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Molecular Mechanisms of Memory Formation and Persistence
in the Adult and Aged Brain

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“Science is the poetry of reality”

Professor Richard Dawkins

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List of Abbreviations and Acronyms

A3SS	Alternative 3′ splice sites
A5SS	Alternative 5′ splice sites
AC	Adenylyl cyclase
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AP-1	Activator protein 1
Arc	Activity regulated cytoskeleton
ATF	Activating transcription factor
Atl2	Atlastin GTPase 2
ATP	Adenosine triphosphate
BDNF	Brain derived neurotrophic factor
BER	Base excision repair
BLAST	Basic Local Alignment Search Tool
CaMK	Ca^{2+}/calmodulin-dependent protein kinase
CamKIIα	Calcium/calmodulin-dependent protein kinase type II subunit alpha
CaMKIV	Calcium/calmodulin-dependent protein kinase IV
cAMP	Activation cyclic adenosine monophosphate
cDNA:	Complementary deoxyribonucleic acid
ChIP-seq	Chromatin immunoprecipitation DNA-sequencing
CICM	CO_2-independent culture medium
Co-REST	REST Co-repressor
CRE	cAMP response element
cre/loxP	Cre recombinase/ locus of x-over, P1
CREB	cAMP response element-binding protein
DAS	Differential alternative splicing
DAVID	Database for annotation, visualization and integrated discovery
DEG	Differential gene expression
dHPC	Dorsal hippocampus
DIV	Day in vitro
DL-APV	DL-2-amino-5-phosphonopentanoic acid
Dnajb5	DnaJ Heat Shock Protein Family (Hsp40) Member B5
DNMT	DNA methyltransferase
Egr1	Early growth response protein 1
ERK	Extracellular signal-regulated kinase
ES	Exon skipping
FBJ	C-Finkel–Biskis–Jinkins
FDR	False discovery rates
FFluc	Firefly luciferase
Fhl1	Four And A Half LIM Domains 1
Fos	FBJ osteosarcoma oncogene
$GABA_A$	γ-aminobutyric acid A

Gabrg2	Gamma-aminobutyric acid (GABA) A receptor, subunit gamma 2
Gadd45	Growth Arrest and DNA Damage
GEO	Gene expression omnibus
Gls:	Glutaminase
GluA1	Glutamate ionotropic receptor AMPA type subunit 1
GluN1	NMDA type subunit 1
GO	Gene ontology
Gria3:	Glutamate ionotropic receptor AMPA type subunit 3
GTP	Guanosine triphosphate
HA	Human influenza hemagglutinin
HC	Home-cage
HEK	Human embryonic kidney
Homer1	*Homer* protein homolog 1
hrGFP	Humanized renilla reniformis green fluorescent protein
$_{i}[Ca^{2+}]$	Intracellular calcium
IEGs	Immediate early genes
ip	Intra-peritoneal
IR:	Intron retention
Irf7:	Interferon regulatory factor 7
JNK	c-Jun N-terminal kinases
JNK	c-Jun N-terminal kinases
KID	Kinase inducible domain
KO	Kusabira Orange
LRGs	Late-response genes
LTD	Long-term depression
LTM	Long-term memory
LTP	Long-term potentiation
Luc7l3:	LUC7 like 3 pre-MRNA splicing factor
Map4:	Microtubule-associated protein 4
MAPK	Mitogen-activated protein kinase
Marchf7:	Membrane associated ring-CH-type finger 7
MATS	Multivariate analysis of transcript splicing
MBD	Methyl-CpG-binding domain
ME:	Mutually exclusive exons
MeCP2	Methyl-CpG-binding protein 2
MEK	Mitogen-activated protein kinase kinase
mEPSC	Miniature excitatory postsynaptic current
mGluR	Metabotropic glutamate receptor
MSK	Mitogen and stress activated protein kinase
Narp	Neuronal activity-regulated pentraxin
NCBI	National Center for Biotechnology Information
NMDA	N-Methyl-D-aspartic acid
Npas4	Neuronal PAS domain protein 4

Nptx2	Pentraxin-2
Nr3c1:	Nuclear receptor subfamily 3, group C, member 1
Nr4a1	Nuclear receptor subfamily 4 group A member 1
Nrcam:	Neuron-glia related cell adhesion molecule
Osmr:	Oncostatin M receptor
P2rx6:	P2X purinoceptor 6
P90RSK	90 kDa ribosomal s6 kinases
PCR:	Polymerase chain reaction
PI3K	Phosphoinositide-3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PKG	Protein kinase g
q-RT-PCR	Quantitative reverse-transcription PCR
rAVV	Recombinant adeno-associated viruses
RE1	Transcriptional repressor element-1
REST	Silencing transcription factor
Rluc	Renilla luciferase
RNA-seq:	RNA sequencing
RNA:	Ribonucleic acid
RT-PCR:	Reverse transcription polymerase chain reaction
RTT:	Rett Syndrome
rtTA	Transactivator
SAM	S-adenosyl-methionine
shRNAs	Short-hairpin RNAs
Snrnp70:	U1 small nuclear ribonucleoprotein 70
SRF	Serum response factor
SST	Somatostatin
STM	Short-term memory
Synj1:	Synaptojanin 1
TET	Ten-eleven translocation
TetR	Tetracycline repressor
Tgfb2	Transforming growth factor-beta 2
TPA	2-O-Tetradecanoylphorbol-13-acetate
Tra2b:	Transformer-2 protein homolog beta
TRE	tetracycline responsive promoter
Trmt1:	TRNA (guanine(26)-N(2))-dimethyltransferase
U2af1l4:	U2 small nuclear RNA auxiliary factor 1-like 4
YB-1:	Y-box binding protein 1
Zmynd8:	Zinc finger, MYND-type containing 8
ΔPSI	Delta “percent spliced in”

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Abstract

Memory formation relies on an orchestrated activation of cellular and molecular mechanisms that drive acquisition and consolidation of memories into a stable form. Research of the past decades has begun to elucidate how activation at the synapse promotes communication to the nucleus, where learning-induced gene expression takes place. This genomic response, that also includes post-transcriptional alterations in the newly-synthetized transcripts, is required for long-term memory formation. In in the first sections of this work, we uncovered a role for the Growth Arrest and DNA Damage γ (Gadd45γ) in memory formation and age-related cognitive decline. We showed that Gadd45γ mediates synapse-to-nucleus communication required for induction of activity-dependent gene expression and memory formation. We further identified that the expression of this protein is tightly regulated in hippocampal neurons. Decreasing or increasing Gadd45γ expression compromised activity-dependent gene expression and memory formation. We further discovered that during murine and human aging the hippocampal expression of this protein is dysregulated, suggesting that it may be involved in age-related cognitive decline. In the next section, we identified the alternative splicing program associated with spatial memory formation. We showed that the DNA methylation reader methyl-CpG-binding protein 2 (MeCP2) is required for the accurate induction of alternative splicing in baseline conditions and after learning. These findings place MeCP2 as a key regulator of adult brain function, particularly cognitive abilities. Lastly, we investigated molecular mechanisms underlying memory persistence. We showed that neuronal PAS domain protein 4 (Npas4) expression is induced several hours after persistent memory learning, but not when mice form short-lasting memories. This delayed expression is dependent on N-Methyl-D-aspartic acid (NMDA) receptor activation several hours after learning. Interestingly, artificially inducing Npas4 expression in a short-lasting memory protocol, further accelerated memory decay. These findings suggest that late Npas4 expression might be a mechanism associated with forgetting. Altogether, in this work we discovered new molecular mechanisms of memory formation and persistence in the adult and aged brain.

KEYWORDS:
Aging, alternative splicing, Gadd45, learning, MeCP2, memory, Npas4.

Zusammenfassung

Die Gedächtnisbildung beruht auf einer orchestrierten Aktivierung von zellulären und molekularen Mechanismen, die den Erwerb und die Konsolidierung von Erinnerungen in eine stabile Form treiben. Die Forschung der letzten Jahrzehnte begann aufzuklären, wie die Aktivierung der Synapse die Kommunikation zum Zellkern antreibt, wo die lerninduzierte Genexpression stattfindet. Diese genomische Antwort, die auch posttranskriptionelle Veränderungen in den neu synthetisierten Transkripten beinhaltet, ist für die Bildung des Langzeitgedächtnisses erforderlich. Im ersten Abschnitt dieser Arbeit haben wir die Rolle für das Growth Arrest and DNA Damage γ (Gadd45γ) bei der Gedächtnisbildung und dem altersbedingten kognitiven Abbau aufgedeckt. Wir zeigen, dass Gadd45γ die Kommunikation zwischen der Synapse und dem Zellkern- vermittelt, die für die Induktion der aktivitätsabhängigen Genexpression und die Gedächtnisbildung erforderlich ist. Weiterhin konnten wir zeigen, dass die Expression dieses Proteins in hippocampalen Neuronen eng reguliert wird und die Verringerung oder Erhöhung der Gadd45γ-Expression beeinträchtigte die aktivitätsabhängige Genexpression und die Gedächtnisbildung. Wir entdeckten außerdem, dass die Expression dieses Proteins im Hippocampus von Mäusen und Menschen während der Alterung dysreguliert ist, was darauf hindeutet, dass Gadd45y am altersbedingten kognitiven Verfall beteiligt sein könnte. Im nächsten Teil identifizierten wir das alternative Spleißprogramm, das mit der Bildung des räumlichen Gedächtnisses verbunden ist. Wir zeigten, dass der DNA-Methylierungsleser Methyl-CpG-bindendes Protein 2 (MeCP2) für die präzise Induktion des alternativen Spleißens im Grundzustand und nach dem Lernen erforderlich ist. Diese Ergebnisse weisen MeCP2 eine Schlüsselrolle bei der Regulierung der Gehirnfunktion im Erwachsenenalter zu, insbesondere bei kognitiven Fähigkeiten. Schließlich untersuchten wir die molekularen Mechanismen, die der Gedächtnispersistenz zugrunde liegen. Wir konnten zeigen, dass die Expression des neuronalen PAS-Domänenproteins 4 (Npas4) mehrere Stunden nach dem Erlernen persistierender Erinnerungen induziert wird, jedoch nicht, wenn Mäuse kurzzeitige Erinnerungen bilden. Diese verzögerte Expression ist abhängig von der Aktivierung des N-Methyl-D-Asparaginsäure (NMDA)-Rezeptors einige Stunden nach dem Lernen. Interessanterweise beschleunigte die künstliche Induktion von Npas4 in dem Protokoll, das zu kürzer-anhaltenden Erinnerungen führt, den Gedächtnisverfall weiter. Diese Befunde deuten darauf hin, dass die späte Npas4-Expression ein Mechanismus sein könnte, der mit dem „Vergessen“

verbunden ist. Insgesamt haben wir in dieser Arbeit neue molekulare Mechanismen der Gedächtnisbildung und -persistenz im erwachsenen und gealterten Gehirn entdeckt.

SCHLÜSSELWÖRTER:

Alternatives Spleißen, Altern, Gadd45, Gedächtnis, Lernen, MeCP2, Npas4.

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Chapter 1. Introduction

1.1. Classes of memory

The study of learning and memory falls into the domains of psychology and neurobiology. In the early nineteenth century, psychological research pioneered memory categorization (James, 1890) before neurobiologists gained access to tools to study the molecular mechanisms underlying different memory types. It was only in 1923, however that McDougall (McDougall, 1923) provided the distinction between explicit and implicit memory, a definition that is still accepted today. Non-declarative or implicit memory is dispositional and recalled through daily performance rather than conscious recollection. In opposition, explicit or declarative memory is knowledge-based and available as a conscious recollection, usually associated to facts or events. Non-declarative memory, although inaccessible by conscious recall, is essential for daily performance and is shaped by past events. Examples include skills and habits, emotional or motor responses and reflex pathways. Declarative memory depends on several brain structures particularly hippocampus and the adjacent entorhinal, perirhinal, and parahippocampal cortices (Squire and Zola-Morgan, 1991). These structures work together to form different components of declarative memory for example the encoding of objects (perirhinal cortex), spatial information (parahippocampal cortex) and importantly the integration between all of these components (hippocampus) (Staresina et al., 2011).

Declarative memory can also be classified according to its stability into working memory, short-term memory (STM), long-term memory (LTM) and remote memory. There is however a distinction between the fields of cognitive neuroscience and cellular and molecular neuroscience. In cognitive neuroscience the term working memory has largely been fully replaced by STM to refer to the capacity for holding a small amount of information in mind for a short time, such as to remember a phone number that has just been listed. In cellular and molecular neuroscience, working memory is used to refer to a memory that is recalled in the scale of milliseconds to minutes, while STM is able to persist up to hours in rodents (Kandel, 2012; Kandel

et al., 2014). From hereinafter STM will be used as defined in cellular and molecular neuroscience. Memories that are consolidated and persist for days to years are classified as LTM. Remote memory is a subtype of LTM that refers to memory that lasts weeks to a lifetime and depend on additional consolidation mechanisms (please refer to section 1.3. “Molecular mechanisms underlying memory persistence”).

In the next section I will introduce the main cellular and molecular mechanisms that distinguish the formation and consolidation of STM and LTM.

1.2. Molecular mechanisms underlying memory formation

Declarative memories depend on specific molecular processes in the hippocampus and its connections to establish LTM. During a learning event information is first acquired and transformed in a labile memory that is prone to disruption and depends on mechanisms that allow its stabilization. This consolidation process takes several hours to occur (Izquierdo and Medina, 1997), which led to an hypothesis pioneered by William James in 1890 (James, 1890). This idea stated that while LTM formation is taking place and is being stabilized one or several STMs are holding the memory in place. This hypothesis was later supported and further developed by others (Mansuy et al., 1998; Greve et al., 2010). If such would be the case it would mean that STMs would be an intermediate process that could lead to LTM formation and not separate mechanisms of memory storage. In 1993 this question started to be addressed by Thomas Carew that showed that long-term facilitation could be normally expressed in the absence of short-term facilitation in Aplysia (Emptage and Carew, 1993). These findings suggested that STM might be a separate entity, molecularly distinguishable from LTM. Nevertheless, it was only in 1998 that this question was answered by Iván Izquierdo and Jorge Medina (Izquierdo et al., 1998). In their study they infused distinct pharmacological inhibitors into the CA1 region of the hippocampus or entorhinal cortex of rats that underwent inhibitory avoidance training, a form of aversive learning. The animals were tested for retained STM (1.5h) and retested for LTM (24h). They concluded that hippocampal serotonin 1A receptor activity and entorhinal α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

(AMPA), γ-aminobutyric acid A ($GABA_A$) activity is required for STM but not LTM. These findings elegantly showed that LTM is able to be formed in the absence of STM and therefore are, at least in part, parallel and distinct events (Izquierdo et al., 1999; Abel and Lattal, 2001; Izquierdo et al., 2002). Since this initial study more detailed molecular pathways have been described to be selectively required for STM or LTM, independently (Izquierdo et al., 2002).

The key feature that distinguishes LTM is its dependence on *de novo* protein synthesis that results on enduring plasticity changes in neurons, a process not required for STM. Fexner and collogues where the first to identify that pharmacological inhibition of translation induces memory impairments in mice (Flexner et al., 1962). Since this initial observation it is well accepted that LTMs mostly require *de novo* protein synthesis, despite extensive debate on off-target effects of the protein synthesis inhibitors used in initial studies (Hernandez and Abel, 2008). This, however does not seem to be an absolute requirement for all types of LTMs (Zhao et al., 2019).

In the next sections I will focus on known molecular mechanisms that orchestrate *de novo* gene expression and subsequential de novo protein synthesis required for memory processes.

1.2.1 Synapse-to-nucleus signaling

1.2.1.1. Signal transduction

It is generally accepted that synapses communicate with the nucleus via signaling pathways that induce gene expression required for long-term structural and functional changes (Cohen and Greenberg, 2008). Sensory experience such as learning promotes neurotransmitter release at the synaptic cleft. This in turn activates synaptic receptors inducing membrane depolarization in the postsynaptic terminal. Calcium influx serves as a second messenger system in neurons by activating calcium-dependent kinases, which mediate posttranslational modifications and the induction of mRNA transcription and translation (Flavell and Greenberg, 2008). Particularly four main signaling pathways are thought to mediate

calcium-dependent signaling to the nucleus, the cAMP, Ca^{2+}/calmodulin-dependent protein kinase (CaMK), Ras and p38 signaling pathways (Figure 1.1).

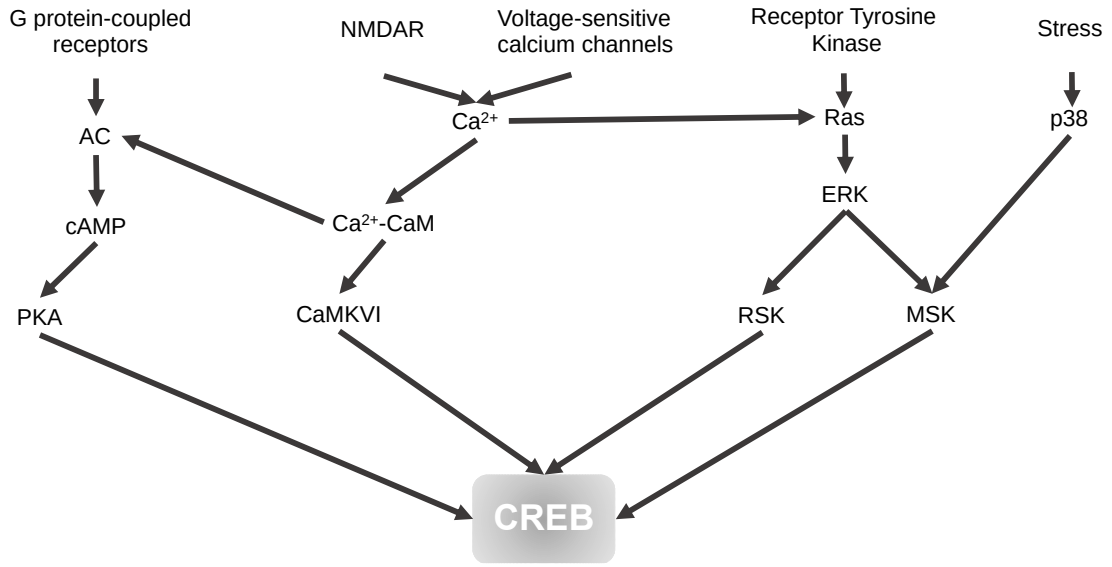


Figure 1.1. Molecular pathways converging in activity-dependent transcription

In the presence of elevated intracellular calcium ($_i[Ca^{2+}]$), adenylyl cyclase (AC) converts ATP into cyclic AMP by activation of calmodulin (CaM). cAMP is then able to bind to regulatory subunits present in the protein kinase A (PKA) which causes it to undergo a conformational change. Upon this change, the active catalytic subunits of PKA are released from the previous active auto-inhibitory domain. This active form of PKA is then able to translocate into the nucleus, where it phosphorylates and activates transcription factors required for activity-dependent gene expression.

In a similar mechanism, intracellular calcium binds to CaM in four calcium binding sites forming Ca^{2+}-CaM complex. This complex is able to change the conformation of several target proteins, such as the AC, but also the (CaMKIV). The binding of Ca^{2+}-CaM to CaMKIV activates its kinase activity. Activated CaMKIV phosphorylates downstream transcription factors in the nucleus, thus inducing transcription activation. This signaling cascade is required for induction of long-term potentiation (LTP) and long-term depression (LTD) (Mulkey et al., 1994).

The increase in $_i[Ca^{2+}]$ also promotes activation of guanosine triphosphate (GTP)ases Ras such as Raf, a mitogen-activated protein kinase (MAPK) kinase kinase. These proteins phosphorylate mitogen-activated protein kinase kinase

(MEK), that in turn phosphorylate MAPK such as ERK1/2. Phosphorylated ERK1/2 targets several proteins such as 90 kDa ribosomal s6 kinases (P90RSK) and mitogen and stress activated protein kinase (MSK) which phosphorylate transcription factors required for gene expression.

Lastly a less studied pathway that under neuronal stress conditions activates synapse-to-nucleus communication is the p38 signaling cascade. Upon neuronal stress, activated forms of p38 are able to directly phosphorylate MSK1/2, a downstream target also targeted by ERK1/2. Therefore, the p38 pathway shows some level of redundancy to signaling that leads to ERK1/2 activation, such as Ras (Tan et al., 1996; Deak et al., 1998).

It is now believed that STM depends on post-translational events regulated by signaling cascades in the cytoplasm of neurons. These events are triggered by initial activation of glutamate AMPA, N-Methyl-D-aspartic acid (NMDA), and metabotropic receptors in the hippocampus. These lead to intracellular influx of sodium and calcium and activation cyclic adenosine monophosphate (cAMP) signaling that activates downstream PKA, phosphoinositide-3-kinase (PI3K), Tyr-kinases, guanylyl cyclase, protein kinase g (PKG), protein kinase C (PKC)α/βII activation, which some are proposed to be specifically required for STM (Kandel, 2001; Izquierdo et al., 2002). Recent evidence also shown that STM and LTM depend on MAPK activity. The MAPK family included the c-Jun N-terminal kinases (JNK), the extracellular signal-regulated kinase (ERK) and p38. JNK proteins phosphorylate synaptic proteins and therefore regulate the insertion of AMPA receptors in dendrites (Coffey, 2014). Similarly, p38 is required for AMPA receptor trafficking associated with metabotropic glutamate receptor (mGluR)-induced LTD and NMDA receptor (NMDAR)-induced LTP (Correa and Eales, 2012). JNK and p38 are phosphorylated upon learning stimuli *in vivo* suggesting a role in this process (Giese and Mizuno, 2013). In fact, pharmacological inhibition of p38 immediately after learning impairs both STM and LTM formation (Alonso et al., 2003). Both short- and long-lasting neuronal adaptive responses have been suggested to require JNK/p38 activity (Alonso et al., 2003; Bevilaqua et al., 2003; Guan et al., 2003; Sherrin et al., 2010;

Giese and Mizuno, 2013; Morel et al., 2018). However, identification of proteins that regulate activation MAPK during plasticity and memory is still lacking.

Besides these classical signaling cascades other proteins have been suggested to regulate synapse-to-nucleus communication. The Growth Arrest and DNA Damage (Gadd45) family is comprised of three family members, Gadd45α, Gadd45β and Gadd45γ, which are small proteins (~18 KDa) with both nuclear and cytoplasmic expression (Tamura et al., 2012). The genes coding each family member were first identified in cell lines following irradiation stress and interleukin treatment (Fornace et al., 1988; Abdollahi et al., 1991; Beadling et al., 1993). Gadd45 family members regulate signaling cascades such as the MAPK cascade, cell-cycle control and DNA repair mechanisms and have been extensively studied during tumorigenesis and cellular stress responses (Moskalev et al., 2012). Gadd45 regulation of MAPK signaling cascades has only been described in tissues outside of the nervous system, through the interaction with kinases upstream of JNK and p38 MAPKs, but not ERK1/2 (Takekawa and Saito, 1998; Tornatore et al., 2008). Recently it was shown that Gadd45 proteins contain a MEKK4 binding domain (Miyake et al., 2007). MEKK4 is inactive in the absence of stimuli since its amino and carboxyl-termini are bound together, thus inhibiting the kinase activity. The binding of Gadd45 family members disrupts this termini interaction, allowing its dimerization and activation by phosphorylation (Miyake et al., 2007). MEKK4 once activated can phosphorylate MAPK kinases, such as MEKK4/6, upstream kinases for both p38 and JNK. The potential role for Gadd45 in JNK and p38 activation during neuronal plasticity has not yet been addressed. Only a few studies started to investigate the role of these proteins in plasticity and memory. It is known that Gadd45β and Gadd45γ transcription is induced upon neuronal activity and learning (Ma et al., 2009). Two studies indicated that Gadd45β regulates memory formation, however these studies showed contradictory results (Leach et al., 2012; Sultan et al., 2012). More recently Gadd45γ in the prelimbic prefrontal cortex was shown to be necessary for memory consolidation by processes of DNA demethylation and regulation of IEGs (Li et al., 2018). Gadd45α was also recently shown to be required for memory consolidation

by regulating mRNA stability via a transcription-independent mechanism (Aparisi Rey et al., 2019).

In the next section I will discuss mechanisms in the nucleus that are activated by the signaling cascades discussed so far.

1.2.1.2. Activity-dependent gene transcription

Upon neuronal activity, such as learning, synapse-to-nucleus communication mechanisms trigger activation of transcription factors that initiate *de novo* gene expression. The identification and characterization of molecules required for plasticity events is pivotal for ultimately understanding how the brain functions. One major limitation in the past century was that the complexity of these phenomena made it challenging to pinpoint specific molecular players involved. An initially criticized strategy to overcome this problem, was the reductionist approach. This consists on taking a complex problem and dissecting one specific component of it in a great detail, which was exactly what Eric Kandel did in the 1970s. Initially inspired by the findings of Brenda Milner on patient H.M. he found electrophysiological evidence for action potentials in the dendritic trees of hippocampal neurons. He soon realized that the hippocampus, was not the optimal system to study mechanisms of memory formation due to its complexity and limited technology available at the time. He decided to focus on a simple model organism that has rudimentary forms of learning such as the gill-withdrawal reflex in the *Aplysia californica* (Montarolo et al., 1986; Kandel, 2001). This invertebrate has only 20,000 neurons in the central nervous system, that are among the largest in the animal kingdom. Due to this fact, *Aplysia´s* neurons are easy to patch and can be singly traced between animals. This invertebrate also shows several forms of learning such as habituation, dishabituation, sensitization, classical conditioning, and operant conditioning that were later described. Kandel ultimately thought that the basic mechanisms identifiable in this basic organism would be conserved in mammals such as humans, receiving much criticism at the time. In 1976 his laboratory discovered that sensitizing stimuli to the tail of *Aplysia* promoted an increase in synaptic strength and

increased the levels of cAMP (Brunelli et al., 1976). This was performed by injecting cAMP directly into *Aplysia* sensory neurons which resulted in increased cAMP levels and in a transient enhancement of transmitter release in the synaptic connection. His laboratory also provided direct evidence that cAMP-PKA signaling that activates cAMP response element-binding protein (CREB) activity is required for learning-associated plasticity (Dash et al., 1990; Kandel, 2001). This was performed by blocking the binding of CREB to cAMP response element (CRE), which selectively impaired long-term facilitation mechanisms. This and other elegant studies demonstrated both the necessity and sufficiency of the CREB transcriptional pathway for memory (Kandel, 2001). The findings observed in this simple organism showed that the mechanisms gating plasticity and memory also happen in more complex animals and particularly in the hippocampus (Lamprecht, 1999).

CREB belongs to the bZIP family of transcription factors, it contains a c-terminal basic domain that allows binding to cis-regulatory elements in DNA. The CRE binding site, consists of the palindromic consensus sequence TGACGTCA. Although CREB can also bind in the presence of the core sequence CGTCA. The remaining domains present in the CREB protein allow the interaction with coactivators and components of the transcriptional machinery required for *de novo* gene expression. Besides cAMP-PKA activation, other signaling pathways converge into CREB activation (Figure 1.1). CREB activation depends on the phosphorylation of its regulatory site (Ser-133), located within the kinase inducible domain (KID). Phosphorylated CREB acts as a stimulus-dependent transcriptional activator (Lonze and Ginty, 2002).

Similarly, to CREB other constitutively expressed transcription factors have been associated to memory and plasticity mechanisms. The Jun (c-Jun, JunB, and JunD) family homodimerizes or heterodimerizes with proteins of the c-Finkel–Biskis–Jinkins (FBJ) osteosarcoma oncogene (Fos), activating transcription factors (ATF) families (Hai and Hartman, 2001; Alberini, 2009), thereby forming the transcription factor complex activator protein 1 (AP-1). AP-1 induces transcription in both baseline and in activity-dependent conditions of several genes that contain the AP-1

consensus site 5'-TGAG/CTCA3' (2-O-Tetradecanoylphorbol-13-acetate [TPA]-responsive element) in their promoter region.

Memory formation as well as LTP and LTD are dependent on AP-1 activity (Abraham et al., 1993). Nonetheless the majority of studies have focused on its role in response to cellular stress (Alberini, 2009). Additionally, AP-1 can also bind, with lower affinity, to the CRE element, promoting a redundant mechanism in some genes. Moreover, most activity-response genes contain several transcriptional factor binding sites not exclusive to a unique transcription factor. This complexity might serve several purposes such as redundancy that increases the likelihood of a correct response to a stimulus. Another possible advantage of this strategy is that the same transcriptional factors and genes respond to different neuronal stimuli. Meaning that the unique signature of genes is crucial for a proper response such as in learning and stress although showing some degree of overlap. Another factor supporting this redundancy is that ERK1/2 and p38 pathways signaling that leads to CREB induction also induces AP-1 activation. This supports the view that these transcriptional factors might have complementary functions in plasticity mechanisms associated with memory formation. Besides these signaling cascades other proteins have been shown to regulate transcription factor activation.

In the next section, I will discuss mechanisms occurring alongside transcription factor activation that mediate activity-dependent gene expression.

1.2.1.3 Epigenetic regulators

Epigenetics mechanisms refer to biochemical modifications to the DNA or DNA-associated structures, without affecting the DNA sequence directly. These modifications have the ability to induce persistent or transient changes in gene expression (Sweatt, 2013). Several categories of epigenetic mechanisms have been described, such as covalent modification to DNA, histone post-translational modifications, adenosine triphosphate (ATP)-dependent chromatin remodeling, histone subunit exchange, transcriptional repressor element-1 (RE1) silencing transcription factor (REST)/REST Co-repressor (Co-REST)/Sin 3A System and non-

coding RNAs. These modifications fall into two main categories: DNA methylation and regulation of chromatin structure via histone modifications. I will mainly focus on DNA methylation as a regulatory mechanism for memory formation since the remaining mechanisms have been described in detail elsewhere (Borrelli et al., 2008; Champagne and Curley, 2009; Brito and Gulmez Karaca, 2018) and go beyond the scope of this study.

Initially cytosine 5'-methylation was referred to as the prime dogma of epigenetics, as it has the potential to regulate in a permanent way gene expression, seen for instance during cellular fate determination. DNA methylation occurs mostly at cytosine-guanine dinucleotide sequences in the DNA (CpG sites) and is mainly associated with repression of gene expression. Although this seems to be a general rule, in recent years evidence has emerged that DNA methylation can also occur at non-CpG sites and that cytosine methylation can promote gene expression activation (Guo et al., 2011). It was initially thought that DNA methylation marks placed during development did not undergo DNA demethylation. The exception being mitotic cells that upon cellular division momentarily loose DNA methylation in the newly synthetized DNA strand. This idea implied that post-mitotic cells such as neurons, carried out DNA methylation marks permanently, as this would be essential to maintain their cellular identity (Holliday, 2006). More recently it has been shown that neurons can undergo active DNA demethylation, where previously-methylated cytosine can be reconverted into their unmethylated state. Interestingly this seems to be limited to a few tissues in the organism such as the adult brain and the fertilized zygote.

DNA methylation-dependent mechanisms occur by the coordinated activity of three classes of enzymes: DNA methylation writers, readers and erasers. DNA methylation writers catalyze the covalent binding of a methyl group from the methyl donor S-adenosyl-methionine (SAM) to the 5′ position on the cytosine-pyrimidine ring. These enzymes are the DNA methyltransferases (DNMT) which either mediate *de novo* methylation (DNMT3a and 3b) or maintain previous methylation marks (DNMT1) (Tollefsbol, 2017). Upon establishment of methylation marks a group of

proteins, DNA methylation readers, that contain a methyl-CpG-binding domain (MBD) are recruited. Five MBD proteins have been identified in mammals the MBD1–MBD4 and methyl-CpG-binding protein 2 (MeCP2) (Baubec et al., 2013). After recruitment and CpG binding, these proteins promote nucleosome remodeling and activity of histone deacetylase complexes, which results in decreased transcriptional activation at these loci (Nan et al., 1998). DNA methylation erasers can promote active DNA demethylation, independent of cell division. This family of proteins are the ten-eleven translocation (TET) proteins comprised of three members TET1-3, that throughout a series of direct and indirect biochemical steps convert 5-methylcytosine into its demethylated form (Alaghband et al., 2016; Bayraktar and Kreutz, 2018). The Gadd45 proteins have also been shown to promote active demethylation by recruiting demethylation machinery components and activation base excision repair (BER), that catalyzes the substitution of 5mC to unmodified cytosine (Gavin et al., 2012; Li et al., 2015) or by direct interaction with TET proteins (Kienhofer et al., 2015). In accordance, knock-down of Gadd45β expression in the neonatal rat amygdala results in impaired expression of MeCP2, Reelin and brain derived neurotrophic factor (BDNF) possibly by processes of active DNA demethylation (Kigar et al., 2015).

LTMs require distinct rounds of gene expression. Constitutively expressed transcription factors initiate transcription of immediate early genes (IEGs) directly after the onset of learning. Among IEGs there are several transcription factors that regulate the expression of late-response genes (LRGs). The transcription of LRGs is induced several hours later as it is dependent on *de novo* protein synthesis.
In the next sections I will discuss the downstream activation of genes associated to plasticity and memory events.

1.2.1.4. Immediate early genes

IEGs are a group of genes that in response to stimulation are rapidly and transiently upregulated in neuronal cells. IEGs transcription is initiated rapidly after neuronal activity/LTM as seen by the presence of their mRNA within minutes after stimulation.

Their transcription is protein synthesis independent and generally transient. This set of about one hundred genes (Nedivi et al., 1993; Lacar et al., 2016) are generally expressed at low levels in the absence of activity. For this reason, IEGs usually contain in their promoter regions binding motifs for constitutively expressed transcription factors such as CREB, AP-1 and serum response factor (SRF) and others. Therefore, IEG expression is used as a marker for activated neurons during a learning event (Okuno, 2011) and their promoters or cis-regulatory regions have been genetically engineered to identify and/or manipulate the neuronal population activated during learning (Kawashima et al., 2013; Sorensen et al., 2016). The enrichment for transcriptional factor binding in their regulatory elements suggests a redundant regulation (Healy et al., 2013). These genes include many transcription factors responsible for generating a secondary response to activity required for LTM to form. Arguably the two most well studied IEGs are the activity regulated cytoskeleton (Arc) protein and Fos, nonetheless other examples include the neuronal PAS domain protein 4 (Npas4), early growth response protein 1 (Egr1) and the nuclear receptor subfamily 4 group A member 1 (Nr4a1). Fos also acts as an activity-induced transcription factor. As discussed before c-fos expression depends on cAMP and Ca^{2+} through the activation of CREB. Fos does not directly act as a transcription factor as it needs to dimerize with members of the Jun family to form the AP-1 complex. Early studies using global Fos knock-out mouse models showed that this protein is required for complex forms of learning and memory (Paylor et al., 1994). Later more sophisticated methods were used such as the Cre recombinase/ locus of x-over, P1 (cre/loxP) system to selectively knock-out fos in the central nervous system. These mice showed robust hippocampus-dependent and associative learning deficits, indicating that fos is required for these processes (Fleischmann et al., 2003). Until recently it was thought that AP-1 binds preferentially at promoter sites of late-response genes, although recent chromatin immunoprecipitation DNA-sequencing (ChIP-seq) evidence has revealed that AP-1 binds mostly to activity-regulated enhancer regions (96% of fos binding sites) (Malik et al., 2014).

Npas4 is another example of an IEG with transcription factor activity. Npas4 has however unique characteristics that distinguishes it from other IEGs. Particularly Npas4 expression is exclusively neuronal, it is selectively activated by neuronal activity but not by other external stimuli such as growth factors or neurotrophins. Moreover, it was suggested to control the expression of a large number of activity-regulated genes (Sun and Lin, 2016). Supporting its function in memory, blockade of Npas4 expression after learning in the CA3 of the hippocampus and the basolateral amygdala impairs memory formation (Ploski et al., 2011; Ramamoorthi et al., 2011; Weng et al., 2018; Sun et al., 2020). Recently it was shown that action potential bursting and excitatory postsynaptic potentials promote two distinct molecular responses. Different Npas4 heterodimers form in the CA1 region of the hippocampus in response to these stimuli leading to molecularly distinct binding in the genome (Brigidi et al., 2019). These recent findings place Npas4 as a decoder of synaptic stimuli and suggest that it might fine-tune high order cognitive events.

Arc is an example of an IEG that is not a transcription factor, but a protein with synaptic function. Upon transcription *Arc* mRNA is transported into dendrites as part of the post-synaptic density (Moga et al., 2004). Arc is required for plasticity mechanisms such as LTP and LTD (Korb and Finkbeiner, 2011). At dendrites Arc interacts with protein complexes to dynamically regulate AMPA receptor endocytosis. This was shown by using mutant forms of Arc lacking interacting domains with these protein complexes, that resulted in abolished Arc-dependent decrease in the AMPA receptor-mediated miniature excitatory postsynaptic current (mEPSC) amplitude (Shepherd et al., 2006; DaSilva et al., 2016). Interestingly Arc can also be localized at the nucleus, where it regulates glutamate ionotropic receptor AMPA type subunit 1 (*GluA1*) expression and homeostatic plasticity (Korb et al., 2013). Unexpected findings revealed that Arc can form virus-like particles that enclose RNA and are transported trans-synaptically (Pastuzyn et al., 2018). More research is needed to pinpoint how this mechanism may be required for memory processes.

1.2.1.5. Late response genes

LRG transcription is induced hours after IEG induction (Hong et al., 2004). As discussed, IEG largely encode transcription factors which are required for induction of LRG. Therefore, this secondary genomic response is mostly dependent on *de novo protein* synthesis, although specific LRGs have been reported to be protein synthesis independent (Tullai et al., 2007). LRGs usually encode effector proteins with distinct neuronal functions such as dendritic growth, spine maturation, synapse elimination and the development of proper excitatory/inhibitory balance (Yap and Greenberg, 2018). Based on genome-wide studies there have been identified about 300-500 LRGs dependent on Fos and Npas4 binding (Kim et al., 2010; Malik et al., 2014; Benito and Barco, 2015), although this number is possibly much larger as other IEGs are expressed upon learning. Examples include the neuronal pentraxin-2/ neuronal activity-regulated pentraxin Nptx2/Narp and the transforming growth factor-beta 2 (Tgfb2). Moreover, the specific induction of LRG seems to be a largely cell- and stimulus specific event (Yap and Greenberg, 2018). Due to this complexity, research has mainly focused on understanding the functions of individual LRGs, as their general contribution is mostly tested by manipulating specific transcriptional factors that mediate their transcription. As discussed above many transcription factors have redundant activities, thus challenging the understanding on how LRGs act as a whole.

1.2.3. Alternative splicing

Post-transcriptional mechanisms provide an additional layer of regulation of protein function. Alternative splicing expands the diversity of the genome by potentially generating thousands of distinct transcript isoforms from single genes (Schreiner et al., 2014). Transcriptomic studies have revealed that ~95% of the human pre-mRNAs are subject to alternative splicing editing (Pan et al., 2008; Wang et al., 2008). Splicing is catalyzed by the spliceosome, a macromolecular RNA-protein complex that recognizes sequence elements on target pre-mRNAs and mostly removes introns. This remains a major unanswered question in Molecular Biology, as it can be perceived as a very inefficient system of keeping introns in a gene only

to transcribe and remove them during splicing. Alternative splicing processes pre-mRNA transcripts to generate mRNA isoforms with different stability or coding segments (Nilsen and Graveley, 2010). This process is regulated by cis-acting RNA elements and by splicing regulatory RNA-binding proteins. These proteins are highly complex as they cannot be grouped into positive and negative splicing regulators, since their function depends on the location of the binding site (Fu and Ares, 2014). Nonetheless recent findings in neurons have shown that DNA methylation in gene bodies directly affects splicing efficiency at these loci particularly through MeCP2 activity (Maunakea et al., 2013; Cheng et al., 2017). Importantly a study revealed that mice that underwent contextual-fear conditioning showed activity-dependent alternative splicing during several stages of memory consolidation (Poplawski et al., 2016). This finding suggests that alternative splicing might be a process involved in cognition. Indeed, several proteins with well-established functions in memory and plasticity have been shown to be regulated by alternative splicing. For example, the *Grin1* gene that encodes for the glutamate ionotropic receptor NMDA type subunit 1 (GluN1), a subunit ubiquitous present in AMPA receptors, undergoes differential splicing (Nakanishi et al., 1992). Mice that express a version that lacks one exon that can be skipped by alternative splicing have enhanced long-term potentiation in the hippocampus compared to mice that constitutively expressed this exon (Sengar et al., 2019a). Importantly no differences in basal synaptic transmission were observed, suggesting that alternative splicing specifically mediated a mechanism involved in synaptic plasticity. Moreover, mice with skipped version of this exon have a better learning and memory performance compared to mice that expressed the exon (Sengar et al., 2019a). This illustrates a scenario where alternative splicing regulation is required for memory formation. It is therefore possible that in instances where global transcript levels of a particular gene are not affected, transcript isoforms generated by alternative splicing are. This is observed in forms of autism spectrum disorders, where forms of homeostatic plasticity are impaired due to alternative splicing malfunction (Thalhammer et al., 2020). IEGs can also be regulated by alternative splicing mechanisms. For example, specific splicing forms of the *Homer* protein homolog 1 (Homer1) are induced after synaptic plasticity

events. This gene generates long (Homer1b, Homer1c, and Homer1d) and short (Homer1a and Ania3) splice isoforms, where only Homer1a and Ania3 have characteristics of IEGs (Thalhammer et al., 2020). This is due to a regulatory element within the intronic region between exons 5 and 6 that leads to alternative poly(A) sites resulting in a premature transcription termination. This process is essential at the function level, as the long isoforms contain domains that allow the biding to multiple postsynaptic proteins. The activity-generated isoforms that lack these domains act as dominant-negative regulators, disrupting the binding between Homer1 long isoforms and their target proteins. Therefore, incorrect regulation of specific isoform expression might lead to alterations in mechanisms of homeostatic plasticity, which have been identified in diseases such as schizophrenia (Matosin et al., 2016). The current understanding of alternative splicing mechanisms in memory formation is however at its infancy.

In this section I summarized the molecular mechanisms that underly long-term memory storage. I introduced signaling pathways that disseminate synaptic activity into the cell nucleus and initiate plasticity-associated gene expression mediated by the regulation of transcription factors and epigenetic regulators. Moreover, I covered how the initial gene expression response modulates a secondary transcriptional pool of transcription. Lastly, I discussed the emerging field of activity-dependent alternative splicing as a contributor to plasticity and memory events.

In the next section I will provide an overview on mechanisms involved in remote memory formation, a form of long-term memory that can last from weeks to the whole lifespan of an organism.

1.3. Molecular mechanisms underlying memory persistence

Early psychological studies showed that memories formed recently were more sensitive to disruption than memories formed remotely in time. These findings originated the Ribot's law of retrograde amnesia (Ribot and Smith, 1882). This law

states that with time memories become more resistant to disruption due to consolidation mechanisms in the brain. This hypothesis was supported by findings from patients with hippocampal damage that displayed both anterograde amnesia and a form of retrograde amnesia that was restricted to more recently formed memories but spared remote memories (Milner and Penfield, 1955; Scoville and Milner, 1957). These early observations lead to the idea that the hippocampus was essential for the formation and early recall of recent memories, but over time memories became hippocampus-independent. It is now largely accepted that consolidation involves two major stages: synaptic or cellular consolidation and systems consolidation. Cellular consolidation mechanisms last from minutes to hours and happen locally in each brain area involved in a particular memory. Ultimately these processes (mainly discussed in the previous sections) restructure synaptic connections in activated neurons (Quillfeldt, 2019). On the other hand, classical systems consolidation is a gradual process that happens within weeks in rodents but it has been suggested to happen during months or even years in humans (Quillfeldt, 2019). It states that at the time of learning, the hippocampus and cortical regions are activated, but only weakly linked together. Reinforcement processes such as replay and sleep strengthen these cortico-hippocampal connections until a remote memory is fully consolidated. This classical theory proposes that remote memories are able to be evoked independently of the hippocampus, merely by the newly formed cortico-cortical connections. Recent technological advances have allowed to test this theory with higher precision. This led to the development of other theories that state a role of the hippocampus even in remote memory recall (Goshen et al., 2011; Tonegawa et al., 2018; Quillfeldt, 2019; Yonelinas et al., 2019). A still largely unanswered question is what mechanisms occur during the cellular consolidation phase that allows the transition to the systems consolidation phase? Particularly considering that not all long-term memories are consolidated into remote memories. This suggests that early mechanisms that are specific to long-lasting memories need to happen early on in regions such as the hippocampus and possibly cortical regions during the initial cellular phase.

In 1999 the first study that showed that long-lasting activity in the hippocampus is required for LTM consolidation was published. Riedel and colleagues revealed that during several days after learning the AMPA/kainate glutamate activity is required in the hippocampus for memories to persist (Riedel et al., 1999). After, independent studies have replicated and expanded on this finding by showing that AMPA and mGluRs are upregulated in the hippocampus after learning for several days, possibly involved in the maintenance of memory consolidation (Katche et al., 2013). It seems that this increase in receptor expression is not exclusive to AMPAR, as NMDA receptor reactivation in the CA1 is required in the first days after learning in order for memories to persist (Shimizu et al., 2000). This increase on long-term activation of the hippocampus possibly depends on input from other brain regions. Indeed, a pioneering study showed that dopaminergic activation is required in order for memories to undergo long-term consolidation (Rossato et al., 2009). Using a pharmacological approach, the authors inhibited D1 dopamine receptor activity in the dorsal hippocampus 12h after memory acquisition. These animals showed a fast-decaying LTM suggesting that mechanisms underlying memory persistence were impaired. Moreover, using a behavior paradigm that would induce a short-lived memory, the authors pharmacologically enhanced D1 dopamine receptor activity, which converted this memory into a persistent one. More recently it was shown that remote memories depend on local activation of parvalbumin interneurons in two distinct phases (Karunakaran et al., 2016). The first phase (0–5h after learning) depends on D1/5 dopamine receptor signaling and entails downstream activation of cAMP signaling in these cells. A second phase (12–14h) is associated with parvalbumin activation which promotes activity of pyramidal neurons in the hippocampus and leads to memory persistence. These findings indicate that late and recurring activity in the hippocampus in diverse cell types is associated with mechanism of systems consolidation.

Neuronal activity needs to generate, at the molecular level, changes in plasticity. In the previous sections, I discussed molecular mechanisms associated with long-term memory formation. These processes arise almost immediately after learning. Particularly induction of signaling cascades that orchestrate a genomic IEG

response. It appears that the learning associated-gene expression required for remote memory consolidation occurs at remote time points. Nonetheless, many of the same signaling events and downstream gene expression seem to arise. Particularly studies show two waves of ERK1/2 activation after learning. The first is transient and rapidly induced after learning, while the second is delayed and persists for several hours after training (Trifilieff et al., 2006; Trifilieff et al., 2007; Bekinschtein et al., 2008). An alternative way of inducing memory persistence is by a reconsolidation process. This involves the conversion of a labile memory into a restabilized state by memory reactivation. Interestingly hippocampal activation of ERK1/2 occurs up to 3h after reinforcement. Abolishing this activation impaired remote memory, but spared 24h memory after reinforcement (Krawczyk et al., 2016). Altogether these intriguing findings suggest that different strategies for remote memory formation share common molecular mechanisms.

Late activation of signaling cascades such as ERK1/2 likely induce gene expression required for remote memory formation. Indeed, several studies have shown that delayed waves of gene expression happen several hours after learning in brain regions associated with memory encoding. An important distinction from the initial induction of IEGs is that few genes seem to be activated, contrasting with the induction of hundreds of genes upon the initial learning event. This might be in part due to active processes of repression which have been shown to occur (Cho et al., 2015). One example is *Arc* expression that has been proposed to be reactivated after learning. Upon spatial exploration two waves or Arc expression occur at 30 min to 2h and 8 to 24h (Ramirez-Amaya et al., 2005), these findings were later replicated by the same group (Ramirez-Amaya et al., 2013). It is unclear however, if this expression was related to memory persistence, as animals were exposed only to a 5min exploration session. More recently an elegant study addressed this question by identifying a delayed expression of Arc 12h after contextual fear conditioning (Nakayama et al., 2015). The authors also showed that the neuronal subpopulation expressing this delayed wave was the same that initially expressed Arc after leaning. Lastly, they showed that this delayed expression was required for memory persistency and not for memory formation. Another study revealed that Arc

expression in the hippocampus mediates cortical mechanisms required for strengthening memories, but not memory retrieval per se (Ye et al., 2017).

Besides Arc other genes were reported to undergo delayed transcriptional activation such as BDNF. Expression of BDNF is upregulated 12h after inhibitory avoidance training in the rat hippocampus (Bekinschtein et al., 2007). Similarly to Arc, inhibition of this late phase of expression selectively hindered memory persistency, but not formation. In a follow up study, the authors showed that artificial induction of late BDNF expression was sufficient to induce memory persistence, in a process dependent on ERK1/2 signaling (Bekinschtein et al., 2008). Later, an independent group also observed a late BDNF expression in mice that underwent contextual fear conditioning training (Mizuno et al., 2012). These findings suggest that BDNF upregulation is a mechanism common in different forms of aversive learning and rodent species.

Besides Arc and BDNF other genes have been identified to undergo similar reactivation patterns required for memory persistence. Particularly Egr-1 (Katche et al., 2012) and Fos (Katche et al., 2010) and more recently the neuron-specific nucleosome remodeling factor BAF53b (Yoo et al., 2020). For a more detailed overview please refer to Katche et al. 2013, which summarizes most of the identified molecules at the time.

These findings suggest that the molecular signature of memory persistence is somewhat similar to global mechanisms during early consolidation. Possibly such is the case to promote a reinforcement of encoded information that allows remote memory storage. A major challenge in this field is to pinpoint the correct time to analyze when remote memory consolidation take place. Contrarily to memory acquisition and recall, when these mechanisms start to occur and what drives them is still an open question. It is tempting to speculate that early events triggered by remote memory training during memory acquisition/early consolidation potentiate signaling mechanisms required for LTM and simultaneous events that will trigger memory endurance. These mechanisms need to be specific for long-lasting memories and possibly generate signaling cascades within brain regions associated

with memory formation that later on will induce appropriate communication and maturation during systems consolidation.

1.4. Molecular mechanisms underlying age-related cognitive decline

In the previous sections I summarized processes underlying proper cognitive function in the brain. Although there are numerous examples where cognitive function is compromised (i.e., neuronal degeneration), this chapter will focus on cognitive aging, a physiological decay of memory performance. Age-related cognitive decline is observed across multiple species, including rodents and humans (Erickson and Barnes, 2003). In humans, about 40% of individuals 65 years old or above experience some form of memory loss (Small, 2002; Aigbogun et al., 2017), even though deterioration of cognitive functions may start earlier (Singh-Manoux et al., 2012). Aged rodents (Dunnett et al., 1988; Winocur, 1988), monkeys (Bartus et al., 1978; Rapp et al., 1997) and humans exhibit similar age-dependent memory impairments (Erickson and Barnes, 2003; Burke et al., 2012). During heathy aging (in the absence of brain disorders), selective forms of cognition are compromised. Aging does not affect the rate of at which elderly humans acquire skills, learn procedures, form simple associations or even long-term retention or performance of skills acquired previously (Foster et al., 2012). Aging disturbs primarily memory formation of recent events associated with a spatial component or contextual memory. In rodent animal models, spatial memory is also sensitive to age, and similarly to humans' other types of procedural skills are spared (Cassel et al., 2007). The reason for this selectivity is that the hippocampus is a brain region particularly affected by the aging process together with other areas such as the neocortex (Ryan et al., 2019a) and the cerebellum (Woodruff-Pak et al., 2010; Kennard et al., 2013). Memories that require intact hippocampal function are specially compromised during aging (Burke and Barnes, 2006). In all species, forms of spatial memory are particularly vulnerable to aging and in aged individuals memories are retained for shorter periods of time (Kennard and Woodruff-Pak, 2011). However, despite these conserved age-related cognitive impairments, not much is known about the molecular mechanisms underlying these changes.

It is well established in rats (Dunnett et al., 1988; Winocur, 1988), monkeys (Bartus et al., 1978; Rapp et al., 1997) and Humans (Flicker et al., 1984) that during ageing recognition short-term memory impairments are observed in a delay-specific manner (Erickson and Barnes, 2003; Burke et al., 2012). If tested early after training no memory deficits are observed, only when aged animals are tested upon greater intervals short-term memory impairments arise. On the contrary contextual fear memory, is less vulnerable to aging (Foster et al., 2012). Although testing contextual-fear memory in aged humans has been challenging in the past (Foster et al., 2012), recent evidence suggests that contextual-fear memory acquisition and recall are not affected during aging (Battaglia et al., 2018).

Calcium dependent-signaling pathways seems to be dysfunctional in the hippocampus during aging. Age-associated changes in CREB activity and expression have been suggested and there is evidence indicating that upregulation of CREB levels or activity ameliorates age-related memory deficits (Bach et al., 1999; Yu et al., 2017b). These results are in line with studies showing that learning-induced phosphorylation of CREB is compromised in mice (Porte et al., 2008) and rats (Kudo et al., 2005; Monti et al., 2005). Conflicting results have emerged regarding the possible age-associated effects of baseline CREB levels. Studies have suggested no effects (Porte et al., 2008), decreases (Foster et al., 2001; Hattiangady et al., 2005) or increases (Monti et al., 2005) which hinder consensus. Alterations in the levels of transcriptional factors associated with memory formation might lead to compromised gene expression. Indeed impaired expression of IEGs and LRGs, such as Arc and Nptx2/Narp, have been shown by several studies (Ryan et al., 2019b). Additional studies have also identified impairments in the regulation of other genes associated calcium signaling processes such as Arc, Egr1, Egr2, Nr4a1, Nr4a2, and Nr4a3 (Rowe et al., 2007; Penner et al., 2016) Recently, comparison of the hippocampal transcriptome of young adult and aged mice revealed that AP-1-associated gene expression is also affected during aging (Stilling et al., 2014). Hence these changes suggest that molecular mechanisms that regulate both CREB and AP-1 transcriptional activity might be disrupted during aging.

There is however high variability among studies in which genes seem to be consistently altered during aging. This heterogeneity might be due to compensatory mechanisms that differ among animals or different stages of the aging process that might differently impact gene expression. Accordingly, age-related changes in the brain transcriptome of different mammalian species are poorly correlated, thus challenging the discovery of mechanisms that lead to the well-established converging memory phenotypes (Zahn et al., 2007; Loerch et al., 2008).

1.5. Aim of the study

In this thesis, we aimed to uncover the basic cellular and molecular mechanisms of short- and long-term memory storage as well and remote memory formation under physiological conditions and during cognitive aging. First, we sought out to uncover a putative role of Gadd45 proteins in processes of short and long-term memory formation and how these mechanisms might be affected during murine and human aging (Chapters 3.1, 3.2). Next, we investigated if DNA methylation-related mechanisms regulate alternative splicing events and their implications on memory-associated gene expression (Chapter 3.3). Lastly, we investigated the contribution of an activity-dependent transcription factor activity for mechanisms of memory persistence (Chapter 3.4).

Chapter 2. Materials and methods

2.1. Subjects

3-months-old or 18-month-old C57BL/6J male mice (Janvier, Saint Berthevin, France) and male C57BL/6N mice (Charles River, Sulzfeld, Germany) that were 3-months-old at the time of behavior experiments were used. Mice were always group-housed (3-4 mice per cage), unless severe fighting occurred, and were housed on a 12h light/dark cycle with *ad libitum* access to water and food, 22 ± 1°C, 55 ± 10% relative humidity. Cannulated animals were kept singly-housed after surgeries to avoid cannula removal. All behavioral experiments took place during the light phase. Sick and/or injured mice from cage-mate fighting were excluded from this study. Animals were randomly assigned to experimental groups and blinded analysis was performed. All procedures were carried out in accordance with German guidelines for the care and use of laboratory animals and with the European Community Council Directive 86/609/EEC.

2.2. Postmortem human samples

The use of human samples was conducted in accordance with the Helsinki Declaration as well as Portuguese and German ethical guidelines. Protocols were approved by the Local Ethics Committee and the National Data Protection Committee. The biospecimens were obtained 36h *postmortem* from healthy aged (60–65 years old) and young (21–22 years old) individuals (Temido-Ferreira et al., 2018). The tissue was processed and preserved for molecular analyses as previously described (Pliassova et al., 2016). Sample acquisition and processing until cDNA production was performed by the laboratory of Dr. Luisa Vaqueiro Lopes, Lisbon, Portugal.

2.3. Primary hippocampal cultures

Hippocampal cultures from newborn C57Bl/6N mice (Charles River, Sulzfeld, Germany) were prepared by Iris Bünzli‐Ehret and maintained as previously described (Gulmez Karaca et al., 2020), except that growth medium was supplemented with B27 (Invitrogen/BRL,MA, USA) and 1% rat serum (vol/vol). Briefly, mice hippocampi were dissociated at P0 by papain digestion and plated onto

tissue culture dishes coated with poly-D-lysine and laminin (Sigma-Aldrich, Munich, Germany). The primary cultures were maintained for 8 days in Neurobasal-A medium (GibcoTM) supplemented with 1% rat serum (Biowest), 0.5mM L-glutamine (Sigma-Aldrich, Munich, Germany) and B27 (Gibco™), followed by incubation in salt-glucose-glycine solution (10 mM HEPES, pH 7.4, 114 mM NaCl, 26.1 mM NaHCO3, 5.3 mM KCl, 1 mM MgCl2, 2 mM CaCl2, 30 mM glucose, 1 mM glycine, 0.5 mM C3H3NaO3, and 0.001% phenol red) and phosphate-free Eagle's minimum essential medium (9:1 v/v), supplemented with insulin (7.5 µg/ml), transferrin (7.5 µg/ml), and sodium selenite (7.5 ng/ml) (ITS Liquid Media Supplement, Sigma-Aldrich, Munich, Germany) and penicillin-streptomycin. rAAV infection of cultures occurred on day *in vitro (*DIV) 4. Infection rates, were determined by analyzing the respective transgene expression which ranged from 80-90%. Experiments were performed on DIV 10-11. To induce action potential bursting, cultures were treated with 50 µM bicuculline (Enzo Life Sciences, Germany) or 100 µM gabazine (Biotrend, Germany) during calcium imaging. DNA co-transfection was performed after a culturing period of 8 DIV using Lipofectamine 2000 (Invitrogen, CA, USA) as described previously (Pruunsild et al., 2011; Pruunsild et al., 2017). Doxycycline hyclate (25µM, Sigma-Aldrich) was introduced in the medium at DIV 8. N numbers represent independent cell preparations.

2.4. Recombinant adeno-associated virus (rAAVs)

Viral particles were produced and purified as described previously (Zhang et al., 2007). Briefly, rAAVs were produced by co-transfection of human embryonic kidney (HEK) cell line 293 (ATCC, Manassas, Virginia) with the target AAV plasmid and helper plasmids (pFΔ6, pRV1and pH21) using standard calcium phosphate precipitation. 60 h after transfection, HEK 293 cells were harvested and lysed. Finally, the viral particles were purified using heparin affinity columns (HiTrap Heparin HP; GE Healthcare, Uppsala, Sweden) and concentrated using Amicon Ultra-4 centrifugal filter devices (Millipore, Bedford, MA). Full list of viral constructs is shown in Table 2.1. For expression of shRNAs, we used a vector containing the U6 promoter upstream of the shRNA sequence (control, Gadd45β or Gadd45γ-specific). The Control-shRNA, Gadd45β-shRNA, Gadd45γ-shRNA1 and Gadd45γ-

shRNA2. Both the Control-shRNA and Gadd45γ-shRNA1 sequences have been previously described and validated (Zhang et al., 2009; Oliveira et al., 2012). Overexpression of Gadd45γ was achieved by using a viral vector that contained the mouse CamKIIα promoter upstream of the Gadd45γ full-length mouse cDNA sequence. As a control vector, we used a construct containing the CamKIIα promoter driving the expression of GFP. For the knockdown of MeCP2, we used a vector containing the U6 promoter upstream of the shRNAs (MeCP2-specific or control) sequence and a CamKIIα promoter driving mCherry expression (as an infection marker) as described previously (Gulmez Karaca et al., 2018). The dual-component TetON-based system containes the driver plasmid (developed and provided by Dr. Sidney Cambridge, Institute of Anatomy, University of Heidelberg) that expresses under the control of a neuron-specific promoter (hSynapsin), the transactivator (rtTA), the tetracycline repressor (TetR) and the fluorescent protein Kusabira Orange (KO) that serves as an infection marker. In the second construct human influenza hemagglutinin (HA)-tagged eGFP or full length Npas4 expression cassette is under the control of the tetracycline responsive promoter (TRE). For each virus batch produced, the infection rate, toxicity, viral titer and effectiveness of knockdown were checked before starting of experiments. Viral titers obtained after production of all viruses were similar and were matched to obtain final working concentrations of 10^{10-12} viral particles/mL. At DIV10, infected hippocampal cultures were imaged using identical microscope settings for infection rate and toxicity analysis. The quantification was performed using Fiji (Schindelin et al., 2012). None of the viral batches used induced cell death.

Table 2.1. Viral constructs used in the study.

Name	Construct
Control-shRNA	rAAV-U6-ACTACCGTTGTTATAGGTGCG::CMV/CBA-hrGFP-WPRE/bGH polyA
Gadd45β-shRNA	rAAV-U6-GCAGATTCACTTCACCCTGAT-CMV/CBA-hrGFP-WPRE/bGH polyA-ITR

Gadd45γ-shRNA1	rAAV-U6-TTGAAAGAGCAGTGCAGTCG-CMV/CBA-hrGFP-WPRE & bGHpolyA
Gadd45γ-shRNA2	rAAV-U6-ATAGCGCTGCAGATCCATTTC-CMV/CBA-hrGFP-WPRE/bGH polyA
jRGECO1a	rAAV-hSyn- jRGECO1a-WPRE/bGH polyA
GFP	rAAV-CamKIIα-GFP-WPRE/bGH polyA-ITR
Gadd45γ-OE	rAAV-CamKIIα-Gadd45γ-WPRE/bGH polyA-ITR
Control-shRNA	rAAV-U6-ACTACCGTTGTTATAGGTGCG-CamKIIα-mCherry-WPRE/bGH polyA-ITR
MeCP2-shRNA	rAAV-U6-GTCAGAAGACCAGGATCTC-CamKIIα-mCherry-WPRE/bGH polyA-ITR
Driver	rAAV-hSyn-rtTA-T2A-TetR-KO
TRE- eGFP	rAAV-TRE-eGFP-HA
TRE-Npas4	rAAV-TRE-HA-Npas4

2.5. Stereotaxic surgery

For dorsal hippocampus targeting AAVs were injected at the following coordinates relative to Bregma: − 2 mm anteroposterior, ± 1.5 mm medio-lateral, − 1.7, − 1.9 and − 2.1 mm dorsoventral. A total volume of 1.5 μl was injected per hemisphere at 200 nl/min; a 2:1 mixture of viral solution and 20% mannitol was used. Following injections at each individual site, the needle was left in place for 60s. For dorsal CA1 targeting AAVs were injected at the following coordinates relative to Bregma: − 2 mm anteroposterior, ± 1.5 mm medio-lateral, − 1.2 mm dorsoventral. A total volume of 0.5 μl (1:1 ratio of Driver and eGFP or Npas4) was injected per hemisphere at 100 nl/min. Before and after injections at each individual site, the needle was left in place for 7min. At the time of behavioral experiments, the experimenter was blind to the identity of the virus injected into each mouse. Behavioral experiments started 2-3 weeks after rAAVs delivery. After behavior, histological analysis was performed to confirm correct targeting and tissue and cellular integrity. Mice that showed absence or miss targeting of viral expression were excluded. For histological analysis, mice

were perfused with 4% paraformaldehyde (Sigma, Munich, Germany). The brains were collected and post-fixed in the same solution overnight, then placed into a 30% sucrose solution. Brain slices were cut at a thickness of 30 µm and incubated in Hoechst 33258 (2µg/ml, Serva, Heidelberg, Germany) for 5 min and mounted on glass slides. Slices were imaged with 10x objective mounted on a fluorescence microscope (Leica Microsystems). Identical microscope settings were used between different experimental groups.

2.6. Behavior paradigms

Before all behavioral tests, mice were habituated to the experimenter and behavioral room by handling for 3 or 5 consecutive days, 1.5-2 minute per mouse. Object-location test and contextual fear conditioning were performed as previously described (Oliveira et al., 2012; Oliveira et al., 2016). Different mice cohorts were used to test long-term memory (24h), early short-term memory (5min) or late short-term memory (1h). If in one experimental batch during the object-location test control animals did not show a preference, due to day effects, the whole set of animals was excluded from this analysis but was still included in contextual fear conditioning data.

2.6.1. Object-place preference test

First mice underwent a habituation session (6min), where they were placed in the training arena in the absence of objects. During training, the animals were exposed to two distinct objects (a glass bottle and a metal tower) and allowed to explore for 6min during 3 trials with 3min inter trial intervals. During the testing session, that occurred 24h, 1h or 5min later, one object was moved to a new location and exploration of objects was scored for 6min.

2.6.2. Open field test

The open field test was carried within the first session of the object-place recognition training as previously described (Gulmez Karaca et al., 2018). Briefly this test was carried out using the Smart Video Tracking Software (Panlab, Harvard Apparatus). The center of the open field was defined as 33% of the total area of the apparatus (50cmx50cmx50cm), and the rest was defined to be periphery. Anxiety-like behavior of the mice was quantified as the percentage of the time spent in the center zone

($100\times\frac{T(center)}{T(total)}$) and locomotion of the mice was defined as the total distance travelled during training session. Open field software analysis was performed by Kübra Gülmez Karaca.

2.6.3. Contextual fear conditioning

Mice were allowed to explore the conditioning chamber for 2min and 28s until a 0.5mA foot shock was administered for 2s, then animals remained for 30s before returning to their home cage. Remote memory training consisted on for 2min and 28s of exploration and 3x 0.7mA shocks for 2s each, spaced for 2min and 28s, then animals remained for 30s before returning to their home cage. Recent memory training consisted in 2min and 28s of exploration until a 0.2mA footshock was administered for 2s, then animals remained for 30s before returning to their home cage. The testing session consisted on exposing the animals to the conditioning chamber for 5min in the absence of any shock. Mice tested for both behavior paradigms first underwent object-location test and one week later contextual fear conditioning was performed.

2.7. Cannulation surgeries and pharmacology

Mice were implanted with 26-gauge double guide cannula cut 1mm below pedestal (C235G-3.0/Spc, Plastics One, Bilaney) aimed at the CA1 region of the dorsal hippocampus at the stereotaxic coordinates: − 2 mm anteroposterior, ± 1.5 mm medio-lateral, − 1.2 mm dorsoventral. Cannulas were placed using HY-bond polycarboxylate cement (9917-1, Shofu) and 2-4 screws (00-96x1/16, Plastics One, Bilaney) and left to dry for 25min. After a dummy cannula without projection (c235g-3 Plastics One, Bilaney) was placed inside the guide cannula to avoid clogging. The animals were allowed to recover from surgery for 7 days before experiments. At the time of drug delivery, internal infusion cannula (C235G-3, Plastics One, Bilaney) were tightly fitted into the guides and injections (0.5µl/side) of DL-2-amino-5-phosphonopentanoic acid (DL-APV) (5µg/µl, 22.8mM), SCH23390 (5µg/µl, 15.4mM) or saline were performed at 200nl/min speed with a microinjection pump. The infusion cannulas were left in place for 60 additional seconds to minimize backflow. The placement of the cannulas was verified postmortem during tissue

microdissection. Only data from animals with correct implants were analyzed. Validation of cannula projection length and volume of drug administration was performed during a pilot study by infusing 0.2% methylene-blue and evaluating diffusion of the stained tissue over the hippocampal axis, the used amounts consistently targeted the dorsal CA1 region (data not shown). Intraperitoneal doxycycline (2.5mg in 500uL saline solution, 100mg/kg, Sigma) injections were performed 16h before contextual fear conditioning training.

2.8. Luciferase reporter assays

Assays were performed as described with alterations (Pruunsild et al., 2017). We used the following Firefly Luciferase expression vectors: pGL4.29[luc2P/CRE/Hygro] (Promega) that contains four CRE cis-elements and a minimal promoter (4×CRE-pmin), a pGL4.44[luc2P/AP1 RE/Hygro] (Promega) that contains six copies of an AP-1 response element and a minimal promoter (AP-1 RE-pmin), the Npas4 reporter plasmid consisted in four Npas4 Responsive Element (TCGTG), a consensus binding motif for Npas4 and a minimal promoter [kindly provided by Dr.Yingxi Lin (Sun et al., 2020)]. Additionally, the plasmid pGL4.83h[RlucP/Puro] (Promega) that contains the human EF1a promoter in front of Renilla luciferase (Rluc) was used for normalization. On DIV 8, mouse primary hippocampal cultures in 48-well plates were changed to transfection medium. Lipofectamine 2000 (Thermo Fisher Scientific) was used for transfection according to the manufacturer's instructions. Neurons were co-transfected with one of the following constructs: Control-shRNA, Gadd45β-shRNA, Gadd45γ-shRNA-1, Gadd45γ-shRNA-2 and 4×CRE-pmin or AP-1 RE-pmin (all 1 µg/well), together with Rluc (75 ng/well). DNA (µg): Lipofectamine 2000 (µl) ratio of 1:2 in total of 25 µl/well. On DIV 10, Dual-Glo Luciferase Assay System (Promega) was used to measure Firefly luciferase (FFluc) and Renilla luciferase (Rluc) activity levels. Background signal measured from non-transfected cells was subtracted and FFluc levels were normalized to Rluc levels. Each condition was done in triplicate. Data is presented as fold change relative to the Control-shRNA condition treated with bicuculline.

2.9. Quantitative reverse-transcription PCR (TaqMan)

RNA was isolated using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) with additional on-column DNase I digestion, according to the manufacturer's instructions. For RNA isolation from mouse hippocampal tissue, the tissue was rapidly dissected and placed in RNAlater (Sigma, Munich, Germany). For the generation of complementary DNA, RNA was reverse transcribed with the High-Capacity complementary DNA reverse-transcription kit (Applied Biosystems, Foster City, CA, USA). Quantitative reverse-transcription PCR (q-RT-PCR) was performed on a Step One Plus Real Time PCR System (Applied Biosystems, Foster City, CA, USA) using TaqMan gene expression assays (Applied Biosystems, Foster City, CA, USA) for the following mouse genes: *Arc* (Mm00479619_g1), *Egr1* (Mm00656724_m1), *c-Fos* (Mm00487425_m1), *FosB* (Mm00500401_m1), *Gadd45α* (Mm00432802_m1), *Gadd45β* (Mm00345123_m1), *Gadd45γ* (Mm00442225_m1), *Npas4* (Mm00463644_m1), *Nptx2/Narp* (Mm00479438_m1) and *Tgfb2* (Mm00436955_m1). Expression levels of target genes were normalized to the expression of the housekeeping gene *GusB* (Mm00446953_m1). Results were further normalized to uninfected conditions. In case of early-response genes (*Arc* and *c-Fos*) or late-response genes (*Nptx2/Narp* and *Tgfb2*) data was normalized to uninfected conditions at the expression peak, 2h or 6h of bicuculline treatment, respectively. Controls were used to exclude the possibility of DNA or RNA contaminations. Total RNA from human tissue was extracted and cDNA produced as previously described (Temido-Ferreira et al., 2018). The following TaqMan probes were used: *Gadd45α* (Hs00169255_m1), *Gadd45β* (Hs00169587_m1), *Gadd45γ* (Hs00198672_m1). Expression levels of target genes were normalized to the expression of the housekeeping *β*-actin (Hs01060665_g1). Controls were used to exclude the possibility of DNA or RNA contaminations.

2.10. Quantitative reverse-transcription PCR (SYBR Green)

RNA samples were obtained in our previous study (Gulmez Karaca et al., 2018) and were used for qRT-PCR validation. These included the samples analyzed by RNA-seq and independent biological replicates. Total RNA was isolated from hippocampal tissue using the RNeasy Plus Mini Kit (Qiagen) with additional on-

column DNase I digestion, according to the manufacturer's instructions. cDNA was synthesized from 400 ng RNA using the Applied Biosystems High-Capacity Complementary DNA Reverse Transcription Kit (ThermoFisher Scientific). qRT-PCR was performed with the Power SYBR Green PCR Master Mix (Applied Biosystems) on a StepOnePlus Real-Time PCR System (Applied Biosystems). PCR reactions were run as technical triplicates in 10 µL reactions (96-well format) with a final primer concentration of 0.5 µM (each). 2 µL of diluted cDNA (~ 1.25 ng) were used per reaction. The following settings were used for thermo-cycling: 10 min 95°C, 40 cycles of 10s each 95°C, 60°C, 72°C followed by 15s incubation at 95°C. Melt curves were determined by subsequent heating from 60°C to 90°C with a ramp speed of 0.6°C/min. Relative expression levels of each target transcript were determined by the ΔΔCt method using beta-actin mRNA expression levels as reference/normalizer. In vivo samples were obtained by myself and Kübra Gülmez Karaca. Primer design, validation and RT-qPCRs were performed by Janina Kupke and Lukas Frank.

2.11. Reverse transcription polymerase chain reaction

PCR reactions were performed using Q5 High-Fidelity Polymerase (NEB) according to the manufacturer's instructions with a final primer concentration of 0.5 µM (each) and ~1.25 ng of diluted cDNA. The following settings were used for thermo-cycling: 30s 98°C, 30 cycles of 15s of 98°C, 20 s of 60°C, 20s of 72°C followed by 2min 72°C. PCR products were visualized using 2% agarose gels. These experiments were performed by Janina Kupke.

2.12. Western blotting

Hippocampal cultures infected on DIV 4 were lysed on DIV 10 in boiling SDS sample buffer (160 mM Tris-HCl (pH 6.8), 4% SDS, 30% glycerol, 10 mM dithiothreitol, and 0.02% bromophenol blue. The cell lysates were loaded into a 12% acrylamide gel and blotted onto a nitrocellulose membrane (GE Healthcare, Buckinghamshire, UK). In the case of western blotting of tissue samples, the dorsal hippocampus was microdissected from mouse brain and homogenized in RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) supplemented with 1% protease inhibitor cocktail (Sigma-Aldrich, Munich, Germany) and 1% phosphatase inhibitor cocktail II and III (Sigma-Aldrich, P5726, P0044), the

whole procedure was performed at 4°C. Protein concentration was measured by Bradford assay and 20 µg of protein was loaded on a 10% polyacrylamide gel after being denatured at 95°C for 5 min. After SDS-PAGE, gels were blotted onto a nitrocellulose membrane (GE Healthcare, Buckinghamshire, UK) and later blocked in 5% milk and probed with the following antibodies (Table 2.2.). Antibodies were diluted in 5% milk in PBS-T or in 5% BSA in PBS-T, if the antibodies targeted a phospho-variant. Next, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies and later analyzed using a ChemiDoc™ Imaging System (Bio-Rad). Data is presented as ratio of phosphorylated/total protein normalized internally to each uninfected condition. Wester blots were mostly performed by myself with exceptions that are stated in figure legends. Contributions included Janina Kupke and Franziska Mudlaff.

Table 2.2. Antibodies used for western blot analysis.

Antibody	**Molecular Weight (kDa)**	**Dilution**	**Company**
β-Actin	43	1:1000	Santa Cruz, #SC-47778
pATF2	70	1:2500	Cell Signaling #9221
ATF-2	70	1:250	Santa Cruz sc-242
Arc	55	1:6000	Synaptic Systems #156 003
pCREB	46	1:10000 in vitro or 1:6000 in vivo	Millipore #05-667
CREB	46	1:5000	Cell Signaling, #4820
pERK	42/44	1:2500	Cell Signaling #9106
ERK	42/44	1:5000	Cell Signaling, #9102

Fos	62	1:1000	Cell Signaling, #2250
HA	Not applicable	1:1000	Biolegend MMS-101R
pJNK	46/54	1:5000	Cell Signaling #4671
JNK	46/54	1:5000	Cell Signaling, #9258
pc-Jun	48	1:1000	Cell Signaling, #9261s
c-Jun	48	1:250	Santa Cruz, sc-74543
Npas4	100	1:500 1:1000	Santa Cruz sc-168789 Activity Signaling # not available
pp38	38	1:750	BD #612288
p38	38	1:3000	Cell Signaling, #92125s
αTubulin	55	1:400000	Sigma #t9026

2.13. Calcium Imaging

For calcium imaging experiments we used a viral vector that contained the human Synapsin promoter upstream of the recombinant calcium indicator jRGECO1a (Dana et al., 2016). On DIV 4, primary hippocampal cultures were infected with jRGECO1a alone or co-infected with Control-shRNA, Gadd45β-shRNA, Gadd45γ-shRNA1 or Gadd45γ-shRNA2. On DIV 10-11, individual coverslips were transferred to a dish containing CO_2-independent culture medium (CICM), at room temperature. Fluorescence was detected using a cooled charge-coupled device camera (iXon, Andor) through a 20x water immersion objective (LUMPlanFl/IR, Olympus) on an

upright microscope (BX51W1, Olympus). jRGECO1a-infected cells were identified by the presence of a strongly red fluorescent soma (excitation ~560). Data were collected using proprietary software (cellR, Olympus) and analyzed using ImageJ and IgorPro (Wavemetrics, Lake Oswego, OR). During imaging, cells on coverslips were transferred to an imaging chamber containing room-temperature CICM, and the $GABA_A$ receptor antagonist gabazine (100μM), was applied to induce reliable action potential bursting and associated intracellular calcium rises. F_{max} was measured in CICM containing 10μM ionomycin, F_{min} was measured in CICM for 2 min before gabazine treatment. GFP^+ cells were used to draw, with confidence, regions of interest for the analysis of somatic calcium responses. Calcium responses were quantified as a fraction of the binding affinity: $[Ca^{2+}]/K_d=\frac{(F-Fmin)}{(Fmax-F)}$ (Mauceri et al., 2015). A total of 120 coverslips from 6 independent neuronal preparations were analyzed.

2.14. RNA-Sequencing

30 min after training in spatial object recognition task, the infected dorsal hippocampal tissue (identified by mCherry expression) was micro-dissected for RNA-seq analysis. Home-cage mice were not subjected to training, but dissected simultaneously with trained mice to account for time of the day differences. Total RNA was isolated using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) with additional on-column DNase I digestion according to the manufacturer's instructions and 1 μg of total RNA from each sample was used for RNA-seq. Both differential gene expression (DEG) and differential alternative splicing (DAS) expression analysis was initially performed by GATC Biotech (Inview Transcriptome Discover, GATC Biotech AG, Constance, Germany) as previously described (Shen et al., 2012; Gulmez Karaca et al., 2018). In brief, paired-end sequencing libraries prepared from total RNA (see above) were sequenced on an Illumina HiSeq4000 platform. After removal of poor quality and single reads without mates, reads were aligned to the GRCm38/mm10 mouse genome assembly with TopHat/Bowtie (Langmead et al., 2009) guided by the Ensembl v85 transcript annotation, yielding between ~73-101 million mapped reads per sample. DEG analysis was conducted

using Cufflinks/Cuffdiff (Trapnell et al., 2012) which computes per gene FPKM values to then test for differential expression between conditions. DAS analysis was conducted using multivariate analysis of transcript splicing (MATS) (Shen et al., 2012). TopHat/Bowtie aligned reads were used as input for MATS which compares splicing patterns by considering exon-exon junction read counts for transcript variants of a gene. For differential alternative splicing and statistical testing, MATS relies on a multivariate uniform prior to model the between-sample correlation in exon splicing patterns, and a Markov chain Monte Carlo method coupled with a simulation-based adaptive sampling procedure to calculate the p-values and false discovery rates (FDR). A $P_{adjusted}<0.05$ (FDR adjusted P-value) was used as a cut-off for DAS. DASs above the cutoff were analyzed for enrichment of gene ontology (GO) terms and pathways using database for annotation, visualization and integrated discovery (DAVID) v6.8 (Huang da, Sherman, & Lempicki, 2009a, 2009b). Default settings of DAVID were chosen except that the background database was restricted to the pool of genes annotated in our RNA-seq analysis (Gulmez Karaca et al., 2018). Only gene enriched terms with a $-\log_{10}$ P-value<3 ($p_{value}<0.001$) were considered significant. Delta “percent spliced in” (ΔPSI) distribution for two groups considered only DAS events detectable in both conditions tested as DAS events detected only in one of the comparisons were not categorized as unique or common. The DEGs identified in our previous study (Gulmez Karaca et al., 2018) were used for overlap analysis between DEGs and DAS.

2.15. Gene expression omnibus (GEO) accession

The RNA-seq data for alternative splicing analyzed in this study is publicly available at the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus with the accession number GSE107004.

2.16. Primer design

Splice variant specific qRT-PCR and gel shift assay primers were designed with Primer3 (http://primer3.ut.ee) using the RefSeq curated (if available) or the GENCODE VM23 comprehensive transcript annotation and the GRCm38/mm10 mouse genome assembly. Primer specificity and amplicon product sizes were confirmed by BLAST Search and *in silico* PCR (UCSC Genome Browser, mm10).

Primer pair efficiencies and product melting curves were experimentally validated by qRT-PCR on serially diluted cDNA from primary mouse hippocampal cultures. Primers were designed by Janina Kupke and Lukas Frank.

2.17. Experimental Design and Statistical Analysis

Each set of experiments contained mice injected with control or experimental viruses and were randomized per cage (i.e., each cage of four mice contained mice injected with control or experimental viruses). After stereotaxic surgery and until the end of each experiment, the experimenter was blind to the identity of the virus injected into each mouse. For normally distributed data sets, two-tailed unpaired Student's t tests were used to compare two groups. If more than two groups were analyzed simultaneously, a one- or two-way ANOVA was used followed by appropriate multiple comparison *post hoc* tests to control for multiple comparisons as specified. Normally distributed significant data was marked with *. In case of a non-Gaussian distribution, two-tailed Mann-Whitney tests were used to compare two distinct groups or a Kruskal-Wallis test followed by Dunn´s *post hoc* test to compare more than 2 groups. Non-normal distributed significant data was marked with $^{\#}$. The sample size was determined based on similar experiments carried-out in the past and in the literature. All plotted data represent mean ± SEM. Statistical analysis was performed using GraphPad Prism for Mac OS X, version 8. For behavioral experiments the investigators were blind to group allocation during data collection and analysis. All behavioral sessions were video recorded and manual scoring by an experimenter blind to the group identity was performed to determine the exploration of objects during training and testing phases or freezing behavior. For *in vitro* experiments no blinding was performed since the outcome was dependent on software analysis and not manual scoring. All statistical details of experiments can be found in the results section.

Chapter 3. Results

In this thesis, we aimed to uncover molecular mechanisms required for proper cognitive function under physiological conditions and during cognitive aging. To address this question, we first investigated the functional role of the Gadd45 family of proteins in plasticity and memory and during murine aging (Section 3.1). Next, we evaluated changes in expression of Gadd45 proteins during human aging and its consequences to memory processes in the mouse brain (Section 3.2). Afterwards, we examined the requirement for a DNA methylation reader in the mouse hippocampus for learning-associated alternative splicing (Section 3.3). Lastly, we probed the function of a memory-induced transcription factor for memory persistence (Section 3.4).

3.1. Murine age-associated Gadd45γ decrease is linked to cognitive and signaling impairments in mice

In this section we hypothesized that Gadd45 proteins might contribute to age-associated cognitive decline. We first analyzed possible changes in expression of these proteins during murine aging and later using rAAVs to model changes in Gadd45 expression in the hippocampus of young adult mice. This strategy allowed to uncover the physiological role of Gadd45 proteins in cognition by loss-of-function approaches, while simultaneously generating translational data to the aged brain. The results presented in this section are based on (Brito et al., 2020b) and were originally written by the author of this thesis.

3.1.1. Aging reduces Gadd45γ expression in the mouse hippocampus

To investigate a possible link between Gadd45 family members and aging-related cognitive decline, we examined whether aging induces alterations in the expression of Gadd45 family members. We compared Gadd45α, Gadd45β and Gadd45γ mRNA expression in the dorsal hippocampus of young adult and aged mice, which we have previously shown to have impaired long-term memory (Oliveira et al., 2012).

Specifically, we performed qRT-PCR analysis from isolated dorsal hippocampus (dHPC) tissue of young and aged mice that underwent training in an object recognition test or remained in home-cage (HC) conditions (Figure 3.1A). Young adult mice showed similar *Gadd45α* baseline and trained levels. In contrast, training induced the expression of *Gadd45β* and *Gadd45γ* in young adult mice (Figure 3.1B-D). These findings are in agreement with previous studies showing that the expression of Gadd45β and Gadd45γ is regulated by neuronal activity (Ma et al., 2009; Leach et al., 2012; Sultan et al., 2012). Interestingly, we found that dHPC *Gadd45γ* levels were reduced in aged mice, both in HC and trained conditions (Figure 3.1D). This impairment was *Gadd45γ* specific as *Gadd45α* and *Gadd45β* levels were similar in young adult and aged mice. Both groups decreased their exploration during the training sessions and presented comparable total object exploration times (Figure 3.1E,F). Thus, these findings reveal that Gadd45γ expression is compromised in the dorsal hippocampus of aged mice and suggest a role in age-related cognitive decline.

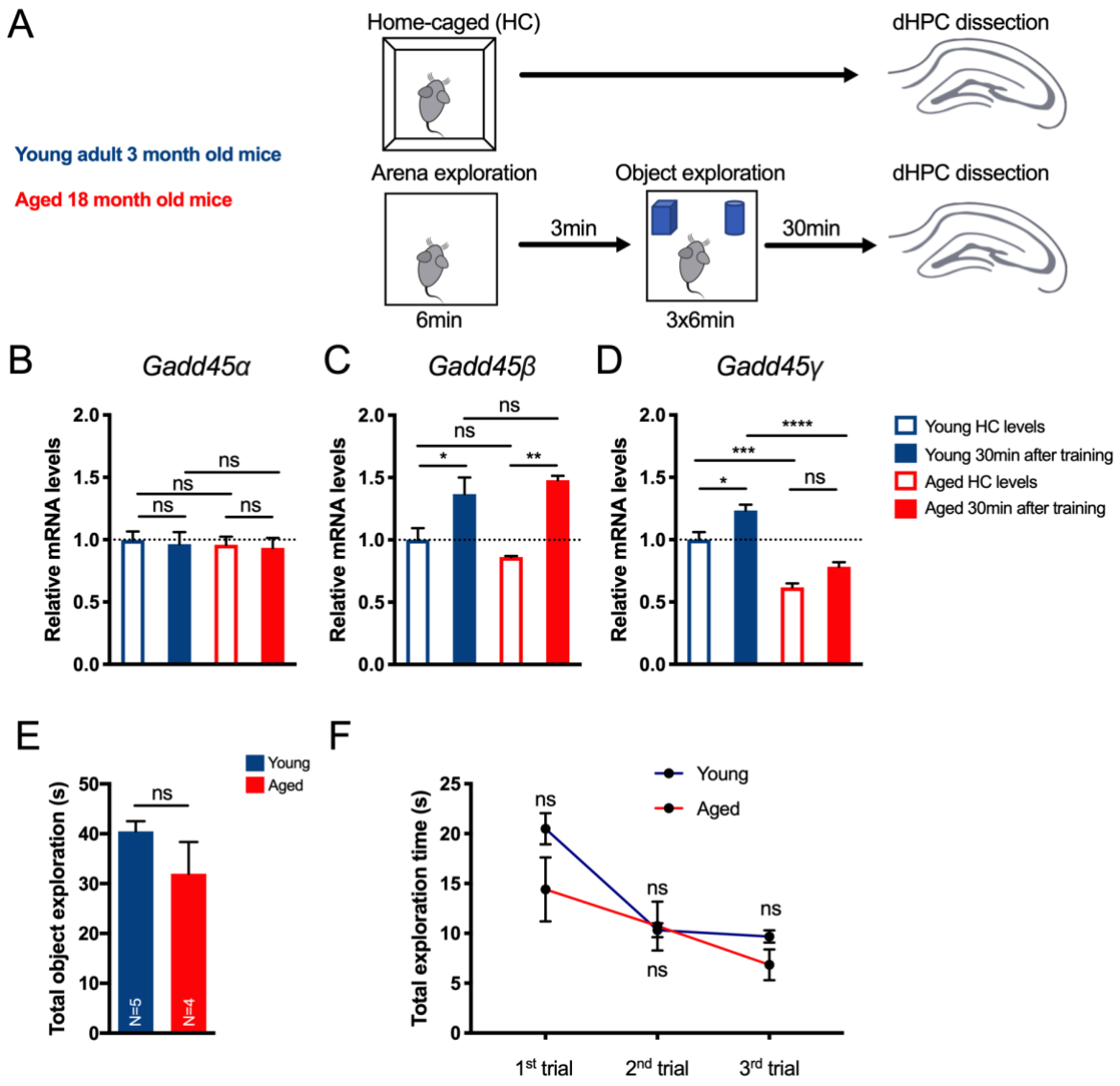


Figure 3.1. Gadd45γ expression is decreased in the hippocampus of aged mice. A) Schematic representation of the experimental design. HC: home cage, dHPC: dorsal hippocampus. **B-D)** qRT-PCR analysis of **B)** Gadd45α, **C)** Gadd45β and **D)** Gadd45γ expression in the dorsal hippocampus of young adult and aged mice in home-cage (HC) conditions or 30 minutes after training in the object location test. Young HC (n=5 mice), Young trained (n=5 mice), Aged HC (n=3 mice), Aged trained (n=4 mice). Expression levels are normalized to young HC levels (dashed line). **E)** Total exploration time during object placement task. **F)** Total exploration time of both objects during each trial session of the object placement task. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 by one-way ANOVA test followed by Bonferroni post hoc test with pairwise comparisons, ns: not significant. Error bars represent SEM.

3.1.2 Reduced Gadd45γ levels impair memory formation in young adult mice

Given our finding that *Gadd45γ* expression is reduced in the hippocampus of cognitively impaired aged mice, we hypothesized that reducing Gadd45γ expression in the hippocampus of young adult mice would promote memory deficits. To explore this hypothesis, we generated recombinant adeno-associated viruses (rAVV) to deliver a control shRNA (Control-shRNA), or a previously characterized Gadd45γ-specific shRNA (Gadd45γ-shRNA1). We used the rAAV1/2 serotype to preferentially knock down Gadd45γ in neurons given its established neuronal tropism (Xu et al., 2001). Additionally, a Gadd45β-specific shRNA sequence (Gadd45β-shRNA) was designed to compare the functions of the two family members. The viral constructs also contained an expression cassette for the humanized renilla reniformis green fluorescent protein (hrGFP) under control of the chicken β-actin promoter that served as an infection marker (Figure 3.2A). Infection rates in primary hippocampal cultures were determined by analyzing the percentage of $hrGFP^+$ cells, which ranged from 80-90% (Figure 3.2B). qRT-PCR analysis revealed that both Gadd45β-shRNA- and Gadd45γ-shRNA1-infected neurons showed a significant and specific decrease in *Gadd45β* and *Gadd45γ* mRNA expression compared to control conditions. This effect was present both in baseline conditions and upon induction of action potential bursting by treatment with the $GABA_A$ receptor antagonist, bicuculline (Figure 3.2D and E). Having established the effectiveness of our knockdown tools, we sought to test if Gadd45β or Gadd45γ knockdown affects locomotor activity or anxiety-like behavior (tendency to thigmotaxis). We employed stereotaxic surgery to deliver rAAV-Control-shRNA, rAAV-Gadd45β-shRNA or Gadd45γ-shRNA1 into the dHPC of young adult mice and performed an open field test two weeks later (Figure 3.2F). We confirmed robust viral-mediated expression in the dHPC of injected animals by assessing GFP expression (Figure 3.2G). No gross anatomical and histological brain changes were observed in Gadd45β or Gadd45γ knockdown conditions. We did not find a significant difference between the three groups in the total distance travelled or the percentage of the time spent in the central zone (Figure 3.2H-J). Thus, our

model allows the evaluation of cognitive functions without confounds originating from impaired locomotion or altered anxiety-like behavior.

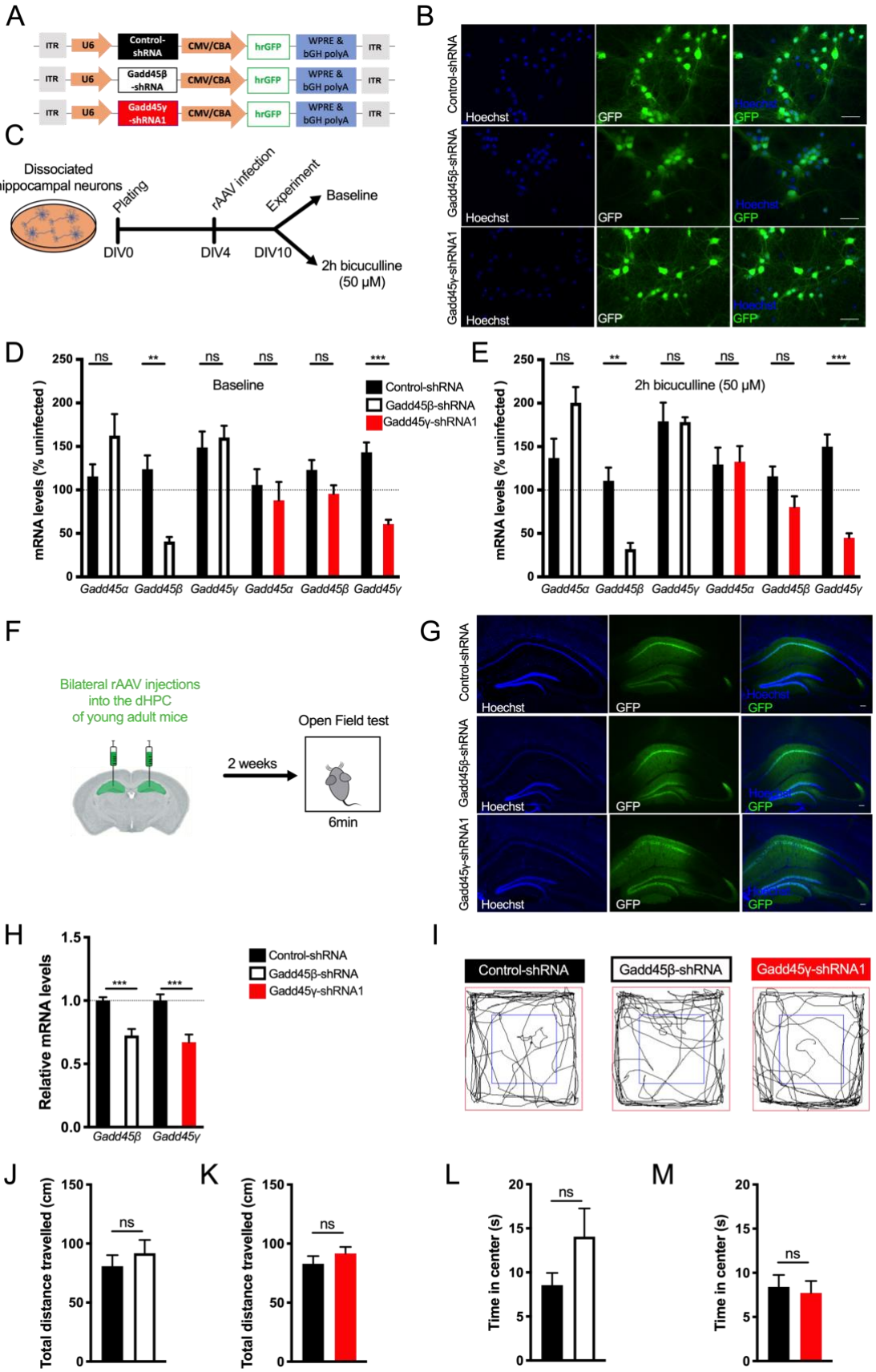

A
ITR
U6
Control-shRNA
Gadd45β-shRNA
Gadd45γ-shRNA1
CMV/CBA
hrGFP
WPRE & bGH polyA
ITR
B
Control-shRNA
Gadd45β-shRNA
Gadd45γ-shRNA1
Hoechst
GFP
C
Dissociated hippocampal neurons
Plating
rAAV infection
Experiment
Baseline
2h bicuculline (50 μM)
DIV0
DIV4
DIV10
D
Baseline
Control-shRNA
Gadd45β-shRNA
Gadd45γ-shRNA1
mRNA levels (% uninfected)
ns
**
***
Gadd45α
Gadd45β
Gadd45γ
E
2h bicuculline (50 μM)
F
Bilateral rAAV injections into the dHPC of young adult mice
2 weeks
Open Field test
6min
G
H
Relative mRNA levels
I
Control-shRNA
Gadd45β-shRNA
Gadd45γ-shRNA1
J
Total distance travelled (cm)
K
L
Time in center (s)
M

Figure 3.2. Characterization of rAAV constructs for shRNA efficiency. A) Schematic representation of the viral constructs used. The viral vector contains a U6 promoter driving either Gadd45β-specific (Gadd45β-shRNA), Gadd45γ-specific (Gadd45γ-shRNA1) or control (Control-shRNA) shRNA sequences and a cytomegalovirus CMV/chicken β-actin (CBA) hybrid promoter driving GFP as an infection marker. bGH polyA: Bovine growth hormone polyadenylation signal. ITR: inverted terminal repeat, WPRE: Woodchuck Hepatitis virus post-transcriptional regulatory element. **B)** Representative images of GFP+ cultured hippocampal cells infected with Control-shRNA, Gadd45β-shRNA or Gadd45γ-shRNA1. Scale bar=40μm. **C)** Schematic representation of the in vitro experimental design. qRT-PCR analysis of Gadd45α, Gadd45β, and Gadd45γ expression **D)** under baseline conditions (n=3-5 independent neuronal cultures) or **E)** after 2h bicuculline treatment (n=3-4 independent neuronal cultures). Expression levels were normalized to the corresponding baseline or bicuculline treated uninfected controls (dashed line). DIV: day in vitro, **p<0.01, ***p<0.001 by Two-tailed unpaired Student's t-test. **F)** Schematic representation of the in vivo experimental design. dHPC: dorsal hippocampus. **G)** Representative images of the dorsal hippocampus of Control-shRNA, Gadd45β-shRNA, or Gadd45γ-shRNA1 groups 4 weeks after stereotaxic surgery. Scale bar=100μm. **H)** qRT-PCR analysis of Gadd45β and Gadd45γ expression in the dorsal hippocampus of Gadd45β-shRNA- (n=8 mice) or Gadd45γ-shRNA1- (n=9 mice) injected mice 30 minutes after object-location training normalized to Control-shRNA (n=9 or 10 mice (dashed line). **I)** Representative exploration traces of all groups during the open field test. Locomotion analysis of **J)** Control-shRNA (n=11 mice) vs. Gadd45βsh-RNA groups (n=12, mice) and **K)** Control-shRNA (n=14, mice) vs Gadd45γ-shRNA groups (n=14, mice), quantified as the total distance travelled during the open field test. Anxiety-like behavior, quantified as the percentage of time spent in the center of the arena during the open field test of **L)** Control-shRNA (n=12 mice) vs. Gadd45βsh-RNA groups (n=12 mice) and **M)** Control-shRNA (n=14 mice) vs Gadd45γ-shRNA groups (n=14 mice). Two-tailed unpaired Student's t-test; ns: not significant. Error bars represent SEM. Open field analysis was performed by Kübra Gülmez Karaca.

Since aging is associated with hippocampus-dependent memory impairments (Erickson and Barnes, 2003; Weber et al., 2015), we subjected mice to hippocampus-dependent memory tasks (Figure 3.3A). Decreasing Gadd45β levels did not change the preference for the displaced object 24h after training in the object-place recognition test (Figure 3.3B left graph). In contrast, Gadd45γ-shRNA1 animals showed an impaired preference for the displaced object 24h after training (Figure 3.3B right graph). This impairment was not due to altered habituation patterns during the training trial sessions or to differences in object exploratory behavior (data not shown). Next, we tested the same cohort of animals in contextual

fear conditioning. Similarly, to what we observed in the object-place recognition test, the Gadd45β-shRNA group did not show long-term memory impairments (Figure 3.3C left graph). Knockdown of Gadd45γ in the dHPC of young adult mice, however, promoted long-term memory deficits in contextual fear memory 24h after training (Figure 3.3D right graph). This impairment was not a result of distinct responsiveness to the shock administration between the groups during the training session. Hence, this set of experiments shows that Gadd45γ is required for hippocampus-dependent long-term memory formation.

We next subjected mice to the same hippocampus-dependent memory tasks as above, but this time employed shorter time delays between training and testing to evaluate short-term memory (1h or 5min delay after training) (Figure 3.3F). In line with the above-mentioned findings, Gadd45β knockdown mice showed intact memory compared to control animals in both tasks (Figure 3.3G,I). In contrast, mice transduced with Gadd45γ-shRNA1 showed short-term memory impairments in locating the displaced object in the object location test 1h after training (Figure 3.3H). These effects were specific to object location memory since no deficits were observed in contextual fear conditioning (Figure 3.3J). Knocking-down Gadd45γ did not promote memory impairments when a recall session was performed 5min after object-place recognition training or contextual fear conditioning, showing that memory acquisition and early short-term memory are not compromised in Gadd45γ-shRNA1 animals (Figure 3.3K,L). Altogether this set of experiments indicates that hippocampal Gadd45γ is required for long-term memory and late stages of short-term recognition memory. Interestingly, mimicking the aging-associated reduction in Gadd45γ expression levels in the young adult mouse hippocampus seems to phenocopy previously described age-like memory impairments (Erickson and Barnes, 2003; Kennard and Woodruff-Pak, 2011; Burke et al., 2012). Rodents, monkeys, and humans show task-specific and delay-specific short-term memory impairments during aging (Erickson and Barnes, 2003; Burke et al., 2012). In contrast to contextual fear condition, tasks that assess forms of recognition memory are more sensitive to aging (Kennard and Woodruff-Pak, 2011).

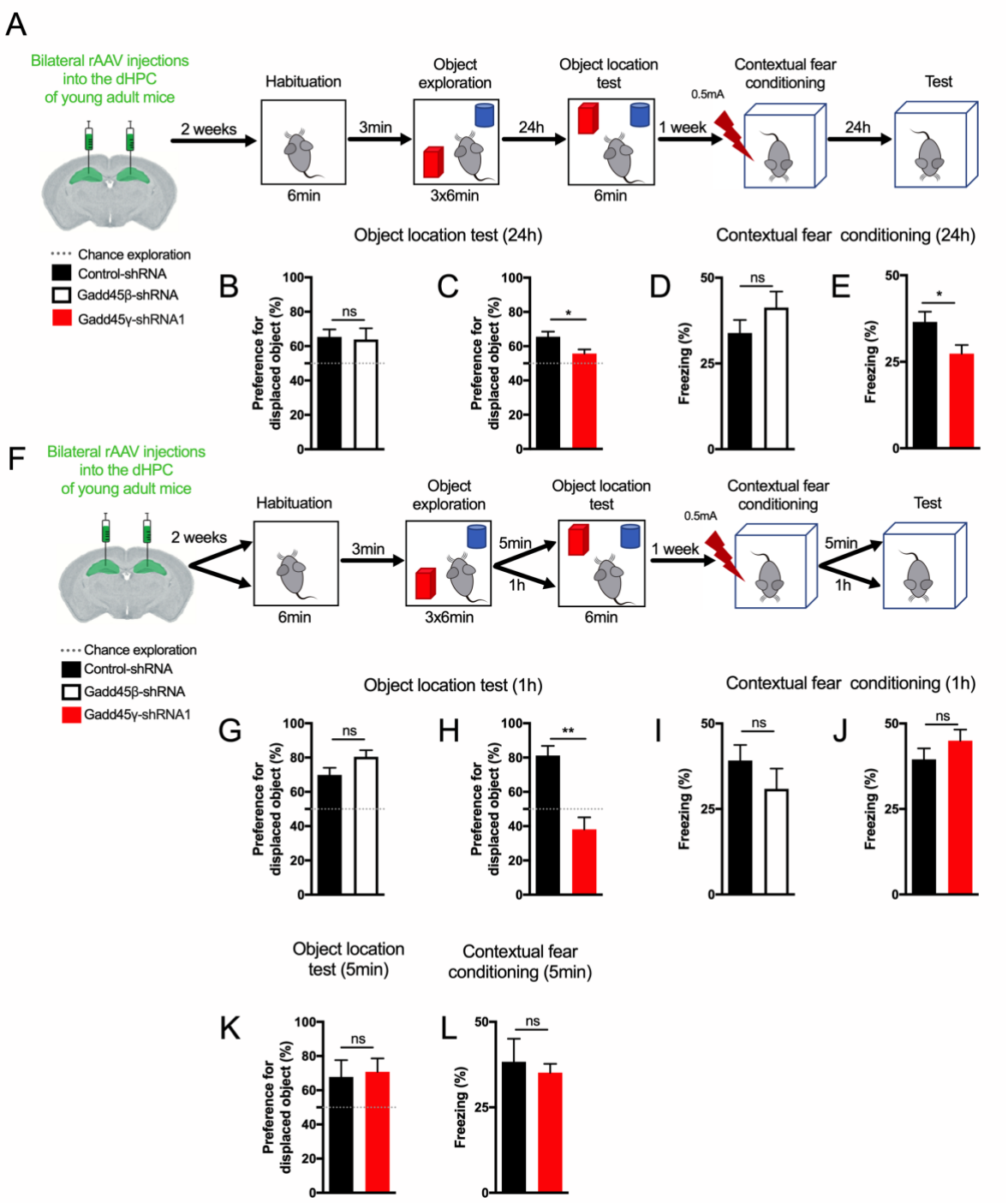


Figure 3.3. **Reducing hippocampal Gadd45γ levels in young adult mice impairs long-memory and short-term memory in a delay-specific manner. A)** Schematic representation of the experimental design for long-term memory tests. dHPC: dorsal hippocampus. 24h object location memory test of young adult mice expressing **B)** Control-shRNA (n=8 mice), Gadd45β-shRNA (n=8 mice), or **C)** Control-shRNA (n=12 mice) Gadd45γ-shRNA1 (n=13 mice) in the dHPC. The dashed

line represents equal preference for either object (chance preference). 24h contextual fear memory test of young adult mice expressing **D)** Control-shRNA (n=8, mice), Gadd45β-shRNA (n=8, mice), or **E)** Control-shRNA (n=16, mice), Gadd45γ-shRNA1 (n=15, mice) in the dHPC. **F)** Schematic representation of the experimental design for short-term memory tests. dHPC: dorsal hippocampus. 1h object location memory test of young adult mice expressing **G)** Control-shRNA (n=7 mice), Gadd45β-shRNA (n=7 mice), or **H)** Control-shRNA (n=5 mice), Gadd45γ-shRNA1 (n=5 mice) in the dHPC. 1h contextual fear memory test of young adult mice expressing **I)** Control-shRNA (n=7 mice), Gadd45β-shRNA (n=8 mice), or **J)** Control-shRNA (n=8 mice), Gadd45γ-shRNA1 (n=8, mice) in the dHPC. 5min object location memory test **K)** and contextual fear conditioning test **L)** of young adult mice expressing Control-shRNA (n=6 mice) or Gadd45γ-shRNA1 (n=6 mice) in the dHPC. *p<0.05, **p<0.01 by two-tailed unpaired Student's t-test, ns: not significant. Error bars represent SEM.

3.1.3 Gadd45γ regulates MAPK signaling and downstream transcription factor activation

Gadd45 family members are known to regulate MAPK signaling cascades in several tissues outside of the nervous system through the interaction with kinases upstream of JNK and p38 MAPK, but not ERK (Takekawa and Saito, 1998; Tornatore et al., 2008). Considering that both short- and long-lasting neuronal adaptive responses have been suggested to require JNK/p38 activity (Alonso et al., 2003; Bevilaqua et al., 2003; Guan et al., 2003; Sherrin et al., 2010; Giese and Mizuno, 2013; Morel et al., 2018), and Gadd45γ regulates these pathways, we next asked whether Gadd45 regulates MAPK signaling in hippocampal neurons. We addressed this by knocking down Gadd45β or Gadd45γ expression in primary hippocampal cultures and by measuring the phosphorylation levels of JNK, p38 and ERK in response to increased neuronal activity (Figure 3.4A). To rule out possible off-target effects we confirmed our findings with a second Gadd45γ-specific shRNA sequence (Gadd45γ-shRNA2). Infection rates and functional knock-down of this rAAV were similar to previously validated shRNAs (Figure 3.4B-D). Reduction of Gadd45β expression did not affect JNK, p38 and ERK phosphorylation levels compared to control conditions (Figure 3.4E-H). In contrast, reduced Gadd45γ resulted in impaired JNK and p38 phosphorylation (Figure 3.4E-G), whereas ERK phosphorylation was not affected (Figure 3.4E,H).

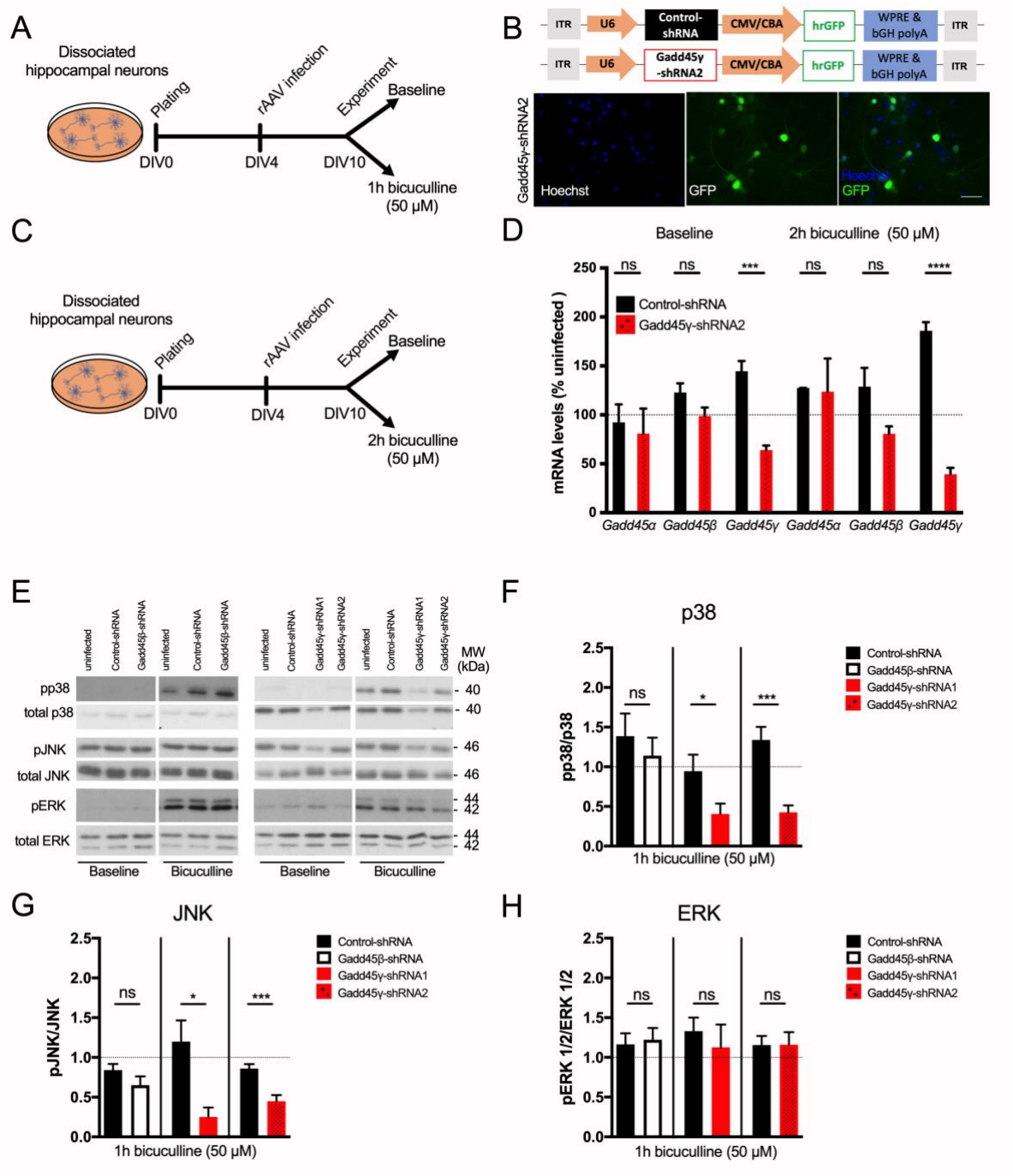


Figure 3.4. Gadd45γ regulates MAPK signaling in mouse hippocampal cultures. A) Schematic representation of the experimental design. DIV: day *in vitro.* **B)** Schematic representation of Gadd45γ-shRNA2 viral construct. The viral vector contains a U6 promoter driving Gadd45γ-specific (Gadd45γ-shRNA2) and a cytomegalovirus (CMV)/chicken β-actin (CBA) hybrid promoter driving GFP as an infection marker. Representative images of cultured hippocampal cells infected with this virus are shown. Scale bar=40µm. bGH polyA: Bovine growth hormone polyadenylation signal. ITR: inverted terminal repeat, WPRE: Woodchuck Hepatitis virus post-transcriptional regulatory element. **C)**

Schematic representation of the experimental design. DIV: day *in vitro.* **D)** qRT-PCR analysis of Gadd45α (n=2 independent neuronal cultures), Gadd45β (n=5 independent neuronal cultures), and Gadd45γ (n=5 independent neuronal cultures) expression levels in hippocampal cultured cells infected with Control-shRNA or Gadd45γ-shRNA2 under basal conditions (left) or after 2h bicuculline treatment (right). Expression levels were normalized to the corresponding uninfected controls in baseline conditions or upon bicuculline treatment (dashed line). **E)** Representative immunoblot scans using phosphospecific and total protein antibodies in hippocampal cultures infected with rAAV expressing Control-shRNA, Gadd45β-shRNA, Gadd45γ-shRNA1 or Gadd45γ-shRNA2. Hippocampal cultures were harvested at baseline conditions or after 1h of bicuculline treatment. Immunoblot quantification of **F)** p38 (n=5-8 independent neuronal cultures) **G)** JNK (n=5-8 independent neuronal cultures) and **H)** ERK (n=4-7 independent neuronal cultures) presented in ratios of phosphorylated / total protein normalized to corresponding uninfected 1h bicuculline condition (dashed line). *p<0.05, ***p<0.001, ****p<0.0001 by two-tailed unpaired Student's t test, ns: not significant. Error bars represent SEM. Western Blots were partially performed by Janina Kupke.

To further explore the functional consequences of Gadd45γ reduction, we analyzed the phosphorylation of transcription factors downstream of JNK and p38, such as ATF-2/CREB2, c-Jun, and CREB (Figure 3.5A). We observed that in Gadd45β-shRNA conditions, the phosphorylated forms of these transcription factors were similar to controls (Figure 3.5B-E). However, the phosphorylation of c-Jun, ATF-2 and CREB was significantly diminished when Gadd45γ levels were reduced (Figure 3.5B-E). These findings provide evidence that Gadd45γ regulates MAPK signaling in neurons.

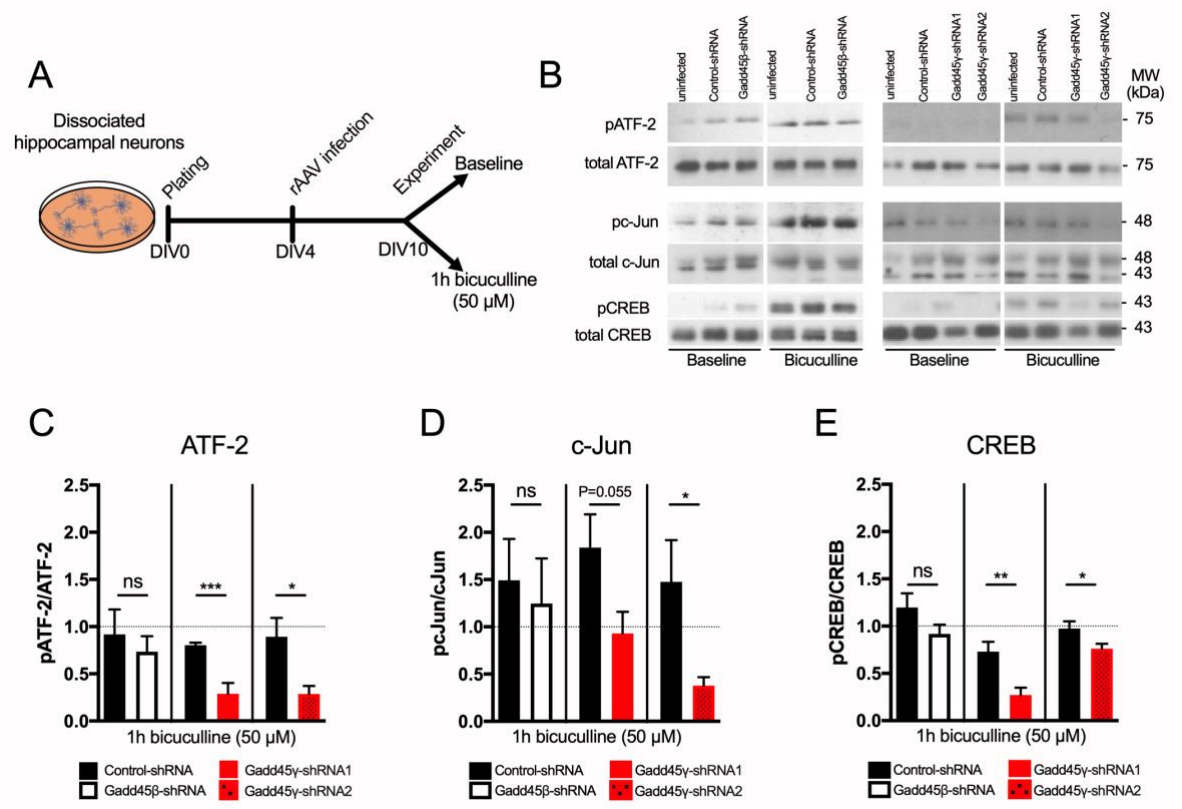


Figure 3.5. Gadd45γ regulates the phosphorylation of downstream transcription factors in hippocampal mouse neuronal cultures. A) Schematic representation of the experimental design. DIV: day *in vitro.* **B)** Representative immunoblot scans using phosphospecific and total protein antibodies in hippocampal cultures infected with rAAV expressing Control-shRNA, Gadd45β-shRNA, Gadd45γ-shRNA1 or Gadd45γ-shRNA2. Hippocampal cultures were harvested at baseline conditions or after 1h of bicuculline treatment. Immunoblot quantification of **C)** ATF-2 (n=4-6 independent neuronal cultures) **D)** c-Jun (n=5-6 independent neuronal cultures) and **E)** CREB (n=5-7 independent neuronal cultures) presented in ratios of phosphorylated / total protein normalized to uninfected 1h bicuculline condition (dashed line). *p<0.05, **p<0.01, ***p<0.001 by two-tailed unpaired Student's t test, ns: not significant. Error bars represent SEM. Western Blots were partially performed by Janina Kupke.

3.1.4 Gadd45γ regulates CRE and AP-1 transcriptional activity and downstream activity-dependent gene transcription

Given the disruption in the phosphorylation of CREB and in the components of AP-1 complex [c-Jun, ATF-2, Fos (Alberini, 2009)], we next aimed at testing whether Gadd45γ reduction would impair AP-1- and CREB-dependent transcriptional activity.

This analysis was further motivated by the established roles of CREB and AP-1 in memory formation (Alberini, 2009) and by studies linking CREB dysfunction and aging (Benito and Barco, 2010; Oliveira and Bading, 2011; Yu et al., 2017b; Yu et al., 2017a). We performed luciferase reporter assays using a reporter plasmid containing the firefly luciferase under the control of CRE or AP-1 binding sites in primary hippocampal cultures (Figure 3.6A). To this end, we co-transfected constructs encoding CRE-luciferase or AP-1-luciferase and Control-shRNA, Gadd45β-shRNA, Gadd45γ-shRNA1, or Gadd45γ-shRNA2 (Figure 3.6B). Luciferase activity was assessed both under basal conditions and after induction of neuronal activity (Figure 3.6C,D). In agreement with our data showing regulation of MAPK signaling specifically by Gadd45γ, Gadd45β reduction did not affect CRE or AP-1 transcriptional activity (Figure 3.6C,D). In contrast, reducing Gadd45γ expression significantly impaired luciferase expression in both assays (Figure 3.6C,D). These results revealed that Gadd45γ expression is required for baseline AP-1 and activity-induced AP-1 and CRE transcriptional activity. To determine whether Gadd45γ regulates the expression of endogenous CRE and AP-1 target genes, we monitored the expression of CREB- (Impey et al., 2004) (*Arc* and *c-Fos*) and AP-1-dependent genes (Malik et al., 2014) (*Nptx2*/*Narp* and *Tgfb2*) that are regulated by synaptic activity and which expression has been shown to be dysregulated with aging (*Arc* and *Nptx2*/*Narp*) (Ryan et al., 2019b) (Figure 3.6E). CREB- and AP-1-dependent gene expression was disrupted in response to increased neuronal activity in Gadd45γ knockdown conditions (Figure 3.6F-I). Moreover, in line with luciferase assays, CREB target genes were predominantly decreased in an activity-dependent manner while AP-1-target genes showed consistent baseline and activity-dependent decreases when Gadd45γ was reduced. Gadd45β knockdown did not promote any changes in gene expression compared to control conditions. These results show that Gadd45γ regulates CREB- and AP-1-dependent gene expression in neurons. It should be noted that cross-talk between both signaling pathways may occur since we detected deficits in *c-Fos* expression, a component of the AP-1 complex (Alberini, 2009).

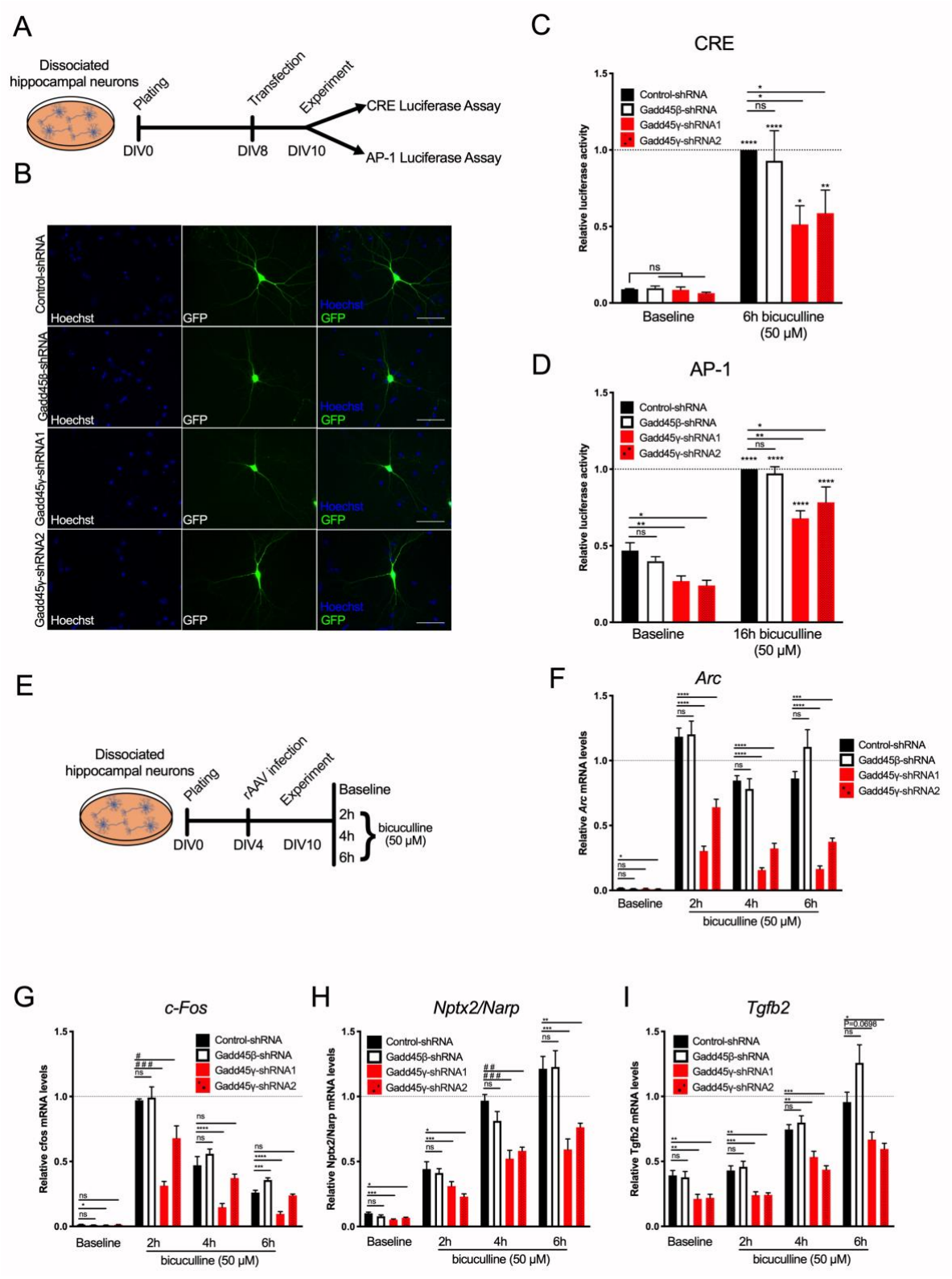


Figure 3.6. Gadd45γ regulates CREB and AP-1 activity and downstream gene expression in mouse hippocampal cultures. A) Schematic representation of the experimental design. DIV: day *in*

vitro. **B)** Representative images of hippocampal cultured cells co-transfected with Control-shRNA, Gadd45β-shRNA, Gadd45γ-shRNA1, or Gadd45γ-shRNA2 and luciferase reporter plasmids. Scale bar=40µm. Luciferase reporter assays were performed using dissociated hippocampal cultures co-transfected with Control-shRNA, Gadd45β-shRNA, Gadd45γ-shRNA1, or Gadd45γ-shRNA2 constructs, and reporter plasmids expressing firefly luciferase (FFluc) under the control of **C)** CRE (n=5 independent neuronal cultures) or **D)** AP-1 (n=7 independent neuronal cultures) transcriptional activity. **C,D)** Shown is luciferase activity as measured from untreated hippocampal cultures and cultures treated for 6h or 18h with bicuculline. Values are normalized to Control-shRNA condition treated with bicuculline for the respective time duration (6h or 8h), (dashed line). **E)** Schematic representation of the experimental design for qRT-PCR analysis ofthe expression of the CREB-dependent genes **F)** *Arc* (n=7-8 independent neuronal cultures) and **G)** *c-Fos* (n=6-8, independent neuronal cultures) or the AP-1-dependent genes **H)** *Nptx2*/*Narp* (n=6-7 independent neuronal cultures) and **I)** *Tgfb2* (n=6-7 independent neuronal cultures). Hippocampal cultures were harvested at baseline conditions or after 2h, 4h or 6h of bicuculline treatment. Uninfected controls treated with bicuculline were used for normalization at the corresponding peak of gene expression: 2h (*Arc* and *c-Fos*) or 6h (*Nptx2*/*Narp* and *Tgfb2*) (dashed line). DIV: day *in vitro,* *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 by one-way ANOVA test followed by Sidak's or Dunnett's *post hoc* test with pairwise comparisons and $^{\#}$p<0.05, $^{\#\#}$p<0.01 and $^{\#\#\#}$p<0.001 Kruskal-Wallis test followed by Dunn´s post hoc test was used for non-gaussian distribution. ns: not significant. Error bars represent SEM.

To confirm that the effects seen in MAPK signaling and downstream transcription factor activity were directly dependent on Gadd45γ function and not due to an overall disruption of neuronal responses and/or calcium-dependent signaling pathways, we assessed calcium rises triggered by neuronal activity. We measured intracellular calcium dynamics using the genetically encoded calcium indicator jRGECO1a (Figure 3.7A) in primary hippocampal cultures co-infected with the above-described shRNA constructs), which has high sensitivity in dissociated neurons. Co-expression of shRNA constructs and jRGECO1a was reliably determined using GFP/mCherry fluorescence, and only GFP^+ cells were analyzed. Neurons were treated with the $GABA_A$ antagonist gabazine to induce action potential bursting (Figure 3.7B-D). No differences in the amplitude (Figure 3.7E) or frequency (Figure 3.7F) of action potential bursts were detected in Gadd45β-shRNA-, Gadd45γ-shRNA1-, or Gadd45γ-shRNA2-infected neurons. This set of experiments indicates that deficits in MAPK signaling and activity-dependent gene expression promoted by Gadd45γ reduction are not explained by altered calcium responses to neuronal activity. This

supports the hypothesis that, similar to its function in other cell types (Takekawa and Saito, 1998; Tornatore et al., 2008), Gadd45γ directly regulates MAPK signaling in neurons.

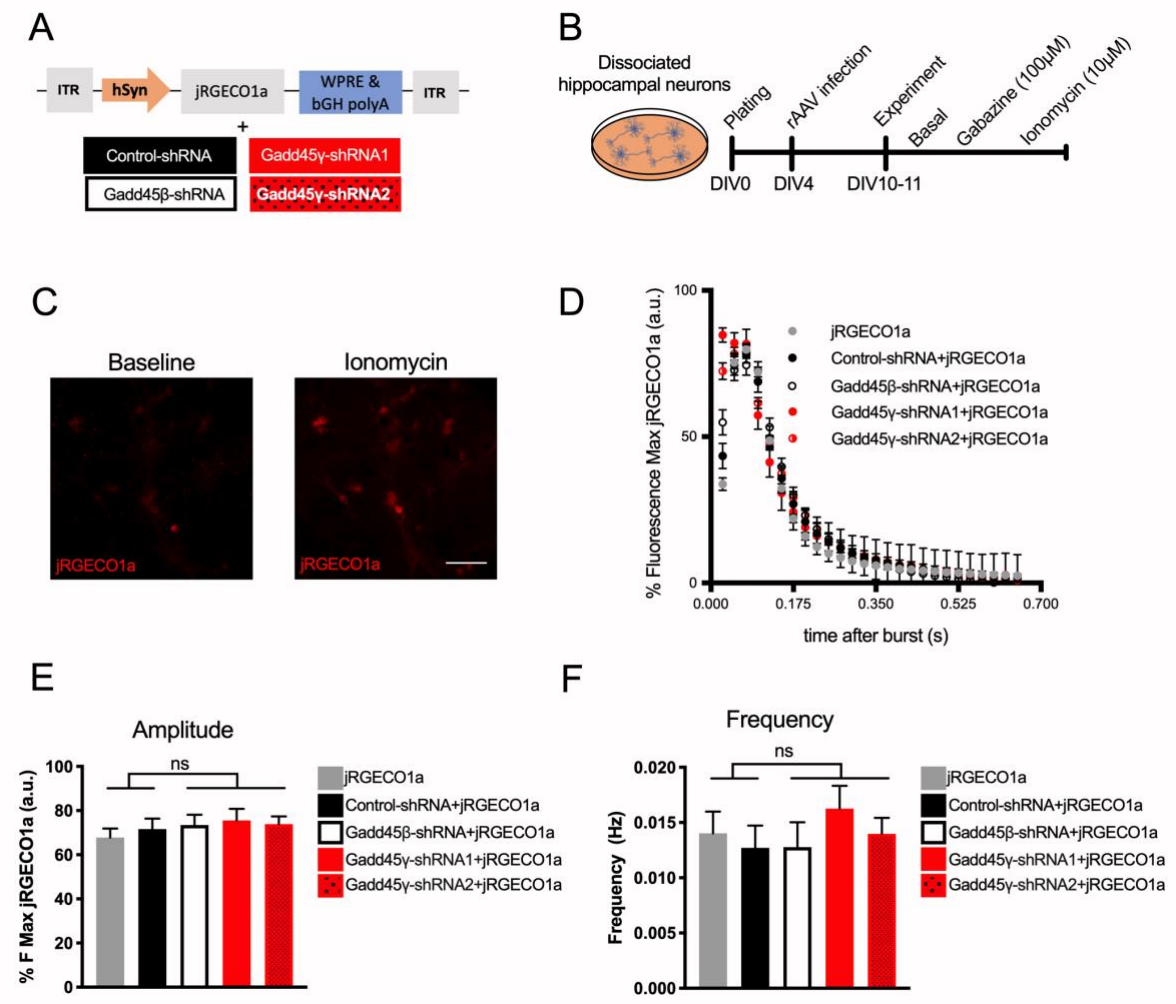


Figure 3.7. Gadd45γ reduction does not alter calcium dynamics in response to neuronal activity. A) Schematic representation of the viral constructs used. The viral vector driving jRGECO1a expression does so under control of the human synapsin (hSyn) promoter. Neuronal cultures were either singly infected with jRGECO1a or co-infected with jRGECO1a and Control-shRNA, Gadd45β-shRNA, Gadd45γ-shRNA1, or Gadd45γ-shRNA2. bGH polyA: Bovine growth hormone polyadenylation signal. ITR: inverted terminal repeat, WPRE: Woodchuck Hepatitis virus post-transcriptional regulatory element. **B)** Schematic representation of the experimental design. DIV: day *in vitro.* **C)** Images of representative cultured hippocampal cells infected with jRGECO1a at baseline or after ionomycin treatment. Scale bar=40μm. **D)** Representative traces showing the first gabazine-evoked burst of all groups. **E)** Amplitude (n=6 independent neuronal cultures) and **F)** frequency (n=6 independent neuronal cultures) of evoked action potential bursts during gabazine treatment in all

groups. One-way ANOVA test followed by Sidak's Multiple Comparisons Test with pairwise comparisons was used, ns: not significant. Error bars represent SEM.

3.2. Human age-related Gadd45γ increase is associated with cognitive and plasticity impairments in mice

In this section we evaluated if Gadd45 expression is compromised similarly to what we detected in the mouse brain. First using post-mortem hippocampal tissue from young and aged subjects we analyzed changes in Gadd45 expression and modeled detected changes in the hippocampus of young adult mice. This strategy revealed that similarly to our previous findings (Brito et al., 2020b) Gadd45 expression is altered in Human aging which is associated with impaired memory and plasticity events. The results presented in this section are based on (Brito et al., 2020c) and were originally written by the author of this thesis.

3.2.1. Aging increases Gadd45γ expression in the human hippocampus

Aberrant gene expression patterns are an evolutionarily conserved hallmark of aging. However, no overall correlation between age-associated gene expression in mice and humans has been detected (Zahn et al., 2007). We asked whether Gadd45γ expression in human aged hippocampus would be compromised as observed in mice (Brito et al., 2020b). We analyzed the expression of Gadd45 family members in young and aged human hippocampi as previously described (21–65 years old) (Temido-Ferreira et al., 2018) (Figure 3.8A). We did not find any correlation between age and *Gadd45α* expression. Interestingly, we found a significant positive correlation between age and increased hippocampal *Gadd45β* and *Gadd45γ* levels (~4.8 and ~8.6 fold, respectively). This result suggests that age-related Gadd45 expression changes in the hippocampus is not conserved in mice and humans.

3.2.2. Increase in Gadd45γ expression leads to impairments in spatial recognition memory

Next, we aimed to model the human-specific aging conditions in the mouse hippocampus and determine the cellular and behavioral consequences of neuronal Gadd45γ overexpression. We focused on Gadd45γ given previous studies that showed a selective function for Gadd45γ in memory formation (Li et al., 2018; Brito et al., 2020b). We delivered by stereotaxic surgery a viral vector containing the

mouse CamKIIα promoter driving the expression of Gadd45γ or GFP as a control into the dorsal hippocampus (dHPC) of young adult mice (Figure 3.8B). We validated dHPC viral expression by assessing GFP expression and *Gadd45γ* mRNA levels (Figure 3.8C-D). Neither groups showed anatomical or histological brain abnormalities. Two weeks after stereotaxic surgery, before assessing cognitive function, we conducted an open field test (Figure 3.8E) to verify whether Gadd45γ overexpression affects locomotor activity or anxiety-like behavior. Total distance travelled and the percentage of the time spent in the central zone were similar between groups (Figure 3.8F-H). Next, we assessed long-term memory in the object-place recognition test and contextual fear conditioning. Increasing Gadd45γ expression in the dHPC of young mice impaired preference for the displaced object 24h after learning (Figure 3.8I). In contrast, Gadd45γOE mice showed intact long-term memory in contextual fear conditioning (Figure 3.8J). Impairment in the object-place recognition test was not due to altered habituation patterns during the training trial sessions or altered object exploratory behavior (Figure 3.8K-L). Importantly, both groups presented similar responses to shock administration during contextual-fear conditioning training (Figure 3.8N).

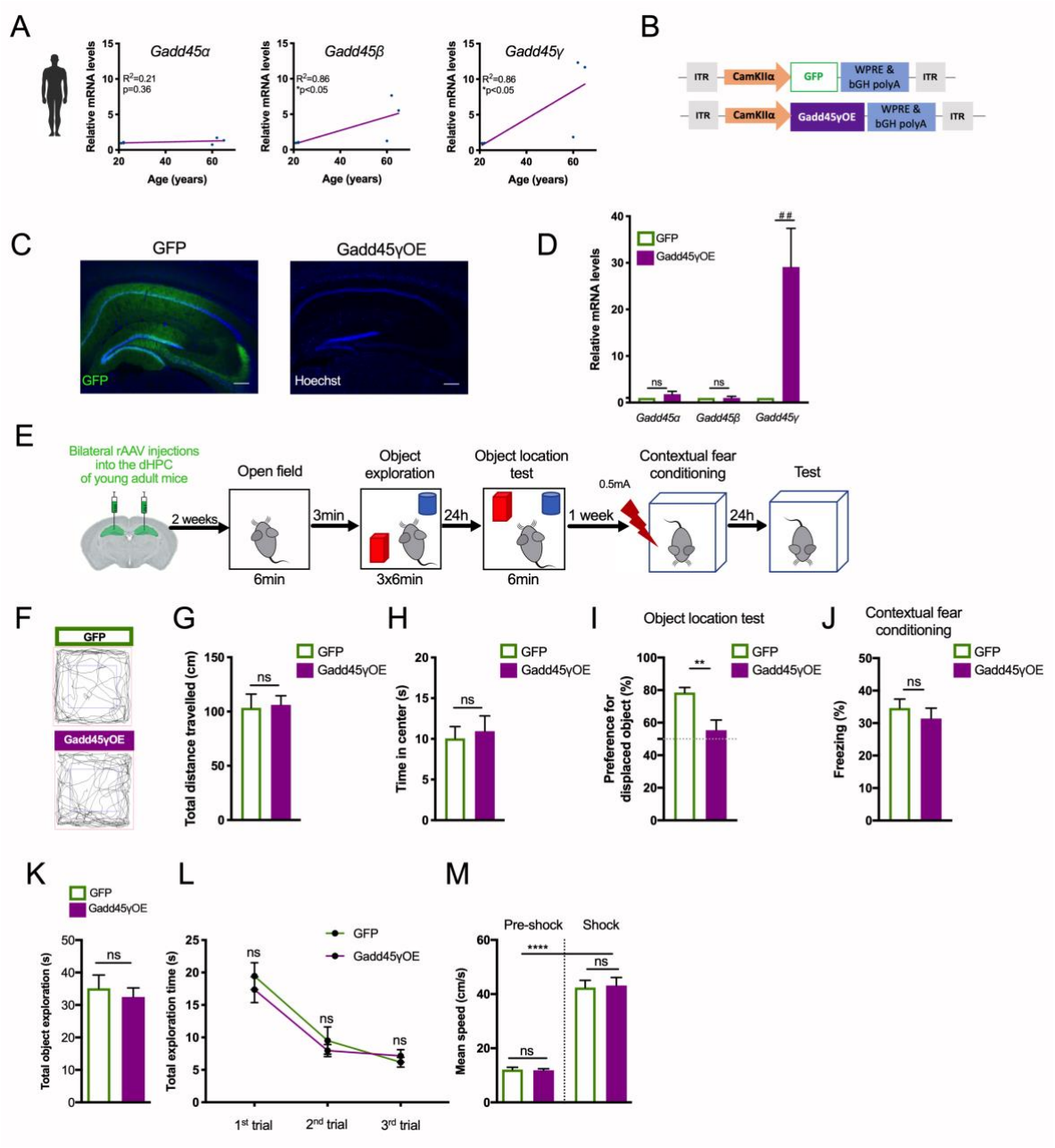


Figure 3.8. Human hippocampal Gadd45γ expression is increased during aging and overexpressing Gadd45γ in the mouse hippocampus impairs object location memory. A) Correlational analysis between the expression of *Gadd45α, Gadd45β* and Gadd45γ in human *postmortem* hippocampal tissue and donors' age (N=6). Correlation analysis was performed using Pearson correlation coefficient or Spearman correlation. **B)** Schematic representation of the viral constructs used. The viral vector contains a CamKIIα promoter driving Gadd45γ overexpression (Gadd45γOE) or GFP as a control (GFP). **C)** Representative images of the dorsal hippocampus injected with viruses leading to either Gadd45γ-specific overexpression (Gadd45γOE) or the control expression of GFP, 4 weeks after stereotaxic surgery. Scale bar=100µm. **D)** qRT-PCR analysis of

Gadd45α, *Gadd45β*, and *Gadd45γ* expression levels in cultured hippocampal cells infected with GFP or Gadd45γOE in baseline conditions (N=6 independent cell preparations). **E)** Schematic representation of the experimental design for behavioral tests. **F)** Representative exploration patterns of all groups during open field test. **G)** Locomotion analysis of the different groups measured as the total distance travelled during the open field test (N=8-9). **H)** Anxiety-like behavior analysis measured as percentage of time spent in the center of the arena during the open field test (N=8-9). **I)** 24h object location memory test of young adult mice expressing GFP or Gadd45γOE in the dHPC (N=13-15). Dashed line represents equal preference for either object (chance preference). **J)** 24h contextual fear memory test of young adult mice expressing GFP or Gadd45γOE in the dHPC (N=9). **K)** Total object exploration time during the training session of the object-location task. One-way repeated measure ANOVA was used (N=9). **L)** Total object exploration time during each trial of the training session compared to the first trial. Similar habituation patterns were observed between groups (N=9). Two-tailed unpaired Student's t-test was used. **M)** Mean speed during the different phases of the contextual fear conditioning training, showing similar performance between groups. A one-way ANOVA followed by a Bonferroni's Multiple Comparisons Test was used (N=9). ns: not significant; **p<0.01 by two-tailed unpaired Student's t-test. Data are normalized to the uninfected control. Kruskal-Wallis Test followed by a Dunn's Multiple Comparisons Test was used. $^{\#\#}$p<0.01, **p<0.01 and ****p<0.0001. Error bars represent SEM. Open field analysis was performed by Kübra Gülmez Karaca.

3.2.3. Increase in Gadd45γ expression disrupts activity-dependent CREB signaling and gene expression

As in the previous section we showed that Gadd45γ regulates CREB activity (Brito et al., 2020b), we next investigated if Gadd45γ overexpression would impact this cellular response. First, we assessed whether Gadd45γ overexpression in the hippocampus of young adult mice (Figure 3.9A) affects the levels of phosphorylated CREB in baseline conditions. In agreement with a role for Gadd45γ in signaling pathways that regulate CREB activation (Brito et al., 2020b), overexpression of Gadd45γ resulted in increased levels of phosphorylated CREB (Figure 3.9B,C). Next using primary hippocampal cultures, we analyzed the phosphorylation of CREB in baseline conditions and in response to increased neuronal activity (Figure 3.9D). Similar to our in vivo findings (Figure 3.9A-C), Gadd45γ overexpression in primary hippocampal cultures led to increased levels of phosphorylated CREB in basal conditions. Moreover, this effect appeared to blunt a response to neuronal activity as CREB phosphorylation in response to neuronal activity did not reach controls

levels (Figure 3.9E,F). We next assessed the expression of the CREB-dependent genes *Arc*, *FosB*, *c-Fos, Egr1 and Npas4* (Impey et al., 2004; Rao-Ruiz et al., 2019) in basal conditions and upon neuronal activity (Figure 3.9G). Hippocampal neuronal cultures infected with rAAV-Gadd45γOE revealed a global disruption of CREB-dependent gene expression in response to increased neuronal activity compared to control conditions (Figure 3.9H-L). Taken together, these findings demonstrate that an increase in Gadd45γ above physiological levels disrupts the expression of memory-related genes and cognitive function.

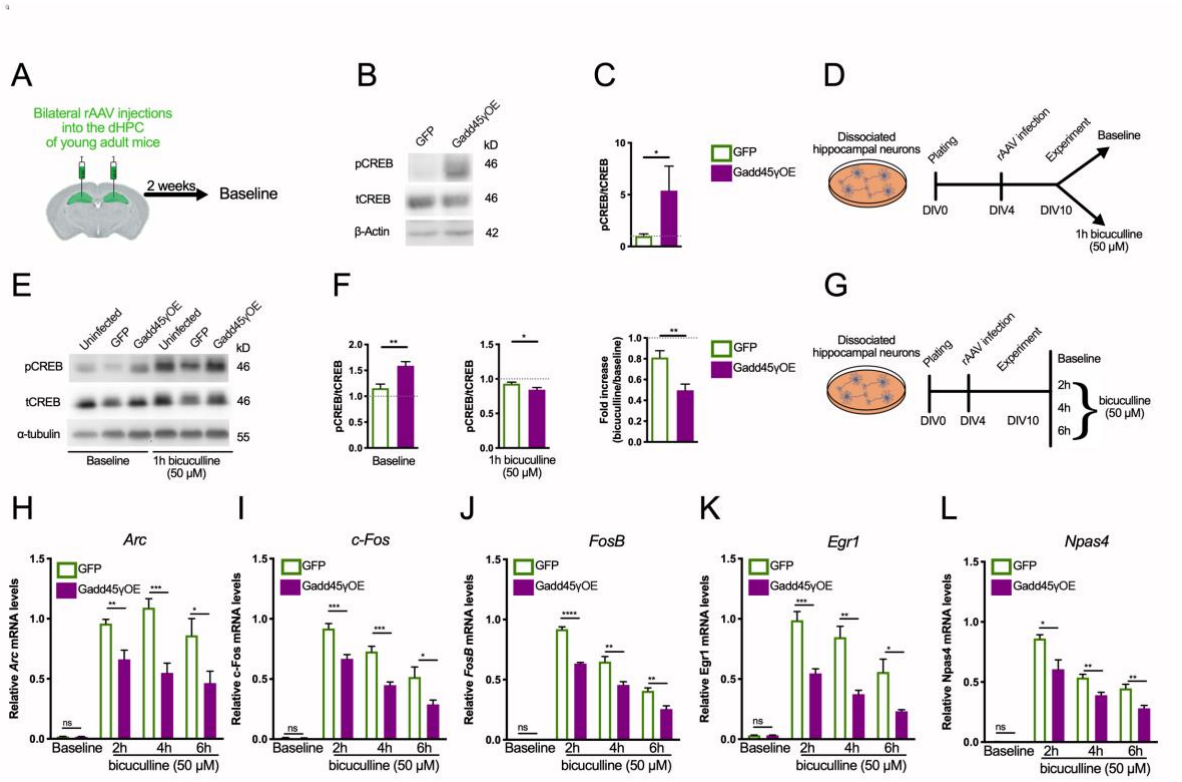


Figure 3.9. Increased Gadd45γ expression dysregulates hippocampal CREB phosphorylation and activity-dependent gene expression. A) Schematic representation of the experimental design for western blot analysis of CREB activation *in vivo*. **B)** Representative immunoblots of hippocampal tissue from young adult mice infected with rAAVs expressing GFP or Gadd45γOE using phosphospecific (pCREB) and total CREB (tCREB) antibodies. **C)** Immunoblot quantification shown as ratios of phosphorylated/total protein normalized to GFP control (N=5-6). **D)** Schematic representation of the experimental design for western blot analysis of CREB activation in mouse hippocampal cultures. **E)** Representative immunoblot scans of hippocampal cultures infected with rAAV expressing GFP or Gadd45γOE using phosphospecific and total CREB antibodies. **F)** Immunoblot quantification shown as ratios of phosphorylated/total protein normalized to uninfected

control in baseline or bicuculline-treated conditions (left and middle graphs) or pCREB fold increase (bicuculline/baseline) for each condition and normalized to uninfected control (right graph) (N=7-8 independent cell preparations). **G)** Schematic representation of the experimental design for qRT-PCR analysis of the expression of CREB-dependent genes (N=5-6 independent cell preparations) **H)** *Arc*, **I)** *c-Fos* **J)** *FosB* **K)** *Egr1* and **L)** *Npas4* in hippocampal cultures. Hippocampal cultures were harvested at baseline conditions or after 2h, 4h, or 6h of bicuculline treatment. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 by two-tailed Student's t-test. ns: not significant. Error bars represent SEM. In vitro western blots and qRT-PCR were partially performed by Franziska Mudlaff and Janina Kupke, respectively.

3.3. MeCP2 coordinates baseline and learning-induced alternative splicing in the mouse hippocampus

In this section we studied the contribution of the DNA methylation reader MeCP2 for induction of alternative splicing. First, we analyzed the genome-wide alternative splicing profile of mice that underwent spatial learning or were kept in baseline conditions. Next, we evaluated using a loss-of-function approach how MeCP2 alters this profile. The results presented in this section are based on (Brito et al., 2020a) and were originally written by the author of this thesis.

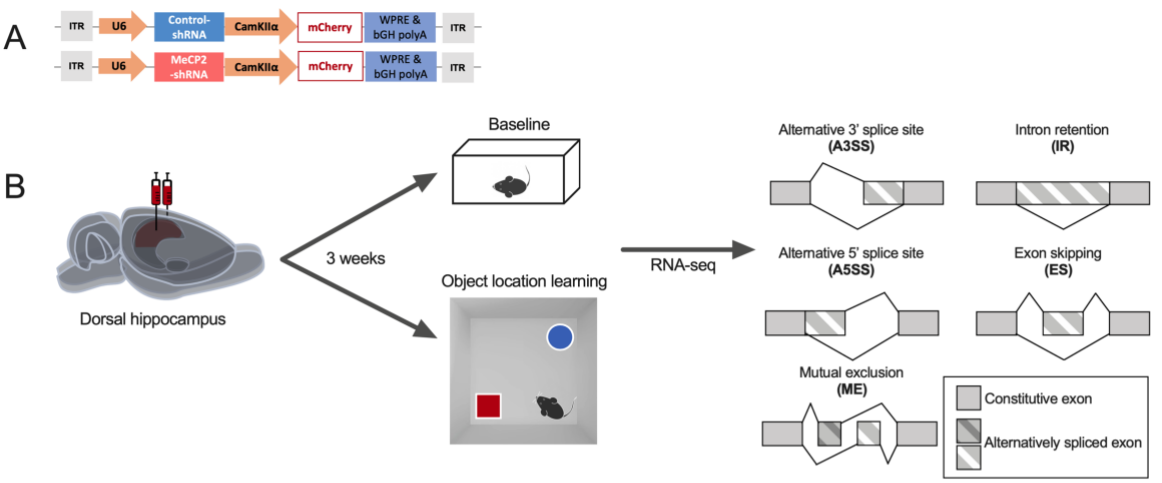
3.3.1. Spatial learning induces alternative splicing events that are impaired in MeCP2 knockdown mice

In this study, we sought to investigate whether MeCP2 regulates alternative splicing events in the adult hippocampus in basal conditions as well as after spatial learning. To this end, we delivered rAAV into the adult dorsal hippocampus encoding either a control (Control-) or a MeCP2-specific shRNA sequence (Figure 3.10A). We knocked down MeCP2 in neurons by using an AAV serotype (rAAV1/2) that displays predominant neuronal tropism (Xu et al., 2001; Burger et al., 2004). The viral construct also contained an expression cassette for mCherry under the control of the CamKIIα promoter (Figure 3.10A) that served as an infection marker. This strategy allowed us to investigate MeCP2 function in the adult hippocampus without confounds originating from impaired postnatal neurodevelopment. We previously confirmed that this tool significantly decreases MeCP2 mRNA and protein levels selectively in the hippocampus. Moreover MeCP2-shRNA mice displayed impairments in hippocampus-dependent long-term memory without exhibiting broad neurological impairments, such as motor deficits or anxiety-like behavior (Gulmez Karaca et al., 2018) that typically occur in animal models with disrupted MeCP2 expression from early developmental stages. Thus, this experimental strategy was chosen for gene expression analysis. In this experiment, half of the mice were kept in their homecage (baseline), whereas the remaining underwent spatial object location training (learning) (Figure 3.10B). This behavioral protocol allowed us to detect hippocampal transcriptional changes associated with novel environment

exposure and learning the location of objects in space. 30 min after the end of the task, a time point with robust transcriptomic changes after learning (Gulmez Karaca et al., 2018), we performed genome-wide differential alternative splicing analysis of the mouse dorsal hippocampal tissue expressing either MeCP2-shRNA or Control-shRNA in baseline conditions and after learning. RNA-seq analysis allowed the identification of five distinct mRNA splicing events: alternative 3′ splice sites (A3SS), alternative 5′ splice sites (A5SS), mutually exclusive exons (ME), intron retention (IR) and exon skipping (ES) events (Figure 3.10B).

We started by identifying the DAS events induced by spatial learning in control mice, and asked whether MeCP2 knockdown alters this learning-induced alternative splicing program. To determine this, we compared the alternative splicing profile of each treatment condition (Control- or MeCP2-shRNA) in basal conditions versus after learning (Figure 3.10C and Figure 3.11A). We observed that object location learning induced 28 differential alternative splicing events in Control-shRNA-injected mice hippocampi, that consisted predominantly of ES events (42.8%) followed by A5SS (21.4%) and IR (17.9%), A3SS (17.9%) while no ME events were detected (Figure 3.10E-D). Some of the genes identified were previously described to undergo alternative splicing during memory consolidation or recall in a contextual fear conditioning paradigm (*Dnajb5* and *Marchf7, Zfp207, Gls, Fuz*, respectively) (Poplawski et al., 2016). In contrast, in MeCP2-shRNA expressing hippocampi, 13 learning-triggered DAS events were detected. Furthermore, MeCP2-shRNA mice showed a clear shift towards more IR events (53.8%) and a reduced occurrence of ES (23.1%) and ME (7.7%), A5SS (7.7%) and A3SS (7.7%) in response to learning compared to the controls (Figure 3.10D and Figure 3.11B-D). These findings indicate that MeCP2 reduction impaired DAS events in the adult hippocampus in response to spatial learning. Next, we analyzed whether there is a change in the fraction of the included or excluded isoforms in Control- or MeCP2-shRNA expressing mice using the delta “percent spliced in” (ΔPSI). The ΔPSI represents the difference between the ratio of transcripts that retain or exclude an intron/exon in two conditions. A ΔPSI value above or below 0% indicates an increased or reduced inclusion of alternative introns/exons, respectively. This parameter allows to

investigate whether MeCP2 regulates the inclusion of introns/exons in alternatively spliced transcripts. Although MeCP2 reduction altered inclusion (ΔPSI>0) and exclusion (ΔPSI<0) events of each splicing subtype (Figure 3.10E), we focused on IR and ES events since the majority of DAS belonged to these splicing categories, and MeCP2 induced an ES-IR switch. We found that MeCP2-shRNA animals showed a mild shift towards excluded IR events (14.3% included vs. 85.7% excluded) compared to controls (20% included vs. 80% excluded) and a decrease of ES (control: 16.7% included vs 83.3% excluded; MeCP2-shRNA: 33.3% included vs 66.7%. excluded) (Figure 3.10E) Similarly, hippocampal knockdown of MeCP2 led to alterations on A3SS, A5SS and ME inclusion/exclusion events (Figure 3.11B-D). The majority of splicing events occurred in the same direction, that is inclusion or exclusion, in both control and MeCP2-shRNA animals (Figure 3.10G). Nonetheless, we also detected a subset of alternative splicing events that occurred in opposite directions, meaning that they underwent increased inclusion in MeCP2-shRNA mice and increased exclusion in Control-shRNA mice or vice versa (e.g. *Gls, Osmr, Trmt1, Irf7*). Remarkably, only 2 of the 13 DAS events observed in MeCP2-shRNA mice overlapped with the DAS events detected in Control-shRNA mice. This indicates that in MeCP2 knockdown conditions DAS events that occur in control conditions were no longer present (e.g. *Zmynd8, Nr3c1, P2rx6, Dnajb5)* and new spliced isoforms were generated (11 unique DAS; e.g. *Atl2, Fhl1, Marchf7*) (Figure 3.10G) None of these events (neither overlapping nor unique) showed a bias towards any particular splicing type (Figure 3.11E-I). Statistical analysis of all the DAS events detected in Control- or MeCP2-shRNA mice in response to learning did not show a statistically significant difference between the groups (Figure 3.10H and Figure 3.11J,K).


A
ITR
U6
Control-shRNA
CamKIIα
mCherry
WPRE & bGH polyA
ITR
ITR
U6
MeCP2-shRNA
CamKIIα
mCherry
WPRE & bGH polyA
ITR
B
Dorsal hippocampus
3 weeks
Baseline
Object location learning
RNA-seq
Alternative 3' splice site (A3SS)
Intron retention (IR)
Alternative 5' splice site (A5SS)
Exon skipping (ES)
Mutual exclusion (ME)
Constitutive exon
Alternatively spliced exon

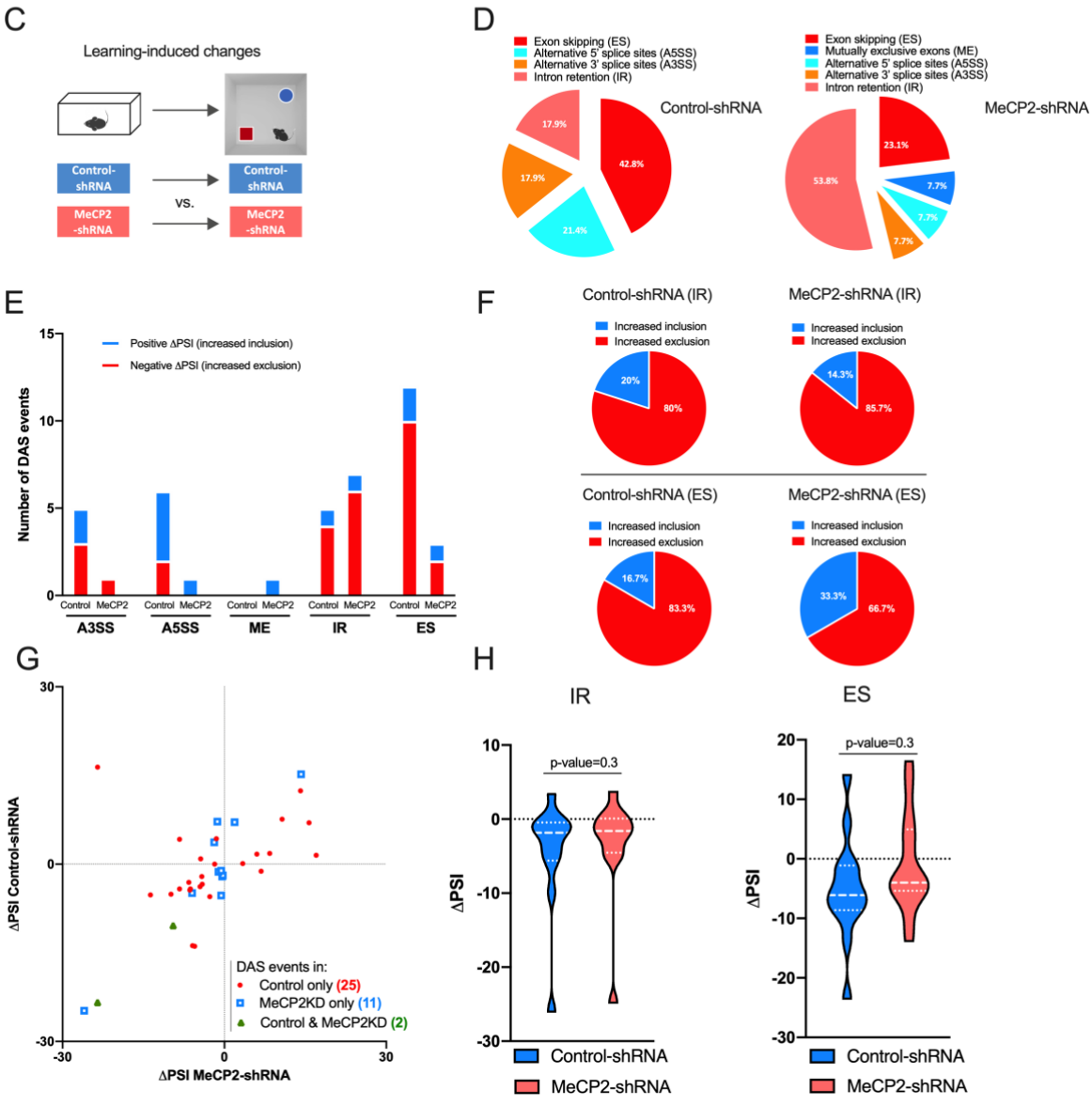

C
Learning-induced changes
Control-shRNA
MeCP2-shRNA
vs.
Control-shRNA
MeCP2-shRNA
D
Exon skipping (ES)
Alternative 5' splice sites (A5SS)
Alternative 3' splice sites (A3SS)
Intron retention (IR)
Control-shRNA
17.9%
42.8%
17.9%
21.4%
Exon skipping (ES)
Mutually exclusive exons (ME)
Alternative 5' splice sites (A5SS)
Alternative 3' splice sites (A3SS)
Intron retention (IR)
MeCP2-shRNA
23.1%
53.8%
7.7%
7.7%
7.7%
E
Positive ΔPSI (increased inclusion)
Negative ΔPSI (increased exclusion)
Number of DAS events
Control MeCP2
A3SS
A5SS
ME
IR
ES
F
Control-shRNA (IR)
MeCP2-shRNA (IR)
Increased inclusion
Increased exclusion
20%
80%
14.3%
85.7%
Control-shRNA (ES)
MeCP2-shRNA (ES)
16.7%
83.3%
33.3%
66.7%
G
ΔPSI Control-shRNA
ΔPSI MeCP2-shRNA
DAS events in:
Control only (25)
MeCP2KD only (11)
Control & MeCP2KD (2)
H
IR
ES
p-value=0.3
p-value=0.3
ΔPSI
Control-shRNA
MeCP2-shRNA

Figure 3.10. Spatial learning induces alternative splicing events that are altered in MeCP2 knockdown mice. **A)** The viral vector contains a U6 promoter driving expression of MeCP2-shRNA or a control-shRNA and mCherry expression under the CamKIIα promoter. **B)** Schematic representation of the experimental design and alternative splicing events analyzed in this study. Three weeks after the delivery of recombinant AAVs into the dorsal hippocampus, mice remained either in home-cage (Baseline) or trained in object location learning. 30 minutes after training mice dorsal hippocampi were micro dissected and RNA-seq and alternative splicing analysis was performed. **C)** Schematic representation of the comparisons used. **D)** Proportion of each differential alternative splicing events (DAS) in control conditions (left) or in MeCP2 knock-down conditions (right) in learning-induced conditions. **E)** Number of inclusion (positive ΔPSI, blue) and exclusion (negative ΔPSI, red) events for each type of alternative splicing modality in Control-shRNA (Control) and MeCP2-shRNA (MeCP2) mice (q-value<0.05). **F)** Pie charts showing the proportion of inclusion and exclusion events for intron retention (IR) and exon skipping (ES) in Control-shRNA and MeCP2-shRNA mice. **G)** Scatter plots showing changes in IR and ES events in Control-shRNA (Control) and MeCP2-shRNA (MeCP2KD) mice upon learning. Red dots and blue squares represent alternative splicing events that occurred in either Control or MeCP2 knockdown hippocampi (q-value<0.05), respectively. Green triangles represent alternative splicing events that occurred in both conditions (q-value<0.05). **H)** Violin plots showing the ΔPSI distribution of IR (left) and ES (right) events in Control-shRNA and MeCP2-shRNA hippocampi after learning. The P-values are based on paired two-tailed Student's t test or Wilcoxon test and are indicated at the top of each panel. ΔPSI: delta "percent spliced in". bGH polyA: Bovine growth hormone polyadenylation signal. ITR: inverted terminal repeat, WPRE: Woodchuck Hepatitis virus post-transcriptional regulatory element.

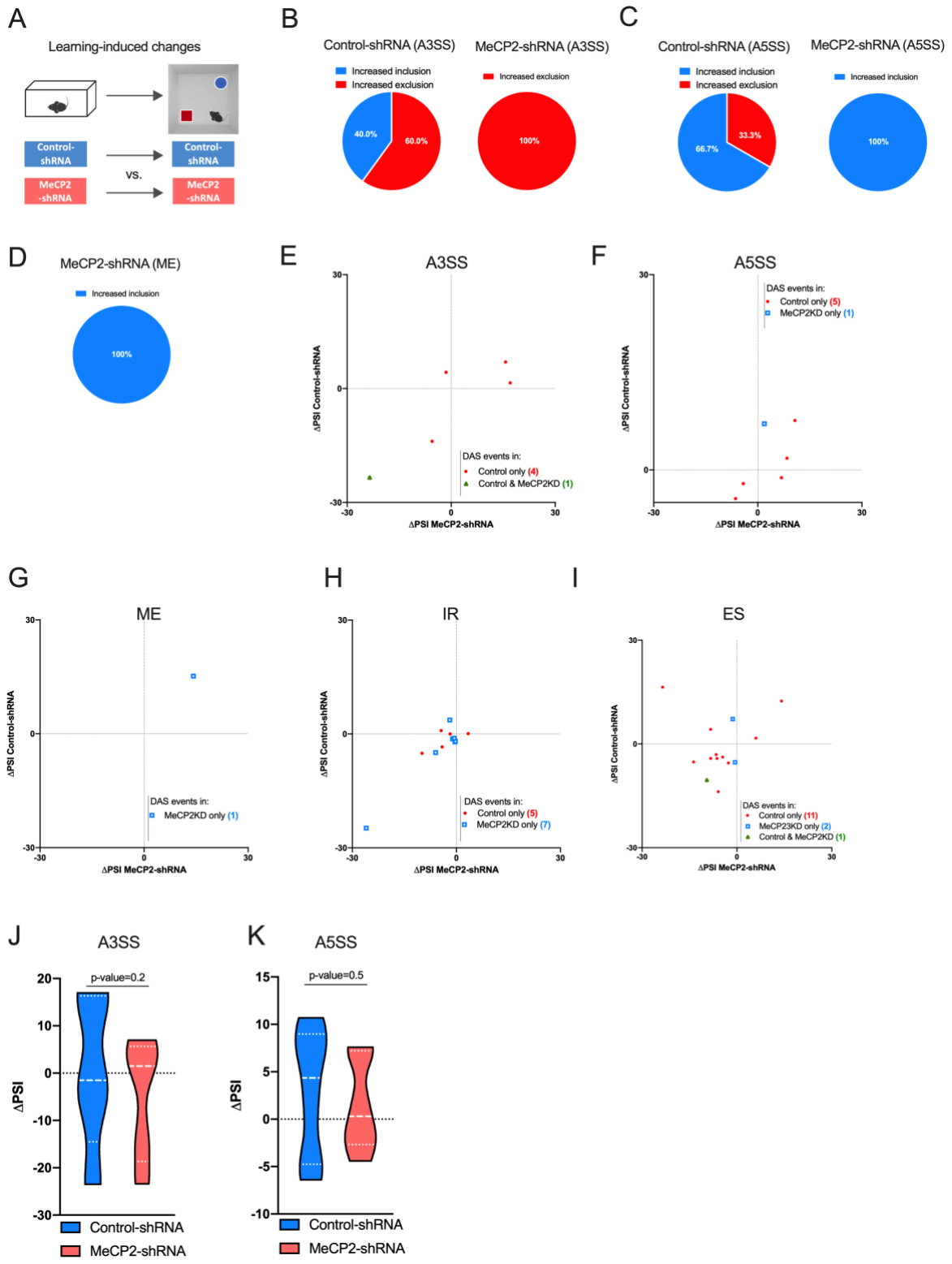


Figure 3.11. Alternative splicing event-specific changes in MeCP2 knockdown mice upon spatial learning. A) Schematic representation of the comparisons used. **B-D)** Pie charts showing the proportion of learning-induced inclusion and exclusion events for **B)** alternative 3′ splice sites (A3SS),

C) alternative 5′ splice (A5SS) and **D)** mutually exclusive exons (ME) in Control-shRNA and MeCP2-shRNA mice. E-I) Scatter plots showing changes in **E)** A3SS, (F) A5SS, **G)** ME, **H)** intron retention (IR) and **I)** exon skipping (ES) events in Control-shRNA (Control) and MeCP2-shRNA (MeCP2KD) mice upon learning. Red dots and blue squares represent alternative splicing events occurred in either Control or MeCP2-knock-down (MeCP2KD) (q-value<0.05), respectively. Green triangles represent alternative splicing events that occurred in both conditions (q-value<0.05). **J-L)** Violin plots showing the ΔPSI distribution of A3SS **J)**, A5SS **K)** events in Control-shRNA and MeCP2-shRNA hippocampi after learning. The P-values are based on paired two-tailed Student's t test or Wilcoxon test and are indicated at the top of each panel. ΔPSI: delta "percent spliced in".

To gain further insight into the functional categories of identified learning-induced DAS, we performed gene ontology (GO) analysis. For this, we carried out two separate evaluations for inclusion and exclusion DAS events (Figure 3.12A,B). We found that in control animals that underwent learning, GO terms associated with "Dendritic spine" and "Positive regulation of spine development" were predominantly enriched in the inclusion group ($-\log_{10}$P value<3), whereas terms associated with "Alternative splicing" and "Splice variant" showed a non-significant trend for enrichment in the exclusion cohort ($-\log_{10}$P value<3). These findings suggest that learning-induced alternative splicing events in the hippocampi of control mice are associated with dendritic spine regulation. Notably, in MeCP2-shRNA mice, there was no enrichment detected for the inclusion group, and the exclusion DAS cohort showed a non-significant trend for enrichment for terms associated with "Methylation", "Splice variant", "Alternative splicing" and "Compositionally bias region: Arg/Ser-rich". This data suggests that MeCP2 reduction compromises predominantly the learning-triggered processes associated with dendritic function.

Considering that MeCP2 is required for optimal expression and alternative splicing of several genes, we asked to which degree these two gene populations (DEGs and DASs) overlap. This analysis indicates whether MeCP2 uses these two regulatory mechanisms on similar or different genes. Since the same tissue was used for DAS and for the previously published differential gene expression analysis (Gulmez Karaca et al., 2018), the two datasets could be directly compared. To this end, we identified genes that underwent alternative splicing, and compared this gene population to the previously identified differentially expressed genes (DEGs) in the

same conditions (Gulmez Karaca et al., 2018) (learning-induced DEGs were compared to learning-induced DASs in Control- or MeCP2-shRNA expressing mouse hippocampus) (Figure 3.12C). We found that in control group, only 3 DEGs showed also DAS events in response to learning (out of 134 DEGs and 26 DAS) (Figure 3.12C,D). In MeCP2-shRNA animals, the differentially expressed genes in response to learning and the learning-induced differential alternative splicing events did not overlap (Figure 3.12D). Taken together, this data indicates that learning induces changes in the expression levels and in the predominance of specific alternatively spliced variants of distinct gene populations. Furthermore, our results implicate a requirement for MeCP2 in both processes.

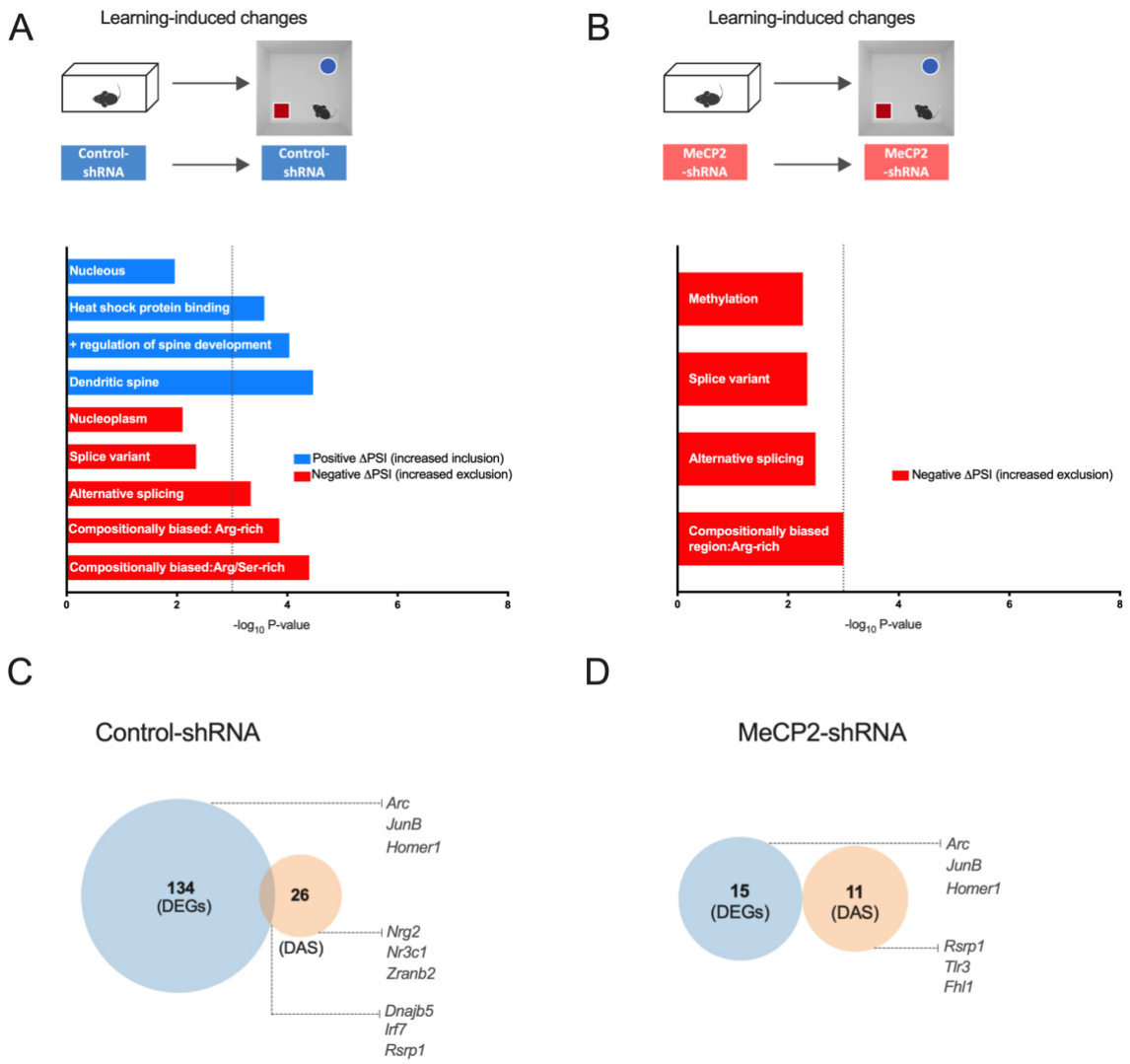


Figure 3.12. Analysis of genes that underwent differential alternative splicing events upon spatial learning. A-B) Schematic representation of comparisons used (top). Gene ontology (GO) analysis for genes that underwent differential alternative splicing in the dorsal hippocampi of Control-shRNA **A)** and MeCP2-shRNA **B)** mice upon learning. Enriched GO terms (Fisher's exact test P<0.001) for genes that underwent inclusion or exclusion (q-value < 0.05) events, upon learning. The blue and red bars represent $-\log_{10}$ (P-value) of the GO enrichment for inclusion and exclusion events, respectively. The vertical dashed line serves as a marker for P-value = 0.001 [$-\log_{10}$ (P-value) =3]. Absence of a colored bar means that genes of that GO term were not enriched in that specific category. **C)** Venn diagram showing overlap between total number of differentially expressed genes (DEGs) and genes that underwent differential alternative splicing events (DAS) in home-cage (baseline) conditions when MeCP2 was knocked down in the adult dorsal hippocampus. **D)** Venn diagram showing overlap between total number of differentially expressed genes and genes that

underwent differential alternative splicing events in learning state (learning) conditions when MeCP2 was knocked down in the adult dorsal hippocampus.

3.3.2. Decrease in MeCP2 alters splicing events in baseline and learning states

To gain a deeper understanding of how MeCP2 regulates alternative splicing events, we asked whether acute MeCP2 reduction influences DAS events already in baseline and/or after learning conditions. To this end, we compared the alternative splicing profile of Control- versus MeCP2-shRNA mice in basal conditions, as well as after learning (hereafter, learning state) (Figure 3.13A and Figure 3.14A). We identified a total of 156 DAS events (q-value < 0.05) in baseline conditions upon MeCP2 disruption in the hippocampus; ES events were predominant (75%), followed by IR (10.3%), ME (6.4%), A5SS (4.5%) and A3SS (3.8%) (Figure 3.13B). Altered alternative splicing genes partially overlapped with findings observed in the hippocampus of *Mecp2-null* mice *(*i.e. *Zfp207, Map4* and *Ppfia2*) (Osenberg et al., 2018). Similarly, DAS profile of MeCP2-shRNA hippocampus after learning was different from the controls. We identified 94 DAS events (q-value < 0.05) in MeCP2-shRNA mice in learning state, in which ES events were predominant (70.2%), followed by IR (25.5%) and A3SS (4.3%) whereas no A5SS and ME events were detected (Figure 3.13B). Next, we determined the change in the fraction of the included or excluded events of each splicing subtype in baseline or learning in MeCP2-knockdown conditions (Figure 3.13C). We found that MeCP2-shRNA animals have preferentially decreased IR in baseline conditions (31.2% inclusion vs. 68.8% exclusion) while in learning state, the relative abundance of inclusions/exclusions in MeCP2-shRNA mice shifted predominantly towards included introns (66.7% inclusion vs. 33.3% exclusion) (Figure 3.13D). Interestingly, the total number of ES events in MeCP2-disrupted hippocampus decreased by learning (Figure 3.13D), the proportion of inclusions/exclusions among the total ES events remained similar in baseline and learning state (baseline: 32.5% included vs. 67.5% excluded; learning 37.9%: vs. 62.1%) (Figure 3.13D). Similarly, hippocampal knockdown of MeCP2 lead to alterations on A3SS, A5SS and ME inclusion/exclusion events in baseline and in learning conditions (Figure 3.14B-D). Next, we checked

the common DAS events in baseline and learning state in MeCP2-shRNA conditions. We found that hippocampal MeCP2 reduction led to 131 and 75 unique DAS events, in baseline (e.g. *Gabrg2*, *Synj1*, *Map4*) and in learning state (e.g. *U2af1l4*, *Nrcam*, *P2xrx6*, *Gria3*), respectively (Figure 3.13E). Only 19 DAS events occurred in both conditions, suggesting that learning induces distinct alternative splicing events. Notably, the majority of DAS events detected in baseline or after learning happened in the same direction in MeCP2-disrupted and control hippocampi, although a small subset of splicing events occurred in opposite ways (Figure 3.13E). Deeper analysis revealed that the oppositely regulated DAS subset showed no bias for a particular splicing event type (Figure 3.14E-I). Cumulative analysis of all DAS events in MeCP2-disrupted hippocampus showed a significant increase in retained introns (Figure 3.13F), a decrease in skipped exons (Figure 3.13F) and either a significant or a non-significant trend for increase of A5SS and ME inclusion events in learning state compared to baseline (Figure 3.14J-L). No change was detected for A3SS events (Figure 3.14K). These results show that MeCP2 reduction induces a distinct DAS profile in baseline and upon learning. Thus, indicating that the differences found in the learning state do not only reflect changes in basal conditions, but also a requirement for MeCP2 in learning-dependent alternative splicing.

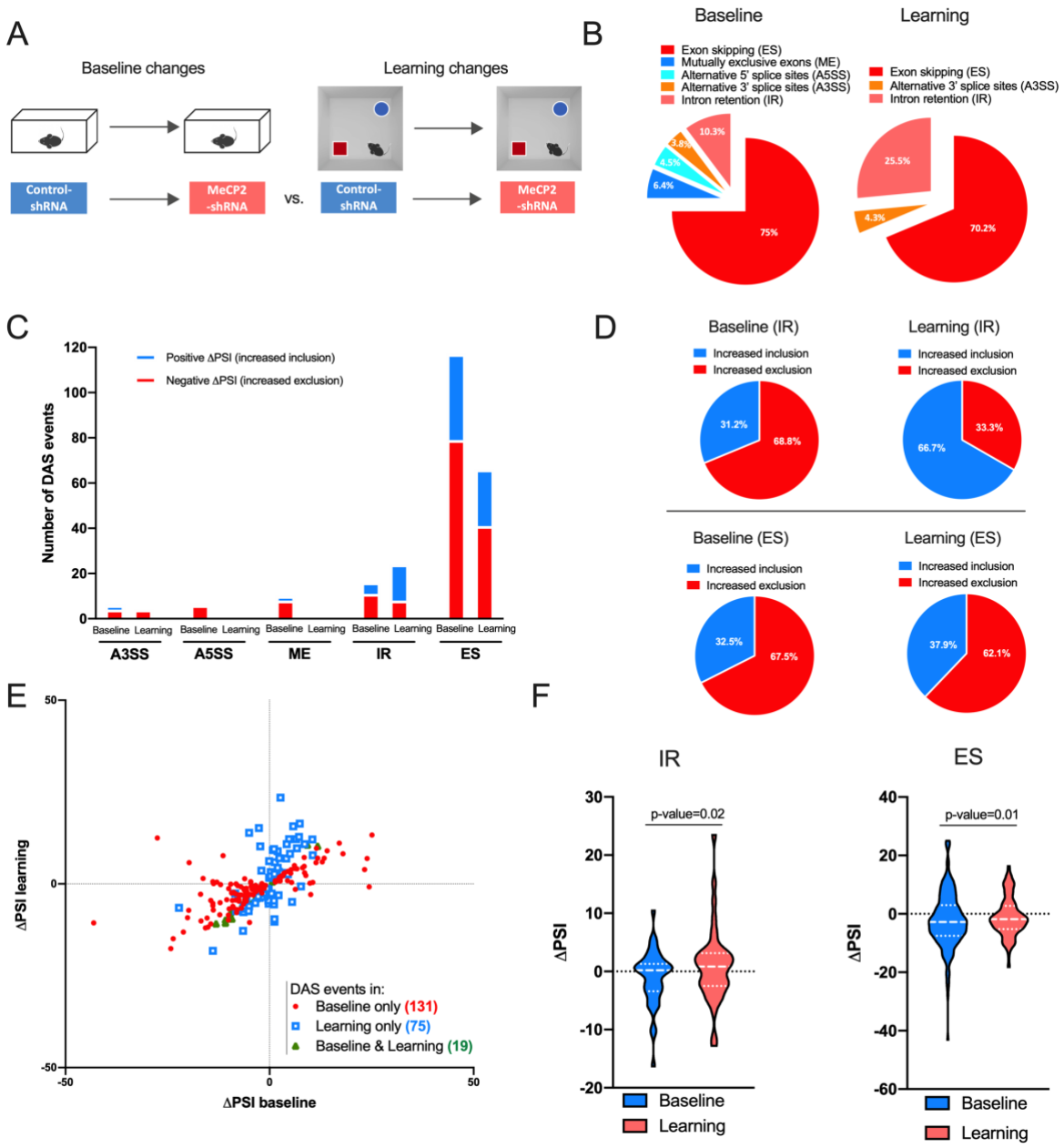


Figure 3.13. MeCP2 knockdown changes baseline and learning-associated splicing events. A) Schematic representation of the comparisons used. **B)** Proportion of each differential alternative splicing events (DAS) in baseline conditions (left) or in learning state (right) in MeCP2-shRNA conditions. **C)** Number of inclusion (positive ΔPSI, blue) and exclusion (negative ΔPSI, red) events for each type of alternative splicing modality in MeCP2 knockdown during baseline and learning state conditions (q-value<0.05). **D)** Pie charts showing the proportion of inclusion and exclusion events for intron retention (IR) and exon skipping (ES) in baseline and learning state conditions. **E)** Scatter plots showing changes in IR and ES events in home-cage (Baseline) and learning state (learning) conditions by MeCP2 knockdown. Red dots and blue squares represent alternative splicing events

occurred in either baseline or learning state (q-value<0.05) conditions, respectively. Green triangles represent alternative splicing events that occurred in both conditions (q-value<0.05). **F)** Violin plots showing the ΔPSI distribution of IR (left) and ES (right) events in baseline and learning state in the hippocampi of MeCP2-shRNA mice. The P-values are based on paired two-tailed Student's t test or Wilcoxon test and are indicated at the top of each panel.

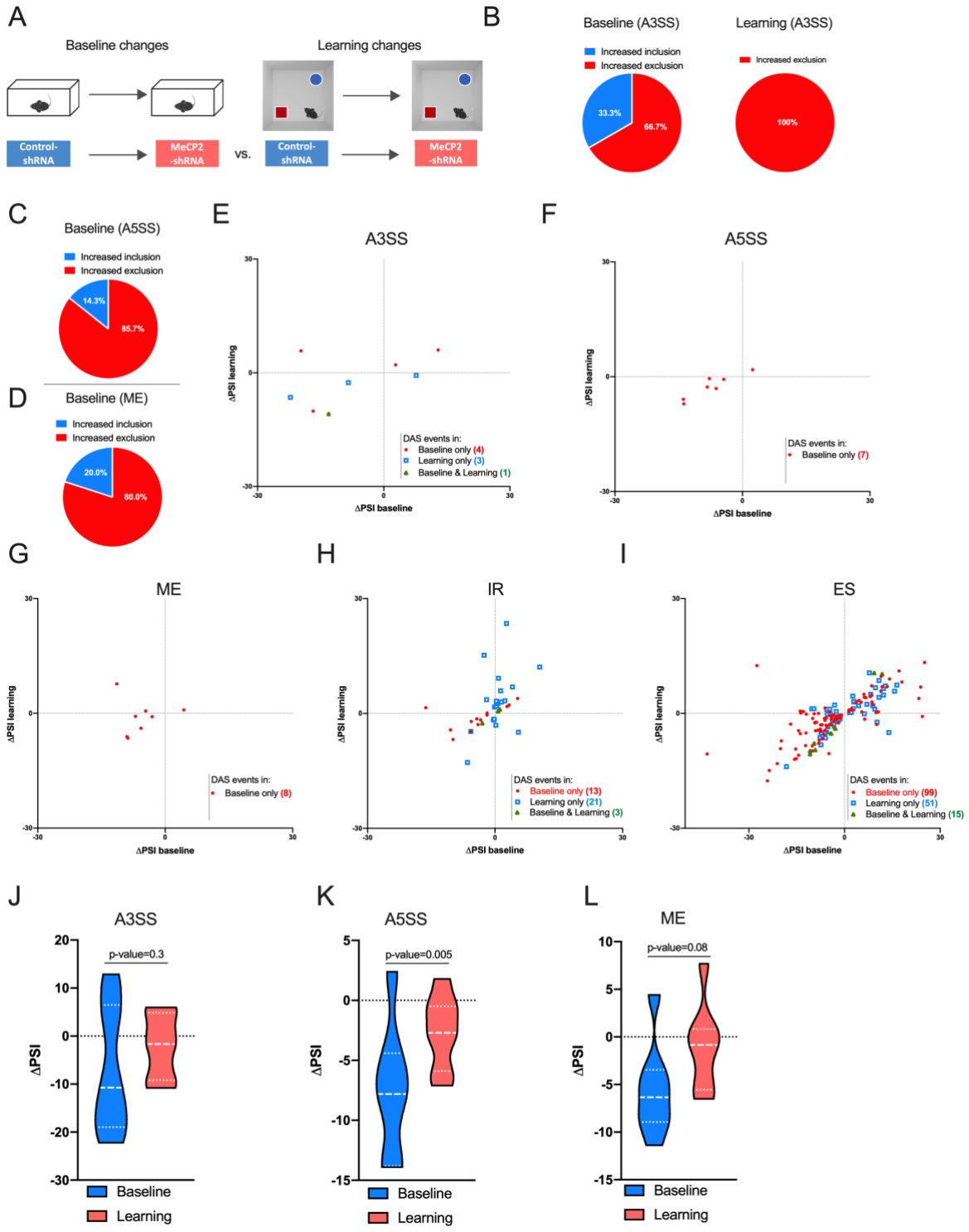


Figure 3.14. Alternative splicing event-specific changes in MeCP2 knock-down mice during baseline and learning state conditions. A) Schematic representation of the comparisons used. **B-D)** Pie charts showing the proportion of inclusion and exclusion events for **B)** alternative 3′ splice sites (A3SS), and **C)** alternative 5′ splice (A5SS) and **D)** mutually exclusive exons (ME) in MeCP2-shRNA

mice during baseline and learning-state conditions. Note that no ME changes were detected by MeCP2 knockdown in learning-state. E-I) Scatter plots showing changes in **E)** A3SS, (F) A5SS, **G)** ME, **H)** intron retention (IR) and **I)** exon skipping (ES) events in home-cage (Baseline) and learning state (Learning) conditions in MeCP2-shRNA mice compared to the controls. Red dots and blue squares represent alternative splicing events occurred in either baseline or learning conditions (q-value<0.05), respectively. Green triangles represent alternative splicing events that occurred in both conditions (q-value<0.05). **J-L)** Violin plots showing the ΔPSI distribution of **J)** A3SS, **K)** A5SS and **L)** ME events in baseline and learning state conditions in the dorsal hippocampi of MeCP2-shRNA mice. The P-values are based on paired two-tailed Student's t test or Wilcoxon test. and are indicated at the top of each panel. ΔPSI: delta "percent spliced in".

Next, to gain functional insight into the categories of the genes that require MeCP2 for alternative splicing in baseline or learning states, we performed GO analysis. This was applied to both conditions (baseline or learning) and were divided into inclusion (ΔPSI>0) and exclusion (ΔPSI<0) events. We found that DAS inclusions in MeCP2 reduction in baseline conditions were enriched for terms such as "Phosphoprotein", "Alternative splicing" and "Cytoskeleton", whereas DAS exclusions in MeCP2-shRNA mice were associated with the functional categories termed "Alternative splicing", "Clathrin vesicle coat", "Tubulin binding" ($-\log_{10}$P value<3) (Figure 3.15A). After learning, only enrichment for "Alternative Splicing" for inclusion events and "Cell-cell adherent junctions", "Neuronal cellular homeostasis" and "Positive regulation of protein binding" for increased exclusion events were found (Figure 3.15B). These results indicate that both in baseline and after learning conditions MeCP2 regulates DAS events associated with general neuronal function processes despite that DAS events are generally distinct in both conditions.

Next, we compared DEGs and DASs in MeCP2-reduced hippocampi in baseline or learning states. We found that only 17 differentially expressed genes in MeCP2 knockdown also showed altered alternative splicing (out of 1948 DEGs and 130 DAS) in baseline conditions (Figure 3.15C), whereas this number was as low as 7 genes in learning state (out of 884 DEGs and 82 DAS) (Figure 3.15D). Altogether, these findings indicate that MeCP2 regulates the predominance of specific alternatively spliced variants mostly without affecting the overall level of transcripts coded by that gene both in baseline conditions and after learning.

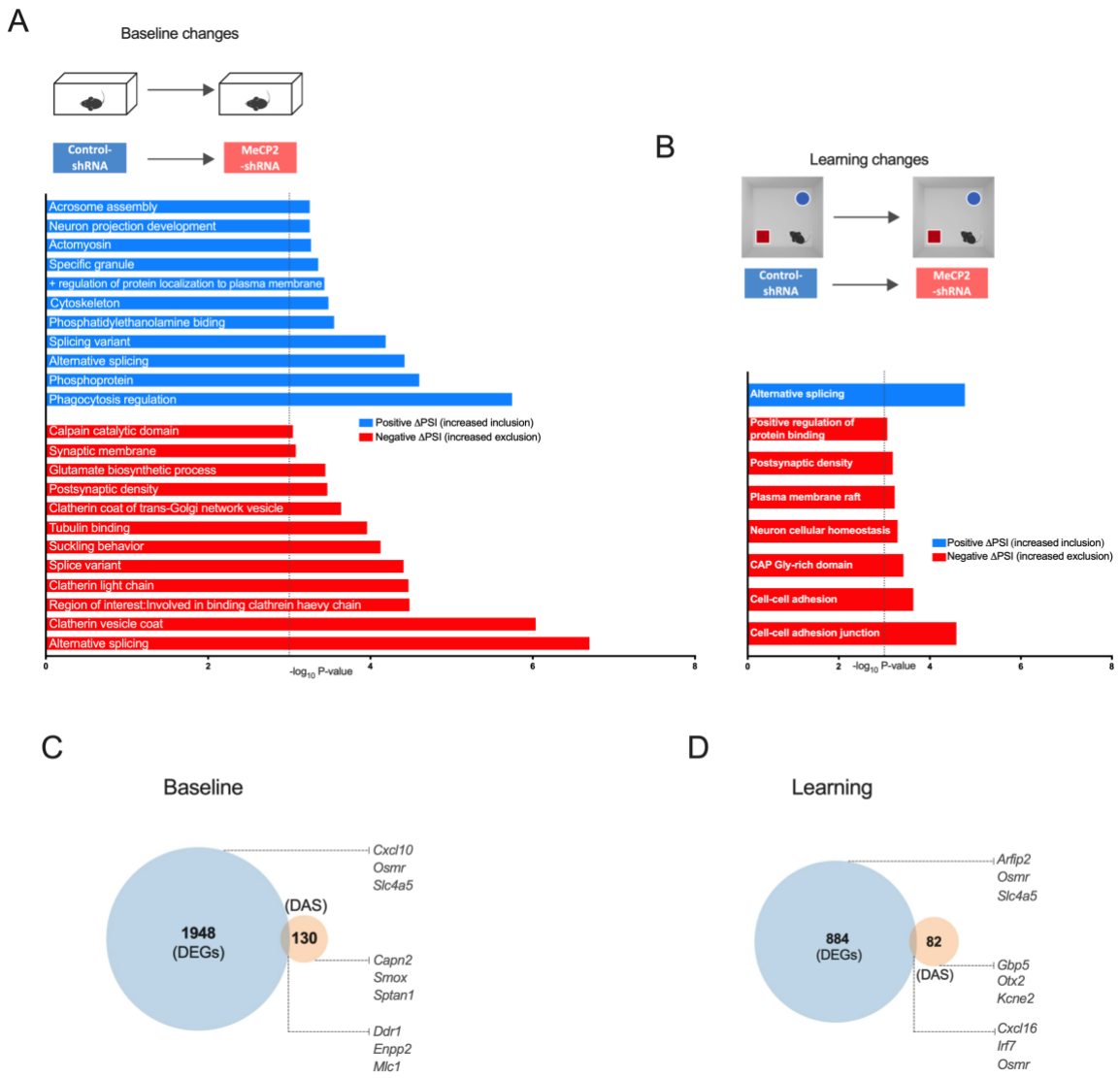


Figure 3.15. Analysis of genes that underwent differential alternative splicing events in baseline and in learning state upon MeCP2 knock-down. A-B) Schematic representation of comparisons used (top). Gene ontology (GO) analysis for genes that underwent differential alternative splicing in the dorsal hippocampi MeCP2-shRNA mice in baseline **A)** and learning state **B)** conditions. Enriched GO terms (Fisher's exact test P<0.001) for genes that underwent inclusion or exclusion (q-value < 0.05) events. The blue and red bars represent $-\log_{10}$ (P-value) of the GO enrichment for inclusion and exclusion events, respectively. The vertical dashed line serves as a marker for P-value = 0.001 [$-\log_{10}$ (P-value) =3]. Absence of a colored bar means that genes of that GO term were not enriched in that specific category. ΔPSI: delta "percent spliced in". **C)** Venn diagram showing overlap between total number of differentially expressed genes and genes that underwent differential alternative splicing events in learning-induced conditions in the adult dorsal hippocampus of control mice (control-shRNA). **D)** Venn diagram showing overlap between total number of differentially expressed genes and genes that underwent differential alternative splicing events in

learning-induced conditions when MeCP2 was knocked down in the adult dorsal hippocampus (MeCP2-shRNA).

3.3.3. Validation of RNA-seq data by quantitative and semi-quantitative RT-PCR

Finally, we aimed to validate our RNA-seq data using additional biological replicates and two independent methods. We stereotaxically delivered rAAV expressing Control or MeCP2-shRNA to a new cohort (N=4-6) of animals as previously performed (Figure 3.10A-B). In combination with the samples used for RNA-seq we validated 12 DAS events by qRT-PCR and 4 DAS events by RT-PCR. The majority of DAS events that we analyzed by qRT-PCR (Figure 3.16A) or RT-PCR (Figure 3.16B) showed an effect consistent with the RNA-seq data. This additional independent analysis thus supports the findings obtained by RNA-seq.

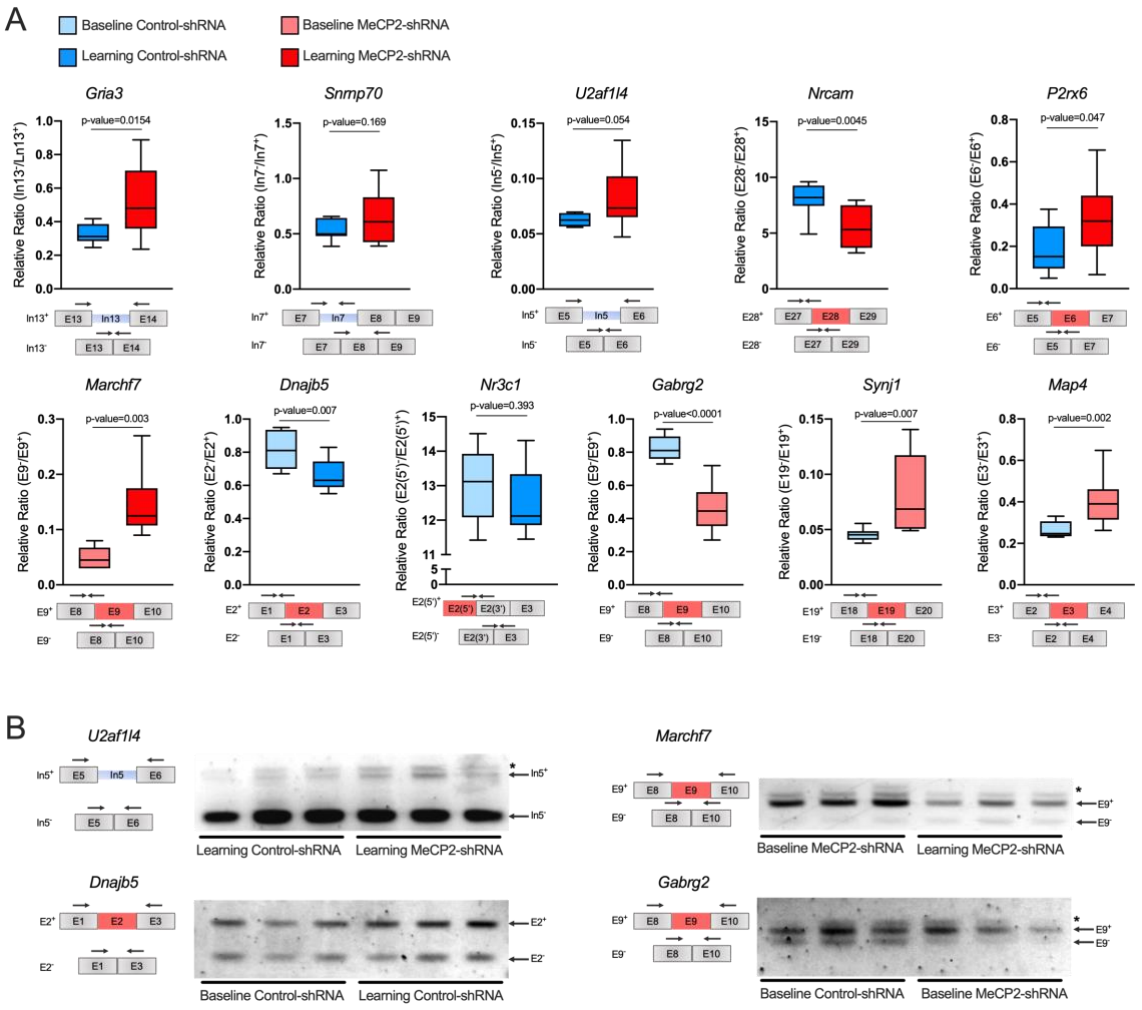


Figure 3.16. Validation of genes that underwent differential alternative splicing. A) Quantitative RT-PCR for differential alternative splicing events. Box plots show the relative ratio of retained vs. excised introns (IR) or included vs. excluded exons (ES, A5SS) between conditions (N=8-10). The diagram below each individual gene shows the two possible isoforms (included/excluded exon or intron in red or blue, respectively) with arrows indicating the location of the primers used. The P-values are based on unpaired two-tailed Student's t test or Mann-Whitney test indicated at the top of each panel. **B)** Semiquantitative RT-PCR for genes that underwent differential alternative splicing. Gel images show the retained vs. spliced isoform intensity. Diagram next to gene name shows the location of the primers used. Three biological replicates are shown per condition. Error bars represent SEM. These experiments were performed by Janina Kupke and Lukas Frank.

3.4. Late Npas4 expression as a mechanism underlying memory strength

In this section we studied the contribution of the activity-dependent transcription factor Npas4 for mechanisms of memory persistence and strength. First, we established behavior paradigms of short- and long-lasting memories and found that Npas4 expression is associated with memory strength. Next, we investigated which type of neuronal signaling drives this late expression. Lastly, we sought out to artificially induce a delayed expression of Npas4 in a behavior protocol of short-lasting memory.

3.4.1. Persistent memory is associated with late Npas4 expression

In order to understand what molecular mechanisms are associated with memory persistence and strength we established two fear-conditioning protocols (Figure 3.17A-B). The behavior paradigms were designed to promote LTM (24h) and either to allow short-lasting (1x0.2mA foot shock) or persistent (3x0.7mA foot shocks) memory over weeks. Mice were trained in these two tasks or were solely exposed to the chamber in the absence of any shock (context-only) which did not induce any contextual-fear memory. Animals trained in both protocols displayed LTM although with distinct strengths (Figure 3.17C) compared to context-only mice. At 3 weeks after learning mice trained in the short-lasting protocol did not exhibit freezing levels distinguishably from controls (Figure 3.17D). In contrast, mice trained in the persistent memory protocol displayed robust freezing levels at 3 weeks and even 2 months after learning (Figure 3.17D-E). These set of experiments show that both protocols can be used as models to study molecular mechanisms underlying memory strength and duration.

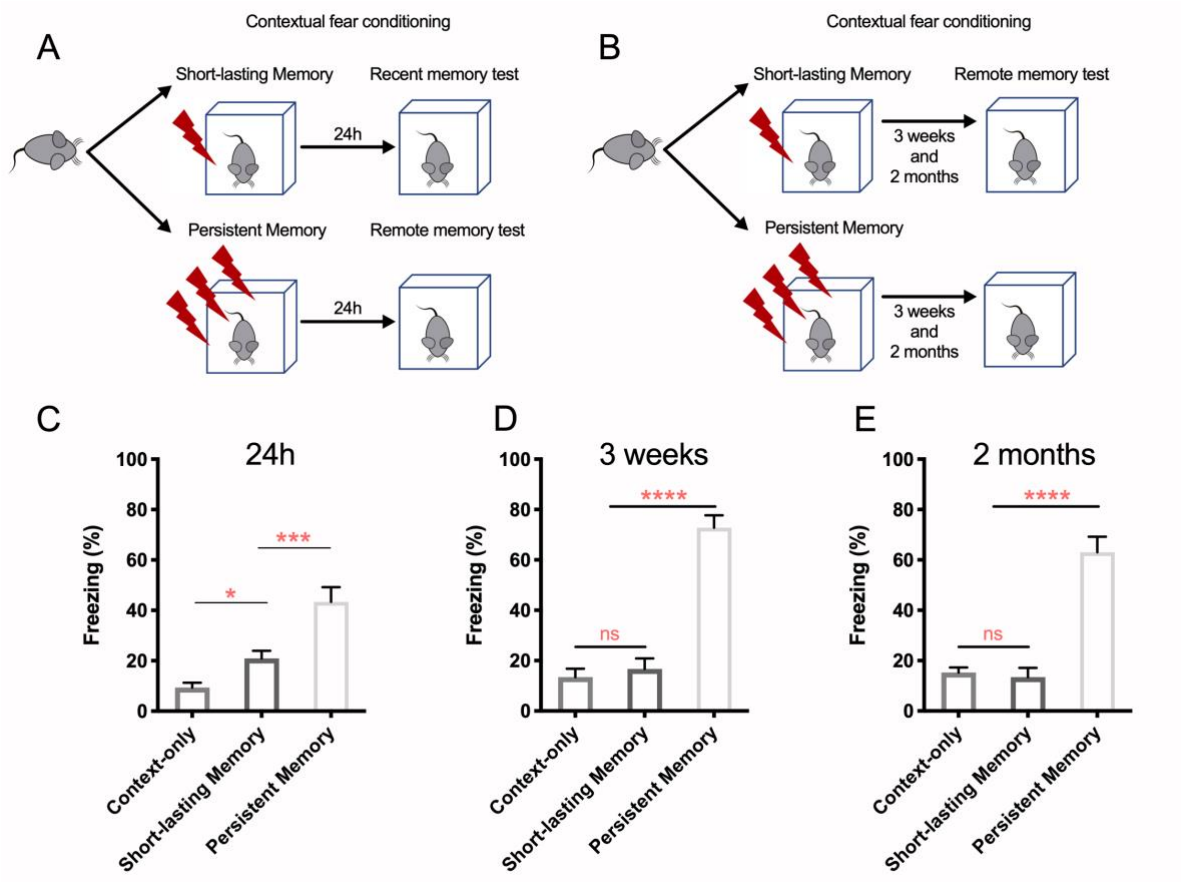


Figure 3.17. Validation of protocols inducing short- or persistent memory. A-B) Schematic illustration of the behavior protocols used. Mice underwent contextual-fear conditioning training for short-lasting (1x 0.2mA foot shock) or persistent memory (3x 0.7mA foot shock). **C)** A cohort of animals was used to test LTM (24h) or **D-E)** remote memory (3 weeks and 2 months) (N=8-14). *p<0.05, ***p<0.001, ****p<0.0001 by one-way ANOVA test followed by Sidak's post hoc test with pairwise comparisons. Error bars represent SEM.

After establishment of these protocols, we investigated changes in learning-dependent gene expression that would be unique to short-lasting or persistent memories. Specifically, we analyzed the expression of 3 IEGs (*Arc*, *cFos* and *Npas4*) in the dorsal CA1 region of the hippocampus. Mice were trained independently in these two behavior protocols (Figure 3.18A) and their hippocampus was dissected for mRNA extraction at distinct time points ranging from 15m to 24h. We controlled for potential circadian fluctuations in gene expression by having HC controls for each time group (i.e., HC: 15m, 30m, 1h, 4h; HC:8h; HC:12h; HC:15h; HC:18h and HC:24h). We observed that all genes in both protocols displayed an early increase

in the expression in early time points after learning as expected from their established role as IEGs (Figure 3.18B-G). We did detect however that the induction kinetics were visibly altered between mice trained in either protocol. Short-lasting memory training induced a more transient gene expression profile than persistent memory training at early phases of gene expression (Figure 3.18B-G). We found that 4h after persistent memory training *Npas4* expression was increased to levels comparable to the early expression observed after training (Figure 3.18F). This late expression was specific to persistent memory training as mice that underwent short-lasting training showed levels similar to HC animals (Figure 3.18G). We also observed at 24h that *Arc* expression was increased in mice that were trained in the persistent memory protocol (Figure 3.18B) but not in the short-lasting memory paradigm (Figure 3.18D). This increase was milder compared to the induction observed early after training. Lastly, we observed a general gene repression in late timepoints after learning, which suggests a restricted window for gene expression after learning.

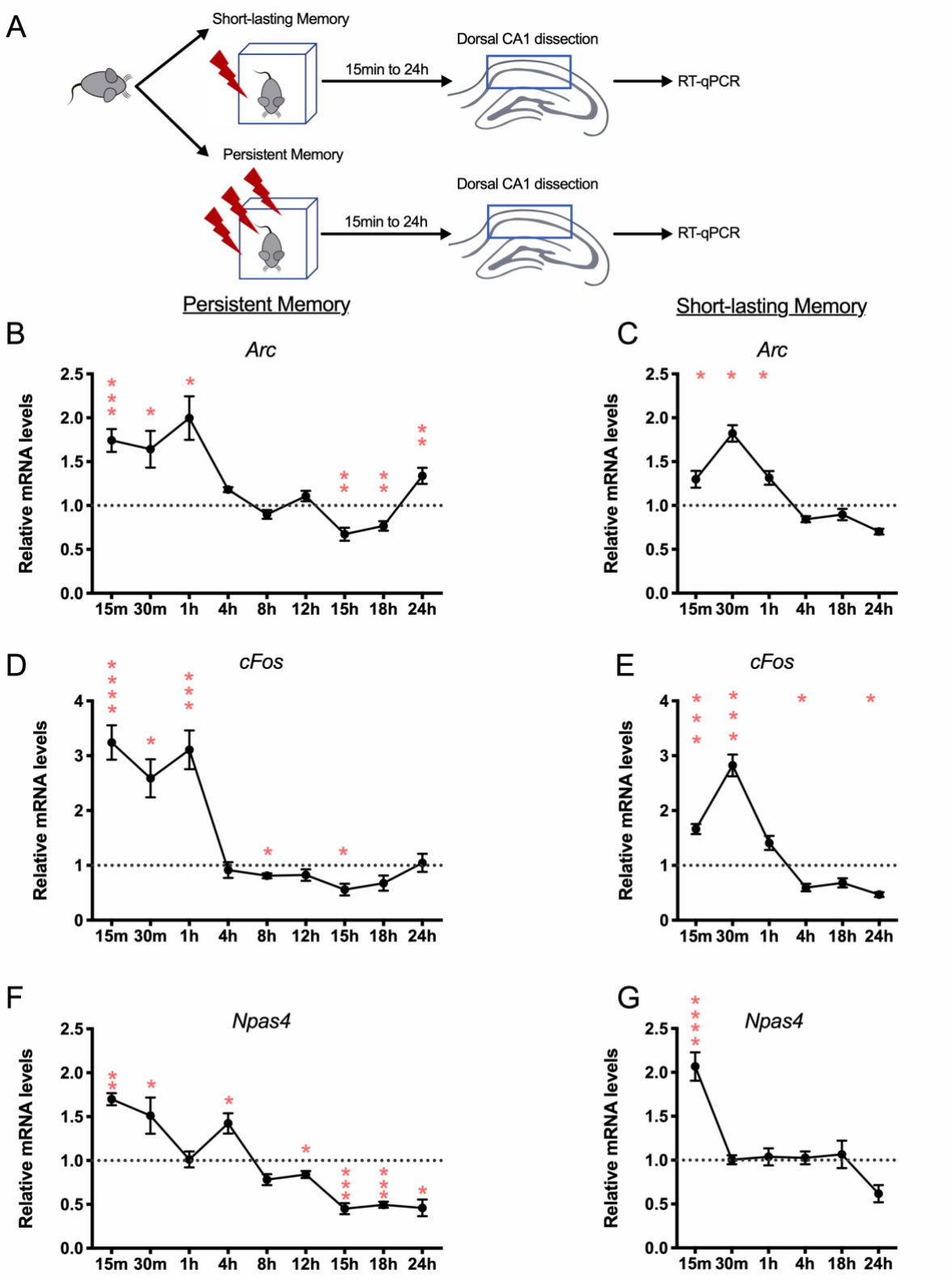


Figure 3.18. Memory strength/persistence is associated with late *Npas4* mRNA expression. A) Schematic illustration of the experimental design. Mice underwent short-lasting or persistent memory

training and their dorsal CA1 was dissected 15m to 24h after training. RT-qPCR was performed to evaluate gene expression changes (N=6-13). **B-G)** mRNA expression of **B-C)** Arc, **D-E)** cFos and **F-G)** Npas4 in the dorsal CA1 of mice that underwent short-lasting or persistent memory training. Gene expression is normalized to the reference gene *Gusb* and to their respective HC control (dashed line). *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 by two-tailed Student's t-test. Error bars represent SEM.

Considering the induction levels of Npas4 expression at 4h and its established role as a memory-induced transcription factor we focused on evaluating its contribution to memory persistence and strength. Next, we evaluated if this delayed expression was also observed at the protein level. We trained mice in both protocols (Figure 3.19A) and evaluated the protein levels of Arc, Fos and Npas4 at 30m, 2h, 4h and 6h after learning by western blot. In agreement with the mRNA kinetics observed we found that both protocols led to the increase of these proteins after learning, reaching baseline levels 2-4h after training (Figure 3.19B-I). We found that at 6h after persistent memory training, mice exhibited increased Npas4 levels (Figure 3.19F,H). This increase was specific as mice that underwent short-lasting training did not show any trend for increase in Npas4 levels in this time-point. These results show that the late Npas4 mRNA expression observed at 4h is detectable at the protein level. This suggests a functional role for Npas4 expression in this time-window in memory persistence mechanisms.

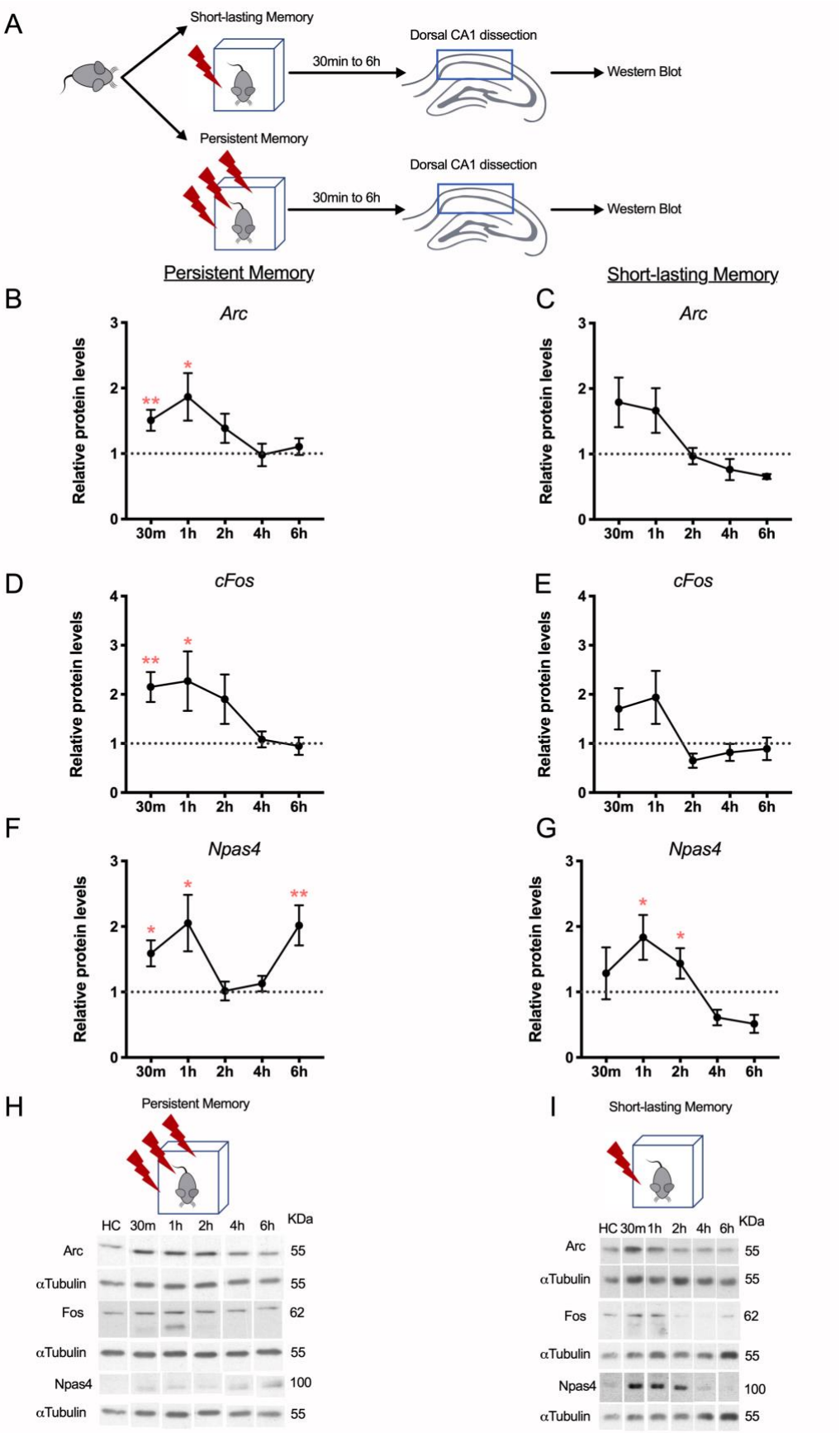

A
Short-lasting Memory
30min to 6h
Dorsal CA1 dissection
Western Blot
Persistent Memory
30min to 6h
Dorsal CA1 dissection
Western Blot
Persistent Memory
Short-lasting Memory
B
Arc
Relative protein levels
30m 1h 2h 4h 6h
C
Arc
Relative protein levels
30m 1h 2h 4h 6h
D
cFos
Relative protein levels
30m 1h 2h 4h 6h
E
cFos
Relative protein levels
30m 1h 2h 4h 6h
F
Npas4
Relative protein levels
30m 1h 2h 4h 6h
G
Npas4
Relative protein levels
30m 1h 2h 4h 6h
H
Persistent Memory
HC 30m 1h 2h 4h 6h KDa
Arc 55
αTubulin 55
Fos 62
αTubulin 55
Npas4 100
αTubulin 55
I
Short-lasting Memory
HC 30m 1h 2h 4h 6h KDa
Arc 55
αTubulin 55
Fos 62
αTubulin 55
Npas4 100
αTubulin 55

Figure 3.19. Memory strength/persistence is associated with late *Npas4 protein expression.* **A)** Schematic illustration of the experimental design. Mice underwent short-lasting or persistent memory training and their dorsal CA1 was dissected 30m to 6h after training. Western blot analysis was performed to evaluate protein expression (N=8-13). **B-G)** protein expression of **B-C)** *Arc*, **D-E)** *cFos* and **F-G)** *Npas4* in the dorsal CA1 of mice that underwent short-lasting or persistent memory training. Protein expression is normalized to the reference protein αTubulin and to their respective HC control (dashed line). **H-I)** Representative immunoblot scans for all conditions tested. *p<0.05, **p<0.01by two-tailed Student's t-test. Error bars represent SEM.

3.4.2. Late Npas4 expression is dependent on NMDA receptor activity

Next, we questioned if the delayed Npas4 expression is an autonomous mechanism independently driven by early neuronal activity or if its dependent on late activation of the dorsal CA1. To test this, we used a pharmacological approach by infusing the competitive NMDA receptor antagonist DL-APV or the D_1 receptor antagonist halobenzazepine (SCH23390) into the dorsal CA1 of mice that underwent persistent memory training (Figure 3.20A). The infusions were performed 3.5h after training to evaluate the possible contribution of these receptors to the induction of Npas4 at 4h (mRNA). The dorsal CA1 of these mice was dissected at 6h after training, the timepoint that overlapped with Npas4 expression at the protein level (Figure 3.19F). We found that protein expression of *Arc* and *cFos* was similar to vehicle-infused animals when NMDA or D_1 receptor function was blocked (Figure 3.20B,C,E,F). On the contrary, Npas4 expression at 6h was reduced compared to trained vehicle-infused animals when NMDA receptor function was blocked (Figure 3.20D,G). These results indicate that late Npas4 expression is dependent on NMDA receptor activation.

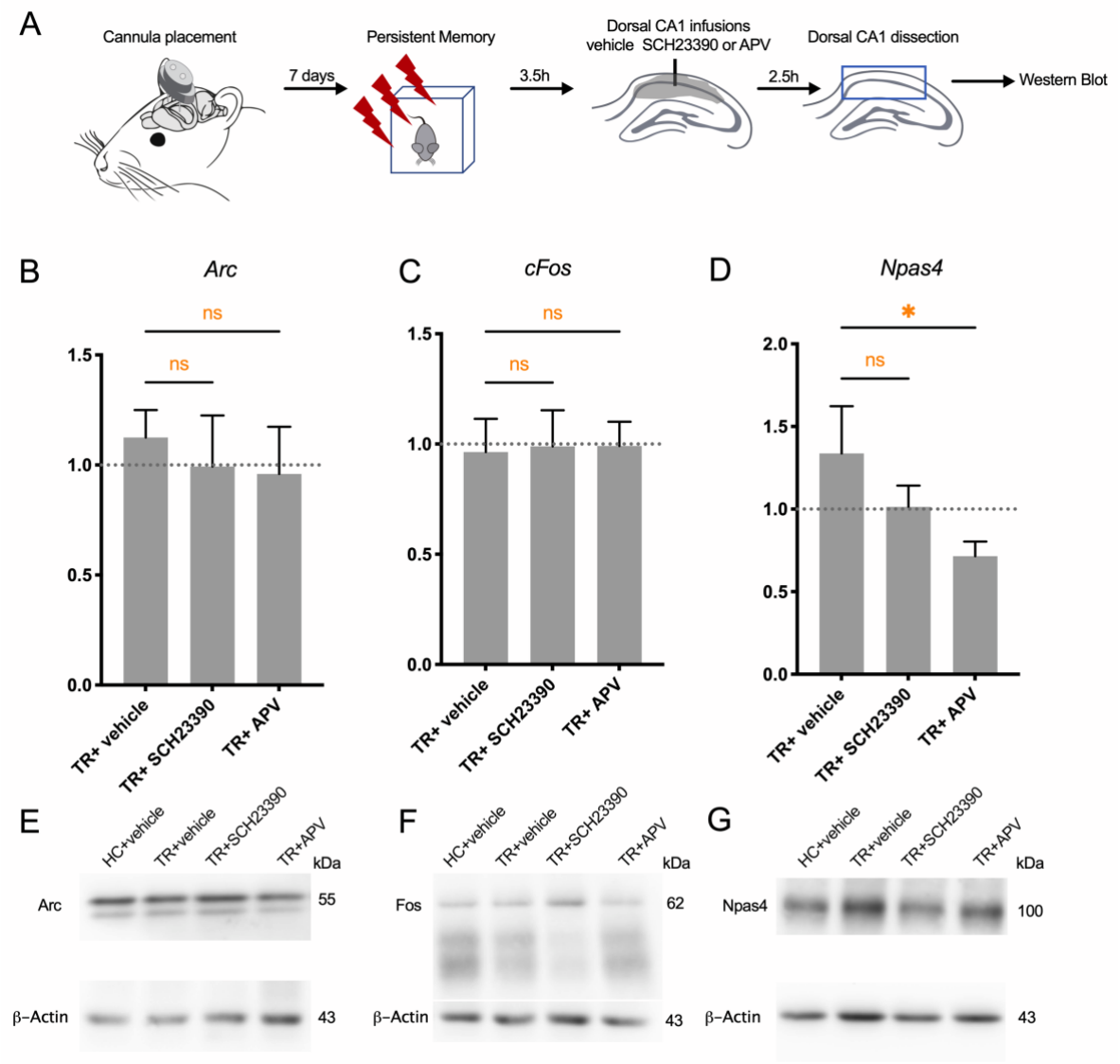


Figure 3.20. Late Npas4 protein expression is dependent on NMDA receptor activity. A) Schematic illustration of the experimental design. Cannulas targeting the dorsal CA1 region were placed into mice to allow drug infusions. Seven days after surgery mice underwent persistent memory training (TR) and 3.5h later vehicle, DL-APV or SCH23390 was infused into the dorsal CA1. At 6h-post training the dorsal CA1 was microdissected and western blot analysis was performed (N=10-13). **B-G)** protein expression of **B)** Arc, **C)** cFos and **D)** Npas4 in the dorsal CA1 of mice that underwent persistent memory training. Protein expression is normalized to the reference protein β-actin and to their respective HC control (dashed line). **E-G)** Representative immunoblot scans for all conditions tested. *p<0.05 by one-way ANOVA followed by a Dunn´s multiple comparison test. Error bars represent SEM.

3.4.3. Induction of late Npas4 expression induces memory decay

In order to evaluate the functional significance of the late expression of Npas4, we developed tools to artificially induced the expression of this protein in the short-lasting protocol. We used a dual-component TetON-based system that consists of a driver plasmid which expresses under the control of hSynapsin, the rtTA, the TetR and a fluorescent protein marker (KO). The second construct drives the expression of HA-tagged eGFP (TRE-eGFP) or full length Npas4 (TRE-Npas4) in a doxycycline dependent manner (under the control of the TRE promoter) (Figure 3.21A). The expression of eGFP or Npas4 is actively repressed by TetR in the absence of doxycycline and activated in the its presence. We infected dissociated hippocampal cultures with rAAV carrying these plasmids and 2 days before protein harvesting treated cultures with doxycycline to evaluate the expression of this system (Figure 3.21B). In the absence of doxycycline, we did not detect expression of eGFP or Npas4 by western blot analysis using an anti-HA antibody (Figure 3.21C). On the contrary, doxycycline treated conditions showed expression of these two proteins. We confirmed that the expression of the exogenous HA-Npas4 is detectable with both HA and Npas4 antibodies and presents the expected molecular weight (Figure 3.21C). After validating that the expression of exogenous Npas4 is dependent on doxycycline treatment we evaluated if its expression in the absence of neuronal activity (baseline conditions) would induce Npas4-dependent transcriptional activity. We used primary hippocampal cultures to perform luciferase reporter assays using a reporter plasmid containing the firefly luciferase under the control of CRE or the Npas4 binding sites (Figure 3.21D). This assay reveled that in doxycycline conditions expression of TRE-Npas4 but not TRE-eGFP leads to Npas4-specific transcriptional activity (Figure 3.21E). Moreover, this effect was specific as no CRE-dependent activity was detected. Next, we aimed to characterize the *in vivo* expression kinetics of this system. We stereotaxically delivered rAAV expressing the driver plasmid and TRE-Npas4 into the mouse dorsal CA1 (Figure 3.21F). We designed this experiment to uncover two time-points 4h apart where expression of TRE-Npas4 was neglectable or already detectable, respectively. The reasoning for this criterion is that we intended to artificially induce Npas4 expression at 4h after learning without

expressing this protein during the contextual-fear conditioning training. We found that intra-peritoneal (ip) injection of doxycycline 16h before animals were analyzed for immunohistochemistry was not sufficient to trigger TRE-Npas4 expression (Figure 3.21G). On the contrary 20h after IP injection of doxycycline animals displayed expression of TRE-Npas4 in the dorsal CA1, evaluated by HA-staining. Altogether, these experiments show that this system is able to induce a time-specific expression of functionally active Npas4 in hippocampal neurons.

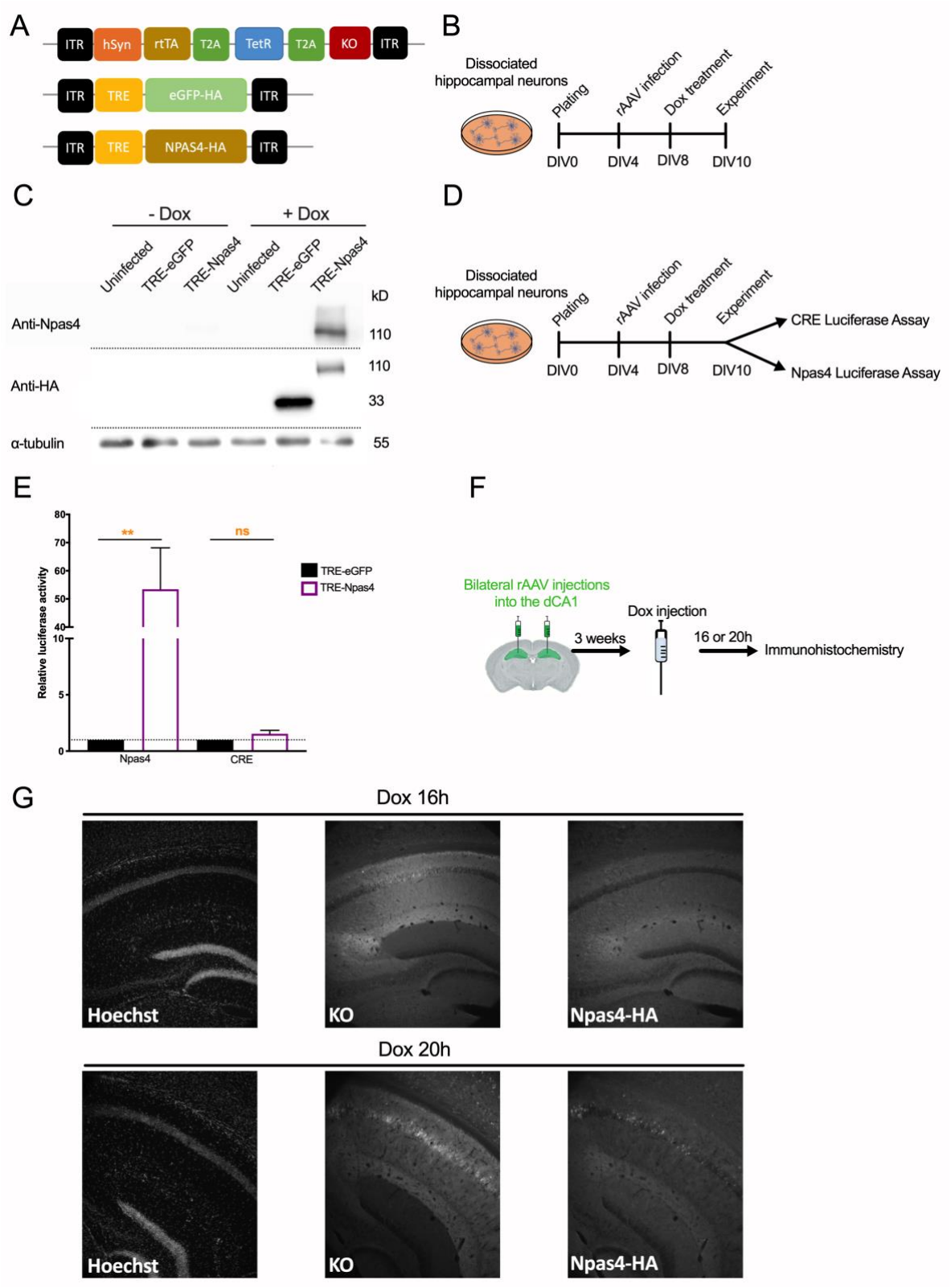


Figure 3.21. Validation of transient Npas4 expression in dissociated hippocampal neurons and *in vivo*. A) Schematic representation of the viral constructs used. The viral vector contains a human

synapsin (hSyn) promoter driving the transactivator (rtTA), the tetracycline repressor (TetR) and the fluorescent protein Kusabira Orange (KO) as an infection marker. In the second construct HA-tagged eGFP or full length Npas4 expression is under the control of the tetracycline responsive promoter (TRE). **B)** Schematic illustration of the experimental design used. **C)** Representative immunoblot scans from hippocampal cultures infected with rAAV expressing TRE-eGFP or TRE-Npas4 in combination with driver plasmid. Hippocampal cultures were treater with doxycycline at DIV 8 and harvested at baseline conditions at DIV10. **D)** Schematic illustration of the experimental design used. Luciferase reporter assays were performed using dissociated hippocampal cultures co-transfected with TRE-eGFP or TRE-Npas4 and driver plasmid and reporter plasmids expressing firefly luciferase (FFluc) under the control of CRE or Npas4 (n=5 independent neuronal cultures) transcriptional activity. Values are normalized to the TRE-eGFP condition treated (dashed line). DIV: day *in vitro* **E)** Schematic illustration of the experimental design used. Mice were stereotaxically injected in the dorsal CA1 with rAAV encoding RE-eGFP or TRE-Npas4 in combination with driver plasmid. Three weeks later mice received a ip injection of doxycycline and were sacrificed for immunohistochemistry 16 or 20h later. **G)** Representative images of the dorsal hippocampus of TRE-Npas4 animals 4 weeks after stereotaxic surgery that were injected 16 or 20h before sacrifice with doxycycline. **p<0.01 by two-tailed Student's t-test. Error bars represent SEM.

Next, we aimed to evaluate the behavior consequences of inducing Npas4 expression 4h after mice were trained in a short-lasting memory behavior protocol. Mice underwent stereotaxic delivery of rAAVs encoding TRE-eGFP or TRE-Npas4 and driver plasmids in the dorsal CA1 (Figure 3.22A). Three weeks after surgeries both groups received an ip injection of doxycycline in order to induce TRE-eGFP or TRE-Npas4 expression 4h after contextual-fear conditioning. Both groups displayed similar reactions to foot shock administration (Figure 3.22B). We evaluated LTM by testing mice 24h after training. Both groups showed similar freezing levels suggesting that 4h-post training Npas4 induction did not affect LTM (Figure 3.22C). Surprisingly, TRE-Npas4 mice displayed a memory impairment 3 weeks after learning compared to TRE-eGFP mice (Figure 3.22D). This result indicates that induction of late Npas4 expression in a protocol that naturally does not show this induction accelerates memory decay.

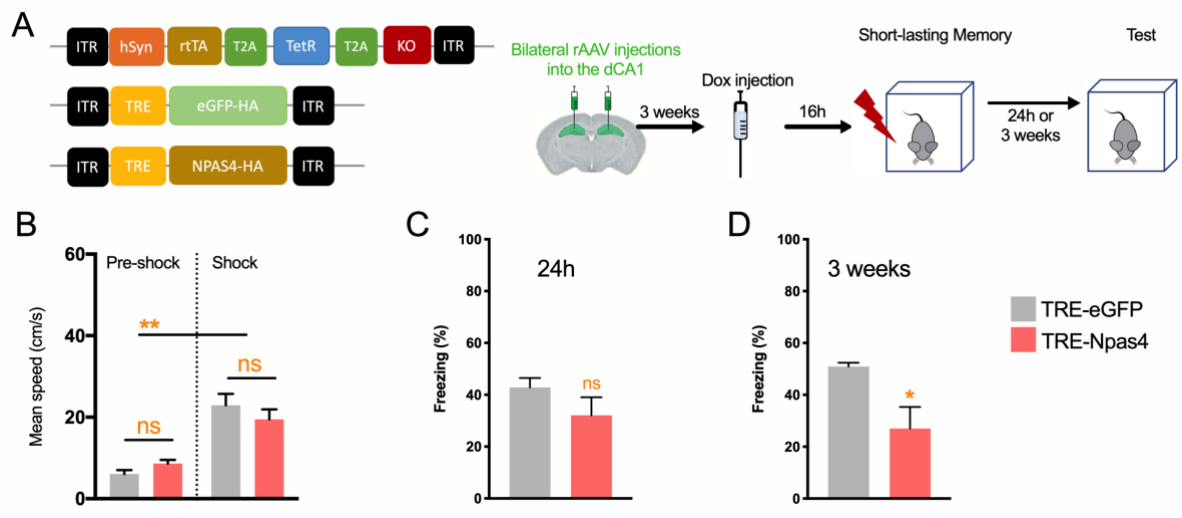


Figure 3.22. Induction of late Npas4 expression induces fast memory decay. A) Left: Schematic representation of the viral constructs used. The viral vector contains a human synapsin (hSyn) promoter driving the transactivator (rtTA), the tetracycline repressor (TetR) and the fluorescent protein Kusabira Orange (KO) as an infection marker. In the second construct HA-tagged eGFP or full length Npas4 expression is under the control of the tetracycline responsive promoter (TRE). Right: Schematic illustration of the experimental design used. Mice underwent stereotaxic delivery of rAAVs encoding TRE-eGFP or TRE-Npas4 and driver plasmids. Three weeks later mice received an ip injection of doxycycline 16h before short-lasting memory training (20h before expression of exogenous proteins). **B)** Mean speed during the different phases of the contextual fear conditioning training, showing similar performance between groups. A one-way ANOVA followed by a Bonferroni's Multiple Comparisons Test was used (N=5). Mice were tested 24h or 3 weeks after training. A cohort of animals was used to test **C)** LTM (24h) or **D)** remote memory (3 weeks and 2 months) (N=4-5). Two-tailed unpaired Student's t test. *p<0.05, **p<0.01. Error bars represent SEM.

Chapter 4. Discussion

In this study, we identified molecular mechanisms that contribute to memory storage and persistence. Additionally, we uncovered novel proteins linked to age-related cognitive decline in mice and humans. First, we discovered that Gadd45γ is required for the formation of different types of memories and that its expression is compromised during murine aging (Section 3.1). Next, we revealed that human aging is accompanied by aberrant Gadd45γ expression which is associated with impaired memory formation (Section 3.2). Next, we showed that learning induces alternative splicing events which require MeCP2 function (Section 3.3). Lastly, we showed that late Npas4 expression is associated with memory persistence mechanisms in the mouse brain (Section 3.4).

4.1. Age-related transcriptional changes associated with impaired cognitive abilities

In the first two sections of this thesis, we demonstrated that mammalian aging is associated with alterations in Gadd45γ expression and that bidirectional dysregulation of hippocampal Gadd45γ levels in young adult mice negatively impacts cognitive functions. This study expands the view that age-related gene expression alterations in the hippocampus are heterogeneous across species despite possible common phenotypes. Furthermore, we showed that Gadd45γ regulates synapse-to-nucleus signaling and AP-1- and CREB-regulated genomic programs. These findings provide insight into how gene expression changes may underlie conserved short- and long-term memory deficits during mammalian aging.

Previous studies on the functions of Gadd45 family members in the brain focused mainly on Gadd45β. Knocking out Gadd45β promoted enhanced long-term memory in one study (Sultan et al., 2012) and deficient hippocampus-dependent long-term memory in another (Leach et al., 2012). Considering that both reports applied a global knock-out strategy, possible confounds originating from developmental compensatory mechanisms and/or differential functions in different brain regions that bias conclusions about the function of Gadd45β in hippocampus-dependent memory may have been present. Here, we acutely decreased the expression of Gadd45β in

the dorsal hippocampus of adult mice and found no differences in long- or short-term memory. Moreover, knockdown of Gadd45β in primary hippocampal cultures did not affect stimulus-induced MAPK signaling, transcription factor activity, or gene expression. This result suggests that previously reported effects of Gadd45β knockout are probably not a direct consequence of Gadd45β-associated functions in the hippocampus.

Here we describe that, analogous to its role in other cell types (Tamura et al., 2012), Gadd45γ functions as a regulator of MAPK signaling pathways in hippocampal neurons. Specifically, we found that Gadd45γ regulates the activation of p38 and JNK MAPKs. Short- and long-term forms of plasticity require JNK and p38 activity. JNK targets synaptic proteins that regulate the insertion of AMPA receptors (Coffey, 2014). Similarly, p38 is required for AMPA receptor trafficking associated with mGluR-induced LTD and NMDAR-induced LTD (Correa and Eales, 2012). *In vivo* studies revealed that both JNK and p38 are activated upon learning (Giese and Mizuno, 2013). Furthermore, pharmacological inhibition of JNK activity in the hippocampus resulted in enhanced and impaired short- and long-term memories, respectively (Bevilaqua et al., 2003; Sherrin et al., 2010), whereas inhibition of p38 immediately after learning impaired both short- and long- term memory formation (Alonso et al., 2003). MAPK signaling cascades regulate the activity of downstream transcription factors with established roles in memory formation (Alberini, 2009). Here we found that Gadd45γ regulates the transcriptional activity of CREB and AP-1 and downstream gene expression. Thus, our data suggests that Gadd45γ mediates the formation of both short- and long-term memory through the regulation of these pathways. Recently it has been suggested that Gadd45γ can act as a DNA demethylase (Grassi et al., 2017; Li et al., 2018) and in this way regulate gene expression. Our findings show a novel mechanism by which Gadd45γ regulates gene expression in neurons. This is supported by our study using luciferase assays that demonstrate the ability of Gadd45γ to regulate CREB- and AP-1-dependent transcription independently of the epigenome. Moreover, we observed a striking similarity in the magnitude of the effects obtained in the luciferase assays and the analysis of expression of CREB- and AP-1 endogenous target genes. Additionally,

in both assays, AP-1-dependent transcription required Gadd45γ both in basal and induced conditions, whereas CREB-dependent gene expression required Gadd45γ only after stimulation, thus suggesting that the regulation of CREB- and AP-1-target genes depends on Gadd45γ mediated synapse-to-nucleus communication. It is possible that these functions of Gadd45γ and its reported DNA demethylase activity work in conjunction in the fine tuning of genomic responses underlying memory formation.

Interestingly, we found that both Gadd45γ loss- and gain-of-function manipulations culminated in similar *in vitro* and *in vivo* phenotypes. We observed that mimicking the human aging-related increase in Gadd45γ expression in the mouse hippocampus promoted memory deficits and impairments in CREB-dependent transcription. These findings are in agreement with another study showing that either Gadd45γ loss- or gain-of-function disrupts neural development (Kaufmann and Niehrs, 2011). Thus, our data together with the work of others, suggests that proper cellular function requires the tight regulation of Gadd45γ levels. Importantly, neither knockdown nor overexpression of Gadd45γ promoted changes in anxiety-like behavior, supporting the claim that Gadd45γ selectively regulates cognitive abilities. Several studies demonstrated that altered CREB activity during aging has been linked to dysregulated calcium homeostasis (Oliveira and Bading, 2011). We found, however, that Gadd45γ knockdown, which mimics age-associated changes in Gadd45γ levels during rodent aging, impacts CREB phosphorylation independently of changes in calcium dynamics. This suggests that during aging, dysregulation of Gadd45γ-dependent regulatory mechanisms may be another factor contributing to altered CREB function.

The mechanisms underlying dysregulation of Gadd45γ expression during mammalian aging are unknown. Neuroinflammation is a hallmark of the aging brain that occurs both in rodents and humans (Barrientos et al., 2015). Age-associated inflammatory processes in the hippocampus are known to initiate JNK and p38 signaling (Barrientos et al., 2015) and to impair gene expression required for memory (Bonow et al., 2009). One inflammatory signal, the transforming growth factor beta (TGFβ) is dysregulated during aging (Rawji et al., 2016) and has been shown to

regulate Gadd45γ expression (Grassi et al., 2017). Thus, it is tempting to speculate that dysfunctional Gadd45γ expression may be a consequence of age-related changes in neuroinflammatory processes.

We found that Gadd45γ reduction impairs short-term object-place recognition in a delay-dependent manner that mimics well established aging-dependent cognitive deficits observed across species. To our knowledge this is the first report of a molecular alteration that mimics this deficit. Moreover, a disruption of contextual fear memory was limited to long-term memory impairments in the case of Gadd45γ reduction and was not present in the case of Gadd45γ overexpression. Intriguingly, these findings are in line with rodent studies showing that associative memory, in particular contextual fear memory, is less vulnerable to aging (Foster et al., 2012). Studies in humans also revealed that aged subjects present memory deficits in spatial navigation through virtual environments (Foster et al., 2012; Leal and Yassa, 2015). Although testing contextual fear memory in aged humans has been challenging in the past (Foster et al., 2012), recent evidence suggests that contextual fear memory acquisition and recall are not affected during aging (Battaglia et al., 2018). Thus, the manipulations of hippocampal Gadd45γ levels achieved in this study seem to phenocopy described memory impairments associated with aging.

Aberrant gene transcription patterns occur as a consequence of aging in the hippocampus (Verbitsky et al., 2004; Burger, 2010; Ianov et al., 2017). These changes do not overly correlate across species (Zahn et al., 2007; Loerch et al., 2008), thus limiting the translational potential of animal models. Studies comparing cross-species alterations in gene expression generally focus on shared changes. The similar consequences we uncovered for the observed bidirectional impairments in Gadd45γ expression levels suggest that this approach may neglect functionally relevant and seemingly disparate age-associated transcription changes. Using *in vivo* and *in vitro* models we show that hippocampal levels of Gadd45γ are tightly regulated and that either a decrease or an increase in Gadd45γ can both dysregulate plasticity-associated gene expression and cause cognitive impairments. Accordingly, our findings illustrate a scenario in which diverging age-related transcriptional programs in mice and humans result in converging phenotypes.

In conclusion, our data demonstrates that Gadd45γ is a critical regulator of cognitive functions in the mouse hippocampus. These results further implicate Gadd45γ as a molecular candidate that may underlie cognitive impairments in aging-associated pathological conditions. Identifying the underlying causes that lead to Gadd45γ dysfunction will allow for the design of strategies that may prevent or delay the onset of age-associated memory deficits across species.

4.2. Learning-induced alternative splicing in coupled with memory formation

In the next section of this thesis, we showed that adult hippocampal MeCP2 is required for the regulation of alternative splicing events during memory consolidation. We demonstrated that MeCP2 preserves the *in vivo* alternative splicing profile of mature hippocampal neurons and regulates learning-dependent splicing of genes important for neuronal structure and function. Therefore, our findings show that MeCP2 regulates the levels of expression of memory-related genes and the relative abundance of specific alternatively spliced isoforms, thus uncovering another mechanism by which MeCP2 impacts neuronal functional and structural properties during memory consolidation. This highlights a multifactorial requirement for MeCP2 in adult cognitive processes.

MeCP2 has well-established functions during neurodevelopment as evidenced by the severe neurological impairment's characteristic of Rett syndrome (RTT), a neurodevelopmental disorder caused by mutations in the *Mecp2* gene (Amir et al., 1999; Ip et al., 2018; Gulmez Karaca et al., 2019). Furthermore, several lines of evidence also support an important function during adulthood; MeCP2 is expressed at high levels in the adult brain (Cheval et al., 2012) and is required for its function (Gemelli et al., 2006; Moretti et al., 2006; Skene et al., 2010; McGraw et al., 2011; Cheval et al., 2012; Nguyen et al., 2012; Gulmez Karaca et al., 2018). Mounting evidence indicates that long-lasting synaptic remodeling important for memory consolidation is supported not only by learning-triggered changes in transcription, but also in the post-transcriptional profile (Poplawski et al., 2016) of neurons. In this study, we investigated the regulatory function of MeCP2 in alternative splicing mechanisms. We selectively decreased MeCP2 levels in adult hippocampal neurons (Gulmez Karaca et al., 2018), this way, dissecting the impact of MeCP2 disruption on the alternative splicing profile of mature hippocampal neurons without confounds altered neurodevelopment. We found that reducing MeCP2 expression of mature hippocampal neurons led to abnormal alternative splicing. This finding is in line with previous studies that demonstrated a role for MeCP2 in alternative splicing regulation in other settings (Young et al., 2005; Cheng et al., 2014; Li et al., 2016;

Wong et al., 2017). Several studies analyzed genome-wide gene expression changes in response to learning and have shown the requirement for MeCP2 for this learning-dependent gene expression (Chahrour et al., 2008; Deng et al., 2010; Gulmez Karaca et al., 2018). In contrast, alternative splicing changes on a genome-wide scale upon learning have been less explored. Poplawski and colleagues (2016) were the first to investigate genome-wide alternative splicing changes in the hippocampus after a contextual-fear learning and after memory recall and identified novel alternative splicing isoforms that may be critical for memory consolidation (Poplawski et al., 2016). Our observations support and further expand these findings providing a novel set of alternative splicing events triggered by a non-aversive object-location learning. Therefore, showing that learning-induced alternative splicing is likely a general mechanism required for several forms of learning and memory.

The mechanisms through which MeCP2 regulate learning-dependent alternative splicing events, particularly in mature neurons, are poorly understood. Osenberg and colleagues (2018) studied activity-dependent gene expression and alternative splicing in a mouse model of RTT. The authors elicited neuronal activity in *Mecp2-null* (Mecp2$^{-/y}$) mice through the administration of kainic acid and identified genome-wide alternative splicing changes in the hippocampus in response to this neuronal stimulation. They found an aberrant global pattern of gene expression and alternative splicing events. Here, we used an adult-onset knockdown of MeCP2 and induced neuronal activity by a physiological and memory-relevant stimulus, novel environment exposure. We found that MeCP2 knockdown led to an increase in intron retention and decreased excluded exons. Notably, Wong and colleagues (2017) showed that decreased MeCP2 binding near splice junctions facilitates intron retention via reduced recruitment of splicing factors, such as the splicing factor transformer-2 protein homolog beta (Tra2b), and stalling of RNA polymerase II (Wong et al., 2017). In MeCP2 depletion conditions, like the one in our study, intron retention is favored possibly through the enabling of Tra2b activity. Importantly, this was not associated with an altered Tra2b expression in MeCP2-shRNA mice (Gulmez Karaca et al., 2018). Moreover, intragenic DNA methylation and MeCP2

binding promote exon recognition and consequently MeCP2 ablation results in aberrant exon skipping events (Maunakea et al., 2013). Overall, the demonstrated involvement of MeCP2 in these splicing modalities together with the shift towards increased retained introns and exons in MeCP2 knockdown conditions observed in our study, suggest that MeCP2 contributes to learning-induced alternative splicing through these mechanisms. Although aberrations in these splicing events were predominant, we identified learning-induced changes in other forms of alternative splicing in the hippocampus of MeCP2-shRNA mice. This indicates that MeCP2 may regulate other forms of splicing through mechanisms not yet identified.

In this study, we analyzed alternative splicing events in response to learning in control or MeCP2-shRNA hippocampi as well as in baseline or learning states. This combinatorial analysis allowed us to conclude that the differences found in the learning state do not only reflect changes in basal conditions, but also a requirement for MeCP2 in learning-dependent alternative splicing. Therefore, this indicates that the contribution of MeCP2 to synaptic plasticity and memory is likely two-fold. On the one hand, MeCP2 regulates the neuronal basal transcriptome which may impact neuronal properties such as synaptic transmission and intracellular signal transduction, and additionally may regulate directly stimulus-dependent transcriptional and post-transcriptional events in the nucleus.

MeCP2 is essential for the maintenance of structural and functional properties of neuronal circuits as demonstrated in RTT mouse models (Kishi and Macklis, 2004; Chapleau et al., 2009; Na et al., 2013; Ip et al., 2018). We found that MeCP2 regulates alternative splicing of learning-regulated genes relevant for synaptic plasticity. Noteworthy examples are the P2X purinoceptor 6 (*P2rx6*), the neuron-glia related cell adhesion molecule (*Nrcam*) and the Glutamate Ionotropic Receptor AMPA Type Subunit 3 (*Gria3*). The P2X receptors are ligand-gated ion channels activated by extracellular ATP. Seven P2X receptor subunits have been identified (P2X1-P2X7) that form trimeric receptors of homomeric or heteromeric composition. P2X6 is present in the rodent hippocampus predominantly at glutamatergic synapses (Rubio and Soto, 2001). The activity of P2X receptors induces fast excitatory postsynaptic currents and has multiple modulatory effects on synaptic

plasticity. In the hippocampus, P2X receptors contribute to synaptic strength modulation through its critical role in the regulation of the trafficking of AMPA receptors at the postsynaptic membrane (Kaczmarek-Hajek et al., 2012; Pougnet et al., 2014; Diaz-Hernandez et al., 2015; Pougnet et al., 2016). Interestingly, alternative splicing regulation of P2X receptors affects its expression pattern and possibly function (Masin et al., 2006; da Silva et al., 2007; Kaczmarek-Hajek et al., 2012). Moreover, P2X6 subunits have been shown to translocate to the nucleus in hippocampal neurons, where they are able to interact with members of the spliceosome (Diaz-Hernandez et al., 2015) leading to altered splicing activity. Hence, in the absence of MeCP2, the cellular function may be compromised due to a change in the relative abundance of alternatively spliced P2X6 forms and/or altered splicing activity.

Nrcam is required for cognitive function (Moy et al., 2009). This protein is expressed at the synapse and regulates synapse formation and remodeling (Sakurai, 2012; Demyanenko et al., 2014). We found that *Nrcam* exon skipping was decreased in learning state in MeCP2-shRNA mice. In agreement with our findings, exon skipping of *Nrcam* is induced following exposure to novel objects supporting a functional role in this process (Scott et al., 2017). These results suggest that reduced splicing of *Nrcam* might contribute to the memory impairments observed in MeCP2-shRNA mice (Gulmez Karaca et al., 2018).

It is well established that glutamate receptor subunits are highly regulated by alternative splicing. RNA editing generates flip/flop variants that dynamically regulate conductance of AMPA receptors (Sommer et al., 1990; La Via et al., 2013). Moreover, alternative splicing of AMPA receptor subunits is induced by neuronal activity in the hippocampus (Balik et al., 2013). We found that MeCP2-shRNA mice showed increase intron retention of the Glutamate Ionotropic Receptor AMPA Type Subunit 3 (*Gria3*) during learning state. Retained intron sequences present in Gria3 mRNA are responsible for its dendritic localization (Buckley et al., 2011). Importantly our findings are in agreement with a study that detected altered expression of *Gria3* splicing variants in the cortex of *Mecp2* KO mice (Li et al., 2016) indicating that MeCP2 regulates *Gria3* alternative splicing also in the mouse hippocampus

specifically in learning conditions. Altogether this data suggests that alterations in the relative amounts of splicing isoforms of genes supporting functional and structural plasticity changes after learning may contribute to the cognitive deficits observed in MeCP2 knock-down mice (Gulmez Karaca et al., 2018). It is noteworthy that acute disruptions of adult hippocampal MeCP2 did not alter the dendritic complexity and spine density of CA1 neurons in baseline conditions (Gulmez Karaca et al., 2018). This is in line with our observations that DAS in MeCP2 knockdown in baseline conditions was not enriched for genes functionally relevant to “dendritic spine regulation”. Our findings therefore suggest that MeCP2 regulates alternative splicing of the genes associated with dendritic spines mostly in response to learning, which may cause selective impairments in learning-dependent spine remodeling (Moser et al., 1994; Sanders et al., 2012; Attardo et al., 2015). Whether MeCP2 disruptions alter learning-dependent structural remodeling in mature hippocampal neurons remains to be investigated.

We found that at baseline conditions MeCP2 reduction promoted an overall increase in IR and a decrease in skipped exons, particularly in genes functionally linked to general neuronal functions. Specifically, the abundance of spliced isoforms relevant for neurotransmitter synthesis (glutaminase (*Gls*)), vesicle recycling (synaptojanin 1 (*Synj1*)) and neurotransmitter receptors (gamma-aminobutyric acid (GABA) A receptor, subunit gamma 2 (*Gabrg2*), glutamate ionotropic receptor NMDA Type Subunit 1 (*Grin1*)) was altered in MeCp2 knockdown conditions. Interestingly, the *Grin1* gene gives rise to 8 splice variants and recently it has been shown that the selective expression of different GluN1 isoforms determines long-term potentiation in the hippocampus and spatial memory performance (Sengar et al., 2019b). Moreover, the relative abundance of some spliced isoforms of GluN1 subunit is associated with increased seizure susceptibility in adult mice (Liu et al., 2019). Taken together, these findings suggest that altered alternative splicing events observed in MeCP2-shRNA mice at baseline might impact proper neuronal function and consequently contribute to cognitive deficits and excitation/inhibition imbalance reminiscent of RTT. Furthermore, we found aberrant splicing and/or expression of splicing regulators in resting and learning conditions. In particular, MeCP2-shRNA

mice during the learning state displayed changes in the abundance of U1 small nuclear ribonucleoprotein 70 (Snrnp70) and U2 small nuclear RNA auxiliary factor 1-like 4 (U2af1l4) spliced variants, two components of the spliceosome. In baseline conditions, MeCP2 regulates the expression of the Small the Nuclear Ribonucleoprotein U4/U6.U5 Subunit 27 (Snrnp27) and the Polypyrimidine tract-binding protein 1 (Ptbp1) (Gulmez Karaca et al., 2018). These findings are in agreement with a previous study that also observed alterations in the expression and splicing of splicing regulators as a consequence of MeCP2 ablation (Osenberg et al., 2018). It is plausible that aberrant expression and/or splicing levels of splicing mediators may induce a second wave of impairments in downstream splicing events, such as in response to learning as observed in MeCP2-shRNA mice. Furthermore, as MeCP2 interacts not only with transcription factors but also with regulators of alternative splicing (Young et al., 2005; Maunakea et al., 2013; Cheng et al., 2014; Lev Maor et al., 2015; Li et al., 2016; Wong et al., 2017), loss of MeCP2 may thus impair their recruitment and promote the disruption of alternative splicing events observed in MeCP2-shRNA mice.

Overall, in this section, we found that spatial learning induces alternative splicing events of transcripts with relevant functions for neuronal structure and function. Moreover, our findings implicated MeCP2 in the regulation of this process. We showed that the reduction of MeCP2 levels in adult hippocampus promoted aberrant alternative splicing patterns both in baseline and learning states. This study uncovered another factor that likely contributes to the neuronal dysfunctions that characterize RTT.

4.3 Delayed transcriptional induction associated with memory duration and strength

In the last section of this thesis, we showed that the formation of memories that are short-lasting or persistent induce different patterns of Npas4 expression. We demonstrated that short-lasting memories promote Npas4 transcription immediately after learning while persistent memories induce a second delayed expression of this transcription factor. Moreover, we show that the late expression of this protein is dependent on NMDA receptor activation. Lastly, we demonstrated that artificial induction of Npas4 expression accelerates remote memory decay. These results place Npas4 as a novel regulator of mechanisms that dictate the memory persistence and strength.

Npas4 expression is selectively induced by calcium influx in neurons but not in other cell types (Lin et al., 2008; Ramamoorthi et al., 2011). This contrasts with constitutively expressed or activity- regulated transcription factors (e.g. CREB or Fos, respectively) that are activated by other stimuli such as neurotrophic signaling. Another seemingly unique characteristic of Npas4 is that it can be induced in glutamatergic and GABAergic neurons.

In excitatory neurons, reduction of Npas4 levels leads to a decrease in inhibitory synaptic contact onto these neurons, while overexpression of Npas4 increases their number (Lin et al., 2008). These findings were also confirmed in organotypic hippocampal slices where reduced Npas4 levels decrease inhibition and increase excitation, while a higher Npas4 level results in more inhibition and less excitation. These results suggest that the levels of Npas4 in excitatory neurons dynamically regulate the amount of inhibitory input they receive.

In inhibitory neurons, *in vitro* and *in vivo* Npas4 deletion in somatostatin (SST)-expressing GABAergic neurons, reduces the number of excitatory synapses on these neurons, without altering the total number of GABAergic synapses (Spiegel et al., 2014). These findings are consistent with the role of Npas4 in excitatory neurons, as its expression seems to negatively regulate the overall activity level of neural circuits in response to activity. For this reason it has been hypothesized that Npas4 expression may regulate homeostatic plasticity mechanisms (Maya-Vetencourt,

2013) by orchestrating excitatory/inhibitory balance of neural circuits. Particularly by engaging inhibitory synapses onto excitatory neurons, and excitatory synapses onto inhibitory neurons in response to increased activity (Spiegel et al., 2014). It is tempting to speculate that the delayed wave of Npas4 observed when mice undergo consolidation of a persistent memory might act as a downscaling mechanism to regulate memory intensity and duration.

Most studies have focused on the processes underlying memory formation and consolidation and therefore memory persistence. However recent evidence highlights the importance of forgetting mechanisms associated to memory transience. The short-lasting memory protocol used in this study is an example of learning that does not induce a persistent memory, resulting in passive forgetting over time. Nonetheless other types of forgetting have been proposed [for review see (Davis and Zhong, 2017)]. One of these mechanisms is intrinsic forgetting where activated signaling pathways compete with consolidation mechanisms to determine if a memory will persist or decay. Daily we are exposed to a constant of new information and learning events that trigger increases in neuronal excitation. Intrinsic forgetting has been proposed to be a homeostatic mechanism to bring the brain back to its basal state (Davis and Zhong, 2017). Potentially by acting as a filter to induce forgetting of memories that are not required and that do not promote molecular mechanisms of consolidation that can override active forgetting activation. Memory persistence is associated with long-lasting increases in synaptic strength between neurons upon learning. These increases are prolonged over long periods of time such as in mechanisms associated with systems consolidation. On the other hand, it has been proposed that forgetting occurs when modified synapses are destabilized (Richards and Frankland, 2017). Specifically, by overturning potentiated or depressed synaptic connections or eliminating newly formed synaptic connections during learning. The established role of Npas4 in downscaling activity-dependent neuronal activity might suggest it might act as a player in intrinsic forgetting. Indeed, we found that artificial induction of late Npas4 expression resulted in remote memory impairments. This result might be interpreted as an increase in memory decay or induction of intrinsic forgetting.

Moreover, we found that induction of delayed Npas4 expression is dependent on NMDA receptor activation and not activation of D1 receptors. This result is consistent with the exclusive calcium-dependent induction of Npas4 expression. Interestingly it has been reported that induction of forgetting mechanisms depend on NMDA receptor activation (Sachser et al., 2016). The authors showed that forgetting of object recognition memory depends on calcium influx induced partially by activation of NMDA receptors after learning has taken place. Rats that were injected with a NMDAR antagonist 6h after training, and subsequential daily injections, showed a decrease in forgetting. These results suggest that post-acquisition NMDAR activation induces memory decay. This finding has also been shown in rats that were trained in Morris water maze (Shinohara and Hata, 2014). Chronic infusion of APV into the hippocampus 24h after training induces a reduction in forgetting mechanisms. It is tempting to speculate that intrinsic forgetting mechanisms that depend on NMDAR activation induce Npas4 expression which in turn might downscale synaptic inputs in the hippocampus. This hypothesis is supported by the dependence of NMDAR activation for Npas4 expression at 4h post-training. An obvious follow-up question is if inhibition of NMDAR activity at 4h will result in a more stable memory. This experiment would indicate if the late Npas4 expression has a bidirectional role in regulating memory stability over time. If such would be the case, it is possible that expression of Npas4 induces remodeling of CA1 circuitry by inhibitory-excitatory synaptic inputs resulting in synaptic downscaling. This intriguing possibility raises the question on the impact of late Npas4 expression on systems consolidation mechanisms associated with remote memory formation. Particularly if Npas4-mediated downscaling would hinder hippocampal-cortical interactions required for the stability of remote memories.

Overall, in this section, we found that persistent memory induces late expression of Npas4 in an NMDAR-dependent manner. Moreover, we showed that this expression is associated with a faster memory decay which might be a mechanism of intrinsic forgetting.

Chapter 5. References

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