereotaxic injection of Trodusquemine (1 μg/side/ 0.4 μl) to the amygdala extinguished anxiety phenotypes of LMO4KO mice. Behavior tests were conducted 1 day (elevated plus maze), 3 days (light/dark preference), and 5 days (open field) after treatment. Mean ± SEM, n= 8 mice per group. *, $p < 0.05$.

(G) The anxiolytic effect of Trodusquemine was abolished by the CB1 antagonist AM251. The elevated plus maze test was conducted 24 hours after intra-amygdalar co-injection of Trodusquemine (1 μg/side/ 0.4 μl) together with vehicle or AM251 (0.8 μg/side/0.4 μl). Mean ± SEM, n= 5-8 mice per group. *, $p < 0.05$.

6). These results further demonstrated that PTP1B activity affects mGluR5 phosphorylation and signaling.

The DHPG-induced depolarization that was markedly attenuated in LMO4KO BLA neurons (Fig. 3.3A) was restored by addition of Trodusquemine to the intracellular recording solution (Fig. 3.4B), as was the DHPG-induced increase in the PPR (Fig. 3.4C), indicating that eCB production in response to mGluR5 activation was also restored.

Since Trodusquemine restored mGluR5 function and mGluR5-dependent eCB signaling in LMO4KO BLA neurons, we asked whether Trodusquemine might also affect the anxiety phenotype of LMO4KO mice. Trodusquemine was delivered by bilateral stereotactic injection to the amygdala (Fig. 3.4D) and this effectively blocked excess PTP1B activity in LMO4KO amygdala (Fig. 3.4E). Trodusquemine completely extinguished the anxiety phenotype of LMO4KO mice assessed in all 3 anxiety behavioral tests (Fig. 3.4F). Of note, this dose of Trodusquemine had no effect on PTP1B activity or behavior in littermate controls. The anxiolytic effect of Trodusquemine was also observed following systemic intra-peritoneal injection (Supplementary Fig. 3.1). Importantly, the anxiolytic effect of Trodusquemine was blocked by the CB1 antagonist AM251 (Fig. 3.4G), demonstrating that the anxiolytic effect of Trodusquemine works by restoring eCB signaling.

Knock-down of PTP1B relieves anxiety in LMO4KO mice. We next used lentiviral vectors expressing PTP1B-specific shRNA to knock-down PTP1B expression in the amygdala of LMO4KO mice. A bilateral stereotaxic injection of lentiviral vectors expressing PTP1B shRNA and GFP ($1x10^9$ TU/ml, 0.4µl/side) into the amygdala (Fig. 3.5A) effectively reduced PTP1B protein expression (Fig. 3.5B) and PTP1B activity (Fig. 3.5C) in amygdala

S 3.1 A single intra-peritoneal injection of Trodusquemine extinguished anxiety phenotypes of LMO4KO mice

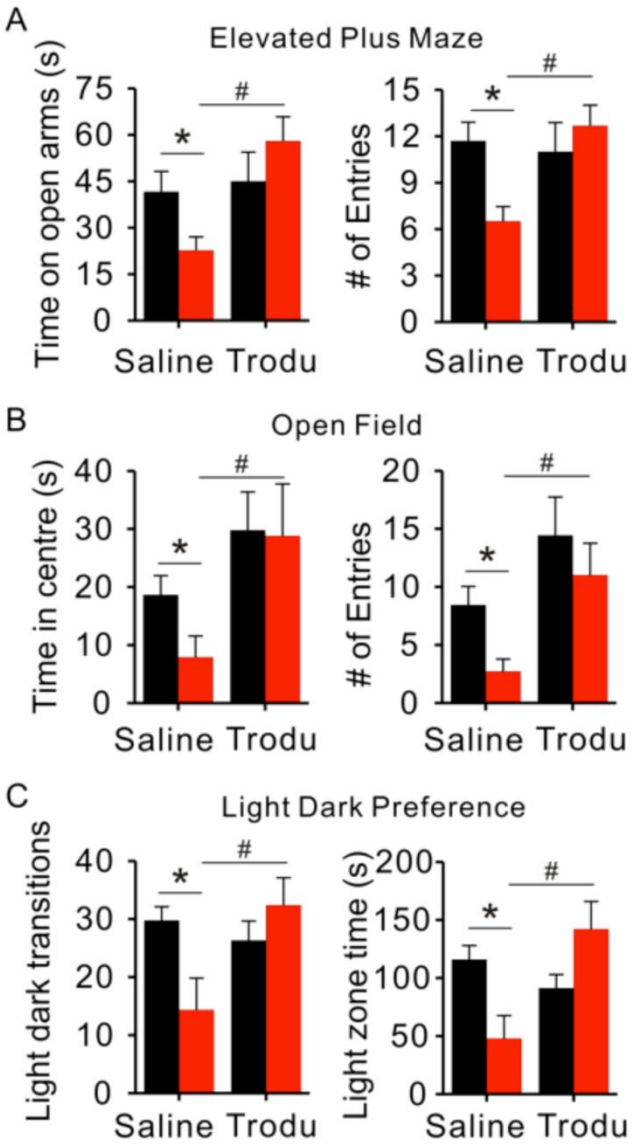

Figure S 3.1 A single intra-peritoneal injection of Trodusquemine extinguished anxiety phenotypes of LMO4KO mice

Behavior tests were conducted 1 hour (elevated plus maze), 3 days (light/dark preference), and 5 days (open field) after Trodusquemine (Trodu, 5 mg/kg weight) treatment. Mean ± SEM, n= 8 mice per group. *, $p < 0.05$.

Figure 3.5 Intra-amygdala injection of lentiviral PTP1B shRNA alleviates the anxiety phenotype of LMO4KO mice.

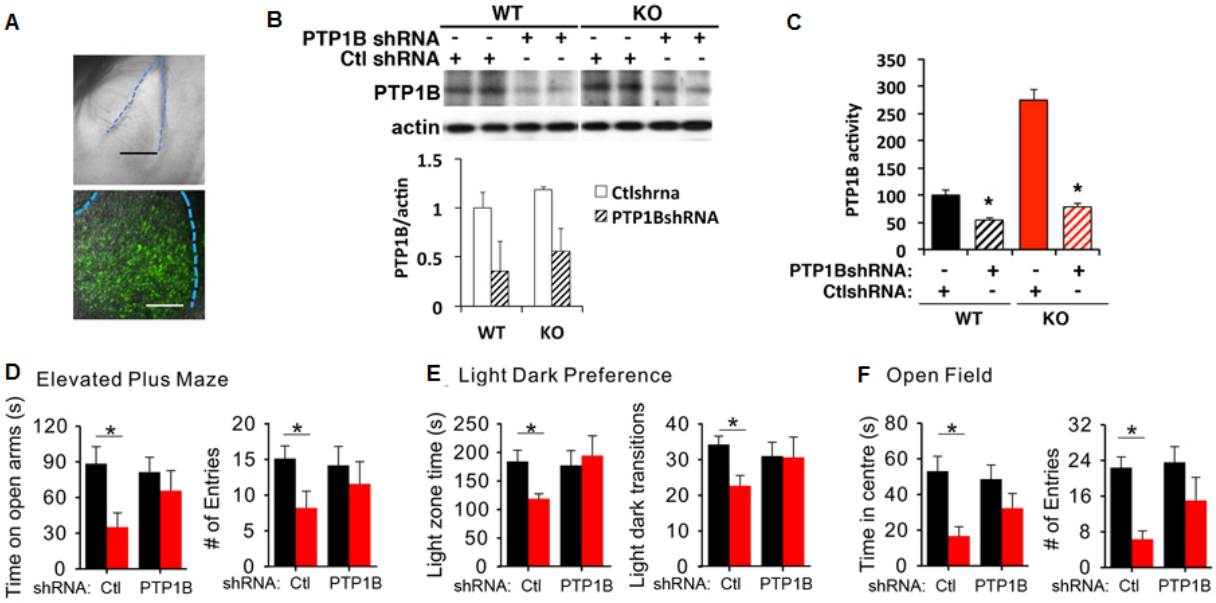

Figure 3.5 Intra-amygdala injection of lentiviral PTP1B shRNA alleviates the anxiety phenotype of LMO4KO mice.

(A) Representative live images of GFP-positive BLA neurons 21 days after intra-amygdalar injection of lentiviral vectors expressing GFP reporter and PTP1B shRNA. Upper panel, bright field; lower panel, merged DIC and fluorescence. Scale bars: top, 0.3 mm; bottom, 0.1 mm.

(B) Western blot of amygdalar extract revealed a reduction in PTP1B protein expression 21 days after intra-amygdalar injection of lentiviral vector expressing PTP1B shRNA compared to control shRNA (CtlshRNA). Blots are quantified in the lower panel.

(C) PTP1B activity assay 21 days after lentiviral vector injection.

(D-F) Bilateral stereotaxic injection of PTP1B shRNA (1x109 TU/ml, 0.4 μ l/side) to the amygdala extinguished anxiety phenotypes of LMO4KO mice (red bars) and had no effect in WT mice (black bars). Behavior tests were conducted 12 days (D, elevated plus maze), 14 days (E, light/dark preference), and 16 days (F, open field) after treatment. n= 8 mice per group. *, $p < 0.05$. Error bars represent s.e.m.

extracts 21 days after injection. Prior to sacrificing, mice were subjected to behaviour tests. PTP1B shRNA extinguished the anxiety phenotypes of LMO4KO mice in the elevated plus maze test at 12 days (Fig. 3.5D), the light/dark preference test at 14 days (Fig. 3.5E), and the open field test at 16 days (Fig. 3.5F) after injection. The control lentiviral vector-injected LMO4KO mice remained anxious in all tests (Fig. 3.5D-F). Together with the Trodusquemine results, these studies show that inhibition of PTP1B relieves anxiety in LMO4KO mice.

PTP1B activity is elevated in stressed wild type mice. We next asked whether elevated PTP1B activity might also account for increased anxiety caused by another experimental anxiety paradigm (in addition to the genetic LMO4KO model). Wild type mice were subjected to a daily 30-minute regimen of restraint stress for 9 days that caused a significant increase in the levels of the glucocorticoid stress hormone corticosterone in the blood (Fig. 3.6A). These stressed mice had elevated PTP1B activity (Fig. 3.6B) that was not due to elevated PTP1B expression, but rather was associated with reduced levels of the oxidized inactive form of PTP1B (Fig. 3.6C) in the amygdala. This is similar to what we see in LMO4KO amygdala, suggesting that loss of LMO4-dependent inhibition of PTP1B may also contribute to anxiety in stressed wild type mice.

Previously we reported that LMO4-dependent inhibition of PTP1B activity requires the palmitoylation and ER-retention of LMO4 (Pandey et al., 2013). We found that oxidative stress prevents LMO4 palmitoylation and ER-retention, resulting in the loss of LMO4-dependent inhibition of PTP1B (Pandey et al., 2013). Glucocorticoids are known to elevate ROS production and cause oxidative stress in the hippocampus (You et al., 2009; Yu et al., 2011). Indeed, stressed wild type mice showed markedly lower levels of palmitoylated LMO4

Figure 3.6 PTP1B activity is elevated in repeatedly stressed wild type mice or in cultured neurons exposed to the stress hormone corticosterone.

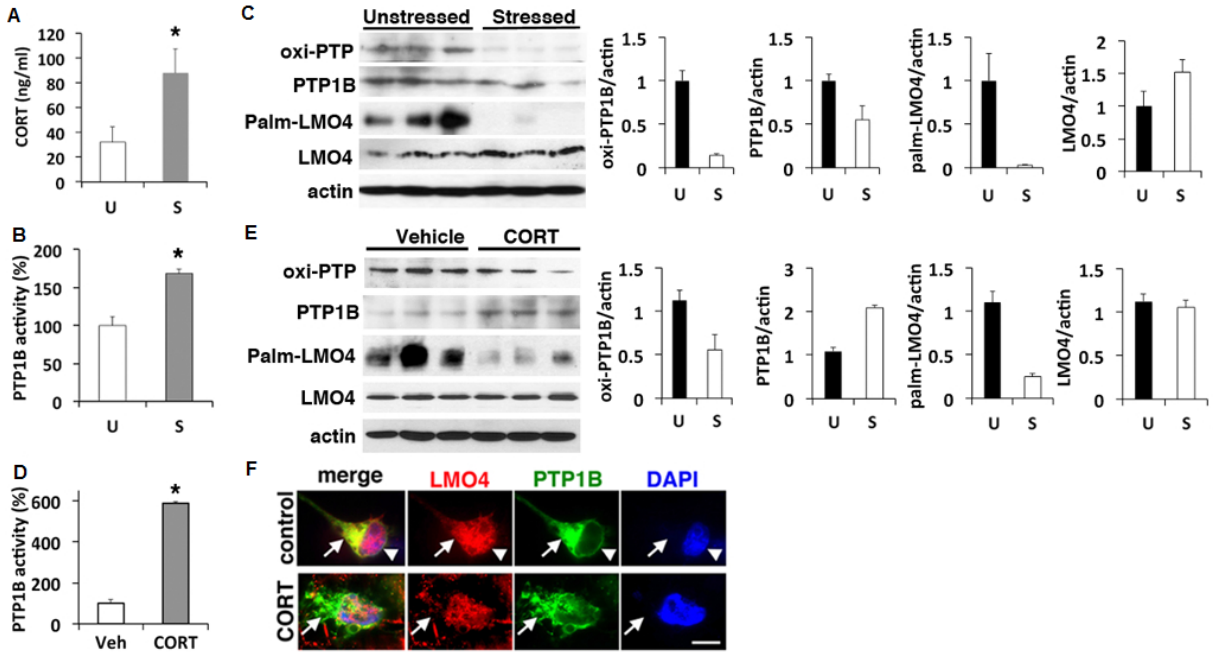

Figure 3.6 PTP1B activity is elevated in repeatedly stressed wild type mice or in cultured neurons exposed to the stress hormone corticosterone.

(A) Elevated CORT in repeatedly (9 days) immobilization stressed (s) wild type male mice compared to unstressed (u) mice. (B) Elevated PTP1B activity in amygdala of repeatedly (9 days) immobilization stressed wild type male mice, compared to un-stressed mice. n= 5-8 mice per group for (A) and (B).

(C) Immunoblots showed lower levels of oxi-PTP and palmitoylated LMO4 (Palm-LMO4) in amygdala of repeatedly immobilization stressed mice. n=3 mice per genotype. Blots are quantified in the right panels.

(D) PTP1B activity is elevated in F11 neuronal cells after 6 hours treatment with CORT (1 μM). n= 6 per group.

(E) Immunoblots showed lower levels of oxi-PTP and Palm-LMO4 in CORT-treated F11 cells. n=3 experiments per condition. Blots are quantified in the right panels.

(F) Immunofluorescent staining showed that CORT treatment caused LMO4 to translocate from the cytoplasm to the nucleus, thereby preventing its interaction with YFP-tagged PTP1B at the cytoplasm. Scale bar, 15 μM. *, $p < 0.05$. Error bars represent s.e.m.

in the amygdala (Fig. 3.6C), consistent with elevated circulating glucocorticoids. Similarly, we found that F11 neuronal cells exposed to corticosterone (CORT, 1 μM = 346 ng/ml) had nearly a 6-fold increase in PTP1B activity (Fig. 3.6D). Accordingly, levels of palmitoylated LMO4 were reduced by CORT treatment (Fig. 3.6E). CORT treatment caused LMO4 to translocate from the cytoplasm to the nucleus and prevented it from interacting with and inhibiting PTP1B at the cytoplasm (Fig. 3.6F). Together, these results suggest that stress-induced elevation of glucocorticoids causes a loss of LMO4-dependent inhibition of PTP1B activity and this may constitute a general mechanism underlying anxiety phenotypes.

3.6 Discussion

In the present study, we identified a novel mechanism that accounts for a profound anxiety phenotype observed in mice with glutamatergic neuron-specific ablation of LMO4. Our electrophysiological studies revealed a deficit in synaptic modulation by eCB signaling in BLA neurons of LMO4KO mice. Since CB1 receptor agonist alleviated the anxiety in these mice, this finding argued for impaired eCB production/release from BLA neurons. eCB production is induced by activation of type 1 metabotropic glutamate receptors and tyrosine phosphorylation of these receptors has been reported to sustain their activity (Orlando et al., 2002; Tozzi et al., 2001). Here, we showed for the first time that PTP1B binds to and dephosphorylates mGluR5. We found that PTP1B phosphatase activity was markedly elevated in the BLA of LMO4KO mice. Even wild type mice subjected to repeated restraint stress showed a similar elevation of PTP1B activity in the amygdala, arguing that this is a general mechanism underlying anxiety. Importantly, direct injection of the PTP1B inhibitor Trodusquemine to the amygdala alleviated anxiety by restoring eCB signaling. From these findings emerges a novel mechanism whereby LMO4 in the BLA is essential to maintain eCB signaling by restraining PTP1B activity and anxiety in mice.

Activation of ryanodine receptors (RyR) and calcium-induced calcium release (CICR) is another key mechanism that drives eCB production (Isokawa and Alger, 2006). We have reported that LMO4 is necessary to maintain RyR expression and CICR in the hippocampus (Qin et al., 2012). Whether RyR expression is also affected in the BLA of LMO4KO mice remains to be determined. Of note, tyrosine phosphorylation of RyR increases channel opening and sensitivity to CICR (Zhang et al., 2004). Thus, elevated PTP1B activity in BLA

neurons of LMO4KO mice might limit eCB production by dephosphorylating RyR. This also remains to be determined.

Our study does not exclude the possibility that increased degradation of eCB in post-synaptic neurons also contributes to impaired eCB-dependent synaptic plasticity and increased anxiety in LMO4KO mice. For example, increased activity of FAAH associated with lower levels of anandamide has been detected in the amygdala in mouse models of anxiety (Bowles et al., 2012; Hill et al., 2012). FAAH is phosphorylated at several tyrosine residues (Y329, Y335 and Y526) of the catalytic domain (http://www.phosphosite.org/). Stimuli that elevate PTP1B protein levels (Lu et al., 2008) have also been associated with elevated FAAH activity (Thors et al., 2010). Thus, FAAH activity could also be regulated by PTP1B. No matter whether reduced eCB signaling occurred from reduced mGluR5-mediated or CICR-dependent eCB production, or from increased eCB degradation, our study showed that the salutary anxiolytic effect of PTP1B inhibition by Trodusquemine worked by restoring eCB signaling, since the CB1R antagonist AM251 blocked this effect.

It was startling that a single dose of Trodusquemine (either intra-amygdalar or intraperitoneal) in LMO4KO mice could produce an immediate (1 hour after administration) anxiolytic effect lasting up to 1 week (the longest time point examined). This anxiolytic effect was accompanied by normalized PTP1B activity measured 1 week after Trodusquemine treatment. The same dose administered to wild type mice had no measurable effect on PTP1B activity or anxiety. This observation suggests that endogenous LMO4 already inhibits PTP1B activity to minimal levels and that Trodusquemine does not produce a further measureable inhibition in wild type mice.

The importance of PTP1B in the central nervous system has been well documented in the hypothalamus, where PTP1B regulates leptin signaling to control feeding behavior and metabolic homeostasis (Bence et al., 2006). Mice with disruption of hippocampal PTP1B function or expression have improved learning ability associated with altered dendritic spine morphology (Fuentes et al., 2012). It remains to be seen whether eCB signaling is enhanced and accounts for enhanced learning in these mice since eCB signaling-mediated synaptic plasticity is important for learning and memory (Mazzola et al., 2009). It will be interesting to test whether mice with glutamatergic neuron-specific ablation of PTP1B are resistant to stress-induced anxiety.

Pharmacological therapies to relieve generalized anxiety disorder include benzodiazepines that enhance GABA(A) receptor signaling, monoamine oxidase inhibitors, serotonin and norepinephrine reuptake inhibitors (Baldwin et al., 2010). Many of these drugs have side effects, may require higher doses to achieve the same effect (habituation) or only have an effect after an extended period of use. Trodusquemine is a spermine metabolite of cholesterol that was originally isolated from the dogfish shark liver during a search for naturally occurring antimicrobial compounds. The unexpected effect of Trodusquemine to cause fat-specific weight loss in mice with diet-induced obesity led to its identification as a PTP1B-specific inhibitor (Ahima et al., 2002; Lantz et al., 2010). The use of PTP1B inhibition to (restore mGluR5 function and) restore normal eCB production has an important mechanistic advantage. As opposed to using agonist ligands to force the action of CB1 receptors or mGluR5, PTP1B inhibition would target restoration of endogenous function of mGluR5, so the action of eCB signaling should occur at the right place and time as in healthy, non-anxious individuals. An interim report of a phase I clinical trial testing the PTP1B

inhibitor Trodusquemine for appetite and obesity control reported no adverse effects of the drug, but unfortunately the final outcome of this clinical trial was not reported (Nguyen et al., 2012). As Trodusquemine readily crosses the blood brain barrier (Lantz et al., 2010; Zasloff et al., 2001), and we observed an anxiolytic effect after intra-peritoneal injection, oral administration may be an option for future therapy for anxiety. In summary, our study suggests that selective PTP1B inhibitors might be an effective means to treat anxiety disorders in humans.

3.7 Methods

Animals. CamK2αCre/LMO4flox (LMO4 KO) and LMO4flox/flox (WT) mice were bred on a CD-1 background as described previously (Qin et al., 2012; Schock et al., 2008b; Zhou et al., 2012). All procedures for animal use were approved by the University of Ottawa Animal Care and Veterinary Service and carried out according to institutional guidelines and in accordance with those of the Canadian Council on Animal Care.

Drugs and anxiety behavior tests. 6-7 weeks old mice were anesthetized with isoflurane and given 1 mL of saline subcutaneously to prevent dehydration before they were placed in a stereotaxic frame for intra-amygdalar injection. Saline or Trodusquemine (1µg in 0.4µl saline with 25% FluoSpheres® carboxylate-modified microspheres (0.04 µm, red fluorescent 580/605, Invitrogen) was infused bilaterally by using 28-Gauge injection needles connected with Hamilton 10 µl syringe into the BLA at a rate of 0.1µL/min [coordinates: anteroposterior (AP), −1.4 mm from bregma; mediolateral (ML), ±2.75 mm from the midline; dorsoventral (DV), −4.75 mm from skull surface; and incisor bar −2.4 mm from interaural] according to the

atlas of Franklin and Paxinos. After surgery, the mice were allowed to recover for 1-2 days before carrying out any behavioral tests. For co-treatment, Trodusquemine (1 µg/0.4µl/side) and AM251 (0.8 µg/0.4 µl/side) or vehicle control was injected to the amygdala and behavior test was conducted next day. Systemic delivery was achieved by intra-peritoneal injection of a CB1 agonist HU210 (10 µg/kg), antagonist AM251 (3 mg/kg) or Trodusquemine (5 mg/kg). HU210 and AM251 were dissolved in a vehicle containing DMSO, Tween80 and saline in a ratio as described (Han et al., 2012). All drugs were freshly prepared and administered 20 minutes (HU210 and AM251) or 1 hour (Trodusquemine) before behavior tests. In most cases, the same set of mice was used for different anxiety behavioral tests; elevated plus maze, light dark preference and open field were performed in a sequence with two recovery days between each testing.

-Elevated Plus Maze. The elevated 4-arm maze has two open arms (6cm x 35cm) and two closed arms (6cm x 35cm x 20cm) that extend from a central platform (6cm x 6cm) and are elevated approximately 75cm above the floor. Mice were placed individually in the center of the maze facing the open arms and allowed to explore the maze for 10 minutes. The session was recorded using a video camera and the results were analyzed with a computerized tracking system (Ethovision 8, Noldus IT, The Netherlands). Parameters measured included number of open arm entries as well as the amount of time spent in open arms.

-Light/Dark Box. The light/dark box apparatus consisted of an automated activity monitor chamber that was fitted with a light/dark box insert (Med Associates) and was enclosed in a sound attenuating chamber. The light side of the compartment was brightly lit (200 Lux). Mice were placed in the bright chamber facing the dark compartment and their activity was

recorded for 10 minutes using a photobeam-based tracking system. Transition and time spent in each of the compartments were recorded.

-Open field test. Mice were placed in an open field box (45 cm x 45 cm x 45cm; MED Associates, St. Albans, VT). Lights of 300 lux intensity were used for this test to enhance the anxiety associated with being in the center of the box. Mice were videotaped for 10 minutes from a mounted camera above the box and the images were projected to a video screen so that the animals could be monitored throughout the testing session using video tracking software from Noldus (Ethovision). The amount of time they spent at the center and the periphery of the chamber was recorded.

Stereotaxic injection of lentivirus. Lentiviral vectors expressing GFP reporter control and PTP1B shRNA were produced at the titer of 1 × 109 transduction units per milliliter (TU/ml). Stereotaxic injection of lentiviral vectors into the amygdala was performed by using similar coordinates and procedures as described in the previous section. A total volume of 0.4 μl of lentivirus was administered at a rate of 0.05 μl/min at each site. At 21 days after injection, the amygdala was isolated for examining the expression of GFP and PTP1B via fluorescence live imaging and western blotting, respectively.

Stress protocol. 6-7 weeks old male mice were brought into the restraint room daily and subjected to 30 minutes of tube restraint in modified 50 ml conical tubes for 9 consecutive days (between 10 and 12 am). On termination of the stressor, mice were placed back in their home cage. Control mice were left undisturbed in their home cages except for necessary handling to maintain tail markings throughout the 9-day protocol. Conical tubes were washed

with soap and water, and then rinsed in 70% ethanol after each stress episode. On the last day, all mice were subjected to corticosterone measurement and amygdala sample collection

Corticosterone radioimmunoassay. In the morning, mice were decapitated no more than 1 minute after being removed from their home cage. Plasma corticosterone was measured by radioimmunoassay following the manufacturer's protocol using the Corticosterone ^{125}I RIA kits (Cat# 07-120102, MP Biomedicals, LLC, NY, USA).

Cell culture and transfections. F11 neuronal cells, a hybridoma between dorsal root ganglion neuron and neuroblastoma were maintained and transfected using Lipofectamine2000 (Invitrogen) with LMO4 cDNA, control scrambled shRNA or LMO4-specific shRNA expression vectors as described previously(Chen et al., 2007a; Chen et al., 2007b; Duquette et al., 2010; Gomez-Smith et al., 2010).

PTP1B phosphatase activity assay. PTP1B phosphatase activity was measured with the PhosphoSeek™ PTP1B Assay Kit (BioVision Inc.) in extracts from amygdalar wedges or F11 cells according to the manufacturer's instructions with PTP1B enzyme and phosphatase inhibitor as positive and negative controls, respectively.

RNA extraction and quantitative RT-PCR. Total mouse amygdalar RNA was extracted and purified from 1 month old mutant and control mice as previously described (Qin et al., 2012). Each sample was quantitated in triplicate. A two-tailed *t* test was used to determine statistical significance at $p<0.05$.

In situ hybridization. Tissues were prepared for *in situ* hybridization with digoxigenin-labeled antisense or sense riboprobes, as previously described (Duquette et al., 2010).

GST pull down, immunoprecipitation and immunoblot analysis. F11 cells transfected with Flag-LMO4 together with GST-PTP1B wild type or a substrate-trapping mutant (D181A, D/A) (gifts from Dr. Michel Tremblay, McGill University (Stuible et al., 2008)) were harvested in lysis buffer and pulled down with glutathione beads according to the manufacturer's protocol (Invitrogen). To detect oxi-PTP1B, PTP1B was immunoprecipitated rabbit anti-PTP1B antibody (Cell Signaling) conjugated to protein G-Sepharose and immunoblotted against a mouse antibody specific to oxidized PTP active site (R&D Systems). Antibodies specific to: phosphorylated (T202/Y204)-ERK1/2, ERK1/2 (Cell Signaling), phosphorylated Tyrosine (Invitrogen), LMO4 (custom-made, Zymed (Chen et al., 2002a)). Immunoblots were performed as described previously (Gomez-Smith et al., 2010).

Palmitoylation assay. This was carried out according to a biotin exchange protocol (Drisdel et al., 2006) kindly provided by Dr. William Green's laboratory (University of Chicago).

Electrophysiology. Brain sections (300 µm) containing amygdala were prepared from 3 to 5 week-old mice as described in details (Qin et al., 2012). ACSF contained (in mM): 119 NaCl, 26.2 $NaHCO_3$, 11 glucose, 2.5 KCl, 2.5 $CaCl_2$, 1.3 $MgSO_4$, $1NaH_2PO_4$, and was equilibrated with 95% O_2 and 5% CO_2 at room temperature. Recording electrodes were filled with a solution of (in mM): 100 $CsCH_3SO_3$, 60 CsCl, 10 HEPES, 0.2 EGTA, 1 $MgCl_2$, 5 QX-314

chloride, 1 Mg-ATP, and 0.3Na_3GTP (pH 7.3, 275 mOsM) as described (Wilson and Nicoll, 2001). Whole-cell recordings were obtained from pyramidal neurons of the basolateral amygdala. In some experiments, the stimulation bipolar electrode was inserted into the amygdala region close to external capsule.

(2R)- amino-5-phosphonopentanoate (APV, 50µM), 2, 3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX, 5µM), (S)-3, 5-dihydroxyphenylglycine (DHPG, 50µM), 2-methyl-6-(phenylethynyl) pyridine (MPEP, 30µM) were purchased from abCam biochemicals. HU210 (1, 100, 250, 500nM) was obtained from Toronto Research Chemicals and JZL195 (1µM) was provided by Dr Xia Zhang at the Ottawa Royal Hospital Mental Health Institute. Trodusquemine (10µM in pipet solution) was produced by Dr Jean Michel Brunel lab.

The AMPA receptor antagonist NBQX and NMDA receptor antagonist APV were included to isolate inhibitory postsynaptic events unless otherwise indicated. Testing stimuli of 0.067 Hz, 10 µs duration were adjusted to evoke approximately 300 pA inhibitory postsynaptic current (eIPSC). A Multiclamp 700B amplifier connected to a Digidata 1440 interface (Molecular Devices) was used for whole cell recordings and analyzed using pClampfit 10.1. Recordings were filtered at 3 kHz, digitized at 10 kHz. Access resistance was continuously monitored by applying a 75 ms, 5 mV hyperpolarizing pulses 250 ms before the eIPSCs, and cells were used for recording only if the access resistance was below 20 MΩ.

Statistical analysis. Data acquisition and analyses were performed using a combination of pClamp10.1, Sigma Stat 3.5 (Systat Software Inc., San Jose, CA, USA), and the Statistical Package for the Social Sciences version 14 (SPSS). Student's two-tailed t tests were used for

comparisons. Differences between treatments were analyzed using Student's t test for paired data or one-way repeated measures ANOVA followed by Bonferroni's post hoc test for comparisons. Significance was accepted if $p<0.05$.

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CHAPTER 4

GENERAL DISCUSSION

My studies using behavioural electrophysiological and biochemical approaches in combination with genetic and pharmacological manipulation provide strong evidence that LMO4 functions as a protein binding interface to mediate brain function associated with memory and emotion in the hippocampus and amygdala, respectively. LMO4 is not only a cofactor involved in transcriptional regulation, it also participates in post-translational modification.

4.1 LMO4 and its Role in Learning and Memory

Our understanding of the function of LMO4 in the adult brain is not very extensive. Most previous studies ascribed a function of LMO4 to its transcriptional role either in a positive or negative manner (Joseph et al., 2013; Kadrmas and Beckerle, 2004; Kashani et al., 2006; Matthews et al., 2013; Schock et al., 2008a; Stokes et al., 2013; Wang et al., 2007; Zheng and Zhao, 2007), so did I in my first study (Qin et al., 2012). I found that LMO4 is an important positive regulator of the molecular machinery controlling CICR in the hippocampus (Qin et al., 2012). Lack of LMO4 in neurons is associated with reduced promoter activity, mRNA and protein expression of RyR2, suggesting the involvement of LMO4 in transcriptional regulation. Functional assessment of the role of LMO4 in controlling CICR was carried out by monitoring several electrophysiological responses known to be modulated by CICR, such as AHP, calcium rise from internal stores and glutamate release probability. These changes were accompanied with impaired hippocampal

LTP and hippocampal-dependent spatial learning ability. However the mechanism whereby LMO4 regulates RyR2 gene expression is uncertain. LMO4 is known to form a complex with the transcription factor CREB which regulates activity-dependent gene expression in response to synaptic stimulation and calcium influx through both L-type voltage sensitive calcium channels and NMDA receptors (Kashani et al., 2006). In addition, CREB can activate the RyR2 promoter (Ziviani et al., 2011). Thus, we reason that LMO4 may cooperate with CREB to regulate RyR2 expression and subsequently to tune the gain of CICR therefore modulating neuronal function. Furthermore, LMO4 has been shown to regulate the GluR1 expression in nucleus accumbens (Acb) which accounts for cocaine sensitivity (Heberlein et al., 2009). However, the transcriptional target of LMO4 for glutamate receptor expression is unclear. Nevertheless, LMO4 might be responsible at least in part for the plasticity of neuronal networks by activity-dependent alteration of excitatory synaptic strength which is widely recognized to underlie cognitive functions such as learning and memory.

In terms of the regulatory role of LMO4 in synaptic plasticity, we can not exclude other candidates. For example, a number of studies have shown that LMO4 interacts with histone deacetylase 2 (HDAC2) to prevent its recruitment and increase target gene transactivation (Gomez-Smith et al., 2010; Singh et al., 2005; Wang et al., 2007). HDAC2 is well known as a negative regulator of hippocampus-associated synaptogenesis and synaptic plasticity (Guan et al., 2009; Haettig et al., 2011; Stefanko et al., 2009; Vecsey et al., 2007). Compared with the other subtype HDAC1, HDAC2 was more enriched in the promoters of genes implicated in synaptic plasticity/remodeling or genes regulated by neuronal activity such as c-Fos, CREB, CREB-binding protein (CBP), BDNFI/II, CamKIIα and NMDA

receptor subunits (Guan et al., 2009). This raises the possibility that LMO4 might act as a suppressor for HDAC2-dependent transrepression of those target genes. As a result, LMO4 facilitates memory formation and synaptic plasticity. This hypothesis is indeed supported by previous observations (Kashani et al., 2006; Qin et al., 2012). However it awaits more experimental validation. In another example, LMO4 was shown to physically and functionally interact with peroxisome proliferator-activated receptor gamma (PPARγ), a nuclear receptor transcription factor. PPARγ was identified as the key regulator of glucose and lipid metabolism as well as having anti-inflammatory and neuroprotective functions (Daynes and Jones, 2002). A recent study revealed that the PPARγ agonist (rosiglitazone) attenuates learning and memory deficits in Tg2576 Alzheimer mice (Landreth et al., 2008), providing indirect evidence of memory improvement by PPARγ activation. The memory enhancement effect was later found during activation of other PPAR isoforms, such as PPARα (Mazzola et al., 2009) and PPARβ/δ (Kuang et al., 2012). More interestingly, Mazzola et al showed that the improvement of memory acquisition on rat passive-avoidance task was either achieved by administration of PPARα agonist WY14643 or by the FAAH inhibitor URB597 that increases eCB. However, this effect was fully blocked by application of a PPARα antagonist MK886. This is the first evidence to demonstrate a novel mechanism for memory enhancement that couples PPAR and eCB signaling. In fact, eCBs have been widely reported as endogenous ligands for PPARs in non-neuronal cells (Bouaboula et al., 2005; O'Sullivan, 2007; O'Sullivan and Kendall, 2010; O'Sullivan et al., 2011). Therefore, it would be tempting to examine whether and how PPARs, working with their potential partners- LMO4 and eCB, besides their common metabolic and neuroprotective roles, exert brain functions in regulating gene expression involved in neurotransmission and

subsequently in many complex processes, such as memory formation and stress response.

4.2 LMO4 and its Role in Anxiety/depression Relief

In a second study, a novel role of LMO4 in the cytoplasm has been identified. We confirmed that LMO4 inhibits PTP1B activity in the amygdala, consistent with our previous observation in the hypothalumus (Pandey et al., 2013). Moreover, we showed that mGluR5 is a substrate of PTP1B which accounts for dephosphorylation on tyrosine residue(s). Elevated PTP1B phosphatase activity observed in mice with glutamatergic neuron-specific ablation of LMO4 decreased mGluR-eCB signaling, resulting in a profound anxiety phenotype. We reasoned that this might be a general mechanism underlying anxiety since wild type mice subjected to repeated stress showed a similar elevation of PTP1B activity in the amygdala. The potential clinical value became more promising when we observed that intra-amygdalar injection of the PTP1B inhibitor Trodusquemine or a PTP1B shRNA alleviated anxiety by restoring eCB signaling. This study provides an emerging role of LMO4 as a mood regulator to maintain mGluR-mediated eCB signaling in the amygdala by restraining PTP1B activity.

However, the precise domains where and how LMO4 inhibits PTP1B activity are unclear. One possibility is that LMO4 stabilizes the oxidized inactivated form of PTP1B. Indeed, oxi-PTP1B levels were reduced in neuronal cells following knockdown of LMO4 and increased with over-expression of LMO4 (Pandey et al., 2013). LMO4 contains four cysteine-rich zinc fingers (Chen et al., 2002) that are known to be highly susceptible to oxidation at cysteine residues (Webster et al., 2001). Whether transient ROS production upon insulin stimulation could oxidize cysteines of the zinc fingers and cause stabilization of the PTP1B/LMO4 complex and retention of LMO4 at the endoplasmic reticulum needs further

examination. The precise domains where and how mGluR5 interacts with PTP1B also remain to be elucidated.

The possibility of LMO4 as a mood regulator might also derive from its interaction with Deaf1, a transcription factor that was shown to bind the promoter of serotonin-1A (5-HT_{1A}) receptor that is implicated in major depression and mood disorders (Lacivita et al., 2012). It is known that LMO4 and Deaf1 have common properties to regulate development and breast cancer (Barker et al., 2008; Hahm et al., 2004; Hermanson et al., 1999; Kenny et al., 1998; Wang et al., 2004). The binding motif for LMO4 is $Deaf1_{404–438/457–479}$ (Cubeddu et al., 2012; Joseph et al., 2013; Sugihara et al., 1998). LMO4 was shown to modulate activity of the Deaf1 nuclear export signal (NES), causing nuclear accumulation by blocking its export and thus favoring Deaf1 transcriptional regulation on target gene expression (Cubeddu et al., 2012). 5-HT_{1A} is known to be one of Deaf1 target genes. The 5-HT_{1A} receptor is the most widespread 5-HT receptor and highly expressed in the central nervous system including cerebral cortex, hippocampus, amygdala, raphe nucleus. Deaf1 displays dual activity : 1) suppressing presynaptic 5-HT_{1A} autoreceptor expression in serotonergic raphe cells ; 2) enhancing postsynaptic 5-HT_{1A} heteroreceptor expression in nonserotonergic neurons (Czesak et al., 2006). It is suggested that suicide and depression appear to result from upregulation of presynaptic 5-HT_{1A} autoreceptors, leading to reduced serotonergic activity (Bayliss et al., 1997; Stockmeier et al., 1998). Thus LMO4 working through its partner Deaf1 may have capability to become antidepressants in term of the role in increasing serotonin levels by downregulation of serotonergic 5-HT_{1A} autoreceptors and activation of postsynaptic 5-HT_{1A} receptors that regulate mood and emotion (Czesak et al., 2012; Czesak et al., 2006; Jacobs and Azmitia, 1992; Jann, 1988). Ablation of LMO4 may disrupt Deaf1 and 5-HT_{1A} receptor

function, leading to depression and suicide.

4.3 Other Potential Targets of PTP1B/LMO4 Associated with Synaptic Plasticity and Neurological Disorders

The role of cadherin and the associated catenin is well documented in synaptic function both *in vivo* and *in vitro* (Arikkath and Reichardt, 2008; Tai et al., 2008; Takeichi and Abe, 2005). N-cadherin and catenin signaling complex is critically regulated by tyrosine phosphorylation/ dephosphorylation (Lilien and Balsamo, 2005). Importantly, PTP1B dephosphorylation of β-catenin at the residue Tyr-654 ensures association of β-catenin with N-cadherin for normal hippocampal synapse formation. Impaired hippocampal PTP1B function or expression leads to elongation of dendritic filopodia and an active remodeling of synapse that could enhance learning and memory (Fuentes et al., 2012). This *in vivo* observation is consistent with previous findings that tyrosine phosphorylation of β-catenin at Tyr-654 reduced its affinity for cadherin (Roura et al., 1999; Xu et al., 2002; Xu et al., 2004). In fact, PTP1B phosphorylation at tyrosine 152 by tyrosine kinase Fer is critical for its binding ability to the cytoplasmic domain of cadherin and maintenance of β-catenin in a dephosphorylated state for complex stability at the cell surface (Xu et al., 2004). In addition, β-catenin seems to be dynamically modulated at both the transcriptional and post-transcriptional levels with fear learning in the amygdala where it is highly expressed (Maguschak and Ressler, 2008). β-catenin Tyr-654 phosphorylation in the amygdala showed alterations during fear memory consolidation, including two phase of increases during the first 30 minutes and 2 hours after fear training as well as one phase of decrease between those time points. Amygdala- specific ablation of β-catenin prevented the transfer of newly learned fear

into long term memory. It will be interesting to examine whether LMO4, as an endogenous inhibitor of PTP1B, can enhance hippocampal and amygdala synaptic plasticity by increasing phosphorylation of β-catenin. In addition, there is also evidence that LMO4 is an essential corepressor for SNAIL-mediated cadherin expression in neuroblastoma and neural crest cells (Ferronha et al., 2013). It remains to be tested whether LMO4 regulates the cadherin/catenin complex at both the transcriptional and post-translational levels.

As both immediate and delayed repressors of protein phosphorylation, protein phosphatases can influence both signal amplitude and duration and exert a powerful dominant effect on the regulation of signal duration in comparison with kinases that mainly affect amplitude (Heinrich et al., 2002; Nguyen et al., 2012). PTP1B has already been identified as a target for developing drugs to treat obesity and diabetes. A potent PTP1B inhibitor, Trodusquemine, has undergone testing in its clinical trials for characterization of anti-obesity and anti-diabetic properties, likely via restoration of insulin signaling in obese patients (Nguyen et al., 2012). Insulin receptors belong to tyrosine kinase receptors that mediate their activity by tyrosine phosphorylation. Insulin binds to and activates insulin receptor and its downstream signal cascade insulin receptor substrate 1 (IRS1) and 2 (IRS2). Evidence has shown that insulin signaling, besides regulation of glucose metabolism (Zinker et al., 2002), is important for regulation of cell surface receptor expression and thus synaptic plasticity (van der Heide et al., 2005; Wan et al., 1997). For instance, insulin activates IRS1 and stimulates tyrosine phosphorylation of the GluR2 subunits of native AMPA receptor. The elevated tyrosine phosphorylation is essential for insulin-induced AMPA receptor internalization and LTD (Ahmadian et al., 2004). It is promising to test whether PTP1B inhibitors such as LMO4

and Trodusquemine are capable of potentiating synaptic plasticity during the process of insulin activation.

Based on their induction mechanism, there are two widely-studied forms of LTD: group I mGluR-LTD (DHPG-LTD) and NMDA-LTD, which can be induced by DHPG and NMDA respectively (Collingridge et al., 2010). mGluR-LTD likely involves G-protein activation, tyrosine dephosphorylation of GluR2, and AMPAR endocytosis whereas NMDA-LTD involves Ca^{2+} rise, serine dephosphorylation of the GluR1 subunit at Ser845 and AMPA receptor internalization (Gladding et al., 2009a; Gladding et al., 2009b; Huang and Hsu, 2006; Moult et al., 2006; Moult et al., 2002). Surprisingly, GluR2 was shown to interact with N-cadherin by its N-terminal domain and GluR2 was indispensable for mGluR-LTD in the hippocampal excitatory synapse involving in an N-cadherin-dependent and cofilin-mediated actin reorganization (Zhou et al., 2011). Whether PTP1B and LMO4 participate in the function of cadherin/GluR2 complex required for mGluR-LTD waits for further studies. It is meaningful to acquire a better understanding of the molecular architecture and function of complexes composed of AMPARs and interacting proteins. LMO4 might be just one such potential candidate playing a role in AMPAR trafficking, subunit distribution or downstream protein synthesis as well as participating in the dynamics of the dendritic cytoskeleton.

Our studies are the first to suggest a role for PTP1B associated with mGluR in modulating eCB production/signaling in the BLA. *In vivo* data showed that elevated PTP1B is responsible for reduced eCB signaling in the BLA and the anxiety phenotype. Intra-amygdala administration of a PTP1B inhibitor Trodusquemine or PTP1B shRNA abrogated the anxiety phenotype in LMO4KO mice. The anxiolytic effect was blocked by intra-amygdala application of the CB1 receptor antagonist AM251, indicating its eCB-dependency. The

notion that inhibition of PTP1B in the BLA relieves anxiety via restoring mGluR-driven eCB release is also supported by in *vitro* study of GABAergic transmission. As we discussed, PTP1B/LMO4 likely involves AMPAR trafficking and mGluR-LTD in glutamatergic synapses is well known to involve the interaction with both AMPAR and NMDAR (Cho et al., 2000; Collingridge et al., 2010; Zhou et al., 2011). In order to narrow down factors affecting PTP1B-mediated postsynaptically-originated mGluR-LTD and focus on examination of mGluR5-driven retrograde eCB-dependent signaling, we decided to examine the DHPG-induced LTD for GABA transmission in BLA. In addition, eCBs contribute to a large portion of presynaptic LTDs and CB1 receptors are highly expressed in GABAergic interneurons. This form of eCB-LTD has been reported in various brain regions (Alger, 2009; Azad et al., 2004; Chevaleyre et al., 2007; Isokawa and Alger, 2005; Maccarrone et al., 2008; Maccarrone et al., 2010; Zhu, 2006) and has important clinical implications for associative learning and anxiety disorders (Heifets and Castillo, 2009; Zhang and Alger, 2010).

Studies by Hill's group have demonstrated that eCB signaling at the BLA regulates the basal tonic activity of the HPA axis under non-stressed conditions (Hill et al., 2009a). Therefore, characterization of PTP1B-mediated eCB signaling in the BLA may help to identify the important molecules for the basal tonic activity of the HPA axis and these may be therapeutic targets for the treatment of anxiety disorders. We found that mGluR5 is a target of PTP1B and that impaired mGluR5 function caused by elevated PTP1B activity contributes to reduced eCB production and signaling, leading to anxiety phenotype. Besides mGluR5, mGluR1 and RyR2 are also involved in eCB production and their activities are influenced by tyrosine phosphorylation (Guse et al., 2001; Tozzi et al., 2001). mGluR1 and RyR2 are also implicated in psychological disorders and cognitive dysfunction (Liu et al., 2012; Niswender

and Conn, 2010; Zalk et al., 2007). Whether mGluR1 and RyR2 are targets of PTP1B remains to be tested. In addition, it will be also interesting to check whether PTP1B affects the activity of the major enzymes involved in the eCB synthesis and degradation, such as DAGL, FAAH, MAGL, etc (Ahn et al., 2008). In fact, besides the established NAPE-PLD pathway (Di Marzo et al., 1994; Okamoto et al., 2004), an alternative pathway for AEA synthesis, identified in the RAW264.7 mouse macrophage cell line, involves NAPE hydrolysis by a phospholipase C to yield phosphoanandamide (pAEA) which is then dephosphorylated by phosphatases, including the putative tyrosine phosphatase PTPN22 (Liu et al., 2006). This PLC/phosphatase-dependent pathway was found in both mouse brain and macrophages. This novel pathway has been shown to be responsible for the LPS-induced increase in AEA biosynthesis in macrophages (Liu et al., 2006). However, it remains to be tested whether this alternative pathway occurs in neurons during mGluR1/5 activation and which phosphatase accounts for the dephosphorylation process from pAEA to AEA. It goes without saying that direct measurement of altered eCB release during DHPG-induced LTD would provide strong evidence to support my current hypotheses. Collaboration is being pursued to this end.

Finally, it can't be neglected to see whether PTP1B affects the dynamic regulation of $GABA_A$ receptor functionality and trafficking from and to the neuronal surface as well as lateral movement between synapses and extrasynapses. Trodusquemine was suggested to be capable of binding to the positive allosteric neurosteroid site of $GABA_A$ receptor and increasing channel gating-efficacy in hypothalamic neurons (Chernova et al., 2005) and thus likely affecting synaptic GABA channels and neuronal excitability (Bianchi and Macdonald, 2003). However, it was not experimentally tested if Trodusquemine was playing a role as a phosphatase inhibitor or as a neurosteroid. In addition, LMO4 has been shown to have an

essential role in directing a balanced generation of inhibitory and excitatory neurons in the ventral spinal cord (Joshi et al., 2009). Therefore, it raises the possibility that LMO4 may affect basal tone of GABA transmission and this awaits further examination. Nevertheless, effect of LMO4 on $GABA_A$ receptor component, trafficking and functionality, which were not elucidated in my studies, is also of considerable interest and has also been implicated in various neurological disorders, such as anxiety, depression, epilepsy and schizophrenia (Jacob et al., 2008).

Regarding the role of PTP1B/LMO4 as a therapeutic target for reducing of stress, it was found that amygdalar PTP1B activity was elevated in association with increased corticosterone levels in wild type mice subjected to a daily 30 minutes regimen of restraint stress for 9 days. The elevated PTP1B activity was associated with the reduced PTP1B oxidation. The oxidized form of PTP1B is inactive and dependent on LMO4 palmitoylation and ER-retention as reported (Pandey et al., 2013). Consistent with observations in the hypothalamus, stressed mice also showed lower levels of palmitoylated LMO4 in the amygdala. The promising preliminary findings lead us to address the following questions in future experiments: 1) Is PTP1B elevation a generalized mechanism for chronic stress models? 2) Does a PTP1B inhibitor like Trodusquemine reduce stress-induced anxiety? 3) Are mice (CamK2αCrePTP1Bflox) with postnatal glutamatergic neuron-specific ablation of PTP1B by using the promoter CamK2α in cre loxp system resistant to chronic stress or have fewer symptoms of anxiety, are double KO mice (CamK2αCrePTP1B/LMO4flox) normal like control WT mice? 4) Are mice with amygdala-specific knockdown of PTP1B or LMO4 anxiolytic or anxious, respectively? 5) Can forced expression of an LMO4 with an ER-tail anchoring sequence effectively block PTP1B activity for maintenance of intact eCB signaling

even during chronic stress conditions or treatment with high glucocorticoids? 6) Is the basic tone of eCB different in these transgenic mice? 7) Are eCB levels consistently altered with PTP1B activity, in a tonic or a phasic manner? All these questions are interesting and remain to be addressed.

My studies propose a new generalized mechanism that subsequently reduces eCB signaling under stress condition. Considering the risk for drug abuse, the limitations and side effects of commonly-used medications (benzodiazepines, antidepressant and beta-blockers) for anxiety disorders, identifying a better therapeutic target becomes more attractive. eCBs modulate synaptic transmission and control anxiety. Control of eCB production and its temporal and spatial action may offer a better therapy. A PTP1B inhibitor via enhancement of mGluR-driven eCB release may serve as a new candidate to treat anxiety.

4. 4 General Conclusions

My studies identify a crucial role of LMO4 in controlling central synaptic function and that disruption of these functions results in important severe behavioural deficits. A proposed working model depicting the role of LMO4 on synaptic function and mechanism accountable for defective synaptic function in LMO4KO in the hippocampus and amygdala was illustrated in Fig. 4.1 and Fig. 4.2, respectively. In glutamatergic hippocampal synapses, I showed that LMO4 is an important regulator of the CICR machinery by positively controlling RyR2 expression. Several electrophysiological responses known to be modulated by CICR were analyzed in LMO4KO hippocampal acute slices and organotypic hippocampal slices in culture. These characterizations revealed a severely-compromised release of calcium from internal stores and the subsequently reduced glutamate release probability and LTP, leading to

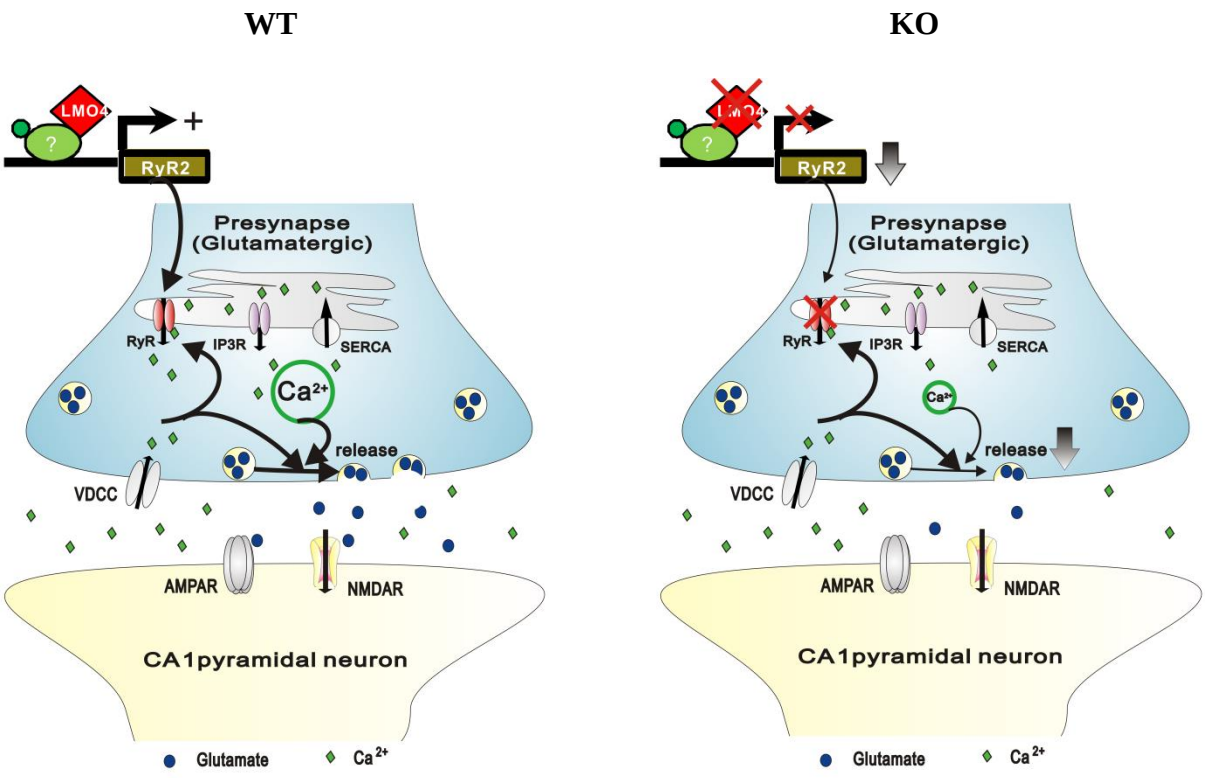

Figure 4.1 A proposed model depicting the role of LMO4 on hippocampal synaptic function

In the hippocampal glutamatergic synapses, LMO4 is an important regulator of the CICR machinery by positively controlling RyR2 expression. Upon stimulation calcium concentration at the presynaptic terminal, is drastically elevated due to calcium influx through VGCCs and calcium induced calcium release (CICR) from intracellular stores, which is mediated through ryanodine receptors and IP3 receptors (not discussed in my study). Loss of LMO4 function in the presynaptic terminal specifically disrupts ryanodine receptor-mediated Ca release from the ER store, thus reducing CICR and decreasing calcium rise induced by action potentials in the presynaptic terminal. This reduction in calcium increases impairs the probability of neurotransmitter release, and the decreased glutamate release causes LTP impairment in LMO4 KO mice at presynaptic terminals.

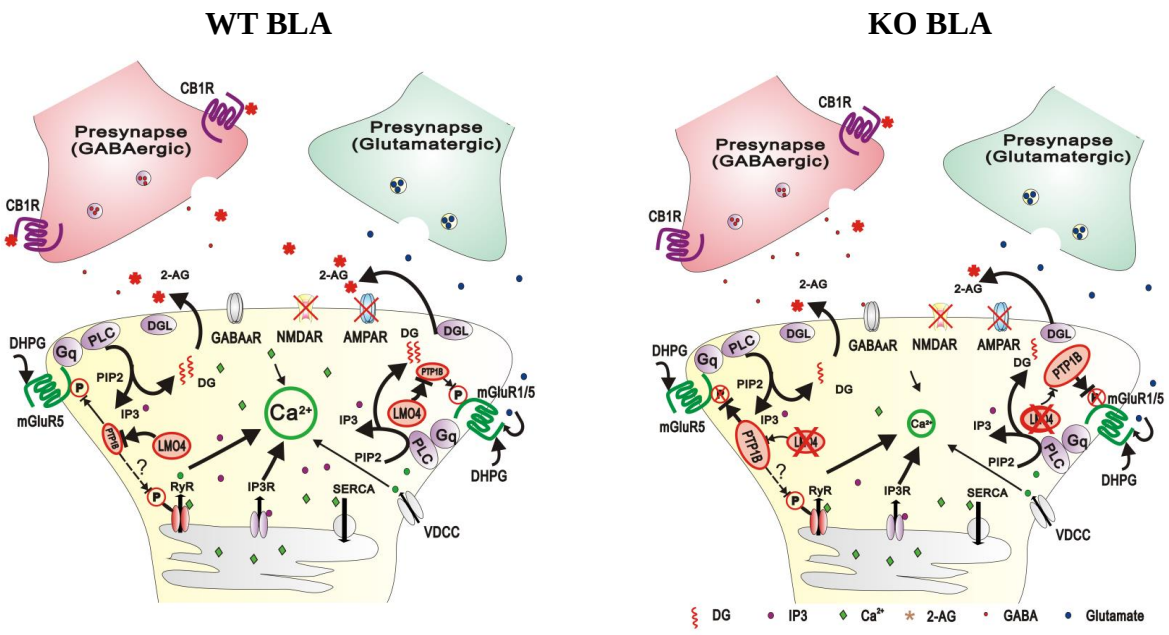

Figure 4.2 A proposed model depicting the role of LMO4 on amygdalar synaptic function

In the basolateral amygdalar GABAergic synapses, LMO4 negatively regulated PTP1B activity. Lack of LMO4 in BLA increased PTP1B activity, which transiently enhanced dephosphorylation of tyrosine residues in the mGluR1/5. Strong activation of mGluR1/5 by DHPG (glutamate in the physiological condition) stimulates PLC, which hydrolyzes PIP2 into DG and inositol 1,4,5-trisphosphate (IP3). 2-AG is then produced from DG by DGL, and released from the postsynaptic neuron. It is suggested that upon mGluR1/5 dephosphorylation, both 2-AG and IP3 production are reduced, resulting in impaired eCB retrograde signaling and neurotransmitter release. The released 2-AG binds to presynaptic CB1 receptors (CB1R) and suppresses neurotransmitter release. eCB signaling may require calcium influx through VDCC or efflux from ER through IP3R or RyR. PTP1B may also affect RyR dephosphorylation in the similar manner of mGluR1/5, thus indirectly regulating Ca^{2+} rise from internal store. Only 2-AG but not AEA is included for simplification of mechanism.

the markedly behavioural deficits in hippocampus-dependent spatial learning. In GABAergic BLA synapses, I demonstrated that LMO4 negatively regulated protein tyrosine phosphatase 1B (PTP1B) activity that targets mGluR5 and is associated with the emotion state. Loss of LMO4 in BLA in LMO4KO mice or reduction of LMO4 expression in chronically stressed wild type mice would increase PTP1B activity and transiently enhance dephosphorylation of tyrosine residues in mGluR5. LMO4KO mice display the dramatic anxiety through PTP1B-mediated mGluR/eCB signaling pathway. Presumably, elevated mGluR1/5 de-phosphorylation reduced eCB production, reflected by inefficient eCB-dependent inhibition of GAABA transmission upon DHPG treatment. In addition, PTP1B-mediated mGluR/eCB pathway associated with anxiety in LMO4KO mice is also supported by the fact that relief of anxiety by PTP1B inhibitor Trodusquemine was dependent on eCB signaling since the anxiolytic effect of Trodusquemine was attenuated by CB1 receptor antagonist AM251.

Here I report that LMO4, as a novel key regulator of CICR and PTP1B in the central nervous system, plays a key role in synaptic function. Intriguedly, PTP1B, for the first time, is identified as a therapeutic target for anxiety besides its original role for obesity and diabetes. Furthermore, amygdala-specific inhibition of PTP1B by pharmacological or genetic approach rescued the innate anxiety in LMO4KO mice. Moreover, this PTP1B-mediated anxiety may be applicable for chronically-stressed animals. Collectively, these data identify that LMO4 plays an essential role for non-genomic or genomic regulation in both central presynaptic and postsynaptic neurons, providing a mechanism for LMO4 to modulate a wide range of neuronal functions and behavior.

REFERENCE

Ahima, R.S., Patel, H.R., Takahashi, N., Qi, Y., Hileman, S.M., and Zasloff, M.A. (2002). Appetite suppression and weight reduction by a centrally active aminosterol. Diabetes *51*, 2099-2104.

Ahmadian, G., Ju, W., Liu, L., Wyszynski, M., Lee, S.H., Dunah, A.W., Taghibiglou, C., Wang, Y., Lu, J., Wong, T.P., Sheng, M., Wang, Y.T. (2004). Tyrosine phosphorylation of GluR2 is required for insulin-stimulated AMPA receptor endocytosis and LTD. Embo J *23*, 1040-1050.

Ahn, K., McKinney, M.K., and Cravatt, B.F. (2008). Enzymatic pathways that regulate endocannabinoid signaling in the nervous system. Chem Rev *108*, 1687-1707.

Aizawa, H., Hu, S.C., Bobb, K., Balakrishnan, K., Ince, G., Gurevich, I., Cowan, M., and Ghosh, A. (2004). Dendrite development regulated by CREST, a calcium-regulated transcriptional activator. Science *303*, 197-202.

Akita, T., and Kuba, K. (2000). Functional triads consisting of ryanodine receptors, Ca(2+) channels, and Ca(2+)-activated K(+) channels in bullfrog sympathetic neurons. Plastic modulation of action potential. J Gen Physiol *116*, 697-720.

Alger, B.E. (2009). Endocannabinoid signaling in neural plasticity. Curr Top Behav Neurosci *1*, 141-172.

Alger, B.E., and Nicoll, R.A. (1980). Epileptiform burst afterhyperolarization: calcium-dependent potassium potential in hippocampal CA1 pyramidal cells. Science *210*, 1122-1124.

Alvarado, D.M., Veile, R., Speck, J., Warchol, M., and Lovett, M. (2009). Downstream targets of GATA3 in the vestibular sensory organs of the inner ear. Dev Dyn *238*, 3093-3102.

Amunts, K., Kedo, O., Kindler, M., Pieperhoff, P., Mohlberg, H., Shah, N.J., Habel, U., Schneider, F., and Zilles, K. (2005). Cytoarchitectonic mapping of the human amygdala, hippocampal region and entorhinal cortex: intersubject variability and probability maps. Anat Embryol (Berl) *210*, 343-352.

Andrade, R., and Nicoll, R.A. (1987). Pharmacologically distinct actions of serotonin on single pyramidal neurones of the rat hippocampus recorded in vitro. J Physiol *394*, 99-124.

Anwyl, R. (2009). Metabotropic glutamate receptor-dependent long-term potentiation. Neuropharmacology *56*, 735-740.

Arikkath, J., and Reichardt, L.F. (2008). Cadherins and catenins at synapses: roles in synaptogenesis and synaptic plasticity. Trends Neurosci *31*, 487-494.

Armeanu-Ebinger, S., Bonin, M., Habig, K., Poremba, C., Koscielniak, E., Godzinski, J., Warmann, S.W., Fuchs, J., and Seitz, G. (2011). Differential expression of invasion promoting genes in childhood rhabdomyosarcoma. Int J Oncol *38*, 993-1000.

Asprer, J.S., Lee, B., Wu, C.S., Vadakkan, T., Dickinson, M.E., Lu, H.C., and Lee, S.K. (2011). LMO4 functions as a co-activator of neurogenin 2 in the developing cortex. Development *138*, 2823-2832.

Azad, S.C., Monory, K., Marsicano, G., Cravatt, B.F., Lutz, B., Zieglgansberger, W., and Rammes, G. (2004). Circuitry for associative plasticity in the amygdala involves endocannabinoid signaling. J Neurosci *24*, 9953-9961.

Baldwin, D.S., Ajel, K.I., and Garner, M. (2010). Pharmacological treatment of generalized anxiety disorder. Curr Top Behav Neurosci *2*, 453-467.

Balschun, D., Wetzel, W., Del Rey, A., Pitossi, F., Schneider, H., Zuschratter, W., and Besedovsky, H.O. (2004). Interleukin-6: a cytokine to forget. FASEB J *18*, 1788-1790.

Barker, H.E., Smyth, G.K., Wettenhall, J., Ward, T.A., Bath, M.L., Lindeman, G.J., and Visvader, J.E. (2008). Deaf-1 regulates epithelial cell proliferation and side-branching in the mammary gland. BMC Dev Biol *8*, 94.

Bassell, G.J., and Warren, S.T. (2008). Fragile X syndrome: loss of local mRNA regulation alters synaptic development and function. Neuron *60*, 201-214.

Bayliss, D.A., Li, Y.W., and Talley, E.M. (1997). Effects of serotonin on caudal raphe neurons: activation of an inwardly rectifying potassium conductance. J Neurophysiol *77*, 1349-1361.

Bear, M.F., and Abraham, W.C. (1996). Long-term depression in hippocampus. Annu Rev Neurosci *19*, 437-462.

Beglopoulos, V., Sun, X., Saura, C.A., Lemere, C.A., Kim, R.D., and Shen, J. (2004). Reduced beta-amyloid production and increased inflammatory responses in presenilin conditional knock-out mice. J Biol Chem *279*, 46907-46914.

Béïque, J.C., and Andrade, R. (2003). PSD-95 regulates synaptic transmission and plasticity in rat cerebral cortex. J Physiol *546*, 859-867.

Béïque, J.C., Imad, M., Mladenovic, L., Gingrich, J.A., and Andrade, R. (2007). Mechanism of the 5-hydroxytryptamine 2A receptor-mediated facilitation of synaptic activity in prefrontal cortex. Proc Natl Acad Sci U S A *104*, 9870-9875.

Béïque, J.C., Lin, D.T., Kang, M.G., Aizawa, H., Takamiya, K., and Huganir, R.L. (2006). Synapse-specific regulation of AMPA receptor function by PSD-95. Proc Natl Acad Sci U S A *103*, 19535-19540.

Bekkers, J.M., and Stevens, C.F. (1990). Presynaptic mechanism for long-term potentiation in the hippocampus. Nature *346*, 724-729.

Bellone, C., Luscher, C., and Mameli, M. (2008). Mechanisms of synaptic depression triggered by metabotropic glutamate receptors. Cell Mol Life Sci *65*, 2913-2923.

Ben-Ari, Y., Gaiarsa, J.L., Tyzio, R., and Khazipov, R. (2007). GABA: a pioneer transmitter that excites immature neurons and generates primitive oscillations. Physiol Rev *87*, 1215-1284.

Bence, K.K., Delibegovic, M., Xue, B., Gorgun, C.Z., Hotamisligil, G.S., Neel, B.G., and Kahn, B.B. (2006). Neuronal PTP1B regulates body weight, adiposity and leptin action. Nat Med *12*, 917-924.

Berridge, M.J. (1998). Neuronal calcium signaling. Neuron *21*, 13-26.

Bianchi, M.T., and Macdonald, R.L. (2003). Neurosteroids shift partial agonist activation of GABA(A) receptor channels from low- to high-efficacy gating patterns. J Neurosci *23*, 10934-10943.

Bliss, T.V., and Lomo, T. (1973). Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path. J Physiol *232*, 331-356.

Bong, Y.S., Lee, H.S., Carim-Todd, L., Mood, K., Nishanian, T.G., Tessarollo, L., and Daar, I.O. (2007). ephrinB1 signals from the cell surface to the nucleus by recruitment of STAT3. Proc Natl Acad Sci U S A *104*, 17305-17310.

Borgdorff, A.J., and Choquet, D. (2002). Regulation of AMPA receptor lateral movements. Nature *417*, 649-653.

Bouaboula, M., Hilairet, S., Marchand, J., Fajas, L., Le Fur, G., and Casellas, P. (2005). Anandamide induced PPARgamma transcriptional activation and 3T3-L1 preadipocyte differentiation. Eur J Pharmacol *517*, 174-181.

Bourdeau, A., Dube, N., and Tremblay, M.L. (2005). Cytoplasmic protein tyrosine phosphatases, regulation and function: the roles of PTP1B and TC-PTP. Curr Opin Cell Biol *17*, 203-209.

Bowles, N.P., Hill, M.N., Bhagat, S.M., Karatsoreos, I.N., Hillard, C.J., and McEwen, B.S. (2012). Chronic, noninvasive glucocorticoid administration suppresses limbic endocannabinoid signaling in mice. Neuroscience *204*, 83-89.

Bredt, D.S., and Nicoll, R.A. (2003). AMPA receptor trafficking at excitatory synapses. Neuron *40*, 361-379.

Brodkin, J., Bradbury, M., Busse, C., Warren, N., Bristow, L.J., and Varney, M.A. (2002). Reduced stress-induced hyperthermia in mGluR5 knockout mice. Eur J Neurosci *16*, 2241-2244.

Byrne, J.H. (2008). Learning and memory:a comprehensive reference London : Academic.

Campolongo, P., Roozendaal, B., Trezza, V., Hauer, D., Schelling, G., McGaugh, J.L., and Cuomo, V. (2009). Endocannabinoids in the rat basolateral amygdala enhance memory consolidation and enable glucocorticoid modulation of memory. Proc Natl Acad Sci U S A *106*, 4888-4893.

Carlsen, J. (1988). Immunocytochemical localization of glutamate decarboxylase in the rat basolateral amygdaloid nucleus, with special reference to GABAergic innervation of amygdalostriatal projection neurons. J Comp Neurol *273*, 513-526.

Carter, A.G., Vogt, K.E., Foster, K.A., and Regehr, W.G. (2002). Assessing the role of calcium-induced calcium release in short-term presynaptic plasticity at excitatory central synapses. J Neurosci *22*, 21-28.

Carty, N.C., Xu, J., Kurup, P., Brouillette, J., Goebel-Goody, S.M., Austin, D.R., Yuan, P., Chen, G., Correa, P.R., Haroutunian, V., *et al.* (2012). The tyrosine phosphatase STEP: implications in schizophrenia and the molecular mechanism underlying antipsychotic medications. Transl Psychiatry *2*, e137.

Casanova, E., Fehsenfeld, S., Mantamadiotis, T., Lemberger, T., Greiner, E., Stewart, A.F., and Schutz, G. (2001). A CamKIIalpha iCre BAC allows brain-specific gene inactivation. Genesis *31*, 37-42.

Castillo, P.E., Younts, T.J., Chavez, A.E., and Hashimotodani, Y. (2012). Endocannabinoid signaling and synaptic function. Neuron *76*, 70-81.

Catterall, W.A., and Few, A.P. (2008). Calcium channel regulation and presynaptic plasticity. Neuron *59*, 882-901.

Cederquist, G.Y., Azim, E., Shnider, S.J., Padmanabhan, H., and Macklis, J.D. (2013). Lmo4 establishes rostral motor cortex projection neuron subtype diversity. J Neurosci *33*, 6321-6332.

Chakroborty, S., Goussakov, I., Miller, M.B., and Stutzmann, G.E. (2009). Deviant ryanodine receptor-mediated calcium release resets synaptic homeostasis in presymptomatic 3xTg-AD mice. J Neurosci *29*, 9458-9470.

Chen, H.H., Schock, S.C., Xu, J., Safarpour, F., Thompson, C.S., and Stewart, A.F. (2007a). Extracellular ATP-dependent upregulation of the transcription cofactor LMO4 promotes neuron survival from hypoxia. Exp Cell Res *313*, 3106-3116.

Chen, H.H., Tourtellotte, W.G., and Frank, E. (2002a). Muscle spindle-derived neurotrophin 3 regulates synaptic connectivity between muscle sensory and motor neurons. J Neurosci *22*, 3512-3519.

Chen, H.H., Xu, J., Safarpour, F., and Stewart, A.F. (2007b). LMO4 mRNA stability is regulated by extracellular ATP in F11 cells. Biochem Biophys Res Commun *357*, 56-61.

Chen, H.H., Yip, J.W., Stewart, A.F., and Frank, E. (2002b). Differential expression of a transcription regulatory factor, the LIM domain only 4 protein Lmo4, in muscle sensory neurons. Development *129*, 4879-4889.

Chernova, M.N., Vandorpe, D.H., Clark, J.S., Williams, J.I., Zasloff, M.A., Jiang, L., and Alper, S.L. (2005). Apparent receptor-mediated activation of Ca2+-dependent conductive Cl- transport by shark-derived polyaminosterols. Am J Physiol Regul Integr Comp Physiol *289*, R1644-1658.

Chevaleyre, V., Heifets, B.D., Kaeser, P.S., Sudhof, T.C., and Castillo, P.E. (2007). Endocannabinoid-mediated long-term plasticity requires cAMP/PKA signaling and RIM1alpha. Neuron *54*, 801-812.

Chevaleyre, V., Takahashi, K.A., and Castillo, P.E. (2006). Endocannabinoid-mediated synaptic plasticity in the CNS. Annu Rev Neurosci *29*, 37-76.

Cho, K., Kemp, N., Noel, J., Aggleton, J.P., Brown, M.W., and Bashir, Z.I. (2000). A new form of long-term depression in the perirhinal cortex. Nat Neurosci *3*, 150-156.

Ciocchi, S., Herry, C., Grenier, F., Wolff, S.B., Letzkus, J.J., Vlachos, I., Ehrlich, I., Sprengel, R., Deisseroth, K., Stadler, M.B., *et al.* (2010). Encoding of conditioned fear in central amygdala inhibitory circuits. Nature *468*, 277-282.

Collingridge, G.L., Isaac, J.T., and Wang, Y.T. (2004). Receptor trafficking and synaptic plasticity. Nat Rev Neurosci *5*, 952-962.

Collingridge, G.L., Peineau, S., Howland, J.G., and Wang, Y.T. (2010). Long-term depression in the CNS. Nat Rev Neurosci *11*, 459-473.

Coutinho, V., and Knopfel, T. (2002). Metabotropic glutamate receptors: electrical and chemical signaling properties. Neuroscientist *8*, 551-561.

Cubeddu, L., Joseph, S., Richard, D.J., and Matthews, J.M. (2012). Contribution of DEAF1 structural domains to the interaction with the breast cancer oncogene LMO4. PLoS One *7*, e39218.

Cummings, J.A., Mulkey, R.M., Nicoll, R.A., and Malenka, R.C. (1996). Ca2+ signaling requirements for long-term depression in the hippocampus. Neuron *16*, 825-833.

Czesak, M., Le Francois, B., Millar, A.M., Deria, M., Daigle, M., Visvader, J.E., Anisman, H., and Albert, P.R. (2012). Increased serotonin-1A (5-HT1A) autoreceptor expression and reduced raphe serotonin levels in deformed epidermal autoregulatory factor-1 (Deaf-1) gene knock-out mice. J Biol Chem *287*, 6615-6627.

Czesak, M., Lemonde, S., Peterson, E.A., Rogaeva, A., and Albert, P.R. (2006). Cell-specific repressor or enhancer activities of Deaf-1 at a serotonin 1A receptor gene polymorphism. J Neurosci *26*, 1864-1871.

Daynes, R.A., and Jones, D.C. (2002). Emerging roles of PPARs in inflammation and immunity. Nat Rev Immunol *2*, 748-759.

de la Calle-Mustienes, E., Lu, Z., Cortes, M., Andersen, B., Modolell, J., and Gomez-Skarmeta, J.L. (2003). Xenopus Xlmo4 is a GATA cofactor during ventral mesoderm formation and regulates Ldb1 availability at the dorsal mesoderm and the neural plate. Dev Biol *264*, 564-581.

Deng, W., Aimone, J.B., and Gage, F.H. (2010). New neurons and new memories: how does adult hippocampal neurogenesis affect learning and memory? Nat Rev Neurosci *11*, 339-350.

Derkach, V.A., Oh, M.C., Guire, E.S., and Soderling, T.R. (2007). Regulatory mechanisms of AMPA receptors in synaptic plasticity. Nat Rev Neurosci *8*, 101-113.

Desai, M.A., McBain, C.J., Kauer, J.A., and Conn, P.J. (1994). Metabotropic glutamate receptor-induced disinhibition is mediated by reduced transmission at excitatory synapses onto interneurons and inhibitory synapses onto pyramidal cells. Neurosci Lett *181*, 78-82.

Di Marzo, V. (2009). The endocannabinoid system: its general strategy of action, tools for its pharmacological manipulation and potential therapeutic exploitation. Pharmacol Res *60*, 77-84.

Di Marzo, V. (2011). Endocannabinoid signaling in the brain: biosynthetic mechanisms in the limelight. Nat Neurosci *14*, 9-15.

Di Marzo, V., Fontana, A., Cadas, H., Schinelli, S., Cimino, G., Schwartz, J.C., and Piomelli, D. (1994). Formation and inactivation of endogenous cannabinoid anandamide in central neurons. Nature *372*, 686-691.

Di, S., Malcher-Lopes, R., Halmos, K.C., and Tasker, J.G. (2003). Nongenomic glucocorticoid inhibition via endocannabinoid release in the hypothalamus: a fast feedback mechanism. J Neurosci *23*, 4850-4857.

Dobrunz, L.E., and Stevens, C.F. (1997). Heterogeneity of release probability, facilitation, and depletion at central synapses. Neuron *18*, 995-1008.

Dolen, G., Osterweil, E., Rao, B.S., Smith, G.B., Auerbach, B.D., Chattarji, S., and Bear, M.F. (2007). Correction of fragile X syndrome in mice. Neuron *56*, 955-962.

Dolmetsch, R.E., Pajvani, U., Fife, K., Spotts, J.M., and Greenberg, M.E. (2001). Signaling to the nucleus by an L-type calcium channel-calmodulin complex through the MAP kinase pathway. Science *294*, 333-339.

Drisdel, R.C., Alexander, J.K., Sayeed, A., and Green, W.N. (2006). Assays of protein palmitoylation. Methods *40*, 127-134.

Dube, N., Bourdeau, A., Heinonen, K.M., Cheng, A., Loy, A.L., and Tremblay, M.L. (2005). Genetic ablation of protein tyrosine phosphatase 1B accelerates lymphomagenesis of p53-null mice through the regulation of B-cell development. Cancer Res *65*, 10088-10095.

Duquette, P.M., Zhou, X., Yap, N.L., MacLaren, E.J., Lu, J.J., Wallace, V.A., and Chen, H.H. (2010). Loss of LMO4 in the retina leads to reduction of GABAergic amacrine cells and functional deficits. PLoS One *5*, e13232.

Ehrlich, I., Humeau, Y., Grenier, F., Ciocchi, S., Herry, C., and Luthi, A. (2009). Amygdala inhibitory circuits and the control of fear memory. Neuron *62*, 757-771.

Emptage, N.J., Reid, C.A., and Fine, A. (2001). Calcium stores in hippocampal synaptic boutons mediate short-term plasticity, store-operated Ca2+ entry, and spontaneous transmitter release. Neuron *29*, 197-208.

Endo, M. (1977). Calcium release from the sarcoplasmic reticulum. Physiol Rev *57*, 71-108.
Esteban, J.A. (2003). AMPA receptor trafficking: a road map for synaptic plasticity. Mol Interv *3*, 375-385.

Etkin, A., Prater, K.E., Schatzberg, A.F., Menon, V., and Greicius, M.D. (2009). Disrupted amygdalar subregion functional connectivity and evidence of a compensatory network in generalized anxiety disorder. Arch Gen Psychiatry *66*, 1361-1372.

Faber, D.S., and Korn, H. (1991). Applicability of the coefficient of variation method for analyzing synaptic plasticity. Biophys J *60*, 1288-1294.

Ferraguti, F., and Shigemoto, R. (2006). Metabotropic glutamate receptors. Cell Tissue Res *326*, 483-504.

Ferronha, T., Rabadan, M.A., Gil-Guinon, E., Le Dreau, G., de Torres, C., and Marti, E. (2013). LMO4 is an essential cofactor in the Snail2-mediated epithelial-to-mesenchymal transition of neuroblastoma and neural crest cells. J Neurosci *33*, 2773-2783.

Fill, M., and Copello, J.A. (2002). Ryanodine receptor calcium release channels. Physiol Rev *82*, 893-922.

Fitzjohn, S.M., Palmer, M.J., May, J.E., Neeson, A., Morris, S.A., and Collingridge, G.L. (2001). A characterisation of long-term depression induced by metabotropic glutamate receptor activation in the rat hippocampus in vitro. J Physiol *537*, 421-430.

Flavell, S.W., and Greenberg, M.E. (2008). Signaling mechanisms linking neuronal activity to gene expression and plasticity of the nervous system. Annu Rev Neurosci *31*, 563-590.

Freund, T.F., Katona, I., and Piomelli, D. (2003). Role of endogenous cannabinoids in synaptic signaling. Physiol Rev *83*, 1017-1066.

Freyd, G., Kim, S.K., and Horvitz, H.R. (1990). Novel cysteine-rich motif and homeodomain in the product of the Caenorhabditis elegans cell lineage gene lin-11. Nature *344*, 876-879.

Fuentes, F., Zimmer, D., Atienza, M., Schottenfeld, J., Penkala, I., Bale, T., Bence, K.K., and Arregui, C.O. (2012). Protein tyrosine phosphatase PTP1B is involved in hippocampal synapse formation and learning. PLoS One *7*, e41536.

Gallagher, S.M., Daly, C.A., Bear, M.F., and Huber, K.M. (2004). Extracellular signal-regulated protein kinase activation is required for metabotropic glutamate receptor-dependent long-term depression in hippocampal area CA1. J Neurosci *24*, 4859-4864.

Ganguly, K., Schinder, A.F., Wong, S.T., and Poo, M. (2001). GABA itself promotes the developmental switch of neuronal GABAergic responses from excitation to inhibition. Cell *105*, 521-532.

Ganon-Elazar, E., and Akirav, I. (2009). Cannabinoid receptor activation in the basolateral amygdala blocks the effects of stress on the conditioning and extinction of inhibitory avoidance. J Neurosci *29*, 11078-11088.

Gasparini, F., and Spooren, W. (2007). Allosteric modulators for mGlu receptors. Curr Neuropharmacol *5*, 187-194.

Gee, C.E., Benquet, P., and Gerber, U. (2003). Group I metabotropic glutamate receptors activate a calcium-sensitive transient receptor potential-like conductance in rat hippocampus. J Physiol *546*, 655-664.

Gladding, C.M., Collett, V.J., Jia, Z., Bashir, Z.I., Collingridge, G.L., and Molnar, E. (2009a). Tyrosine dephosphorylation regulates AMPAR internalisation in mGluR-LTD. Mol Cell Neurosci *40*, 267-279.

Gladding, C.M., Fitzjohn, S.M., and Molnar, E. (2009b). Metabotropic glutamate receptor-mediated long-term depression: molecular mechanisms. Pharmacol Rev *61*, 395-412.

Goebel-Goody, S.M., Baum, M., Paspalas, C.D., Fernandez, S.M., Carty, N.C., Kurup, P., and Lombroso, P.J. (2012). Therapeutic implications for striatal-enriched protein tyrosine phosphatase (STEP) in neuropsychiatric disorders. Pharmacol Rev *64*, 65-87.

Gomez-Ospina, N., Tsuruta, F., Barreto-Chang, O., Hu, L., and Dolmetsch, R. (2006). The C terminus of the L-type voltage-gated calcium channel Ca(V)1.2 encodes a transcription factor. Cell *127*, 591-606.

Gomez-Smith, M., Qin, Z., Zhou, X., Schock, S.C., and Chen, H.H. (2010). LIM domain only 4 protein promotes granulocyte colony-stimulating factor-induced signaling in neurons. Cell Mol Life Sci *67*, 949-957.

Green, B., Kavanagh, D., and Young, R. (2003). Being stoned: a review of self-reported cannabis effects. Drug Alcohol Rev *22*, 453-460.

Grueter, B.A., Gosnell, H.B., Olsen, C.M., Schramm-Sapyta, N.L., Nekrasova, T., Landreth, G.E., and Winder, D.G. (2006). Extracellular-signal regulated kinase 1-dependent metabotropic glutamate receptor 5-induced long-term depression in the bed nucleus of the stria terminalis is disrupted by cocaine administration. J Neurosci *26*, 3210-3219.

Grutz, G., Forster, A., and Rabbitts, T.H. (1998). Identification of the LMO4 gene encoding an interaction partner of the LIM-binding protein LDB1/NLI1: a candidate for displacement by LMO proteins in T cell acute leukaemia. Oncogene *17*, 2799-2803.

Guan, J.S., Haggarty, S.J., Giacometti, E., Dannenberg, J.H., Joseph, N., Gao, J., Nieland, T.J., Zhou, Y., Wang, X., Mazitschek, R., *et al.* (2009). HDAC2 negatively regulates memory formation and synaptic plasticity. Nature *459*, 55-60.

Guse, A.H., Tsygankov, A.Y., Weber, K., and Mayr, G.W. (2001). Transient tyrosine phosphorylation of human ryanodine receptor upon T cell stimulation. J Biol Chem *276*, 34722-34727.

Haettig, J., Stefanko, D.P., Multani, M.L., Figueroa, D.X., McQuown, S.C., and Wood, M.A. (2011). HDAC inhibition modulates hippocampus-dependent long-term memory for object location in a CBP-dependent manner. Learn Mem *18*, 71-79.

Hahm, K., Sum, E.Y., Fujiwara, Y., Lindeman, G.J., Visvader, J.E., and Orkin, S.H. (2004). Defective neural tube closure and anteroposterior patterning in mice lacking the LIM protein LMO4 or its interacting partner Deaf-1. Mol Cell Biol *24*, 2074-2082.

Haller, J., Varga, B., Ledent, C., and Freund, T.F. (2004). CB1 cannabinoid receptors mediate anxiolytic effects: convergent genetic and pharmacological evidence with CB1-specific agents. Behav Pharmacol *15*, 299-304.

Han, J., Kesner, P., Metna-Laurent, M., Duan, T., Xu, L., Georges, F., Koehl, M., Abrous, D.N., Mendizabal-Zubiaga, J., Grandes, P., *et al.* (2012). Acute cannabinoids impair working memory through astroglial CB1 receptor modulation of hippocampal LTD. Cell *148*, 1039-1050.

Hashimotodani, Y., Ohno-Shosaku, T., Watanabe, M., and Kano, M. (2007). Roles of phospholipase Cbeta and NMDA receptor in activity-dependent endocannabinoid release. J Physiol *584*, 373-380.

Hebb, D.O. (1949). The organization of behavior: a neuropsychological theory
New York : Wiley, 1-335.

Heberlein, U., Tsai, L.T., Kapfhamer, D., and Lasek, A.W. (2009). Drosophila, a genetic model system to study cocaine-related behaviors: a review with focus on LIM-only proteins. Neuropharmacology *56 Suppl 1*, 97-106.

Heifets, B.D., and Castillo, P.E. (2009). Endocannabinoid signaling and long-term synaptic plasticity. Annu Rev Physiol *71*, 283-306.

Heine, M., Groc, L., Frischknecht, R., Béïque, J.C., Lounis, B., Rumbaugh, G., Huganir, R.L., Cognet, L., and Choquet, D. (2008). Surface mobility of postsynaptic AMPARs tunes synaptic transmission. Science *320*, 201-205.

Heinrich, R., Neel, B.G., and Rapoport, T.A. (2002). Mathematical models of protein kinase signal transduction. Mol Cell *9*, 957-970.

Hermanson, O., Sugihara, T.M., and Andersen, B. (1999). Expression of LMO-4 in the central nervous system of the embryonic and adult mouse. Cell Mol Biol (Noisy-le-grand) *45*, 677-686.

Hessler, N.A., Shirke, A.M., and Malinow, R. (1993). The probability of transmitter release at a mammalian central synapse. Nature *366*, 569-572.

Hill, M.N., Kambo, J.S., Sun, J.C., Gorzalka, B.B., and Galea, L.A. (2006). Endocannabinoids modulate stress-induced suppression of hippocampal cell proliferation and activation of defensive behaviours. Eur J Neurosci *24*, 1845-1849.

Hill, M.N., Karatsoreos, I.N., Hillard, C.J., and McEwen, B.S. (2010a). Rapid elevations in limbic endocannabinoid content by glucocorticoid hormones in vivo. Psychoneuroendocrinology *35*, 1333-1338.

Hill, M.N., Kumar, S.A., Filipski, S.B., Iverson, M., Stuhr, K.L., Keith, J.M., Cravatt, B.F., Hillard, C.J., Chattarji, S., and McEwen, B.S. (2012). Disruption of fatty acid amide hydrolase activity prevents the effects of chronic stress on anxiety and amygdalar microstructure. Mol Psychiatry *18*, 1125-1135.

Hill, M.N., and McEwen, B.S. (2009). Endocannabinoids: The silent partner of glucocorticoids in the synapse. Proc Natl Acad Sci U S A *106*, 4579-4580.

Hill, M.N., McLaughlin, R.J., Bingham, B., Shrestha, L., Lee, T.T., Gray, J.M., Hillard, C.J., Gorzalka, B.B., and Viau, V. (2010b). Endogenous cannabinoid signaling is essential for stress adaptation. Proc Natl Acad Sci U S A *107*, 9406-9411.

Hill, M.N., McLaughlin, R.J., Morrish, A.C., Viau, V., Floresco, S.B., Hillard, C.J., and Gorzalka, B.B. (2009a). Suppression of amygdalar endocannabinoid signaling by stress contributes to activation of the hypothalamic-pituitary-adrenal axis. Neuropsychopharmacology *34*, 2733-2745.

Hill, M.N., Miller, G.E., Carrier, E.J., Gorzalka, B.B., and Hillard, C.J. (2009b). Circulating endocannabinoids and N-acyl ethanolamines are differentially regulated in major depression and following exposure to social stress. Psychoneuroendocrinology *34*, 1257-1262.

Hill, M.N., Patel, S., Campolongo, P., Tasker, J.G., Wotjak, C.T., and Bains, J.S. (2010c). Functional interactions between stress and the endocannabinoid system: from synaptic signaling to behavioral output. J Neurosci *30*, 14980-14986.

Holbro, N., Grunditz, A., and Oertner, T.G. (2009). Differential distribution of endoplasmic reticulum controls metabotropic signaling and plasticity at hippocampal synapses. Proc Natl Acad Sci U S A *106*, 15055-15060.

Hotson, J.R., and Prince, D.A. (1980). A calcium-activated hyperpolarization follows repetitive firing in hippocampal neurons. J Neurophysiol *43*, 409-419.

Hou, L., and Klann, E. (2004). Activation of the phosphoinositide 3-kinase-Akt-mammalian target of rapamycin signaling pathway is required for metabotropic glutamate receptor-dependent long-term depression. J Neurosci *24*, 6352-6361.

Huang, C.C., and Hsu, K.S. (2006). Sustained activation of metabotropic glutamate receptor 5 and protein tyrosine phosphatases mediate the expression of (S)-3,5-dihydroxyphenylglycine-induced long-term depression in the hippocampal CA1 region. J Neurochem *96*, 179-194.

Huang, Z., Kawase-Koga, Y., Zhang, S., Visvader, J., Toth, M., Walsh, C.A., and Sun, T. (2009). Transcription factor Lmo4 defines the shape of functional areas in developing cortices and regulates sensorimotor control. Dev Biol *327*, 132-142.

Humeau, Y., Reisel, D., Johnson, A.W., Borchardt, T., Jensen, V., Gebhardt, C., Bosch, V., Gass, P., Bannerman, D.M., Good, M.A., *et al.* (2007). A pathway-specific function for different AMPA receptor subunits in amygdala long-term potentiation and fear conditioning. J Neurosci *27*, 10947-10956.

Iino, M. (1989). Calcium-induced calcium release mechanism in guinea pig taenia caeci. J Gen Physiol *94*, 363-383.

Inan, M., and Crair, M.C. (2007). Development of cortical maps: perspectives from the barrel cortex. Neuroscientist *13*, 49-61.

Ishikawa, T., Kaneko, M., Shin, H.S., and Takahashi, T. (2005). Presynaptic N-type and P/Q-type Ca2+ channels mediating synaptic transmission at the calyx of Held of mice. J Physiol *568*, 199-209.

Isokawa, M., and Alger, B.E. (2005). Retrograde endocannabinoid regulation of GABAergic inhibition in the rat dentate gyrus granule cell. J Physiol *567*, 1001-1010.

Isokawa, M., and Alger, B.E. (2006). Ryanodine receptor regulates endogenous cannabinoid mobilization in the hippocampus. J Neurophysiol *95*, 3001-3011.

Ito-Ishida, A., Kakegawa, W., and Yuzaki, M. (2006). ERK1/2 but not p38 MAP kinase is essential for the long-term depression in mouse cerebellar slices. Eur J Neurosci *24*, 1617-1622.

Ito, M., Sakurai, M., and Tongroach, P. (1982). Climbing fibre induced depression of both mossy fibre responsiveness and glutamate sensitivity of cerebellar Purkinje cells. J Physiol *324*, 113-134.

Jacob, T.C., Moss, S.J., and Jurd, R. (2008). GABA(A) receptor trafficking and its role in the dynamic modulation of neuronal inhibition. Nat Rev Neurosci *9*, 331-343.

Jacobs, B.L., and Azmitia, E.C. (1992). Structure and function of the brain serotonin system. Physiol Rev *72*, 165-229.

Jann, M.W. (1988). Buspirone: an update on a unique anxiolytic agent. Pharmacotherapy *8*, 100-116.

Jiang, J., Suppiramaniam, V., and Wooten, M.W. (2006). Posttranslational modifications and receptor-associated proteins in AMPA receptor trafficking and synaptic plasticity. Neurosignals *15*, 266-282.

Joels, M., and Baram, T.Z. (2009). The neuro-symphony of stress. Nat Rev Neurosci *10*, 459-466.

Joels, M., Fernandez, G., and Roozendaal, B. (2011). Stress and emotional memory: a matter of timing. Trends Cogn Sci *15*, 280-288.

Jorntell, H., and Hansel, C. (2006). Synaptic memories upside down: bidirectional plasticity at cerebellar parallel fiber-Purkinje cell synapses. Neuron *52*, 227-238.

Joseph, S., Kwan, A.H., Mackay, J.P., Cubeddu, L., and Matthews, J.M. (2013). Backbone and side-chain assignments of a tethered complex between LMO4 and DEAF-1. Biomol NMR Assign. [Epub ahead of print]

Joshi, K., Lee, S., Lee, B., Lee, J.W., and Lee, S.K. (2009). LMO4 controls the balance between excitatory and inhibitory spinal V2 interneurons. Neuron *61*, 839-851.

Joshi, P.S., Molyneaux, B.J., Feng, L., Xie, X., Macklis, J.D., and Gan, L. (2008). Bhlhb5 regulates the postmitotic acquisition of area identities in layers II-V of the developing neocortex. Neuron *60*, 258-272.

Jung, K.M., Mangieri, R., Stapleton, C., Kim, J., Fegley, D., Wallace, M., Mackie, K., and Piomelli, D. (2005). Stimulation of endocannabinoid formation in brain slice cultures through activation of group I metabotropic glutamate receptors. Mol Pharmacol *68*, 1196-1202.

Kadrmas, J.L., and Beckerle, M.C. (2004). The LIM domain: from the cytoskeleton to the nucleus. Nat Rev Mol Cell Biol *5*, 920-931.

Kandel, E.R. (2013). Principles of Neural Science (5th edition).New York : McGraw-Hill, Medical, 2013 1-1709.

Kano, M., Ohno-Shosaku, T., Hashimotodani, Y., Uchigashima, M., and Watanabe, M. (2009). Endocannabinoid-mediated control of synaptic transmission. Physiol Rev *89*, 309-380.

Karachaliou, N., Costa, C., Gimenez-Capitan, A., Molina-Vila, M.A., Bertran-Alamillo, J., Mayo, C., Massuti, B., Majem, M., Carcereny, E., Moran, T., *et al.* (2013). BRCA1, LMO4, and CtIP mRNA expression in erlotinib-treated non-small-cell lung cancer patients with EGFR mutations. J Thorac Oncol *8*, 295-300.

Karlsson, O., Thor, S., Norberg, T., Ohlsson, H., and Edlund, T. (1990). Insulin gene enhancer binding protein Isl-1 is a member of a novel class of proteins containing both a homeo- and a Cys-His domain. Nature *344*, 879-882.

Karst, H., Berger, S., Erdmann, G., Schutz, G., and Joels, M. (2010). Metaplasticity of amygdalar responses to the stress hormone corticosterone. Proc Natl Acad Sci U S A *107*, 14449-14454.

Kashani, A.H., Qiu, Z., Jurata, L., Lee, S.K., Pfaff, S., Goebbels, S., Nave, K.A., and Ghosh, A. (2006). Calcium activation of the LMO4 transcription complex and its role in the patterning of thalamocortical connections. J Neurosci *26*, 8398-8408.

Katona, I., and Freund, T.F. (2008). Endocannabinoid signaling as a synaptic circuit breaker in neurological disease. Nat Med *14*, 923-930.

Kemp, N., and Bashir, Z.I. (2001). Long-term depression: a cascade of induction and expression mechanisms. Prog Neurobiol *65*, 339-365.

Kenny, D.A., Jurata, L.W., Saga, Y., and Gill, G.N. (1998). Identification and characterization of LMO4, an LMO gene with a novel pattern of expression during embryogenesis. Proc Natl Acad Sci U S A *95*, 11257-11262.

Kessler, R.C., Chiu, W.T., Demler, O., Merikangas, K.R., and Walters, E.E. (2005). Prevalence, severity, and comorbidity of 12-month DSM-IV disorders in the National Comorbidity Survey Replication. Arch Gen Psychiatry *62*, 617-627.

Kim, J.J., and Diamond, D.M. (2002). The stressed hippocampus, synaptic plasticity and lost memories. Nat Rev Neurosci *3*, 453-462.

Kim, S.H., Markham, J.A., Weiler, I.J., and Greenough, W.T. (2008). Aberrant early-phase ERK inactivation impedes neuronal function in fragile X syndrome. Proc Natl Acad Sci U S A *105*, 4429-4434.

Kisilevsky, A.E., and Zamponi, G.W. (2008). Presynaptic calcium channels: structure, regulators, and blockers. Handb Exp Pharmacol, 45-75.

Klein, R. (2004). Eph/ephrin signaling in morphogenesis, neural development and plasticity. Curr Opin Cell Biol *16*, 580-589.

Klein, R. (2012). Eph/ephrin signalling during development. Development *139*, 4105-4109.
Krasne, F.B., Fanselow, M.S., and Zelikowsky, M. (2011). Design of a neurally plausible model of fear learning. Front Behav Neurosci *5*, 41.

Kreitzer, A.C., and Regehr, W.G. (2001). Retrograde inhibition of presynaptic calcium influx by endogenous cannabinoids at excitatory synapses onto Purkinje cells. Neuron *29*, 717-727.

Krishnan, N., Fu, C., Pappin, D.J., and Tonks, N.K. (2011). H2S-Induced sulfhydration of the phosphatase PTP1B and its role in the endoplasmic reticulum stress response. Sci Signal *4*, ra86.

Kuang, G., He, Q., Zhang, Y., Zhuang, R., Xiang, A., Jiang, Q., Luo, Y., and Yang, J. (2012). Modulation of Preactivation of PPAR-beta on Memory and Learning Dysfunction and Inflammatory Response in the Hippocampus in Rats Exposed to Global Cerebral Ischemia/Reperfusion. PPAR Res *2012*, 209794.

Kuba, K., Morita, K., and Nohmi, M. (1983). Origin of calcium ions involved in the generation of a slow afterhyperpolarization in bullfrog sympathetic neurones. Pflugers Arch *399*, 194-202.

Lacivita, E., Di Pilato, P., De Giorgio, P., Colabufo, N.A., Berardi, F., Perrone, R., and Leopoldo, M. (2012). The therapeutic potential of 5-HT1A receptors: a patent review. Expert Opin Ther Pat *22*, 887-902.

Landreth, G., Jiang, Q., Mandrekar, S., and Heneka, M. (2008). PPARgamma agonists as therapeutics for the treatment of Alzheimer's disease. Neurotherapeutics *5*, 481-489.

Lantz, K.A., Hart, S.G., Planey, S.L., Roitman, M.F., Ruiz-White, I.A., Wolfe, H.R., and McLane, M.P. (2010). Inhibition of PTP1B by trodusquemine (MSI-1436) causes fat-specific weight loss in diet-induced obese mice. Obesity (Silver Spring) *18*, 1516-1523.

Lasek, A.W., Gesch, J., Giorgetti, F., Kharazia, V., and Heberlein, U. (2011). Alk is a transcriptional target of LMO4 and ERalpha that promotes cocaine sensitization and reward. J Neurosci *31*, 14134-14141.

Lasek, A.W., Kapfhamer, D., Kharazia, V., Gesch, J., Giorgetti, F., and Heberlein, U. (2010). Lmo4 in the nucleus accumbens regulates cocaine sensitivity. Genes Brain Behav.

LeDoux, J.E. (2000). Emotion circuits in the brain. Annu Rev Neurosci *23*, 155-184.

Lee, S.K., Jurata, L.W., Nowak, R., Lettieri, K., Kenny, D.A., Pfaff, S.L., and Gill, G.N. (2005). The LIM domain-only protein LMO4 is required for neural tube closure. Mol Cell Neurosci *28*, 205-214.

Leuba, G., Vernay, A., Vu, D., Walzer, C., Belloir, B., Kraftsik, R., Bouras, C., and Savioz, A. (2004). Differential expression of LMO4 protein in Alzheimer's disease. Neuropathol Appl Neurobiol *30*, 57-69.

Lilien, J., and Balsamo, J. (2005). The regulation of cadherin-mediated adhesion by tyrosine phosphorylation/dephosphorylation of beta-catenin. Curr Opin Cell Biol *17*, 459-465.

Lim, B.K., Matsuda, N., and Poo, M.M. (2008). Ephrin-B reverse signaling promotes structural and functional synaptic maturation in vivo. Nat Neurosci *11*, 160-169.

Lisman, J. (1989). A mechanism for the Hebb and the anti-Hebb processes underlying learning and memory. Proc Natl Acad Sci U S A *86*, 9574-9578.

Liu, J., Wang, L., Harvey-White, J., Osei-Hyiaman, D., Razdan, R., Gong, Q., Chan, A.C., Zhou, Z., Huang, B.X., Kim, H.Y., *et al.* (2006). A biosynthetic pathway for anandamide. Proc Natl Acad Sci U S A *103*, 13345-13350.

Liu, L., Wong, T.P., Pozza, M.F., Lingenhoehl, K., Wang, Y., Sheng, M., Auberson, Y.P., and Wang, Y.T. (2004). Role of NMDA receptor subtypes in governing the direction of hippocampal synaptic plasticity. Science *304*, 1021-1024.

Liu, X., Betzenhauser, M.J., Reiken, S., Meli, A.C., Xie, W., Chen, B.X., Arancio, O., and Marks, A.R. (2012). Role of leaky neuronal ryanodine receptors in stress-induced cognitive dysfunction. Cell *150*, 1055-1067.

Llano, I., Gonzalez, J., Caputo, C., Lai, F.A., Blayney, L.M., Tan, Y.P., and Marty, A. (2000). Presynaptic calcium stores underlie large-amplitude miniature IPSCs and spontaneous calcium transients. Nat Neurosci *3*, 1256-1265.

Long, J.Z., Nomura, D.K., Vann, R.E., Walentiny, D.M., Booker, L., Jin, X., Burston, J.J., Sim-Selley, L.J., Lichtman, A.H., Wiley, J.L., *et al.* (2009). Dual blockade of FAAH and MAGL identifies behavioral processes regulated by endocannabinoid crosstalk in vivo. Proc Natl Acad Sci U S A *106*, 20270-20275.

Lu, X., Malumbres, R., Shields, B., Jiang, X., Sarosiek, K.A., Natkunam, Y., Tiganis, T., and Lossos, I.S. (2008). PTP1B is a negative regulator of interleukin 4-induced STAT6 signaling. Blood *112*, 4098-4108.

Lu, Y., Fukuyama, S., Yoshida, R., Kobayashi, T., Saeki, K., Shiraishi, H., Yoshimura, A., and Takaesu, G. (2006a). Loss of SOCS3 gene expression converts STAT3 function from anti-apoptotic to pro-apoptotic. J Biol Chem *281*, 36683-36690.

Lu, Z., Lam, K.S., Wang, N., Xu, X., Cortes, M., and Andersen, B. (2006b). LMO4 can interact with Smad proteins and modulate transforming growth factor-beta signaling in epithelial cells. Oncogene.

Lujan, R., Nusser, Z., Roberts, J.D., Shigemoto, R., and Somogyi, P. (1996). Perisynaptic location of metabotropic glutamate receptors mGluR1 and mGluR5 on dendrites and dendritic spines in the rat hippocampus. Eur J Neurosci *8*, 1488-1500.

Luscher, C., and Huber, K.M. (2010). Group 1 mGluR-dependent synaptic long-term depression: mechanisms and implications for circuitry and disease. Neuron *65*, 445-459.

Maccarrone, M., Rossi, S., Bari, M., De Chiara, V., Fezza, F., Musella, A., Gasperi, V., Prosperetti, C., Bernardi, G., Finazzi-Agro, A., *et al.* (2008). Anandamide inhibits metabolism and physiological actions of 2-arachidonoylglycerol in the striatum. Nat Neurosci *11*, 152-159.

Maccarrone, M., Rossi, S., Bari, M., De Chiara, V., Rapino, C., Musella, A., Bernardi, G., Bagni, C., and Centonze, D. (2010). Abnormal mGlu 5 receptor/endocannabinoid coupling in mice lacking FMRP and BC1 RNA. Neuropsychopharmacology *35*, 1500-1509.

Mackie, K. (2008). Cannabinoid receptors: where they are and what they do. J Neuroendocrinol *20 Suppl 1*, 10-14.

Madden, D.R. (2002). The structure and function of glutamate receptor ion channels. Nat Rev Neurosci *3*, 91-101.

Madison, D.V., and Nicoll, R.A. (1984). Control of the repetitive discharge of rat CA 1 pyramidal neurones in vitro. J Physiol *354*, 319-331.

Madison, D.V., and Nicoll, R.A. (1986). Cyclic adenosine 3',5'-monophosphate mediates beta-receptor actions of noradrenaline in rat hippocampal pyramidal cells. J Physiol *372*, 245-259.

Maejima, T., Hashimoto, K., Yoshida, T., Aiba, A., and Kano, M. (2001). Presynaptic inhibition caused by retrograde signal from metabotropic glutamate to cannabinoid receptors. Neuron *31*, 463-475.

Maguschak, K.A., and Ressler, K.J. (2008). Beta-catenin is required for memory consolidation. Nat Neurosci *11*, 1319-1326.

Mahadev, K., Zilbering, A., Zhu, L., and Goldstein, B.J. (2001). Insulin-stimulated hydrogen peroxide reversibly inhibits protein-tyrosine phosphatase 1b in vivo and enhances the early insulin action cascade. J Biol Chem *276*, 21938-21942.

Maiya, R., Kharazia, V., Lasek, A.W., and Heberlein, U. (2012). Lmo4 in the basolateral complex of the amygdala modulates fear learning. PLoS One *7*, e34559.

Makino, H., and Malinow, R. (2009). AMPA receptor incorporation into synapses during LTP: the role of lateral movement and exocytosis. Neuron *64*, 381-390.

Malenka, R.C. (2003). Synaptic plasticity and AMPA receptor trafficking. Ann N Y Acad Sci *1003*, 1-11.

Malenka, R.C., Lancaster, B., and Zucker, R.S. (1992). Temporal limits on the rise in postsynaptic calcium required for the induction of long-term potentiation. Neuron *9*, 121-128.

Malinow, R., and Malenka, R.C. (2002). AMPA receptor trafficking and synaptic plasticity. Annu Rev Neurosci *25*, 103-126.

Malinow, R., and Tsien, R.W. (1990). Presynaptic enhancement shown by whole-cell recordings of long-term potentiation in hippocampal slices. Nature *346*, 177-180.

Mameli, M., Balland, B., Lujan, R., and Luscher, C. (2007). Rapid synthesis and synaptic insertion of GluR2 for mGluR-LTD in the ventral tegmental area. Science *317*, 530-533.

Manabe, T., Wyllie, D.J., Perkel, D.J., and Nicoll, R.A. (1993). Modulation of synaptic transmission and long-term potentiation: effects on paired pulse facilitation and EPSC variance in the CA1 region of the hippocampus. J Neurophysiol *70*, 1451-1459.

Manetopoulos, C., Hansson, A., Karlsson, J., Jonsson, J.I., and Axelson, H. (2003). The LIM-only protein LMO4 modulates the transcriptional activity of HEN1. Biochem Biophys Res Commun *307*, 891-899.

Mannaioni, G., Marino, M.J., Valenti, O., Traynelis, S.F., and Conn, P.J. (2001). Metabotropic glutamate receptors 1 and 5 differentially regulate CA1 pyramidal cell function. J Neurosci *21*, 5925-5934.

Maren, S. (2001). Neurobiology of Pavlovian fear conditioning. Annu Rev Neurosci *24*, 897-931.

Maren, S. (2005). Synaptic mechanisms of associative memory in the amygdala. Neuron *47*, 783-786.

Maren, S., and Quirk, G.J. (2004). Neuronal signalling of fear memory. Nat Rev Neurosci *5*, 844-852.

Marie, H., Morishita, W., Yu, X., Calakos, N., and Malenka, R.C. (2005). Generation of silent synapses by acute in vivo expression of CaMKIV and CREB. Neuron *45*, 741-752.

Marrion, N.V., and Tavalin, S.J. (1998). Selective activation of Ca2+-activated K+ channels by co-localized Ca2+ channels in hippocampal neurons. Nature *395*, 900-905.

Marsicano, G., Wotjak, C.T., Azad, S.C., Bisogno, T., Rammes, G., Cascio, M.G., Hermann, H., Tang, J., Hofmann, C., Zieglgansberger, W., *et al.* (2002). The endogenous cannabinoid system controls extinction of aversive memories. Nature *418*, 530-534.

Martin, E.D., and Buno, W. (2003). Caffeine-mediated presynaptic long-term potentiation in hippocampal CA1 pyramidal neurons. J Neurophysiol *89*, 3029-3038.

Massey, P.V., Johnson, B.E., Moult, P.R., Auberson, Y.P., Brown, M.W., Molnar, E., Collingridge, G.L., and Bashir, Z.I. (2004). Differential roles of NR2A and NR2B-containing NMDA receptors in cortical long-term potentiation and long-term depression. J Neurosci *24*, 7821-7828.

Matthews, J.M., Lester, K., Joseph, S., and Curtis, D.J. (2013). LIM-domain-only proteins in cancer. Nat Rev Cancer *13*, 111-122.

Mazzola, C., Medalie, J., Scherma, M., Panlilio, L.V., Solinas, M., Tanda, G., Drago, F., Cadet, J.L., Goldberg, S.R., and Yasar, S. (2009). Fatty acid amide hydrolase (FAAH) inhibition enhances memory acquisition through activation of PPAR-alpha nuclear receptors. Learn Mem *16*, 332-337.

McDonald, A.J. (1982). Cytoarchitecture of the central amygdaloid nucleus of the rat. J Comp Neurol *208*, 401-418.

McGaugh, J.L. (2004). The amygdala modulates the consolidation of memories of emotionally arousing experiences. Annu Rev Neurosci *27*, 1-28.

McIntyre, C.K., Hatfield, T., and McGaugh, J.L. (2002). Amygdala norepinephrine levels after training predict inhibitory avoidance retention performance in rats. Eur J Neurosci *16*, 1223-1226.

Mechoulam, R., and Parker, L.A. (2013). The endocannabinoid system and the brain. Annu Rev Psychol *64*, 21-47.

Melis, M., Pistis, M., Perra, S., Muntoni, A.L., Pillolla, G., and Gessa, G.L. (2004). Endocannabinoids mediate presynaptic inhibition of glutamatergic transmission in rat ventral tegmental area dopamine neurons through activation of CB1 receptors. J Neurosci *24*, 53-62.

Minichiello, L. (2009). TrkB signalling pathways in LTP and learning. Nat Rev Neurosci *10*, 850-860.

Montanez-Wiscovich, M.E., Seachrist, D.D., Landis, M.D., Visvader, J., Andersen, B., and Keri, R.A. (2009). LMO4 is an essential mediator of ErbB2/HER2/Neu-induced breast cancer cell cycle progression. Oncogene *28*, 3608-3618.

Mori, F., Fukaya, M., Abe, H., Wakabayashi, K., and Watanabe, M. (2000). Developmental changes in expression of the three ryanodine receptor mRNAs in the mouse brain. Neurosci Lett *285*, 57-60.

Moser, E.I. (2011). The multi-laned hippocampus. Nat Neurosci *14*, 407-408.

Moult, P.R., Gladding, C.M., Sanderson, T.M., Fitzjohn, S.M., Bashir, Z.I., Molnar, E., and Collingridge, G.L. (2006). Tyrosine phosphatases regulate AMPA receptor trafficking during metabotropic glutamate receptor-mediated long-term depression. J Neurosci *26*, 2544-2554.

Moult, P.R., Schnabel, R., Kilpatrick, I.C., Bashir, Z.I., and Collingridge, G.L. (2002). Tyrosine dephosphorylation underlies DHPG-induced LTD. Neuropharmacology *43*, 175-180.

Mousses, S., Bubendorf, L., Wagner, U., Hostetter, G., Kononen, J., Cornelison, R., Goldberger, N., Elkahloun, A.G., Willi, N., Koivisto, P., *et al.* (2002). Clinical validation of candidate genes associated with prostate cancer progression in the CWR22 model system using tissue microarrays. Cancer Res *62*, 1256-1260.

Murakoshi, H., Wang, H., and Yasuda, R. (2011). Local, persistent activation of Rho GTPases during plasticity of single dendritic spines. Nature *472*, 100-104.

Murphy, N.C., Scarlett, C.J., Kench, J.G., Sum, E.Y., Segara, D., Colvin, E.K., Susanto, J., Cosman, P.H., Lee, C.S., Musgrove, E.A., *et al.* (2008). Expression of LMO4 and outcome in pancreatic ductal adenocarcinoma. Br J Cancer *98*, 537-541.

Murray, E.A. (2007). The amygdala, reward and emotion. Trends Cogn Sci *11*, 489-497.

Neu, A., Foldy, C., and Soltesz, I. (2007). Postsynaptic origin of CB1-dependent tonic inhibition of GABA release at cholecystokinin-positive basket cell to pyramidal cell synapses in the CA1 region of the rat hippocampus. J Physiol *578*, 233-247.

Nguyen, L.K., Matallanas, D., Croucher, D.R., von Kriegsheim, A., and Kholodenko, B.N. (2013). Signalling by protein phosphatases and drug development: a systems-centred view. FEBS J *280*,751-65.

Niswender, C.M., and Conn, P.J. (2010). Metabotropic glutamate receptors: physiology, pharmacology, and disease. Annu Rev Pharmacol Toxicol *50*, 295-322.

Nosyreva, E.D., and Huber, K.M. (2005). Developmental switch in synaptic mechanisms of hippocampal metabotropic glutamate receptor-dependent long-term depression. J Neurosci *25*, 2992-3001.

Novotny-Diermayr, V., Lin, B., Gu, L., and Cao, X. (2005). Modulation of the interleukin-6 receptor subunit glycoprotein 130 complex and its signaling by LMO4 interaction. J Biol Chem *280*, 12747-12757.

Nunez, E., Benito, C., Pazos, M.R., Barbachano, A., Fajardo, O., Gonzalez, S., Tolon, R.M., and Romero, J. (2004). Cannabinoid CB2 receptors are expressed by perivascular microglial cells in the human brain: an immunohistochemical study. Synapse *53*, 208-213.

O'Sullivan, S.E. (2007). Cannabinoids go nuclear: evidence for activation of peroxisome proliferator-activated receptors. Br J Pharmacol *152*, 576-582.

O'Sullivan, S.E., and Kendall, D.A. (2010). Cannabinoid activation of peroxisome proliferator-activated receptors: potential for modulation of inflammatory disease. Immunobiology *215*, 611-616.

O'Sullivan, S.E., Kendall, P.J., and Kendall, D.A. (2012). Endocannabinoids and the cardiovascular response to stress. J Psychopharmacol *26*,71-82.

Ochoa, S.D., Salvador, S., and LaBonne, C. (2012). The LIM adaptor protein LMO4 is an essential regulator of neural crest development. Dev Biol *361*, 313-325.

Oddo, S., Caccamo, A., Shepherd, J.D., Murphy, M.P., Golde, T.E., Kayed, R., Metherate, R., Mattson, M.P., Akbari, Y., and LaFerla, F.M. (2003). Triple-transgenic model of Alzheimer's disease with plaques and tangles: intracellular Abeta and synaptic dysfunction. Neuron *39*, 409-421.

Offord, D.R., Boyle, M.H., Campbell, D., Goering, P., Lin, E., Wong, M., and Racine, Y.A. (1996). One-year prevalence of psychiatric disorder in Ontarians 15 to 64 years of age. Can J Psychiatry *41*, 559-563.

Ohno-Shosaku, T., Hashimotodani, Y., Ano, M., Takeda, S., Tsubokawa, H., and Kano, M. (2007). Endocannabinoid signalling triggered by NMDA receptor-mediated calcium entry into rat hippocampal neurons. J Physiol *584*, 407-418.

Ohno-Shosaku, T., Maejima, T., and Kano, M. (2001). Endogenous cannabinoids mediate retrograde signals from depolarized postsynaptic neurons to presynaptic terminals. Neuron *29*, 729-738.

Ohno-Shosaku, T., Shosaku, J., Tsubokawa, H., and Kano, M. (2002). Cooperative endocannabinoid production by neuronal depolarization and group I metabotropic glutamate receptor activation. Eur J Neurosci *15*, 953-961.

Okamoto, Y., Morishita, J., Tsuboi, K., Tonai, T., and Ueda, N. (2004). Molecular characterization of a phospholipase D generating anandamide and its congeners. J Biol Chem *279*, 5298-5305.

Orlando, L.R., Dunah, A.W., Standaert, D.G., and Young, A.B. (2002). Tyrosine phosphorylation of the metabotropic glutamate receptor mGluR5 in striatal neurons. Neuropharmacology *43*, 161-173.

Padua, R.A., Yamamoto, T., Fyda, D., Sawchuk, M.A., Geiger, J.D., and Nagy, J.I. (1992). Autoradiographic analysis of [3H]Ryanodine binding sites in rat brain: regional distribution and the effects of lesions on sites in the hippocampus. J Chem Neuroanat *5*, 63-73.

Pandey, N.R., Zhou, X., Qin, Z., Zaman, T., Gomez-Smith, M., Keyhanian, K., Anisman, H., Brunel, J.M., Stewart, A.F., and Chen, H.H. (2013). The LIM domain only 4 protein is a metabolic responsive inhibitor of protein tyrosine phosphatase 1B that controls hypothalamic leptin signaling. J Neurosci *33*:12647-55.

Paoletti, P., and Neyton, J. (2007). NMDA receptor subunits: function and pharmacology. Curr Opin Pharmacol *7*, 39-47.

Pasquale, E.B. (2010). Eph receptors and ephrins in cancer: bidirectional signalling and beyond. Nat Rev Cancer *10*, 165-180.

Passafaro, M., Piech, V., and Sheng, M. (2001). Subunit-specific temporal and spatial patterns of AMPA receptor exocytosis in hippocampal neurons. Nat Neurosci *4*, 917-926.

Peng, Y. (1996). Ryanodine-sensitive component of calcium transients evoked by nerve firing at presynaptic nerve terminals. J Neurosci *16*, 6703-6712.

Perea, G., Navarrete, M., and Araque, A. (2009). Tripartite synapses: astrocytes process and control synaptic information. Trends Neurosci *32*, 421-431.

Pertwee, R.G., Howlett, A.C., Abood, M.E., Alexander, S.P., Di Marzo, V., Elphick, M.R., Greasley, P.J., Hansen, H.S., Kunos, G., Mackie, K., *et al.* (2010). International Union of Basic and Clinical Pharmacology. LXXIX. Cannabinoid receptors and their ligands: beyond CB and CB. Pharmacol Rev *62*, 588-631.

Petralia, R.S., and Wenthold, R.J. (1992). Light and electron immunocytochemical localization of AMPA-selective glutamate receptors in the rat brain. J Comp Neurol *318*, 329-354.

Pfeffer, M., Muller, C.M., Mordel, J., Meissl, H., Ansari, N., Deller, T., Korf, H.W., and von Gall, C. (2009). The mammalian molecular clockwork controls rhythmic expression of its own input pathway components. J Neurosci *29*, 6114-6123.

Pineda, J.C., Galarraga, E., and Foehring, R.C. (1999). Different Ca2+ source for slow AHP in completely adapting and repetitive firing pyramidal neurons. Neuroreport *10*, 1951-1956.

Pineda, J.C., Waters, R.S., and Foehring, R.C. (1998). Specificity in the interaction of HVA Ca2+ channel types with Ca2+-dependent AHPs and firing behavior in neocortical pyramidal neurons. J Neurophysiol *79*, 2522-2534.

Pinheiro, P.S., and Mulle, C. (2008). Presynaptic glutamate receptors: physiological functions and mechanisms of action. Nat Rev Neurosci *9*, 423-436.

Pitler, T.A., and Alger, B.E. (1992). Postsynaptic spike firing reduces synaptic GABAA responses in hippocampal pyramidal cells. J Neurosci *12*, 4122-4132.

Polleux, F., Ince-Dunn, G., and Ghosh, A. (2007). Transcriptional regulation of vertebrate axon guidance and synapse formation. Nat Rev Neurosci *8*, 331-340.

Qin, Z., Zhou, X., Gomez-Smith, M., Pandey, N.R., Lee, K.F., Lagace, D.C., Beique, J.C., and Chen, H.H. (2012). LIM domain only 4 (LMO4) regulates calcium-induced calcium release and synaptic plasticity in the hippocampus. J Neurosci *32*, 4271-4283.

Racevskis, J., Dill, A., Sparano, J.A., and Ruan, H. (1999). Molecular cloning of LMO41, a new human LIM domain gene. Biochim Biophys Acta *1445*, 148-153.

Rae, M.G., Martin, D.J., Collingridge, G.L., and Irving, A.J. (2000). Role of Ca2+ stores in metabotropic L-glutamate receptor-mediated supralinear Ca2+ signaling in rat hippocampal neurons. J Neurosci *20*, 8628-8636.

Roche, K.W., O'Brien, R.J., Mammen, A.L., Bernhardt, J., and Huganir, R.L. (1996). Characterization of multiple phosphorylation sites on the AMPA receptor GluR1 subunit. Neuron *16*, 1179-1188.

Rodriguez de Fonseca, F., Del Arco, I., Bermudez-Silva, F.J., Bilbao, A., Cippitelli, A., and Navarro, M. (2005). The endocannabinoid system: physiology and pharmacology. Alcohol Alcohol *40*, 2-14.

Ronesi, J.A., Collins, K.A., Hays, S.A., Tsai, N.P., Guo, W., Birnbaum, S.G., Hu, J.H., Worley, P.F., Gibson, J.R., and Huber, K.M. (2012). Disrupted Homer scaffolds mediate abnormal mGluR5 function in a mouse model of fragile X syndrome. Nat Neurosci *15*, 431-440, S431.

Roovers, K., Klein, E.A., Castagnino, P., and Assoian, R.K. (2003). Nuclear translocation of LIM kinase mediates Rho-Rho kinase regulation of cyclin D1 expression. Dev Cell *5*, 273-284.

Rosenmund, C., Clements, J.D., and Westbrook, G.L. (1993). Nonuniform probability of glutamate release at a hippocampal synapse. Science *262*, 754-757.

Roura, S., Miravet, S., Piedra, J., Garcia de Herreros, A., and Dunach, M. (1999). Regulation of E-cadherin/Catenin association by tyrosine phosphorylation. J Biol Chem *274*, 36734-36740.

Rowan, M.J., Klyubin, I., Cullen, W.K., and Anwyl, R. (2003). Synaptic plasticity in animal models of early Alzheimer's disease. Philos Trans R Soc Lond B Biol Sci *358*, 821-828.

Sah, P. (1996). Ca(2+)-activated K+ currents in neurones: types, physiological roles and modulation. Trends Neurosci *19*, 150-154.

Sah, P., and McLachlan, E.M. (1991). Ca(2+)-activated K+ currents underlying the afterhyperpolarization in guinea pig vagal neurons: a role for Ca(2+)-activated Ca2+ release. Neuron *7*, 257-264.

Sanchez-Garcia, I., and Rabbitts, T.H. (1994). The LIM domain: a new structural motif found in zinc-finger-like proteins. Trends Genet *10*, 315-320.

Sapolsky, R.M. (2000). Glucocorticoids and hippocampal atrophy in neuropsychiatric disorders. Arch Gen Psychiatry *57*, 925-935.

Sapolsky, R.M. (2003). Stress and plasticity in the limbic system. Neurochem Res *28*, 1735-1742.

Sara, S.J. (2009). The locus coeruleus and noradrenergic modulation of cognition. Nat Rev Neurosci *10*, 211-223.

Schaffar, G., Taniguchi, J., Brodbeck, T., Meyer, A.H., Schmidt, M., Yamashita, T., and Mueller, B.K. (2008). LIM-only protein 4 interacts directly with the repulsive guidance molecule A receptor Neogenin. J Neurochem *107*, 418-431.

Schmeichel, K.L., and Beckerle, M.C. (1994). The LIM domain is a modular protein-binding interface. Cell *79*, 211-219.

Schock, S.C., Xu, J., Duquette, P.M., Qin, Z., Lewandowski, A.J., Rai, P.S., Thompson, C.S., Seifert, E.L., Harper, M.E., and Chen, H.H. (2008a). Rescue of neurons from ischemic injury by peroxisome proliferator-activated receptor-gamma requires a novel essential cofactor LMO4. J Neurosci *28*, 12433-12444.

Schock, S.C., Xu, J., Duquette, P.M., Qin, Z., Rai, P.S., Lewandowski, A.J., Thompson, C.S., Seifert, E.L., Harper, M.E., and Chen, H.H. (2008b). Rescue of neurons from ischemic injury by PPARgamma requires a novel essential cofactor LMO4. J Neurosci *28*, 12433-12444.

Schwartzkroin, P.A., and Stafstrom, C.E. (1980). Effects of EGTA on the calcium-activated afterhyperpolarization in hippocampal CA3 pyramidal cells. Science *210*, 1125-1126.

Scoville, W.B., and Milner, B. (1957). Loss of recent memory after bilateral hippocampal lesions. J Neurol Neurosurg Psychiatry *20*, 11-21.

Selye, H. (1950). Stress and the general adaptation syndrome. Br Med J *1*, 1383-1392.

Shankar, G.M., Li, S., Mehta, T.H., Garcia-Munoz, A., Shepardson, N.E., Smith, I., Brett, F.M., Farrell, M.A., Rowan, M.J., Lemere, C.A., *et al.* (2008). Amyloid-beta protein dimers isolated directly from Alzheimer's brains impair synaptic plasticity and memory. Nat Med *14*, 837-842.

Sharp, A.H., McPherson, P.S., Dawson, T.M., Aoki, C., Campbell, K.P., and Snyder, S.H. (1993). Differential immunohistochemical localization of inositol 1,4,5-trisphosphate- and ryanodine-sensitive Ca2+ release channels in rat brain. J Neurosci *13*, 3051-3063.

Shepherd, J.D., and Huganir, R.L. (2007). The cell biology of synaptic plasticity: AMPA receptor trafficking. Annu Rev Cell Dev Biol *23*, 613-643.

Singh, R.R., Barnes, C.J., Talukder, A.H., Fuqua, S.A., and Kumar, R. (2005). Negative regulation of estrogen receptor alpha transactivation functions by LIM domain only 4 protein. Cancer Res *65*, 10594-10601.

Sloniowski, S., and Ethell, I.M. (2012). Looking forward to EphB signaling in synapses. Semin Cell Dev Biol *23*, 75-82.

Stefanko, D.P., Barrett, R.M., Ly, A.R., Reolon, G.K., and Wood, M.A. (2009). Modulation of long-term memory for object recognition via HDAC inhibition. Proc Natl Acad Sci U S A *106*, 9447-9452.

Stockmeier, C.A., Shapiro, L.A., Dilley, G.E., Kolli, T.N., Friedman, L., and Rajkowska, G. (1998). Increase in serotonin-1A autoreceptors in the midbrain of suicide victims with major depression-postmortem evidence for decreased serotonin activity. J Neurosci *18*, 7394-7401.

Stokes, P.H., Liew, C.W., Kwan, A.H., Foo, P., Barker, H.E., Djamirze, A., O'Reilly, V., Visvader, J.E., Mackay, J.P., and Matthews, J.M. (2013). Structural basis of the interaction of the breast cancer oncogene LMO4 with the tumour suppressor CtIP/RBBP8. J Mol Biol *425*, 1101-1110.

Stoppini, L., Buchs, P.A., and Muller, D. (1991). A simple method for organotypic cultures of nervous tissue. J Neurosci Methods *37*, 173-182.

Stuible, M., Dube, N., and Tremblay, M.L. (2008). PTP1B regulates cortactin tyrosine phosphorylation by targeting Tyr446. J Biol Chem *283*, 15740-15746.

Stutzmann, G.E., Smith, I., Caccamo, A., Oddo, S., Laferla, F.M., and Parker, I. (2006). Enhanced ryanodine receptor recruitment contributes to Ca2+ disruptions in young, adult, and aged Alzheimer's disease mice. J Neurosci *26*, 5180-5189.

Sugihara, T.M., Bach, I., Kioussi, C., Rosenfeld, M.G., and Andersen, B. (1998). Mouse deformed epidermal autoregulatory factor 1 recruits a LIM domain factor, LMO-4, and CLIM coregulators. Proc Natl Acad Sci U S A *95*, 15418-15423.

Sum, E.Y., Peng, B., Yu, X., Chen, J., Byrne, J., Lindeman, G.J., and Visvader, J.E. (2002). The LIM domain protein LMO4 interacts with the cofactor CtIP and the tumor suppressor BRCA1 and inhibits BRCA1 activity. J Biol Chem *277*, 7849-7856.

Sum, E.Y., Segara, D., Duscio, B., Bath, M.L., Field, A.S., Sutherland, R.L., Lindeman, G.J., and Visvader, J.E. (2005). Overexpression of LMO4 induces mammary hyperplasia, promotes cell invasion, and is a predictor of poor outcome in breast cancer. Proc Natl Acad Sci U S A *102*, 7659-7664.

Szumlinski, K.K., Kalivas, P.W., and Worley, P.F. (2006). Homer proteins: implications for neuropsychiatric disorders. Curr Opin Neurobiol *16*, 251-257.

Tai, C.Y., Kim, S.A., and Schuman, E.M. (2008). Cadherins and synaptic plasticity. Curr Opin Cell Biol *20*, 567-575.

Takasu, M.A., Dalva, M.B., Zigmond, R.E., and Greenberg, M.E. (2002). Modulation of NMDA receptor-dependent calcium influx and gene expression through EphB receptors. Science *295*, 491-495.

Takeichi, M., and Abe, K. (2005). Synaptic contact dynamics controlled by cadherin and catenins. Trends Cell Biol *15*, 216-221.

Tancredi, V., D'Antuono, M., Cafe, C., Giovedi, S., Bue, M.C., D'Arcangelo, G., Onofri, F., and Benfenati, F. (2000). The inhibitory effects of interleukin-6 on synaptic plasticity in the rat hippocampus are associated with an inhibition of mitogen-activated protein kinase ERK. J Neurochem *75*, 634-643.

Taniwaki, M., Daigo, Y., Ishikawa, N., Takano, A., Tsunoda, T., Yasui, W., Inai, K., Kohno, N., and Nakamura, Y. (2006). Gene expression profiles of small-cell lung cancers: molecular signatures of lung cancer. Int J Oncol *29*, 567-575.

Tasker, J.G. (2006). Rapid glucocorticoid actions in the hypothalamus as a mechanism of homeostatic integration. Obesity (Silver Spring) *14 Suppl 5*, 259S-265S.

Tasker, J.G., and Herman, J.P. (2011). Mechanisms of rapid glucocorticoid feedback inhibition of the hypothalamic-pituitary-adrenal axis. Stress *14*, 398-406.

Thors, L., Bergh, A., Persson, E., Hammarsten, P., Stattin, P., Egevad, L., Granfors, T., and Fowler, C.J. (2010). Fatty acid amide hydrolase in prostate cancer: association with disease severity and outcome, CB1 receptor expression and regulation by IL-4. PLoS One *5*, e12275.

Tomita, S., Chen, L., Kawasaki, Y., Petralia, R.S., Wenthold, R.J., Nicoll, R.A., and Bredt, D.S. (2003). Functional studies and distribution define a family of transmembrane AMPA receptor regulatory proteins. J Cell Biol *161*, 805-816.

Torres, G.E., Arfken, C.L., and Andrade, R. (1996). 5-Hydroxytryptamine4 receptors reduce afterhyperpolarization in hippocampus by inhibiting calcium-induced calcium release. Mol Pharmacol *50*, 1316-1322.

Tozzi, A., Guatteo, E., Caputi, L., Bernardi, G., and Mercuri, N.B. (2001). Group I mGluRs coupled to G proteins are regulated by tyrosine kinase in dopamine neurons of the rat midbrain. J Neurophysiol *85*, 2490-2497.

Treiman, M., Caspersen, C., and Christensen, S.B. (1998). A tool coming of age: thapsigargin as an inhibitor of sarco-endoplasmic reticulum Ca(2+)-ATPases. Trends Pharmacol Sci *19*, 131-135.

Triller, A., and Choquet, D. (2005). Surface trafficking of receptors between synaptic and extrasynaptic membranes: and yet they do move! Trends Neurosci *28*, 133-139.

Tse, E., Smith, A.J., Hunt, S., Lavenir, I., Forster, A., Warren, A.J., Grutz, G., Foroni, L., Carlton, M.B., Colledge, W.H., *et al.* (2004). Null mutation of the Lmo4 gene or a combined null mutation of the Lmo1/Lmo3 genes causes perinatal lethality, and Lmo4 controls neural tube development in mice. Mol Cell Biol *24*, 2063-2073.

Tsetsenis, T., Younts, T.J., Chiu, C.Q., Kaeser, P.S., Castillo, P.E., and Sudhof, T.C. (2011). Rab3B protein is required for long-term depression of hippocampal inhibitory synapses and for normal reversal learning. Proc Natl Acad Sci U S A *108*, 14300-14305.

Tulving, E. (2006). Are there 256 kinds of memory. In: Name J (ed) The Foundation of Remembering: Essays in Honor of Henry L Roediger III, 39-52.

Tulving, E.a.C.F.I.M. (2000). The Oxford handbook of memory Oxford ; New York : Oxford University Press.

Twitchell, W., Brown, S., and Mackie, K. (1997). Cannabinoids inhibit N- and P/Q-type calcium channels in cultured rat hippocampal neurons. J Neurophysiol *78*, 43-50.

Tye, K.M., Prakash, R., Kim, S.Y., Fenno, L.E., Grosenick, L., Zarabi, H., Thompson, K.R., Gradinaru, V., Ramakrishnan, C., and Deisseroth, K. (2011). Amygdala circuitry mediating reversible and bidirectional control of anxiety. Nature *471*, 358-362.

Tzingounis, A.V., Heidenreich, M., Kharkovets, T., Spitzmaul, G., Jensen, H.S., Nicoll, R.A., and Jentsch, T.J. (2010). The KCNQ5 potassium channel mediates a component of the afterhyperpolarization current in mouse hippocampus. Proc Natl Acad Sci U S A *107*, 10232-10237.

Valenti, O., Conn, P.J., and Marino, M.J. (2002). Distinct physiological roles of the Gq-coupled metabotropic glutamate receptors Co-expressed in the same neuronal populations. J Cell Physiol *191*, 125-137.

van der Heide, L.P., Kamal, A., Artola, A., Gispen, W.H., and Ramakers, G.M. (2005). Insulin modulates hippocampal activity-dependent synaptic plasticity in a N-methyl-d-aspartate receptor and phosphatidyl-inositol-3-kinase-dependent manner. J Neurochem *94*, 1158-1166.

Van Sickle, M.D., Duncan, M., Kingsley, P.J., Mouihate, A., Urbani, P., Mackie, K., Stella, N., Makriyannis, A., Piomelli, D., Davison, J.S., *et al.* (2005). Identification and functional characterization of brainstem cannabinoid CB2 receptors. Science *310*, 329-332.

van Strien, N.M., Cappaert, N.L., and Witter, M.P. (2009). The anatomy of memory: an interactive overview of the parahippocampal-hippocampal network. Nat Rev Neurosci *10*, 272-282.

Varma, N., Carlson, G.C., Ledent, C., and Alger, B.E. (2001). Metabotropic glutamate receptors drive the endocannabinoid system in hippocampus. J Neurosci *21*, RC188.

Vecsey, C.G., Hawk, J.D., Lattal, K.M., Stein, J.M., Fabian, S.A., Attner, M.A., Cabrera, S.M., McDonough, C.B., Brindle, P.K., Abel, T., *et al.* (2007). Histone deacetylase inhibitors enhance memory and synaptic plasticity via CREB:CBP-dependent transcriptional activation. J Neurosci *27*, 6128-6140.

Visvader, J.E., Venter, D., Hahm, K., Santamaria, M., Sum, E.Y., O'Reilly, L., White, D., Williams, R., Armes, J., and Lindeman, G.J. (2001). The LIM domain gene LMO4 inhibits differentiation of mammary epithelial cells in vitro and is overexpressed in breast cancer. Proc Natl Acad Sci U S A *98*, 14452-14457.

Viveros, M.P., Marco, E.M., and File, S.E. (2005). Endocannabinoid system and stress and anxiety responses. Pharmacol Biochem Behav *81*, 331-342.

Vyas, A., Mitra, R., Shankaranarayana Rao, B.S., and Chattarji, S. (2002). Chronic stress induces contrasting patterns of dendritic remodeling in hippocampal and amygdaloid neurons. J Neurosci *22*, 6810-6818.

Wamsteeker, J.I., Kuzmiski, J.B., and Bains, J.S. (2010). Repeated stress impairs endocannabinoid signaling in the paraventricular nucleus of the hypothalamus. J Neurosci *30*, 11188-11196.

Wan, Q., Xiong, Z.G., Man, H.Y., Ackerley, C.A., Braunton, J., Lu, W.Y., Becker, L.E., MacDonald, J.F., and Wang, Y.T. (1997). Recruitment of functional GABA(A) receptors to postsynaptic domains by insulin. Nature *388*, 686-690.

Wang, N., Kudryavtseva, E., Ch'en, I.L., McCormick, J., Sugihara, T.M., Ruiz, R., and Andersen, B. (2004). Expression of an engrailed-LMO4 fusion protein in mammary epithelial cells inhibits mammary gland development in mice. Oncogene *23*, 1507-1513.

Wang, N., Lin, K.K., Lu, Z., Lam, K.S., Newton, R., Xu, X., Yu, Z., Gill, G.N., and Andersen, B. (2007). The LIM-only factor LMO4 regulates expression of the BMP7 gene through an HDAC2-dependent mechanism, and controls cell proliferation and apoptosis of mammary epithelial cells. Oncogene *26*, 6431-6441.

Wang, Z., Edwards, J.G., Riley, N., Provance, D.W., Jr., Karcher, R., Li, X.D., Davison, I.G., Ikebe, M., Mercer, J.A., Kauer, J.A., *et al.* (2008). Myosin Vb mobilizes recycling endosomes and AMPA receptors for postsynaptic plasticity. Cell *135*, 535-548.

Waung, M.W., and Huber, K.M. (2009). Protein translation in synaptic plasticity: mGluR-LTD, Fragile X. Curr Opin Neurobiol *19*, 319-326.

Waung, M.W., Pfeiffer, B.E., Nosyreva, E.D., Ronesi, J.A., and Huber, K.M. (2008). Rapid translation of Arc/Arg3.1 selectively mediates mGluR-dependent LTD through persistent increases in AMPAR endocytosis rate. Neuron *59*, 84-97.

Way, J.C., and Chalfie, M. (1988). mec-3, a homeobox-containing gene that specifies differentiation of the touch receptor neurons in C. elegans. Cell *54*, 5-16.

Weick, J.P., Groth, R.D., Isaksen, A.L., and Mermelstein, P.G. (2003). Interactions with PDZ proteins are required for L-type calcium channels to activate cAMP response element-binding protein-dependent gene expression. J Neurosci *23*, 3446-3456.

Wilson, R.I., and Nicoll, R.A. (2001). Endogenous cannabinoids mediate retrograde signalling at hippocampal synapses. Nature *410*, 588-592.

Xu, G., Arregui, C., Lilien, J., and Balsamo, J. (2002). PTP1B modulates the association of beta-catenin with N-cadherin through binding to an adjacent and partially overlapping target site. J Biol Chem *277*, 49989-49997.

Xu, G., Craig, A.W., Greer, P., Miller, M., Anastasiadis, P.Z., Lilien, J., and Balsamo, J. (2004). Continuous association of cadherin with beta-catenin requires the non-receptor tyrosine-kinase Fer. J Cell Sci *117*, 3207-3219.

Yang, N., and Mizuno, K. (1999). Nuclear export of LIM-kinase 1, mediated by two leucine-rich nuclear-export signals within the PDZ domain. Biochem J *338 (Pt 3)*, 793-798.

Yang, Y., Wang, X.B., Frerking, M., and Zhou, Q. (2008a). Delivery of AMPA receptors to perisynaptic sites precedes the full expression of long-term potentiation. Proc Natl Acad Sci U S A *105*, 11388-11393.

Yang, Y., Wang, X.B., Frerking, M., and Zhou, Q. (2008b). Spine expansion and stabilization associated with long-term potentiation. J Neurosci *28*, 5740-5751.

Yin, H.H., Davis, M.I., Ronesi, J.A., and Lovinger, D.M. (2006). The role of protein synthesis in striatal long-term depression. J Neurosci *26*, 11811-11820.

You, J.M., Yun, S.J., Nam, K.N., Kang, C., Won, R., and Lee, E.H. (2009). Mechanism of glucocorticoid-induced oxidative stress in rat hippocampal slice cultures. Canadian journal of physiology and pharmacology *87*, 440-447.

Yu, J., Ohuchida, K., Nakata, K., Mizumoto, K., Cui, L., Fujita, H., Yamaguchi, H., Egami, T., Kitada, H., and Tanaka, M. (2008). LIM only 4 is overexpressed in late stage pancreas cancer. Mol Cancer *7*, 93.

Yu, S., Yang, S., Holsboer, F., Sousa, N., and Almeida, O.F. (2011). Glucocorticoid regulation of astrocytic fate and function. PLoS One *6*, e22419.

Zabolotny, J.M., Bence-Hanulec, K.K., Stricker-Krongrad, A., Haj, F., Wang, Y., Minokoshi, Y., Kim, Y.B., Elmquist, J.K., Tartaglia, L.A., Kahn, B.B., *et al.* (2002). PTP1B regulates leptin signal transduction in vivo. Dev Cell *2*, 489-495.

Zalk, R., Lehnart, S.E., and Marks, A.R. (2007). Modulation of the ryanodine receptor and intracellular calcium. Annu Rev Biochem *76*, 367-385.

Zasloff, M., Williams, J.I., Chen, Q., Anderson, M., Maeder, T., Holroyd, K., Jones, S., Kinney, W., Cheshire, K., and McLane, M. (2001). A spermine-coupled cholesterol metabolite from the shark with potent appetite suppressant and antidiabetic properties. Int J Obes Relat Metab Disord *25*, 689-697.

Zhang, C., Wu, B., Beglopoulos, V., Wines-Samuelson, M., Zhang, D., Dragatsis, I., Sudhof, T.C., and Shen, J. (2009). Presenilins are essential for regulating neurotransmitter release. Nature *460*, 632-636.

Zhang, L., and Alger, B.E. (2010). Enhanced endocannabinoid signaling elevates neuronal excitability in fragile X syndrome. J Neurosci *30*, 5724-5729.

Zhang, Q., Kohler, M., Yang, S.N., Zhang, F., Larsson, O., and Berggren, P.O. (2004). Growth hormone promotes Ca(2+)-induced Ca2+ release in insulin-secreting cells by ryanodine receptor tyrosine phosphorylation. Mol Endocrinol *18*, 1658-1669.

Zheng, Q., and Zhao, Y. (2007). The diverse biofunctions of LIM domain proteins: determined by subcellular localization and protein-protein interaction. Biol Cell *99*, 489-502.

Zhou, X., Gomez-Smith, M., Qin, Z., Duquette, P.M., Cardenas-Blanco, A., Rai, P.S., Harper, M.E., Tsai, E.C., Anisman, H., and Chen, H.H. (2012). Ablation of LMO4 in glutamatergic neurons impairs leptin control of fat metabolism. Cell Mol Life Sci *69*, 819-828.

Zhou, Z., Hu, J., Passafaro, M., Xie, W., and Jia, Z. (2011). GluA2 (GluR2) regulates metabotropic glutamate receptor-dependent long-term depression through N-cadherin-dependent and cofilin-mediated actin reorganization. J Neurosci *31*, 819-833.

Zhu, P.J. (2006). Endocannabinoid signaling and synaptic plasticity in the brain. Crit Rev Neurobiol *18*, 113-124.

Zhu, P.J., and Lovinger, D.M. (2005). Retrograde endocannabinoid signaling in a postsynaptic neuron/synaptic bouton preparation from basolateral amygdala. J Neurosci *25*, 6199-6207.

Zinker, B.A., Rondinone, C.M., Trevillyan, J.M., Gum, R.J., Clampit, J.E., Waring, J.F., Xie, N., Wilcox, D., Jacobson, P., Frost, L., *et al.* (2002). PTP1B antisense oligonucleotide lowers PTP1B protein, normalizes blood glucose, and improves insulin sensitivity in diabetic mice. Proc Natl Acad Sci U S A *99*, 11357-11362.

Ziviani, E., Lippi, G., Bano, D., Munarriz, E., Guiducci, S., Zoli, M., Young, K.W., and Nicotera, P. (2011). Ryanodine receptor-2 upregulation and nicotine-mediated plasticity. Embo J *30*, 194-204.

Zucker, R.S. (1999). Calcium- and activity-dependent synaptic plasticity. Curr Opin Neurobiol *9*, 305-313.

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Education

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Academic Experience

Graduate Research Assistant (2009–2013)
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- Identifying the role of LMO4 on anxiety and learning/memory;
- Identifying protein tyrosine phosphatase 1B as a novel therapeutic target for the relief of anxiety.

Lab technician (2008-2009)
Ottawa Health Research Institute, Centre for Stoke Recovery

- Identifying a novel neural protective role of LMO4 in brain ischemia;
- Lab management and mouse colonies maintainance.

Graduate Research Assistant (2005-2007)
University of Ottawa, Canada

- Completed the project of CO2 chemosensitivity in branchial neuroepithelial cells of zebrafish, concerning identification of chemoreceptors and sensory pathways;
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Publications

Pandey, N.R., Zhou, X., **Qin, Z.**, Zaman, T., Gomez-Smith, M., Keyhanian, K., Anisman, H., Brunel, J.M., Stewart, A.F., and Chen, H.H. (2013). LMO4 is a metabolic responsive inhibitor of PTP1B that controls hypothalamic leptin signaling. J Neurosci *33*:12647-55.

Qin, Z., Zhou, X., Gomez-Smith, M., Pandey, N.R., Lee, K.F., Lagace, D.C., Beique, J.C., and Chen, H.H. (2012). LIM domain only 4 (LMO4) regulates calcium-induced calcium release and synaptic plasticity in the hippocampus. J Neurosci *32*, 4271-4283.

Zhou, X., Gomez-Smith, M., **Qin, Z.**, Duquette, P.M., Cardenas-Blanco, A., Rai, P.S., Harper, M.E., Tsai, E.C., Anisman, H., and Chen, H.H. (2012). Ablation of LMO4 in glutamatergic neurons impairs leptin control of fat metabolism. Cell Mol Life Sci *69*, 819-828.

Qin, Z., Lewis, J.E., Vulesevic, B., Duff, D.W., Whitfield, N.L., Yang, G., Wang, R., and Perry, S.F. (2010). Zebrafish (Danio rerio) gill neuroepithelial cells are sensitive chemoreceptors for environmental CO2. J Physiol *588 (5)*, 861-872.

Gomez-Smith, M., **Qin, Z.**, Zhou, X., Schock, S.C., and Chen, H.H. (2010). LIM domain only 4 protein promotes granulocyte colony-stimulating factor-induced signaling in neurons. Cell Mol Life Sci *67*, 949-957.

Schock, S.C., Xu, J., Duquette, P.M., **Qin, Z.**, Lewandowski, A.J., Rai, P.S., Thompson, C.S., Seifert, E.L., Harper, M.E., and Chen, H.H. (2008). Rescue of neurons from ischemic injury by peroxisome proliferator-activated receptor-gamma requires a novel essential cofactor LMO4. J Neurosci *28*, 12433-12444.

Olson, K.R., Healy, M.J., **Qin, Z.**, Skovgaard, N., Vulesevic, B., Duff, D.W., Whitfield, N.L., Yang, G., Wang, R., and Perry, S.F. (2008). Hydrogen sulfide as an oxygen sensor in trout gill chemoreceptors. Am J Physiol Regul Integr Comp Physiol 295, R669-680.

Scholarships and Awards

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Travel Award at the 8th World Congress of International Brain Research Organization (IBRO) in Florence, Italy (2011)

Ontario Graduate Scholarship and University of Ottawa Excellent Scholarship (2010)

Ontario Graduate Scholarship and University of Ottawa Excellent Scholarship (2007) declined

George Holeton Book Prize at the 46th Annual Meeting of Canadian Society of Zoologists (2007)

University of Ottawa Admission Scholarship in 2005 & 2009

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- **Figure 1.2.2 The main hippocampal circuitry (page 12)**

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Deng, W., Aimone, J.B., and Gage, F.H. (2010). New neurons and new memories: how does adult hippocampal neurogenesis affect learning and memory? Nat Rev Neurosci *11*, 339-350.

Figure 1. The neural circuitry in the rodent hippocampus

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- **Figure 1.4.1 A general model for endocannabinoid-based retrograde signaling (page 29)**

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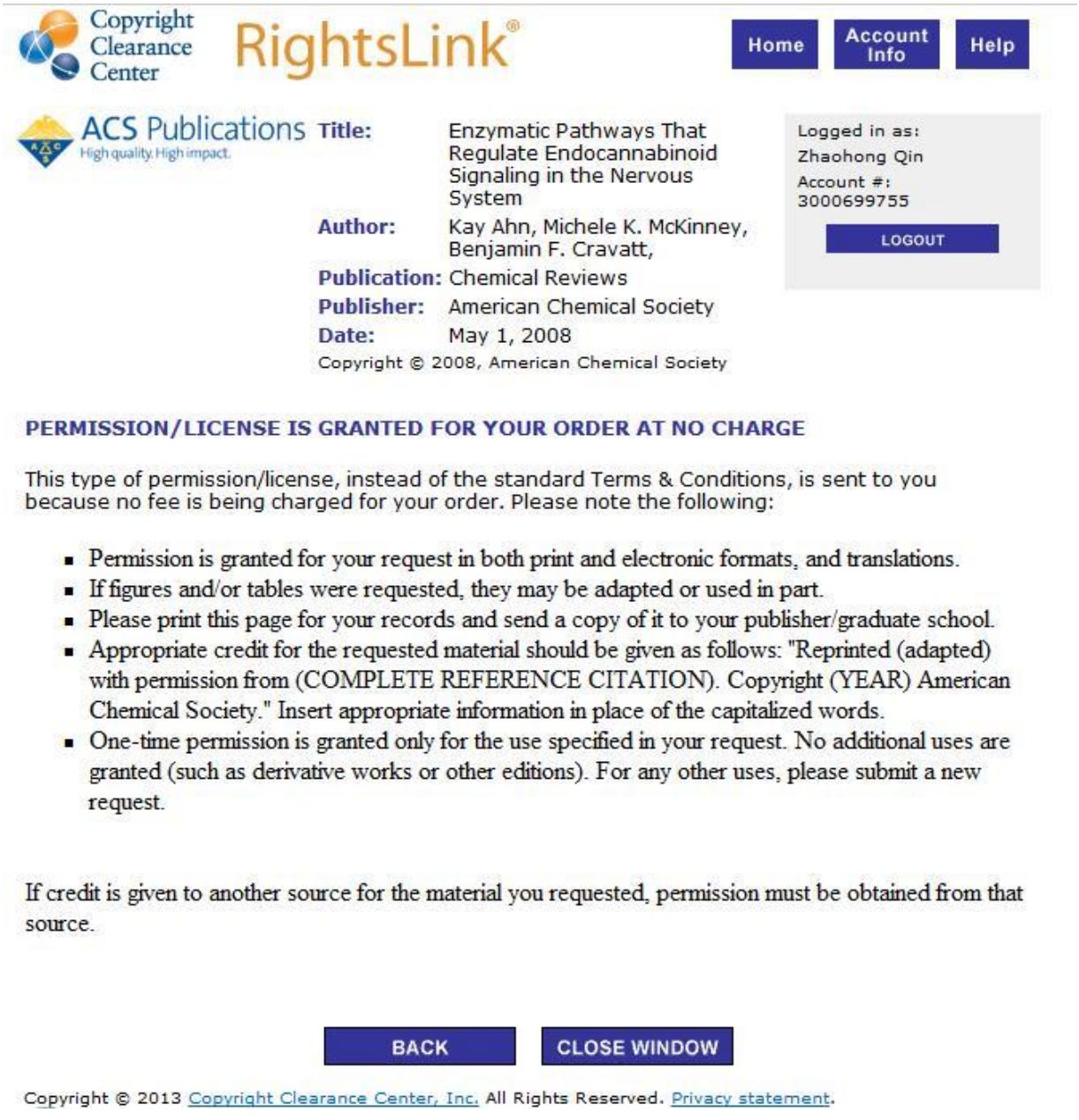
Ahn, K., McKinney, M.K., and Cravatt, B.F. (2008). Enzymatic pathways that regulate endocannabinoid signaling in the nervous system. Chem Rev *108*, 1687-1707.
Figure 2. General model for endocannabinoid-based retrograde signaling.



- **All figures in Chapter 2. Manuscript #1 (page 61, 63, 67, 71, 74, 77, 80, 84, 87)**

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LIM domain only 4 (LMO4) regulates calcium-induced calcium release and synaptic plasticity in the hippocampus. Journal of Neuroscience 2012; 32, 4271-4283.