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Nuclear calcium regulates dendrite maintenance, memory formation and fear extinction.

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Für

Mama, Papa & Alex

1 Acknowledgments

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2 Summary

Throughout our entire life, memory is central to our ability to carry out everyday tasks. Therefore, diseases that affect cognition have severe consequences, that influence patients' daily lives. However, not only do deficits in forming or recalling memories impact peoples' lives but also the inability to forget traumatic events or fear towards specific stimuli, such as with post-traumatic stress disorder (PTSD), anxiety disorders and phobias. Hence, it is essential to understand the cellular and molecular mechanisms in order to help people with any of these disorders.

In the first study of this thesis, I investigated if nuclear calcium, which is known to control cAMP response element binding protein (CREB)/CREB binding protein (CBP)-mediated transcription (Bading (2013)), is required for the formation of memory. Indeed, I found that inhibition of hippocampal nuclear calcium impairs memory formation in two hippocampus-dependent tasks. I further investigated the possible mechanisms that contribute to these deficits and found that dysregulation of nuclear calcium leads to alterations in dendritic morphology. I was able to rescue these morphological alterations via overexpression of the vascular endothelial growth factor D (VEGFD). VEGFD is regulated by nuclear calcium under basal conditions and is known to be important for the maintenance of dendritic morphology (Mauceri et al. (2011)). Additionally, I could show that overexpression of VEGFD rescued memory deficits caused by nuclear calcium inhibition, probably due to the restoration of the dendritic architecture.

In the second study, we aimed to investigate whether DNA methyltransferases (DNMTs) are required for memory formation. We found that DNMT3a2 is regulated by nuclear calcium and is induced upon neuronal activity. Additionally, we were able to show that the level of DNMT3a2 determines memory performance in mice. Downregulation of DNMT3a2 caused memory deficits and, in addition, a decrease in DNMT3a2 expression was associated with age-dependent memory decline. Further, restoring the level of DNMT3a2 in aged mice rescued memory impairments. Additionally, we showed that DNMT3a2 expression correlates with global methylation levels, and we were able to identify two of the target genes of DNMT3a2, namely, activity-regulated cytoskeleton-associated protein (ARC) and brain-derived neurotrophic factor (BDNF) (Oliveira et al. (2012)).

In the third part of this thesis, I studied if nuclear calcium is also required for fear memory extinction, which is routinely used as a model of PTSD. It is widely accepted that during fear extinction a new memory is built up that inhibits the previous, acquired memory. Though it is known that memory formation and fear extinction share common mechanisms, studies investigating the role of transcription in fear extinction are partly controversial (Mamiya et al. (2009), Lin et al. (2003), Vianna et al. (2003)). Here, I have shown that nuclear calcium is

involved in fear extinction, pointing to a requirement of gene transcription. I have shown in the first study of this thesis that nuclear calcium maintains the dendritic architecture. Therefore, alterations in the dendritic tree, probably also contribute to fear memory extinction.
In summary, I have shown in this thesis that nuclear calcium mediates two forms of cognition, memory formation and fear extinction. I provide evidence that nuclear calcium-regulated VEGFD maintains the dendritic structure, which is important for the permissiveness of the neuron to process information required for long-term adaptations. Additionally, nuclear calcium regulates DNMT3a2, and we show that the level of DNMT3a2 has an impact on memory formation. Overexpression of DNMT3a2 restored the level of DNMT3a2 in aged mice and rescued age-dependent memory deficits.

3 Zusammenfassung

Unser Gedächtnis spielt für uns täglich eine wichtige Rolle, da wir die Fähigkeit uns an Dinge zu erinnern für die Ausübung von Alltagsaufgaben nutzen. Deshalb haben Krankheiten, die das Gedächtnis beeinträchtigen, schwerwiegende Folgen für die Patienten. Nicht nur Defizite bei der Gedächtnisbildung oder Erinnerung haben dabei einen Einfluss auf das Leben der Patienten,auch die Störung, dass traumatische Ereignisse nicht mehr vergessen werden können, wirken sich auf deren Leben aus. Dies ist zum Beispiel der Fall bei Patienten mit posttraumatischen Bewusstseinsstörungen, Angststörungen oder Phobien. Um Patienten in Zukunft besser helfen zu können, ist es daher von besonderer Wichtigkeit, die zellulären und molekularen Mechanismen der Gedächtnisbildung und posttraumatischen Bewusstseinsstörung genauer zu verstehen.
Im ersten Teil meiner Arbeit habe ich untersucht, ob Kernkalziumsignale für die Gedächtnisbildung benötigt werden. Es ist bekannt, dass Kernkalziumsignale die CREB/CBP-abhängige Gentranskription regulieren (Bading (2013)). Ich habe gezeigt, dass in zwei verschiedenen Erinnerungstests die Inhibierung von hippokampalen Kernkalziumsignalen zum Gedächtnisverlust führt. Des Weiteren habe ich detaillierter untersucht, auf welche Weise die Inhibierung der Kernkalziumsignale zum Gedächtnisverlust beiträgt. Ich habe herausgefunden, dass die Dysregulation der Kernkalziumsignale zu Veränderungen der Dendritenstruktur führt. Diese Veränderungen konnten durch die Überexpression des Signalmoleküls VEGFD (aus dem Englischen: vascular endotheliar growth factor D) wiederhergestellt werden. VEGFD wird unter basalen Bedingungen durch Kernkalziumsignale reguliert. Zusätzlich ist bekannt, dass VEGFD für die Erhaltung der Dendritenstruktur notwendig ist (Mauceri et al. (2011)). Ich konnte außerdem zeigen, dass Gedächtnisverluste, die durch die Inhibierung von Kernkalziumsignalen ausgelöst wurden, durch die Überexpression von VEGFD, rückgängig gemacht werden konnten. Dies ist wahrscheinlich durch die Wiederherstellung der Dendritenstruktur erzeugt worden.
Im zweiten Abschnitt meiner Arbeit, haben wir die Rolle von DNA Methyltransferasen (DNMTs) bei der Gedächtnisbildung untersucht. Wir haben herausgefunden, dass die DNMT3a2 von Kernkalziumsignalen reguliert wird und durch neuronale Aktivität induziert wird. Des Weiteren konnten wir zeigen, dass die Menge von DNMT3a2 die Gedächtnisbildung in Mäusen bedingt. Eine Senkung der DNMT3a2 Expression hat zu Gedächtnisverlusten geführt und altersabhängige Gedächtnisdefizite gingen mit einer geringeren Menge an DNMT3a2 einher. Nachdem die Menge von DNMT3a2 in alten Mäusen wiederhergestellt wurde, verbesserte sich auch die Gedächtnisfunktion. Außerdem konnten wir zeigen, dass die Expression von DNMT3a2 mit der Menge der globalen Methylierung korreliert. Zusätzlich konnten wir mit ARC und BDNF zwei der Zielgene von DNMT3a2 bestimmen (Oliveira et al. (2012)).

Im dritten Teil meiner Arbeit, habe ich untersucht, ob Kernkalziumsignale auch bei der Angstextinktion eine Rolle spielen. Angstextinktion wird routinemäßig als ein Model der posttraumatischen Bewusstseinsstörung genutzt. Es ist weitgehend akzeptiert, dass während der Extinktion von Angst eine neue Erinnerung aufgebaut wird, die die vorherige Erinnerung unterdrückt. Es ist bereits bekannt, dass sowohl bei der Gedächtnisbildung als auch bei der Angstextinktion einige gleiche Mechanismen auftreten. Studien, die sich mit der Notwendigkeit der Gentranskription während der Angstextinktion beschäftigen, zeigen jedoch kontroverse Ergebnisse (Mamiya et al. (2009), Lin et al. (2003), Vianna et al. (2003)). In meiner Arbeit habe ich gezeigt, dass hippocampale Kernkalziumsignale bei der Angstextinktion eine wichtige Rolle spielen. Dieses Resultat weisst daraufhin, dass Gentranskription im Hippocampus für die Extinktion der Angst benötigt wird. Berücksichtigt man zusätzlich das Ergebnis des ersten Teilabschnittes meiner Arbeit, der zeigt dass Kernkalziumsignale für die Erhaltung der Dendritenstruktur wichtig sind, so ist es außerdem wahrscheinlich, dass der Erhalt der Architektur des Dendritenbaumes eine weitere Voraussetzung für die Angstextinktion darstellt. Zusammenfassend habe ich in dieser Arbeit gezeigt, dass Kernkalziumsignale für zwei kognitive Prozesse, nämlich der Gedächtnisbildung und der Angstextinktion, eine essenzielle Rolle spielen. Des Weiteren habe ich Hinweise geliefert, dass VEGFD, welches durch Kernkalziumsignale reguliert wird, zur Aufrechterhaltung der Dendritenstruktur beiträgt. Der Erhalt der Dendritenstruktur erlaubt es der Nervenzelle Informationen zu verarbeiten, die für langfristige Anpassungen benötigt werden. Zusätzlich regulieren Kernkalziumsignale DNMT3a2 und wir haben gezeigt, dass die Menge von DNMT3a2 einen Einfluss auf die Gedächtnisbildung bei Mäusen hat. Altersabhängige Gedächtnisdefizite konnten durch Überexpression von DNMT3a2 wiederhergestellt werden.

4 Introduction

'We are what we are because of
what we learn and what we remember.'
Eric Kandel, Nobel prize winner

In our everyday life, we depend on our ability to learn and memorize things. Knowing how to ride the bike, to remember which ingredients we just added while preparing a meal or to recall that we need to go to the grocery store after work are just a few examples. Some of the memories we recall unconsciously (implicit memory), such as how to ride a bike, while we remember others consciously (explicit memories). Explicit memories can be further divided in episodic and semantic memories. Episodic memories are memories about an event (remembering your first day in school), whereas semantic memories are memories about a fact (the birthday of your best friend). As memories have such a great impact on our life, it is not surprising that, for many decades, researchers have been trying to understand the mechanisms that underlie memory formation. But even after many years of research, we are still only beginning to understand how we encode, store and retrieve information. However, diseases that affect peoples' memory such as Alzheimer's disease, remind us of the importance to continue with our studies on memory processes.
In addition to understanding the mechanisms involved in how we learn, it is also of great relevance to investigate how we forget or extinguish memory. This is especially important for people who develop post-traumatic stress disorder (PTSD) after traumatic events and for people with phobias. Though some therapies, such as exposure therapy, can partly help the patients to reduce fear and anxiety, those therapies are often short-lived, as the fear or anxiety is usually only suppressed but not erased (Bouton (1988)).
This thesis aims to further investigate aspects of memory formation and fear extinction memory, the latter serving as a model to study PTSD. In the following, molecular and structural mechanisms underlying long-term memory (LTM) and fear extinction memory will be described.

4.1 Learning and memory- a focus on calcium signalling

How do we form memories? How does a memory become long-lasting? What is required to remember things not only for a short time but for durations up to a lifetime? Two important features that distinguish short-term from long-term memory are gene transcription and protein synthesis. Short-labile memories do not require gene expression, however in order to form LTM,

we need the production of new proteins (Barondes and Cohen (1968),Montarolo et al. (1986), Katche et al. (2013)). Calcium signals are key mediators that regulate these processes and help to transfer information from the synapse to the nucleus. Calcium influx to the cell, as well as its release from internal stores, can increase the calcium concentration. This increase can differ in duration, magnitude and localisation and, in turn, can affect gene transcription (Bading et al. (1993), Hardingham et al. (1997)). In the following, I will describe signalling pathways that are activated by increases in calcium concentration and whose activation lead to long-term potentiaiton (LTP) or memory formation.

4.1.1 Signalling pathways regulating gene transcription in long-term potentiation

Initial studies investigating the underlying molecular and cellular mechanisms of memory mainly analysed long-term potentiation (LTP), an in vitro homologue for memory consolidation. It was shown that synaptic activity can induce two forms of LTP, an early phase (E-LTP) that does not require protein synthesis and a late phase (L-LTP) that does require protein synthesis (Krug et al. (1984), Frey et al. (1988)). It is now widely accepted that the formation of L-LTP requires communication from the synapse to the nucleus, using calcium as a mediator in this process. As an initial step, calcium has to enter the cell, either through ligand-gated N-methyl-D-aspartate (NMDA) receptors or voltage-gated calcium channels (VGCCs) (Bading et al. (1993), Bading et al. (1995)). In order to allow calcium to pass through NMDA receptors, two things have to happen simultaneously; hence, the NMDA receptor is often referred to as a coincidence detector (Bliss and Collingridge (1993)). Glutamate released from the presynaptic terminal needs to bind to the receptor, and additionally magnesium, which blocks the NMDA receptor under resting conditions, needs to be expelled (Mayer et al. (1984), Nowak et al. (1984)). The latter is achieved by sodium and potassium flow through α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, resulting in prolonged membrane depolarization. Upon depolarization, L-type VGCCs also get activated and calcium can enter through both L-type VGCCs and NMDA receptors (Nowak et al. (1984)). Intracellular calcium can, in turn, bind to calmodulin (CaM) and can hence initiate a chain of signalling cascades. Calcium-bound CaM can activate, for example, the enzyme adenylyl cyclase (AC), leading to the activation of the cAMP-dependent protein kinase A (PKA). Calcium and calcium-bound CaM can further activate mitogen-activated protein kinase (MAPK), protein phosphatase and calcium/calmodulin-dependent protein kinases (CaMKs) (Rosen et al. (1994), West et al. (2002), Bengtson and Bading (2012)). Of those calcium signalling pathways, mainly MAPK and CaMKs are known to phosphorylate cyclic AMP (cAMP) response element binding protein (CREB) at Ser133 (Chawla et al. (1998), Hardingham et al. (1999)). This is an important step,

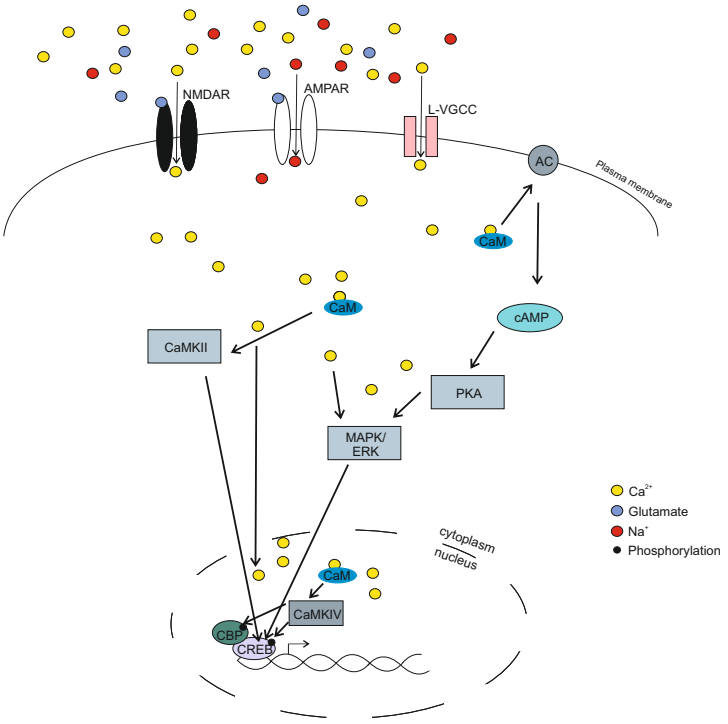

Figure 1: Calcium-regulated signalling pathways lead to gene transcription required for long-term potentiation and long-term memory formation. Calcium enters the cells via NMDA receptors and L-type VGCCs. Inside the cell, calcium can bind to calmodulin, thereby activating several signalling cascades, leading to the phosphorylation of CREB. Inside the nucleus, CaMKIV gets activated via nuclear calcium/calmodulin. CaMKIV is the only known kinase that can phosphorylate CBP and, therefore, can initiate CREB/CBP-dependent transcription.
N-methyl-D-aspartate receptors, NMDAR; α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors, AMPAR; L-type voltage-gated calcium channels, L-type VGCC; adenylyl cyclase, AC; calmodulin, CaM; calcium/calmodulin-dependent protein kinase, CaMK; cyclic AMP, cAMP; protein kinase A, PKA; mitogen-activated protein kinase/extracellular-signal-regulated kinase, MAPK/ERK; cAMP response element binding protein, CREB; CREB binding protein, CBP.

as LTP and LTM require the initiation of gene transcription via CREB phosphorylation and CREB binding protein (CBP) phosphorylation. While CREB phosphorylation can be initiated by several kinases, CBP phosphorylation at Ser301 requires nuclear calcium regulated CaMKIV (Chawla et al. (1998), Hardingham et al. (1999), Bading (2013)). This underlies the importance of nuclear calcium signalling and its relevance in synaptic activity and learning-induced gene expression (Figure 1). A variety of genes associated with cognition are among those genes that are regulated by CREB/CBP-mediated transcription. These include activity-regulated cytoskeleton-associated protein (ARC), brain-derived neurotrophic factor (BDNF), nuclear receptor subfamily 4, group A, member 1 (Nr4a1) and neuronal PAS domain protein 4 (NPAS4) (Zhang et al. (2009)). Nevertheless, nuclear calcium signalling also regulates gene expression and mechanisms known to play a role in memory formation in a CREB/CBP independent manner. Nuclear calcium regulates, for example, the shuttling of histone deacetylases (HDACs) (Schlumm et al. (2013)) and is required for phosphorylation of methyl CpG binding protein 2 (MeCP2) at serine 421 (Buchthal et al. (2012)). Further examples of memory relevant genes that are regulated by nuclear calcium are vascular endothelial growth factor D (VEGFD) (Mauceri et al. (2011)) and DNA methyltransferase 3a2 (DNMT3a2) (Oliveira et al. (2012)).

4.1.2 Calcium-regulated signalling cascades required for memory formation

At this point, an overview of the current knowledge regarding activity-dependent gene transcription in LTP has been given. Though LTP does not equal memory, many mechanisms required for LTP are also necessary for the formation of memory. First, studies have shown that NMDA receptors are required for a variety of memory types, such as spatial memory (Morris et al. (1986)), novel object recognition and fear memory (Tang et al. (1999), Kim et al. (1991)). Even though there are currently studies reassessing the functions of hippocampal NMDA receptors in spatial memory, the general idea that NMDA receptors are involved in certain memory functions is currently no matter of debate. L-type VGCCs have also been reported to be relevant for fear memory and remote spatial memory (Bauer et al. (2002), White et al. (2008)), though other studies report that L-type VGGS are not required for fear memory (Cain et al. (2002)). Genetic studies have revealed that the L-type VGCC subtype $Ca_v1.3$ (McKinney and Murphy (2006)), but not the subtype $Ca_v1.2$ (McKinney et al. (2008)), is important for the consolidation of fear memory. However, hippocampal $Ca_v1.2$ has been reported to be important for spatial memory (Moosmang et al. (2005)). The controversial findings for both NMDA receptors and VGCCs may be explained by different behavioural protocols applied, pharmacological versus genetic approaches used or different brain areas that were investigated. Hence, further studies have to be conducted, which may then contribute to our understanding of the functional role of calcium

channels in different forms of memory.
The AMPA receptor subunit GluR1 is important for retention performance following aversive learning (Mesches et al. (1996)) and retention performance in spatial memory (Lee et al. (2003)), for spatial working, but not spatial reference memory (Reisel et al. (2002)) and for fear conditioning learning (Humeau et al. (2007)). It was further suggested that regulation of GluR2-dependent AMPA receptor trafficking is important for maintaining memories (Migues et al. (2010)). Therefore, AMPA receptor GluR1 and GluR2 subunits have both been suggested to be involved in memory tasks, though executing distinct functions.
Activity-dependent influx of calcium through NMDA receptors and VGCCs regulates a variety of down-stream events, important not only for LTP but also for the formation of memory. Dysregulation of AC 1 and 8 leads to behavioural alterations, for example, in passive avoidance, fear conditioning and spatial memory (Wong et al. (1999), Wu et al. (1995), Sindreu et al. (2007)). Further the kinases MAPK, PKA, CaMKII and CaMKIV have all been implicated in playing a role in associative or spatial memory (Silva et al. (1992), Mayford et al. (1996), Atkins et al. (1998), Abel et al. (1997), Giese and Mizuno (2013)). Of these kinases, CaMKII is presumably the best studied kinase. Dysregulation of its autophosphorlyation at Thr 286, Thr305 and Thr306 and mutation of the isoform CaMKIIα cause memory deficits in a variety of tasks (Giese et al. (1998), Elgersma et al. (2002), Silva et al. (1992)).
Memory formation that is dependent on CREB/CBP dependent transcription requires nuclear signalling events. As mentioned previously, CREB/CBP-mediated transcription depends on nuclear CaMKIV activation by calcium/calmodulin (Bading (2013)). Studies have shown that inhibition of nuclear calcium/calmodulin signalling impaired the formation of memory (Weislogel et al. (2013), Limbäck-Stokin et al. (2004)). Further, the level of phosphorylated CREB (pCREB) and expression of target genes was reduced. Studies elucidating the role of CaMKIV have shown that CaMKIV is important for spatial memory (Kang et al. (2001)), however, findings on its role in fear conditioning are not consistent (Wei et al. (2002), Fukushima et al. (2008), Kang et al. (2001)). Given its role in gene transcription, it is not surprising that disruption of the CREB function itself has been shown in a variety of studies to impair LTM. The role of CREB/CBP-regulated transcription in memory formation will be discussed in more detail in the following section.

4.1.3 CREB/CBP-mediated transcription is essential for long-term memory formation

As discussed above, CREB phosphorylation at Ser133 and CBP phosphorylation at Ser301 are crucial for CREB/CBP-mediated transcription. Several studies have demonstrated the

importance of CREB and CBP in memory formation, however especially for the function of CREB, results are partly controversial. First studies on the role of CREB in memory formation were done in *Aplysia*. Dash et al. (1990) showed that long-term facilitation, but not short-term facilitation, was blocked by CRE oligonucleotide injections into the nucleus of sensory neurons. In mice, CRE oligonucleotide injections into the CA1 region of the dorsal hippocampus resulted in memory deficits in contextual fear conditioning (Athos et al. (2002)). A variety of studies have confirmed the requirement of CREB in LTM (Impey et al. (1998),Kida et al. (2002), Pittenger et al. (2002)). Mice deficient in the two CREB isoforms α and δ displayed impairments in contextual and cued fear memory as well as in spatial memory (Bourtchuladze et al. (1994)). However, another study reported no memory impairments in mice deficient of the two isoforms (Gass et al. (1998)). These controversial findings may be explained by different genetic backgrounds used (Lipp and Wolfer (1998)). Studies on two additional mouse mutants support the idea that CREB is necessary for LTM formation. Mice expressing a CREB mutation of Ser133 to alanine (CREBSer133A) (Kida et al. (2002)), as well as mice expressing a CREB mutant that fails to bind with CRE, therefore inhibiting DNA binding (KCREB) (Pittenger et al. (2002)), have deficits in contextual and cued fear memory or deficits in object recognition and spatial memory, respectively. KCREB mice however did not show deficits in contextual and cued fear conditioning (Pittenger et al. (2002)). The function of CREB was further investigated by generating three different mouse strains, which display a CREB gain of function. Surprisingly, one strain showed deficits in spatial memory (Barco et al. (2002), Viosca et al. (2009)). It was suggested that expression of CREB in these mice, is much higher than under physiological conditions, therefore leading to memory abnormalities (Kida and Serita (2014)). Studies with the other two strains showed enhancements in social recognition, contextual fear and spatial memory (Suzuki 2011). Notably, in all three mouse strains expression of at least one CREB target gene associated with memory formation, i.e. c-FOS or BDNF, was shown to be enhanced (Barco et al. (2005), Suzuki et al. (2011)).
In addition, dysregulation of CBP or the homolog p300, resulted in behavioural abnormalities in object recognition (Korzus et al. (2004), Wood et al. (2006), Oliveira et al. (2007)) and passive avoidance (Oike et al. (1999)). Transgenic mice expressing CBP, in which the HAT activity is reduced, showed additionally deficits in spatial memory, though contextual fear memory was intact (Korzus et al. (2004)). However, among others, mice heterozygous for a null mutation of CBP (Alarcón et al. (2004)) and mice having an inactivating mutation in the CREB-binding (KIX) domain of CBP, displayed impairments in contextual fear conditioning (Wood et al. (2006)). It is worth noting that interference of genes, which are targets of nuclear calcium signalling and/or CREB/CBP-dependent transcription, including BDNF, ARC and NPAS4, have additionally been shown to impair memory (Linnarsson et al. (1997), Guzowski et al. (2000), Ramamoorthi et al. (2011)). Overall, CREB and CBP are strongly suggested to be

important for memory formation.
In summary, these studies and the ones mentioned in the previous section, indicate that LTP and LTM share common mechanisms. Though a variety of discrepancies were found in studies investigating the requirement of receptors, kinases and transcriptional regulators, a general involvement in memory formation seems likely, though their contributions may differ by task and brain region. Dysregulation of calcium, and specifically nuclear calcium signalling, therefore has severe consequences on learning-induced gene transcription. It can affect the expression of activity-regulated genes and, moreover, it can also alter the structural features of a neuron.

4.1.4 Neuronal morphology impacts memory formation - nuclear calcium and VEGFD as key players

Previous work in our lab has identified that nuclear calcium signalling has a crucial impact on neuronal morphology (Mauceri et al. (2011) and unpublished data). Morphological, including dendritic length and complexity, as well as spine density and spine length are important for the formation of new memories. In multiple studies, learning induced changes in spine remodelling, density and morphology have been reported (Patel and Stewart (1988), Leuner et al. (2003), Yang et al. (2009)). The dendritic arbour, however, is less dynamic during adulthood; instead, its maintenance is a critical feature for memory formation (Mizrahi and Katz (2003), Koleske (2013)). The dendritic tree receives and propagates synaptic inputs and, further, influences the output signal that is produced. Therefore, its integration into the neuronal network is of great relevance for proper information processing. The importance of the neuronal architecture in learning and memory becomes evident in a variety of studies investigating brain morphology in neurological diseases. Among many others, Rett syndrome and Alzheimer's disease are both known to impair cognition and have been reported to show alterations in dendritic morphology (Armstrong et al. (1995), Chapleau et al. (2009), Kaufmann and Moser (2000), Kulkarni and Firestein (2012)).
The underlying mechanisms that cause the changes in dendritic morphology are not well understood. As mentioned above, *in vitro* studies have demonstrated that nuclear calcium may be one possible candidate that regulates the maintenance of the dendritic tree. Inhibition of nuclear calcium signalling has led to a decrease in spine density and a reduction in dendritic length and complexity (Mauceri et al. (2011) and unpublished data). Supplementation of VEGFD rescued changes in dendritic length and arborisation caused by nuclear calcium dysregulation. However, VEGFD was not able to reverse the density of spines. VEGFD is a mitogen that has been implicated in angiogenesis and lymphatic vasculature and is regulated by nuclear calcium. It has further been shown that VEGFD rescues nuclear calcium-

mediated morphological changes via the activation of p38 MAP kinase. Suppression of VEGFD underlined its importance in dendritic morphology, as CA1 pyramidal cells infected with a short hairpin RNA (shRNA) against VEGFD revealed a reduction in dendritic complexity and length. Additionally, these alterations resulted in spatial and LTM deficits. Hence, nuclear calcium signalling influences dendritic morphology at least partially via the regulation of VEGFD (Mauceri et al. (2011)). It is noteworthy that nuclear calcium signalling controls the transcription of multiple other genes that are reported to have a role in regulating spine morphology. Among those are, for example, ARC, BDNF, NPAS4, NR4a1 and C1q (Peebles et al. (2010), Horch et al. (1999), An et al. (2008), Orefice et al. (2013), Bloodgood et al. (2013), Chen et al. (2014), Simonetti et al. (2013)).
Thus, nuclear calcium signalling regulates features of a neuron's morphology which are important for the consolidation of memory.

4.1.5 Aim I

Nuclear calcium signals have been shown to be crucial for various long-term adaptations. These include not only neuroadaptations, such as acquired neuroprotection (Zhang et al. (2009)) and development of chronic pain (Simonetti et al. (2013)), but they further control memory formation in mice (Limbäck-Stokin et al. (2004), Oliveira et al. (2012)) and in *Drosophila melanogaster* (Weislogel et al. (2013)). In this study, I aimed at further investigating nuclear calcium signalling as a central mechanism in memory formation by targeting the calcium buffer parvalbumin to the nucleus. Next, I analysed if memory impairments in mice deficient in nuclear calcium signalling are caused by alterations in the neuronal architecture, as previous *in vitro* studies have suggested that nuclear calcium also regulates neuronal morphology (Mauceri et al. (2011), Li et al. (2014a)). Additionally, I investigated if nuclear calcium-regulated VEGFD, which links basal neuronal activity to neuronal morphology and is important for memory formation, can rescue morphological and cognitive changes that are induced by inhibition of nuclear calcium signalling.

Plasmids for parvalbumin.NLS were kindly provided by Drs B.Ehrlich and M.Nathanson. rAAV-VEGFD was kindly provided by Dr. Daniela Mauceri.

4.2 Epigenetic mechanisms in memory formation - a focus on DNA methylation

In the past sections, I have discussed that memory consolidation requires gene transcription and indicated that nuclear calcium is a key mediator. During the last years, epigenetic modifications have additionally been identified as crucial regulators of gene transcription in the context of memory formation. Epigenetic mechanisms change the expression of genes without altering the DNA sequence and, hence, are reversible. Beside its emerging role in memory formation, earlier studies elucidated a crucial role in processes such as cellular differentiation and development and imprinting. The most studied epigenetic processes with respect to memory formation are probably histone modifications, such as histone methylation and acetylation, as well as DNA methylation. With regards to the content of this thesis, I will exclusively focus on DNA methylation.

4.2.1 DNA methyltransferases

DNA methylation is catalysed by DNA methyltransferases (DNMTs). DNMTs add a methyl group to the 5' position of a cytosine in the CpG nucleotide taking S-adenosyl-L-methionine as a methyl group donor (Jeltsch (2002)). To date, the DNMTs DNMT1, DNMT3b and DNMT3a have been identified to be catalytically active (Margot et al. (2003)). DNMT1 is most abundant (Hermann et al. (2004)) and maintains the methylation pattern established early in development. DNMT1 preferentially methylates hemimethylated DNA and, hence, is also called maintenance DNMT. As DNMT3a and DNMT3b do not show such a preference, they are also referred to as *de novo* methyltransferases. Whereas DNMT3a is expressed during embryogenesis, as well as in postmitotic neurons during postnatal development (Feng et al. (2005)), DNMT3b is mainly expressed during a limited time window in early stages of embryogenesis (Watanabe et al. (2002)). It was long believed that DNMTs are restricted to dividing cells and diminish after terminal differentiation, however recently, it has become clear that DNMTs are also expressed in adult tissue, including the brain (Szyf et al. (1991), Yen et al. (1992), Goto et al. (1994)). Several transcripts of DNMT3b exist that result from alternative splicing (Robertson et al. (1999), Xie et al. (1999)). In contrast, only two isoforms of DNMT3a are currently known, namely DNMT3a1 and DNMT3a2. DNMT3a2 is transcribed from an intronic promoter and lacks the N-terminal 219 amino acids of the full length protein. Interestingly, DNMT3a1 localizes mainly to transcriptionally silent heterochromatin, which is associated with gene repression. However, DNMT3a2 localizes to euchromatin, which is thought to be transcriptionally active (Chen et al. (2002)). Traditionally, DNA methylation was believed

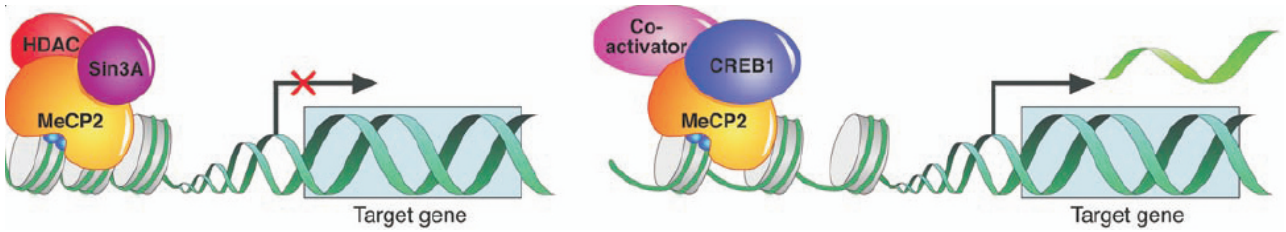

Figure 2: DNA methylation has been shown to inactivate gene transcription via recruitment of HDACs by the methyl-binding protein MeCP2. However, DNA methylation has also recently been shown to activate gene transcription. It was suggested that MeCP2 recruits CREB1, which then leads to a more open chromatin structure and, hence, to gene transcription (Figure adopted from Chahrour et al. (2008)).

to be involved in gene repression, either by directly interfering with transcription factor binding (Watt and Molloy (1988)) or via methyl-CpG binding domain (MBD) proteins, such as MeCP2 (Boyes and Bird (1991), Nan et al. (1998), Jones et al. (1998)). MeCP2 can recruit HDACs that cause deacetylation of histones, which leads to a more condensed chromatin formation, resulting in transcriptional silencing (Nan et al. (1998), Jones et al. (1998)). However, during the last years, this idea was challenged by the finding that MeCP2 can also recruit transcriptional activators, such as CREB1, leading to an open chromatin formation and, consequently, gene activation (Chahrour et al. (2008))(Figure 2). Additionally, it has been shown that DNA methylation in the gene bodies can increase gene transcription (Wu et al. (2010)).

4.2.2 DNA methylation in memory formation

Due to the finding that DNMTs are expressed in the adult brain and existing evidence shows that other epigenetic marks play a role in memory formation, Levenson et al. (2006) investigated if DNMTs are involved in synaptic plasticity. They found that inhibition of DNMTs impaired LTP induction and altered cytosine methylation in the promoters of Reelin and BDNF, genes known to be important for the induction of synaptic plasticity. Studies elucidating the role of DNMTs in memory formation reported that DNMT3a is upregulated after contextual and cued fear conditioning, whereas DNMT3b was only upregulated after contextual fear conditioning (Miller and Sweatt (2007), Monsey et al. (2011)). Pharmacological inhibition of DNMTs impaired memory formation (Miller and Sweatt (2007), Monsey et al. (2011), Sui et al. (2012)) and reduced methylation at the promoter of the memory suppressor gene protein phosphatase 1 (PP1) and, hence, increased its expression (Miller and Sweatt (2007)). The induction of PP1 likely contributes at least partially to the deficits in memory formation. However, the

expression of the memory activator reelin also increases, providing the possibility that reelin abolishes the effect of the increase in PP1 expression. Therefore, future studies need to address this question further, to better understand the effect of DNA methylation on gene expression. Genetic studies have supported the idea that DNA methylation regulates memory formation, though it is not yet clear, which DNMTs are required. One study demonstrated that mice with conditional deletions of DNMT1 and DNMT3a exhibit memory deficits, though deletion of DNMT1 or DNMT3a alone did not have that effect, pointing to a redundancy of these two DNMTs (Feng et al. (2010)). However, a different study showed that deletion of DNMT3a alone, but not DNMT1, is enough to cause impairments in memory consolidation (Morris et al. (2014)). Though, some of the findings seem to be controversial and reveal that we are just beginning to understand the role of DNA methylation in memory formation. Currently, the general finding that DNMTs are involved in learning and memory is widely accepted. This becomes apparent as DNA methylation has also been shown to be involved in remote memory (Miller et al. (2010)) and reward learning (Day et al. (2013)) and has further been implicated to be crucial also for learning in different organisms, such as for associative LTM in honey bees (Lockett et al. (2010)). Moreover, MBD proteins, MBD1 and MeCP2, have been associated with defects in cognition (Zhao et al. (2003), Moretti et al. (2006)). Additionally, not only DNA methylation but also DNA demethylation, via ten-eleven translocation methylcytosine dioxygenase 1 (Tet1) and growth arrest and DNA-damage-inducible 45 β (Gadd45β), has been reported to have an impact on memory formation (Kaas et al. (2013), Zhang et al. (2013), Leach et al. (2012), Sultan et al. (2012)).

4.2.3 Aim II

Studies investigating the role of DNMT3a in memory formation have previously not distinguished between the two isoforms DNMT3a1 and DNMT3a2. However, as DNMT3a1 and DNMT3a2 associate with hetereochromatin and euchromatin, respectively, we aimed to investigate whether the two isoforms play different roles in memory formation. We found that only DNMT3a2, not DNMT3a1 is upregulated by neuronal activity. Hence, we focused mainly on DNMT3a2 in this project and studied if the level of DNMT3a2 is critical for memory formation in young and aged mice. It has been previously demonstrated that cognitively impaired aged mice show changes in DNA methylation levels at the promoter and intragenic region of the ARC gene (Penner et al. (2011)). Moreover, it has been reported that 5-methylcytosine levels decrease with normal aging in mice (Wilson et al. (1987)) and that age-dependent methylation changes occur in the human cerebral cortex (Siegmund et al. (2007)). Therefore, we hypothesised that changes in the level of DNMT3a2 may be one of the underlying

causes of memory impairments observed in aging.

Experiments of this study have been conducted by Dr. Ana M. M. Oliveira and myself. In the results part of this thesis, I will indicate which of the experiments were done personally or by Dr. Ana M. M. Oliveira. Iris Bïnzli-Ehert prepared dissociated hippocampal cultures for this project.
The results of this part of my thesis have been published: Ana M. Oliveira, Thekla J. Hemstedt, and Hilmar Bading (2012) Rescue of aging-associated decline in Dnmt3a2 expression restores cognitive abilities. Nature Neuroscience 15(8): p. 1111-3.

4.3 Signalling pathways that are important for fear extinction

Fear extinction serves as a model to study the molecular mechanisms that underlie anxiety disorders, such as PTSD and phobias. During fear extinction, a repetitive non-reinforced presentation of a conditioned stimulus leads to a reduction of a previously acquired fear response. It is widely accepted that during fear extinction, the old memory is not erased but, rather, inhibited by a memory that has newly formed during extinction. Therefore, it is not surprising that memory consolidation and memory extinction share mechanisms that regulate both forms of learning (Lin et al. (2003), Szapiro et al. (2003), Orsini and Maren (2012)). As for fear conditioning, NMDA receptors are key players for the extinction of fear. Several studies have used pharmacological application to inhibit NMDA receptor activation and reported impairments in fear extinction (Falls et al. (1992), Baker and Azorlosa (1996), Szapiro et al. (2003)). In contrast, the NMDA receptor agonist D-cycloserine facilitated extinction (Walker et al. (2002)). Fear extinction was also enhanced in a study using a potentiator of AMPA receptors, whereas dysregulation of AMPA receptor trafficking has been indicated to cause deficits in extinction (Dalton et al. (2008) , Clem and Huganir (2010)). Most studies agree that NMDA receptors and AMPA receptors are essential in the regulation of fear extinction, however, literature is more controversial on the function of L-type VGCCs. A variety of pharmacological studies injecting VGCC blockers systemically (Cain et al. (2002), Suzuki et al. (2004)) or locally (Davis and Bauer (2012)) have reported fear extinction deficits. Genetic studies reported, however, that deletion of either of the two L- type VGCC subtypes, $Ca_v1.2$ or $Ca_v1.3$ (McKinney and Murphy (2006), McKinney et al. (2008)), did not interfere with memory extinction. Side effects of the L-VGCC blocker nifedipine have been reported (McKinney et al. (2008)) and may explain the discrepancy of the studies. However, compensatory effects between the two isoforms $Ca_v1.2$ and $Ca_v1.3$ can also not be ruled out. Little is known about the contribution of AC in memory extinction. To my best knowledge, AC 3 is the only one reported so far that is required for the extinction of contextual fear (Wang et al. (2011)). Among the kinases that are involved in memory formation, MAPK is the best studied with regards to fear extinction. Pharmacological (Lu et al. (2001), Szapiro et al. (2003),Matsuda et al. (2010), Davis and Bauer (2012)) and genetic studies (Ryu et al. (2008)) have shown either directly or indirectly, via upstream manipulations, a functional role of MAPK in fear extinction. Some studies suggested, though, that the underlying mechanisms may differ in memory consolidation and extinction, providing data that show different activation and localisation patterns of phosphorylated extracellular signal-regulated kinase (pERK) within hippocampal neurons in these two tasks (Fischer et al. (2007)). Additionally, segregated populations of principal CA1 neurons were predominantly c-FOS positive or pERK positive after memory consolidation

and fear extinction, respectively. Activation of MAPK does not seem to be required in the entorhinal cortex (Bevilaqua et al. (2006)) and in taste aversion extinction (Berman and Dudai (2001)). Fear memory extinction also seems to depend on CaMKII activation in the amygdala and hippocampus, as shown in both pharmacological and genetic approaches (Szapiro et al. (2003), Bevilaqua et al. (2006), Kimura et al. (2008), Myskiw et al. (2010)). As mentioned for memory consolidation, for processes involving gene transcription, the activation of these kinases is not sufficient. The nuclear-localised kinase CaMKIV needs to be activated, and the downstream targets CREB and CBP have to be phosphorylated. Though it is widely accepted that LTM formation requires gene transcription and protein synthesis, their requirement in fear extinction is not as clear. CREB-mediated gene transcription was reported to be required in the prefrontal cortex (PFC) and in the amygdala, though it was not required in the hippocampus (Mamiya et al. (2009)). On the other hand, studies using transcription inhibitors did not detect extinction deficits when injected in the amygdala (Lin et al. (2003)) but, rather, when injected into the hippocampus (Vianna et al. (2003)). Further, studies inhibiting protein synthesis in the insular cortex and amygdala or hippocampus have seen extinction deficits in the conditional taste aversion and one-trial inhibitory avoidance task, respectively (Berman and Dudai (2001), Vianna et al. (2001), Bahar et al. (2003)). However, when the protein synthesis inhibitor anisomycin was injected subcutaneously, no extinction deficits could be detected in spatial memory extinction and fear extinction (Lattal and Abel (2001)).

Some genes that have a known function in memory consolidation have also been shown to be regulated during extinction. Early growth response protein 1 (EGR1) was reported to be upregulated in the medial PFC and amygdala after extinction training (Herry and Mons (2004)). Further, a recent study has shown that downregulation of ARC in the amygdala causes impairments in fear extinction. The best studied gene in the context of fear extinction is probably BDNF. Its requirement for fear extinction has been shown in several brain areas such as the amygdala (Chhatwal et al. (2006)), the hippocampus (Heldt et al. (2007)) and the medial PFC (Peters et al. (2010)). Moreover, a single nucleotide polymorphism in the BDNF gene has been reported to impair extinction in humans (Soliman et al. (2010)). In contrast, the role of c-FOS in fear extinction is controversial. Some studies have seen an induction of c-FOS after extinction training (Herry and Mons (2004)), and others reported decreased expression in c-FOS after extinction training in comparison to mice that were fear conditioned but did not undergo extinction training (Tronson et al. (2009)). In conclusion, it becomes apparent that memory consolidation and fear extinction share a variety of molecular mechanisms, but differences between these two tasks nonetheless exist. However, many studies on fear extinction come to contradictory conclusions, which can be probably best explained by differences in the protocols applied. Among the critical factors are, presumably, whether within-session or between session protocols are used, the duration of the extinction training and the brain region

studied.

4.4 Aim III

Our aim was to study if nuclear calcium, which is known to regulate CREB/CBP-dependent gene transcription, is important for fear memory extinction. I interfered with hippocampal nuclear calcium signalling using the nuclear-targeted calcium buffer Parvalbumin, and subsequently tested mice in contextual fear extinction. Current studies show diverse findings regarding if gene transcription and/or protein synthesis are required in the hippocampus for memory extinction. Therefore, this study aims, additionally, at further understanding the molecular mechanisms involved in extinction of fear memory.

The Parvalbumin.NLS plasmid was kindly provided Drs B.Ehrlich and M.Nathanson. The fear extinction protocol was established together with Dr. Ana MM Oliveira.

5 Materials and Methods

5.1 Hippocampal cell preparation and maintenance

Dissociated hippocampal neurons were cultured from new born wild type C57BL/6 mice. Hippocampi were dissected in a mixture of Ky/Mg solution (Table 1) and dissociation medium (DM) (Table 2) at 1:9 volume ratio and ,afterwards, were transferred to Papain (Papain latex 10 Units/ml, 3.7 mM L-Cysteine in Ky/My/DM solution) for 20 min at 37°C to dissociate. The solution was homogenized by stirring it gently every 5 min. The Papain treatment was done twice and enzyme activity was stopped by supplementation of trypsin (1%, w/v trypsin inhibitor in Ky/ My/ DM solution). The inhibition solution was changed twice with complete inhibition solution and incubated each time for 5 min at 37°C. The cells were washed three times with growth medium (Table 3) and then were triturated by pipetting to achieve a single cell suspension. Afterwards, the cell suspension was diluted in Opti-MEM (supplemented with 20 mM glucose) for a concentration of 0.8 hippocampus/2 ml. Then, 2 ml were given to each poly-D-lysine/laminin (BD Biosciences)-coated-35-mm plastic dish. The medium was changed to neurobasal- A-medium (NBA, Invitrogen, 2ml/dish) 2.5 hours after plating. The dishes were stored in the incubator at 37°C supplied with 5% CO2. On day in vitro (DIV) 3, 2.8μM AraC (cytosine β-D-arabinofuranoside, Sigma-Aldrich, C1768) was added to inhibit the proliferation of non-neuronal cells. On DIV8, the medium was changed to transfection medium (Tables 4 and 5).

5.1.1 Treatment of hippocampal cultures

Neurons were infected on DIV4. Bicuculline was used at a concentration of 50 μM (Alexis Biochemicals). For NMDA receptor inhibition, 10μm MK-801 (Tocris Bioscience) was added to the cells 30 min prior to bicuculline stimulation. Table 6 shows the depolarization solution used for KCl stimulation.

5.1.2 Hippocampal culture solutions

Table 1: Ky/Mg solution

Component [1]	Final Concentration	Stock Concentration	For 80 ml
Kynurenic acid	10 mM	Poweder	158.56 mg
Phenol Red	0.5 %	100 %	0.4 ml
NaOH	12.5 mM	1 N	1 ml
HEPES	5 mM	1M	0.4 ml
$MgCl_2$	100 mM	2 M	4 ml
H_2O			up to 80 ml

[1] All chemicals are from Sigma-Aldrich Chemie GmbH, München, Germany.

Table 2: Dissociation medium

Component [1]	Final Concentration	Stock Concentration	For 80ml
Na_2SO_2	81.8 mM	1 M	20.45 ml
K_2SO_4	30 mM	0.25 M	30 ml
$MgCl_2$	5.85 mM	1.9 M	0.77 ml
$CaCl_2$	0.25 mM	1 M	0.063 ml
HEPES	1 mM	1 M	0.25 ml
Phenol Red	0.2 %	100 %	0.5 ml
Glucose	0.36 %	45 %	2 ml
H_2O			up to 250 ml

[1] All chemicals are from Sigma-Aldrich Chemie GmbH, München, Germany.

Table 3: Growth medium

Component [1]	Volume (ml)
Neurobasal A-medium	97 ml
B27	2 ml
Rat serum	1 ml
L-Glutamin (200mM)	0.25 ml
Penicillin/Streptomycin	0.5 ml

[1] sterile filtered through 0.22-μm Millipore filter and stored at +4°C.

Table 4: Salt Glucose Glycine solution (SGG)

Component [1]	Final Concentration	Stock Concentration	For 80 ml
NaCl	114 mM	5 M	11.4 ml
$NaHCO_3$	0.22% mM	7.5%	14.6 ml
KCl	5.29 mM	3 M	0.882 ml
$MgCl_2$	1 mM	1.9 mM	0.264
$CaCl_2$	2 mM	1 M	1 ml
HEPES	10 mM	1 M	5 ml
Glycine	1 mM	1 mM	0.5 ml
Glucose	0.54 %	45 %	6 ml
Sodium Pyruvate	0.5 mM	0.1 mM	2.5 ml
Phenol Red	0.2 %	100 %	1 ml
H_2O			up to 500 ml

[1] All chemicals are from Sigma-Aldrich Chemie GmbH, München, Germany.

Table 5: Transfection medium

Component [1]	Volume (ml)
SGG	88 ml
MEM (Without glutamine)	10 ml
Insulin-Transferrin-Selenium	1.5 ml
Penicillin/Streptomycin	0.5 ml

[1] sterile filtered through 0.22-μm Millipore filter and stored at +4°C.

Table 6: Depolarization solution

Component	Final Concentration	Stock Concentration	For 100 ml
KCl	170 mM	3 mM	5.67 ml
$CaCl_2$	2 mM	1 M	200 μl
$MgCl_2$	1 mM	1.9 mM	53 μl
HEPES	10 mM	1M	1 ml
Phenol Red	0.05 %	100 %	200 μl
TM			up to 100 ml

5.2 AAV production

5.2.1 HEK cell culturing

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 7). Cells were split by a dilution factor of 1:5 and passaged every 3 to 4 days. In order to split cells, culturing medium was removed and cells were washed twice with phosphate buffered saline (PBS). Afterwards, 0.05% Trypsin-EDTA (Gibco®) was added to the cells directly and cells were incubated for 5 min 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 min at room temperature (RT) and medium was replaced by fresh DMEM-complete for resuspension. The cells were then plated in a new flask.

5.2.2 HEK cell transfection

HEK293 cells were transfected using the calcium phosphate precipitation method. Cells were plated onto 15-cm dishes (TC Dish, NUNC) containing DMEM-complete. Medium was changed for each plate (60-70% confluent) 2-3 hours prior to transfection in Iscove's Modified Dulbecco Medium (IMDM; Life Technologies/Invitrogen, Table 8). Five dishes were used for producing one virus. The reagents listed in Table 9 were used for producing one virus. The mixture was filtered through a 0.2 μm syringe filter and 13 ml 2x HeBs (Table 10) was added slowly while vortexing for 15 sec. The transfection mixture was added drop-wise to the cells. The medium was replaced with fresh DMEM 16-22 h after transfection.

5.2.3 Virus harvesting

About 60 hours after transfection, HEK293-cells were washed once with pre-warmed PBS, harvested, pelleted (5 min at 800 x g) and re-suspended in 100 mM NaCl and 10 mM Tris-HCl (pH 8.0). The cells were then lysed with 0.5% sodium deoxycholate monohydrate (Sigma-Aldrich) and 50 U/ml Benzonase® Nuclease (Sigma- Aldrich) for 1 hour at 37°C and afterwards pelleted at 4°C for 15 min at 3000g. Cell pellets were then frozen at -20°C until virus purification.

5.2.4 Virus purification

Cell pellets were thawed and centrifuged at 3000g for 15 min at 4°C. The virus was purified with heparin affinity HiTrapTM Heparin HP Columns (GE Healthcare). Columns were pre-equilibrated with 10 ml 150 mM NaCl/20 mM Tris (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 mM 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).

5.2.5 Virus production solutions

Table 7: Dulbecco's Modified Eagle Medium-complete (DMEM-complete)

Component [1]	Volume (ml)
DMEM (including 4.5 g/l glucose)	500
Fetal calf serum [2]	50
Non-essential amino acids	5
Sodium pyruvate	5
Pennicilin/streptomycin	2.5

[1] All from GIBSCO®
[2] Heat inactivated (30 min at 56°C)

Table 8: 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 min at 56°C)

Table 9: Transfection mixture

Component	Amount
H_2O	15 ml
$CaCl_2$ (2.5 M)	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 10: 2x HeBS

Component	Final Concentration	For 500 ml
HEPES	50 mM	5.957 g
NaCl	280 mM	8.182 g
Na_2HPO_4	1.5 mM	0.1065 g
H_2O		to 500 ml

[1] pH adjusted to 7.05
[2] sterile filtered through 0.22-μm Millipore filter and stored at +4°C.

5.3 RNA extraction, cDNA synthesis and qPCR analysis

The RNA from hippocampal cultures was extracted under RNase free conditions using the RNeasy Total RNA isolation kit (Qiagen, Roche) according to manufacturer's instructions, including DNAse treatment with RNase-free DNase set (Qiagen).
For complementary DNA (cDNA) synthesis, a total of 1μg RNA per sample was reversed transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative real time PCR (qRT-PCR) was performed on an ABI7300 thermal cycler. The universal qRT-PCR master mix with the following TaqMan Gene Expression Assays (Applied Biosystems) was used: Dnmt1 (Mm00599763_m1), Dnmt3a1 (Mm00432870_m1), Dnmt3a2 (Mm00463987_m1), Dnmt3b (Mm00599800_m1), Arc (Mm00479619_g1), Bdnf (Mm00432069_m1), Nurr77 (Mm00439358_m1),Fos (Mm00487425_m1) and
Egr1 (Mm00656724_m1). Gene Ct-values were normalised to GusB, used as the endogenous control gene.

5.4 Immunohistochemistry

Mice were perfused with 10% formalin (Sigma-Aldrich, Munich, Germany). The brains were post-fixed in the same solution overnight. For cryoprotection, brains were transferred to a solution containing 30% sucrose in 0.1 M PBS containing 0.04% thimerosal (Sigma). Brains were cut at a thickness of 30μm. Slices were collected and stored in PBS containing 0.04% thimerosal until further process. Slices were then washed in PBS for 5 min and permeabilized with 0.1% Triton X-100 in PBS. For 5-methylcytosine staining, the slices were permeabilized with methanol for 6 min at -20°C and incubated with 1 M HCl for 2 hours at RT. All slices were then blocked in 8% normal goat serum (NGS) or, normal donkey serum (NDS) for doublecortin (DCX) staining, with 0.3% Triton X-100 in PBS for 50 min at RT. Slices were washed three times for five minutes with PBS and were then incubated with the primary antibody, diluted in PBS containing 2% NGS and 0.3% Triton X-100 overnight at 4°C. For DCX staining slices were incubated with the primary antibody in PBS containing 3% NDS. The next day, slices were washed with PBS three times for five minutes (for DCX staining PBS contained 0.1% Triton) and were incubated in the secondary antibody diluted in PBS for 2 hours in the dark at RT. Slices were washed again and incubated in Hoechst 33258 (1:5000, Serva) for 5 min. The slices were washed with PBS and mounted on glass slides (Marienfeld, Germany).
The quantification was done with ImageJ (US National Institutes of Health). The infected area was defined as the region of interest, and the 5-methylcytosine signal intensity was measured in this region. The mean fluorescence was measured and plotted as an arbitrary

Table 11: List of antibodies

Antibody	Species	Immunocyto-chemistry	Immunohisto-chemistry	Company
5-methylcytosine	mouse	1:100	1:100	Calbiochem, 16233D3
Doublecortin	goat	-	1:500	Santa Cruz Technology, sc-8066
HA	rabbit	1:250	1:100-1:250	Santa Cruz Technology, sc-805
HA	mouse	-	1:100	Covance, MMS-101R
Parvalbumin	mouse	-	1:3000	Sigma, P3088
Mouse-IgG (Dylight488 conjugated)	goat	1:500	1:500	Dianova
Rabbit-IgG (Dylight488 conjugated)	goat	1:500	1:500	Dianova
Rabbit-IgG (Cy3-conjugated)	goat	1:500	1:500	Dianova
Goat-IgG (Alexa555 conjugated)	donkey	-	1:1000	Dianova

value. Additionally, the background fluorescence was assessed for each picture and subtracted from the 5-methylcytosine signal intensity value.

5.5 Immunocytochemistry

On DIV10, medium was removed from the cells and the cells were fixed with 4% PFA for 15 min. For 5-methylcytosine staining, the cells were washed 3x with PBS and afterwards permeabilised with methanol for 6 min at -20°C. After washing the cells again twice with PBS, they were incubated with 1 M HCl for 2 hours at RT. All cover slips were washed 4x for 5 min in PBS. Cells were blocked with 10% NGS in PBS for 1 hour at RT. The cells were washed twice in PBS and incubated with the primary antibody in PBS containing 2% Bovine serum albumin (BSA) and 0.1% Triton ON at 4°C. The following day, the coverslips were washed four times with PBS and incubated with the secondary antibody in 2% BSA and 0.1% Trition in PBS. The incubation time was 1 hr at RT. The cells were washed 3x with PBS, incubated with Hoechst stain for 5 min at RT and washed again twice with PBS. The cover slips were rinsed in water and mounted on slides with Mowiol. For analysis, different areas of the coverslip were imaged and microscope settings were identical for all conditions. ImageJ (US National Institutes of Health) was used for quantification. For measuring the 5-methylcytosine signal intensity, the nucleus of an infected cell was defined as the region of interest. The mean fluorescence was measured and plotted as an arbitrary value.

5.6 Mice

We used 8-week-old-male C57Bl/6J mice from Charles River. For the aging studies, we used 8-week-old (young) and 18-month-old (aged) mice from Janvier. Mice were group housed in a controlled environment (12 hour light dark cycle, 22±1°C) with water and food *ad libitum*. All procedures were carried out in accordance with German guidelines for the care and use of laboratory animals and with the European Community Council Directive 86/609/EEC.

5.7 Stereotaxic surgery

rAAVs were delivered into the dorsal hippocampus of mice by stereotaxic injection. Mice were randomly grouped and anaesthesied with a sleep mix (8μl/g; mix containing 5.5 ml NaCl isotonic, 0.5 ml Dormitor, 1 ml Dormicum and 1 ml Fentanyl). A total volume of 1.5μl virus per hemisphere, at a speed of 200 nl/min through a 33G needle, was injected. For infection with only one virus, a 2:1 mixture of viral solution and 20% Mannitol was used. In experiments which required infection of two viruses a mixture of 1:1:1 for viral solutions and mannitol was injected. If a single infection was conducted as a reference in the same experiment as double infections, a mixture of 1:1:1 of viral solution, PBS and Mannitol was injected. The following coordinates relative to Bregma were used: anteriorposterior; -2 mm, mediolateral; ±1.5 mm, dorsoventral; -1.7, -1.9 and -2.1 mm from the skull surface. The injection needle remained an additional 60 s in place to allow the fluid to diffuse. After the surgeries, mice were given pain killers (4.8μl /g; mix containing 4.75ml NaCl isotonic and 250μl Temgesic) and a wake-up mix (8.4μl/g; mix containing 0.5 ml Antisedan, 5 ml Anexate and 3 ml Naloxon). Behavioural experiments and morphological analyses were performed 3 weeks after stereotaxic delivery of rAAVs, unless stated otherwise.

5.8 Kainic acid administration

Mice were injected intraperitoneally with kainic acid (20 mg per kg of body weight, Biotrend) or saline. Mice were monitored continuously, and the occurrence of epileptic seizures was noted. For analysis, only animals that exhibited immobility and rigid posture were included. The hippocampus was dissected at different time points after injection. Until tissues were processed further, they were stored in RNAlater (Ambion) at -80°C.

5.9 Behavioural protocols

5.9.1 Contextual fear conditioning

Mice were habituated to the conditioning chamber (23x23x35 cm, TSE) for 148 s before receiving a mild 2-s foot shock. Mice were removed from the box 30 s after shock termination. After 1 hour or 24 hours (STM and LTM, respectively), mice were placed back for 5 min into the chamber. The time in which the mice spent freezing, defined as the absence of movements except respiration, was recorded continuously and manually.

5.9.2 Trace fear conditioning

Mice were habituated to the conditioning chamber (c.f. Contextual fear conditioning) for 165 s before a 30-s tone (conditioned stimulus, 10 kHz, 75 dB, pulsed 5 Hz) was played, 15 s later mice received a 2-s foot shock (unconditioned stimulus, 0.7 mA). Mice were placed back in their home cage 30 s after the shock terminated. For testing, mice were placed in an altered context 1 hour or 24 hours later (STM and LTM, respectively). To alter the context, a grey plastic cover was placed on the grid floor, the box was divided with an A4 paperboard to make it triangular, cues were placed on the box, the light was dimmed from 200 lux to 20 lux and the box was sprayed with a lemon scent (lemon soap, Leonti, 50%). During the first 180 s, no tone was presented (pre-conditioned stimulus), while during the following 180 s, a tone was presented (conditioned stimulus). Freezing was measured as explained above (c.f. Contextual fear conditioning). In order to normalise for the generalised freezing, the freezing percentage during the pre-conditioned stimulus was subtracted from the freezing percentage during the conditioned stimulus.

5.9.3 Fear memory extinction

Mice were conditioned as described in Contextual fear conditioning using a 0.7 mA foot shock. For fear extinction training, mice were placed back into the conditioning chamber 2 weeks after fear conditioning. Mice were extinguished for 20 min (recorded by the software as 4 x 5 min sessions). For extinction testing, mice were placed again into the chamber 24 hours later and remained there for 5 min. During all sessions, freezing was measured as described above (c.f. Contextual fear conditioning).

5.9.4 Spatial object recognition

Mice were placed in an open arena (50 cm x 50 cm x 50 cm) with a visual cue located on the wall of the arena. Mice were habituated to the arena for 6 min, and 3 min later, mice were trained for 3 x 6 min with a 3-min 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), one of the objects was displaced to a new location and mice were allowed to explore the objects for 6 min. The time the mice spent exploring both objects was measured manually in all sessions. As a behavioural read-out, the percentage the mice spent exploring the displaced object was measured as followed: $\text{Time}_{\text{displaced}}$ /($\text{Time}_{\text{nondisplaced}}$ + $\text{Time}_{\text{displaced}}$) * 100

5.10 Golgi stain

Golgi impregnation was done using the Rapid Golgi Stain Kit (FD Neuro Technologies) according to the manufacturer's protocol. Briefly, brains were left in solution A+B for 2 weeks and were then placed into solution C for 3-4 days. Brains were quickly frozen on dry ice and then cut with a cryostat (Leica CM1950) in 100-μm sections. Sections were mounted on Gelatin Couted Slides (LabScientific). Within three days, slices were stained according to the manufacturer's protocol.

5.11 Morphometric analysis

Z-stacks of Golgi-stained CA1 neurons were taken at the Nikon Imaging Center of the University of Heidelberg. Pictures were imported to Fiji (Schindelin et al. (2012)), calibrated and manually traced using the simple neurite tracer plugin (Longair et al. (2011)). As a read-out, total dendritic length was computed. A plugin available in Fiji was used for Sholl analysis (SHOLL (1953)). The shell interval was set to 5μm. Between three to five animals per condition were analysed.

5.12 Statistical analysis

For comparison of two distinct groups two-tailed unpaired Student's t-tests were applied. For comparison within the same group paired Student's t-test was used. One-way ANOVA, followed by Tukey's *post hoc* test (Section 6.1) or by Bonferroni *post hoc* test (Section 6.2), was used

for comparisons of more than two groups. For extinction training and acquisition in the Morris water maze, repeated-measure ANOVA was applied.

6 Results

6.1 Nuclear calcium-regulated dendritic architecture controls neuronal permissiveness for long-term memory formation

6.1.1 Nuclear calcium is required for long-term memory formation

In order to study the role of nuclear calcium in memory formation, I used the nuclear targeted calcium buffer Parvalbumin (Pusl et al. (2002), Rodrigues et al. (2007), Guatimosim et al. (2008), Schlumm et al. (2013),Li et al. (2014a)). A recombinant adeno-associated virus (rAAV) was produced containing an expression cassette of Parvalbumin-NLS fused to mCherry (rAAV-PV.NLS-mC). mCherry targeted to the nucleus (rAAV-mC.NLS) was used as the control. The rAAVs were delivered via stereotaxic surgery to the dorsal hippocampus of mice. Figure 3A shows the expression pattern of mCherry three weeks after stereotaxic delivery. To validate expression of Parvalbumin, slices were stained for Parvalbumin. Further, confocal images were taken to ensure that expression of Parvalbumin was restricted to the nucleus, thus

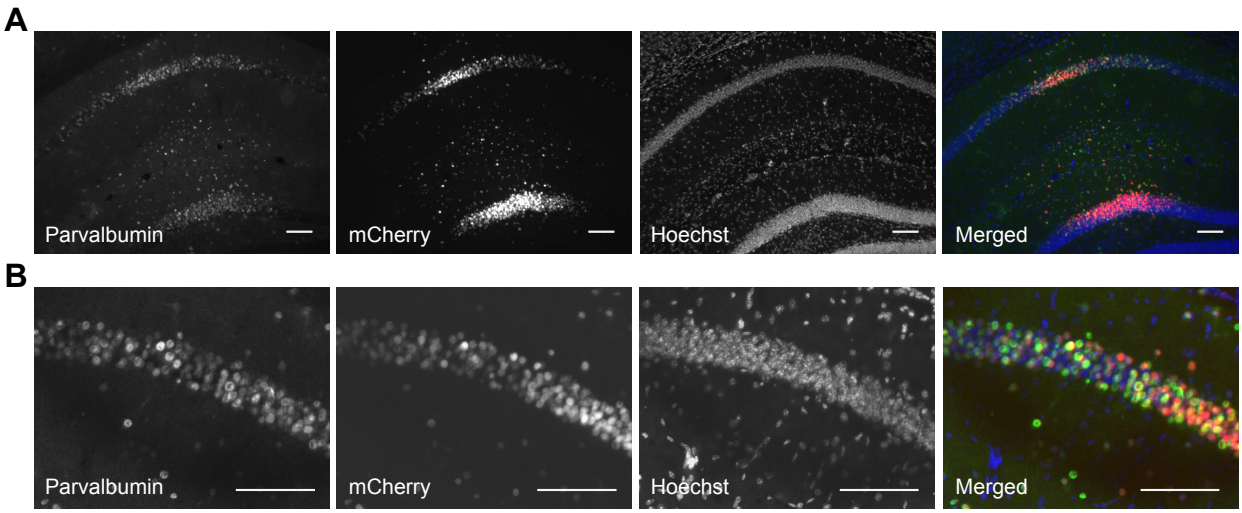

Figure 3: Validating rAAV-PV.NLS-mC expression *in vivo*.
(A) Representative pictures of rAAV-PV.NLS-mC expression in the dorsal mouse hippocampus three weeks after sterotaxic delivery. Slices were stained against Parvalbumin to ensure that Parvalbumin and mCherry are expressed in the same cells.
(B) Magnified image of (A) assuring that rAAV-PV.NLS-mC is exclusively expressed in the nucleus. Scale bar=100 μm.

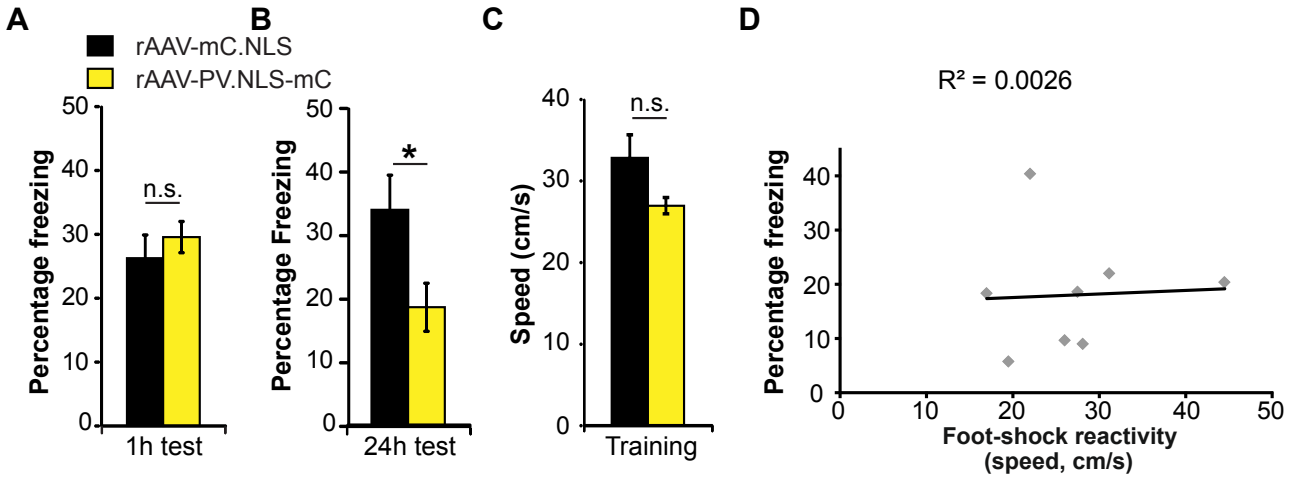

Figure 4: Nuclear calcium is required for fear memory.
(A) Inhibition of nuclear calcium does not impair short-term memory (n=12 per group).
(B) Long-term contextual fear memory is impaired in mice expressing rAAV-PV.NLS-mC, indicated by decreased freezing rates in comparison to control injected mice (rAAV-mC.NLS n=10, rAAV-PV.NLS-mC n=9, t_{17}=2.317, *p<0.05 by two-tailed student's t-test).
(C) Mice expressing rAAV-PV.NLS-mC react slightly less to the foot-shock.
(D) However, there is no correlation between foot-shock reactivity and memory performance.
Not significant, n.s. Data are shown as mean ± S.E.M.

excluding that obtained results are due to interference with cytosolic calcium (Figure 3B). Memory consolidation was tested in two hippocampus-dependent tasks, three weeks after rAAV injections. In contextual fear conditioning, mice expressing rAAV-PV.NLS-mC displayed reduced freezing compared to control animals when tested 24 hours after training, indicating LTM deficits in this task (Figure 4B). There was no difference in the freezing rate between both groups when tested 1 hour after training (Figure 4A). This suggests that STM was not altered by nuclear calcium inhibition. Though rAAV-PV.NLS-mC mice reacted slightly less to the foot-shock than mice expressing rAAV-mC.NLS, this difference was statistically not significant (Figure 4C) and additionally there was no correlation between foot-shock reactivity and memory performance (Figure 4D). The finding that nuclear calcium inhibition affects LTM was confirmed in the spatial object recognition task. Mice expressing rAAV-mC.NLS showed a preference for the displaced object 24 hours after the training session, whereas mice injected with rAAV-PV.NLS-mC did not spend more time exploring the displaced object (Figure 5B). However, rAAV-PV.NLS-mC-expressing mice showed a preference for the displaced object when tested 1 hour after training, indicating that STM was intact and memory acquisition was not

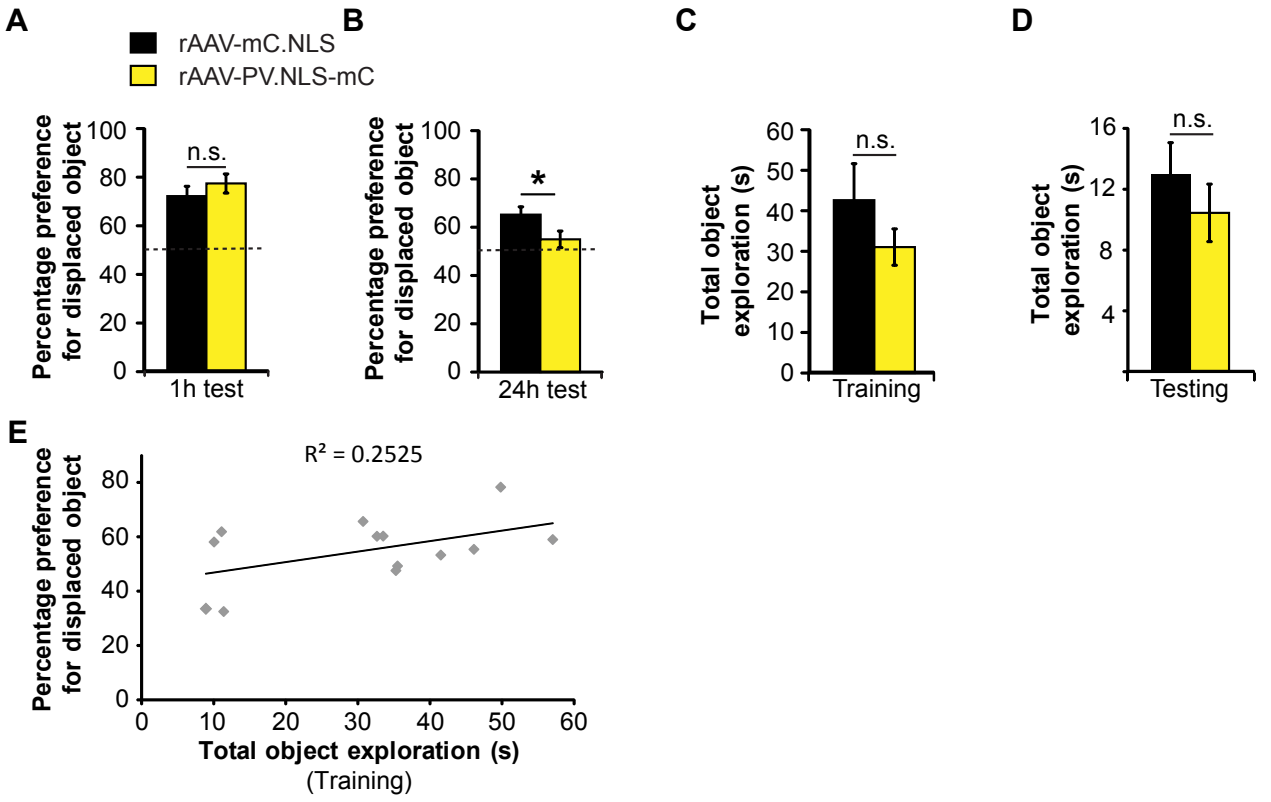

Figure 5: Inhibition of nuclear calcium causes deficits in spatial object recognition memory.

(A) Mice expressing rAAV-PV.NLS-mC or rAAV-mC.NLS display a preference for the displaced object, when tested 1 hour after training (rAAV-mC.NLS n=7, rAAV-PV.NLS-mC n=8).

(B) Mice expressing rAAV-PV.NLS-mC show significantly less preference for the displaced object compared to control injected mice, when tested 24 hours after training (n=13/group, t_{24}=2.168, *p<0.05 by two-tailed student's t-test)

(C) In rAAV-PV.NLS expressing mice, total exploration time is slightly decreased in the training and

(D) in the testing session. However, this decrease was statistically not significant.

(E) Additionally, there was no correlation between total exploration time and memory performance.

The dashed line at 50% indicates chance level to explore either of the two objects. Not significant, n.s. Data is shown as mean ± S.E.M.

impaired (Figure 5A). Further, both objects were explored equivalently in the training session and total exploration time for both objects did not differ between rAAV-mC.NLS- and rAAV-PV.NLS-mC-expressing mice (Figure 5C-E). Here, we show that hippocampal nuclear calcium is required for the formation of long-term, but not short-term, memory.

6.1.2 VEGFD overexpression reverses morphological alterations caused by interference with nuclear calcium signalling

I aimed at identifying mechanisms that can explain how nuclear calcium controls memory consolidation. As it is known that the neuronal morphology is important for memory formation, I studied if memory deficits in mice expressing rAAV-PV.NLS were due to an altered dendritic morphology. Previously, it has been shown in our lab that nuclear calcium regulates dendritic architecture *in vitro*. Transfecting hippocampal cultures either with CaMBP4, a nuclear protein that inactivates the nuclear calcium/calmodulin complex by binding to it, or with PV.NLS-mC reduced dendritic length and complexity (Mauceri et al. (2011) and unpublished date). In order to investigate if nuclear calcium regulates the dendritic morphology also *in vivo*, I analysed Golgi-stained pyramidal neurons expressing either rAAV-PV.NLS-mC or rAAV-mC.NLS in the CA1 region of the hippocampus. The basal dendrites were significantly shortened in length, and the complexity of the dendritic tree was decreased in mice expressing rAAV-PV.NLS-mC (Figure 6A-C). Earlier studies in our lab have demonstrated that nuclear calcium-regulated VEGFD controls neuronal morphology *in vitro* and *in vivo*. Further, it was shown *in vitro* that a loss in dendritic length and complexity caused by dysregulation of nuclear calcium could be rescued when recombinant VEGFD was supplemented (Mauceri et al. (2011)). Therefore, I next aimed at investigating *in vivo*, if alterations in dendritic architecture that result from nuclear calcium inhibition can also be reversed by VEGFD overexpression. Thus, I co-expressed rAAV-PV.NLS.mC with a rAAV containing an expression cassette for hemagglutinin (HA)-tagged VEGFD (rAAV-VEGFD) in the mouse hippocampus (Figure 7). The analysis revealed that neurons expressing rAAV-PV.NLS-mC and rAAV-VEGFD had significantly longer dendrites than neurons expressing only rAAV-PV.NLS-mC, indicating a rescue in dendritic length (Figure 6B). This rescue was only partial, as rAAV-PV.NLS-mC- and rAAV-VEGFD-expressing neurons did not reach the same dendritic length as control infected neurons, i.e., rAAV-mC.NLS or rAAV-mC.NLS and rAAV-VEGFD. However, this difference was statistically not significant. Further, neurons expressing rAAV-PV.NLS also demonstrated reduced dendritic complexity in comparison to cells infected with either rAAV-mC.NLS or rAAV-mC.NLS and rAAV-VEGFD (Figure 6C). There is a strong trend for a rescue in dendritic complexity in neurons expressing rAAV-PV.NLS-mC and rAAV-VEGFD, compared to neurons expressing rAAV-PV.NLS-mC alone, but this trend was statistically not significant. In conclusion, I have shown that nuclear calcium is important for maintaining the dendritic architecture. Nuclear calcium dysregulation causes alterations in dendritic geometry, however, these changes can be partially rescued by increasing VEGFD expression.

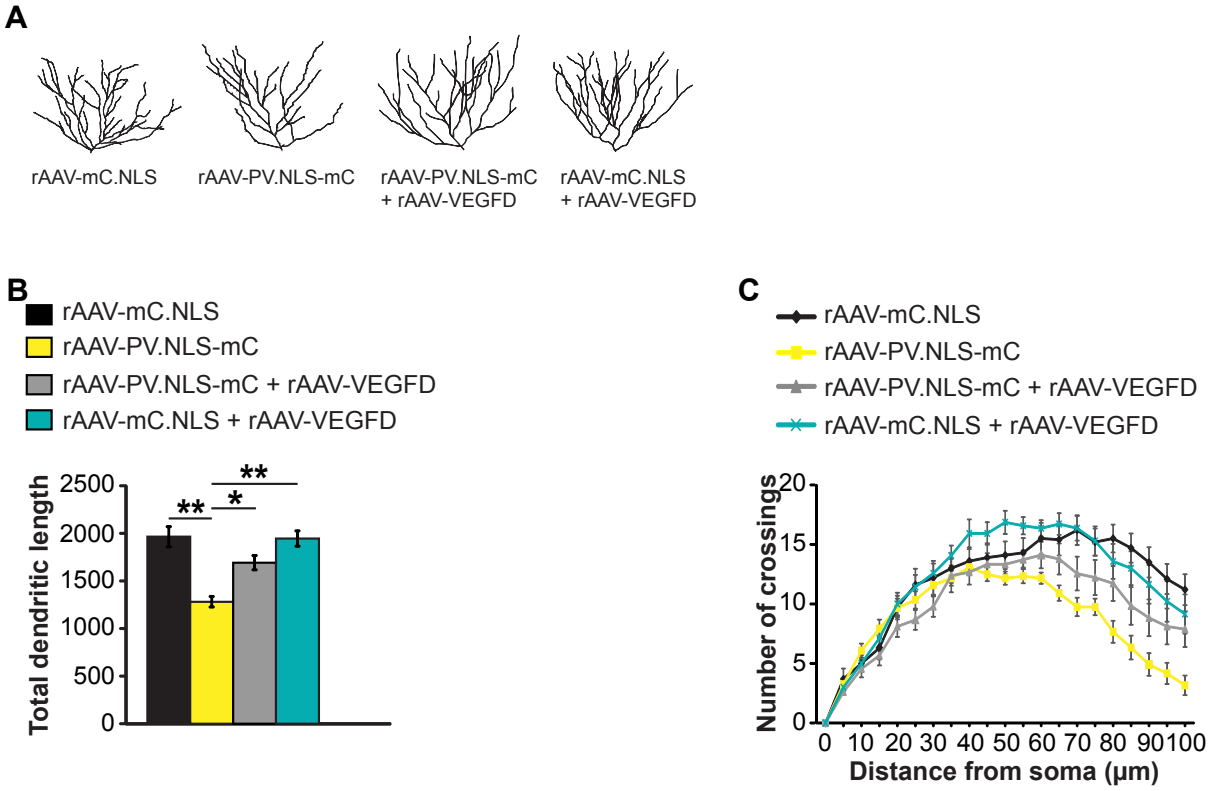

Figure 6: Inhibition of nuclear calcium causes morphological alterations which can be reversed by VEGFD overexpression.

(A) Representative tracings of basal dendrites in CA1 pyramidal cells.

(B) Pyramidal CA1 neurons expressing rAAV-PV.NLS-mC show a reduction in dendritic length in comparison to control infected neurons, i.e., neurons that are either infected with rAAV-mC.NLS or with rAAV-mC.NLS and rAAV-VEGFD. These changes can be partially rescued when rAAV-PV.NLS-mC expressing neurons were co-infected with rAAV-VEGFD (rAAV-mC.NLS n=10, rAAV-PV.NLS-mC n=12, rAAV-PV.NLS-mC & rAAV-VEGFD n=18, rAAV-mC.NLS & rAAV-VEGFD n=14, $F_{[3,53]}$ =13.848, *p<0.05, **p<0.001 by one-way ANOVA followed by Tukey's *post hoc* test)

(C) rAAV-PV.NLS-mC-infected neurons show a significant decrease in dendritic complexity compared to rAAV-mC.NLS and neurons co-infected with rAAV-mC.NLS & rAAV-VEGFD (For statistics see Table 12). There is a trend for a rescue in dendritic complexity in rAAV-PV.NLS-mC- & rAAV-VEGFD-infected neurons, however statistically, this difference is not significant.

Data are shown as mean ± S.E.M.

Table 12: Statistical analysis of dendritic complexity (p-values)

distance	mC PBS			PV PBS			PV VEGFD			mC VEGFD		
from soma (μm)	PV PBS	PV VEGFD	mC VEGFD	mC PBS	PV VEGFD	mC VEGFD	mC PBS	PV PBS	mC VEGFD	mC PBS	PV PBS	PV VEGFD
0	1	1	1	1	1	1	1	1	1	1	1	1
5	0,913	0,324	0,719	0,913	0,71	0,979	0,324	0,71	0,904	0,719	0,979	0,904
10	0,704	0,963	1	0,704	0,311	0,646	0,963	0,311	0,951	1	0,646	0,951
15	0,604	0,952	0,908	0,604	0,207	0,916	0,952	0,207	0,529	0,908	0,916	0,529
20	1	0,633	0,996	1	0,606	0,994	0,633	0,606	0,404	0,996	0,994	0,145
30	0,977	0,317	0,994	0,977	0,525	0,893	0,317	0,525	0,134	0,994	0,893	0,134
35	0,969	0,984	0,931	0,969	0,999	0,674	0,984	0,999	0,691	0,931	0,674	0,691
40	0,988	0,921	0,442	0,988	0,991	0,227	0,921	0,991	0,081	0,442	0,227	0,081
45	0,783	0,976	0,499	0,783	0,917	0,071	0,976	0,917	0,168	0,499	0,071	0,168
50	0,526	0,935	0,196	0,526	0,78	0,004*	0,935	0,78	0,022*	0,196	0,004*	0,022*
55	0,444	0,963	0,288	0,444	0,618	0,005*	0,963	0,618	0,311	0,939	0,024*	0,311
65	0,073	0,762	0,874	0,073	0,272	0,005*	0,762	0,272	0,216	0,874	0,005*	0,216
70	0,009*	0,19	1	0,009*	0,358	0,003*	0,19	0,358	0,101	1	0,003*	0,101
75	0,047*	0,392	1	0,047*	0,506	0,023*	0,392	0,506	0,279	1	0,023*	0,279
80	0,004*	0,247	0,796	0,004*	0,153	0,024*	0,247	0,153	0,737	0,796	0,024*	0,737
85	0,003*	0,105	0,866	0,003*	0,299	0,012*	0,105	0,299	0,347	0,866	0,012*	0,347
90	0,003*	0,142	0,841	0,003*	0,225	0,014*	0,142	0,225	0,472	0,841	0,014*	0,472
95	0,007*	0,255	0,834	0,007*	0,219	0,032*	0,255	0,219	0,697	0,834	0,032*	0,697
100	0,007*	0,39	0,787	0,007*	0,139	0,049*	0,39	0,139	0,911	0,787	0,049*	0,911

* indicates p-value <0.05 by one-way Anova followed by Tukey's post hoc test

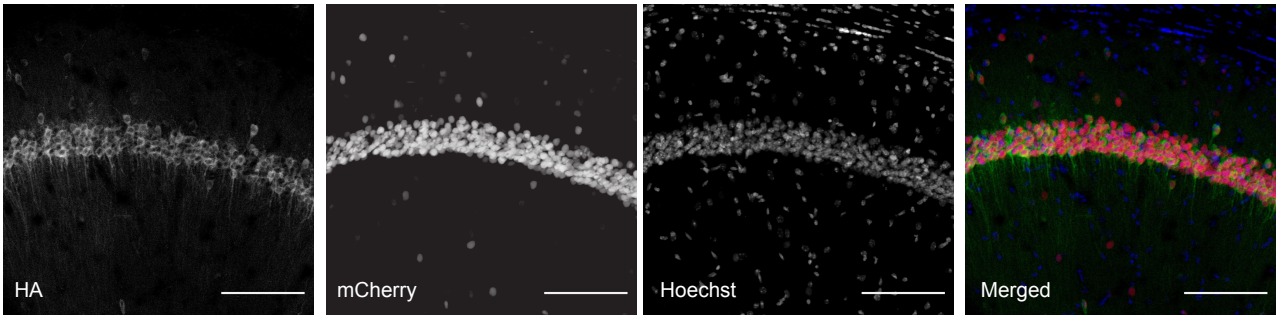

Figure 7: Co-expression of rAAV-PV.NLS-mC and rAAV-VEGFD. Representative pictures of rAAV-PV.NLS-mC and rAAV-VEGFD co-expressing mice three weeks after stereotaxic delivery.

6.1.3 Nuclear calcium dysregulation leads to memory impairments that can be reversed by VEGFD overexpression

Previous studies have shown that VEGFD is required for memory consolidation (Mauceri et al. (2011)). Therefore, I next determined if overexpression of VEGFD can rescue behavioural deficits that are caused by inhibition of nuclear calcium signalling. In order to investigate if behavioural alterations can be rescued, I tested mice in the spatial object recognition task. Three weeks prior to experiments, a mixture of rAAV-PV.NLS-mC and rAAV-VEGFD was injected into the dorsal hippocampus of mice. As a control, I used rAAV-mC.NLS or a mixture of rAAV-mC.NLS and rAAV-VEGFD. As shown in section 6.1.1., mice expressing rAAV-PV.NLS-mC did not display a preference towards the displaced object. However, mice expressing rAAV-PV.NLS-mC and rAAV-VEGFD had a preference for the displaced object that was comparable to control injected animals (Figure 8A). It is important to note that expression of rAAV-VEGFD alone had no effect on memory consolidation. Further, in the training session, all groups explored both objects to the same amount (Figure 8B) and total exploration time did not differ between groups (Figure 8C-D). Thus, I have shown that VEGFD overexpression can rescue memory deficits induced by nuclear calcium dysregulation, presumably via reversing morphological alterations.

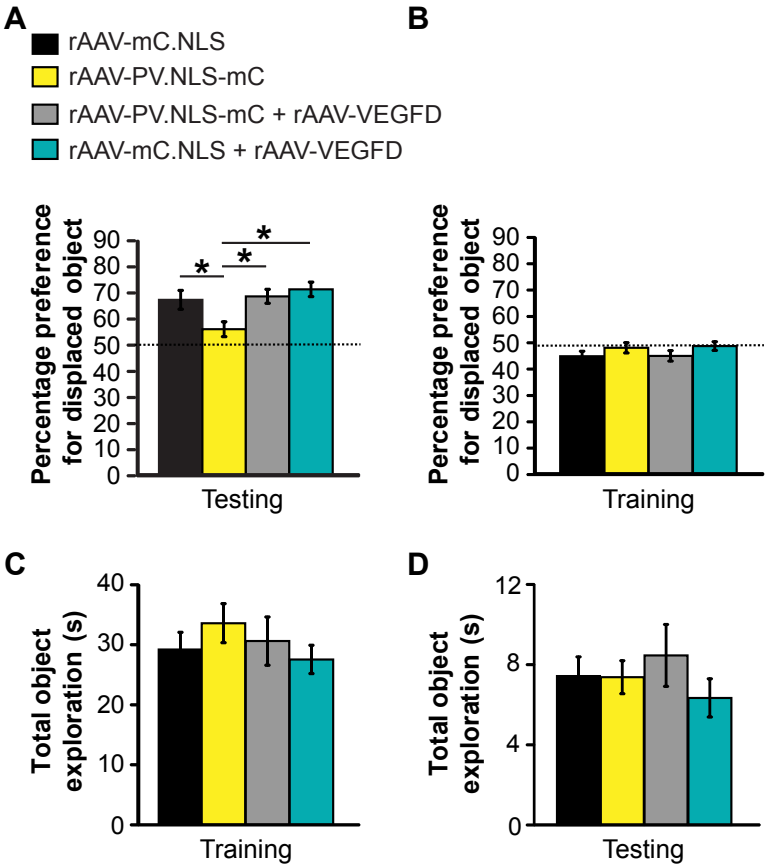

Figure 8: Nuclear calcium-regulated VEGFD can rescue behavioural alterations.
(A) Memory deficits observed in rAAV-PV.NLS-mC injected mice are rescued by VEGFD overexpression (rAAV-mC.NLS n=16, rAAV-PV.NLS-mC n=16, rAAV-PV.NLS-mC & rAAV-VEGFD n=16, rAAV-mC.NLS & rAAV-VEGFD n=15, $F_{[3,59]}$ =5.028, *p<0.05 by one-way ANOVA followed by Tukey's *post hoc* test).
(B) All four groups explored the two objects equivalent in the training session.
(C) There were no differences in total exploration time in the training and
(D) in the testing session.
The dashed line at 50% indicates chance level to explore either of the two objects. Data are shown as mean ± S.E.M.

6.2 Rescue of aging-associated decline in DNMT3a2 expression restores cognitive abilities

This part of my thesis includes data that were collected by Dr. Ana M.M.Oliveira. In the following, I will indicate in the text after every figure reference if the experiments were done personally (TH) or by Dr. Ana M.M. Oliveira (AO). Additionally, data that I already obtained during my Master thesis are indicated by 'MT'.

6.2.1 DNMT3a2 is induced by neuronal activity

It has been previously reported that DNMT3a and DNMT3b are induced after learning (Miller and Sweatt (2007)). To investigate the regulation of DNMTs by neuronal activity in more detail, we induced action potential bursting using the γ-aminobutyric acid ($GABA_A$) receptor antagonist bicuculline for 2 hours. As shown in Figure 9A (AO, TH during MT), DNMT3a2 is induced upon stimulation, whereas DNMT3b is downregulated. In contrast, DNMT1 and DNMT3a1 are not regulated by activity. The changes in induction are reversed when the NMDA-receptor inhibitor MK-801 was provided to the cells, indicating that DNMT3a2 is regulated in an NMDA receptor-dependent manner (Figure 9A). As DNMT3a2 is the only DNMT that is induced by activity, we focused in the following studies mainly on DNMT3a2. We investigated if DNMT3a2 is a target of nuclear calcium signalling. The neurons were infected with a rAAV containing an expression cassette of CaMBP4 fused to mCherry (rAAV-CaMBP4-mC). CaMBP4 is a nuclear protein which binds to and, hence, inactivates the nuclear calcium/calmodulin complex. As a control, the cells were either left untreated or were infected with a rAAV containing an expression cassette of mCherry targeted to the nucleus (rAAV-mC.NLS). Inhibition of nuclear calcium signalling partially reduces the induction of DNMT3a2, whereas the basal level of DNMT3a2 is unaltered (Figure 9B; TH during MT). Furthermore, we studied the kinetics of DNMT3a2 after stimulation. We found that DNMT3a2 expression peaks at 4 hours after stimulation and is similarly induced as c-FOS, which is a known classical immediate early gene (Figure 9C; TH). To additionally show that DNMT3a2 is regulated robustly by neuronal activity, we used different stimulation methods *in vitro* and *in vivo*. *In vitro*, supplementation of KCl, which leads to an influx of calcium in the cells and hence causes depolarization, induces DNMT3a2 expression at different time points, leaving the other DNMTs unchanged (Figure 10A; TH). *In vivo*, neuronal activity in the hippocampus was induced either by intraperitoneal injections of kainic acid or by training the mice in the spatial object recognition task. Hippocampal tissue was collected either 1 hour, 2 hours or 4 hours after injection of kainic acid. As a control, we used mice that were injected with PBS. To study

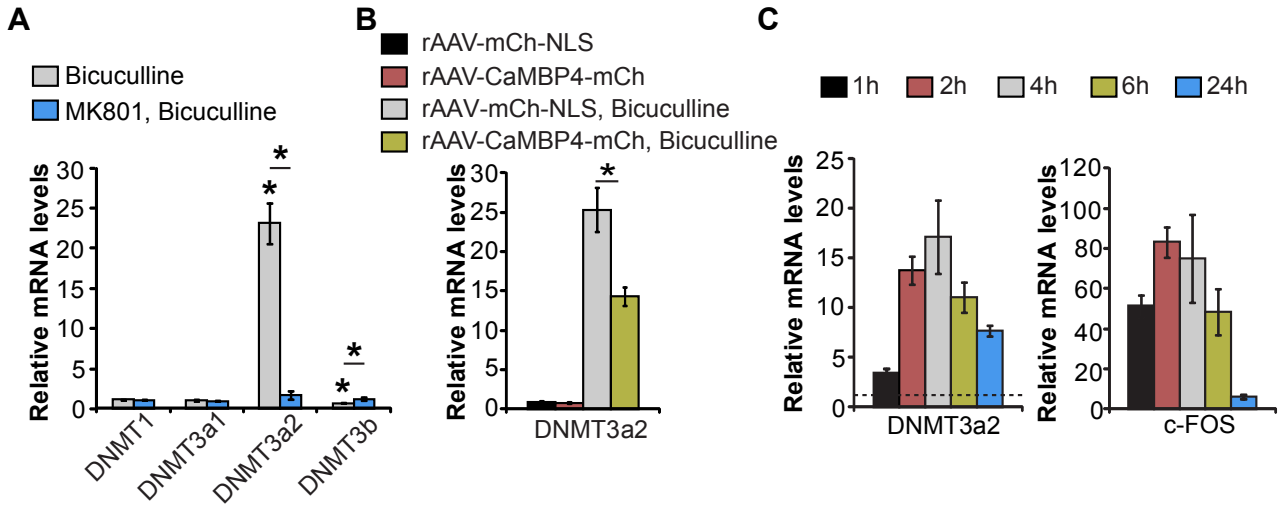

Figure 9: Nuclear calcium regulates DNMT3a2 via NMDA receptors *in vitro*.
(A) qRT-PCR analysis reveals that DNMT3a2 is upregulated and DNMT3b is downregulated upon neuronal stimulation with bicuculline (n=4; DNMT3a2, t_6=9.67; DNMT3b, t_6=11.97, *p<0.05 by two-tailed student's t-test) in an NMDA receptor-dependent manner (n= 4; DNMT3a2, t_6=9.23; DNMT3b, t_6=4.98, *p<0.05 by two-tailed student's t-test).
(B) qRT-PCR analysis shows that DNMT3a2 expression is regulated by nuclear calcium in stimulated conditions. Inhibition of nuclear calcium, using rAAV-CaMBP4-mCh, shows a significant decrease of DNMT3a2 expression in hippocampal cultures treated with bicuculline for 2 hours (n=5; $F_{(4,20)}$=66.35, *p<0.05 by one-way ANOVA followed by Bonferroni *post hoc* test).
(C) qRT-PCR data reveals relative mRNA levels of DNMT3a2 and c-FOS in a time course analysis (n≥3; dashed line represents unstimulated control condition).
Data are shown as mean ± S.E.M.

whether DNMT3a2 is induced after training in the spatial object recognition task, we collected the tissue 4 hours after training and compared trained mice to mice that were only handled and otherwise left in the home cage. In both *in vivo* methods, DNMT3a2, but neither of the other DNMTs, was induced at the different time points that we explored (Figure 10B; AO & TH; Figure10C; AO).

Together, these results indicate that DNMT3a2 is robustly regulated by neuronal activity. The activity-induced expression of Dnmt3a2 is mediated by NMDA receptors and nuclear calcium signalling.

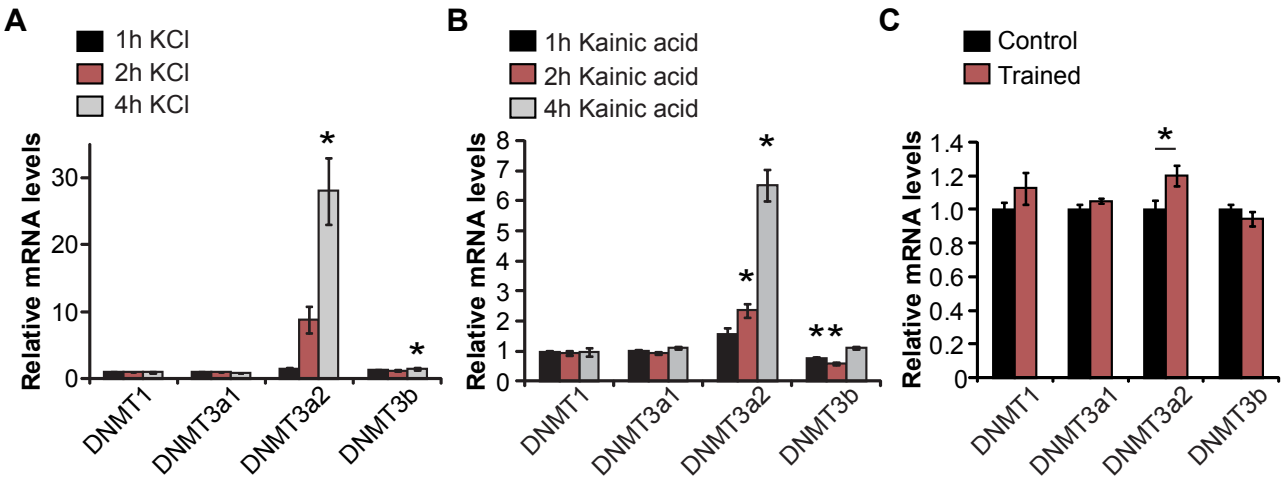

Figure 10: DNMT3a2 is robustly activated by neuronal activity.
(A) qRT-PCR analysis shows that DNMT3a2 is strongly induced 4 hours upon KCl stimulation in primary hippocampal cultures. DNMT3b is induced mildly after 4 hours of KCl stimulation. DNMT1 and DNMT3a1 are not regulated by stimulation of KCl (n=7; DNMT3a2, $F_{(3,24)}$=22.66; DNMT3b, $F_{(3,24)}$=5.78, *p<0.05 by one-way ANOVA followed by Bonferroni *post hoc* test).
(B) qRT-PCR analysis reveals upregulation of DNMT3a2 relative mRNA levels in the hippocampus 2 hours and 4 hours after kainic acid injections. DNMT3b is mildly downregulated 1 hour and 2 hours after kainic acid administration. Expression of DNMT1 and DNMT3a1 is unaltered (n=3 in 1 hour and 4 hour time point, n=4 in saline and 2 hour time point; DNMT3a2, $F_{(3,10)}$=77.63; DNMT3b, $F_{(3,10)}$=62, *p<0.05 by one way ANOVA followed by Bonferroni *post hoc* test).
(C) Hippocampal DNMT3a2 expression is induced 4 hours after training in the spatial object recognition task, as revealed by qRT-PCR analysis. Expression levels of DNMT1, DNMT3a1 and DNMTb were not changed (n=8/group; DNMT3a2, t_{14}=2.47, *p<0.05 by two-tailed student's t-test).
Data are shown as mean ± S.E.M.

6.2.2 A decrease in DNMT3a2 expression leads to memory deficits

As DNMT3a2 expression increases after learning, we further investigated if DNMT3a2 is important for memory formation. Therefore, we produced a rAAV containing a DNMT3a2-specific shRNA and an expression cassette of green fluorescent protein (GFP) (rAAV-sh3a2-GFP). For the control, we used a rAAV containing a universal negative control sequence and an expression cassette of GFP (rAAV-UNC) (Figure 11A). We infected hippocampal cultures with either rAAV-sh3a2-GFP or rAAV-UNC. In basal as well as in induced conditions, neurons expressing rAAV-sh3a2-GFP have a reduced level of DNMT3a2 expression compared to uninfected controls and rAAV-UNC-expressing cells. However, the expression level of DNMT3a1 was not affected (Figure 12A; AO). Next, we injected rAAV-sh3a2-GFP and rAAV-UNC into the dorsal hippocampus of mice (Figure 11B; AO & TH). Mice expressing rAAV-sh3a2-GFP show decreased levels of DNMT3a2, but not DNMT3a1, compared to control injected mice (Figure 12B; AO & TH). As our rAAV-sh3a2-GFP specifically downregulates DNMT3a2 *in vitro* and *in vivo*, we next investigated if mice with reduced levels of DNMT3a2

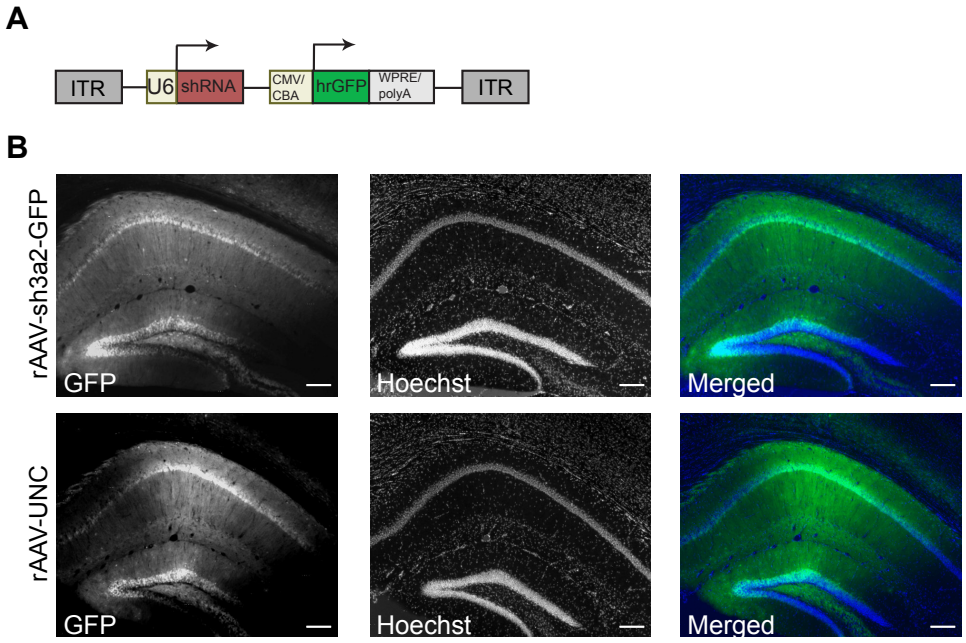

Figure 11: Assessing viral infection *in vivo*.
(A) Schematic representation of the design of rAAV-sh3a2-GFP. The shRNA for DNMT3a2 or control UNC is driven by the U6 promoter. To assess viral infection, GFP was additionally driven by a chicken-β-actin promoter.
(B) Representative pictures of rAAV-sh3a2-GFP and rAAV-UNC expression in the dorsal hippocampus three weeks after sterotaxic delivery. Scale bar=100μm.

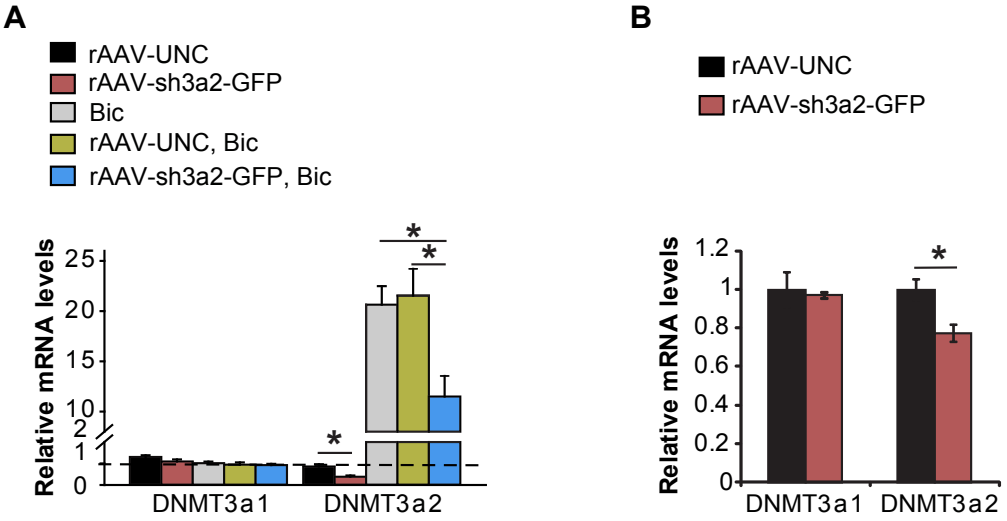

Figure 12: Downregulation of DNMT3a2 levels via rAAV-sh3a2-GFP infection.
(A) *In vitro* validation of shRNA-mediated downregulation of DNMT3a2. DNMT3a2 expression is downregulated upon infection of hippocampal neuronal cultures with rAAV-sh3a2-GFP in basal (n=6/condition, $F_{(2,11)}$=37.92, *p<0.05 by one-way ANOVA followed by Bonferroni *post hoc* test) and stimulated ($F_{(2,14)}$=13.52, *p<0.05 by one-way ANOVA followed by Bonferroni *post hoc* test) conditions. DNMT3a1 expression level is not altered. The dashed line represents the mRNA expression level of the uninfected, unstimulated control condition.
(B) *In vivo* validation of shRNA-mediated downregulation of DNMT3a2. The level of hippocampal DNMT3a2 is reduced in mice expressing rAAV-sh3a2-GFP (n≥5, t_9=3.09, p<0.05 by two-tailed student's t-test), without affecting endogenous DNMT3a1 expression.
Data are shown as mean ± S.E.M.

show any behavioral alteration. We assessed memory performance in four hippocampal dependent tasks, namely, trace fear conditioning, contextual fear conditioning (data not shown), spatial object recognition and Morris water maze. In trace fear conditioning, mice show comparable freezing during the pre-Conditioning Stimulus (preCS) phase. However, during the presentation of the tone (Conditioned Stimulus; CS), control mice exhibited an increase in fear response, which was significantly higher compared to the fear response of rAAV-sh3a2-GFP-injected mice (Figure 13B; AO). This effect was not due to differences in shock reactivity (Figure 13C; AO). Further, the observed memory deficits were specific to LTM, as both groups performed equally well when tested 1 hour after training (STM) (Figure 13A; AO). In addition, mice with reduced DNMT3a2 expression exhibit deficits in memory formation compared to control injected mice in the spatial object recognition task (Figure 14A; AO). In the training session, both groups demonstrated equal preference for both objects (Figure 14B) and spent

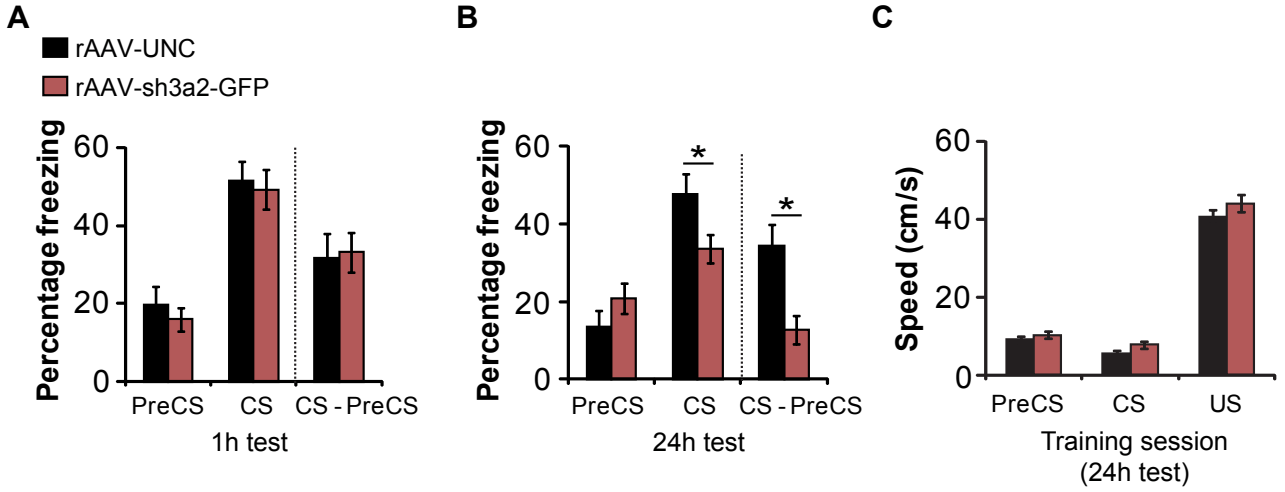

Figure 13: Memory deficits in trace fear conditioning due to a reduction in DNMT3a2 expression.
(A) Downregulation of DNMT3a2 expression does not affect short-term memory (1 h test, n=9 per group).
(B) Mice expressing rAAV-sh3a2-GFP display significantly less freezing upon CS presentation compared to control mice, suggesting long-term memory deficits (24h test; rAAV-UNC n=14, rAAV-sh3a2-GFP n=15; CS, t_{27}=2.28; CS-preCS, t_{27}=3.24, *p<0.05 by two-tailed student's t-test).
(C) Both groups show similar activity and foot-shock reactivity in the training session.
Pre-conditioned stimulus, PreCS; conditioned stimulus, CS. Data are shown as mean ± S.E.M.

comparable time exploring the objects (Figure 14C; AO). Control mice developed a preference for the displaced object in the test session. In contrast, mice with reduced DNMT3a2 expression had no preference towards the displaced object (Figure 14A; AO). Performance in the Morris water maze task was also altered in mice injected with rAAV-sh3a2-GFP, compared to control mice. Both groups learned the task during the acquisition phase (Figure 15A; TH). Probe trial one, which was conducted after 4 days of training, revealed that at this early time point, both groups have not yet learned the location of the platform (Figure 15B; TH). However, after 8 days of training, mice expressing rAAV-UNC spent significantly more time in the target quadrant than in the other three quadrants. Mice with reduced DNMT3a2 levels did not spend more time in the target quadrant, suggesting that they did not learn the spatial location of the platform (Figure 15C; TH). As these mice learned the visible version of this test, differences in motivation and sight can be ruled out (Figure 15D; TH). To exclude the possibility that memory deficits were caused by viral interference with neurogenesis, we performed a doublecourtin (DCX) staining. DCX is a marker for immature neurons. Hence, in case immature neurons

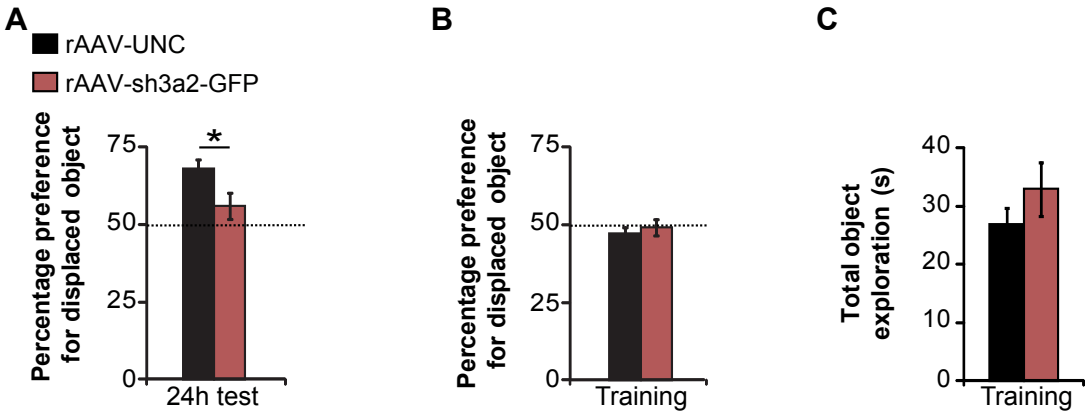

Figure 14: Diminished DNMT3a2 expression results in memory deficits in the spatial object recognition task.
(A) Mice expressing rAAV-sh3a2-GFP showed significantly less preference for the displaced object compared to control injected mice (rAAV-UNC n= 19, rAAV-sh3a2-GFP n=16; t_{33}=2.35, *p<0.05 by two-tailed student's t-test).
(B) The two groups explored both objects equivalently in the training session.
(C) Total exploration time was similar in both groups.
The dashed line at 50% indicates chance level of object exploration. Data are shown as mean ± S.E.M.

are affected by our viral infections, neurons should be positive for DCX and GFP. We analysed the dentate gyrus, the region in which neurogenesis takes place, but could not find any neuron that was double-positive. Therefore, we ruled out the possibility that behavioural alterations are the result of interference with neurogenesis (Figure 16).
Here, we demonstrate that a reduction in the hippocampal Dnmt3a2 level causes impairments in associative and spatial learning tasks.

6.2.3 Aged mice exhibit memory deficits and reduced levels of DNMT3a2

Numerous studies have previously shown that memory performance during aging is impaired (Bach et al. (1999), Blank et al. (2003)). As we have shown that a decrement of DNMT3a2 expression leads to memory deficits, we aimed to investigate if aging-associated memory impairments progress in line with diminishing DNMT3a2 expression. First, we show that aged mice show memory deficits in two hippocampal tasks, trace fear conditioning and spatial object recognition. In trace fear conditioning, both groups show comparable freezing during the onset of the tone (CS). However, taken into account the freezing level before the tone onset (preCS), it becomes apparent that aged mice are generally more anxious. Therefore, we subtracted the

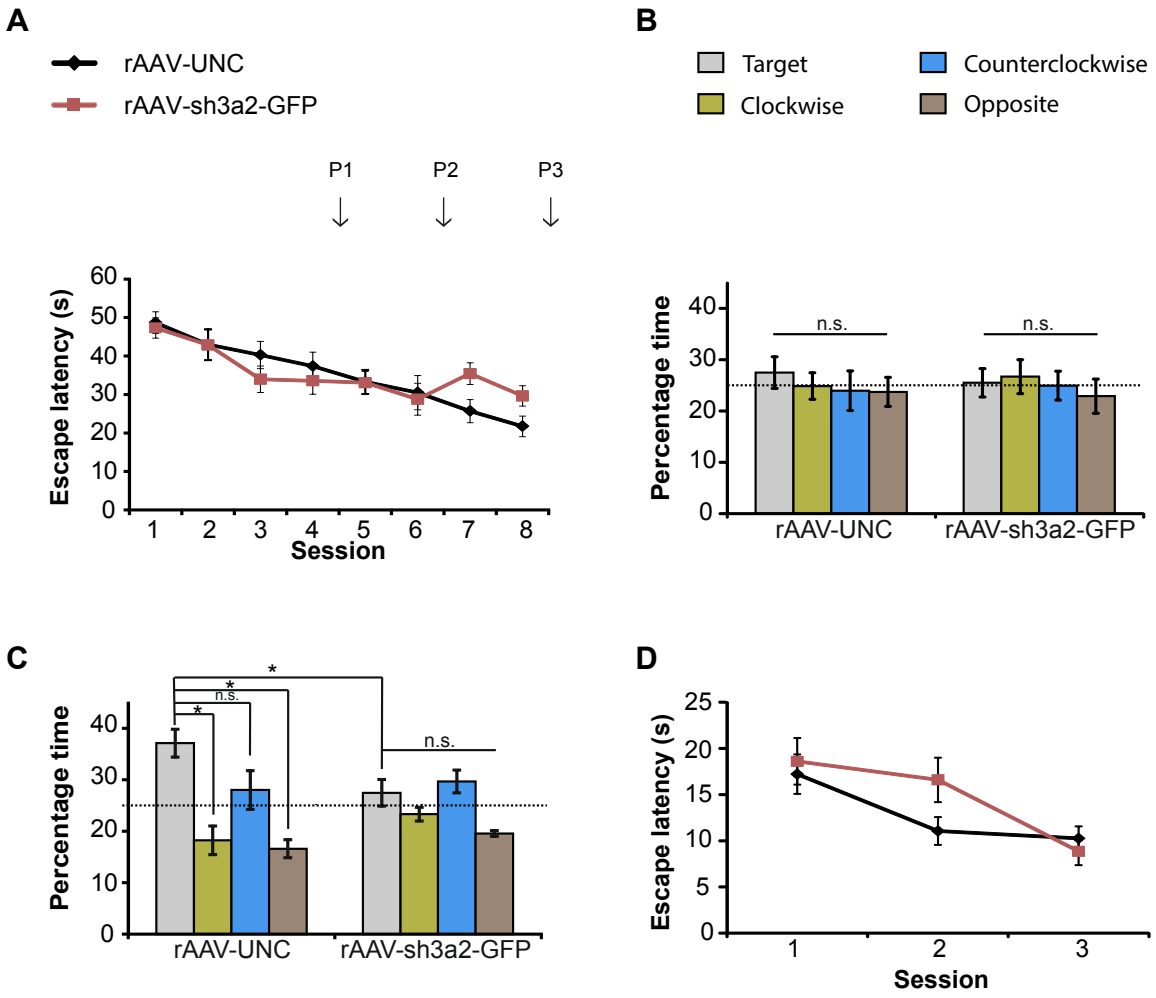

Figure 15: Downregulation of DNMT3a2 causes memory deficits in the Morris water maze.

(A) rAAV-UNC- and rAAV-sh3a2-GFP-expressing mice show no statistically significant differences in escape latency during the acquisition phase. It is noteworthy, however, that escape latency starts to plateau from day 3 onwards in the rAAV-sh3a2-GFP group, whereas escape latency still decreases slightly in the rAAV-UNC group.

(B) Both groups did not learn the location of the platform after 4 days of training, as indicated in probe trial 1.

(C) In probe trial 3, control mice spend significantly more time in the target quadrant compared to the other quadrants (n=14, $F_{[3,52]}=12.74$, *p<0.05 by one-way ANOVA followed by Newman Keuls *post hoc* test) and rAAV-sh3a2-GFP injected mice (rAAV-UNC n=14, rAAV-sh3a2-GFP n=11, $t_{13}=2.53$, *p<0.05 by two-tailed student's t-test). Mice with a reduction in DNMT3a2 expression did not spend more time in the target quadrant.

(D) Both groups performed equivalently in the visible-platform version of the Morris water maze.

The dashed line at 25% indicates chance level of spending time in each quadrant. Not significant, n.s. Data are shown as mean ± S.E.M.

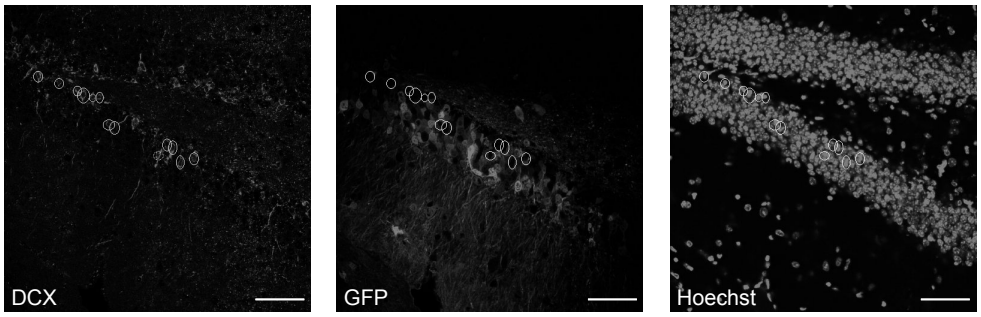

Figure 16: Postmitotic neurons are exclusively infected by rAAVs.
To rule out that behavioural deficits were due to interference with neurogenesis, we analysed if infected cells are also positive for doublecortin (DCX). DCX is a marker for immature neurons. Four mice were infected with our rAAV vectors, and we analysed 8 slices in total. We did not find any cell that was positive for GFP and DCX. Scale bar=100μm.

freezing time during preCS from the time mice spent freezing during the CS to normalise for basal freezing (Figure 17A; TH during MT). Though aged mice reacted significantly stronger to the shock, aged mice display significantly less freezing in the test session (CS-preCS), suggesting they are impaired in memory consolidation (Figure 17B; TH during). Due to the high anxiety of aged mice, we performed an additional task that is independent of aversive reinforcement. The spatial object recognition task confirmed our finding that aged mice have cognitive deficits, as aged mice did not show a preference for the displaced object (Figure 18A; AO). It is worth noting that total exploration time in aged mice was not altered (Figure 18B; AO). Next, we dissected hippocampal tissue from young and aged mice and compared DNMT3a2 mRNA levels. Aged mice show significantly lower levels of DNMT3a1 and DNMT3a2, but not DNMT1 and DNMT3b (Figure 18C; AO). Additionally, the training-induced increase of DNMT3a2 observed in young mice is missing in aged mice (Figure 18D; AO). Therefore, we provide a causal link between memory performance and DNMT3a2 expression in young and aged mice.

6.2.4 Memory deficits in aged mice can be rescued by restoring DNMT3a2 expression

To investigate whether restoring DNMT3a2 expression rescues memory deficits in aged mice, we produced a rAAV containing an expression cassette for HA-epitope-tagged DNMT3a2 (rAAV-HA-Dnmt3a2) and a control rAAV containing an expression cassette for GFP (rAAV-GFP) (Figure 19A). Mice that were injected with rAAV-HA-DNMT3a2 (Figure 19B; AO & TH)

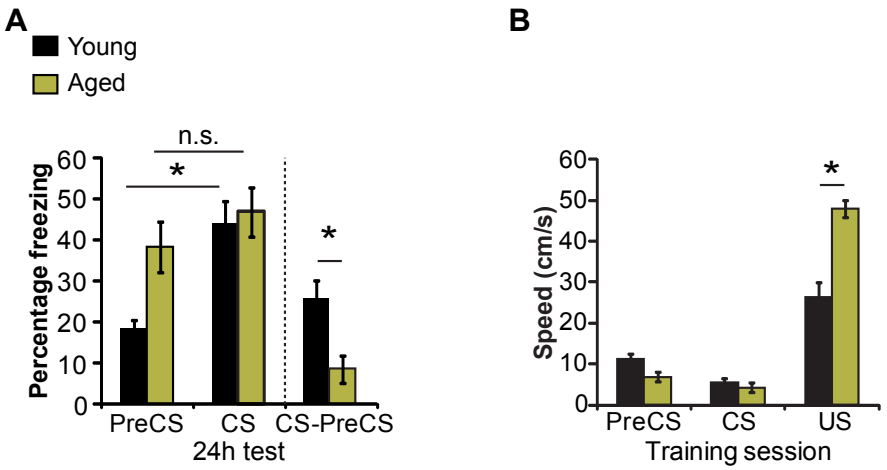

Figure 17: Aged mice display memory deficits in trace fear conditioning.
(A) Young mice show an increase in freezing rate during presentation of the CS (n=6 per group; PreCS vs CS, t_4=4.57; $p<0.05$ by paired student's t-test). Though aged mice freeze equivalently during CS, compared to young mice, they are generally more anxious, as indicated by high freezing rates during preCS. Therefore, subtracting the baseline freezing (preCS) from the freezing during CS reveals a memory deficit in aged mice (CS-preCS) (t_9=2.61, $p<0.05$ by two-tailed student's t-test).
(B) Basal moving speed (preCS) in the training session was decreased in aged mice (n=6 per group, t_{10}=2.56, $p<0.05$ by two-tailed student's t-test), however, no freezing was measured in both groups. Aged mice displayed higher foot-shock reactivity (t_{10}=5.22, $p<0.05$ by two-tailed student's t-test), compared to young mice.
Not significant, n.s.; pre-conditioned stimulus, PreCS; conditioned stimulus, CS. Data are shown as mean ± S.E.M.

showed significantly higher freezing during the tone presentation in trace fear conditioning, in comparison to aged control animals, indicating improved memory performance (Figure 20A; AO). One must note that mice expressing rAAV-HA-DNMT3a2 exhibited stronger shock reactivity (Figure 20B; AO), however, there was no correlation between foot-shock reactivity and memory performance (Figure 20C; AO). We confirmed our findings in the spatial object recognition task. Mice injected with rAAV-HA-DNMT3a2 expressed a preference for the displaced object, which was not observed in aged littermates (Figure 21A; AO & TH). The two objects were equally explored in the training session (Figure 21B; AO & TH), and total exploration time did not differ between the groups (Figure 21C; AO & TH). Hence, we show that cognitive deficits in aged mice can be rescued by restoring the level of DNMT3a2.

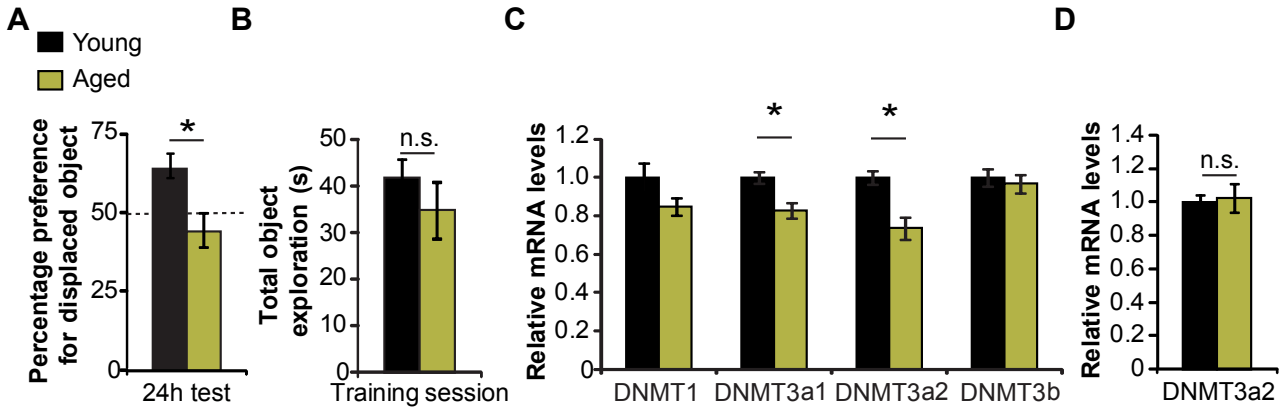

Figure 18: Aged mice are impaired in spatial object recognition.
(A) Young mice, but not aged mice, show a preference for the displaced object 24 hours after training (aged n=12, young n=11, t_{21}=3.57, *p<0.05 by two-tailed student's t-test), suggesting a memory deficit in aged mice. The dashed line at 50% indicates the chance level preference for the displaced object.
(B) Total exploration time of both objects did not differ in young and aged animals.
(C) qRT-PCR analysis revealed that DNMT3a1 and DNMT3a2 expression, but not DNMT1 and DNMT3b expression, is decreased in the hippocampus of aged mice (n=6 per group; DNMT3a1, t_9=3.82, DNMT3a2, t_8=4.08, *p<0.05 by two-tailed student's t-test).
(D) qRT-PCR analysis reveals that DNMT3a2 expression is not increased at 4 hours after training in the spatial object recognition task. This is in contrast to results obtained for young mice (Figure 10C).
Not significant, n.s. Data are shown as mean ± S.E.M.

6.2.5 Global DNA methylation levels are altered by DNMT3a2

Next, we studied if global methylation levels are affected by changes in DNMT3a2 levels. We infected cells with rAAV-sh3a2-GFP and rAAV-HA-Dnmt3a2 and used rAAV-UNC and rAAV-GFP as control, respectively. To detect global methylation, we stained the cells against 5-methylcytosine. As can be seen in Figure 22A (AO), global methylation levels were decreased in cells expressing rAAV-sh3a2-GFP, compared to control cells, whereas infection with rAAV-HA-DNMT3a2 led to an increase in global methylation levels (Figure 22B; AO). We further confirmed our findings *in vivo*, i.e., mice infected with rAAV-HA-DNMT3a2 displayed higher global methylation levels compared to rAAV-GFP control injected animals (Figure 22C; TH). Hence, altering the levels of DNMT3a2 consecutively changes the global methylation level.

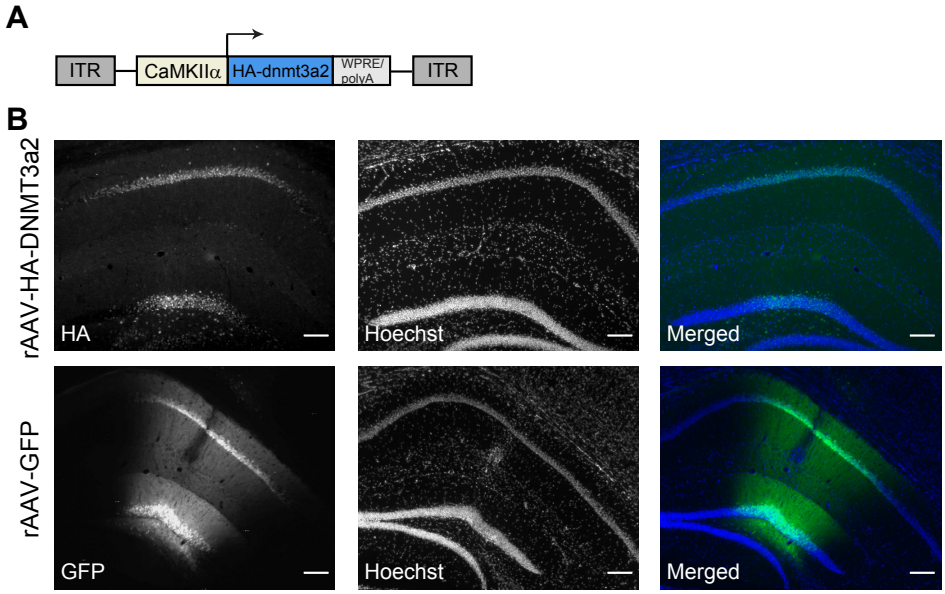

Figure 19: Validation of rAAV-HA-DNMT3a2 *in vivo*.
(A) Schematic representation of the design of rAAV-HA-DNMT3a2. The rAAV contains an HA eptiope-tagged DNMT3a2 driven by a CaMKIIα promoter.
(B) Representative pictures of rAAV-HA-DNMT3a2 and rAAV-GFP expression in the dorsal hippocampus three weeks after stereotaxic delivery. Scale bar=100μm.

6.2.6 Memory relevant genes are regulated by DNMT3a2

We next aimed at identifying genes that are regulated by DNMT3a2. We infected hippocampal cultures either with rAAV-sh3a2-GFP or rAAV-UNC and compared mRNA expression of a variety of genes known to be induced by synaptic activity and learning. In cells that were infected with rAAV-sh3a2-GFP, BDNF and ARC, mRNA expression was significantly reduces 3 hours and 6 hours after stimulation in comparison to cells infected with rAAV-UNC (Figure 23A-B; TH, partially during MT). There was also a trend towards a reduction in relative expression levels for c-FOS, Nr4a1 and EGR1 (Figure 23C-E; TH, partially during MT), however, this effect was not significant mainly due to high variability between independent samples. Thus, expression levels of DNMT3a2 and certain memory genes show a positive correlation, suggesting that regulation of DNMT3a2 is crucial for proper expression of memory relevant genes.

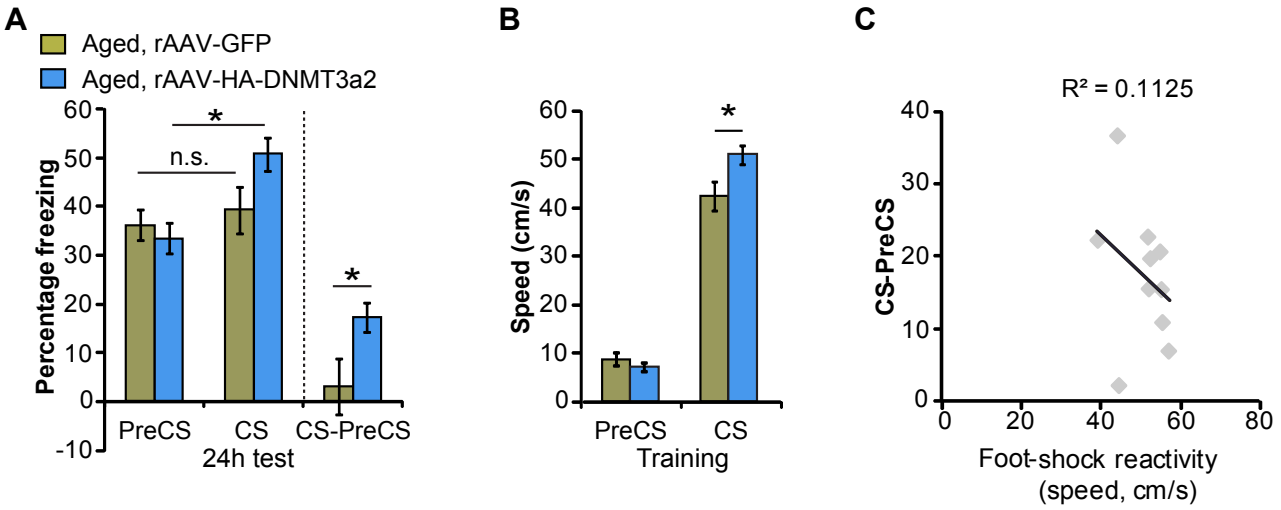

Figure 20: Memory deficits in aged mice in trace fear conditioning can be rescued by DNMT3a2 overexpression.

(A) Aged mice expressing rAAV-HA-DNMT3a2 show an increase in freezing during the presentation of the CS, unlike the observations of aged control mice (n=10 per group, preCS vs CS, aged rAAV-HA-dnmt3a2, t_9=5.67, *p<0.05 by paired student's t-test). Normalizing for generalized fear further reveals memory improvement in aged mice expressing rAAV-HA-DNMT3a2 (CS-preCS, t_{14}=2.51, *p<0.05 by two-tailed student's t-test).

(B) Aged mice injected with rAAV-HA-DNMT3a2 showed a slight increase in foot-shock reactivity, compared to aged control injected mice (t_{15}=2.4, *p<0.05 by two-tailed student's t-test).

(C) There was no correlation in foot-shock reactivity and memory performance in aged mice injected with rAAV-HA-DNMT3a2. This indicates that improvement in memory performance is not the result of a stronger response to the foot-shock. Not significant, n.s. Data are shown as mean ± S.E.M.

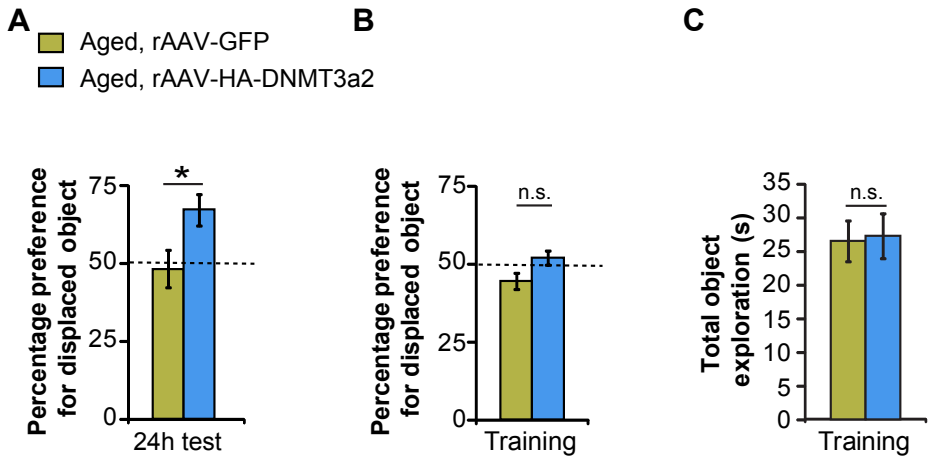

Figure 21: Restoring DNMT3a2 levels rescues age-dependent memory deficits in spatial object recognition.

(A) Mice expressing rAAV-HA-DNMT3a2 display a preference for the displaced object that is not seen in aged control injected littermates (rAAV-GFP n=14, rAAV-HA-DNMT3a2 n=13; t_{25}=2.38, *p<0.05 by two-tailed student's t-test).

(B) rAAV-HA-DNMT3a2- and rAAV-GFP-expressing mice show no preference for either object in the training session.

(C) There was no difference in total exploration time of the two objects in rAAV-HA-DNMT3a2- and rAAV-GFP-injected mice.

The dashed line at 50% indicates exploration of the objects at chance level. Not significant, n.s. Data are shown as mean ± S.E.M.

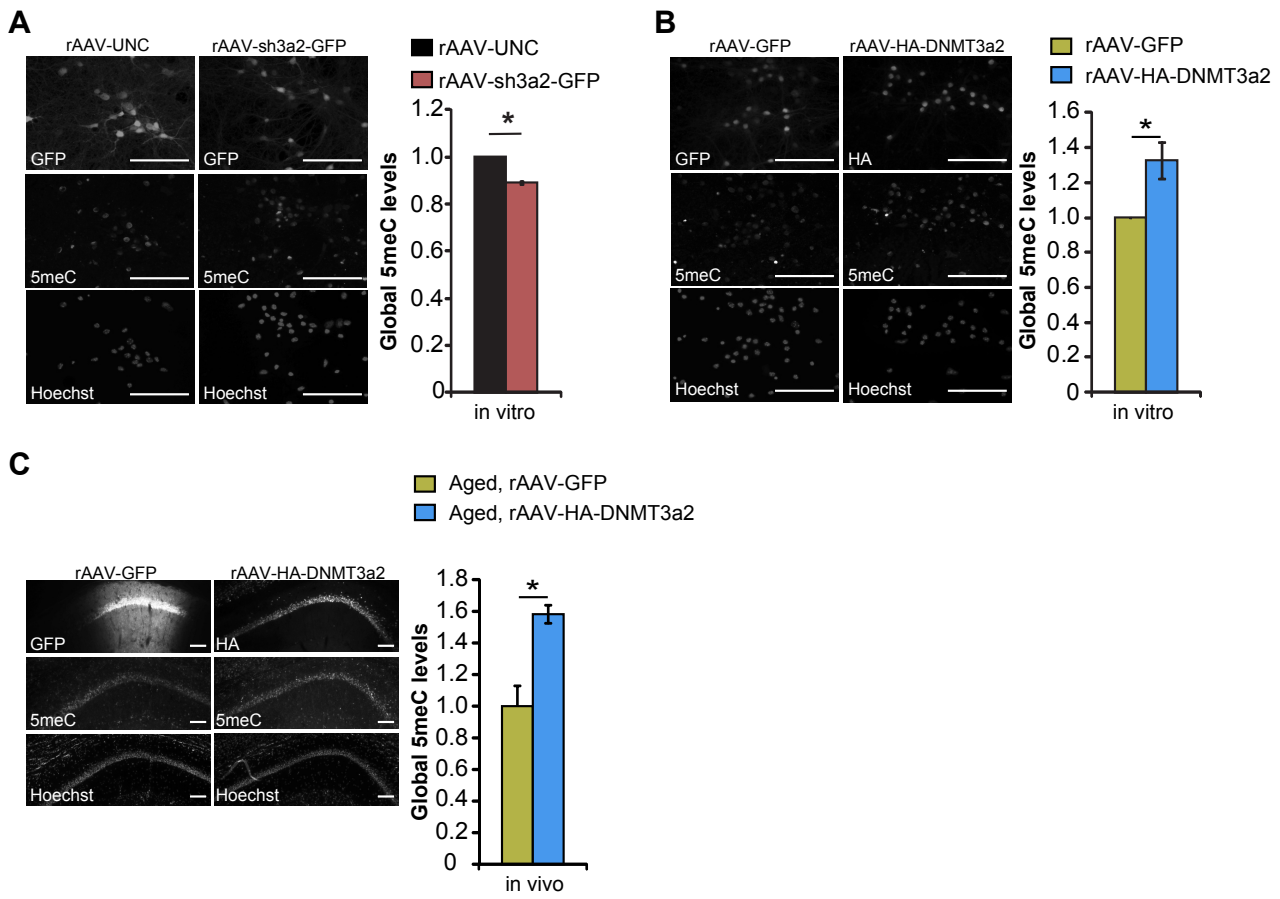

Figure 22: Levels of DNMT3a2 expression positively correlate with global methylation levels.

(A) Representative pictures of cells stained against 5-methylcytosine in hippocampal cultures infected with rAAV-UNC and rAAV-sh3a2-GFP. Quantification of 5-methylcytosine intensity revealed that global methylation levels were decreased in cells expressing rAAV-sh3a2-GFP, compared to control cells (n=4 per group, t_6=22.14, *p <0.05 by two-tailed student's t-test).

(B) Representative pictures of 5-methylcytosine staining in cultured hippocampal neurons expressing rAAV-GFP or rAAV-HA-DNMT3a2. Quantification of 5-methylcytosine intensity showed that global methylation levels are increased in cells overexpressing DNMT3a2 (n=5 per group, t_8=3.05, *p <0.05 by two-tailed student's t-test).

(C) Representative pictures of 5-methylcytosine staining in mice expressing rAAV-GFP or rAAV-HA-DNMT3a2. Quantification of 5-methylcytosine intensity reveals an increase in global methylation levels in mice expressing rAAV-HA-DNMT3a2 (n=6/group, t_{10}=4.12, *p <0.05 by two-tailed student's t-test).

Scale bars=100μm. Data are shown as mean ± S.E.M.

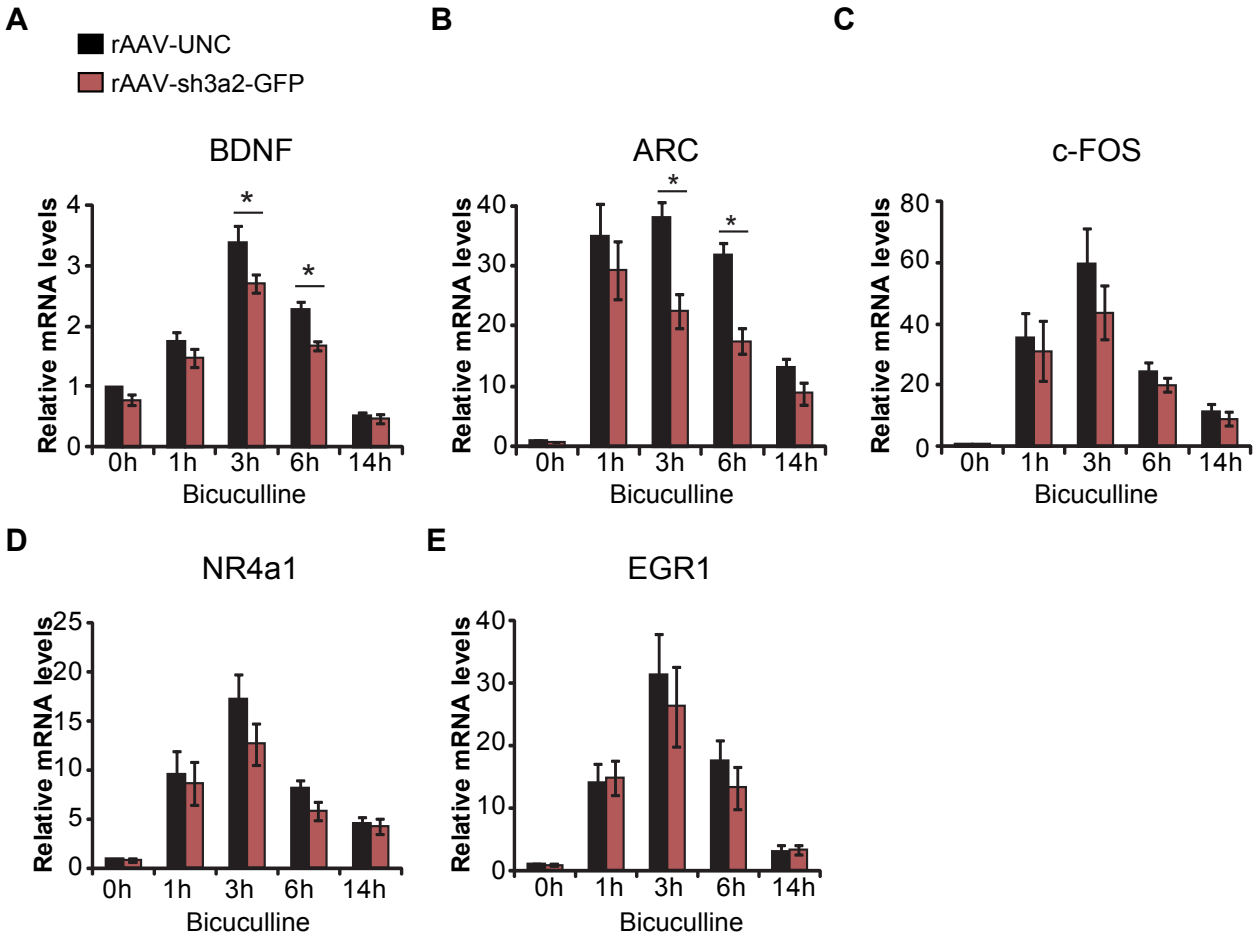

Figure 23: A decrement in DNMT3a2 expression results in decreased expression levels of plasticity-related genes.

qRT-PCR analysis shows relative expression levels of putative target genes of DNMT3a2.

(A) Hippocampal cultures that were infected with rAAV-sh3a2-GFP showed reduced ARC expression at 3 hours ($n \geq 7$; $t_{12}=4.83$, $^*p<0.05$ by two-tailed student's t-test) and 6 hours ($t_{12}=4.71$, $^*p<0.05$ by two-tailed student's t-test) after bicuculline stimulation, compared to control infected cells.

(B) BDNF expression was also reduced 3 hours ($t_{14}=2.34$, $^*p<0.05$ by two-tailed student's t-test) and 6 hours ($t_{14}=3.8$, $^*p<0.05$ by two-tailed student's t-test) after bicuculline treatment in cells infected with rAAV-sh3a2-GFP. There was also a trend for a reduction in (C) Nr4a1 expression, (D) c-FOS expression and (E) EGR1 exprssion at 3 hours and 6 hours after bicuculline treatment in cells infected with rAAV-sh3a2-GFP. However, this difference was statistically not significant. Data are shown as mean ± S.E.M.

6.3 Nuclear calcium regulates fear memory extinction

I aimed at investigating if nuclear calcium not only regulates memory formation but, further, whether it is also required for fear memory extinction. I injected rAAV-PV.NLS-mC and rAAV-mC via stereotaxic delivery into the dorsal hippocampus of mice (c.f. Section 6.1.1. for more information). As expression of rAAV-PV.NLS-mC interferes with fear memory consolidation, I performed contextual fear conditioning already three days after rAAV infections, to assure that memory consolidation *per se* is not affected. After three days, rAAV expression is still too low to be functionally relevant (own unpublished observations). Two weeks after fear conditioning, mice underwent fear extinction training and, 24 hours later, fear extinction testing (Figure 24A). As can be seen in Figure 24B, both groups displayed similar freezing during the initial 5 minutes of the extinction session. This confirms that both groups, indeed, consolidated the fear memory. However, the freezing rate within each group varies greatly in this protocol, and further, it was reported that there are high variations in the dynamics of fear extinction between experiments (Sananbenesi et al. (2007), Agis-Balboa et al. (2011), Bahari-Javan et al. (2012)). Thus, I normalised the data to the first five minutes of extinction training in order to reduce variability and maintain the possibility to compare independent experiments. There was no difference in the extinction learning curve between mice expressing rAAV-PV.NLS and control injected animals, suggesting that both groups are able to extinguish fear memory (Figure 24C). However, when the mice were tested 24 hours after extinction training, only mice expressing rAAV-mC displayed freezing that was comparable to the end of the training session. Mice expressing rAAV-PV.NLS-mC showed increased freezing compared to the final five minutes of the extinction training. Statistical analysis revealed that rAAV-PV.NLS-mC-expressing mice showed significantly higher freezing levels than control injected mice in the testing session, indicating that mice injected with rAAV-PV.NLS-mC did not consolidate fear memory extinction (Figure 24D). Thus, I have shown here that nuclear calcium is required to form new memories, which in turn can interfere with existing memories, as it is the case in fear memory extinction.

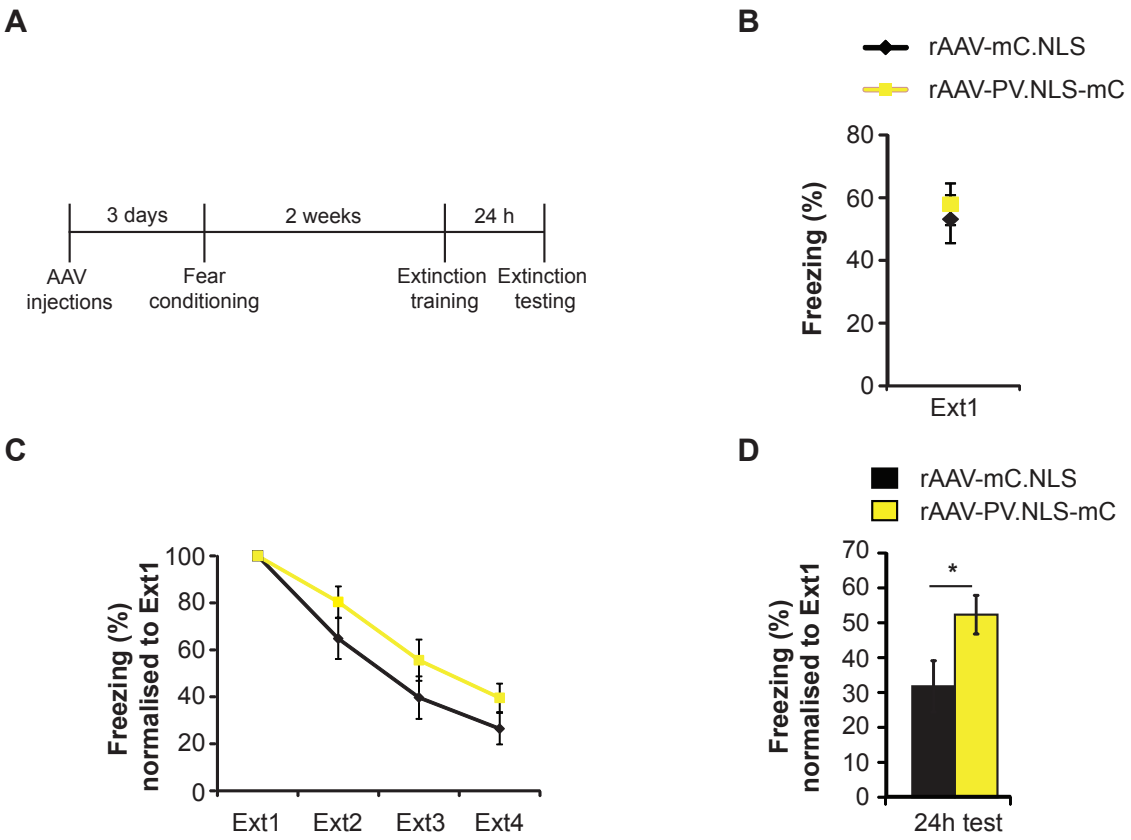

Figure 24: Nuclear calcium is essential for fear memory extinction.
(A) Schematic drawing of experimental procedure.
(B) Both groups show comparable freezing rate during the first 5 minutes of extinction, assuring that memory consolidation is not impaired in rAAV-PV.NLS-mC expressing mice when trained three days after stereotaxic surgery.
(C) Mice expressing either rAAV-PV.NLS-mC or rAAV-mC.NLS extinguish fear in within-session training.
(D) When tested 24 hours later, mice expressing rAAV-PV.NLS-mC show an increase in freezing rate compared to the end of the extinction training session, significantly deviating from control injected animals (rAAV-mC.NLS n=9, rAAV-PV.NLS-mC n=11, t_{18}=2.279, *p<0.05 by two-paired student's t-test). Data are shown as mean ± SEM.

7 Discussion

7.1 Nuclear calcium-regulated dendritic architecture controls neuronal permissiveness for long-term memory formation

In this study, I have demonstrated that hippocampal nuclear calcium is required for memory formation. Further, I have shown that the maintenance of the dendritic tree is important in order to keep the neuron permissive to information processing, required for memory consolidation. VEGFD can rescue alterations of dendritic morphology, resulting from nuclear calcium dysregulation and, further, can restore cognitive abilities.

7.1.1 Memory consolidation requires hippocampal nuclear calcium signalling

Nuclear calcium has previously been reported to be important for memory consolidation. In these studies CaMBP was either expressed in the forebrain of mice (Limbäck-Stokin et al. (2004)) or in the mushroom bodies of flies (Weislogel et al. (2013)). CaMBP binds to and inactivates the nuclear calcium/calmodulin complex and hence inhibits CREB-dependent transcription. However, a recent paper suggests that calcium/calmodulin shuttles from the cytoplasm to the nucleus via the carrier γCaMKII and ,thus, impacts CREB-mediated transcription (Ma et al. (2014)), raising the question if free nuclear calcium is required for memory formation. Here, I show that free nuclear calcium, using the nuclear-targeted calcium binding protein Parvalbumin, is important for proper memory formation in the hippocampus of mice. By buffering nuclear calcium, Parvalbumin alters the functional properties of nuclear calcium transients, which in turn, will probably also alter the activity of the nuclear calcium/calmodulin-dependent kinases CaMKII and CaMKIV. Consequently, the activity of transcriptional regulators important for memory formation, such as CREB (Hardingham et al. (2001), Kida et al. (2002), Pittenger et al. (2002), Kida and Serita (2014)), CBP (Chawla et al. (1998), Oike et al. (1999), Alarcón et al. (2004)) and MeCP2 (Moretti et al. (2006), Zhou et al. (2006), Buchthal et al. (2012)) and the expression of plasticity-related genes such as ARC (Lyford et al. (1995),Guzowski et al. (2000), Plath et al. (2006)), DNMT3a2 (Oliveira et al. (2012)) and Nr4a1 (Malkani and Rosen (2000), von Hertzen and Giese (2005)) that are relevant for memory consolidation, are likely to be changed. In addition, the nuclear calcium-dependent gene VEGFD and the transcriptional regulator HDAC4 have previously been identified to be influenced by the nuclear calcium buffer Parvalbumin (Schlumm et al. (2013), unpublished data) and, further, are known to be important for memory consolidation (Mauceri et al. (2011), Kim

et al. (2012),Sando et al. (2012), Fitzsimons et al. (2013)). Thus, nuclear calcium is a critical mediator for a variety of memory-related processes, and I have shown that it, indeed, affects memory consolidation when expressed in the hippocampus of mice.

7.1.2 VEGFD reverses morphological and behavioural alterations that are due to nuclear calcium dysregulation

As nuclear calcium signalling not only controls activity-related gene expression but also regulates genes during basal neuronal activity, structural features of neurons are also influenced by nuclear calcium. The structural features are important as they keep the neuron in a permissive state that allows it to process information which is relevant for memory consolidation. Though it has been demonstrated that inhibition of nuclear calcium by CaMBP causes morphological alterations *in vitro* (Mauceri et al. (2011), Li et al. (2014a)) and further causes memory deficits and influences CREB phosphorylation and gene expression *in vivo* (Limbäck-Stokin et al. (2004)), a direct link between nuclear calcium, neuronal morphology and memory formation has not yet been reported to my knowledge. Here, I provide evidence that inhibition of nuclear calcium via an increase of its buffer capacity leads to changes in the dendritic arborisation *in vivo*, which in turn, results in memory formation and fear extinction deficits. These changes are specific to LTM as no impairments in STM could be detected. Hence, nuclear calcium is required for the maintenance of the dendritic morphology, and this dendritic architecture is required to form long-term adaptation. A possible candidate that mediates these changes during basal neuronal activity is VEGFD. Suppressing nuclear calcium signalling leads to lower levels of VEGFD in basal conditions (Mauceri et al. (2011)). In addition, it has been shown in our lab that lowering the levels of VEGFD using a shRNA against VEGFD directly causes morphological alterations and memory deficits. Indeed, I could show that, in mice with nuclear calcium dysregulation, supplementation of VEGFD can partially rescue dendritic length. A full rescue in dendritic length and dendritic complexity was not achieved. Nuclear calcium further regulate the expression of other genes important for dendritic morphology, such as MeCP2 (Chapleau et al. (2009)), and therefore other genes presumably contribute to the observed morphological alterations. The fact that I could induce a rescue in LTM formation by overexpressing VEGFD strongly supports the idea that nuclear calcium is important for memory formation via regulating the expression of genes necessary for maintaining the structural features of neurons.

In conclusion, I show that nuclear calcium signalling in the hippocampus is important for contextual memory formation. In addition to the known link of its regulatory role in activity-induced gene transcription and memory formation, here, I show that during basal neuronal

activity, nuclear calcium is necessary to maintain dendritic morphology. These morphological features are important to keep the neurons in a permissive state to consolidate memory. I provide VEGFD as a key player between nuclear calcium signalling, dendritic morphology and memory formation.

7.1.3 Outlook

In this study, I have shown that dysregulation of nuclear calcium signalling leads to alterations in dendritic morphology and memory formation. These alterations can be rescued by over-expression of VEGFD. As previously noted, among others, Rett syndrome and Alzheimer's disease have been reported to show alterations in dendritic morphology and are further known to affect cognition (Armstrong et al. (1995), Chapleau et al. (2009), Kaufmann and Moser (2000), Kulkarni and Firestein (2012)). Thus, regulating nuclear calcium and VEGFD may serve as a therapeutic strategy in these diseases. Besides nuclear calcium-regulated VEGFD, it is likely that other genes regulated by nuclear calcium contribute to morphological alterations and memory deficits. It is known from *in vitro* studies that nuclear calcium also regulates spine morphology. However, VEGFD supplementation does not rescue dendritic spine impairments. Spine morphology has been demonstrated to be essential for memory formation. Among the genes that are regulated by nuclear calcium and further control spine morphology are C1q and ARC. Whether memory deficits which are caused by nuclear calcium inhibition in the hippocampus can be linked to dysregulation of these genes remains to be investigated.

7.2 Rescue of aging-associated decline in DNMT3a2 expression restores cognitive abilities

This study shows that DNMT3a2 is regulated by neuronal activity and that the level of DNMT3a2 expression correlates with memory performance. Aged mice show reduced expression of DNMT3a2, and age-dependent learning deficits are rescued when expression of DNMT3a2 is restored.

7.2.1 Activity-regulated DNMT3a2 is required for memory consolidation

In this study, we have investigated the role of DNMT3a2 in memory formation. Though it has been previously been shown that DNMT3a and DNMT3b are upregulated after contextual fear conditioning (Miller and Sweatt (2007)), the two isoforms DNMT3a1 and DNMT3a2 were not investigated independent from each other. Here, we demonstrate that only DNMT3a2, not DNMT3a1, is robustly regulated by neuronal activity *in vitro* and *in vivo*. The other DNMTs were not induced after any of the stimulation methods applied, however, DNMT3b was reduced after bicuculline treatment *in vitro* and after kainic acid administration *in vivo*. This in contrast to the study of Miller and Sweatt (2007), in which they found DNMT3b to be induced after contextual fear conditioning, suggesting that DNMT3b is differentially regulated, dependent on the stimulation method applied. Induction of DNMT3a2 expression upon activity was regulated in a NMDA receptor and nuclear calcium-dependent manner. Given that DNMT3a2 is highly regulated by activity, we next investigated if it is also relevant for memory formation. Previous studies have injected DNMT inhibitors (Miller and Sweatt (2007)) or used conditional KO mice for DNMT3a and DNMT1 in the forebrain to study the role of DNA methylation in memory formation (Feng et al. (2010)). Injections of DNMT inhibitors into the hippocampus resulted in contextual fear conditioning deficits, however, DNMT inhibitors cannot interfere with the expression of a specific DNMT. Therefore, we applied a viral-mediated approach using a shRNA that specifically downregulates DNMT3a2 mRNA expression. We could show that hippocampal reduction of DNMT3a2 expression causes deficits in spatial and fear memory. Hence, interference with the activity-induced DNMT3a2 alone can cause behavioural alterations. Furthermore, DNMT3a2 is important for LTM, however, it is not required for STM. STM is independent of transcription (Barondes and Cohen (1968),Montarolo et al. (1986), Katche et al. (2013)), suggesting that DNMT3a2 is involved in transcription-dependent processes, which parallels its known function as a transcriptional regulator (Turek-Plewa and Jagodzi?ski (2005)). We cannot rule out that other DNMTs also contribute to the learning deficits observed in the study that used DNMT inhibitors. However, given that only

DNMT3a2 is induced upon learning and taking into account its unique feature of associating with transcriptionally active euchromatin (Chen et al. (2002)), DNMT3a2 demonstrates key characteristics of serving as an integral player in regulating memory formation. It is noteworthy that forebrain deletion of DNMT3a and DNMT1, but not DNMT3a or DNMT1 alone, resulted in spatial and fear memory deficits (Feng et al. (2010)). This suggests that DNMT3a and DNMT1 are redundant in memory formation. Additionally, knockout of DNMT3a affects the expression of both isoforms, DNMT3a1 and DNMT3a2. This seems to be in contrast to our finding that DNMT3a2 expression alone can interfere with memory consolidation, whereas in the other study, deletion of both DNMT3a did not affect memory consolidation. However, differences in protocol and brain region may contribute to the discrepancy of the findings. Moreover, another study recently investigated the role of DNMT3a and DNMT1 and demonstrated that inhibition of DNMT3a expression alone can cause memory deficits (Morris et al. (2014)). Conclusively, we have shown that DNMT3a2 is robustly regulated by neuronal activity and that DNMT3a2 is required for the formation of transcription-dependent LTM.

7.2.2 Age-dependent cognitive decline can be rescued by restoring DNMT3a2 expression

Changes in global methylation levels (Siegmund et al. (2007)) and in methylation levels at the ARC promoter have been reported to coincide with aging (Penner et al. (2011)). Though it has been reported beforehand that 5-methylcytosine levels decrease with normal aging in mice (Wilson et al. (1987)), to our best knowledge, we are the first to demonstrate that DNMT expression in the brain is decreased in aged animals. Here, we show that expression of both DNMT3a isoforms decreases in the hippocampus, and additionally, learning-induced increases in DNMT3a2 expression that occur in young mice are not observed in aged mice. Here, we demonstrate for the first time that aging-associated memory decline can be rescued by restoring the level of DNMT3a2. We do not exclude the possibility that other DNMTs contribute partially to the age-dependent memory decline. However, in young mice, we could only observe an increase of DNMT3a2 expression upon learning, which suggests that a decrement of DNMT3a2 levels is the main underlying cause for alterations in DNA methylation associated with age-dependent memory loss.

It is worth noting that changes in histone acetylation, another epigenetic marker, have previously been observed in aging. Acetylation at histone 4 lysine 12 was altered in aged mice, preventing learning-induced gene expression and, in turn, leading to memory impairments. Injection of an HDAC inhibitor reversed changes in acetylation at H4K12 and further restored the induction of gene expression after learning, which resulted in amelioration of cognitive

functions (Penner et al. (2011)). Together with our data, this shows the importance of epigenetic processes in aging-dependent memory decline. However, we are only at the very beginning of understanding the role of epigenetic mechanisms in memory consolidation in health and disease.

7.2.3 The level of DNMT3a2 affects global methylation levels and expression of target genes

DNA methylation has been implicated in regulating gene expression. Here, we show that global methylation levels positively correlated with DNMT3a2 expression. Further, the expression of target genes was reduced when DNMT3a2 expression was inhibited. These results seem to be counterintuitive with respect to the historical view that DNA methylation causes gene repression. The recent finding, that DNA methylation can activate gene transcription via recruitment of CREB by MBD proteins (Chahrour et al. (2008)), may provide an explanation for our results in which increases in global methylation levels correlated with improved memory consolidation. In contrast, DNMT3a2 inhibition caused a decrease in global methylation levels that coincided with reduced gene expression of DNMT3a2 target genes and, consecutively, impaired memory performance. An alternative explanation is that DNA methylation in gene bodies can increase transcription (Wu et al. (2010)). Another possibility may also be that an increase in DNA methylation decreases the expression of memory suppressor genes, therefore improving memory performance. Additionally, a decrease in global methylation levels may induce the expression of genes that serve as 'intermediates' and negatively regulate memory relevant genes and, hence, impair memory consolidation. The finding that the expression of DNMT3a2 target genes is reduced when DNMT3a2 is inhibited may further be explained by the observation that DNMTs can also demethylate DNA through deamination (Métivier et al. (2008)).
Interestingly, among the target genes regulated by DNMT3a2 is ARC. ARC is known to be important for memory formation (Plath et al. (2006), Czerniawski et al. (2011)) and has been reported to regulate spine density and morphology (Peebles et al. (2010)). Thus, one of the possible mechanisms underlying DNMT3a2's impact on memory is the regulation of ARC expression and, subsequently, altering spine density and morphology. In summary, our study supports the idea that DNA methylation does not exclusively lead to gene repression but can also be associated with gene activation. The exact mechanisms driving modulations in global methylation which can influence gene expression and, hence, lead to memory impairments and improvements remain to be uncovered.

7.2.4 Outlook

In this study, we have shown that age-dependent memory decline is associated with a decrease in DNMT3a2 level and that a rescue in the DNMT3a2 expression restores cognitive abilities. With respect to the aging population worldwide, this is an important finding, as therapeutic intervention becomes more and more important. Further, understanding the molecular mechanisms in age-dependent memory decline may help to understand the mechanisms in pathological conditions that are more complex, as in the case of Alzheimer's disease. Alterations in DNA methylation in Alzheimer's disease patients have already been reported in several studies. In Alzheimer's disease patients, it was shown that DNA was hypomethylated in the enthorihnal cortex (Mastroeni et al. (2010), Chouliaras et al. (2013)) and hypermethylated in the PFC (Bollati et al. (2011)). Additionally, histone hypo- and hyperacetylation have both been reported in mouse models of Alzheimer's disease (Kim et al. (2007), Rouaux et al. (2003)), and an increase in HDAC2 has been observed in Alzheimer's disease patients (Gräff et al. (2012)). We are just beginning to understand the impact of epigenetic dysregulation on diseases, however, these first findings indicate that epigenetic mechanisms presumably play a central role in diseases affecting cognition. Therefore, pharmacological targeting of epigenetic processes may represent a future tool to treat age- and disease-related cognitive impairments.

7.3 Nuclear calcium regulates fear memory extinction

The importance of gene expression and protein synthesis in memory consolidation has been shown in many studies and is widely accepted. Whether gene expression and protein synthesis are also required for memory extinction is, however, less clear. Studies have shown that fear extinction involves CREB/CBP-mediated gene expression in the amygdala and in the PFC, but not in the hippocampus (Mamiya et al. (2009)), whereas other studies have shown that fear extinction does not require the transcription of new mRNA (Lin et al. (2003)) in the amygdala, but, rather, requires transcription in the hippocampus (Vianna et al. (2003)). Here, I show that in the hippocampus, nuclear calcium signalling is important for contextual fear extinction. As CREB is one of the main targets of nuclear calcium and CREB regulates genes known to be important for fear extinction, such as BDNF, one possibility on how nuclear calcium impacts fear extinction is through its effect on CREB phosphorylation and, hence, on CREB/CBP-dependent gene expression. Though this seems to be in contradiction to the study of Mamiya et al. (2009), one must note that it has been previously reported that performance in fear extinction to certain molecular manipulations may be restricted to very specific extinction parameters (Lattal et al. (2006)). Additionally, nuclear calcium might control extinction through its regulation of epigenetic factors which are known to further regulate gene transcription, such as HDACs and DNMTs. It has been previously shown in our lab that nuclear calcium regulates the shuttling from the nucleus to the cytoplasm of a subset of class II HDACs (Schlumm et al. (2013)) and, hence, mediates gene transcription. Further we have shown that nuclear calcium regulates DNMT3a2, which in turn regulates genes such as BDNF (Oliveira et al. (2012)), that play a role in fear extinction. That HDACs have an effect on fear extinction has been elucidated in previous studies (Bredy et al. (2007),Lattal et al. (2007), Bahari-Javan et al. (2012), Itzhak et al. (2012)), and further, a link between the level of DNA methylation and fear extinction has also been reported (Baker-Andresen et al. (2013), Rudenko et al. (2013), Li et al. (2014b)).

7.3.1 Outlook

In this study, I have shown that nuclear calcium regulates fear memory extinction. This is an important finding, as fear extinction is a model for PTSD. Development of drugs that can regulate nuclear calcium may provide the possibility to reduce the likelihood of developing PTSD or even help to treat PTSD. How exactly nuclear calcium regulates fear extinction remains to be investigated. A possible mechanism is through the regulation of gene transcription. However, as I have shown in this thesis that nuclear calcium regulates the

maintenance of the dendritic tree, which is important for memory consolidation, it seems to be likely that the neuron's permissiveness to process information is of great importance in fear extinction. Additionally, as we have shown that DNMT3a2 is regulated by nuclear calcium, and a variaty of other epigenetic processes have been reported to play a role in fear extinction, DNMT3a2 also serves as a candidate to regulate fear exinction. Thus, finding targets of nuclear calcium that are required for fear extinction may offer additional strategies to treat PTSD.

8 List of Abbreviations

AC	Adenylyl cyclase
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ARC	Activity-regulated cytoskeleton-associated protein
BDNF	Brain-derived neurotrophic factor
BSA	Bovine serum albumin
CaM	Calmodulin
CaMBP	Calmodulin Binding Protein
CaMK	Calcium/calmodulin-dependent protein kinase
cAMP	Cyclic adenosine monophosphate
CBP	CREB binding protein
cDNA	complementary DNA
CRE	cAMP response element
CREB	cAMP response element binding protein
CS	Conditioned Stimulus
DCX	Doublecortin
DIV	Day in vitro
DM	Dissociation medium
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
EGR1	Early growth response protein 1
E-LTP	Early phase LTP
GABA	γ-Amino butyric acid
Gadd45β	Growth arrest and DNA-damage-inducible 45 beta
GFP	Green fluorescent protein
HA	Hemagglutinin
HDAC	Histone deacetylase
HEK293	Human embryonic kidney 293 cells
IMDM	Iscove's Modified Dulbecco Medium
LTM	Long-term memory
LTP	Long-term potentiation

L-LTP	Late phase LTP
MAPK	Mitogen-activated protein kinase
MBD	Methyl-CpG binding domain
MeCP2	Methyl CpG binding protein 2
MK801	a NMDA receptor antagonist
mRNA	Messenger RNA
NDS	Normal donkey serum
NGS	Normal goat serum
NLS	Nuclear localised signal
NMDA	N-methyl-D-aspartate
NPAS4	Neuronal PAS domain protein 4
NR4a1	Nuclear receptor subfamily 4, group A, member 1
n.s.	not significant
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PKA	Protein kinase A
rAAV	Recombinant adeno-associated virus
pERK	phosphorylated extracellular signal-regulated kinase
PFC	Prefrontal cortex
preCS	Pre-conditioned stimulus
PP1	Protein phosphatase 1
PTSD	Post-traumatic stress disorder
qRT-PCR	Quantitative real time PCR
RNA	Ribonucleic acid
RT	Room temperature
SEM	Standard error of the mean
shRNA	Short hairpin RNA
SGG	Salt glucose glycine solution
STM	Short-term memory
TET1	Ten-eleven translocation methylcytosine dioxygenase 1
UNC	Universal negative control
VEGFD	Vascular endothelial growth factor D
VGCC	Voltage-gated calcium channel

References

Abel, T., Nguyen, P. V., Barad, M., Deuel, T. A., Kandel, E. R., and Bourtchouladze, R. (1997). Genetic demonstration of a role for pka in the late phase of ltp and in hippocampus-based long-term memory. *Cell*, 88(5):615–626.

Agis-Balboa, R. C., Arcos-Diaz, D., Wittnam, J., Govindarajan, N., Blom, K., Burkhardt, S., Haladyniak, U., Agbemenyah, H. Y., Zovoilis, A., Salinas-Riester, G., Opitz, L., Sananbenesi, F., and Fischer, A. (2011). A hippocampal insulin-growth factor 2 pathway regulates the extinction of fear memories. *EMBO J*, 30(19):4071–4083.

Alarcón, J. M., Malleret, G., Touzani, K., Vronskaya, S., Ishii, S., Kandel, E. R., and Barco, A. (2004). Chromatin acetylation, memory, and ltp are impaired in cbp+/- mice: a model for the cognitive deficit in rubinstein-taybi syndrome and its amelioration. *Neuron*, 42(6):947–959.

An, J. J., Gharami, K., Liao, G.-Y., Woo, N. H., Lau, A. G., Vanevski, F., Torre, E. R., Jones, K. R., Feng, Y., Lu, B., and Xu, B. (2008). Distinct role of long 3' utr bdnf mrna in spine morphology and synaptic plasticity in hippocampal neurons. *Cell*, 134(1):175–187.

Armstrong, D., Dunn, J. K., Antalffy, B., and Trivedi, R. (1995). Selective dendritic alterations in the cortex of rett syndrome. *J Neuropathol Exp Neurol*, 54(2):195–201.

Athos, J., Impey, S., Pineda, V. V., Chen, X., and Storm, D. R. (2002). Hippocampal cre-mediated gene expression is required for contextual memory formation. *Nat Neurosci*, 5(11):1119–1120.

Atkins, C. M., Selcher, J. C., Petraitis, J. J., Trzaskos, J. M., and Sweatt, J. D. (1998). The mapk cascade is required for mammalian associative learning. *Nat Neurosci*, 1(7):602–609.

Bach, M. E., Barad, M., Son, H., Zhuo, M., Lu, Y. F., Shih, R., Mansuy, I., Hawkins, R. D., and Kandel, E. R. (1999). Age-related defects in spatial memory are correlated with defects in the late phase of hippocampal long-term potentiation in vitro and are attenuated by drugs that enhance the camp signaling pathway. *Proc Natl Acad Sci U S A*, 96(9):5280–5285.

Bading, H. (2013). Nuclear calcium signalling in the regulation of brain function. *Nat Rev Neurosci*, 14(9):593–608.

Bading, H., Ginty, D. D., and Greenberg, M. E. (1993). Regulation of gene expression in hippocampal neurons by distinct calcium signaling pathways. *Science*, 260(5105):181–186.

Bading, H., Segal, M. M., Sucher, N. J., Dudek, H., Lipton, S. A., and Greenberg, M. E. (1995). N-methyl-d-aspartate receptors are critical for mediating the effects of glutamate on intracellular calcium concentration and immediate early gene expression in cultured hippocampal neurons. *Neuroscience*, 64(3):653–664.

Bahar, A., Samuel, A., Hazvi, S., and Dudai, Y. (2003). The amygdalar circuit that acquires taste aversion memory differs from the circuit that extinguishes it. *Eur J Neurosci*, 17(7):1527–1530.

Bahari-Javan, S., Maddalena, A., Kerimoglu, C., Wittnam, J., Held, T., Bähr, M., Burkhardt, S., Delalle, I., Kügler, S., Fischer, A., and Sananbenesi, F. (2012). Hdac1 regulates fear extinction in mice. *J Neurosci*, 32(15):5062–5073.

Baker, J. D. and Azorlosa, J. L. (1996). The nmda antagonist mk-801 blocks the extinction of pavlovian fear conditioning. *Behav Neurosci*, 110(3):618–620.

Baker-Andresen, D., Flavell, C. R., Li, X., and Bredy, T. W. (2013). Activation of bdnf signaling prevents the return of fear in female mice. *Learn Mem*, 20(5):237–240.

Barco, A., Alarcon, J. M., and Kandel, E. R. (2002). Expression of constitutively active creb protein facilitates the late phase of long-term potentiation by enhancing synaptic capture. *Cell*, 108(5):689–703.

Barco, A., Patterson, S. L., Patterson, S., Alarcon, J. M., Gromova, P., Mata-Roig, M., Morozov, A., and Kandel, E. R. (2005). Gene expression profiling of facilitated l-ltp in vp16-creb mice reveals that bdnf is critical for the maintenance of ltp and its synaptic capture. *Neuron*, 48(1):123–137.

Barondes, S. H. and Cohen, H. D. (1968). Memory impairment after subcutaneous injection of acetoxycycloheximide. *Science*, 160(3827):556–557.

Bauer, E. P., Schafe, G. E., and LeDoux, J. E. (2002). Nmda receptors and l-type voltage-gated calcium channels contribute to long-term potentiation and different components of fear memory formation in the lateral amygdala. *J Neurosci*, 22(12):5239–5249.

Bengtson, C. P. and Bading, H. (2012). Nuclear calcium signaling. *Adv Exp Med Biol*, 970:377–405.

Berman, D. E. and Dudai, Y. (2001). Memory extinction, learning anew, and learning the new: dissociations in the molecular machinery of learning in cortex. *Science*, 291(5512):2417–2419.

Bevilaqua, L. R., Bonini, J. S., Rossato, J. I., Izquierdo, L. A., Cammarota, M., and Izquierdo, I. (2006). The entorhinal cortex plays a role in extinction. *Neurobiol Learn Mem*, 85(2):192–197.

Blank, T., Nijholt, I., Kye, M.-J., Radulovic, J., and Spiess, J. (2003). Small-conductance, ca2+-activated k+ channel sk3 generates age-related memory and ltp deficits. *Nat Neurosci*, 6(9):911–912.

Bliss, T. V. and Collingridge, G. L. (1993). A synaptic model of memory: long-term potentiation in the hippocampus. *Nature*, 361(6407):31–39.

Bloodgood, B. L., Sharma, N., Browne, H. A., Trepman, A. Z., and Greenberg, M. E. (2013). The activity-dependent transcription factor npas4 regulates domain-specific inhibition. *Nature*, 503(7474):121–125.

Bollati, V., Galimberti, D., Pergoli, L., Valle, E. D., Barretta, F., Cortini, F., Scarpini, E., Bertazzi, P. A., and Baccarelli, A. (2011). Dna methylation in repetitive elements and alzheimer disease. *Brain Behav Immun*, 25(6):1078–1083.

Bourtchuladze, R., Frenguelli, B., Blendy, J., Cioffi, D., Schutz, G., and Silva, A. J. (1994). Deficient long-term memory in mice with a targeted mutation of the camp-responsive element-binding protein. *Cell*, 79(1):59–68.

Bouton, M. E. (1988). Context and ambiguity in the extinction of emotional learning: implications for exposure therapy. *Behav Res Ther*, 26(2):137–149.

Boyes, J. and Bird, A. (1991). Dna methylation inhibits transcription indirectly via a methyl-cpg binding protein. *Cell*, 64(6):1123–1134.

Bredy, T. W., Wu, H., Crego, C., Zellhoefer, J., Sun, Y. E., and Barad, M. (2007). Histone modifications around individual bdnf gene promoters in prefrontal cortex are associated with extinction of conditioned fear. *Learn Mem*, 14(4):268–276.

Buchthal, B., Lau, D., Weiss, U., Weislogel, J.-M., and Bading, H. (2012). Nuclear calcium signaling controls methyl-cpg-binding protein 2 (mecp2) phosphorylation on serine 421 following synaptic activity. *J Biol Chem*, 287(37):30967–30974.

Cain, C. K., Blouin, A. M., and Barad, M. (2002). L-type voltage-gated calcium channels are required for extinction, but not for acquisition or expression, of conditional fear in mice. *J Neurosci*, 22(20):9113–9121.

Chahrour, M., Jung, S. Y., Shaw, C., Zhou, X., Wong, S. T. C., Qin, J., and Zoghbi, H. Y. (2008). Mecp2, a key contributor to neurological disease, activates and represses transcription. *Science*, 320(5880):1224–1229.

Chapleau, C. A., Calfa, G. D., Lane, M. C., Albertson, A. J., Larimore, J. L., Kudo, S., Armstrong, D. L., Percy, A. K., and Pozzo-Miller, L. (2009). Dendritic spine pathologies in hippocampal pyramidal neurons from rett syndrome brain and after expression of rett-associated mecp2 mutations. *Neurobiol Dis*, 35(2):219–233.

Chawla, S., Hardingham, G. E., Quinn, D. R., and Bading, H. (1998). Cbp: a signal-regulated transcriptional coactivator controlled by nuclear calcium and cam kinase iv. *Science*, 281(5382):1505–1509.

Chen, T., Ueda, Y., Xie, S., and Li, E. (2002). A novel dnmt3a isoform produced from an alternative promoter localizes to euchromatin and its expression correlates with active de novo methylation. *J Biol Chem*, 277(41):38746–38754.

Chen, Y., Wang, Y., Ertürk, A., Kallop, D., Jiang, Z., Weimer, R. M., Kaminker, J., and Sheng,

M. (2014). Activity-induced nr4a1 regulates spine density and distribution pattern of excitatory synapses in pyramidal neurons. *Neuron*, 83(2):431–443.

Chhatwal, J. P., Stanek-Rattiner, L., Davis, M., and Ressler, K. J. (2006). Amygdala bdnf signaling is required for consolidation but not encoding of extinction. *Nat Neurosci*, 9(7):870–872.

Chouliaras, L., Mastroeni, D., Delvaux, E., Grover, A., Kenis, G., Hof, P. R., Steinbusch, H. W. M., Coleman, P. D., Rutten, B. P. F., and van den Hove, D. L. A. (2013). Consistent decrease in global dna methylation and hydroxymethylation in the hippocampus of alzheimer's disease patients. *Neurobiol Aging*, 34(9):2091–2099.

Clem, R. L. and Huganir, R. L. (2010). Calcium-permeable ampa receptor dynamics mediate fear memory erasure. *Science*, 330(6007):1108–1112.

Czerniawski, J., Ree, F., Chia, C., Ramamoorthi, K., Kumata, Y., and Otto, T. A. (2011). The importance of having arc: expression of the immediate-early gene arc is required for hippocampus-dependent fear conditioning and blocked by nmda receptor antagonism. *J Neurosci*, 31(31):11200–11207.

Dalton, G. L., Wang, Y. T., Floresco, S. B., and Phillips, A. G. (2008). Disruption of ampa receptor endocytosis impairs the extinction, but not acquisition of learned fear. *Neuropsychopharmacology*, 33(10):2416–2426.

Dash, P. K., Hochner, B., and Kandel, E. R. (1990). Injection of the camp-responsive element into the nucleus of aplysia sensory neurons blocks long-term facilitation. *Nature*, 345(6277):718–721.

Davis, S. E. and Bauer, E. P. (2012). L-type voltage-gated calcium channels in the basolateral amygdala are necessary for fear extinction. *J Neurosci*, 32(39):13582–13586.

Day, J. J., Childs, D., Guzman-Karlsson, M. C., Kibe, M., Moulden, J., Song, E., Tahir, A., and Sweatt, J. D. (2013). Dna methylation regulates associative reward learning. *Nat Neurosci*, 16(10):1445–1452.

Elgersma, Y., Fedorov, N. B., Ikonen, S., Choi, E. S., Elgersma, M., Carvalho, O. M., Giese, K. P., and Silva, A. J. (2002). Inhibitory autophosphorylation of camkii controls psd association, plasticity, and learning. *Neuron*, 36(3):493–505.

Falls, W. A., Miserendino, M. J., and Davis, M. (1992). Extinction of fear-potentiated startle: blockade by infusion of an nmda antagonist into the amygdala. *J Neurosci*, 12(3):854–863.

Feng, J., Chang, H., Li, E., and Fan, G. (2005). Dynamic expression of de novo dna methyltransferases dnmt3a and dnmt3b in the central nervous system. *J Neurosci Res*, 79(6):734–746.

Feng, J., Zhou, Y., Campbell, S. L., Le, T., Li, E., Sweatt, J. D., Silva, A. J., and Fan, G. (2010).

Dnmt1 and dnmt3a maintain dna methylation and regulate synaptic function in adult forebrain neurons. *Nat Neurosci*, 13(4):423–430.

Fischer, A., Radulovic, M., Schrick, C., Sananbenesi, F., Godovac-Zimmermann, J., and Radulovic, J. (2007). Hippocampal mek/erk signaling mediates extinction of contextual freezing behavior. *Neurobiol Learn Mem*, 87(1):149–158.

Fitzsimons, H. L., Schwartz, S., Given, F. M., and Scott, M. J. (2013). The histone deacetylase hdac4 regulates long-term memory in drosophila. *PLoS One*, 8(12):e83903.

Frey, U., Krug, M., Reymann, K. G., and Matthies, H. (1988). Anisomycin, an inhibitor of protein synthesis, blocks late phases of ltp phenomena in the hippocampal ca1 region in vitro. *Brain Res*, 452(1-2):57–65.

Fukushima, H., Maeda, R., Suzuki, R., Suzuki, A., Nomoto, M., Toyoda, H., Wu, L.-J., Xu, H., Zhao, M.-G., Ueda, K., Kitamoto, A., Mamiya, N., Yoshida, T., Homma, S., Masushige, S., Zhuo, M., and Kida, S. (2008). Upregulation of calcium/calmodulin-dependent protein kinase iv improves memory formation and rescues memory loss with aging. *J Neurosci*, 28(40):9910–9919.

Gass, P., Wolfer, D. P., Balschun, D., Rudolph, D., Frey, U., Lipp, H. P., and Schütz, G. (1998). Deficits in memory tasks of mice with creb mutations depend on gene dosage. *Learn Mem*, 5(4-5):274–288.

Giese, K. P., Fedorov, N. B., Filipkowski, R. K., and Silva, A. J. (1998). Autophosphorylation at thr286 of the alpha calcium-calmodulin kinase ii in ltp and learning. *Science*, 279(5352):870–873.

Giese, K. P. and Mizuno, K. (2013). The roles of protein kinases in learning and memory. *Learn Mem*, 20(10):540–552.

Goto, K., Numata, M., Komura, J. I., Ono, T., Bestor, T. H., and Kondo, H. (1994). Expression of dna methyltransferase gene in mature and immature neurons as well as proliferating cells in mice. *Differentiation*, 56(1-2):39–44.

Gräff, J., Rei, D., Guan, J.-S., Wang, W.-Y., Seo, J., Hennig, K. M., Nieland, T. J. F., Fass, D. M., Kao, P. F., Kahn, M., Su, S. C., Samiei, A., Joseph, N., Haggarty, S. J., Delalle, I., and Tsai, L.-H. (2012). An epigenetic blockade of cognitive functions in the neurodegenerating brain. *Nature*, 483(7388):222–226.

Guatimosim, S., Amaya, M. J., Guerra, M. T., Aguiar, C. J., Goes, A. M., Gómez-Viquez, N. L., Rodrigues, M. A., Gomes, D. A., Martins-Cruz, J., Lederer, W. J., and Leite, M. F. (2008). Nuclear ca2+ regulates cardiomyocyte function. *Cell Calcium*, 44(2):230–242.

Guzowski, J. F., Lyford, G. L., Stevenson, G. D., Houston, F. P., McGaugh, J. L., Worley, P. F., and Barnes, C. A. (2000). Inhibition of activity-dependent arc protein expression in the rat hippocampus

impairs the maintenance of long-term potentiation and the consolidation of long-term memory. *J Neurosci*, 20(11):3993–4001.

Hardingham, G. E., Arnold, F. J., and Bading, H. (2001). Nuclear calcium signaling controls creb-mediated gene expression triggered by synaptic activity. *Nat Neurosci*, 4(3):261–267.

Hardingham, G. E., Chawla, S., Cruzalegui, F. H., and Bading, H. (1999). Control of recruitment and transcription-activating function of cbp determines gene regulation by nmda receptors and l-type calcium channels. *Neuron*, 22(4):789–798.

Hardingham, G. E., Chawla, S., Johnson, C. M., and Bading, H. (1997). Distinct functions of nuclear and cytoplasmic calcium in the control of gene expression. *Nature*, 385(6613):260–265.

Heldt, S. A., Stanek, L., Chhatwal, J. P., and Ressler, K. J. (2007). Hippocampus-specific deletion of bdnf in adult mice impairs spatial memory and extinction of aversive memories. *Mol Psychiatry*, 12(7):656–670.

Hermann, A., Gowher, H., and Jeltsch, A. (2004). Biochemistry and biology of mammalian dna methyltransferases. *Cell Mol Life Sci*, 61(19-20):2571–2587.

Herry, C. and Mons, N. (2004). Resistance to extinction is associated with impaired immediate early gene induction in medial prefrontal cortex and amygdala. *Eur J Neurosci*, 20(3):781–790.

Horch, H. W., Krüttgen, A., Portbury, S. D., and Katz, L. C. (1999). Destabilization of cortical dendrites and spines by bdnf. *Neuron*, 23(2):353–364.

Humeau, Y., Reisel, D., Johnson, A. W., Borchardt, T., Jensen, V., Gebhardt, C., Bosch, V., Gass, P., Bannerman, D. M., Good, M. A., Øivind Hvalby, Sprengel, R., and Lüthi, A. (2007). A pathway-specific function for different ampa receptor subunits in amygdala long-term potentiation and fear conditioning. *J Neurosci*, 27(41):10947–10956.

Impey, S., Smith, D. M., Obrietan, K., Donahue, R., Wade, C., and Storm, D. R. (1998). Stimulation of camp response element (cre)-mediated transcription during contextual learning. *Nat Neurosci*, 1(7):595–601.

Itzhak, Y., Anderson, K. L., Kelley, J. B., and Petkov, M. (2012). Histone acetylation rescues contextual fear conditioning in nnos ko mice and accelerates extinction of cued fear conditioning in wild type mice. *Neurobiol Learn Mem*, 97(4):409–417.

Jeltsch, A. (2002). Beyond watson and crick: Dna methylation and molecular enzymology of dna methyltransferases. *Chembiochem*, 3(4):274–293.

Jones, P. L., Veenstra, G. J., Wade, P. A., Vermaak, D., Kass, S. U., Landsberger, N., Strouboulis, J., and Wolffe, A. P. (1998). Methylated dna and mecp2 recruit histone deacetylase to repress transcription. *Nat Genet*, 19(2):187–191.

Kaas, G. A., Zhong, C., Eason, D. E., Ross, D. L., Vachhani, R. V., Ming, G.-L., King, J. R., Song, H., and Sweatt, J. D. (2013). Tet1 controls cns 5-methylcytosine hydroxylation, active dna demethylation, gene transcription, and memory formation. *Neuron*, 79(6):1086–1093.

Kang, H., Sun, L. D., Atkins, C. M., Soderling, T. R., Wilson, M. A., and Tonegawa, S. (2001). An important role of neural activity-dependent camkiv signaling in the consolidation of long-term memory. *Cell*, 106(6):771–783.

Katche, C., Cammarota, M., and Medina, J. H. (2013). Molecular signatures and mechanisms of long-lasting memory consolidation and storage. *Neurobiol Learn Mem*, 106:40–47.

Kaufmann, W. E. and Moser, H. W. (2000). Dendritic anomalies in disorders associated with mental retardation. *Cereb Cortex*, 10(10):981–991.

Kida, S., Josselyn, S. A., Peña de Ortiz, S., Kogan, J. H., Chevere, I., Masushige, S., and Silva, A. J. (2002). Creb required for the stability of new and reactivated fear memories. *Nat Neurosci*, 5(4):348–355.

Kida, S. and Serita, T. (2014). Functional roles of creb as a positive regulator in the formation and enhancement of memory. *Brain Res Bull*, 105:17–24.

Kim, D., Nguyen, M. D., Dobbin, M. M., Fischer, A., Sananbenesi, F., Rodgers, J. T., Delalle, I., Baur, J. A., Sui, G., Armour, S. M., Puigserver, P., Sinclair, D. A., and Tsai, L.-H. (2007). Sirt1 deacetylase protects against neurodegeneration in models for alzheimer's disease and amyotrophic lateral sclerosis. *EMBO J*, 26(13):3169–3179.

Kim, J. J., DeCola, J. P., Landeira-Fernandez, J., and Fanselow, M. S. (1991). N-methyl-d-aspartate receptor antagonist apv blocks acquisition but not expression of fear conditioning. *Behav Neurosci*, 105(1):126–133.

Kim, M.-S., Akhtar, M. W., Adachi, M., Mahgoub, M., Bassel-Duby, R., Kavalali, E. T., Olson, E. N., and Monteggia, L. M. (2012). An essential role for histone deacetylase 4 in synaptic plasticity and memory formation. *J Neurosci*, 32(32):10879–10886.

Kimura, R., Silva, A. J., and Ohno, M. (2008). Autophosphorylation of alphacamkii is differentially involved in new learning and unlearning mechanisms of memory extinction. *Learn Mem*, 15(11):837–843.

Koleske, A. J. (2013). Molecular mechanisms of dendrite stability. *Nat Rev Neurosci*, 14(8):536–550.

Korzus, E., Rosenfeld, M. G., and Mayford, M. (2004). Cbp histone acetyltransferase activity is a critical component of memory consolidation. *Neuron*, 42(6):961–972.

Krug, M., Lössner, B., and Ott, T. (1984). Anisomycin blocks the late phase of long-term potentiation in the dentate gyrus of freely moving rats. *Brain Res Bull*, 13(1):39–42.

Kulkarni, V. A. and Firestein, B. L. (2012). The dendritic tree and brain disorders. *Mol Cell Neurosci*, 50(1):10–20.

Lattal, K. M. and Abel, T. (2001). Different requirements for protein synthesis in acquisition and extinction of spatial preferences and context-evoked fear. *J Neurosci*, 21(15):5773–5780.

Lattal, K. M., Barrett, R. M., and Wood, M. A. (2007). Systemic or intrahippocampal delivery of histone deacetylase inhibitors facilitates fear extinction. *Behav Neurosci*, 121(5):1125–1131.

Lattal, K. M., Radulovic, J., and Lukowiak, K. (2006). Extinction: [corrected] does it or doesn't it? the requirement of altered gene activity and new protein synthesis. *Biol Psychiatry*, 60(4):344–351.

Leach, P. T., Poplawski, S. G., Kenney, J. W., Hoffman, B., Liebermann, D. A., Abel, T., and Gould, T. J. (2012). Gadd45b knockout mice exhibit selective deficits in hippocampus-dependent long-term memory. *Learn Mem*, 19(8):319–324.

Lee, H.-K., Takamiya, K., Han, J.-S., Man, H., Kim, C.-H., Rumbaugh, G., Yu, S., Ding, L., He, C., Petralia, R. S., Wenthold, R. J., Gallagher, M., and Huganir, R. L. (2003). Phosphorylation of the ampa receptor glur1 subunit is required for synaptic plasticity and retention of spatial memory. *Cell*, 112(5):631–643.

Leuner, B., Falduto, J., and Shors, T. J. (2003). Associative memory formation increases the observation of dendritic spines in the hippocampus. *J Neurosci*, 23(2):659–665.

Levenson, J. M., Roth, T. L., Lubin, F. D., Miller, C. A., Huang, I.-C., Desai, P., Malone, L. M., and Sweatt, J. D. (2006). Evidence that dna (cytosine-5) methyltransferase regulates synaptic plasticity in the hippocampus. *J Biol Chem*, 281(23):15763–15773.

Li, B., Jie, W., Huang, L., Wei, P., Li, S., Luo, Z., Friedman, A. K., Meredith, A. L., Han, M.-H., Zhu, X.-H., and Gao, T.-M. (2014a). Nuclear bk channels regulate gene expression via the control of nuclear calcium signaling. *Nat Neurosci*, 17(8):1055–1063.

Li, X., Wei, W., Zhao, Q.-Y., Widagdo, J., Baker-Andresen, D., Flavell, C. R., D'Alessio, A., Zhang, Y., and Bredy, T. W. (2014b). Neocortical tet3-mediated accumulation of 5-hydroxymethylcytosine promotes rapid behavioral adaptation. *Proc Natl Acad Sci U S A*, 111(19):7120–7125.

Limbäck-Stokin, K., Korzus, E., Nagaoka-Yasuda, R., and Mayford, M. (2004). Nuclear calcium/calmodulin regulates memory consolidation. *J Neurosci*, 24(48):10858–10867.

Lin, C.-H., Yeh, S.-H., Lu, H.-Y., and Gean, P.-W. (2003). The similarities and diversities of signal pathways leading to consolidation of conditioning and consolidation of extinction of fear memory. *J Neurosci*, 23(23):8310–8317.

Linnarsson, S., Björklund, A., and Ernfors, P. (1997). Learning deficit in bdnf mutant mice. *Eur J Neurosci*, 9(12):2581–2587.

Lipp, H. P. and Wolfer, D. P. (1998). Genetically modified mice and cognition. *Curr Opin Neurobiol*, 8(2):272–280.

Lockett, G. A., Helliwell, P., and Maleszka, R. (2010). Involvement of dna methylation in memory processing in the honey bee. *Neuroreport*, 21(12):812–816.

Longair, M. H., Baker, D. A., and Armstrong, J. D. (2011). Simple neurite tracer: open source software for reconstruction, visualization and analysis of neuronal processes. *Bioinformatics*, 27(17):2453–2454.

Lu, K. T., Walker, D. L., and Davis, M. (2001). Mitogen-activated protein kinase cascade in the basolateral nucleus of amygdala is involved in extinction of fear-potentiated startle. *J Neurosci*, 21(16):RC162.

Lyford, G. L., Yamagata, K., Kaufmann, W. E., Barnes, C. A., Sanders, L. K., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., Lanahan, A. A., and Worley, P. F. (1995). Arc, a growth factor and activity-regulated gene, encodes a novel cytoskeleton-associated protein that is enriched in neuronal dendrites. *Neuron*, 14(2):433–445.

Ma, H., Groth, R. D., Cohen, S. M., Emery, J. F., Li, B., Hoedt, E., Zhang, G., Neubert, T. A., and Tsien, R. W. (2014). γcamkii shuttles ca^{2+}/cam to the nucleus to trigger creb phosphorylation and gene expression. *Cell*, 159(2):281–294.

Malkani, S. and Rosen, J. B. (2000). Induction of ngfi-b mrna following contextual fear conditioning and its blockade by diazepam. *Brain Res Mol Brain Res*, 80(2):153–165.

Mamiya, N., Fukushima, H., Suzuki, A., Matsuyama, Z., Homma, S., Frankland, P. W., and Kida, S. (2009). Brain region-specific gene expression activation required for reconsolidation and extinction of contextual fear memory. *J Neurosci*, 29(2):402–413.

Margot, J. B., Ehrenhofer-Murray, A. E., and Leonhardt, H. (2003). Interactions within the mammalian dna methyltransferase family. *BMC Mol Biol*, 4:7.

Mastroeni, D., Grover, A., Delvaux, E., Whiteside, C., Coleman, P. D., and Rogers, J. (2010). Epigenetic changes in alzheimer's disease: decrements in dna methylation. *Neurobiol Aging*, 31(12):2025–2037.

Matsuda, S., Matsuzawa, D., Nakazawa, K., Sutoh, C., Ohtsuka, H., Ishii, D., Tomizawa, H., Iyo, M., and Shimizu, E. (2010). d-serine enhances extinction of auditory cued fear conditioning via erk1/2 phosphorylation in mice. *Prog Neuropsychopharmacol Biol Psychiatry*, 34(6):895–902.

Mauceri, D., Freitag, H. E., Oliveira, A. M. M., Bengtson, C. P., and Bading, H. (2011). Nuclear calcium-vegfd signaling controls maintenance of dendrite arborization necessary for memory formation. *Neuron*, 71(1):117–130.

Mayer, M. L., Westbrook, G. L., and Guthrie, P. B. (1984). Voltage-dependent block by mg2+ of nmda responses in spinal cord neurones. *Nature*, 309(5965):261–263.

Mayford, M., Bach, M. E., Huang, Y. Y., Wang, L., Hawkins, R. D., and Kandel, E. R. (1996). Control of memory formation through regulated expression of a camkii transgene. *Science*, 274(5293):1678–1683.

McKinney, B. C. and Murphy, G. G. (2006). The l-type voltage-gated calcium channel cav1.3 mediates consolidation, but not extinction, of contextually conditioned fear in mice. *Learn Mem*, 13(5):584–589.

McKinney, B. C., Sze, W., White, J. A., and Murphy, G. G. (2008). L-type voltage-gated calcium channels in conditioned fear: a genetic and pharmacological analysis. *Learn Mem*, 15(5):326–334.

Mesches, M. H., Bianchin, M., and McGaugh, J. L. (1996). The effects of intra-amygdala infusion of the ampa receptor antagonist cnqx on retention performance following aversive training. *Neurobiol Learn Mem*, 66(3):324–340.

Migues, P. V., Hardt, O., Wu, D. C., Gamache, K., Sacktor, T. C., Wang, Y. T., and Nader, K. (2010). Pkmzeta maintains memories by regulating glur2-dependent ampa receptor trafficking. *Nat Neurosci*, 13(5):630–634.

Miller, C. A., Gavin, C. F., White, J. A., Parrish, R. R., Honasoge, A., Yancey, C. R., Rivera, I. M., Rubio, M. D., Rumbaugh, G., and Sweatt, J. D. (2010). Cortical dna methylation maintains remote memory. *Nat Neurosci*, 13(6):664–666.

Miller, C. A. and Sweatt, J. D. (2007). Covalent modification of dna regulates memory formation. *Neuron*, 53(6):857–869.

Mizrahi, A. and Katz, L. C. (2003). Dendritic stability in the adult olfactory bulb. *Nat Neurosci*, 6(11):1201–1207.

Monsey, M. S., Ota, K. T., Akingbade, I. F., Hong, E. S., and Schafe, G. E. (2011). Epigenetic alterations are critical for fear memory consolidation and synaptic plasticity in the lateral amygdala. *PLoS One*, 6(5):e19958.

Montarolo, P. G., Goelet, P., Castellucci, V. F., Morgan, J., Kandel, E. R., and Schacher, S. (1986). A critical period for macromolecular synthesis in long-term heterosynaptic facilitation in aplysia. *Science*, 234(4781):1249–1254.

Moosmang, S., Haider, N., Klugbauer, N., Adelsberger, H., Langwieser, N., Müller, J., Stiess, M., Marais, E., Schulla, V., Lacinova, L., Goebbels, S., Nave, K.-A., Storm, D. R., Hofmann, F., and Kleppisch, T. (2005). Role of hippocampal cav1.2 ca2+ channels in nmda receptor-independent synaptic plasticity and spatial memory. *J Neurosci*, 25(43):9883–9892.

Moretti, P., Levenson, J. M., Battaglia, F., Atkinson, R., Teague, R., Antalffy, B., Armstrong, D., Arancio, O., Sweatt, J. D., and Zoghbi, H. Y. (2006). Learning and memory and synaptic plasticity are impaired in a mouse model of rett syndrome. *J Neurosci*, 26(1):319–327.

Morris, M. J., Adachi, M., Na, E. S., and Monteggia, L. M. (2014). Selective role for dnmt3a in learning and memory. *Neurobiol Learn Mem*.

Morris, R. G., Anderson, E., Lynch, G. S., and Baudry, M. (1986). Selective impairment of learning and blockade of long-term potentiation by an n-methyl-d-aspartate receptor antagonist, ap5. *Nature*, 319(6056):774–776.

Métivier, R., Gallais, R., Tiffoche, C., Péron, C. L., Jurkowska, R. Z., Carmouche, R. P., Ibberson, D., Barath, P., Demay, F., Reid, G., Benes, V., Jeltsch, A., Gannon, F., and Salbert, G. (2008). Cyclical dna methylation of a transcriptionally active promoter. *Nature*, 452(7183):45–50.

Myskiw, J. C., Fiorenza, N. G., Izquierdo, L. A., and Izquierdo, I. (2010). Molecular mechanisms in hippocampus and basolateral amygdala but not in parietal or cingulate cortex are involved in extinction of one-trial avoidance learning. *Neurobiol Learn Mem*, 94(2):285–291.

Nan, X., Ng, H. H., Johnson, C. A., Laherty, C. D., Turner, B. M., Eisenman, R. N., and Bird, A. (1998). Transcriptional repression by the methyl-cpg-binding protein mecp2 involves a histone deacetylase complex. *Nature*, 393(6683):386–389.

Nowak, L., Bregestovski, P., Ascher, P., Herbet, A., and Prochiantz, A. (1984). Magnesium gates glutamate-activated channels in mouse central neurones. *Nature*, 307(5950):462–465.

Oike, Y., Hata, A., Mamiya, T., Kaname, T., Noda, Y., Suzuki, M., Yasue, H., Nabeshima, T., Araki, K., and Yamamura, K. (1999). Truncated cbp protein leads to classical rubinstein-taybi syndrome phenotypes in mice: implications for a dominant-negative mechanism. *Hum Mol Genet*, 8(3):387–396.

Oliveira, A. M. M., Hemstedt, T. J., and Bading, H. (2012). Rescue of aging-associated decline in dnmt3a2 expression restores cognitive abilities. *Nat Neurosci*, 15(8):1111–1113.

Oliveira, A. M. M., Wood, M. A., McDonough, C. B., and Abel, T. (2007). Transgenic mice expressing an inhibitory truncated form of p300 exhibit long-term memory deficits. *Learn Mem*, 14(9):564–572.

Orefice, L. L., Waterhouse, E. G., Partridge, J. G., Lalchandani, R. R., Vicini, S., and Xu, B. (2013). Distinct roles for somatically and dendritically synthesized brain-derived neurotrophic factor in morphogenesis of dendritic spines. *J Neurosci*, 33(28):11618–11632.

Orsini, C. A. and Maren, S. (2012). Neural and cellular mechanisms of fear and extinction memory formation. *Neurosci Biobehav Rev*, 36(7):1773–1802.

Patel, S. N. and Stewart, M. G. (1988). Changes in the number and structure of dendritic spines 25 hours after passive avoidance training in the domestic chick, gallus domesticus. *Brain Res*, 449(1-2):34–46.

Peebles, C. L., Yoo, J., Thwin, M. T., Palop, J. J., Noebels, J. L., and Finkbeiner, S. (2010). Arc regulates spine morphology and maintains network stability in vivo. *Proc Natl Acad Sci U S A*, 107(42):18173–18178.

Penner, M. R., Roth, T. L., Chawla, M. K., Hoang, L. T., Roth, E. D., Lubin, F. D., Sweatt, J. D., Worley, P. F., and Barnes, C. A. (2011). Age-related changes in arc transcription and dna methylation within the hippocampus. *Neurobiol Aging*, 32(12):2198–2210.

Peters, J., Dieppa-Perea, L. M., Melendez, L. M., and Quirk, G. J. (2010). Induction of fear extinction with hippocampal-infralimbic bdnf. *Science*, 328(5983):1288–1290.

Pittenger, C., Huang, Y. Y., Paletzki, R. F., Bourtchouladze, R., Scanlin, H., Vronskaya, S., and Kandel, E. R. (2002). Reversible inhibition of creb/atf transcription factors in region ca1 of the dorsal hippocampus disrupts hippocampus-dependent spatial memory. *Neuron*, 34(3):447–462.

Plath, N., Ohana, O., Dammermann, B., Errington, M. L., Schmitz, D., Gross, C., Mao, X., Engelsberg, A., Mahlke, C., Welzl, H., Kobalz, U., Stawrakakis, A., Fernandez, E., Waltereit, R., Bick-Sander, A., Therstappen, E., Cooke, S. F., Blanquet, V., Wurst, W., Salmen, B., Bösl, M. R., Lipp, H.-P., Grant, S. G. N., Bliss, T. V. P., Wolfer, D. P., and Kuhl, D. (2006). Arc/arg3.1 is essential for the consolidation of synaptic plasticity and memories. *Neuron*, 52(3):437–444.

Pusl, T., Wu, J. J., Zimmerman, T. L., Zhang, L., Ehrlich, B. E., Berchtold, M. W., Hoek, J. B., Karpen, S. J., Nathanson, M. H., and Bennett, A. M. (2002). Epidermal growth factor-mediated activation of the ets domain transcription factor elk-1 requires nuclear calcium. *J Biol Chem*, 277(30):27517–27527.

Ramamoorthi, K., Fropf, R., Belfort, G. M., Fitzmaurice, H. L., McKinney, R. M., Neve, R. L., Otto, T., and Lin, Y. (2011). Npas4 regulates a transcriptional program in ca3 required for contextual memory formation. *Science*, 334(6063):1669–1675.

Reisel, D., Bannerman, D. M., Schmitt, W. B., Deacon, R. M. J., Flint, J., Borchardt, T., Seeburg, P. H., and Rawlins, J. N. P. (2002). Spatial memory dissociations in mice lacking glur1. *Nat Neurosci*, 5(9):868–873.

Robertson, K. D., Uzvolgyi, E., Liang, G., Talmadge, C., Sumegi, J., Gonzales, F. A., and Jones, P. A. (1999). The human dna methyltransferases (dnmts) 1, 3a and 3b: coordinate mrna expression in normal tissues and overexpression in tumors. *Nucleic Acids Res*, 27(11):2291–2298.

Rodrigues, M. A., Gomes, D. A., Leite, M. F., Grant, W., Zhang, L., Lam, W., Cheng, Y.-C., Bennett,

A. M., and Nathanson, M. H. (2007). Nucleoplasmic calcium is required for cell proliferation. *J Biol Chem*, 282(23):17061–17068.

Rosen, L. B., Ginty, D. D., Weber, M. J., and Greenberg, M. E. (1994). Membrane depolarization and calcium influx stimulate mek and map kinase via activation of ras. *Neuron*, 12(6):1207–1221.

Rouaux, C., Jokic, N., Mbebi, C., Boutillier, S., Loeffler, J.-P., and Boutillier, A.-L. (2003). Critical loss of cbp/p300 histone acetylase activity by caspase-6 during neurodegeneration. *EMBO J*, 22(24):6537–6549.

Rudenko, A., Dawlaty, M. M., Seo, J., Cheng, A. W., Meng, J., Le, T., Faull, K. F., Jaenisch, R., and Tsai, L.-H. (2013). Tet1 is critical for neuronal activity-regulated gene expression and memory extinction. *Neuron*, 79(6):1109–1122.

Ryu, J., Futai, K., Feliu, M., Weinberg, R., and Sheng, M. (2008). Constitutively active rap2 transgenic mice display fewer dendritic spines, reduced extracellular signal-regulated kinase signaling, enhanced long-term depression, and impaired spatial learning and fear extinction. *J Neurosci*, 28(33):8178–8188.

Sananbenesi, F., Fischer, A., Wang, X., Schrick, C., Neve, R., Radulovic, J., and Tsai, L.-H. (2007). A hippocampal cdk5 pathway regulates extinction of contextual fear. *Nat Neurosci*, 10(8):1012–1019.

Sando, R., Gounko, N., Pieraut, S., Liao, L., Yates, J., and Maximov, A. (2012). Hdac4 governs a transcriptional program essential for synaptic plasticity and memory. *Cell*, 151(4):821–834.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J.-Y., White, D. J., Hartenstein, V., Eliceiri, K., Tomancak, P., and Cardona, A. (2012). Fiji: an open-source platform for biological-image analysis. *Nat Methods*, 9(7):676–682.

Schlumm, F., Mauceri, D., Freitag, H. E., and Bading, H. (2013). Nuclear calcium signaling regulates nuclear export of a subset of class iia histone deacetylases following synaptic activity. *J Biol Chem*, 288(12):8074–8084.

SHOLL, D. A. (1953). Dendritic organization in the neurons of the visual and motor cortices of the cat. *J Anat*, 87(4):387–406.

Siegmund, K. D., Connor, C. M., Campan, M., Long, T. I., Weisenberger, D. J., Biniszkiewicz, D., Jaenisch, R., Laird, P. W., and Akbarian, S. (2007). Dna methylation in the human cerebral cortex is dynamically regulated throughout the life span and involves differentiated neurons. *PLoS One*, 2(9):e895.

Silva, A. J., Paylor, R., Wehner, J. M., and Tonegawa, S. (1992). Impaired spatial learning in alpha-calcium-calmodulin kinase ii mutant mice. *Science*, 257(5067):206–211.

Simonetti, M., Hagenston, A. M., Vardeh, D., Freitag, H. E., Mauceri, D., Lu, J., Satagopam, V. P., Schneider, R., Costigan, M., Bading, H., and Kuner, R. (2013). Nuclear calcium signaling in spinal neurons drives a genomic program required for persistent inflammatory pain. *Neuron*, 77(1):43–57.

Sindreu, C. B., Scheiner, Z. S., and Storm, D. R. (2007). Ca2+ -stimulated adenylyl cyclases regulate erk-dependent activation of msk1 during fear conditioning. *Neuron*, 53(1):79–89.

Soliman, F., Glatt, C. E., Bath, K. G., Levita, L., Jones, R. M., Pattwell, S. S., Jing, D., Tottenham, N., Amso, D., Somerville, L. H., Voss, H. U., Glover, G., Ballon, D. J., Liston, C., Teslovich, T., Kempen, T. V., Lee, F. S., and Casey, B. J. (2010). A genetic variant bdnf polymorphism alters extinction learning in both mouse and human. *Science*, 327(5967):863–866.

Sui, L., Wang, Y., Ju, L.-H., and Chen, M. (2012). Epigenetic regulation of reelin and brain-derived neurotrophic factor genes in long-term potentiation in rat medial prefrontal cortex. *Neurobiol Learn Mem*, 97(4):425–440.

Sultan, F. A., Wang, J., Tront, J., Liebermann, D. A., and Sweatt, J. D. (2012). Genetic deletion of gadd45b, a regulator of active dna demethylation, enhances long-term memory and synaptic plasticity. *J Neurosci*, 32(48):17059–17066.

Suzuki, A., Fukushima, H., Mukawa, T., Toyoda, H., Wu, L.-J., Zhao, M.-G., Xu, H., Shang, Y., Endoh, K., Iwamoto, T., Mamiya, N., Okano, E., Hasegawa, S., Mercaldo, V., Zhang, Y., Maeda, R., Ohta, M., Josselyn, S. A., Zhuo, M., and Kida, S. (2011). Upregulation of creb-mediated transcription enhances both short- and long-term memory. *J Neurosci*, 31(24):8786–8802.

Suzuki, A., Josselyn, S. A., Frankland, P. W., Masushige, S., Silva, A. J., and Kida, S. (2004). Memory reconsolidation and extinction have distinct temporal and biochemical signatures. *J Neurosci*, 24(20):4787–4795.

Szapiro, G., Vianna, M. R. M., McGaugh, J. L., Medina, J. H., and Izquierdo, I. (2003). The role of nmda glutamate receptors, pka, mapk, and camkii in the hippocampus in extinction of conditioned fear. *Hippocampus*, 13(1):53–58.

Szyf, M., Bozovic, V., and Tanigawa, G. (1991). Growth regulation of mouse dna methyltransferase gene expression. *J Biol Chem*, 266(16):10027–10030.

Tang, Y. P., Shimizu, E., Dube, G. R., Rampon, C., Kerchner, G. A., Zhuo, M., Liu, G., and Tsien, J. Z. (1999). Genetic enhancement of learning and memory in mice. *Nature*, 401(6748):63–69.

Tronson, N. C., Schrick, C., Guzman, Y. F., Huh, K. H., Srivastava, D. P., Penzes, P., Guedea, A. L., Gao, C., and Radulovic, J. (2009). Segregated populations of hippocampal principal ca1 neurons mediating conditioning and extinction of contextual fear. *J Neurosci*, 29(11):3387–3394.

Turek-Plewa, J. and Jagodzi?ski, P. P. (2005). The role of mammalian dna methyltransferases in the regulation of gene expression. *Cell Mol Biol Lett*, 10(4):631–647.

Vianna, M. R., Igaz, L. M., Coitinho, A. S., Medina, J. H., and Izquierdo, I. (2003). Memory extinction requires gene expression in rat hippocampus. *Neurobiol Learn Mem*, 79(3):199–203.

Vianna, M. R., Szapiro, G., McGaugh, J. L., Medina, J. H., and Izquierdo, I. (2001). Retrieval of memory for fear-motivated training initiates extinction requiring protein synthesis in the rat hippocampus. *Proc Natl Acad Sci U S A*, 98(21):12251–12254.

Viosca, J., Malleret, G., Bourtchouladze, R., Benito, E., Vronskava, S., Kandel, E. R., and Barco, A. (2009). Chronic enhancement of creb activity in the hippocampus interferes with the retrieval of spatial information. *Learn Mem*, 16(3):198–209.

von Hertzen, L. S. J. and Giese, K. P. (2005). Memory reconsolidation engages only a subset of immediate-early genes induced during consolidation. *J Neurosci*, 25(8):1935–1942.

Walker, D. L., Ressler, K. J., Lu, K.-T., and Davis, M. (2002). Facilitation of conditioned fear extinction by systemic administration or intra-amygdala infusions of d-cycloserine as assessed with fear-potentiated startle in rats. *J Neurosci*, 22(6):2343–2351.

Wang, Z., Phan, T., and Storm, D. R. (2011). The type 3 adenylyl cyclase is required for novel object learning and extinction of contextual memory: role of camp signaling in primary cilia. *J Neurosci*, 31(15):5557–5561.

Watanabe, D., Suetake, I., Tada, T., and Tajima, S. (2002). Stage- and cell-specific expression of dnmt3a and dnmt3b during embryogenesis. *Mech Dev*, 118(1-2):187–190.

Watt, F. and Molloy, P. L. (1988). Cytosine methylation prevents binding to dna of a hela cell transcription factor required for optimal expression of the adenovirus major late promoter. *Genes Dev*, 2(9):1136–1143.

Wei, F., Qiu, C.-S., Liauw, J., Robinson, D. A., Ho, N., Chatila, T., and Zhuo, M. (2002). Calcium calmodulin-dependent protein kinase iv is required for fear memory. *Nat Neurosci*, 5(6):573–579.

Weislogel, J.-M., Bengtson, C. P., Müller, M. K., Hörtzsch, J. N., Bujard, M., Schuster, C. M., and Bading, H. (2013). Requirement for nuclear calcium signaling in drosophila long-term memory. *Sci Signal*, 6(274):ra33.

West, A. E., Griffith, E. C., and Greenberg, M. E. (2002). Regulation of transcription factors by neuronal activity. *Nat Rev Neurosci*, 3(12):921–931.

White, J. A., McKinney, B. C., John, M. C., Powers, P. A., Kamp, T. J., and Murphy, G. G. (2008). Conditional forebrain deletion of the l-type calcium channel ca v 1.2 disrupts remote spatial memories in mice. *Learn Mem*, 15(1):1–5.

Wilson, V. L., Smith, R. A., Ma, S., and Cutler, R. G. (1987). Genomic 5-methyldeoxycytidine decreases with age. *J Biol Chem*, 262(21):9948–9951.

Wong, S. T., Athos, J., Figueroa, X. A., Pineda, V. V., Schaefer, M. L., Chavkin, C. C., Muglia, L. J., and Storm, D. R. (1999). Calcium-stimulated adenylyl cyclase activity is critical for hippocampus-dependent long-term memory and late phase ltp. *Neuron*, 23(4):787–798.

Wood, M. A., Attner, M. A., Oliveira, A. M. M., Brindle, P. K., and Abel, T. (2006). A transcription factor-binding domain of the coactivator cbp is essential for long-term memory and the expression of specific target genes. *Learn Mem*, 13(5):609–617.

Wu, H., Coskun, V., Tao, J., Xie, W., Ge, W., Yoshikawa, K., Li, E., Zhang, Y., and Sun, Y. E. (2010). Dnmt3a-dependent nonpromoter dna methylation facilitates transcription of neurogenic genes. *Science*, 329(5990):444–448.

Wu, Z. L., Thomas, S. A., Villacres, E. C., Xia, Z., Simmons, M. L., Chavkin, C., Palmiter, R. D., and Storm, D. R. (1995). Altered behavior and long-term potentiation in type i adenylyl cyclase mutant mice. *Proc Natl Acad Sci U S A*, 92(1):220–224.

Xie, S., Wang, Z., Okano, M., Nogami, M., Li, Y., He, W. W., Okumura, K., and Li, E. (1999). Cloning, expression and chromosome locations of the human dnmt3 gene family. *Gene*, 236(1):87–95.

Yang, G., Pan, F., and Gan, W.-B. (2009). Stably maintained dendritic spines are associated with lifelong memories. *Nature*, 462(7275):920–924.

Yen, R. W., Vertino, P. M., Nelkin, B. D., Yu, J. J., el Deiry, W., Cumaraswamy, A., Lennon, G. G., Trask, B. J., Celano, P., and Baylin, S. B. (1992). Isolation and characterization of the cdna encoding human dna methyltransferase. *Nucleic Acids Res*, 20(9):2287–2291.

Zhang, R.-R., Cui, Q.-Y., Murai, K., Lim, Y. C., Smith, Z. D., Jin, S., Ye, P., Rosa, L., Lee, Y. K., Wu, H.-P., Liu, W., Xu, Z.-M., Yang, L., Ding, Y.-Q., Tang, F., Meissner, A., Ding, C., Shi, Y., and Xu, G.-L. (2013). Tet1 regulates adult hippocampal neurogenesis and cognition. *Cell Stem Cell*, 13(2):237–245.

Zhang, S.-J., Zou, M., Lu, L., Lau, D., Ditzel, D. A. W., Delucinge-Vivier, C., Aso, Y., Descombes, P., and Bading, H. (2009). Nuclear calcium signaling controls expression of a large gene pool: identification of a gene program for acquired neuroprotection induced by synaptic activity. *PLoS Genet*, 5(8):e1000604.

Zhao, X., Ueba, T., Christie, B. R., Barkho, B., McConnell, M. J., Nakashima, K., Lein, E. S., Eadie, B. D., Willhoite, A. R., Muotri, A. R., Summers, R. G., Chun, J., Lee, K.-F., and Gage, F. H. (2003). Mice lacking methyl-cpg binding protein 1 have deficits in adult neurogenesis and hippocampal function. *Proc Natl Acad Sci U S A*, 100(11):6777–6782.

Zhou, Z., Hong, E. J., Cohen, S., Zhao, W.-N., Ho, H.-Y. H., Schmidt, L., Chen, W. G., Lin, Y., Savner, E., Griffith, E. C., Hu, L., Steen, J. A. J., Weitz, C. J., and Greenberg, M. E. (2006). Brain-specific phosphorylation of mecp2 regulates activity-dependent bdnf transcription, dendritic growth, and spine maturation. *Neuron*, 52(2):255–269.