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Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular, realizada sob a orientação científica da Doutora Ana Oliveira (Universidade de Heidelberg) e Professor Doutor Carlos Duarte (Universidade de Coimbra).

David Vilhena Catarino Brito

2015

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“Science is the poetry of reality”

Professor Richard Dawkins

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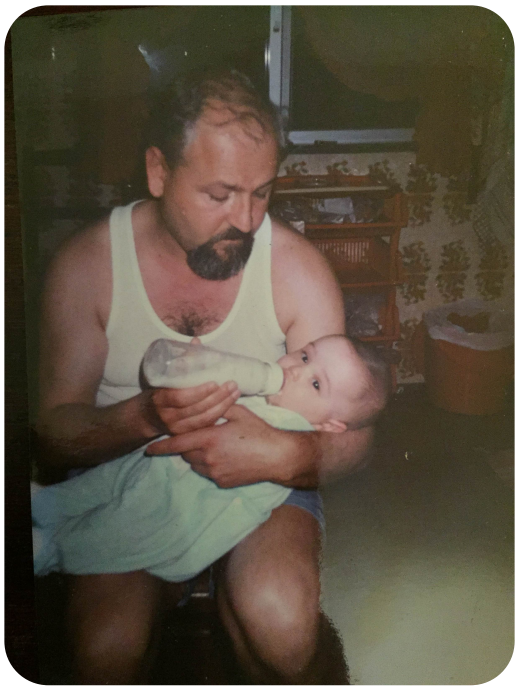
Finally, I acknowledge Maria Amélia Brito my mother, who has supported me in ways which are not writable, with her unconditional love. I also thank my brother Diogo Brito for doing what I cannot do in Portugal, thus providing comfort. Most importantly I thank my late father Alberto Brito to whom this thesis is dedicated.

I thank you all.

David Brito

Dedication

This thesis is dedicated to my late father Alberto Brito. The following text is a poem written in Portuguese.



Ó nobre poeta, de prados verdes do baixo Alentejo chegaste,
vida de entrega e amor a todos, fortemente abraçaste.
Satisfação tua era a felicidade dos outros, tanto ficará para sempre devido...
Prometemos, entre os teus mais queridos, jamais serás esquecido.

O que me ensinaste tornou-me no que hoje sou,
Por mais que tentasse, o que foste acabou.
Ajuda, trabalha e sê racional, coisas maravilhosas acontecerão,
Ainda assim, neste momento lagrimas não secam.

Nada te levou, escolheste este caminho,
O ano de 2014 ensinou-me o que é estar sozinho.
Tudo embora que seja efémero,
Para mim serás sempre eterno.
Em mim vives, tal como em todos os abraçados antes da partida,
Pois esta tese a ti dedicada, foi contigo redigida

David Brito, Julho de 2015

List of Abbreviations and Acronyms

5HMC 5-hydroxymethylcytosine

5HMU 5-hydroxymethyluracil

5MC 5-methylcytosine

A

AC adenylate cyclase

ADP adenosine Di Phosphate

AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate

AraC β-D-arabinofuranoside

Arc activity regulated cytoskeleton associated protein

ARNT2 aryl-hydrocarbon receptor nuclear translocator 2

ATF-2 activated transcriptional factor 2

B

BDNF brain-derived neurotrophic factor

bic bicuculline

BSA bovine serum albumin

C

CA1 *cornu ammonis*-1

Ca^{2+} calcium

CaM calmodulin

CaMKII Ca^{2+} /calmodulin-dependent protein kinase II

CaMKIV Ca^{2+} /calmodulin-dependent protein kinase IV

cAMP cyclic adenosine monophosphate

CBP CREB binding protein

CDC cell division cycle

CH_3 methyl group

C^{me} 5-methylcytosine

CNS Central Nervous System

CpA cytosine-adenine

CpG cytosine-guanine

CRE cAMP response element

CREB cyclic adenosine monophosphate response element-binding protein

CREB cAMP response element binding protein

D

DA deaminase

ddH_2O double-distilled water

DIV day *in vitro*

DM dissociation medium

DNA deoxyribonucleic acid

DNMTs DNA methyltransferases

E

ECL enhanced chemiluminescence substrate

Egr 1 early growth response protein 1

ERK extracellular-signal-regulated kinases

F

F-actin filamentous actin

G

Gadd45 growth arrest and DNA damage 45 protein

Gadd45a Gadd45α

Gadd45b Gadd45β

Gadd45bsh short-hairpin for **Gadd45b**

Gadd45g Gadd45γ

GLY glycosylase

GPCR: G protein–coupled receptor

GusB glucuronidase beta

H

H3K4 histone H3 at lysine 4

HATs histone acetyltransferases

HDACs Histone deacetlyases

HDMs histone demethylases

HMTs histone methyltransferases

HPRT1 hypoxanthine phosphoribosyltransferase 1

HRX histone-lysine N-methyltransferase

I

IEGs immediate early genes

IMDM Iscove's Modified Dulbecco Medium

J

JNKs c-Jun N-terminal kinases

K

KA kainate

L

L-LTP Late LTP

LA laminin

LTD long-term depression

LTM long-term memory

LTP long-term potentiation

M

M molar

MAPK mitogen-activated protein kinase

MAPK: mitogen-activated protein kinase

MAPKK MAPK kinases

MAPKKK MAPKK kinases

MBD methyl-DNA biding domain

Mg^{2+} magnesium

mGluR metabotropic glutamate receptors

Mm millimeters

mM millimolar

mRNA Messenger Ribonucleic acid

N

Na^{+} sodium

NBA neurobasal-A-medium

NMDA: N-methyl-D-aspartate

Npas4 neuronal PAS domain protein 4

Nr4a1 nuclear receptor subfamily 4, group A, member 1

O

ON overnight

ORF open reading frame

P

P0 postnatal day 0

PBS Phosphate buffered saline

PDL Poly-D-lysine

PK protein kinases

PKA: protein kinase A

PKC: protein kinase C

PRMTs protein arginine methyltranferases

PSD95 phosphorylate postsynaptic density protein 95

R

rAAVs Recombinant adenoassociated viruses

RNA Ribonucleic acid

RSK: ribosomal s6 kinase;

RT room temperature

S

s.e.m standard error of the mean

Ser Serine

siRNA small interfering RNA

snRNA small nuclear RNA

STM short-term memory

T

TGY Thr-Gly-Tyr motif

Thr threonine

TRD transcriptional-regulatory domain

TRDMT1 TRNA aspartic acid methyltransferase 1

TrkB tropomyosin receptor kinase B

TSA Trichostatin A

Tyr tyrosine

U

UNC universal control short-hairpin

UV ultraviolet

V

VDCC L-type voltage dependent Ca^{2+} channels

VSCC: voltage sensitive Ca^{2+} channel

List of Illustrations

Chapter 1 Introduction

Chapter 2 Materials and Methods

Chapter 3 Results

Chapter 5 Discussion

List of Tables

Chapter 2 Materials and Methods

Resumo

A adaptação a estímulos ambientais depende de regulação neuroepigenética. Expressão genética de novo é necessária para a formação de memória a longo prazo, sendo facilitada por regulação epigenética. Metilação de ADN é um processo geralmente associado a inativação de genes. Recentemente a reação contrária a este processo foi reportada, num processo referido como demetilação ativa de ADN, sendo promovido pela ação de proteínas como a *growth arrest and DNA damage-inducible protein 45* β (Gadd45b).

No entanto, os mecanismos promovidos pela ação de Gadd45b em genes associados a memória ainda não é clara.

Neste estudo sugerimos que Gadd45b pode se comportar como um facilitador de memória de longo prazo, promovendo alterações necessárias para esta.

Os nossos resultados demonstram que ao fazer *knock-down* de Gadd45b observa-se decréscimos na expressão de genes de resposta rápida e tardia, sendo também detetadas alterações em níveis proteicos. Analise de metilação a nível global aparenta estar aumentada neste modelo, sugerindo que Gadd45b pode-se comportar como uma demetilase.

Importantemente, ratinhos onde a expressão de Gadd45b estava reduzida no hipocampo apresentaram défices em memória de longo prazo, em dois testes comportamentais distintos.

Os nossos resultados demonstram um amplo papel regulatório de Gadd45b em aprendizagem e memória.

Assim, este estudo visa ser uma primeira contribuição para entender como possíveis demetilases de ADN regulam expressão de genes necessários para formação e consolidação de memória.

PALAVRAS CHAVE:

Demetilação de ADN, Gadd45b, Genes associados a memória, Memória de longo prazo.

Abstract

Neuroepigenetic regulation provides adaptive mechanisms to environmental stimuli. Epigenetic mechanisms regulate *de novo* gene expression induction which is required for long-term memory formation. DNA methylation is a process generally associated with gene inactivation. Recently, the active reversal of this mark, DNA demethylation, has received growing attention and has been suggested to depend on proteins action of such as the growth arrest and DNA damage-inducible protein 45 β (Gadd45b). However, precise memory-associated gene expression alterations mediated by Gadd45b are still elusive.

Here we suggest Gadd45b as a long-term memory facilitator, promoting alterations required for long-term memory formation. Our results demonstrate that knocking-down Gadd45b expression promotes impairments in early and late response gene activity, also identified at protein level. Global methylation seems to be increased in this model, suggesting a Gadd45b DNA demethylation action. Importantly, mice with reduced Gadd45b expression showed long-term memory deficits in two distinct memory paradigms.

Our results demonstrate a wide Gadd45b regulatory role in learning and memory.

The present study is a starting point in understanding how possible DNA demethylase players orchestrate gene expression changes required for long-term memory formation and consolidation.

KEYWORDS:

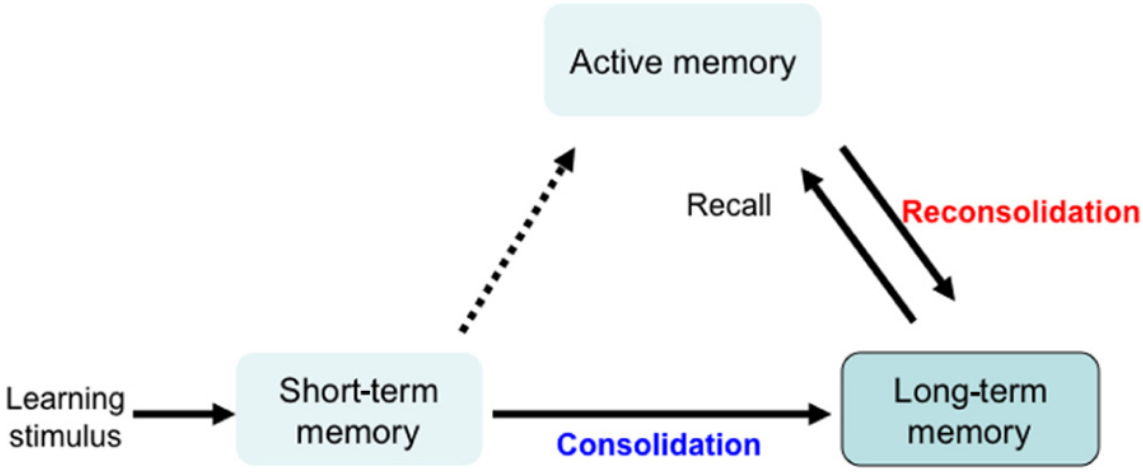
DNA demethylation, Memory-associated genes, Gadd45b, Long-term memory.

Table of Contents

Chapter 1. Introduction

1.1 The Stages of Memory Formation

Memory, the capacity to retain previous learned information in response to external stimuli is undoubtedly an indispensable feature for survival. Memory itself is responsible for behavior adaptations to the surrounding environment. At a unicellular level, several forms of rudimentary memory can be observed throughout the organism i.e. memory B and T lymphocytes in response to diverse antigens, the fate determination of embryonic or adult stem cells. At a systemic level, it is the Central Nervous System (CNS), that is responsible for the formation and consolidation of memory. There are three main phases in which memory storage occurs: (1) memory formation, (2) memory consolidation and (3) memory reconsolidation (**Figure|1.1**) (Wang and Morris, 2010). The first phase consists on promoting the formation of an initial but labile memory, short-term memory (STM), that can range from seconds to on hour. In order to long-term memory (LTM) to form, that lasts hours to years, *de novo* gene transcriptional activation and protein synthesis is required (Alberini, 2009). Particularly, the hippocampus is highly involved in the initial phases of contextual/spatial memory formation and consolidation. Memory consolidation



Figure|2.1 **Phases of memory formation and maintenance.** A learning stimulus potentiates a short-term memory, which lasts from seconds to an hour. Later through a process of consolidation it can be converted into long-term memory which can range from hours to year. Long-term memory can be then reactivated by a memory-cue in a process also *de novo* protein synthesis-dependent, reconsolidation. Adapted from: (Takashima, 2012).

conversely is dependent in systems consolidation, where hippocampal interconnected neurons interplay with the neocortex (Wiltgen et al., 2004).

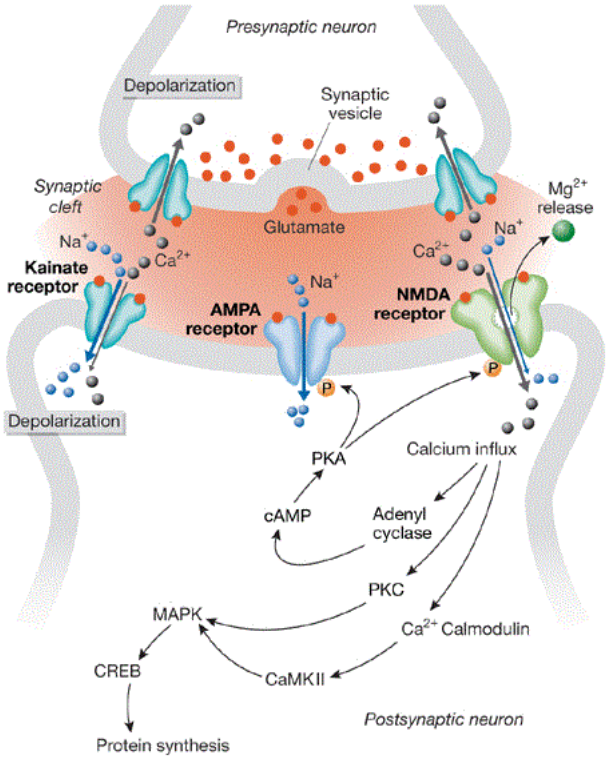
Evidence from the literature highlight that during the consolidation phase, early hypothesized in 1969, memory is resistant to pharmacological disruption (Lewis, 1969). An additional feature is that memory consolidation is optimally engaged during sleep circadian phases. During these stages, molecular pathways involved in learning acquisition and memory formation are reactivated, thus sleep deprivation severely impairs memory storage (Tononi and Cirelli, 2014). These evidence suggest that molecular consolidation is an ongoing process, that continues for at least 24 hours after the learning stimuli (Alberini and Kandel, 2015). Also at a circuitry level, similar patterns of neuronal firing are observed during sleep in post-acquisition phases both in the hippocampus and the neocortex (Kudrimoti et al., 1999). In 1997, a description of a third memory phase was proposed –memory reconsolidation (Przybyslawski and Sara, 1997). Although this stage was described before the turning of the century, within the last 18 years a massive amount of studies aimed to understand the role of this phenomenon. One interesting finding is when animals are exposed to a memory cue, associated to a previously consolidated memory, cue-induced memory reactivation seems to take place (Gisquet-Verrier and Riccio, 2012). In this process, gene transcription is reactivated, promoting a temporary window for memory disruption (Nader and Einarsson, 2010). Thus, when the reinforcement of memory is abolished at this stage by interfering for example with transcriptional activation and/or protein synthesis the newly formed memory is abolish, a process referred to memory extinction. In extinction sessions both performed in rodents and humans, previously acquired memories can be disrupted where a predictive cue is presented alone, immediately after the trial. Performing cue fear conditioning where rodents listen to a tone associated to an electric foot-shock, later developing a fear contextual memory to the tone, if the cue is presented alone, the fear responses will decrease, suggesting a decline in fear-induced memory (Nader and Einarsson, 2010). This process is thought not to be the abolishment of a previous memory, but the formation of a new memory, that inhibits the expression of the first.

Understanding how these phases interplay, and occur at an individual level is a challenging but exciting field of study. Cellular mechanisms, such as signaling

cascades and transcriptional activation are a relevant study target and a first step towards the understanding of memory formation and storage.

1.1.1 Molecular Mechanisms of Learning and Memory

The formation of a new memory in the brain, requires a synchronized set of neuronal responses to a learning stimulus that orchestrate both short and long-term alterations. These changes are commonly referred as synaptic plasticity. The main molecular events that lead to synaptic plasticity are (1) activation of specific neurotransmitter receptors in the postsynaptic terminal, (2) intracellular influx, (3) activation of cytosolic protein kinase cascades, (4) induction of *de novo* gene expression and protein translation and (5) structural and network changes in neurons (**Figure|1.2**).



Figure|1.2 **Gutamate release promotes synaptic plasticity by Ca^{2+} influx.** Impulses that arrive at the presynaptic terminal trigger the release of glutamate, the major excitatory neurotransmitter in the CNS, which binds to glutamate receptors present at the postsynaptic membrane. On activation, AMPA and kainate receptors mediate the entry of Na ions, which initiate postsynaptic membrane depolarization. This change in membrane potential initiate the release of magnesium ions that block NMDA receptors. Ca^{2+} influx through NMDA channels sets off a chain of events that establish long-term potentiation and thus memory-associated plasticity. **AMPA**: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate; **CaMKII**: Ca^{2+}/calmodulin-dependent kinase II; **CREB**: **cAMP** response element binding protein; **MAPK**: mitogen-activated protein kinase; **NMDA**: N-methyl-D-aspartate; **PKA**: protein kinase A; **PKC**: protein kinase C. Adapted from: (Voglis and Tavernarakis, 2006)

In the CNS the most abundant excitatory neurotransmitter is glutamate, which has a predominant role in hippocampal-mediated plasticity. Once released from the pre-synaptic terminal, glutamate activates both ionotropic and metabotropic receptors. The first comprise α-amino-5-hydroxy-3-methyl-4-isoxazole propionic acid (AMPA), N-methyl d-aspartate (NMDA) receptors and kainate (KA) receptors. Most AMPA receptors are permeable only to Na^+, whereas NMDA receptors are both Ca^{2+} and Na^+ permeable. Glutamate firstly activates AMPA receptors, which induce the influx of Na^+. The increase in positively charged ions will depolarize the cell membrane, promoting a conformational change in synaptic NMDA receptors. This voltage-dependent change, potentiates the release of Mg^{2+} from these receptors thus allowing the influx of extracellular Ca^{2+}. NMDA-dependent currents are required for memory formation. In fact, its blockade impairs the formation of long-term potentiation (LTP), the molecular equivalent of learning and memory (Morris et al., 1986). L-type voltage dependent Ca^{2+} channels (VDCC) are also an additional Ca^{2+} source. Other Ca^{2+} sources include intracellular storages as the mitochondria and endoplasmic reticulum.

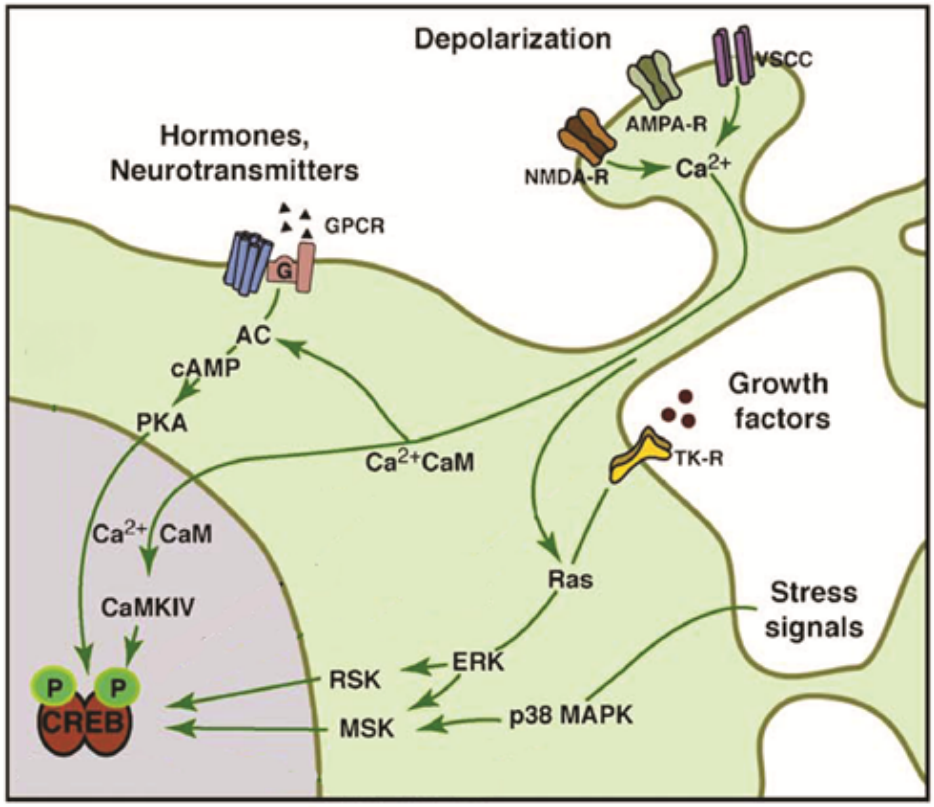
The increased intracellular Ca^{2+} is bound by calmodulin (CaM) (**Figure|1.2**). This Ca^{2+} sensor promotes activation of Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII), a Ser/Thr protein kinase abundant in the CNS. In the absence of Ca^{2+}/CaM complexes, CaMKII is auto-inhibited. After Ca^{2+}/CaM activation, CAMKII autophosphorylation activity is triggered, promoting the phosphorylation of several targets. Interestingly after autophosphorylation the dissociation CaM rate decreases, which allows this enzyme to remain active even when Ca^{2+} influx has decreased (Lučić et al., 2008). This ability of CaMKII to maintain its activity due to autophosphorylation represents a gateway between a transient stimulus and a long-term mechanism. As it will be discussed, CaMKII downstream pathways play roles in LTM consolidation. CaMKII co-localizes with post-synaptic density proteins, where after autophosphorylation can form a complex with the NR2B subunit of NMDA receptors, forming a CaMKII–NMDA receptor complex (Bayer et al., 2001). After activation, CAMKII can potentiate the conductance of AMPA receptors by phosphorylating these receptors (Derkach et al., 1999). This biochemical properties, give CaMKII the potential to work as a memory switch. Studies

showing that synaptic potentiation is induced per se by the administration of Ca^{2+} /CaM in the post-synaptic terminal and the maintenance of this potentiation depends on CaMKII activity (Coultrap and Bayer, 2012).

Besides the CaMKII-dependent signaling, there are other Ca^{2+}-related signaling players. Ca^{2+}/CaM also targets Ca^{2+} /calmodulin-dependent protein kinase IV (CaMKIV) activation. Particularly CaMKK is activated by Ca^{2+}/CaM, after which will phosphorylate CaMKIV, present in the nucleus (Hagenston and Bading, 2011). CaMKIV can phosphorylate the transcription factor cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB). Additionally CaMKIV induced CREB-mediated transcription activation requires additional triggering of coactivator CREB binding protein (CBP) (Wayman et al., 2008). CREB was one of the earliest identified transcription factors required for long-term memory formation, thus being extensively well described as a memory-facilitator (**Figure|1.3**). Most of the upstream signaling cascade leading to CREB activation appears to be conserved through evolution, both in the mammalian brain and in invertebrates. Nevertheless CREB phosphorylation is a complex process where more than 300 different stimuli have been reported to activate CREB (Johannessen et al., 2004). Indeed the overexpression of CREB is enough to potentiate LTM storage, and CREB-dependent pathways have also been shown to be necessary for both LTM formation and long-term synaptic plasticity (Gruart et al., 2012, Alberini and Kandel, 2015). Besides the Ca^{2+} dependent, CaMKIV activation of CREB, excitatory G-protein coupled receptors, i.e metabotropic receptors can activate adenylate cyclase (AC) which produces the second messenger cAMP and activation of protein kinase A (PKA). PKA activation occurs through the catalytic subunits dissociation from the regulatory subunits of this enzyme (Kandel, 2012). PKA can regulate the biochemical activation of several nuclear targets. Moreover, several genes contain the cAMP response element (CRE), CREB is bound to these regions in basal conditions. Upon stimuli, phosphorylated CREB promotes increase or decrease in gene activity.

Activation of CREB-dependent pathways is also dependent on the mitogen-activated protein kinase (MAPK) extracellular-signal-regulated kinases (ERK). Upon activation of protein kinase receptors such as tropomyosin receptor

kinase B (TrkB) receptors by brain-derived neurotrophic factor (BDNF) ERK1/2 is activated by phosphorylation. ERK1/2 activation has been shown to be required for LTM formation but not STM (Adams and Sweatt, 2002). The administration of selective upstream ERK1/2 activator (MEK1/2) blockers, severely impairs fear LTM formation. Where expressing a dominant negative form of MEK 1 also selectively impairs contextual LTM formation but not STM (Atkins et al., 1998, Kelleher et al., 2004). Suggesting a relevant contribution of ERK signaling in memory processes. Recent work also point into a role of other MAPK signaling cascades in memory formation (Yang et al., 2013, Coffey, 2014).



Figure|1.3 **CREB activation is regulated by different neuronal stimuli.** CREB phosphorylation can be triggered by several stimuli such as synaptic activity, hormones, growth factors released during development, hypoxia and stress, (green arrows). Although CREB can bind to genes that contain CRE-sequence, phosphorylation is needed to promote activity. **AC**: adenylate cyclase; **AMPA-R**: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; ; **CaM**: calmodulin; **CaMK**: Ca^{2+}/calmodulin-dependent protein kinase; **cAMP**: Cyclic adenosine monophosphate; **CREB**: cAMP response element-binding protein; **ERK**: extracellular signal-regulated kinases; **GPCR**: G protein–coupled receptor; **MAPK**: mitogen-activated protein kinase; **MSK**: mitogen- and stress-activated protein kinase; **NMDA-R**: N-methyl-D-aspartate receptor; **PKA**: Protein kinase A; **RSK**: ribosomal s6 kinase; **TK-R**: tyrosine kinase receptor; **VSCC**: voltage sensitive Ca^{2+} channel. Adapted from: (Benito and Barco, 2010).

1.1.1.1 The MAPK signaling cascades and plasticity

In the above sections I discussed primarily how plasticity events may be mediated by Ca^{2+} signaling cascades. MAPK are activated through phosphorylation by upstream kinases, the MAPK kinases (MAPKK), and these are target of regulation by MAPKK kinases (MAPKKK) phosphorylation. Although ERK-mediated signaling has been extensively described in plasticity events as a mediator of the communication between the synapse and the nucleus, the involvement of other MAPK family, such as p38 and the c-Jun N-terminal kinases (JNKs), in LTM are still poorly understood.

Similar to other MAPKs, activation of p38 MAPK enzyme occurs by threonine (Thr) and tyrosine (Tyr) phosphorylation in the Thr-Gly-Tyr (TGY) motif, localized within the kinase activation loop by MKK3/6 (Correa and Eales, 2012). In the organism there are four known different genes encoding the p38 MAPK isoforms (p38α, p38β, p38γ, p38δ), which are highly expressed in the mature CNS. Interestingly p38 phosphorylation is increased upon glutamatergic activity (Thomas and Huganir, 2004). In addition there is cellular specificity among the different isoforms in the CNS, where p38α is mainly expressed in neurons in opposition to p38β, which is also present in glia cells (Lee et al., 2000). Also p38 isoforms vary among themselves in cellular distribution in *cornu ammonis-1* (CA1) hippocampal neurons, where p38α distributed throughout various neuronal compartments including dendrites, cytoplasm and nucleus, where p38β is preferentially localized in the nucleus (Lee et al., 2000). Although not yet clear, this difference may underlie different cellular functions for these two isoforms. Indeed the specific nuclear localization of p38β lead to the speculation of a transcriptional regulation role. Nevertheless the plasticity-associated mechanism meditating p38 activation by far most understood is activation of metabotropic glutamate receptors (mGluR) and NMDAR-induced long-term depression (LTD) (Moult et al., 2008, Collingridge et al., 2010). The activation of the MAPK p38 signaling cascade, in mGluR-LTD, is thought to underlie internalization of GluA1/GluA2-containing AMPA receptors, although the specific mechanism still proves illusive (Huang et al., 2004). Besides this established role, the involvement of p38 in LTM formation is poorly addressed.

Most of the available literature focuses in the context of post-training in inhibitory avoidance. It is relevant to note that hippocampal-specific p38 phosphorylation increases immediately after training in this fear-induced memory task, but declines within 30 minutes (Alonso et al., 2003). Although a transient activation of this cascade is sufficient to block both short- and long-term memory formation (Alonso et al., 2003). This was shown by specific inhibition of p38MAPK phosphorylation by SB203580 immediately after training. Overall the data highly suggest a contribution of the p38 MAPK pathway in both synaptic plasticity and LTM and STM formation. Nonetheless available studies are still unclear, not allowing accurate conclusions.

The JNK MAPK signaling cascade also known as stress-activated protein kinases (SAPK) comprises three main isoforms JNK1, JNK2 which are expressed ubiquitously and JNK3 whose expression is restricted to brain, testis and heart. These proteins, similarly to ERK1/2 and p38 are present in neurons, potentiating both survival and neuronal death (Waetzig and Herdegen, 2004). The most well studied downstream target of JNK is c-jun and activated transcriptional factor (ATF) 2, which are regulated primarily by phosphorylation. Phosphorylation of c-jun at the N-terminal Ser63 and Ser73 residues mediates different phenomena, including the increase of transcriptional activity and biding affinity with CBP (Seo et al., 2012). Interestingly c-jun mutant mice, in which the active phosphorylation sites were replaced by alanines, showed that hippocampal c*ornu ammonis-3- cornu ammonis-1* (CA3–CA1) specific LTP was impaired, suggesting modulation of synaptic plasticity by JNK (Seo et al., 2012). Among the three isoforms, JNK1 is the main responsible for JNK activity in the brain (Chen et al., 2005). JNK1 mutant mice show progressive learning impairment and dendritic alterations in the hippocampus. Additionally contextual fear learning selectively increases the expression of JNK1, but not JNK2/3 (Sherrin et al., 2010). This activation occurs 1 hour after, but not 30 minutes after, conditioning and JNK activity returns to baseline within 8 hours post-training. Thus similarly to p38, the activation of this MAPK occurs in a specific time widow. There is evidence that blockade of JNK activity, selectively in the hippocampus, impairs LTM formation but enhances STM (Bevilaqua et al., 2003). Nevertheless JNK protein inhibitor SP600125 released in 2001, has been recently reported to have several off-target effects. In fact, SP600125 can

inhibit other protein kinases with similar or greater potency than JNK (Shuhei Tanemura, 2010). Remarkably the remaining members, JNK2 and JNK3, have been associated to negative effects on LTM. Where administration of pharmacological JNK inhibitors into the hippocampus rescued acute stress-associated memory impairments in contextual fear conditioning. Later on the authors created mutant mice lacking JNK1, JNK2 or JNK3 and found only an involvement of JNK2/3 in stress-memory deficits, but not JNK1 (Sherrin et al., 2010). Altogether, these recent findings suggest that members of the JNK MAPK signaling pathway are involved in plasticity processes and memory formation. It seems that different isoforms of JNK may contribute differently to these processes. Although based on limited information, it is tempting to speculate that JNK1 may have a role in physiological LTM processes, while JNK2/3 may me mediators of response stress signaling.

In future studies it would be relevant to test how both p38 and JNK members regulate mechanisms relevant for STM and LTM, particularly, alterations in transcriptional activity and structural changes.

1.1.1.2 Immediate Early Genes

As mentioned earlier, one requirement for LTM formation is the induction of *de novo* gene transcription and protein synthesis. The first step in this process is the transcription of immediate early genes (IEGs). These genes are activated transiently and rapidly in response to plasticity stimuli, being usually virtually absent in basal conditions. The induction of IEGs can occur within minutes, where more than 500 different neuronal rapid response genes have been identified so far (Perez-Cadahia et al., 2011). IEGs encode products with different roles; these include secreted proteins, cytoplasmic enzymes, cytoskeleton-associated proteins, ligand-dependent transcriptional factors and inducible transcriptional factors. IEG can be divided in: (1) regulatory transcription factors of other genes and (2) effector IEGs, which directly promote changes in neurons that potentiate plasticity effects.

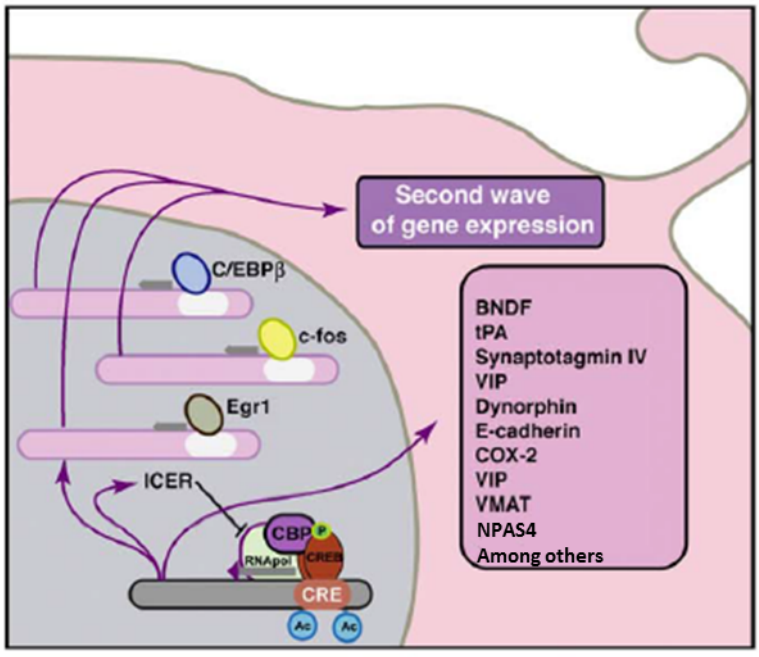
LTP underlies increases in synaptic transmission efficiency, as a result of presynaptic stimulation is thought to be the cellular and molecular equivalent of learning and memory. Not surprisingly late-LTP (L-LTP) is dependent on *de novo* gene transcription. IEGs have been shown to be critical players in this

process (Abraham et al., 1991, Jones et al., 2001). The first IEG class includes genes such as c-fos, early growth response protein 1 (*Egr*-1 or *zif268*), nuclear receptor subfamily 4, group A, member 1 (Nr4a1 or Nur77) and neuronal PAS domain protein 4 (Npas4). Enhanced mRNA expression of both *Egr1* is observed within minutes after induction, where in LTM experiments Egr1 protein is detected upon 12 hours but not in STM (Jones et al., 2001, Katche et al., 2012). In fact, selective Egr-1 knock-out mutants in the dentate gyrus were unable to induce late LTP (L-LTP) (Jones et al., 2001). Moreover this member of the zinc finger family of transcriptional factors regulates the expression of several late response genes involved in plasticity events (Katche et al., 2012). Although the tight involvement between IEG and plasticity events, it is still unclear what are their specific molecular contribution. Lastly IEGs that act as transcriptional factors, open an avenue for a second wave of gene activation (**Figure|1.4**). The study of this second wave of gene expression is still poorly understood, nevertheless it seems to mediate memory maintenance/persistence. Recently, it has been show that the *c-fos* second wave of expression in the dorsal hippocampus, is necessary for the formation of inhibitory avoidance long-term memory (Katche et al., 2010). Moreover transcription inhibition 24 hours after training hinders memory persistence, but not formation (Katche et al., 2010). Additionally Egr-1 protein expression after inhibitory avoidance is shown to be upregulated after training and 12–24 hours after (Katche et al., 2012). Interestingly downregulation of *Egr-1* in the dorsal hippocampus is sufficient to disrupt both LTM formation and maintenance.

Some IEG, however have a less stablished role in memory formation. *Nr4a1*, both a transcriptional factor and member and nuclear receptor is induced in the CA1 after context fear memory consolidation (Pena de Ortiz et al., 2000). *Nr4a1* transcriptional activation is thought to be mainly regulated by histone acetylation. Indeed HDAC inhibitor Trichostatin A (TSA) administration was sufficient to maintain *Nr4a1* expression during memory consolidation (Vecsey et al., 2007). Although there is a clear role for this IEG in memory, recent efforts suggest that *Nr4a1* expression may be associated to specific types of memory (Hawk et al., 2012). Particularly *Nr4a1* showed increase expression in hippocampal neurons, associated with object location memory but no

detectable expression in the perirhinal cortex, associated with object recognition memory (McNulty et al., 2012).

The CA3 is one of the hippocampal regions required for contextual memory encoding. This role has not been extensively explored, nevertheless it has been suggested that *Npas4* may be a master regulator of gene expression in this area (Ramamoorthi et al., 2011). This IEG is expressed following contextual fear conditioning in the CA3 but not in the CA1. This gene is induced by Ca^{2+} signaling in neurons, but not in other cells of the organisms, suggesting a specific function in the brain (Maya-Vetencourt, 2013). Moreover deleting Npas selectively in the CA3 was sufficient to dramatically reduce memory-associated gene expression such as *c-fos*, arc and *Egr-1* (Ramamoorthi et al., 2011). Altogether these exciting new findings start to elucidate a mechanistic long-term genetic regulation. Indeed, the authors of this study hypothesize that Npas4 is required for RNA polymerase II to bind to these target genes regulatory regions. It would be relevant in the future to study how Npas4 activity affected the memory-associated second wave of gene expression.



Figure|1.4 **CREB phosphorylation initiates a transcriptional program.** There are over a thousand of described CREB-activated genes with heterogeneous functions. Some are listed, nevertheless activation of these targets (purple arrows) include transcriptional factors, which upon translation will promote a second wave of gene expression. Adapted from: (Benito and Barco, 2010).

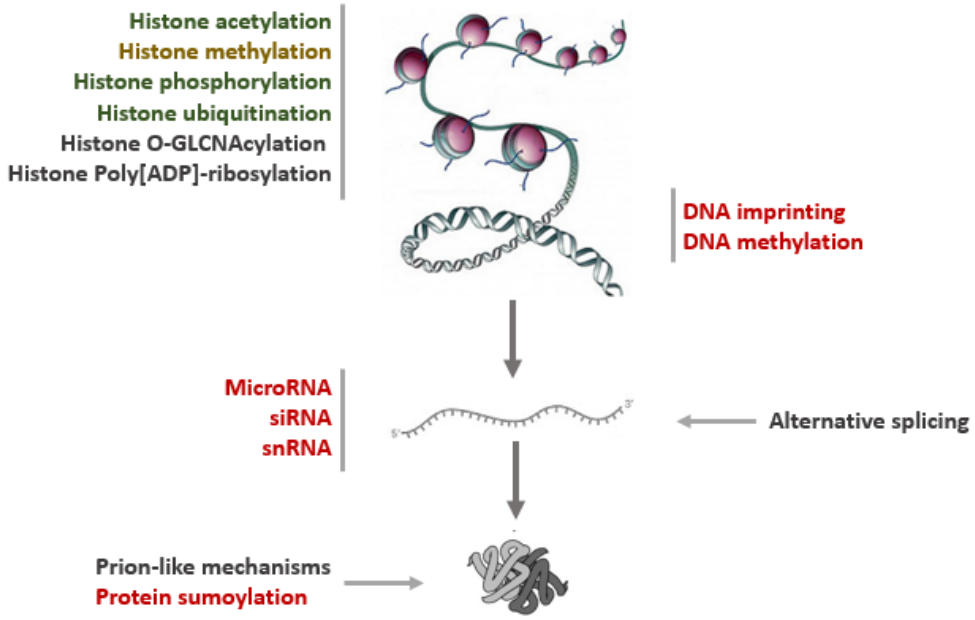
Effector IEGs include among others *Arc*, *Dnmt3a2*, *Homer*, *Cox2*, and *Rheb*. Particularly activity regulated cytoskeleton associated protein (Arc), also known as Arg 3.1 is one of the best studied in the context of memory. Studies where antisense mRNA for Arc was infused into the dentate gyrus led to impairment in L-LTP (Guzowski et al., 2000). Arc expression is induced rapidly, and is dependent on NMDA, but not AMPA receptor activation (Lyford et al., 1995). Moreover, the Arc protein co-precipitates with cytoskeleton proteins such as filamentous actin (F-actin). One interesting feature is that Arc mRNA can travel at high speeds (~300 μm/hour) through dendrites, and this process seems to be independent of protein synthesis (Steward et al., 1998). Also, Arc mRNA is recruited specifically into activated dendritic regions, whereupon Arc protein accumulation occurs and may persist for at least 8 hours (Dong et al., 2003, Ramirez-Amaya et al., 2005). Arc knock-out mice show selective impairments in LTM consolidation, but not STM in several learning tasks (Plath et al., 2006). Together these exciting findings strongly place Arc as an important player in plasticity and LTM consolidation. In addition dendritic Arc has been suggested to play a role in AMPA receptor trafficking. Arc seems to interact with scaffold dendritic proteins and has been suggested to regulate endocytosis of AMPA glutamate receptors (Chowdhury et al., 2006). This finding is in accordance with the LTM effects of Arc and may suggest an involvement in modulating synaptic scaling and memory consolidation.

The study of IEGs prove relevant to understand the interplay between nuclear transcriptional activation and plasticity and memory events. Unfortunately, most studies focus on causal relationship between gene activation and phenotype. Identification of signaling cascades that underlie IEG transcription and downstream mechanisms of their protein products will be a relevant aim in future studies.

1.2 The Neuroepigenetic Field

One of the most intriguing questions in Biology is how a diverse array of cells present in an organism having the same basic genetic material can express/ repress diverse gene pools characteristic of a neuron, myoblast or of an epithelial cell. Surely regulatory events not confined to the gene sequence per

se must exist. Besides playing a critical role in cell fate determination, epigenetic mechanisms have recently been shown to also serve as a link between environment and cellular response (Sweatt, 2013b). Indeed, external stimuli such as behavior experience, learning and stress can trigger epigenetic modifications (**Figure|1.5**) that orchestrate different genetic expression patterns. The alterations produced are capable of generating not only molecular and cellular effects but also functional and behavior alterations.



Figure| 1.5 **Main epigenetic modifications.** There are several layers of epigenetic control which result in altered gene expression. These can act on histone tails, deoxyribonucleic acid chain, regulate messenger ribonucleic acid processing or act directly on protein products. **In green** – Epigenetic marks that mostly result in genetic activation; **in red** – Epigenetic marks that mostly result in genetic repression; in **yellow** – Epigenetic marks that result both in genetic activation and repression; **in gray** – Epigenetic marks that result in increased variability or still unclear outcome. **DNA** -deoxyribonucleic acid; **RNA** - ribonucleic acid; **siRNA** - Small interfering RNA; **snRNA** - small nuclear RNA.

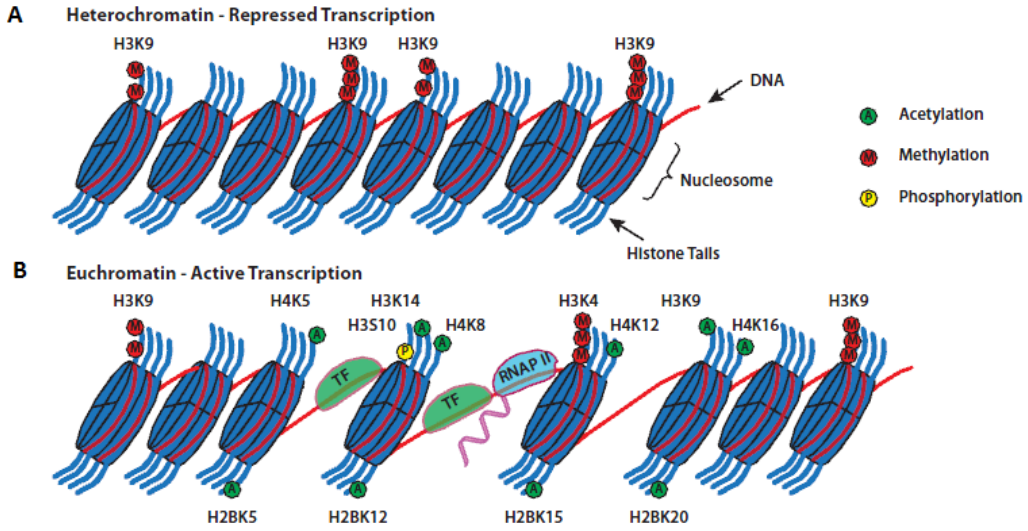
As early as in 1984, Crick postulated that due to the nature of memory formation, epigenetic mechanisms would be suitable candidates to induce self-perpetuating biochemical reactions essential for memory formation and consolidation (Crick, 1984). In the present time, epigenetic alterations associated with learning and memory formation is a subject of extensive research (Zovkic et al., 2013). The past decade provided a wide range of studies that led to the formulation of a new epigenetics subfield, termed neuroepigenetics or behavior epigenetics (Lester et al., 2011). This emerging

field comprises the mechanisms and processes that allow dynamic experience-dependent regulation of the epigenome in non-dividing cells of the nervous system. Different groups have reported a critical role for epigenetic mechanisms in several learning and memory paradigms and across different brain areas. With this in mind, since *de novo* gene expression is a requirement for LTM formation, the study of how epigenetic mechanisms can actively regulate plasticity-associated genes and memory are an exciting and relevant field of study.

1.2.1 Epigenetic Marks

Due to the extensive size of the cellular genome, DNA suffers diverse levels of structural condensation and regulation. Within the nucleus of a cell 146 base pairs of DNA are coiled around an octamer of two pairs of histone dimers (H2A-H2B) and a histone tetramer (H3-H4) resulting in a nucleosome unit (Day and Sweatt, 2010). Adjacent nucleosomes are joined among themselves via the linker histone H1 resulting in a three-dimensional structure called chromatin. This condensed state can come in two different forms according to a second degree of condensation, thus altering transcriptional activity: (1) heterochromatin, a highly condensed state that is not transcription permissive or (2) euchromatin, a state that allows transcriptional activity (**Figure|1.6**). The heterochromatin condensation state is achieved by hypermethylation in chromosome regions.

There are two basic epigenetic mechanisms on current research focus: firstly the regulation of chromatin structure due to post-translational modifications such as histone acetylation, methylation and phosphorylation; and secondly cytosine methylation and demethylation (Feil and Fraga, 2011). There are other relevant mechanisms such as histone ubiquitination, Poly-ADP ribosylation, non-coding RNA inhibition and prion-based traces that still remain unclear (Day and Sweatt, 2012, Sweatt, 2013a, Sweatt, 2013b, Zovkic et al., 2013).



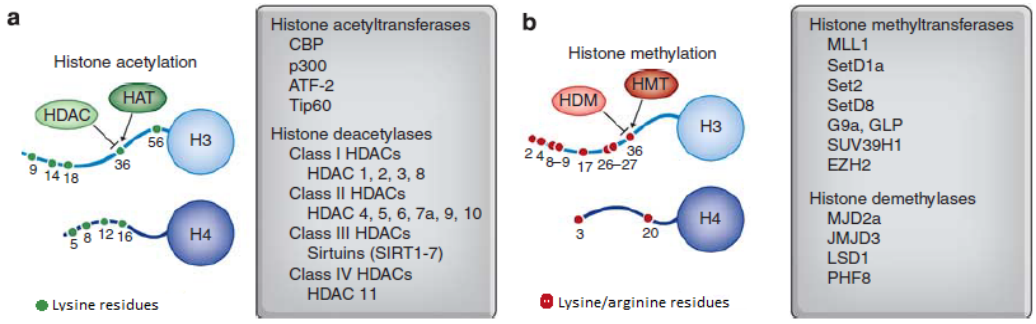
Figure|1.6 **Structure of heterochromatin and euchromatin. A)** The heterochromatin structure is highly condensed and hypermethylated which impairs transcription machinery from accessing genes. **B)** Euchromatin, on the other hand exhibits a more relaxed state due to the presence of epigenetic activation marks such as acetylation and phosphorylation. TF- transcription factor; RNAP II, RNA - polymerase II. Adapted from (Zovkic et al., 2013).

1.2.1.1 Histone Modifications

As mentioned above, histones are partially responsible for the condensed state of DNA in the nucleus, thus allowing/inhibiting the access of the transcriptional machinery to specific gene loci. Although posttranslational modifications of histones can arise after DNA methylation, they can also occur per se by intracellular mechanisms independent of methylation. For the purpose of simplification, here I will only address histone acetylation, methylation and phosphorylation since these represent the most well-known epigenetic marks.

Histone acetylation occurs due to the action of histone acetyltransferases (HATs), which catalyze the transfer of a negatively charged acetyl group from acetyl-CoA to the amine group of lysine residues within histones tails (**Figure|1.7 a**) (Graff and Tsai, 2013). The neutralization of the positive charge in lysine residues promoted by acetylation results mainly in permissive effects on gene transcription. The HAT enzymes are divided into the GNAT, MYST and p300/CBP subfamilies (Berndsen and Denu, 2008). Histone deacetlyases (HDACs) reverse this reaction by removing acetyl groups from lysine residues (**Figure|1.7 a**). The HDAC family members are organized into four different classes and are expressed heterogeneously in the brain (Kouzarides, 2007). The four groups are the zinc-dependent class I, II and IV HDACs, and the NAD-

dependent class III HDACs, also known as sirtuin family members (Haberland et al., 2009). These enzymes play an important pharmacological role in current research strategies, since HDAC inhibitors are currently the main pharmacologic strategy of manipulating the epigenome. In addition, histone acetylation increases after neuronal stimulation, thus being implicated in plasticity events associated to memory formation (Peixoto and Abel, 2013).



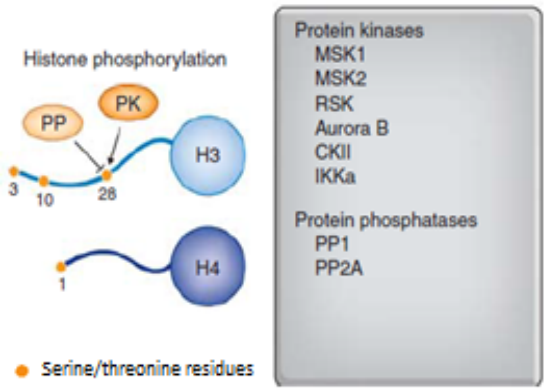
Figure|1.7 **Histone acetylation and methylation**. **(a)** Histone acetylation occurs at lysine residues on histonic tails, histone acetyltransferases (HATs) catalyze the addition of acetyl groups and histone deacetlyases (HDACs) are responsible for the inverse reaction. Histone acetylation generally results in transcriptional activation. **(b)** Histone methylation occurs at lysine and arginine residues on histone tails the adding of methyl groups is catalyzed by histone methyltransferases (HMTs), and demethylation occurs by histone demethylases (HDMs) action. Histone methylation occurs selectively on particular sites of the genome, providing both transcriptional activation and transcriptional repression. Adapted from: (Day and Sweatt, 2012).

Interestingly acetylation changes that occur after diverse types of neurotransmitter stimulation are produced by both HAT stimulation and inhibition of HDAC action (Crosio et al., 2003, Cowansage et al., 2010). This interplay highlights the dynamic nature of epigenetic control associated to neuronal stimulation. Moreover fear-conditioning experiments, a paradigm of hippocampal-dependent memory, produces increased levels of H3 acetylation in the CA1 area of the hippocampus (Levenson et al., 2004). Nevertheless, the current evidence still fail to correlate complex interactions of histone acetylation with other epigenetic marks. Additionally, most of the studies available do not study different neuronal populations, such as GABAergic neurons, glia cells and different brain regions such as the different layers of the cerebral cortex.

After discussing a permissive epigenetic modification, methylation (**Figure|1.7 b**) of histones tails contrary to acetylation generates both transcriptional

activation and repression depending on the lysine/arginine residue methylated and the histone in question. The adding of methyl groups to lysine residues preserves an overall positive change, the opposite effect of acetylation. The enzyme intervenients in this process are the histone methyltransferases (HMTs) which promote methylation of histone at specific residues (**Figure|1.7 b**). Contrarily to HDACs, HMTs such as histone-lysine N-methyltransferase (HRX), may result in transcriptional activation when catalyzing H3K4 methylation (Akbarian and Huang, 2009). The reverse effect, removal of methyl groups from histone tails, is catalyzed by histone demethylases (HDMs). As previously noted methylation can also occur in arginine residues present in histone tails, a reaction catalyzed by the protein arginine methyltranferases (PRMTs). In opposition to other histone epigenetic alterations, residues can be -mono, -di methylated in arginine residues or even tri- methylated on lysine residues (Lester et al., 2011, Day and Sweatt, 2012, Sweatt, 2013a, Peters, 2014). This plastic effect of methylation, produces different repression effects depending on the degree of methylation. Nevertheless, also produces a layer of complexity for understanding the possible combinations of this epigenetic mark. Interestingly, trimethylation of histone H3 at lysine 4 (H3K4) is upregulated in the hippocampus after contextual-fear conditioning. Moreover, mutants that lack specific trimethylation of H3K4 exhibit deficits in hippocampal associated memory, comparing to controls (Gupta et al., 2010). This posttranscriptional modification was also associated to selective increase of the immediate early gene *Zif268* and also *bdnf*, well characterized memory-associated genes. Active DNA demethylation has been also associated to the increased expression of these targets which makes it tempting to hypothesize that different epigenetic modifications may have a redundant effect. Nevertheless, a more interesting hypothesis is that both histone methylation and DNA demethylation events may occur in a synergetic manner. From a mechanistic point of view, studies that correlate these two epigenetic marks would be central in the understanding learning and memory-associated epigenetic mechanisms.

As a third class of the most common epigenetic modifications in histones is phosphorylation. This mark consists in the addition of a phosphate group to serine residues present in histonic tail. This reaction is catalyzed by protein kinases (PK), and the inverse reaction by protein phosphatases (PP) (**Figure|1.8**) (Santos-Rosa and Caldas, 2005, Gelato and Fischle, 2008). Similarly, to histone acetylation,



Figure|1.8 **Histone phosphorylation**. This epigenetic mark is catalyzed by protein kinases (PKs) which occurs at serine/threonine residues. Where the inverse reaction is catalyzed by protein phosphatases (PPs). Similarly, to histone acetylation, phosphorylation is generally associated to transcriptional activation. Adapted from (Day and Sweatt, 2012).

phosphorylation is associated with gene expression enhancements. This epigenetic mark has also been linked to learning and memory paradigms, excitingly by promoting histone deacetylase inhibition, thus enhancing memory-associated gene expression in brain areas such as the hippocampus (Hait et al., 2014).

Contextual fear conditioning experiments, which promote activation of the ERK MAPK pathway also seem to regulate histone phosphorylation. Moreover specific inhibitors of this pathway selectively impair histone H3 phosphorylation in the CA1 area of the hippocampus (Chwang et al., 2006). Studies that demonstrate the interplay between cytosolic pathway activation and epigenetic modifications upon models of learning and memory are key in fully understanding how these actions take place. Likewise, in the light of recent identified epigenetic marks, i.e. dynamic DNA methylation, future investigation that focuses on these non-nuclear mechanisms are crucial.

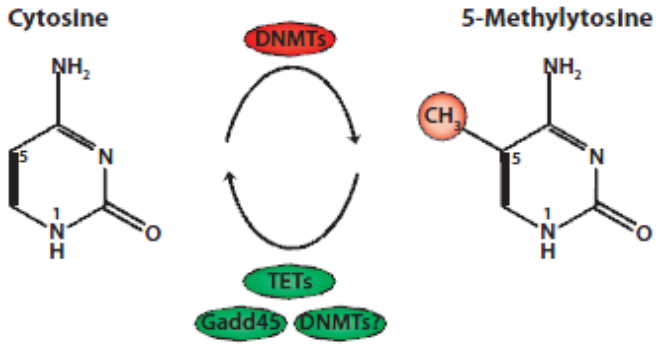
1.2.1.2 DNA Methylation

DNA methylation is an exceptionally powerful regulator of genetic expression, extensively characterized in the past decades (Robertson, 2005, Santos et al., 2005, Miller et al., 2010, Szyf, 2013). This form of regulation has a core silencing role not only in cells of the CNS, but in virtually in all cell types of a mammalian organism. DNA methylation occurs most frequently at cytosine-guanine dinucleotides, the so-called CpG islands although recent findings reveal other possible sites of methylation, at CpH islands (Guo et al., 2014). CpG methylation sites exist both in promoter sites and intragenically; nevertheless, a high number unmethylated CpG islands exist in promoter regions, thus giving an active regulation role to methylation mechanisms. Accordingly, both CpG and cytosine-adenine (CpA) methylation are present in the brain and produce, generally transcriptional repression.

Mechanistically methylation occurs when a methyl group (CH_3) is covalently added to a cytosine at the 5' on the pyramidal ring. This reaction is catalyzed by active DNA methyltransferases (DNMTs) (**Figure|1.9**) (Feil and Fraga, 2011). There are two variants of DNMTs: (1) maintenance DNMTs and (2) *de novo* DNMTs. Both types vary in the conditions of their actions, the first type methylate hemimethylated DNA, in order to maintain gene repression after cell division in mitotic cells. *De novo* DNMTs however, methylate sites where there are no methyl-cytosine groups on either one of the DNA strands. The only known maintenance DNMT is DNMT1 where DNMT3a and 3b are *de novo* DNMTs. All DNMTs are present in most cells in the organism including the CNS with the exception for DNMT3b which exhibits low expression on the adult brain (Feng et al., 2010). Until 2006 there was a fourth member of DNMTs, DNMT2, which share a high homology with the previous cytosine methyltransferase enzymes. Nevertheless, a study by Goll and collaborators showed that DNMT2 did not methylate DNA, instead methylate small RNAs. Currently this enzyme is called TRNA aspartic acid methyltransferase 1 (TRDMT1) (Goll et al., 2006).

Gene repression is the most described functional result of methylation. This process occurs most frequently when CpG islands are located in non-coding areas, usually in upstream promoter regions. Therefore, the effect of cytosine

methylation depends greatly on the localization of the methylated CpG island. If the methyl group transfer occurs in coding regions, i.e. in the open reading frame (ORF) the opposite effect may occur (Metivier et al., 2008). The precise molecular mechanism responsible for this outcome are not fully understood and are an area of active research. The repressive effects of cytosine methylation are better characterized and will be addressed in more detail.



Figure|1.9 **DNA Methylation**. This process involves the transfer of a methyl group to the 5′ position of the cytosine pyrimidine ring by a DNA methyltransferase. The inverse process, active DNA demethylation, can also occur by mechanisms involving members of the Gadd45 family, TET family, and DNMTs themselves, although the precise mechanisms are unclear. Adapted from: (Zovkic et al., 2013).

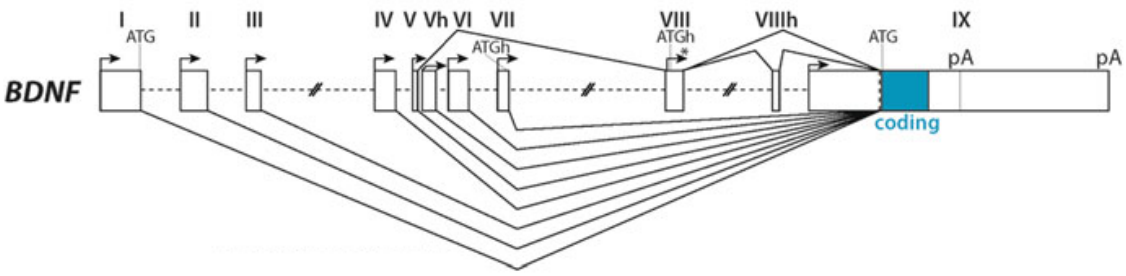
Subsequently to DNA methylation, specific proteins that have a methyl-DNA biding domain (MBD) are recruited to particular sites in the cellular genome. Such members include MeCP2, MBD1, MBD2, MBD4 and Kaiso (Hendrich and Bird, 1998, Prokhortchouk et al., 2001). These regulatory proteins have a transcriptional-regulatory domain (TRD) that induces the recruitment of adapter/scaffold proteins (Zou et al., 2012). Thus, after these proteins recognize the newly transferred methyl group on the DNA chain, HDACs are mobilized to the site. HDACs are responsible for altering chromatin structure by removing acetyl groups from histone core proteins. Taking into account that histone acetylation is an epigenetic mark associated with transcriptional activation, the action of HDACs will promote chromatin compaction resulting in gene repression. In sum, cytosine-methylation occurs by the action of two main components, the DNMTs, methylation ‘writers’, that promote the physical

methylation of DNA mainly at CpG islands, and the 'reader' proteins that have a MBD and recruit HDACs to the site.

In addition to the classical methylation writers and readers exciting new findings report methylation 'eraser' proteins involved in the removal of methyl groups from previously methyl-cytosine (mC) residues, a process called active DNA demethylation.

1.2.1.2.1 Active DNA Demethylation

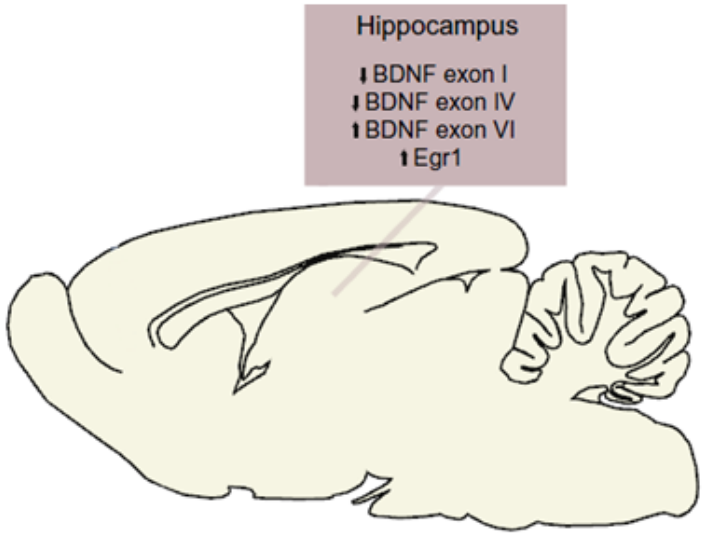
The concept of DNA demethylation has been postulated repeatedly throughout the literature, yet until recent years this elusive phenomena was only associated to methylation loss through cellular division when daughter cells fail to replicate this mark post-mitotically (Robertson and Jones, 2000). The idea that DNA methylation was a stationary process in post-mitotic cells e.g. neurons, generated the hypothesis that DNA methylation could be a memory consolidation mechanism. This hypothesis was refuted since active DNA demethylation of mC has been recently described in neurons (Guo et al., 2011a). The occurrence of a dynamic DNA methylation process opens avenues for research in plasticity processes and memory formation. In fact the gene encoding BDNF, which expression has also been associated with formation and persistence of fear-memories, undergoes changes in DNA methylation patterns, after fear conditioning (Day and Sweatt, 2011) The *bdnf* gene comprises nine different exons (**Figure|1.10**), which are differently regulated during learning and memory.



Figure|1.10 **Structure of the mammalian *bdnf* gene.** The *bdnf* gene consists of different exons linked to promoters that initiate transcription of distinct mRNAs. As a common feature all transcripts contain the open reading frame (ORF, represented in **blue**) that encodes for the pre-pro-BDNF. Human-specific exons that are not present in rodent *bdnf* are marked with the letter "h" following the roman numeral representing the name of the exon brought above the box designating the exon. Horizontal dashed lines

represent introns. Vertical dashed lines inside exons indicate alternative splicing acceptor sites used within that exon. Splicing patterns of neurotrophin mRNAs are shown by lines linking exons. The most upstream transcription start site (TSS) is indicated by an arrow for each exon. The asterisk marking the TSS of BDNF exon VIII stands for a rodent-specific transcription initiation site that has not been detected to be used in human. Introns that are interrupted with double slash are longer and out of this scale. Reproduced from: (West et al., 2014).

Studies where heterozygous mutant BDNF mice were generated show an impairment in hippocampal-mediated tasks such as contextual fear conditioning and Morris water maze (Linnarsson et al., 1997, Liu et al., 2004). Particularly the methylation of CpG islands within BDNF exons I and IV promoter regions seems to decrease in response to contextual fear conditioning (**Figure|1.11**) (Lubin et al., 2008).



Figure|1.11 **BDNF exons present different methylation patterns after fear conditioning.** Fear conditioning promotes methylation changes in the rat hippocampus at CpG islands in the promoter regions of selected genes. Particularly BDNF exon I and IV show a decrease in methylation suggesting that they are activated, compared to BDNF exon VI and Egr-1 which have an increase in promoter methylation. Adapted from: (Day and Sweatt, 2011).

More recently, evidence from the same group highlighted that rats exposed to a novel spatial environment exhibited decreased *bdnf* methylation in the dentate gyrus and increased methylation DNA in CA3 (Roth et al., 2015). Interestingly

the exon I of BDNF was analyzed at individual CG dinucleotides resolution in the dentate gyrus and lower levels of methylated DNA associated with this exon was detected. This studies support an exon-specific regulation of the *bdnf* gene associated to learning and dependent DNA methylation regulation.

Other recent findings in the methylation field include the discovery of 5-hydroxymethyl-cytosine (hmC), a novel DNA base, also present in the CNS (Kriaucionis and Heintz, 2009). The existence of this sixth base, could indeed serve as a precursor form for dynamic demethylation. Since hmC exists both in the totipotent fertilized zygote and the nervous system at high levels, promotes this enthusiastic hypothesis due to the unmethylated nature of embryonic cells. In fact recent studies demonstrated dynamic DNA demethylation in the brain occurring in study models of fear memory (Levenson et al., 2006, Feng et al., 2010).

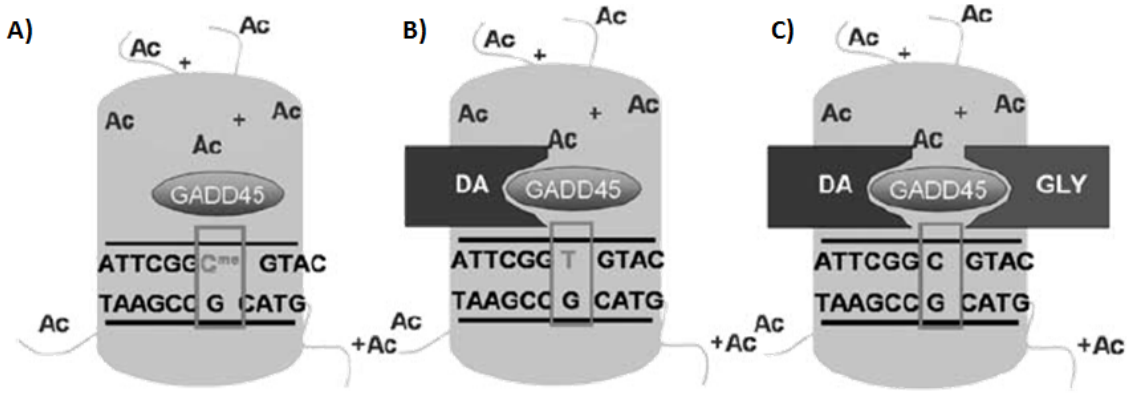
Currently molecular players involved in the conversion of mC to a demethylated state have been identified. The ten-elevated translocation (TET) family of oxidases were the first enzymes associated to this process (Guo et al., 2011b). In the last years other proteins have been linked to active demethylation in the CNS. Both the growth arrest and DNA damage (Gadd45) family of proteins and even DNMTs themselves have been suggested to promote dynamic demethylation (Lester et al., 2011, Moskalev et al., 2012, Chen et al., 2013, Sultan and Sweatt, 2013).

1.3 The Gadd45 Family Members

The growth arrest and DNA damage or Gadd45 family was firstly identified after cell line stimulation with irradiation stress and interleukin treatment. This family includes three members Gadd45α (Gadd45a), Gadd45β/Myd118 (Gadd45b) and Gadd45γ/CR6 (Gadd45g) (Fornace et al., 1988, Abdollahi et al., 1991, Beadling et al., 1993). All members are expressed in a wide range of tissues including the brain, were their response to stress stimuli has been verified. The proteins produced by these family members are relatively small, about 18kDa, and share high homology (55%-57%). Due to this amino acid similarity, different members of the group can form homo-, heterodimers and oligomers, although dimers are the most frequent form (Moskalev et al., 2012). Their most well-known function include an active role in cell-cycle arrest and cellular senescence. Correspondingly these family members are key elements in DNA-damage induced G2/M cell cycle arrest in various cell types (Wang et al., 1999). These actions are achieved by interaction with p53-inducing proteins and cell division cycle (CDC) kinases (Moskalev et al., 2012). As expected, all members of the family are expressed mainly in the cell nucleus, which highlights their genetic associated functions. Importantly Gadd45a and Gadd45b have been identified as mediators of epigenetic control in the brain independent of programed cell death or mitosis checkpoint control (Sultan and Sweatt, 2013).

The gene that encodes Gadd45a originates four transcripts, however only two translate into a protein form (Sultan and Sweatt, 2013). At the present, the different isoforms produced do not have different reported functions, thus experiments that test differential functions could be relevant. It is thought that Gadd45a could only promote DNA demethylation by repair-mediated mechanisms upon DNA damage, which resulted in gene activation (Barreto et al., 2007, Niehrs and Schafer, 2012). Particularly, a TATA-binding protein, TAF12 is thought to recruit Gadd45a into specific methylated promoter sites, were by base-excision repair the cytosine group is removed (Schmitz et al., 2009). In the nervous system, it was shown that after depolarization Gadd45 family members bind to methylated promoter regions proximal to an acetylated histone (**Figure|1.12 A**), then a deaminase is recruited and deaminates 5-methylcytosine (5MC) or 5-hydroxymethylcytosine (5HMC) to for thymine or 5-

hydroxymethyluracil (5HMU), thus leading to a leading to a T:G mismatch (**Figure|1.12 B**) (Gavin et al., 2012). The next step is the excision of the TpG:methyl-CpG or 5HMUpG:CpG mismatch by a DNA glycosylase (**Figure|1.12 C**). This new exciting data gives a dynamic role for DNA methylation as a dynamic process with methylation 'writers' and 'erasers'. Recently the demethylation activity of Gadd45b has been associated to hippocampal-dependent memories; due to the high homology and expression patterns of these two members, it would be interesting to further investigate if Gadd45a has an active role in learning and memory consolidation.



Figure|1.12 **Gadd45b proposed mechanism for active DNA demethylation.** Coupling between 5mC deaminase and G/T mismatch DNA glycosylase is triggered by the presence of Gadd45b. Following depolarization, Gadd45b protein binds to a methylated promoter region proximal to an acetylated histone, next **(A)** Gadd45b recruits a deaminase (DA), which converts 5-methylcytosine (C^{me}) to thymine, leading to a T:G mismatch **(B)**. Gadd45b may then recruit a DNA glycosylase (GLY), which removes thymine from the T:G mismatch by base excision repair. Thymine is later replaced with an unmethylated cytosine **(C)** Adapted from: (Gavin et al., 2012).

The gene for Gadd45g encodes three transcript variants, of which two are protein-coding, and produced by alternative splicing although similarly to Gadd45a these variants are not described to have different physiologic functions, (Moskalev et al., 2012). Although the oncogenic and embryonic functions of this family member are described in some detail, its role in memory consolidation is still quite limited. Indeed Gadd45g transcripts were found to be upregulated in the hippocampus and amygdala after fear induced memory (Sultan et al., 2012). However, the effects of Gadd45g ablation on hippocampal-dependent memory is still a subject to be explored. Since the expression of both

Gadd45b/g is induced in the hippocampus after learning paradigms, taking into account their high structural similarities it is tempting to hypothesize a complementarity action. Fascinatingly unpublished work from the group of Dr. Farah D. Lubin, suggests that the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) can actively regulate Gadd45b expression in the CA1 area of the hippocampus upon fear induced memories. In fact, NF-kB inhibition impaired Gadd45b mediated *bdnf* demethylation and Gadd45g transcription.

Altogether the current knowledge of Gadd45g is still lacking functional and mechanistic detail. Emerging studies put this protein as a possible hippocampal and amygdala memory consolidation mediator, but further investigation is needed to a better understanding of its precise function.

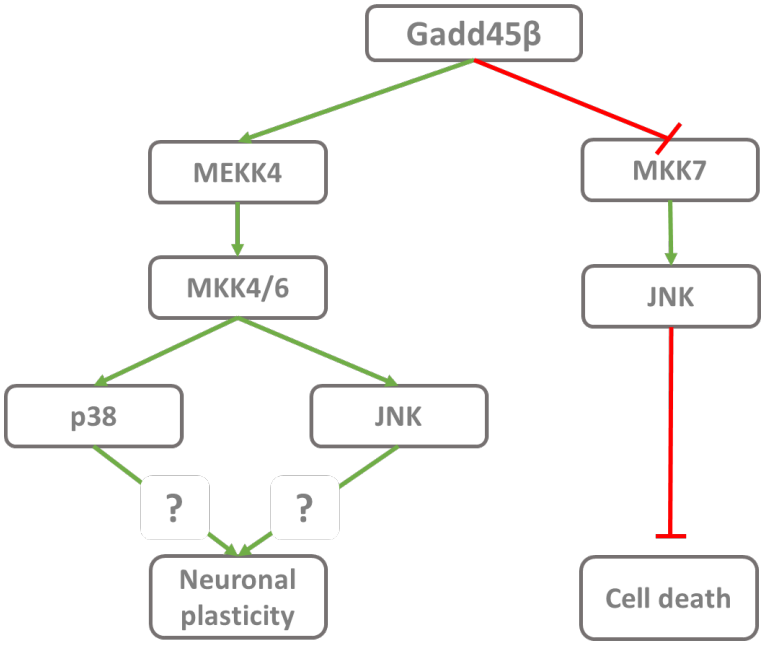
1.3.1 Gadd45β and DNA Demethylation

Comparing to the other members of this family, Gadd45b is the only one that encodes only one protein. This family member has been a recent target studied in terms of its demethylating role associated to hippocampal learning and memory. Firstly, in the CNS, Gadd45b is highly expressed after electroconvulsive therapy particularly in the dentate gyrus. Moreover, demethylation events were detected in the transcriptional starting sites of the BDNF exon IX and FGF-1B genes. These were then abolished in Gadd45b-null mice (Ma et al., 2009). Together these findings reveal a Gadd45b demethylation induced event in genes associated to synaptic plasticity. Interestingly Gadd45b KO mice in models of hippocampal and amygdala dependent memory tasks performed normally compared to controls only in non-hippocampal paradigms. This finding suggest that Gadd45b mediates preferentially hippocampal-dependent memory formation. Controversially, independent studies of Gadd45b knock-outs reported opposite contextual fear conditioning results; memory deficits and enhanced performance in hippocampal- dependent memory (Leach et al., 2012, Sultan et al., 2012). It is worth noting that the models used in both studies were whole knockouts. In future approaches it would be important to use hippocampal-specific Gadd45b deletions or knockdowns to validate further conclusions. Given the wide expression and functions of these family members, this approach would be crucial to isolate region-specific effects. Also, the current targets of Gadd45b action in the hippocampus are not completely understood, methylation assays in knock-down models will be useful to further address this issue.

1.3.1.1 Gadd45β-mediated MAPK activation

Although the role of Gadd45b in neuroepigenetic regulation has only recently started do be addressed, other functions have been demonstrated. Indeed, the Gadd45 family members are thought to selectively activate upstream members (MAPKKK) of the MAPK signaling cascades. Indeed the activation of MEKK4/MTK1 by Gadd45 proteins has been suggested (Takekawa and Saito, 1998). Nonetheless, only recently it was shown that Gadd45b contains a MEKK4 binding domain (Miyake et al., 2007). MEKK4 is usually inactive since the N-terminus is bound to the C-terminus, thus inhibiting the kinase activity. The biding of Gadd45 family members disrupts this C-N termini interaction, thus allowing its dimerization and activation by phosphorylation (Miyake et al., 2007). MEKK4 once activated can phosphorylate MAPKK, such as MEKK4/6, upstream kinases for both p38 and JNK (**Figure|1.13**). Also Gadd45b has been shown to bind directly to MKK7 (Papa et al., 2004). This interaction blocks the catalytic activity of MKK7, which in the context of toxicity is responsible for the JNK-dependent induction of apoptosis (Papa et al., 2004). Nevertheless a role for Gadd45b in JNK activation in the context of neuronal plasticity has not yet been addressed. As discussed beforehand (see “The MAPK signaling cascades and plasticity”), JNK activity can also contribute for alterations that may promote or inhibit long-term memory formation.

To my knowledge there is no data that suggests Gadd45b can promote p38 activation in the CNS. Nevertheless MKK4/6 activation is known to potentiate activation of the p38 MAPK pathway. In fact outside the CNS there is evidence that Gadd45b can in fact promote its activation (Dodeller and Schulze-Koops, 2006, Cho et al., 2010, Salerno et al., 2012). Thus, in order to fully understand the role Gadd45b in neuronal plasticity and memory, studying its potential demethylation role may prove insufficient. A synergetic action of Gadd45b both in the nucleus and cytoplasm may occur, and one could speculate that activation of MAPK pathways may be involved in mediating long-term synaptic alterations for example by contributing to structural changes.



Figure|1.13 **Gadd45b may modulate JNK and p38 MAPK activity in the CNS.** Outside the CNS Gadd45b can modulate the activation of MEKK4 which is both upstream from JNK and p38 MAPK signaling cascades (left cascade). Nevertheless, it is unclear if in neurons Gadd45b may mediate processes relevant for plasticity and even memory thought the activation of these pathways. Also, evidences suggest that Gadd45b may inhibit MKK7 activity which will result in neuroprotective effects (right cascade).

1.4 Aims

In the past decade several studies have arised in an effort to understand how memory formation occurs in the context of Neuroepigenetics. Cumulating evidence points to a role of Gadd45b in learning possibly by mediating changes in transcriptional activity of memory-associated genes. However, the cellular mechanism by which Gadd45b may mediate memory formation is still unclear. In order to extend this knowledge, we propose to test how Gadd45b regulates plasticity-associated genes and protein expression and if Gadd45b is required for memory formation and consolidation. We proposed to analyze using a knock-down model of Gadd45b: (1) the alterations in induction of expression of IEGs and their protein products during plasticity, (2) the involvement of MAPK pathways as possible mediators of Gadd45b action in hippocampal neurons (3) changes in gene methylation patterns and (4) how both short-term and long-term memory is altered in this paradigm.

Chapter 2. Materials and Methods

2.1 *In vitro* approaches

2.1.1 Primary Hippocampal Neurons

Mice hippocampal neurons were prepared from newborn C5BL/6 mice, all experiments were performed after a culturing period of 10-14 days during which hippocampal neurons develop a rich network of processes, express functional NMDA-type and AMPA/kainate-type glutamate receptors, and form synaptic contacts.

2.1.1.1 Preparation of Coverslips

Coverslips were beforehand washed with 70% ethanol for 15 minutes and with absolute ethanol. They were kept at room temperature in an ultrasonic bath for 5 minutes and washed with absolute ethanol. To evaporate the ethanol, glass bottles containing the coverslips were put into a dryer for 2-3 hours. The coverslips were transferred into a new glass bottle and sterilized at 140^{o}C for 4-5 hours.

2.1.1.2 Coating of Culture Dishes

Poly-D-lysine (PDL [2µg/cm^2]) and laminin (LA [1µg/cm^2]) were diluted in millipore H_2O. 2 mL of the PDL/LA solution was added to each 35-mm dish, if containing 4 coverslips 2.5 ml was added. Afterwards dishes were stored for 24 hours in the incubator (37^{o}C, 5% CO_2). The dishes were washed twice with millipore H_2O and dried well for about 2 hours. They were kept under sterile conditions until dissection.

2.1.1.3 Dissection of Hippocampi and Dissociation of Neurons

Dissociated hippocampal neurons were cultured from newborn (P0) wild-type C5BL/6 mice. Hippocampi were dissected in a mixture of Ky/Mg solution (**Table|2.1**) and dissociation medium (DM) (**Table|2.2**) at 1:9 volume ratio, next were transferred to a Papain (Papain latex 10 Units/mL, 3.7 mM L-Cystein in

Ky/My/DM solution) for 20 minutes at 37°C for dissociation. The solution was homogenized by gently stirring every 5 minutes. Papain treatment was performed twice with complete inhibition solution and incubated each time for 5 minutes at 37°C. Hippocampal cultures were washed three times with growth medium (**Table|2.3**) and then triturated by pipetting to achieve a homogeneous cell suspension solution. Afterwards, the cell suspension was diluted inOpti-MEM, supplemented with 20 mM glucose, for a final concentration of 0.8 hippocampus/2 mL. Then, 2mL was given to each poly-D-lysine/laminin (BD Biosciences)-coated-35mm plastic dish alone or containing 4 coverslips. The medium was exchanged to neurobasal-A-medium (NBA, Introgen, 2mL/dish) 2.5 hours after plating. The dishes were stored in a 37°C 5% CO_2 incubator. At day *in vitro* (DIV) 3, 2.8 µM of cytosine β-D-arabinofuranoside (AraC, Sigma-Aldrich C1768) was added to inhibit non-neuronal cell proliferation. On DIV8, the medium was changed to transfection medium (**Table|2.4** and **Table|2.5**). In dishes containing coverslips, these were transferred to a 4 well plate (Thermo Scientific) for transfection medium change.

Table|2.1 Ky/Mg Solution

Component[1]	Final concentration	Stock concentration
Kyneurenic acid	10 mM	Powered
Phenol red	0.5%	100%
NaOH	12.5 mL	1 N
HEPES	5 mL	1 M
$MgCl_2$	100 mL	2 M
H_20		

[1]All chemicals were purchased from Sigma-Aldrich Chemie GmbH, Munich, Germany.

Table|2.2 Dissociation medium

Component[1]	Final concentration	Stock concentration
Na_2SO_2	81.8 mM	1 M
K_2SO_4	30 mM	0.25 M
$MgSO_4$	5.85 mM	1.9 M
$CaCl_2$	0.25 mM	1 M
HEPES	1 mL	1 M
Phenol red	0.2%	100%
Glucose	0.36%	45%
H_20		

[1]All chemicals were purchased from Sigma-Aldrich Chemie GmbH, Munich, Germany.

Table|2.3 Growth medium (L17/1% rat serum)

Component[1]	Volume (mL)
Neurobasal A-medium	97
B27[2]	2
1% Rat serum[3]	1
L-Glutamin (0.5 mM)	0.025
Penicilin/Streptomycin	0.5

[1]All chemicals were purchased from Sigma-Aldrich Chemie GmbH, Munich, Germany except [2] from Introgen GmbH, Karlsruhe, Germany and [3]Biowest S.A.S, Nuaille, France.

Table|2.4 Salt Glucose Glycine solution (SGG)

Component[1]	Final concentration	Stock concentration
NaCl	114 mM	5 M
$NaHCCO_3$	0.22% mM	7.5%
KCl	5.29 mM	3 M
$MgCl_2$	1 mM	1.9 mM
$CaCl_2$	2 mM	1 M
HEPES	10 mM	1 M
Glycine	1 mM	1 mM
Glucose	0.54%	45%
Na^+ Pyrovate	0.5 mM	0.1 mM
Phenol Red	0.2%	100%
H_2O		

[1]All chemicals were purchased from Sigma-Aldrich Chemie GmbH, Munich, Germany.

Table|2.5 Transfection medium

Component[1]	Volume (mL)
SGG	88
MEM (without Glutamine)	10
Insulin-Transferrin-Selenium	1.5
Penicillin/Streptomycin	0.5

[1]All chemicals were purchased from Sigma-Aldrich Chemie GmbH, Munich, Germany except MEM which was purchased from Introgen GmbH, Karlsruhe, Germany.

2.1.2 Treatment of Hippocampal Cultures

Hippocampal cultures were infected in DIV 4. At DIV 10 bicuculline (Alexis Biochemicals®) was used at a concentration of 50 µM to selectively block $GABA_A$ receptors, promoting depolarization of cell cultures.

2.1.3 Virus Constructs and Production

Human embryonic kidney cell line 293 (HEK-293, American Type Culture Collection, CRL1573) was cultured in flasks with Dulbecco's Modified Eagle Medium-complete (DMEM-complete) (**Table|2.6**). Cells were split by a dilution factor of 1:5 and passaged every 3 to 5 days. In order to split cells, culturing medium was removed and cells washed twice with phosphate buffered saline (PBS). Afterword's, 0.05% Trypsin-EDTA (Gibco®) was added to the cells directly and cells were incubated for 5 minutes at 37°C. The same volume of DMEM-complete was supplemented and cells were collected in a Falcon tube for suspension. The cells were spun at 800g for 5 minutes at room temperature (RT) and medium was replaced by fresh DNEM-complete for resuspension. Cells were then plated into a new flask.

Table|2.6 Dulbecco's Modified Eagle Medium-complete (DMEM-complete)

Component[1]	Volume (mL)
DMEM (with 4.5 g/L glucose)	500
Fetal calf serum[2]	50
Non-essential amino acids	5
Na^+ pyruvate	5
Pennicilin/streptomycin	2.5

[1] All from GIBSCO®.

[2]Heat inactivated (30 minutes at 56°C).

HEK293 cells were transfected using the Ca^{2+} phosphate precipitation method. Cells were plated onto 15-cm dishes (TC Dish, NUNC) containing DNEM-complete. Medium was changed for each plate (60-70% confluent) 2-3 hours

prior to transfection in Iscove's Modified Dulbecco Medium (IMDN; Life Technologies/Introgen, **Table|2.7**). Five dishes were used for producing each virus batch. The reagents listed in **Table|2.8** were used for each virus production. The mixture was filtered through a 0.2 µm syringe filter and 13 mL 2x HeBs (**Table|2.9**) was added slowly while vortexing for 15 seconds. The transfection mixture was added drop-wise to the cells. The medium was replaced with fresh DNEM 16-22 hours after transfection.

Table|2.7 Iscove's Modified Dulbecco's Medium-complete (IMDM-complete)

Component[1]	Volume (mL)
IMDM	500
Fetal calf serum[2]	25

[1] All from GIBSCO®.

[2]Heat inactivated (30 minutes at 56°C).

Table|2.8 Transfection Mixture

Component[1]	Amount
H_2O	15 mL
$CaCl_2$	1.65 mL
AAV plasmid	62.5 µg
pFdelta6 (AAV helper)	125 µg
pNLrep (AAV helper)	30 µg
Ph21 (AAV helper)	31.25 µg

[1] Mixture filtered through 0.2-µm syringe filter.

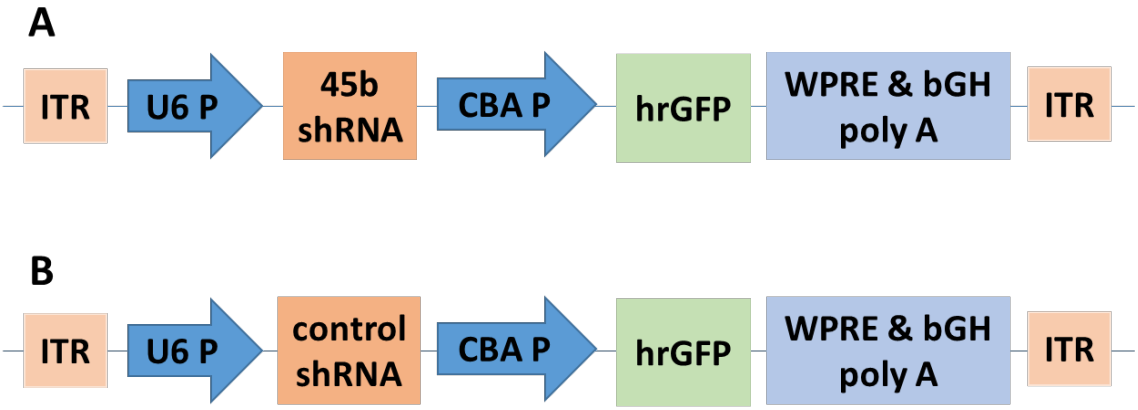
Table|2.9 2x HebS

Component[1,2]	Final concentration
HEPES	50 mM
NaCl	280 mM
Na_2HPO_4	1.5 mM
H_2O	

[1] pH adjusted to 7.05.

2 Sterile filtrated through 0.22-µm Millipore filter and stored at 4°C.

About 60 hours after transfection, HEK293-cells were washed once with pre-warmed PBS, harvested, pelleted (5 minutes at 800g) and resuspended in 100 mM NaCl and 10 mL Tris-HCl (pH 8.0). The cells were then lysed with 0.5% Na^+ deoxycholate monohydrate (Sigma-Aldrich) and 50 U/mL Benzonase® Nuclease (Sigma-Aldrich) for 1 hour at 37°C, afterwards pelleted at 4°C for 15 minutes at 3000g. Lysates were then frozen at -20°C until virus purification.
Lysates were thawed and centrifuged at 3000g for 15 minutes at 4oC. The virus was purified with heparin affinity HiTrapTM Heparin HP Colums (GE Healthcare). Columns were pre-equilibrated with 10 mL 150 mM NaCl/20 mM (pH 8.0). The sample solution was then loaded using a 50-mL syringe and set up on the Harvard infusion pump at 1 mL/min. The column was washed with 20 mL of 100 mM NaCl/20 mM Tris (pH 8.0). All steps were done at a flow rate of 1 mL/min. The column was washed manually again with 1 mL 200 mM NaCl/20 mM Tris (pH 8.0), and 1 mL 300 mM NaCl/20 mM Tris (pH 8.0) and the virus was eluted using an increasing concentration of NaCl/Tris (pH 8.0) buffers sequentially: 1.5 mL 400 mM NaCl/20 mL Tris (pH 8.0), 3 mL 450 mM NaCl/20 mM Tris (pH 8.0) and 1 mL 500 mM NaCl/20 mM Tris (pH 8.0). The virus was concentrated with Amicon® Ultra-4 Centrifugal Filter Units (Millipore, Bedford, MA), and the integrities of viral particles were routinely checked by SDS-PAGE (10% resolving gel). Infection rate and cell death assessment of new viral particles were quantified by standard immunocytochemistry with Hoechst 33258 (1:5000, Serva) staining. Additional target specific effect on mRNA expression was tested by qRT-PCR analysis. The rAAV constructs used are represented in **Figure|2.1**.



Figure|2.1 **Representation of rAAVs constructs used**. Two constructs were synthesized a **(A)** Gadd45bsh and a universal negative control (UNC) virus **(B)**. **bGH**: bovine growth hormone gene; **CBA P**: chicken beta-actin promotor; **ITR**: Inverted terminal repeat; **U6 P**: U6 promoter; **hrGFP**: humanized recombinant green fluorescence protein; **WPRE**: woodchuck hepatitis virus posttranscriptional regulatory element. Constructed validated in: (Zhang et al., 2009, Oliveira et al., 2012).

2.1.4 RNA extraction, cDNA synthesis and qRT-PCR analysis

RNA from dissociated hippocampal neurons was extracted under RNase free conditions using RNeasy Total RNA isolation kit (Qiagen, Roche) according to the manufacturer's inductions, 600µL of Buffer RLT was used and the optional step of DNAse treatment with RNase-free DNase set (Qiagen). For complementary DNA (cDNA) synthesis, a total of 1 µg RNA per sample was reversed transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative real-time PCR (qRT-PCR) was performed on an ABI7300 thermal cycler. For experiments using TaqMan probes the following TaqMan Gene Expression Assays (Applied Biosystems) was used: Mm00446953_m1 GusB, Mm00487425_m1 cFos, Mm00463644_m1 Npas4, Mm00656724_m1 Erg1, Mm00463987_m1 Dnmt3a2, Mm00656724_m1 Egr1, Mm00479619_g1 Arc, Mm00432069_m1 BDNF IV, Mm00439358_m1 Nr4a1, Mm00442225 Gadd45a, Mm00435123_m1 Gadd45b, Mm00432802_m1 Gadd45g. Gene Ct-values were normalized to glucuronidase beta (GusB) or hypoxanthine phosphoribosyltransferase 1 (HPRT1), used as endogenous control genes for TaqMan (Life Technologies_4369542) or SYBR Green (Life Technologies_4367659) qRT-PCR, respectively. SYBR Green *bdnf* primers were purchased as previously described (Kairisalo et al., 2009).

2.1.5 Western Blot

Protein extracts were prepared from hippocampal cultures on DIV 10 in 2x Sample Buffer (**Table|2.10**) (300 µL per dish) supplemented with 10 mM dithiothreitol (DTT). After harvest samples were boiled for 5 minutes and stored at -80^{o}C until experiments. Protein extracts were resolved by SDS-PAGE in 12% polyacrylamide gels at a constant voltage of 80 volts for 30 minutes and 120 volts for the remaining time. Proteins were transferred onto a nitrocellulose membrane (GE Healthcare Life Sciences Whatman^{TM}) by electroblotting (120 volts for 1 hour at 4^{o}C). Effective transfer was determined by Ponceau S solution (Serva_ 33427.01). The membranes were blocked for 1 hour at room temperature in PBS containing 0.1% (v/v) Tween-20 (PBS-T), and 5% (w/v) low-fat milk. Membranes were probed overnight at 4^{o}C, with the primary antibodies diluted in PBST containing 5% (w/v) low-fat milk or 5% (w/v) BSA (**Table|2.11**). Following several washes for a total time of 20 minutes, membranes were incubated for 1 hour with horseradish peroxidase conjugated secondary antibodies (anti-mouse or anti-rabbit, depending on the primary antibody host species) at room temperature, PBS-T washed for 20 minutes and incubated with enhanced high sensitive or low sensitive chemiluminescence substrate (ECL) (BIO-RAD_170-5060; GE Healthcare_ 81863, respectively) for 1 minute at room temperature. Membranes were revealed in high performance chemiluminescence films (GE Healthcare_28906837). When necessary, the membranes were reprobed after a treatment (0.1% Na^{+} azide in PBS for 1.5 hours) that inhibits the first secondary antibody activity.

Table|2.10 2x Sample Buffer

Component	Volume (mL) for 100 mL
Glycerol	30
20% SDS	20
1M Tris pH 6.8	16
0.2% Bromophenol Blue	10
Millipore water	24

Table|2.11 Antibody information

Antibody	Species	Molecular Weight	Dilution WB	Dilution ICC	Company
Arc/ Arg 3.1	Rabbit	55	1:6000	1:2500	Synaptic Systems #156 003
Fos	Rabbit	62	1:2500	1:1000	Cell Signaling #2250
Npas4	Goat	100	1:2000		Santa Cruz sc-168789
pCREB	Mouse	46	1:10.000		Millipore #05-667
pERK	Mouse	42/44	1:2500		Cell Signaling #9106
pH3ser10	Rabbit	18	1:1500		Millipore #06-570
pJNK	Rabbit	46/54	1:5000		Cell Signaling #4671
pp38	Mouse	38	1:500		BD #612288
Tubulin	Mouse	55	1:10000		Sigma #t9026

2.1.6 Immunocytochemistry

On DIV 10 hippocampal neurons plated on coverslips were fixed for 15 min in 4% sucrose and 4% PFA at room temperature. Cells were washed and permeabilized with methanol for 6 minutes at -20^{o}C. Neurons were then incubated in 10% normal goat serum (NGS) in PBS at RT for 1 hour to block nonspecific binding. Afterwards coverslips were incubated in appropriate primary antibody diluted (**Table|2.11**) in 2% (w/v) BSA and 0.1% (w/v) Triton in PBS overnight at 4^{o}C. After washing 4 times with PBS, cells were incubated with the secondary antibody diluted in 2% (w/v) BSA and 0.1% (w/v) Triton in PBS for 1 hour at RT. Coverslips were washed 3 times with PBS and incubated with Hoechst stain for 5 minutes at room temperature and washed twice with PBS. The coverslips were then mounted using homemade Mowiol and let to dry overnight. For analysis, 5 distinct areas of the coverslip were imaged and microscope settings were identical for all conditions. Fiji (Schindelin et al., 2012) was used for quantification analysis. For measuring nuclear targets the nucleus of infected cells was defined as the region of interest using Hoechst staining. For analysis background signal was also subtracted and intensity signals were integrated by area.

2.1.7 5-MeC Dot Blot

Total DNA from dissociated hippocampal neurons was extracted using DNeasy blood and tissue kit (Qiagen, Roche) according to the manufacturer's inductions and stored at -20^{o}C until experiments were performed. First, DNA was diluted in TE (10mM Tris-HCl pH 8, 1mM EDTA pH 8), denaturation solution (2M NaOH, 50 mM EDTA) was added and the resulting solution was denatured at 95°C for 10 minutes. Next, samples were put immediately on ice and neutralization solution (2M ammonium acetate, pH 7.0) was added, and left for 10 minutes. A positively charged nylon membrane (Sigma-Aldrich, 11209299001) was cut to appropriate size and wet in ddH_2O. Afterwards 5 serial dilutions were done in order to obtain samples with 1000 ng, 500 ng, 250 ng, 125 ng and 60 ng of denatured DNA. Vacuum was applied and TE was used to wash the membrane, by application in the wells of the assembled dot blot apparatus (BioRad, 1706545). Samples were loaded and transferred with vacuum to the membrane. Wells were washed with 2x SSC (20x SSC, 3M NaCL, 0.3M triNa citrate). Next

membrane was rinsed in 2x SSC and air-dried. Membrane was then UV cross-linked and stained with methylene blue for 3 minutes. Subsequently membranes were washed with ddH_2O until methylene blue signal was clear for quantification. Membrane was blocked for 1 hour in PBS/5 % milk at room temperature. Next 5-mC antibody (1:1000, #NA81, mouse monoclonal) was diluted in the blocking solution overnight. In the next day membrane was washed 3 times for 5 minutes with PBS and incubated with secondary antibody (anti-mouse) in blocking solution for 1 hour at room temperature. Thereafter membrane was washed six times with PBS and incubated with high sensitive ECL (BIO-RAD_170-5060) for 1 minute at room temperature. Membrane was revealed in high performance chemiluminescence films (GE Healthcare_28906837) and results quantified.

2.2 *In vivo* approaches

2.2.1 Ethics

All of the animal procedures were done in accordance with German guidelines for the care and use of laboratory animals and with the European Community Council Directive 86/609/EEC. No conflicts of interest that may have encourage critical appraisal were detected.

2.2.2 Animals

Animal experiments were conducted on male 9 week-old C57Bl/6N (Charles River) mice. Rodents were housed in groups of 3 in polycarbonate cages with food and water *ad libitum*. After stereotaxic surgeries animals were kept at room temperature of 23 ± 1^{o}C, humidity of 50 ± 5% and 12 hours' light/dark cycles beginning at 6 am. Mice were handled for three consecutive days for individual periods of 1.5 to 2 minutes in each session, prior to contextual fear conditioning or spatial object recognition test.

2.2.3 Stereotaxic Surgery

Recombinant adenoassociated viruses (rAAVs) were delivered into the dorsal hippocampus of mice by stereotaxic injection. Mice were randomly grouped and anaesthetized with a sleep mix (8 µL/g; mix containing 5.5 mL NaCl isotonic, 0.5

mL Dormitor, 1 mL Dormicum and 1 mL Fentanyl). A total volume of 1.5 µL virus per hemisphere, at a speed of 200 nL/minute through a 33G needle, was injected. For infection a 2:1 mixture of viral solution and 20% Mannitol was used. The following coordinates relative to the Bregma were used: anterior-posterior; -2 mm, medio-lateral; ±1.5 mm, dorsoventral; -1.7, -1.9 and -2.1 mm from the skull surface. The injection needle remained an additional 120 seconds between hemispheres in order to allow diffusion an avoid reflux. After the surgeries mice were given pain killers (4.8 µL/g; mix containing 4.75 mL NaCl isotonic and 150 µL Temgesic) and a wake-up mix (8.4 µL/g; mix containing 0.5 mL Antisedan, 5 mL Anexate and 3 mL Naloxon). Behavior experiments were conducted 3 weeks after stereotaxic delivery of AAVs to promote a full post-surgery recovery and allow viral expression.

2.2.4 Contextual Fear Conditioning

Mice were habituated to the conditioning chamber (23x23x35 cm, TSE) for 148 seconds before receiving a 0.5 mA foot shock for 2 seconds. Mice were then removed from the box 30 seconds after shock termination. After one hour or 24 hours (STM and LTM, respectively), mice were placed back for 5 minutes into the chamber. The time spent freezing, defined as the absence of movements except respiration, was recorded manually in a blinded fashion.

2.2.5 Spatial Object Recognition Test

Mice were placed in an open field (50 cm x 50 cm x 50 cm) with a spatial visual cue located on the wall of the area. Mice were habituated to the area for 6 minutes, after a 3-minute interval, mice were trained three times during 6 minutes with a 3-minute interval between sessions. During the training sessions mice were allowed to explore two distinct objects (a glass bottle and a metal tower). After 1 hour or 24 hours (STM and LTM, respectively) the metal tower was displaced into a non-symmetrical localization and mice were allowed to explore the objects for 6 minutes. The time spent exploring the two objects was measured manually in all sessions in a blinded fashion.

2.2.6 Hippocampal mRNA Isolation

Mice were sacrificed by cervical dislocation, after decapitation brains were rapidly placed in a mouse brain matrix (World Precision Instruments_RBMS-

200C), sliced into 1 mm sections and placed in RNAlater (Ambion/Sigma_ R0901). Virus infected areas of the hippocampi were isolated in a florescence microscope and stored at -80^{o}C until isolation. Afterwards mRNA was extracted and purified under RNase free conditions using RNeasy Total RNA isolation kit (Qiagen, Roche) according to the manufacturer's inductions and described previously, with an additional step of tissue homogenization using a rotor homogenizer for 25 seconds and a centrifugation step at full speed for 3 minutes, the supernatant was then isolated.

2.2.7 Brain Perfusion and Slide Preparation

After behavior experiments, mice were heavily anesthetized with Narcoren (0.5µL/g) and perfused with 25 mL of PBS. Next, mice were fixed by perfusion with 25mL 10% formalin and brains were isolated and stored overnight in 10% formalin. Brains were next transferred into a 30% sucrose solution for three days as a dehydration step. Brains were next sectioned (30µm) and stored in PBS-Thimerosal at 4^{o}C. Later selected slices were washed in PBS, Hoechst stained and mounted in home-made Mowiol for later microscope analysis. Animals in which low virus expression or visual cell death was detected in one or two hemispheres were excluded from the dataset.

2.3 Statistical Analysis

All of the experiments expressed continuous outcomes (means), for statistical analysis data was grouped by (1) independent or correlated, (2) comparison between two or more groups and (3) statistical testing for normality. All groups we tested for Gaussian distribution before significance analysis by Kolmogorov-Smirnov test, with the Dallal-Wilkinson-Lillie for corrected P value (for $n>4$), Shapiro-Wilk test ($n>6$) and D'Agostino test ($n>7$). For groups smaller than 4, non-parametric tests were used. The comparison between two independent groups was analyzed by a Student's T-test or Mann-Whitney test, for parametric and non-parametric analysis, respectively. When group comparisons were greater than two, an one-way ANOVA test was used for independent experiments, if groups passed normality tests. When the outcome was statistical significance a Bonferroni post hoc test was performed. For non-parametric analysis between more then two groups Kruskal-Wallis test was

applied. Statistical analysis was performed using GraphPad Prism 6®. Results are presented as the mean ± standard error of the mean (s.e.m) of n experiments. P-values <0.05 were considered to be significant.

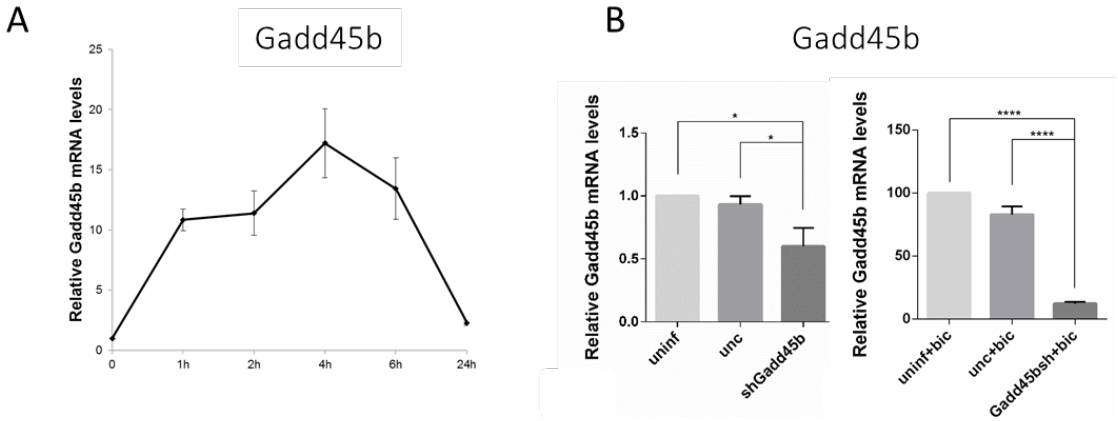
2.4 Author contribution

The author of this thesis performed all of the experiments described in this method section unless stated otherwise in the results. The only exceptions are the virus production and purification which was performed by Benjamin Zeuch and hippocampi isolation, dissociation and plating performed by Iris Bünzli-Ehret.

Chapter 3. Results

3.1 *In vitro* validation of Gadd45bsh

First, we aimed at understanding if Gadd45b expression is required for the induction of plasticity-related genes. In order to test this two different rAAVs were synthetized. The viral constructs expressed a short-hairpin for Gadd45b (Gadd45bsh) (Zhang et al., 2009) or a control short-hairpin (unc, universal control) (Oliveira et al., 2012). To further validate this method, dissociated hippocampal neurons were infected at DIV4 either with Gadd45b, unc or uninfected. At DIV10 neuronal cultures were treated with bicuculline, a selective $GABA_A$ inhibitor, for 4 hours to confirm a Gadd45b knockdown both in basal and stimulated conditions (**Figure|3.1 A**). In uninfected conditions, after 4 hours of stimulation Gadd45b mRNA levels were induced about 18 fold (**Figure|3.1 B**). Neurons infected with Gadd45bsh showed a statistically significant decrease in mRNA levels both in basal and induced conditions for Gadd45b. This data thus validates an mRNA reduction of Gadd45b expression both in basal conditions and upon bicuculline stimulation

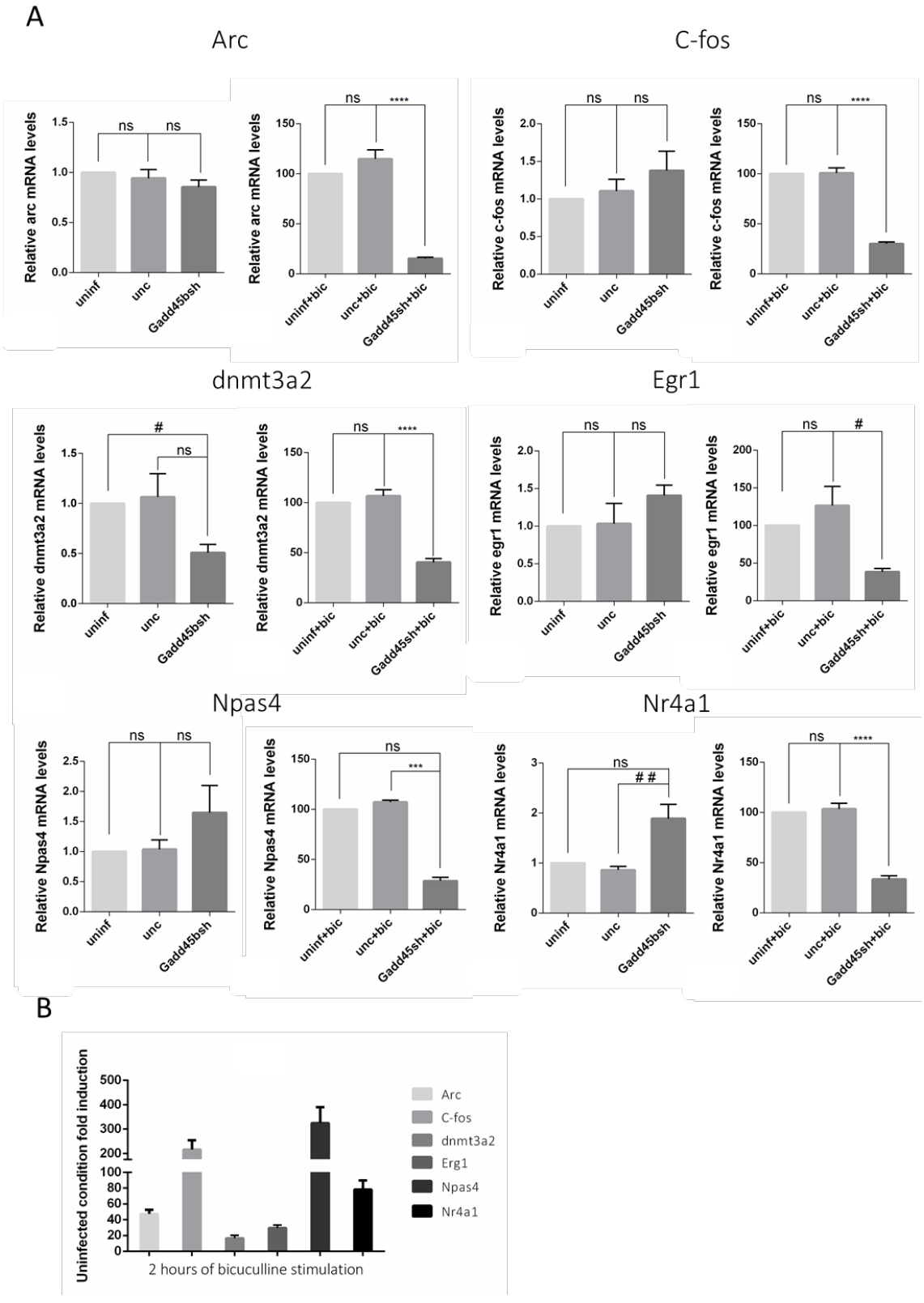


Figure|3.1 **Gadd45b activity-induced expression is blocked by Gaddd45bsh. (A)** Gadd45b expression is induced by bicuculline stimulation (50 μM) in dissociated hippocampal neurons. Expression levels were measured at different time points after Bic addition to the cells. Highest expression is observed at 4 hours (experiment performed by Dr. Ana MM Oliveira). **(B)** Neuronal cultures were infected with rAAVs expressing a control short-hairpin (unc) or a Gadd45b short-hairpin (Gadd45bsh), cells were stimulated with bicuculline for 4 hours. Values were normalized to GusB mRNA levels as an endogenous reference and for the control uninfected control condition, next they were averaged from 5 independent experiments.

Error bars represent s.e.m, the statistical significance was calculated using one-way ANOVA and followed by the Bonferroni's multiple comparisons post-hoc test (*P < 0.05 and ****P < 0.0001).

3.2 Induction of Plasticity-associated genes is impaired by Gadd45b knockdown

After validating our viral constructs we aimed at testing if Gadd45b expression could alter expression patterns of gene expression required for plasticity events and long-term memory formation. We analyzed the mRNA expression of several immediate-early genes, *Arc, c-fos, Dnmt3a2, Erg1, Npas4 and Nr4a1* (**Figure|3.2**), (experiments conducted previously by a laboratory rotation student, Stephanie Rothe). These genes are rapidly induced after stimulation, taking into account the fast kinetics characteristic for these genes, we performed a 2 hours time point of bicuculline stimulation. Indeed, mRNA levels were induced (Fig|3.2B). Nevertheless in all genes tested, reducing the expression of Gadd45b was sufficient to decrease the mRNA synthesis of all the IEG after bicuculline stimulation.



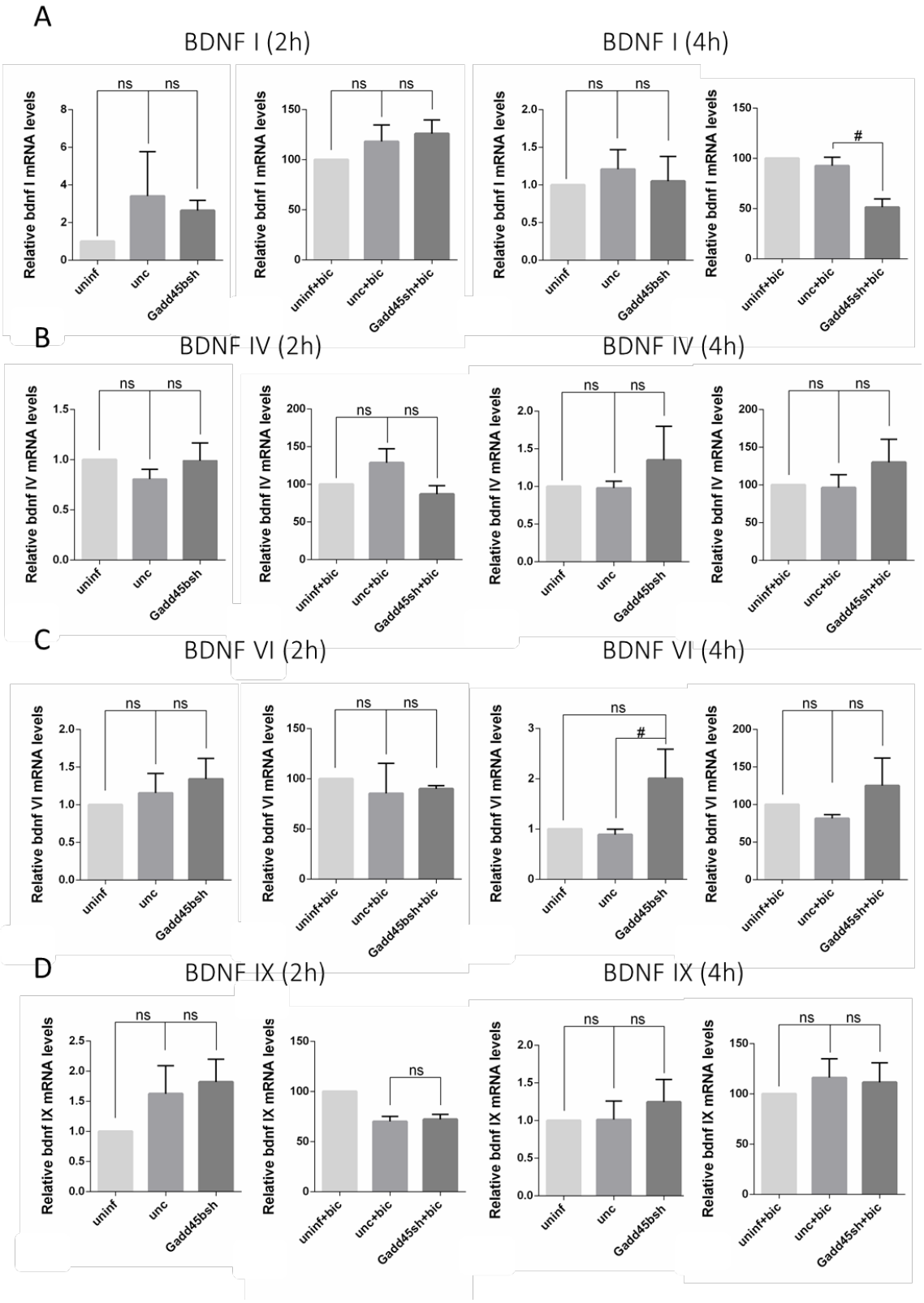
Figure|3.2 **Gadd45bsh impairs mRNA expression of IEG**. **(A)** Dissociated hippocampal neuronal cultures were infected with rAAVs expressing a control short-hairpin (unc) or a Gadd45b short-hairpin (Gadd45bsh), cells were stimulated with bicuculline for 2 hours. IEG mRNA levels were normalized to GusB mRNA levels as an endogenous reference and for the control uninfected control condition or for uninfected condition stimulated with bicuculline. Next they were averaged from 4-5 independent

experiments. **(B)** Fold induction of IEG at 2 hours of bicuculline stimulation are shown normalized to GusB mRNA levels. Error bars represent s.e.m, the statistical significance was calculated using one-way ANOVA and followed by the Bonferroni's multiple comparisons post-hoc test (*P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001). In case of conditions that not passed normality tests Kruskal-Wallis test followed by the Dunn's Multiple Comparison test ($^{\#}$P<0.05 and $^{\#\#}$P<0.01) was performed. Experiments performed by Stephanie Rothe during a Master laboratory rotation.

It is unlikely that the effects observed are due to the Gadd45b that is induced by activity because the time point analyzed is too early for a mechanism that would require *de novo* protein synthesis. Therefore, these results may suggest that the basal pool of Gadd45b regulates a permissive state for gene expression.

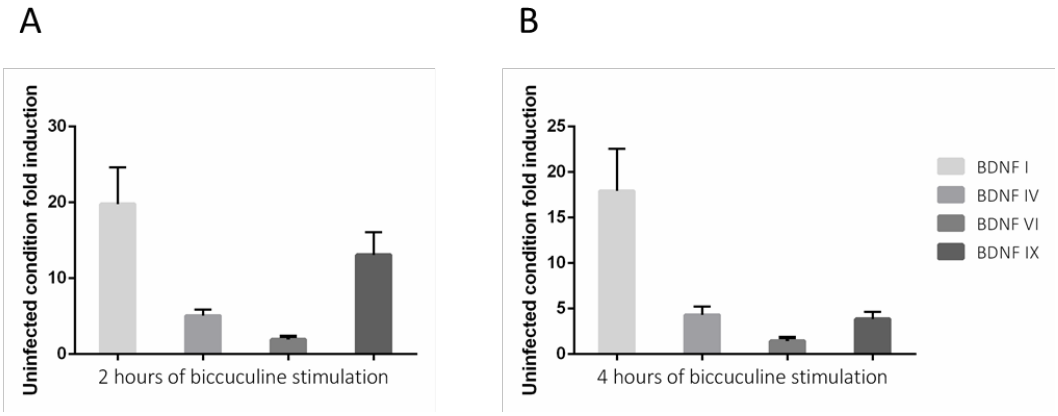
3.2.1 BDNF exon I induction is selectively impaired by Gadd45b knockdown

As discussed previously, recent evidence points to a role of Gadd45b in gene regulation by altering methylation patterns. In the context of adult neurogenesis, mice with a specific Gadd45b deletion showed impairment in *bdnf* expression (Ma et al., 2009). Taking into account our results on IEGs, we hypothesized that *bdnf* induction could be affected by our model of Gadd45b knockdown. Next, , we selected exons of BDNF that have been described to be methylation-regulated and induced by plasticity paradigms (Lubin et al., 2008). Considering the slow induction kinetics of particular BDNF exons compared to IEG, we designed experiments for two distinct bicuculline stimulation time points, 2 and 4 hours for BDNF exons I, IV, VI and IX. We did not find any statistically significant changes in any of the exons at 2 hours of stimulation (**Figure|3.3**). At 4 hours of stimulation, we found a selective decrease in BDNF exon I induction compared to control infected neurons. Moreover these effect seemed to be specific for this exon since for exons IV, VI and IX we did not detect any trend. These results highlight a selective effect of Gadd45b action on gene expression. Additionally it has been suggested that different exons of BDNF may determine cell-compartment specific mechanisms, nonetheless from a functional point of view BDNF exon I is the form that it highly induced (**Figure|3.4**).



Figure|3.3 **Gadd45bsh selectively impairs mRNA expression of BDNF exon I at 4 hours**. Hippocampal neuronal cultures were infected at DIV4 with rAAVs expressing a control short-hairpin (unc) or a Gadd45b short-hairpin (Gadd45bsh), cells were stimulated with bicuculline for 2 hours or 4 hour shown in left or right side of panels, respectively. **(A), (B), (C) and (D)** BDNF exon mRNA levels were normalized to HPRT1 mRNA levels as an endogenous reference and for the control uninfected control condition or for uninfected

condition stimulated with bicuculline. Next they were averaged from 4-7 independent experiments. Error bars represent s.e.m, the statistical significance was calculated using Kruskal-Wallis test followed by the Dunn's Multiple Comparison test ($^{\#}P<0.05$).

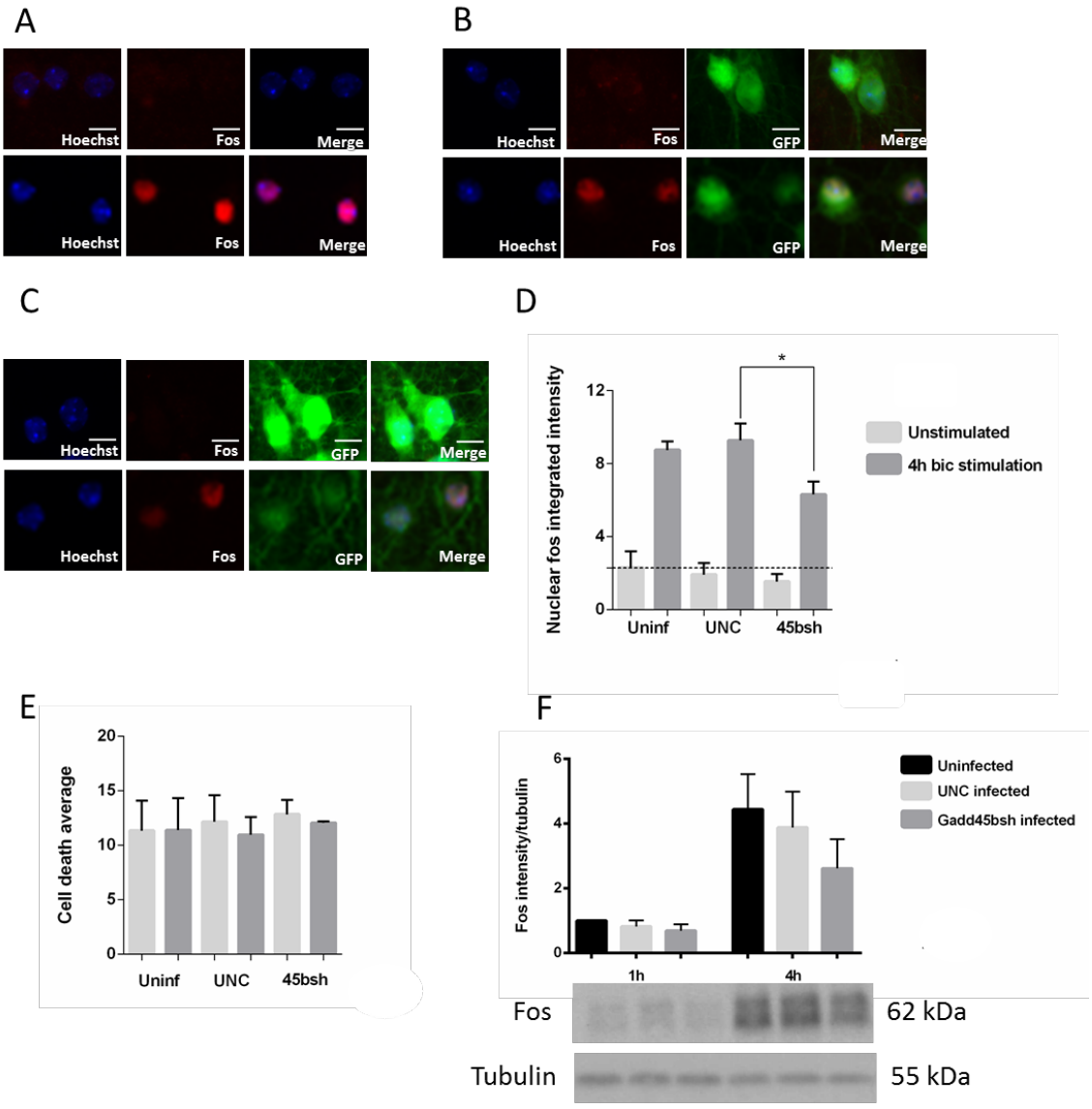


Figure|3.4 **BDNF exon I mRNA is strongly induced both at 2 and 4 hours**. Different BDNF exons fold induction 2 **(A)** and 4 hours **(B)** of bicuculline stimulation are shown normalized to HPRT1 mRNA levels.

3.3 Fos, Npas4 and Arc protein levels are decreased by Gadd45b knockdown

Although we show a regulatory role of Gadd45b in gene induction by accessing mRNA levels it is unclear if changes could be compensated in other layers of regulation in the cell. Indeed, testing at the protein level some of the candidate genes is critical. Thus dissociated hippocampal neurons were infected with rAAVs expressing a Gadd45bsh or unc at DIV4. Cultures were then stained for Hoechst as a nuclear marker and for Fos using a highly specific antibody. Since Fos is a transcriptional factor present in the nucleus, we identified nuclear Fos by co-localization with Hoechst in GFP (infection marker) positive neurons (**Figure|3.5 A, B and C**). For quantification of nuclear Fos we calculated an integrated intensity value per area of nucleus in order to normalize for different sized nuclei (**Figure|3.5 D**). As expected Fos protein levels were highly increased upon 4 hours of bicuculline stimulation. Interestingly we detected a statistically significant decrease in nuclear Fos induction in cultured neurons infected with Gadd45bsh compared to control conditions. In order to be sure that these effects were not a consequence of reduced cell viability, we also assessed cell death in these conditions (**Figure|3.5 E**), nonetheless no alterations in cell death was detected. To further support these conclusions, we

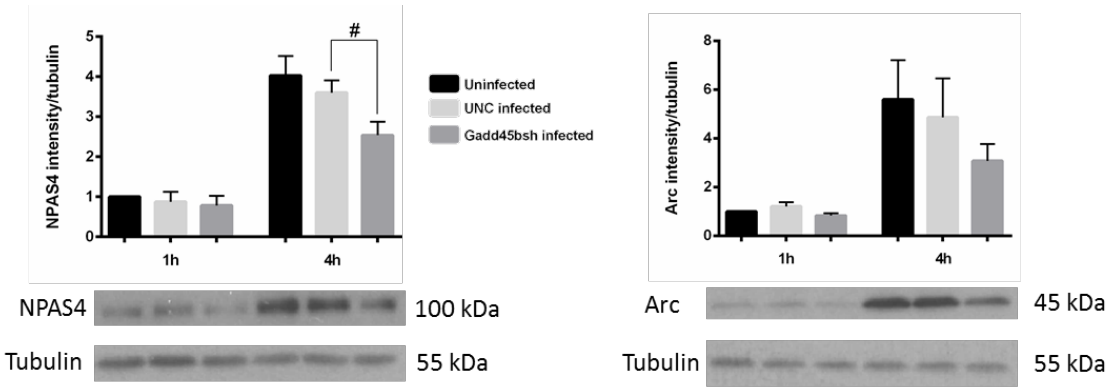
used a different approach to detect alterations in Fos levels. We performed western blot analysis to qualify total levels of Fos and in agreement with the previous experiment, we observed a trend for a decrease in Fos protein levels at 4 hours of bicuculline stimulation but not at 1 hour (**Figure|3.5 F**). Additional statistical power is needed for solid conclusions.



Figure|3.5 **Gadd45bsh decreases nuclear Fos.** Representative immunocytochemical stainings of Fos and hoechst nuclear staining in dissociated hippocampal neurons. Uninfected neurons **(A)** or cultures infected at DIV4 with rAAVs expressing a control short-hairpin (UNC) **(B)** or a Gadd45b short-hairpin (Gadd45bsh) **(C)**, were stimulated with bicuculline for 4 hours (bottom conditions in **A**, **B** and **C**). **(D)** Quantification of nuclear Fos intensity per nucleus positive for GFP (infection marker). **(E)** Nuclei were analyzed for condensation or fragmentation as an indicator of neuronal death. **(F)** Western blot analysis of protein extracts from dissociated hippocampal cultures for total Fos normalized for tubulin and for 1 hour of bicuculline stimulated uninfected condition. Bottom time-points represent bicuculline stimulation for 1 or 4 hours. Color code represents uninfected cultures or infected with control sh (UNC) or Gadd45bsh Error

bars represent s.e.m. Immunocytochemical results were obtained from 3-4 independent experiments, each consisted in nuclei from two coverslips 5 fields in each. Western Blot results were obtained from 5 independent experiments. Scale bars represent 5μm. The statistical significance was calculated using unpaired Students-T test (*P <0.05).

With this first set of protein data, next we aimed in testing if other protein products would be also affected. Since both Fos and Npas4 are well known to be transcription factors involved in plasticity and memory paradigms we performed western blot analysis on Npas4 protein levels (**Figure|3.6**). Indeed, we detected a trend at 4 hours of bicuculline stimulation similarly to Fos.

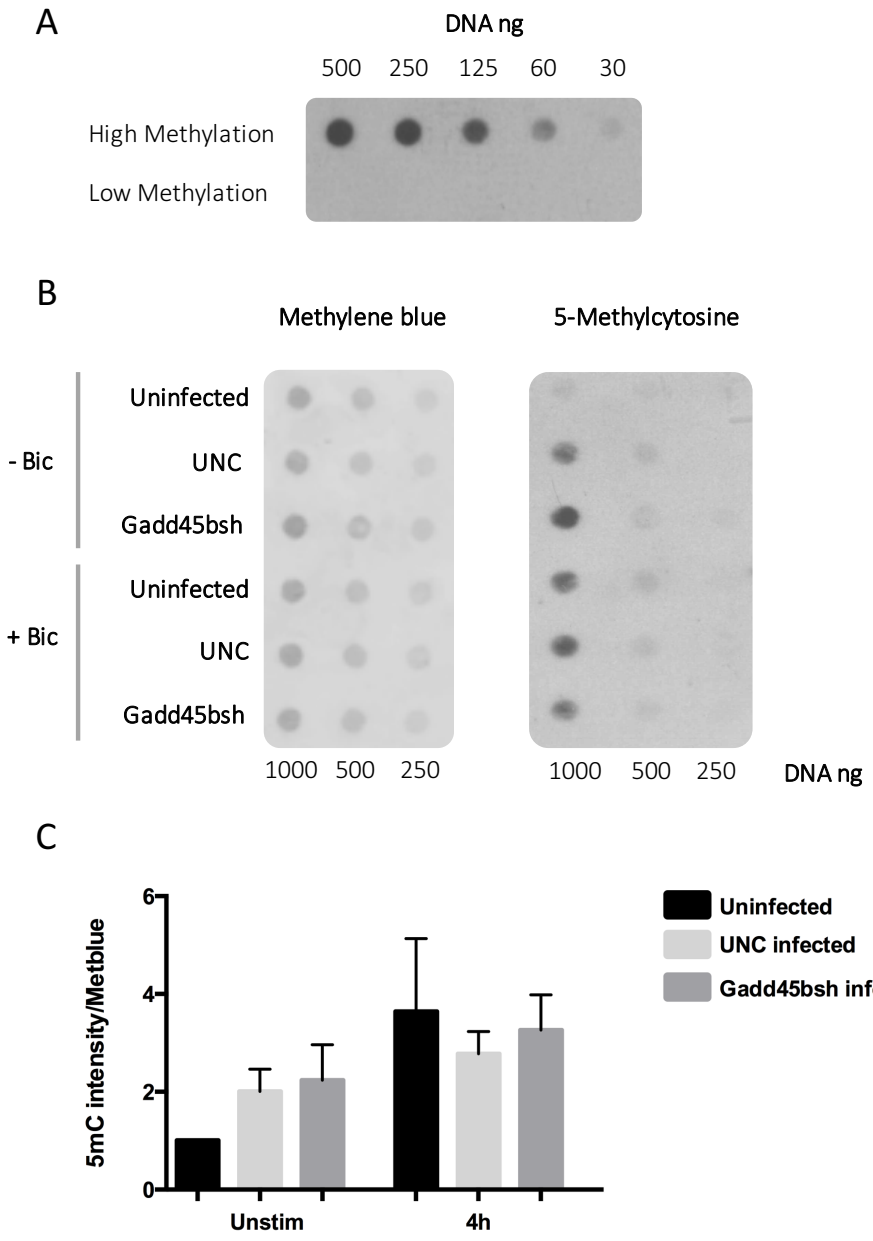


Figure|3.6 **Gadd45bsh induces a tendency for decreased Npas4 and Arc present at 4 hours of stimulation.** Western blot analysis of protein extracts from dissociated hippocampal cultures for Npas4 (left) and Arc (right) normalized for tubulin and for 1 hour of bicuculline stimulated uninfected condition. Bottom time-points represent bicuculline stimulation for 1 hour and 4 hours. Color code represents uninfected cultures or infected with control sh (UNC) or Gadd45bsh. Results were obtained from 5 independent experiments; error bars represent s.e.m. Statistical significance was calculated using Mann-Whitney test ($^{\#}P<0.05$).

It is tempting to hypothesize that these changes in transcriptional factors protein levels may have an effect on late response genes that depend on their regulation (see Discussion section). Keeping in mind that changes in gene expression detected also included genes with described functions outside the nucleus we next tested arc protein levels (**Figure|3.7**). Similarly, to our previous findings we saw a trend for decreased protein levels of Arc specifically at 4 hours of bicuculline stimulation, compared to controls. Overall these set of experiments suggest that gene expression impairment at the mRNA level is also seen at the protein level in targets with different subcellular localizations and functions.

3.4 Gadd45b may promote global DNA methylation changes

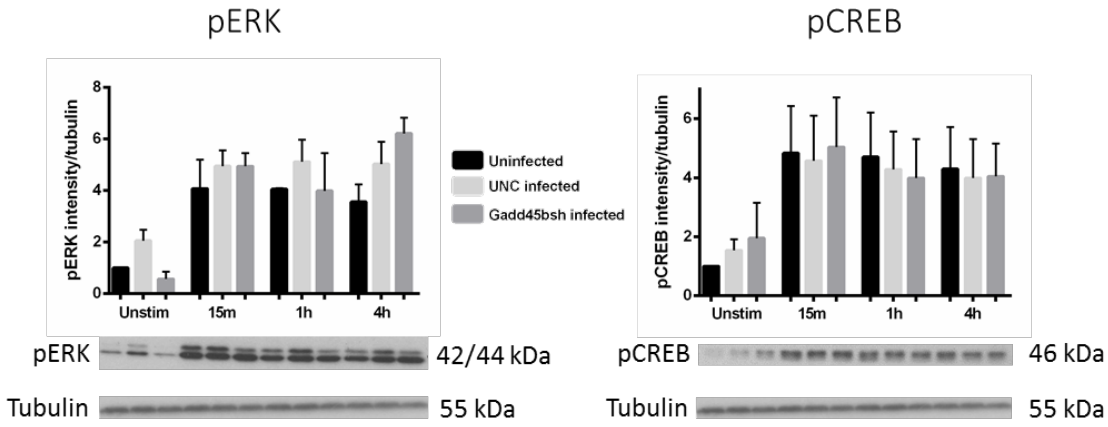
Due to the effects seen in early and late genes mRNA expression in Gadd45b knockdown, we hypothesized Gadd45b could regulate basal levels of methylation this way regulating the permissiveness for transcription activation. As a preliminary approach we aimed at testing if global DNA methylation would be altered. First we validated 5'methylcytosine Dot Blot assay by accessing global methylation staining of standard low or high methylation samples (**Figure|3.8 A**). Indeed, high methylated samples presented elevated staining, contrarily to low methylated samples. Next we stimulated neuronal cultures infected at DIV4 with rAAVs expressing a Gadd45bsh or UNC control 4 hours with bicuculline (**Figure|3.8 B and C**). After DNA purification, 5'methylcytosine Dot Blot assay was performed to test global methylation. After stimulation global methylation levels increased about 4 fold compared to unstimulated conditions. An increase in methylation has also been described in the literature, additionally with gene-specific demethylation (Guo et al., 2011a). Interestingly basal global methylation levels seemed to be increased in Gadd45bsh condition (**Figure|3.8 B and C**). This possible effect was not as pronounced in stimulated conditions, although statistical power is insufficient to give accurate conclusions at this point. This preliminary data is in accordance with a demethylation action of Gadd45b. It is surprising if Gadd45b action *per se* is sufficient to promote such a global alteration. If such is indeed the case it is tempting to speculate that the gene pool identified in this thesis may be just a sample compared to the total Gadd45b-mediated gene alterations.



Figure|3.7 **Gadd45b may promote global DNA methylation changes.** Total DNA was purified from dissociated hippocampal cultures and immunostained for 5-methylcytosine by 5-MeC Dot Blot assay. **(A)** Low and high methylated standard samples were used for initial validation of the assay. **(B)** Before 5-MeC antibody incubation, methylene blue staining was performed as a control for total DNA present. represent Bicuculline stimulation was performed for 4 hours (+bic). **(C)** Total 5-MeC quantification was performed by normalizing meC signal to methylene blue staining and unstimulated uninfected condition. Color code represents uninfected cultures or infected with control sh (UNC) or Gadd45bsh. Results were obtained from 2 independent experiments; error bars represent s.e.m.

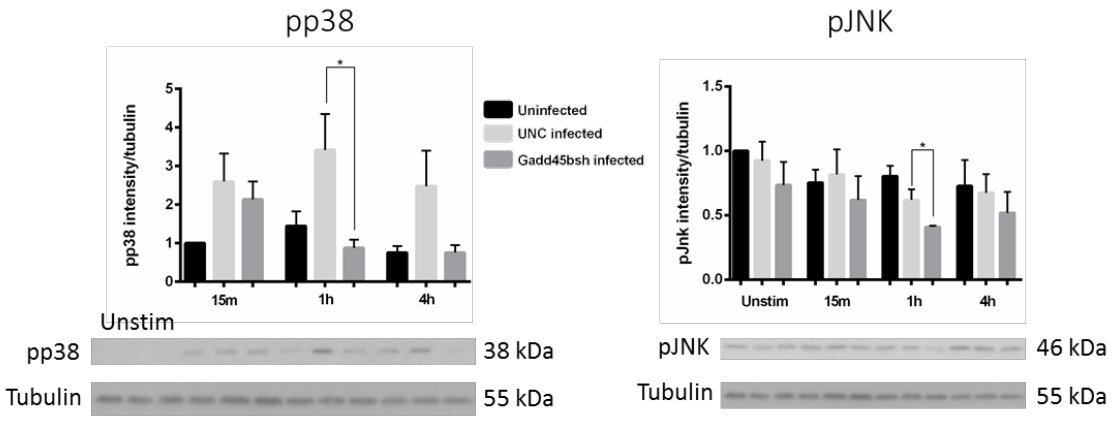
3.5 MAPK signaling pathways are impaired by Gadd45b knockdown

As referred in the Introduction (section 1.3.1.1 Gadd45β-mediated MAPK activation), studies outside the CNS report that Gadd45b can act trough different MAPK signaling pathways. Particularly, Gadd45b activate both JNK and p38 MAPK by activating upstream kinases as MEKK4 or inhibiting MKK7 activity. We hypothesized that these signaling cascades could also be altered by our model of Gadd45b knockdown. Indeed these pathways could mediate functional alterations required for memory processes. To address this hypothesis, we analyzed the phosphorylated versions of JNK, p38 and ERK members and additionally downstream targets of these cascades. Due to the rapid activation of these signaling cascades, we stimulated hippocampal cultures with bicuculline for 15 minutes, 1 hour or 4 hours. Although, to our knowledge, there is no evidence for Gadd45b mediating ERK activity we briefly analyzed both pERK and pCREB a downstream target of ERK activity to highlight a regulatory specificity by Gadd45b. Indeed, both phosphorylated versions of ERK and CREB showed no alteration in the different time points analyzed (**Figure|3.8**).



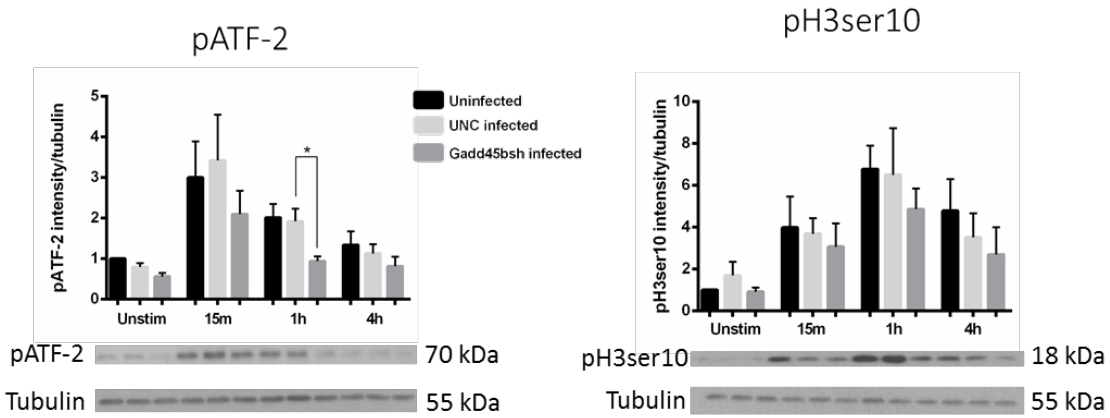
Figure|3.8 **Gadd45bsh does not affect ERK MAPK pathway.** Western blot analysis of protein extracts from dissociated hippocampal cultures for total phosphorylated versions of ERK (left) and CREB (right) normalized for tubulin and for uninfected unstimulated condition. Bottom time-points represent bicuculline stimulation for 15 minutes, 1 hour or 4 hours. Color code represents uninfected cultures or infected with control sh (UNC) or Gadd45bsh. Results were obtained from 2 independent experiments, error bars represent s.e.m.

Next we used a similar approach to access if the p38 or JNK signaling pathways could be altered. We detected statistically significant decreases in both phosphorylated versions of JNK and p38 (**Figure|3.9**). These decreases seemed to be specific for 1 hour of bicuculline stimulation, since changes were not detected in other points of stimulation compared to control conditions.



Figure|3.9 **Gadd45bsh decreases pp38 and pJNK MAPK pathway activity.** Western blot analysis of protein extracts from dissociated hippocampal cultures for total phosphorylated versions of p38 (left) and JNK (right) normalized for tubulin and for uninfected unstimulated condition. Bottom time-points represent bicuculline stimulation for 15 minutes, 1 hour or 4 hours. Color code represents uninfected cultures or infected with control sh (UNC) or Gadd45bsh. Results were obtained from 4-6 independent experiments, error bars represent s.e.m. The statistical significance was calculated using unpaired Student's-T test (*P <0.05).

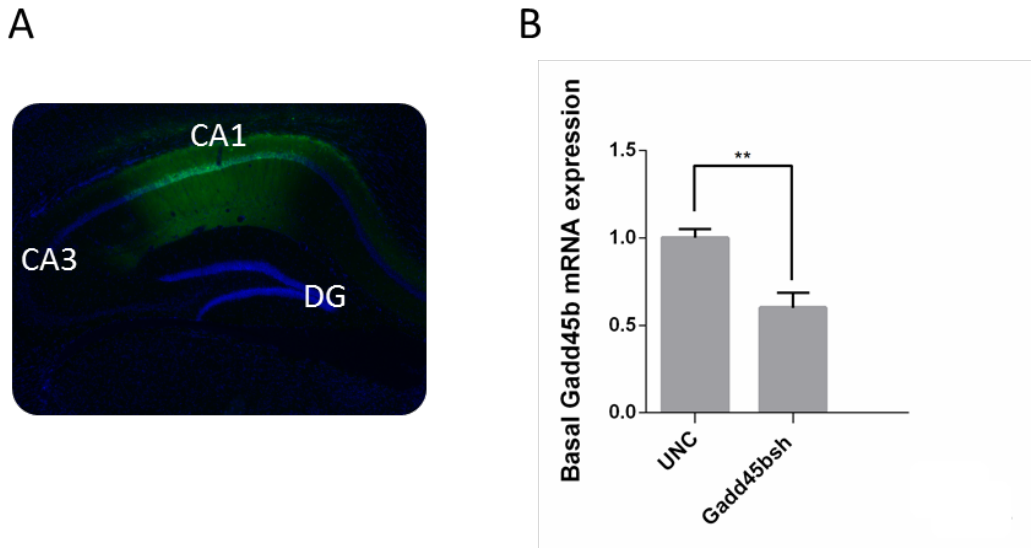
Indeed, if Gadd45b could be relevant for the activation of these signaling pathways at this particular time-window then we should be able to detect a decrease in downstream targets. To test this we analyzed phosphorylated activating transcription factor 2 (pATF-2) (**Figure|3.10**). Indeed, pATF-2 was rapidly induced 15 minutes after bicuculline stimulation, similarly to the previous findings, pATF-2 induction was significantly impaired compared to control conditions selectively at 1 hour of bicuculline stimulation, further supporting a role of Gadd45b in the activation of these signaling cascades. Additionally, phosphorylation of serine 10 in histone 3 was evaluated since it is a marker for chromatin relaxation and transcriptional activation (Wood et al., 2006). Nonetheless, no differences were detected (**Figure|3.10**).



Figure|3.10 **Gadd45bsh decreases pATF-2 at 1 hour of bicuculline stimulation but does not affect pH3ser10.** Western blot analysis of protein extracts from dissociated hippocampal cultures for pATF-2 (left) and pH3ser10 (right) normalized for tubulin and for 1 hour of bicuculline stimulated uninfected condition. Bottom time-points represent bicuculline stimulation for 1 hour and 4 hours. Color code represents uninfected cultures or infected with control sh (UNC) or Gadd45bsh. Results were obtained from 4-5 independent experiments, error bars represent s.e.m. The statistical significance was calculated using unpaired Student's-T test (*P <0.05).

3.6 Long-term memory formation is impaired by Gadd45b knockdown

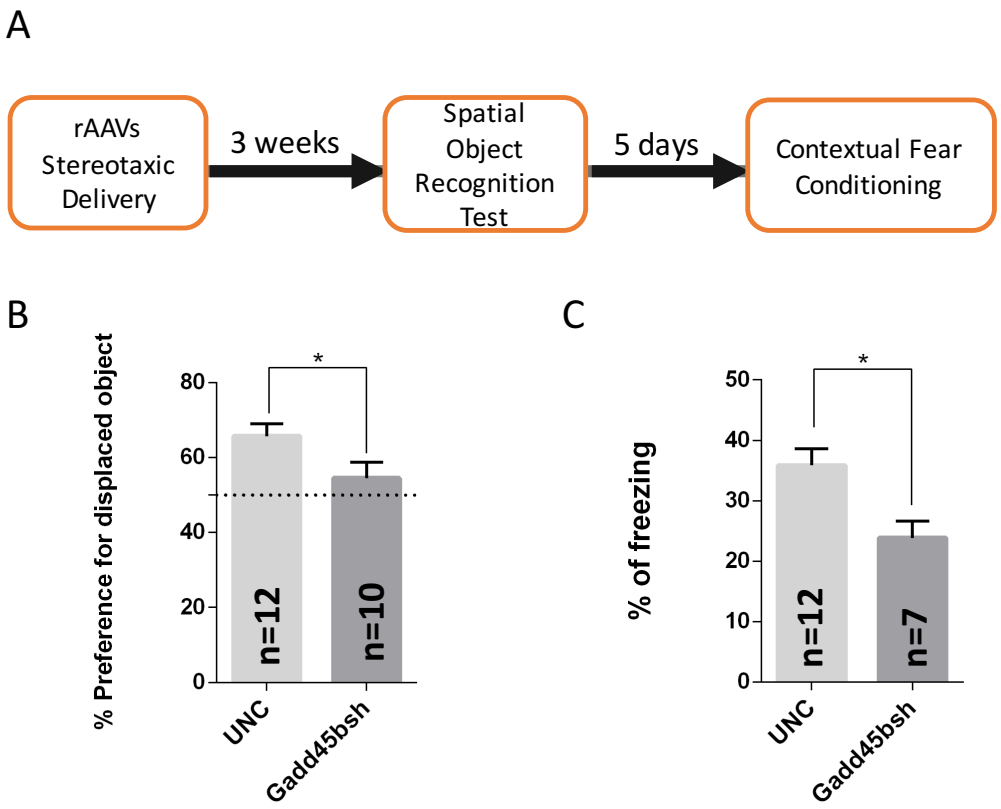
So far our data clearly shows that Gadd45b can promote alterations both at the transcriptional and protein level and modifications in MAPK signaling. Thus a relevant question would be if these changes could underlie a phenotypical alteration in vivo. Next we aimed at determining if long-term memory formation could be altered by a decrease in Gadd45b expression. To test this, we performed stereotaxic delivery of the same rAAVs used in in vitro experiments selectively in the dorsal hippocampus (**Figure|3.11 A**). We further validated the decrease of Gadd45b expression in dissected hippocampal tissue from injected animals.



Figure|3.11 **Gadd45bsh injected mice have decreased Gadd45b mRNA expression in the hippocampus. (A)** rAAVs expressing a control short-hairpin (UNC) or a Gadd45b short-hairpin (Gadd45bsh) were delivered in the dorsal hippocampus of 9 week-old C57Bl/6 mice. CA1 region of the hippocampus expressed GFP as an infection marker (DG- dentate gyrus). (B) Gadd45b mRNA expression was analyzed in the hippocampi of mice. Values were normalized to GusB mRNA levels as an endogenous reference and for the Gadd45b control UNC condition average. Results were obtained from 11 mice (5-6 per group), error bars represent s.e.m. The statistical significance was calculated using unpaired Student's-T test (**P <0.01).

After this *in vivo* validation, we performed a series of behavior tasks with control- and Gadd45b- injected mice. Particularly after stereotaxic delivery of rAAVs mice where given a 3-week period to recover from surgery and express the viral constructs in the dorsal hippocampus (**Figure|3.12 A**). Next, we tested their performance in spatial object recognition test, a hippocampal dependent task. Briefly, in this test the animals are exposed to two different objects and allowed to explore them freely, after a 24-hour time period rodents are placed back in the initial context however, one of the original objects is displaced. The amount of time passed exploring the displaced object versus total time of exploration was measured as an indication of long-term memory formation. Animals injected with Gadd45bsh had a performance impairment in this task compared to control injected animals (**Figure|3.12 B**). Moreover, mean exploration values were 54.5%, which may be interpreted as chance exploration between both objects. To confirm this outcome, we waited for a 5-day period in order to avoid interference between two tests and we tested the performance of animals in contextual-fear behavior paradigm for hippocampal-mediated fear memory. Briefly, animals are exposed in a chamber were they are administered

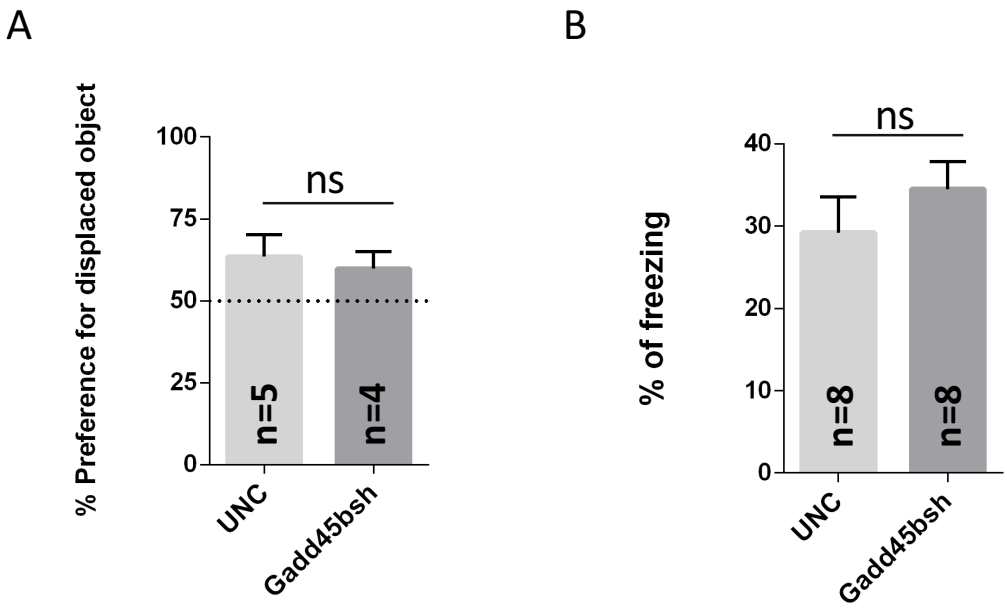
an electric foot-shock, we waited 24 hours and placed animals back in the same contextual environment, the amount of freezing was measured to assess fear-memory. Similarly, to spatial object recognition results, Gadd45sh injected mice showed decreased freezing, suggesting a memory impairment. Indeed, these animals had a statistically significant decrease in performance in this task (**Figure|3.12 C**). Overall, we saw in two distinct paradigms of hippocampal-dependent long-term memory a decrease in performance in Gadd45bsh injected animals. Therefore, our in vitro findings that suggest a role of Gadd45b in plasticity events are supported by a behavior impairment in long-term memory formation.



Figure|3.12 **Gadd45bsh injected mice have impaired LTM formation.** Control short-hairpin (UNC) or a Gadd45b short-hairpin (Gadd45bsh) expressing mice were tested in two paradigms of hippocampal-mediated LTM. **(A)** After rAAVs delivery to the hippocampi of mice, a three-week period anticipated testing, between spatial object recognition test and contextual fear conditioning a 5 day interval was applied. **(B)** In spatial object recognition test a 24-hour interval between training and testing was performed. Exploration time for the displaced object per total exploration time is shown as percentage of preference for displaced object. **(C)** During contextual-fear conditioning training a 0.5 mA foot-shock was applied, a waiting period of 24 hours between training and testing sessions was done, percentage of freezing per total test time is shown. Results were obtained from 19 mice for contextual fear conditioning and 22 mice for spatial object recognition test. Number of animals per group are indicated in the graph. , error bars represent s.e.m. The statistical significance was calculated using unpaired Student's T test (*P <0.05).

3.6.1 Gadd45b may not contribute for short-memory formation

LTM is described to be dependent on gene transcription activation, on the other hand, STM is independent of this transcriptional activation (Alberini, 2009). Since our data suggests that LTM formation is impaired in two distinct *in vivo* paradigms, we tested if these effects are LTM specific. This approach could help understanding the specificity of the Gadd45b role in plasticity. To test this we performed a preliminary study similar to the LTM experiments. Control and Gadd45bsh mice were tested in spatial object recognition test and contextual-fear conditioning (**Figure|3.12 A**), nevertheless between training and test sessions a 1 hour waiting time was adopted for STM assessment. Interestingly both tests did not show any differences between the two groups (**Figure|3.13 A and B**). Although an increase in statistical power is needed to confirm these results. This data suggests that Gadd45b may be a specific LTM mediator not acting in STM formation.



Figure|3.13 **Gadd45bsh injected mice may not show alteration in STM formation.** Control short-hairpin (UNC) or a Gadd45b short-hairpin (Gadd45bsh) expressing mice were tested in two paradigms of hippocampal-mediated STM. After rAAVs delivery to the hippocampi of mice, a three-week period anticipated testing, between spatial object recognition test and contextual fear conditioning a 5-day interval was applied. **(A)** In spatial object recognition test a 1 hour interval between training and testing was performed. Exploration time for the displaced object per total exploration time is shown as percentage for displaced object. **(B)** In contextual-fear conditioning training a 0.5 mA foot-shock was applied, a waiting period of 1 hour between training and testing sessions was done, percentage of freezing per total test time is shown. Results were obtained from 9 mice for spatial object recognition test and 16 mice for contextual fear conditioning. Number of animals per group are indicated in the graph. error bars represent s.e.m. The statistical significance was calculated using unpaired Student's T test.

Chapter 4. Discussion

In this work we demonstrated that Gadd45b is required for memory-associated gene expression. Moreover, we show that an alteration in early-response genes by our Gadd45b knockdown model is accompanied by a selective effect in late-response genes, particularly BDNF exon I. We hypothesize that Gadd45b may regulate the DNA methylation status of these genes, indeed it seems that global methylation is increased in basal conditions in absence of this protein. Next, we suggest that Gadd45b can mediate MAPK signaling activity in neurons trough the JNK and p38 cascades. Indeed, with such relevant alterations detected *in vitro* hippocampal-specific reduction of Gadd45b is sufficient to promote impairments in long-term memory formation and consolidation. Nonetheless, these effect seems to not occur in short-term memory paradigms, thus being LTM specific.

4.1 Gadd45b a mediator of plasticity-associated gene expression

The induction of gene expression is one of the earliest steps for memory formation. Thus, the study of gene expression regulators is crucial for understanding how memory is formed and maintained. Evidence from the literature suggested that Gadd45b, a potential mediator of DNA demethylation, can regulate memory-associated-gene expression particularly BDNF exon IX (Ma et al., 2009). Nevertheless, it was unclear if this regulation was also present in other genes with established roles is memory. With this in mind, we tested 6 different early-response genes, *Arc*, *c-Fos*, *Dnmt3a2*, *Erg-1*, *Npas4* and *Nr4a1*. Surprisingly Gadd45b knockdown was sufficient to promote a robust decrease in mRNA of all of these genes upon 2 hours of bicuculline treatment (**Figure|3.2 A**). These memory players are known to be virtually absent in basal conditions, so it is expected that no alterations would be detected in this state. Indeed, we did not detect an effect of Gadd45b knockdown in most of the unstimulated conditions. One exception to this was *Nr4a1*, which was increased in basal conditions upon treatment with Gadd45bsh. *Nr4a1* has been recently confirmed

to be sensitive to DNMT inhibitors, such treatment promotes an increase in *Nr4a1* expression (Tognini et al., 2015). Since we could detect a reduction of Dnmt3a2 in basal conditions, one could speculate that a basal state of DNA-methylation is compromised in the *Nr4a1* gene, thus resulting in an increased expression. Additionally, since after stimulation *Nr4a1* induction exceeds 80 fold (**Figure|3.2**), we are able to detect an impairment in this stimulated state, possibly due to a more significant demethylation action of Gadd45b compared to DNMT methylation. Altogether, these findings suggested that Gadd45b can regulate crucial players in plasticity and memory. Next, we aimed at testing if in genes with slower kinetics, particularly *Bdnf*, such an effect occurred. Indeed at 2 hours of stimulation no differences were detected for exons of *Bdnf* that have been suggested to be regulated by methylation (Lubin et al., 2008). After 4 hours of stimulation, we detected a specific decrease in *Bdnf* exon I but not on exons IV, VI and IX (**Figure|3.3**). Different *Bdnf* exons can behave as late-response genes, particularly some forms are highly dependent on transcriptional activation by early-response gene protein products. In fact, we detected alterations in the protein levels of Npas4 and Fos, together with a tendency for Arc (**Figures|3.5 and 3.6**). A transcriptional feature of *Bdnf* exon I compared to exon IV it that its transcriptional induction is highly dependent on Npas4 activity compared to CREB activation (West et al., 2014). Indeed Npas4 has shown to form heterodimers with aryl-hydrocarbon receptor nuclear translocator 2 (ARNT2) after a stimuli, thus binding to exon I and inducing its transcription (Pruunsild et al., 2011). This regulatory mechanism is dependent on *de novo* synthesis of Npas4, where abolishment of *Npas4* expression selectively impairs exon I induction. It is tempting to hypothesize that the selective effects seen in *bdnf* may be due to the Npas4 induction impairment promoted by Gadd45b knock-down. To test this hypothesis it would be relevant to perform *Npas4* rescue experiment together with Gadd45bsh treatment. If *bdnf* exon I levels were restored to control levels one could suggest that effects on *Npas4* are responsible for an exon-specific effect on BDNF. From a functional point of view, this particular exon has the highest induction compared to other exons (**Figures|3.4**), since all code for pro-BDNF, a regulation in this exon points to a relevant regulation by Gadd45b. It has been proposed that different forms of Bdnf represent a “spatial code”. Interestingly *bdnf* exon IV, a

particular exon well described in memory, was proposed to be localized in proximal dendrites (Baj et al., 2011). Among the other mRNA transcripts only exon I was also proposed to have this spatial arrangement (Baj et al., 2011). One could speculate that this induction promoted by Gadd45b may contribute to local dendritic rearrangements also proposed for these exons.

Moreover, the effects seen in early-response genes probably are not due to *de novo* Gadd45b reduction, since Gadd45b mRNA activity-regulated expression peaks at 4 hours post induction (**Figures|3.1**). Thus, it would be relevant to see if Gadd45b is responsible for maintaining a basal demethylated state in these genes, which would facilitate the induction of expression upon a learning stimulus. Indeed, it seems that global methylation is increased in Gadd45b knockdown conditions, which suggest a demethylase role (**Figure|3.8 A**). Nevertheless, an effect on global DNA methylation may suggest a wider gene regulation role or Gadd45b. Additionally, gene-specific promoter and enhancer methylation analysis could elucidate if Gadd45b regulates these genes by changes in methylation patterns. Such may also be the case for BDNF exon I with the synergetic effect of Npas4 biding.

4.2 JNK and p38 pathways are possible mediators of Gadd45b memory effects

MAPK signaling pathways have been extensively explored in plasticity and memory paradigms. Particularly the ERK signaling cascade is a known switch for the induction of plasticity-associated gene expression (Adams and Sweatt, 2002). Downstream targets such as ELK1 and CREB upon activation by phosphorylation can induce the expression of target genes such as *c-fos* (West and Greenberg, 2011). Other MAPK signaling cascades as JNK and p38 have a more unstudied role, although findings point to phosphorylated forms of JNK to be required for long-term memory and p38 has been suggested to play a role both in LTM and STM (Alonso et al., 2003, Kelleher et al., 2004, Thomas and Huganir, 2004). Because Gadd45b was shown to modulate activity of the JNK cascade trough activation of MEKK4 outside of the CNS, we hypothesized such could occur also in hippocampal neurons. Interesting a decrease in Gadd45b expression was sufficient to promote an impairment in both JNK and p38

phosphorylation at 1 hour of stimulation (**Figures|3.9**). These findings are in accordance with data from the literature that suggests that these cascades are activated transiently until 30 minutes after training (Alonso et al., 2003, Kelleher et al., 2004, Thomas and Huganir, 2004). It is important to note that our effects in the p38 cascade were based in the comparison between Gadd45bsh conditions versus UNC infected, in which there was an abnormal increase in p38 phosphorylation. Repetition of this experiment with a different sh control would be relevant. We also detected a decrease in ATF-2 phosphorylation at the same time point suggesting that knocking down Gadd45b is sufficient to promote effects on JNK/p38 downstream targets (**Figures|3.10 A**). Furthermore, statistically significant effects from the p38 signaling cascade were obtained from comparison with the universal sh control, which presented a high induction compared to uninfected conditions. In order to conclude with more certainty, a next step would be to repeat experiments with another sh control. It is unclear if the results seen at a transcriptional level are partially due to these signaling alterations. It would be interesting in the future to test if gene induction is impaired before the 1 hour time point of stimulation and immediately after. Also, expand the analysis to other targets of JNK activity such as c-jun would be relevant to further understand how Gadd45b regulates this cascade. Activation of c-jun has been reported to be essential for hippocampal synaptic plasticity (Seo et al., 2012).

We also tested mRNA expression of other Gadd45 family members (data not shown). Interestingly, Gadd45g was also decreased upon Gadd45bsh treatment in basal and stimulated conditions. This effect could suggest a Gadd45g regulation by Gadd45b. Taking into account the homology between Gadd45 b and g, there is also the possibility of a sh off-target effect, to assess these two distinct interpretations, it would be relevant to repeat gene expression analysis with different Gadd45bsh to confirm the specificity of our findings. Additionally, one could speculate that this particular Gadd45b-mediated role could promote other changes at the synapse level such as structural alterations. Indeed, evidences from the literature point that JNKs phosphorylate postsynaptic density protein 95 (PSD95) and AMPAR (Kim et al., 2007, Thomas et al., 2008). Similarly, p38 MAPK is required in the induction of mGluR-induced LTD

(Thomas and Huganir, 2004). One similarity is that Gadd45b has been suggested to play a role in mGluR activation, thus a synergetic effect with active DNA demethylation could occur (Matrisciano et al., 2011).

4.3 Gadd45b is required for LTM formation but possibly not STM

One indispensable requirement for LTM formation is IEG expression, during several peaks after the initial learning stimuli. Taking into account our results, we hypothesized that Gadd45b would be required for LTM formation. Mice injected with Gadd45bsh showed robust LTM deficits in spatial object recognition test and contextual fear conditioning (**Figures|3.12 B and C**). These results clearly suggest Gadd45b as a mediator of hippocampal LTM formation. Future set of experiments could include mRNA analysis in the hippocampi of tested animals to validate *in vitro* findings *in vivo*. One could argue that compensatory mechanisms could occur, thus the phenotype seen could not be directly due to *in vitro* findings. It would be interesting to bridge the cellular mechanisms and behavior with electrophysiological analysis; for example studying if reducing levels of Gadd45b would promote alterations in neuronal transmission. Indeed past findings suggest that Gadd45b$^{-/-}$ hippocampal slices show pair-pulse facilitation deficits (Sultan et al., 2012). Taking into account the behavior impairments seen, it is tempting to suggest that physiological alterations are occurring in the hippocampi of Gadd45bsh injected animals. In addition, validation analysis of Gadd45b mRNA expression after training should be performed. Nevertheless, since p38 activity is required for LTM, our MAPK results could also suggest an impairment on STM. On the contrary to gene expression results since *de novo* gene expression is not required for STM formation. Although greater statistical power is needed to take solid conclusions, it seems that STM is not altered (**Figures|3.13 A and B**). Additionally, phosphorylated JNK results do not take into account different JNK isoforms, thus it is difficult to speculate the precise role of the JNK pathway in Gadd45b-mediated effects. Nevertheless, both JNK and p38 results can be interpreted as parallel mechanisms of Gadd45b action, initially independent but together can promote alterations required for LTM formation. To further explore these hypotheses, it is necessary to test how specific modifications seen in

gene expression and MAPK pathways contribute to alterations at the cellular level.. Although the LTM deficits seem in accordance to *in vitro* data, it would be interesting to perform recue experiments in Gadd45bsh injected animals. Indeed, if these animals upon restoring levels of Gadd45b would perform similar to controls in LTM tasks one could strongly suggest a role for Gadd45b in LTM.

Chapter 5. Conclusion

The findings of the present work present an initial contribution to the understanding of how Gadd45b may mediate memory formation by changes in gene expression and signaling cascades. Our results suggest that Gadd45b can alter expression of several memory-associated early genes. These alterations may be essential for induction of late-response gene expression required for learning and memory. We suggest that alterations in methylation patterns present before learning stimuli, thus regulating the permissiveness for gene-activation, underlie the observed alterations. Future studies assessing gene-specific methylation alterations in different gene-regulatory regions would be relevant to further address this hypothesis. Additionally, Gadd45b role in memory formation may also rely on MAPK activity. MAPK changes identified by Gadd45b knock-down may mediate different neuronal adaptations required for memory. Altogether, we propose Gadd45b as a novel mediator of hippocampal-dependent LTM (**Figures|5.1**).

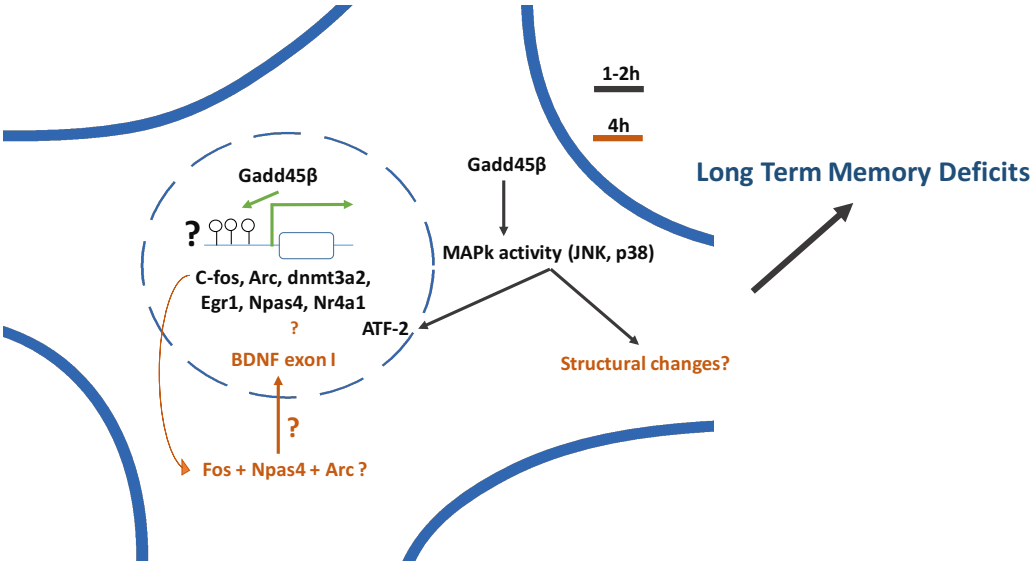


Figure 5.1 **Gadd45b knockdown potentiates LTM formation though gene and MAPK regulation**. Current proposed model: Gadd45 may promote a basal demethylated state in early-response genes important for memory. Once these genes are translated some have transcriptional activity, we hypothesize Npas4 can later regulate the transcription of BDNF exon I. Both Fos and Npas4 proteins were shown to be decrease in reduced levels of Gadd45b, while Arc presented a tendency for decrease. Additionally, Gadd45b can promote alterations in MAPK signaling cascades, JNK and p38. These changes can be observed in downstream targets such as ATF-2. We hypothesize these changes may also promote changes in neuronal architecture, although future experiments are needed. Finally, Gadd45b through these actions and likely others, facilitates LTM formation

Chapter 6. References

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