

Role of Histone Methylation in Cognition and Effects of Different Durations of Environmental Enrichment on Learning and Memory

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Declaration

I herewith declare that I have prepared the dissertation “Role of Histone Methylation in Cognition and Effects of Different Durations of Environmental Enrichment on Learning and Memory” entirely by myself with no other aids and sources than quoted.

Goettingen, 24.02.12

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SUMMARY

Part 1

Formation of stable long-term memories requires novel gene expression, and chromatin modifications have been shown to be involved in mediating the de novo gene expression which is necessary for memory formation. Although the role of histone acetylation and different histone deacetylases (HDACs) and histone acetyltransferases (HATs) in learning and memory has been studied quite extensively, the knowledge concerning the role of histone methylation – another important histone modification – in learning and memory is lacking.

I have characterized the transgenic mouse line in which the *Mll2* gene is conditionally knocked down at adult stage in the forebrain excitatory neurons by the Cre-LoxP system, with Cre recombinase being expressed under CamKII promoter in order to ensure recombination specifically in forebrain glutamatergic neurons knock-out mice being denoted as “F/F CKII” hereafter). I have established that the knock-out (F/F CKII) mice have deficits in learning and memory assessed by novel object recognition, fear conditioning and water maze tests. In addition to that the F/F CKII mice were observed to have increased anxiety assessed by open field and elevated plus maze tests. Both males and females showed the same phenotype in these tests, and there was no genotype vs sex interaction. Moreover, DNA microarray revealed that the KO mice exhibit downregulation in 153 genes in dorsal dentate gyrus when compared to the controls. Interestingly such an extensive downregulation of gene expression is not observed in the dorsal CA region of the hippocampus, which partially may be explained by the observation that *Mll2* expression is slightly higher in the dentate gyrus than in the CA in wild-type mice to begin with. Many of the genes downregulated in KO mice play roles in synaptic plasticity and synaptic transmission which corroborates the behavioural findings. Moreover, among genes selected for qRT-PCR confirmation some of them – *Rab38*, *Car4*, *Adcy5*, *Gabrg3* and *Tpm4* – did not show downregulation in the ventral dentate gyrus of F/F CKII mice. This decrease in gene expression in the dentate gyrus of F/F CKII mice was accompanied by a decrease Histone 3 Lysine 4 tri and dimethylation *but not* in monomethylation at gene promoters. And it was observed that acute SAHA injection did not lead to an increase in expression of those genes neither in control (F/F) nor in F/F CKII mice.

Part 2

In the second part of my PhD work I investigated the effects of different durations of environmental enrichment (EE) on learning and memory. Although it is firmly established that environmental enrichment facilitates learning and memory, not much is known whether its effects would persist once enriched environment is removed and mice are returned to conventional housing.

I have found out that environmental enrichment operates in three stages. If its duration does not exceed a certain level EE does not improve learning at all – this was observed with 1 week of EE where mice did not show any learning improvement. After a certain level of duration EE begins to exert positive effects on learning – 2 weeks of EE were enough to facilitate learning. However, the most interesting observation was that once mice were put back into standard housing after 2 weeks of EE the learning enhancement mediated by prior enrichment soon faded away. This was however not the case if mice were enriched for a much longer time – i.e. the positive effects of 10 weeks of EE persisted for at least up to 2 months after enrichment was ceased. These different levels of persistence were accompanied by differences in gene expression that short-term (2 weeks) and long-term (10 weeks) EE mediated; long-term EE mediated a robust change in gene expression with 62 genes up and 69 genes downregulated, however 2 weeks of EE failed to exert such a drastic effect.

ABBREVIATIONS

Nkap1 – NFkB activating protein-like

Rab38 – RAB38, member of RAS oncogene family

Ap1s3 – adaptor-related protein complex AP-1, sigma 3

Acot4 – acyl-CoA thioesterase 4

Dusp2 – dual specificity phosphatase 2

Adcy5 – adenylate cyclase 5

Ptgr1 – prostaglandin reductase 1

Prkra – protein kinase, interferon inducible double stranded RNA dependent activator

Gabrg3 – gamma-aminobutyric acid (GABA) A receptor, subunit gamma 3

Car4 – carbonic anhydrase 4

Ckap4 – cytoskeleton-associated protein 4

Stxbp2 – syntaxin binding protein 2

Sypl2 – synaptophysin-like 2

Gkap1 – G kinase anchoring protein 1

Tpm4 – tropomyosin 4

Tceal1 – transcription elongation factor A (SII)-like 1

Oxr1 – oxidation resistance 1

Cuedc1 – CUE domain containing 1

Plbd1 – phospholipase B domain containing 1

Tjp2 – tight junction protein 2

Ccl6 – chemokine (C-C motif) ligand 6

Paip1 – polyadenylate binding protein-interacting protein 1

Nnt – nicotinamide nucleotide transhydrogenase

Cml5 – camello-like 5

Ccl8 – chemokine (C-C motif) ligand 8

Ing3 – inhibitor of growth family, member 3

Meox1 – mesenchyme homeobox 1

Kat2a – K(lysine) acetyltransferase 2A

Lpcat2 – lysophosphatidylcholine acyltransferase 2

Klf1 – Kruppel-like factor 1 (erythroid)

Ccl17 – chemokine (C-C motif) ligand 17

Mamdc2 – MAM domain containing 2

Pde6h – phosphodiesterase 6H, cGMP-specific, cone, gamma

INTRODUCTION

Learning and Memory

Learning is regarded as the process of acquiring new skills and knowledge. Memory, in turn, refers to storage and the process of recalling the acquired information and skills. The questions where in the brain memories are formed and stored and what biological mechanisms are behind these processes have long intrigued scientists. With the advancement of science we are in the process of unravelling the biological and molecular mechanisms underlying learning and memory processes.

Memory Storage in the Brain

The first indication that certain parts of the brain are involved in the storage of memories came from the famous patient H.M. His medial temporal lobe was removed to treat the epilepsy that he was suffering from. After the surgery he exhibited severe anterograde amnesia characterized by the inability to form new memories (Scoville and Milner, 1957). However, the memories that he acquired before the operation were intact in a time-dependent fashion – i.e. he still had a partial loss of memories that he acquired shortly before the operation, but the more remote memories tended to be more intact. Moreover, his ability to acquire new motor skills and reasoning abilities remained intact (Corkin, 2002). This indicated for the first time that different kinds of cognitive skills and even different kinds of memories (to be explained below) are subserved by different brain regions. This was later confirmed by many studies.

Forms of Memory

Memories are divided into different subtypes depending on their mode of acquisition, processing and their persistence.

Declarative and Procedural Memories

Memory is generally divided into two major subtypes depending on their modes of acquisition; declarative memory and procedural memory. Declarative memory is commonly referred to as the memory of facts and events (Donaldson and Tulving, 1972); e.g. the date of your birthday, the capital of Germany, the year when you started your PhD etc. This type of memory is acquired consciously and conscious awareness is necessary to recall it later (Donaldson and Tulving, 1972; Eichenbaum, 1997; Ullman, 2004). Due to this reason it is also called as explicit memory – you are explicitly aware of the information. Non-declarative memory, alternatively called procedural memory, is the memory of habits and skills (i.e. memory of a sequence, memory of a procedure); e.g. riding a bike or playing a violin (Fitts, 1954; Ullman, 2001; Ullman, 2004; Ullman and Pierpont, 2005). Acquisition and recall of this memory does not require any conscious awareness. For that reason it is also referred to as implicit memory. Implicit memory is the only type of memory that is manifested by invertebrates, whereas explicit memory is present only in vertebrates. In other words, implicit forms of memory are evolutionarily much older.

Hippocampus is the major brain part responsible for acquisition and storage of declarative memories (Eichenbaum, 2001), whereas formation of procedural memory depends on striatum (Kreitzer, 2009) and cerebellum (Saywell and Taylor, 2008).

Short-term and Long-term Memories

Memory is divided into subtypes also according to the amount of time it remains accessible to recall. Short-term memory lasts only for a short period of time, as the name suggests – from seconds up to several minutes (Cowan, 2001). For example, remembering a phone number until you dial it and then forgetting it. Long-term memory however lasts for much longer time, from days to years up to a lifetime. Another type of memory which is being distinguished from the two mentioned above is working memory. This kind of memory is often confused with short-term memory but lately it has been considered as a distinct subtype. This type of memory remains accessible as long as a

sequence of actions is being performed. It can be described as the memory of the entity in a sequence that comes before the entity currently being dealt with. And as one proceeds with the sequence, the entities that were left behind can be forgotten (Becker and Morris, 1999; Baddeley, 2003).

Recently acquired memories are stored in the hippocampus; however throughout time they become transferred to the cortex (Frankland *et al.*, 2004; Maviel *et al.*, 2004; Frankland and Bontempi, 2005; Teixeira *et al.*, 2006; Restivo *et al.*, 2009). Repeated reactivation of hippocampo-cortical circuitry (esp. during sleep) has been suggested to mediate this process (Pavlidis and Winson, 1989; Wilson and McNaughton, 1994; Kudrimoti *et al.*, 1999; Nadasdy *et al.*, 1999; Maquet *et al.*, 2000; Shimizu *et al.*, 2000; Ribeiro *et al.*, 2002; Huber *et al.*, 2004; Peigneux *et al.*, 2004; Ribeiro *et al.*, 2004; Peigneux *et al.*, 2006; Ji and Wilson, 2007; Euston *et al.*, 2007; Buhry *et al.*, 2011; Schwindel and McNaughton, 2011). Once the memory comes to the stage of being stored in the cortex it begins to be regarded as remote memory.

Testing Memory in Rodents

Associative Memory

This type of memory is encoded through the association of two stimuli occurring in a specific sequence and at a certain interval. The most often used way to study it in rodents is afforded by Pavlovian fear conditioning paradigm (Blanchard and Blanchard, 1969). Here the rodents (either rats or mice) learn to associate a conditioned stimulus (CS), e.g. context or tone, with an unconditioned stimulus (US) which is presented in the form of a mild electrical footshock. Upon a second exposure to the CS, but without the presentation of US, the animal manifests freezing behaviour, which is an inborn behaviour that rodents show when in a potentially threatening situation. The amount of freezing corresponds to the amount of fear that the animal has at that moment, and the amount of fear that the animal manifests corresponds to the strength of the associative memory that it acquired, relating the CS to the expectation of US. Contextual fear conditioning, where CS is a context, is highly dependent on hippocampus (Kim and Fanselow, 1992). Cue-

dependent fear conditioning, where CS is a cue in the form of a tone or an odour, is mediated through amygdala (LeDoux, 1993; LeDoux, 1994).

Spatial Memory

It is the type of memory concerning spatial orientation. Hippocampus is the main brain structure responsible for acquisition and storage of spatial memories (Rossi-Arnaud *et al.*, 1991; Schwegler and Crusio, 1995; Crusio and Schwegler, 2005). Spatial memory in rodents can be tested by Morris water maze test (Morris, 1981). In this test, the animals are introduced into a maze filled with opaque water. Beneath the water there is a hidden platform, which for the mice is the only escape from the water. The animal learns to locate the platform with the help of visual cues provided. And training proceeds it learns the place of the platform and the time that it takes to reach it decreases. After training, the memory is tested by putting the animal into the pool but without platform this time. And the strength of the memory is indicated by the relative amount of time that it spends in the region where the platform was previously located.

Biological and Molecular Mechanisms of Learning & Memory

As mentioned above hippocampus is the main region where new memories are acquired and retained for a certain time. Hippocampus has a regular structure along the longitudinal axis, and is divided into two major subregions – dentate gyrus and CA. CA further consists of two functionally distinct divisions called CA3 and CA1. Hippocampus receives projections from the cortical regions through perforant pathway. Entorhinal cortex sends excitatory projections to dentate gyrus through that pathway. Then granule cells in dentate gyrus send glutamatergic projections to pyramidal neurons in CA3 through mossy fiber pathway. And neurons in CA3 project to pyramidal neurons in CA1 through Schaffer-collateral pathway.

The cellular correlate of memory called long-term potentiation (LTP) was first identified in the perforant pathway of the hippocampus (Bliss and Lomo, 1973). Later the same phenomenon was identified in other glutamatergic synapses in the hippocampus – mossy

fiber and Schaffer collateral synapses (Wheal *et al.*, 1983; Harris *et al.*, 1984; Higashima and Yamamoto, 1985; Reymann *et al.*, 1985; Kamiya *et al.*, 1988; Bradler and Barrioneuvo, 1989; Jaffe and Johnston, 1990) and also in other brain regions like cerebellum and striatum (Crepel and Jaillard, 1991; Calabresi *et al.*, 1992; Shibuki and Okada, 1992; Quinn and Harris, 1995; Salin *et al.*, 1996; Calabresi *et al.*, 1997; Charpier and Deniau, 1997; Kimura *et al.*, 1998; D'Angelo *et al.*, 1999). The phenomenon of long-term potentiation was most extensively studied in Schaffer collateral synapses that CA3 neurons make on CA1 neurons. The basic rationale behind the phenomenon of long-term potentiation (LTP) of synaptic transmission is that stimulations of differing intensity and/or duration can produce qualitatively different outcomes. Any amount of stimulation that is below a certain threshold either will not lead to any changes in the strength of synaptic transmission or will lead to changes that are not long lasting. For example, stimulating the slice with a single 100-Hz train will induce an early, short-term stage of LTP (early LTP), which would last only several minutes before returning to basal levels. However, applying at least four repeated 100-Hz trains will result in a much more persistent form of LTP (late LTP) which will persist from hours to days (Martin *et al.*, 2000). Moreover, another way through which a stimulus that would normally induce only a short lasting change can be made to induce a long-lasting change in synaptic transmission is to pair it with another stimulation. In this regard, the most common method used in slices is to apply a modulatory neurotransmitter (e.g. dopamine) or its analogs together with stimulation (Delanoy *et al.*, 1983; Mochida and Libet, 1990; Huang and Kandel, 1995; Otmakhova and Lisman, 1996; Kusuki *et al.*, 1997).

Principally long lasting forms of LTP differ from short lasting ones by dependence of the former on *de novo* gene expression and protein synthesis (Reymann *et al.*, 1988; Frey *et al.*, 1988; Bourtchuladze *et al.*, 1994; Deisseroth *et al.*, 1996; Frey and Morris, 1997; Impey *et al.*, 1998; Tischmeyer and Grimm, 1999; Martin *et al.*, 2000; Kandel, 2001; Bergado *et al.*, 2003; Ramanan *et al.*, 2005). As a result late LTP involves formation of new synapses and/or strengthening of already existing ones (Bailey and Chen, 1983; Bailey and Kandel, 1993; Malinow *et al.*, 2000; Malinow and Malenka, 2002; Matsuzaki *et al.*, 2004; Nagerl *et al.*, 2004; Hayashi and Majewska, 2005; Segal, 2005). In contrast,

early LTP involves only posttranslational modifications of already existing proteins (Martin *et al.*, 2000; Kandel, 2001; Malenka and Bear, 2004).

Initial studies concerning synaptic plasticity were carried out in marine snail *Aplysia* (Kandel, 2001; Barco *et al.*, 2006). When a light touch is applied to its siphon, the snail responds by withdrawing its siphon and gill. This natural reflex is enhanced when a stimulus is applied also to the tail. Tail stimulus induces the release of serotonin (another modulatory neurotransmitter), which in turn acts on sensory neurons and induces production of cAMP. The increase in cAMP leads to facilitation of excitatory glutamatergic connections that sensory neurons make on motor neurons (Klein *et al.*, 1982; Castelluci *et al.*, 1986; Dale *et al.*, 1988).

In mouse hippocampus, LTP induction depends on the Ca^{+2} influx into the neuron through NMDA receptors (NMDARs) (Malenka, 1991; Malenka and Nicoll, 1993; Blitzer *et al.*, 1995). An important phenomenon underlying LTP induction is that excitatory neurons possess two major kinds of ionotropic glutamate receptors; α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors (AMPARs) and N-methyl-D-aspartic acid (NMDA) receptors (NMDARs). AMPARs are permeable to Na^{+} and K^{+} ions and mediate most of the depolarization that is responsible for synaptic transmission under basal conditions. NMDARs in turn are not readily permeable to ions because of the magnesium block that occludes the channel. However, upon depolarization (which is caused by the passage of cations through AMPRs) the magnesium block is removed and binding of glutamate to the NMDAR activates it leading to an influx not only of Na^{+} but also, most crucially, of Ca^{+2} ions. The latter in turn associate with calmodulin. At low Ca^{+2} concentrations calmodulin is able to activate only CaM-dependent kinase II (Malenka, 1991; Malenka and Nicoll, 1993). And the latter in turn induces early LTP by phosphorylating AMPARs (Martin *et al.*, 2000; Kandel, 2001; Malenka and Bear, 2004). Phosphorylation of AMPARs increases their ion conductivity, which leads to transient potentiation of synaptic transmission (Greengard *et al.*, 1991; Keller *et al.*, 1992; Blackstone *et al.*, 1994; Carvalho *et al.*, 2000). However once Ca^{+2} concentration passes beyond a certain threshold the resulting calcium/calmodulin complex activates CaM-dependent adenylate cyclase (Blitzer *et al.*, 1995), which leads to cAMP production. The produced cAMP activates cAMP-dependent

protein kinase (PKA), which in turn phosphorylates MAPK and results in activation of MAPK/ERK signalling which leads to CREB-dependent gene transcription, which in the end leads to maintenance of late LTP and is crucial for formation of long-term memory (Frey *et al.*, 1993; English and Sweatt, 1996; English and Sweatt, 1997; Atkins *et al.*, 1998; Sweatt, 2001; Sweatt, 2004; Thomas and Huganir, 2004; Reissner *et al.*, 2006). Phosphorylated CREB binds to conserved cAMP response element (CRE) sequences at gene promoters and mediates the first wave of gene expression by inducing transcription of immediate early genes (IEGs) like c-Fos, Zif268/Egr-1 and Egr-2 (Impey *et al.*, 1996; Impey *et al.*, 1998; Taubenfeld *et al.*, 1999; Bito *et al.*, 1996; Deisseroth *et al.*, 1996; Lu *et al.*, 1999). Those IEGs in turn lead to successive waves of gene expression as a result of which the newly synthesized proteins lead to occurrence of new synapses, activation of silent ones and/or strengthening of already existing ones (see above).

Epigenetics and Chromatin Plasticity

In eukaryotic cells, DNA does not exist in the cell as simply bare structure. It is rather wrapped around proteins called histones giving rise to the structure called chromatin. The condensed form of chromatin is called heterochromatin and is inaccessible to transcriptional machinery (Grewal and Moazed, 2003). Therefore the genes located in heterochromatic region usually are repressed and have a low level of transcription. However, euchromatin represents a relatively loose and open state of DNA, and hence the genes located at euchromatic regions are accessible to RNA transcription machinery and are expressed at a relatively high level.

147 bp of DNA is wrapped around an octamer of histone proteins forming the basic unit of chromatin called the nucleosome. This octamer unit consists of two of each four different histone proteins – H2A, H2B, H3 and H4. The histone proteins have an overall positive charge and that is what makes them attracted to negatively charged DNA. Each histone protein is composed of a central globular domain and an N-terminal tail. The latter appear to be crucial since they undergo different chemical modifications at different sites. The N-terminal tails of histones can undergo acetylation, methylation, phosphorylation, ubiquitination and ADP-ribosylation (Strahl and Allis, 2000; Vaquero *et*

al., 2003). And these post-translational modifications on histone tails regulate the attraction between DNA and histones. The modifications that reduce the positive charge of histones loosen the attractive force between histone proteins and DNA; and this in turn makes DNA more accessible to transcriptional machinery. Histone acetylation therefore always serves to activate gene expression (Kurdistani *et al.*, 2004; Li *et al.*, 2007). It occurs on Lysine 9 and 14 residues of H3, and on Lysine 5, 8, 12 and 16 residues of H4. Histone acetylation is catalysed by histone acetyltransferases (HATs), and acetyl marks are removed by histone deacetylases (HDACs) (Strahl and Allis, 2000). Histone methylation (to be discussed in more detail below) can be both activatory and repressive for gene expression depending on which residue it occurs (Shi and Whetstine, 2007; Shilatifard, 2008; Scharf and Imhof, 2011; Justin *et al.*, 2010). It is catalysed by histone methyltransferases (HMTs) and histone methyl marks are removed by histone demethylases (HDMs) (Tachibana *et al.*, 2001; Shi *et al.*, 2004).

Histone Methylation

Like acetylation, histone methylation also occurs on lysine residues of histone tails. However differently from histone acetylation, which always involves addition of only one acetyl residue, histone methylation can occur in monomethylated, dimethylated and trimethylated forms (Schneider *et al.*, 2005; Santos-Rosa *et al.*, 2002; Wood *et al.*, 2007). Each state of histone lysine methylation can either be carried out in a non-processive manner (i.e. monomethylation from non-methylated substrate, dimethylation from monomethylated substrate and trimethylation from dimethylated substrate) or it can be carried out in a processive manner with each level of histone lysine methylation occurring from non-methylated residue as the substrate (Scharf and Imhof, 2011).

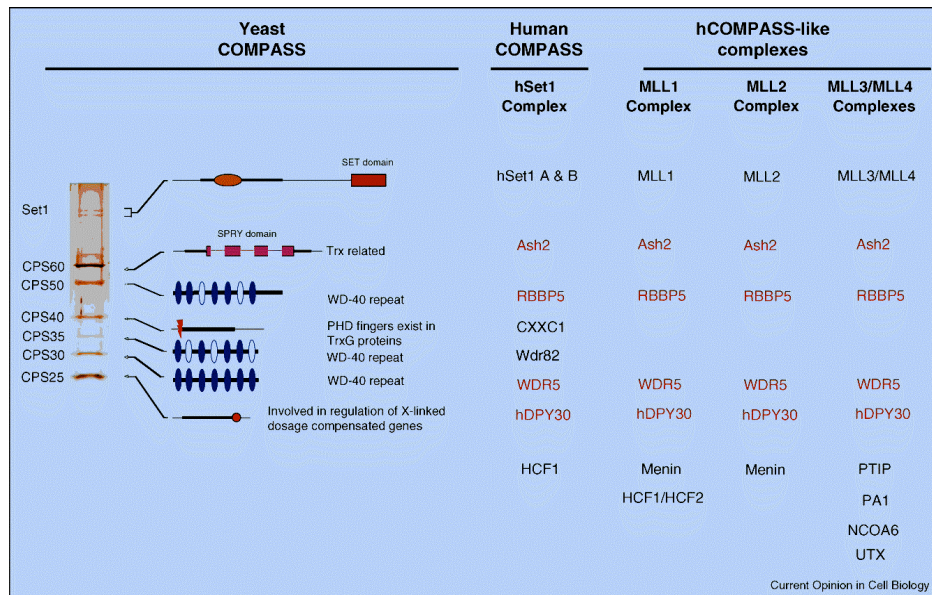
Also differently from acetylation, histone methylation can serve either as an activatory or repressive mark depending on which lysine residue is methylated (Kouzarides, 2007; Shilatifard, 2006; Sims *et al.*, 2003). For example, H3 lysine 9 methylation serves to inhibit transcription and hence is associated mostly with heterochromatic regions (Nakayama *et al.*, 2001; Noma *et al.*, 2001; Peters *et al.*, 2001; Peters *et al.*, 2002; Volpe *et al.*, 2002). However, H3 lysine 4 methylation is exclusively associated with active

transcription (Santos-Rosa *et al.*, 2002; Kouzarides, 2007; Shilatifard, 2006; Sims and Reinberg, 2006). Other most prominent histone methylation sites are H3 lysine 27, which is associated with transcriptional repression (Boyer *et al.*, 2006; Lee *et al.*, 2006; Schwartz *et al.*, 2006; Schüttengruber *et al.*, 2009), and H3K36 which is associated with transcriptional activation (Bell *et al.*, 2007; Lee and Shilatifard, 2007; Wang *et al.*, 2007).

H3K4 methylation: As was mentioned above, H3K4 methylation always acts to facilitate transcription. H3K4 methylation is mostly localized near the transcription start sites of actively transcribed genes (Krogan *et al.*, 2003; Ng *et al.*, 2003) although it has been shown to be present in coding regions as well (Santos-Rosa *et al.*, 2002). Moreover, there exist differences in the function of different levels of H3K4 methylation, with H3K4 trimethylation being responsible for induction of gene expression after stimulation and being more enriched around transcription start sites whereas H3K4 dimethylation being distributed evenly across the coding region and being responsible only for the expression of constitutively active genes (Santos-Rosa *et al.*, 2002; Ng *et al.*, 2003).

COMPASS complex was the first H3K4 methylase identified. It was identified in yeast and consists of the actual methyltransferase Set1 together with other accessory proteins (Miller *et al.*, 2001). Set1 itself is enzymatically active only within the COMPASS complex and is able to monomethylate, dimethylate and trimethylate H3K4 (Miller *et al.*, 2001; Roguev *et al.*, 2001; Nagy *et al.*, 2002). Set1 is the only H3K4 methylase in yeast; however in mammals there exist six Set1-related homologs that are capable of performing H3K4 methylation – MLL1, MLL2, MLL3, MLL4, hSet1A, hSet1B (Shilatifard, 2008; Ansari and Mandal, 2010). The functions of mixed-lineage leukemia (MLL) type of H3K4 methylases consist of cell cycle regulation, development and embryogenesis (through regulating *HOX* gene expression) (Yu *et al.*, 1995; Hess *et al.*, 1997; Yagi *et al.*, 1998; Yu *et al.*, 1998; Terranova *et al.*, 2006; Jude *et al.*, 2007). Moreover, MLLs have been found to be rearranged in different types of cancers, especially leukemias – hence the name (Thomas *et al.*, 2005; Horton and Williams, 2006; Meyer *et al.*, 2009; Marschalek, 2010). All H3K4 methylases consist of a C-terminal catalytic SET domain which performs the actual histone methylation, PHD domains and RING fingers that are involved in protein-protein interactions and HMG domains that are

responsible for DNA binding. Moreover, all of them exist as distinct multiprotein complexes both with common but also complex specific protein subunits. The common subunits include Ash2, Wdr5, Rbbp5 and Dpy30 (Ansari and Mandal, 2010). They are depicted in red in the picture below, whereas complex specific subunits are depicted in black.



Ali Shilatifard, *Current Opinion in Cell Biology*, 2008

MLLs are functional histone methyltransferases only in the context of the other protein components associated with them in multiprotein complexes. And each of the components associated with MLL plays a crucial role in regulating MLL function. For example, knocking down any of the core components of MLL complexes – Wdr5, Rbbp5 or Ash2 – has been shown to decrease its H3K4 methylase activity without affecting the recruitment of MLL to gene promoters (Dou *et al.*, 2005; Wysocka *et al.*, 2005; Steward *et al.*, 2006).

MLL2: *Mll2* gene is a close homolog of *Mll1* through gene duplication (FitzGerald and Diaz, 1999). It is highly expressed throughout development and also in adult tissue (Glaser *et al.*, 2006; McMahon *et al.*, 2007). Like its close homolog *Mll1*, *Mll2* is also involved in regulating *HOX* gene expression (Glaser *et al.*, 2006; Ansari *et al.*, 2008).

Moreover, Mll2 has been shown to mediate transcription at the β -globin locus in erythroid cells (Demers *et al.*, 2007). Mll2 knock-down in mice before E11.5 leads to embryonic lethality, whereas knocking-down Mll2 after that time point does not lead to any notable pathology (Glaser *et al.*, 2009); however loss of Mll2 leads to complete loss of spermatogenesis (Glaser *et al.*, 2009). Furthermore, it associates with estrogen receptor alpha through menin, and regulates estrogen dependent gene expression (Mo *et al.*, 2006; Dreijerink *et al.*, 2006).

Role of Epigenetic Mechanisms in Learning and Memory

Since histone modifications appear to be an important mechanism in regulating gene expression, and since gene expression is crucial for formation and retention of long-term memories neuroscientists have long wondered whether epigenetic mechanisms play a role in memory formation. And indeed, up to date many studies have conclusively shown that histone modifications in different brain regions (especially hippocampus) are important for learning and memory. Most of the studies have used Pavlovian fear conditioning paradigm to investigate this and have shown that it induces an increase in histone acetylation (H3K9, H3K14, H4K5, H4K8 and H4K12) (Levenson *et al.*, 2004; Chwang *et al.*, 2007; Lubin *et al.*, 2008; Peleg *et al.*, 2010), histone methylation (H3K4) (Gupta *et al.*, 2010) and histone phosphorylation (H3S10) (Chwang *et al.*, 2006) in hippocampus. Moreover, apart from acquisition and consolidation, reconsolidation of memory after retrieval and extinction of fear memory were also shown to depend on histone acetylation and phosphorylation (Bredy *et al.*, 2007; Lubin and Sweatt, 2007). Supporting these findings there were also many studies that showed that interfering with the mechanisms mediating chromatin modifications affects long-term potentiation (LTP), which is considered as a biological correlate of memory (see above). Increasing histone acetylation through the usage of HDAC inhibitors has been shown to facilitate memory formation and enhance LTP (Levenson *et al.*, 2004). Moreover mice having their CREB binding protein (CBP) – a HAT – knocked out manifest deficits in learning & memory and LTP (Alarcon *et al.*, 2004; Valor *et al.*, 2011). In line with these findings, mice that

have their HDAC2 deleted manifest memory enhancement and an increase in LTP (Guan et al., 2009). Moreover inhibition of PP1, which dephosphorylates histones, has also been shown to facilitate memory (Koshibu *et al.*, 2009).

Histone Methylation in Learning and Memory

Although the role of histone acetylation in memory formation has been extensively studied, the involvement of histone methylation, another important histone modification, in learning and memory has been relatively ignored. Only recently has its importance for learning & memory and behaviour in general begun to be revealed. For example knock down of H3K9 methyltransferase GLP/G9a in adult neurons in mice leads learning impairment accompanied with decrease in exploratory behaviour and motivation and a decrease in anxiety (Schaefer *et al.*, 2009). Moreover, it has recently been shown that mice having a deletion in one of the alleles of *Mll1* manifest impairment in fear conditioning and increased anxiety (Gupta *et al.*, 2010).

Environmental Enrichment

Environmental enrichment refers to provision of conditions that enhance cognitive, motor and sensory stimulation compared to standard (i.e. control) conditions. Normally, rodents are housed in standard home cages which include *ad libitum* access to food and water, bedding and nesting material. Environmental enrichment is achieved through placing the animals (rats or mice) in more spacious cages containing toys of different size, shape, colour and texture. Moreover, in most studies enriched environment also includes provision of running wheels in order to provide the rodents with an opportunity for voluntary exercise as well, together with cognitive and sensory stimulation provided by the toys. In some cases, social interaction is also utilized as an additional component of the enriched environment, with control animals housed singly in standard home cages and enriched animals housed in groups. However, in many studies, including the one to be presented in this thesis, the effect of social interaction as an additional factor is

eliminated by housing control mice in groups as well. This provides the researcher with an opportunity to discern the effects of only cognitive and motor stimulation on behavioural changes in rodents and on the changes in molecular pathways in their brains. Environmental enrichment as an experimental paradigm was first described by Donald Hebb (Hebb, 1947). He observed that rats that he took home as pets and allowed to roam freely showed improvements in different behavioural tasks when compared to the ones kept in the laboratory. The enriched environment he provided included two most important features which would constitute all the environmental enrichment protocols in subsequent studies, namely novelty and complexity (van Praag *et al.*, 2000; Nithianantharajah and Hannan, 2006). Complexity is achieved by providing different toys of different size, shape, texture and colour (although colour complexity is the least effective due to limitations in rodent vision). Novelty is achieved by changing the toys at a regular basis; this includes removing some of the old toys and replacing them with new ones and rearranging the remaining toys inside the cage. Increased complexity and novelty leads to increased levels of stimulation in different brain regions – e.g. changing the objects and their position provides additional cognitive stimulation by facilitating the formation of new spatial maps in hippocampus (Nithianantharajah and Hannan, 2006). The first studies investigating the effects of enriched environment on rodent brain documented that environmental enrichment increased brain weight and cortical thickness (Bennett *et al.*, 1969; Diamond *et al.*, 1972; Diamond *et al.*, 1976). Later studies showed that enrichment increases dendritic density and the number of synaptic arborizations (Greenough and Volkmar, 1973; Greenough *et al.*, 1985; Turner *et al.*, 1985; Rampon *et al.*, 2000; Faherty *et al.*, 2003; Leggio *et al.*, 2005). In line with induction of increased synaptogenesis, environmental enrichment also increases the expression of post and pre-synaptic proteins, PSD95 and synaptophysin respectively (Frick *et al.*, 2003; Tang *et al.*, 2001). Moreover, environmental enrichment has also been shown to increase the expression of NMDA and AMPA receptors (Tang *et al.*, 2001; Naka *et al.*, 2005) together with enhancing LTP (Foster *et al.*, 2001; Duffy *et al.*, 2001; Artola *et al.*, 2006). Last but not least, it has been shown that environmental enrichment facilitates adult neurogenesis in hippocampus, through both increasing the number of newborn neurons

and also facilitating their survival (Kempermann *et al.*, 1997; Kempermann *et al.*, 1998a; Kempermann *et al.*, 1998b; Bruel-Jungermann *et al.*, 2005).

Through all these, environmental enrichment leads to alterations in behaviour. It facilitates learning and memory in young adult mice (Rampon *et al.*, 2000; Tang *et al.*, 2001; Duffy *et al.*, 2001; Lee *et al.*, 2003), ameliorates memory deficits in aged and Alzheimer's disease model mice (Bennett *et al.*, 2006; Fischer *et al.*, 2007) and decreases anxiety (Roy *et al.*, 2001; Benaroya-Milshtein *et al.*, 2004). It is mostly believed that the effects of environmental enrichment on brain structure and function, and on behaviour are mediated through cellular and molecular mechanisms underlying learning and synaptic plasticity (Rosenzweig and Bennett, 1996; van Praag *et al.*, 2000; Nithianantharajah and Hannan, 2006). The effect of increased adult hippocampal neurogenesis on learning enhancement remains more controversial since some studies have claimed that adult neurogenesis is not crucial for learning & memory (Meshi *et al.*, 2006; Jaholkowsky *et al.*, 2009).

AIMS OF THE PROJECT

Part 1

My PhD consisted of two main parts. In the first part I characterized behaviourally and molecularly transgenic mice having their *Mll2* gene knocked out in forebrain excitatory neurons. *Mll2* is a histone methyltransferase responsible for H3K4 methylation and a close homolog of *Mll1* (see above), the heterozygous knock-out of which has previously been shown to produce deficits in learning and in LTP (Kim *et al.*, 2007; Gupta *et al.*, 2010). However, those studies did not perform a comprehensive analysis of the role that *Mll1* might play in regulating the molecular mechanisms involved in neuronal function and synaptic plasticity. Moreover, the heterozygous knock-out mice used in those studies were straight knock-outs. Therefore, the behavioural and synaptic deficits observed in transgenic mice cannot be attributed solely to the role of *Mll1* in the functioning in adult forebrain excitatory neurons which are the ones mainly involved in sophisticated cognitive functions like learning & memory. Therefore by choosing to study the effect of

Mll2 knock-down in excitatory forebrain neurons in adults I was aiming to achieve two major goals: (i) to extend our knowledge on the role of histone methylation in learning & memory by studying the involvement of another histone methyltransferase in those processes, and (ii) to implement a more specific approach by characterizing the transgenic mice that have their *Mll2* gene knocked-out only in a subset of neurons at adult stage.

Part 2

In the second part of my PhD I set out to investigate the effects of different durations of environmental enrichment on learning & memory with an attempt to uncover the molecular mechanisms behind those effects. Although it has long been known that environmental enrichment facilitates learning, a comprehensive analysis of the effects of different durations of environmental enrichment on learning & memory has never been performed. Moreover, knowledge concerning persistence of the effects of environmental enrichment once enrichment ceases is currently scarce; therefore one of my main aims was to find out what amount of enrichment is enough not only to facilitate learning but also exert persistent effects once it ceases. Last but not least, I also tried to find out whether different durations of environmental enrichment activate different molecular pathways in hippocampus. In my investigations, I concentrated on two different durations of enrichment: (i) 2 weeks (short-term) and (ii) 10 weeks (long-term).

MATERIALS AND METHODS

Animals

Male mice were single-housed and female mice were group-housed in standard home cages (365 x 207 x 140 mm). Food and water were provided *ad libitum*. All experiments were performed according to the animal protection law and were approved by the District Government of Germany. All mice were of C57B/6J background. The generation of *Mll2* F/F and CamKII-Cre mice was described previously (Glaser *et al.*, 2004; Minichiello *et al.*, 1999).

In brief, one week before the start of the behavioural battery the animals were allocated to cages and put into the room where the behavioural experiments were to be performed. Male mice were housed singly while the female mice were housed in groups of 4-5 in order to have their oestrous cycle synchronized. The behavioural battery was always performed in the following sequence: Open Field Test, Novel Object Recognition Test, Elevated Plus Maze, Contextual Fear Conditioning, Morris Water Maze.

Environmental Enrichment

The mice subjected to enriched environment were housed in more spacious cages (595 x 380 x 200 mm), 3-5 mice per cage. Apart from that the mice were provided with different objects (e.g. tunnels, castles, running wheels etc). Every day 2 of the toys were exchanged for the new ones and the ones remaining in the cage were rearranged. Food and water were provided *ad libitum*.

Behavioural Analysis

Open Field Test: In the open field test mice were placed near one of the side walls of a plastic arena (length 1m; width 1m; transparent side walls 20 cm high) and were left to explore it for 5 min. Behaviour was recorded by a camera and analysis was performed

using the VideoMot2 software (TSE Systems). The open field arena was cleaned with 70% ethanol before each mouse was put into the setup. The open field arena was virtually divided into 16 quadrants, and the relative time spent in the central 4 quadrants was plotted as a read-out for anxiety. Moreover, the total distance travelled in the arena was also analyzed.

Elevated Plus Maze: The Plus Maze setup was made of plastic and was 53 cm above the ground level. It consisted of two open and two closed arms 45 cm long and 10 cm wide, which came off from the center region 10 x 10 cm. The closed arms were bordered with sidewalls 29 cm high. Mice were put into the center facing one of the open arms. The behavioural performance was recorded during a 5 min period by VideoMot2 (TSE Systems).

Novel Object Recognition: For the novel object recognition test mice were habituated to an empty plastic arena for 5 min for 2 consecutive days, the exploratory pattern obtained on the first day of habituation being used as a read-out for the open field exploration. For the next two days mice were presented with two white boxes (4.5 x 6.5 x 4 cm), which were spaced 52 cm away from each other, and 18 cm away from the sidewalls. The mice were left to explore them for 5 min. On the training day the mice were introduced to two black cubes and left to explore them for 5 min. After that they were put back into the home cage and retained there for 5 min. After the 5 min retention they were reintroduced into the plastic arena with one of the black cubes being exchanged to a small stone. They were allowed to explore the objects for 5 min. 24 hr later the mice were reintroduced into the plastic arena and presented with the same black cube and a red tape. They were allowed to explore the objects for 5 min. Before the mice were put into the arena, the latter was cleaned with 70% ethanol.

Pavlovian Fear Conditioning: Fear conditioning test was performed using TSE fear conditioning system. The setup consisted of a computer-based control unit, with a shock generator, connected to the training chamber. The latter consisted of metal grids at the bottom, a 10 Watt light at the ceiling, a fan and a loudspeaker. The walls of the chamber

consisted of a Plexiglas box with measurements of 25 x 25 x 25 cm. Mice were allowed to explore the chamber for 3 min after which they received a mild electric foot shock (constant current, 0.5 mA) for 2 sec. The next day (i.e. 24 hr later) they were introduced to the same context for 3 min without receiving a footshock.

Morris Water Maze: The training was performed in a circular pool (diameter 1.2 m) filled with opaque water. A platform (11 x 11 cm) was submerged into the water in the center of one of the quadrants (target quadrant). Moreover there were different visual cues on four sites of the pool. Water level was 35 cm and the temperature was between 18-20°C. The swimming behaviour of the mice was recorded by a camera and was analyzed by VideoMot2 (TSE). At each training session the mice were placed into the maze subsequently from four random points and were allowed to swim (i.e. search for the platform) for 60 sec. If during the 60 sec the mouse failed to find the platform it was gently guided to it. Mice were allowed to stay on the platform for 15 sec. Mice were subjected to the probe test 24 hr after the last training session. In the probe test the platform was removed and the mice were placed into the maze at one of those four points furthest away from the platform. They were allowed to swim for 60 sec.

***Mll2* Genotyping**

DNA Isolation from Tail: A small part of mouse tail tissue was kept in 1.5 ml tubes (Eppendorf, Hamburg, Germany). In order to isolate the tail DNA 200 µl of DirectPCR[®] Lysis Reagent (Peqlab, Erlangen, Germany) were added into the tube together with 0.3 mg/ml Proteinase K (Peqlab, Erlangen, Germany). The tubes were then incubated on a thermomixer (Eppendorf, Hamburg, Germany) at 55°C and shaking for 16 h at 550 rpm. After that the temperature was raised to 85°C and the tubes were incubated at the latter temperature for 45 min. The DNA samples were then briefly spun down and were kept at 4°C throughout. 1 µl of tail DNA was used for subsequent polymerase chain reaction.

Polymerase Chain Reaction (PCR): The PCR to detect the *Mll2* knock-out was performed using the primers encompassing the region containing the exon 2 with the Flox sites at its

borders, the Dream Taq Polymerase (Fermentas, Heidelberg, Germany), Dream Tag Buffer (Fermentas, Heidelberg, Germany) and the dNTP mix (Fermentas, Heidelberg, Germany). In order to detect the Cre transgene the corresponding primers were also added to the reaction mix. The reaction mix together with the tail DNA were pipetted into in 0.2 ml micro test tubes (Nerbe Plus, Winsen/Luhe, Germany), and the reaction was performed in a Mastercycler ep *gradient* S (Eppendorf, Hamburg, Germany). The PCR reaction mix protocol and the program are explained below.

Dream Taq Buffer (Green): **2.5 μ l**
 dNTP Mix (2.5 mM): **2 μ l**
 loxP Primer Forward: **0.25 μ l**
 loxP Primer Reverse: **0.25 μ l**
 CamK-Cre Primer Forward: **0.13 μ l**
 Cam-Cre Primer Reverse: **0.13 μ l**
 PCR-grade Water: **18.54 μ l**
 Dream Taq: **0.2 μ l**
 Tail DNA: **1 μ l**

95°C – 5 min
 95°C – 30 sec
 58°C – 30 sec
 68°C – 1 min
 68°C – 7 min

} 35X

Here are the sequences of the primers used for genotyping:

| Primer | Sequence (5'-3') | Tm° |
|------------------|---------------------------|-----|
| loxPse Forward | gtcctgtgttcagtccaaggtag | 57 |
| loxPas Reverse | ggagaacagttgtgggagatgggtc | 63 |
| CamK-Cre Forward | gggaggttaggaagagcgatg | 56 |
| CamK-Cre Reverse | ccatgagtgaacgaacctgg | 54 |

RNA Isolation and Quantitative real time PCR (qPCR)

RNA Isolation: The dissection of dentate gyrus and CA regions was performed under a stereomicroscope (Motic) as described previously (Hagihara *et al.*, 2009). The RNA from dorsal & ventral dentate gyrus and dorsal & ventral CA were isolated using TRIZOL Reagent from Invitrogen. For RNA isolation from whole hippocampus, prefrontal cortex

and cerebellum the protocol was adjusted with the amounts written in parantheses in red (see also for protein isolation). The tissue was homogenized in 400 (1000) μl of TRIZOL Reagent. After that 80 (400) μl of chloroform was added to the samples and the solutions were left at room temperature (RT) for 15 min. Later the samples were centrifuged at 12000g, at 4°C for 15 min. The resulting aqueous phase was collected, mixed with 200 (500) μl of Isopropanol and kept at -20°C for at least 30 min. The rest (i.e. the interphase and organic phenol-chloroform phase) was frozen at -80°C for subsequent protein isolation (see below). Afterwards the aqueous phase + isopropanol mixture was centrifuged for 30 min at 4°C at 12000g. The pellet was dissolved in 30-50 (100) μl RNase free water.

Reverse Transcription: cDNA was synthesized using the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Mannheim, Germany). 1 μg of RNA was mixed with 2 μl of random hexamers (600 pmol/ μl) and with dH₂O (PCR-grade) up to a volume of 13 μl in a 200 μl tube (Nerbe Plus, Winsen/Luhe, Germany). The mixture was incubated at 65°C for 10 min in a Mastercycler ep *gradient* S (Eppendorf, Hamburg, Germany) to denature the RNA into primary structure. After that 0.5 μl of Reverse Transcriptase (20 U/ μl), 2 μl of Deoxynucleotide Mix (10 mM each), 4 μl of the 5X Transcriptor Reverse Transcriptase Reaction Buffer and 0.5 μl Protector RNase Inhibitor (40 U/ μl) were added to the tube. The final mixture was incubated at 25°C for 10 min followed by incubation at 55°C in the Mastercycler. After that the Reverse Transcriptase was inactivated by incubating at 85°C for 5 min in the same Mastercycler. The cDNA was stored at 4°C.

qPCR: a) With UPL Probes: qPCR was performed in a Roche 480 Light Cycler (Roche Applied Science, Mannheim, Germany) using the primers from the Roche Universal Probe Library (UPL) together with specific fluorescence probes and a Light Cycler PCR Mix (Roche Applied Science, Mannheim, Germany). The primers and the corresponding UPL probes are listed in the table below. 10 μl from each cDNA sample was diluted 10-fold with PCR-grade water. And the rest (10 μl) was pooled into one tube and then diluted serially to yield the following standard dilutions: 1:1; 1:2; 1:4; 1:8; 1:16. The

standard dilutions were used to generate a standard curve of fluorescence intensity versus cDNA concentration. The 1:16 dilution was also used as a positive calibrator. The following were the protocol for PCR mix and the PCR program:

PCR-grade Water: **5.05 μ l**
 Forward Primer (20 μ M): **0.15 μ l**
 Reverse Primer (20 μ M): **0.15 μ l**
 LC480 Probe Master Mix (2X): **7.5 μ l**
 UPL Probe: **0.15 μ l**
 Template cDNA: **2 μ l**
Total Volume: 15 μ l

95°C – 5 min
 95°C – 10 sec
 60°C – 30 sec
 72°C – 10 sec
 40°C – 30 sec

} 45X

The cDNA template and the master mix were pipetted into the wells in a 96-well plate. The plate was then sealed with the cellophane foil and spun at 1250 rpm for 1 min in a centrifuge (Eppendorf, Hamburg, Germany) before being put into the Light Cycler in order to start the PCR reaction. The housekeeping gene hypoxanthine guanine phosphoribosyltransferase 1 (*Hprt1*) was taken as an internal control.

qPCR with this protocol was performed in order to validate the results from DNA microarray and to detect *Mil2* and *Mil1* expression.

| Primer | Sequence (5'-3') | Tm ^o | UPL Probe |
|-------------|---------------------------|-----------------|-----------|
| Nkapl left | cacacctctcaagatgagaaacc | 63.9 | 38 |
| Nkapl right | agccattgctgcaccttc | 64.1 | 38 |
| Rab38 left | ccaaaacttctctctcgcact | 63.3 | 49 |
| Rab38 right | tcatgtttccaaatctttcttgac | 62.9 | 49 |
| Ap1s3 left | ggaccagcagcttcattgac | 64.8 | 17 |
| Ap1s3 right | tgacagcaaaaaataaaactagca | 63.3 | 17 |
| Acot4 left | atgcttcgacatccaaaggt | 63.4 | 17 |
| Acot4 right | ggaagccatgatcagacagac | 63.7 | 17 |
| Dusp2 left | gaagataaccagatggtggagataa | 63.0 | 79 |
| Dusp2 right | cccactattcttcaccgagtctat | 63.3 | 79 |
| Adcy5 left | cgggagaaccagcaacag | 64.6 | 18 |
| Adcy5 right | ctccatggcaacatgacg | 64.1 | 18 |
| Ptgr1 left | gactgagctcccacccttaa | 64.3 | 18 |

| | | | |
|--------------|-------------------------|------|-----|
| Ptgr1 right | gtaaggatccacagagaggaaca | 63.3 | 18 |
| Prkra left | gcgagcaaggctttaacataa | 63.0 | 17 |
| Prkra right | agacactgatactgtccgttg | 63.0 | 17 |
| Gabrg3 left | ggctcactggatcaccaca | 65.1 | 17 |
| Gabrg3 right | ggcactctgcattgatagtgc | 63.7 | 17 |
| Car4 left | aaaccaaggatcctagaagcagt | 63.0 | 1 |
| Car4 right | gacaatggtgatgggggact | 63.6 | 1 |
| Ckap4 left | ggaggaggtccagcaggt | 64.5 | 7 |
| Ckap4 right | ttgcagggattggacctt | 63.1 | 7 |
| Stxbp2 left | tcttgcacatctgtgtaaaatgc | 62.9 | 9 |
| Stxbp2 right | ccgtttgtgatgtctcca | 63.6 | 9 |
| Sypl2 left | tctatggggctggctaact | 64.6 | 99 |
| Sypl2 right | cagcccacaggaagaagttg | 64.8 | 99 |
| Gkap1 left | cagaaggagtcaagggaaga | 64.4 | 94 |
| Gkap1 right | ttcaaacattcagaggtcagc | 63.1 | 94 |
| Tpm4 left | cgaccgcaagtatgaggag | 63.4 | 108 |
| Tpm4 right | tcagatacctccgacctct | 63.8 | 108 |
| E2f1 left | tgccaagaagtccaagaatca | 64.6 | 5 |
| E2f1 right | cttcaagccgcttaccatc | 63.5 | 5 |

b) With SYBR Green: The qPCR was performed in a similar fashion to the one described above with the exception that in this case the reaction mix contained the SYBR Green dye (Roche Applied Science, Mannheim, Germany) was used to detect amplification instead of UPL probes. This protocol was used in order to confirm the loss of exon 2 in F/F CKII mice through recombination and for comparison of histone methylation in the promoter region (for some in the coding region – see ‘Results’) of the genes downregulated in F/F CKII mice, between the latter and F/F mice (see section “Chromatin Immunoprecipitation (ChIP)”). The following protocol and PCR program were used:

PCR-grade Water: **3 μ l**
Forward Primer: **0.75 μ l**
Reverse Primer: **0.75 μ l**
LC480 SYBR Green I Mastermix (2X): **7.5 μ l**
Template cDNA: **3 μ l**

95°C – 5 min
 95°C – 10 sec
 58°C – 15 sec
 72°C – 10 sec
 95°C – 15 sec
 67°C – 30 sec
 95°C – Acquisition
 40°C – 10 sec

45X

The following primers were used for detection of recombination of exon 2 of *Mil2*. The rest will be mentioned in the section “Chromatin Immunoprecipitation (ChIP)”.

| Primer | Sequence (5'-3') | T _m ^o |
|---------------|-----------------------|-----------------------------|
| Primer Exon 1 | cggaggaagagagcagtgagc | 58 |
| Primer Exon 2 | gggaccgaagcgagagc | 57 |

DNA Microarray

DNA microarray was carried out as mono-color experiment. Total RNA was labeled with Cy3 according to Agilent's Low RNA Input Fluorescent Linear Amplification Kit and later hybridized to Agilent Whole Mouse Genome 4x44K G4122F microarrays according to the manufacturer's protocol. Quantity and Cy-dye incorporation rates of the generated target material were assessed using a NanoDrop ND-100. Washes were performed according to the Agilent Technologies SSPE protocol (v2.1) – wash solution 3 was replaced by acetonitrile. After that scanning was performed using an Agilent G2505B scanner. Intensity data were extracted using Agilent's Feature Extraction (FE) software, version 9.5.3.1, and analyzed using the Limma (Smyth, 2004) package of Bioconductor (Gentelman, 2004). The microarray data analysis consisted of three steps; (i) between-array normalization followed by (ii) fitting the data to a linear model and finally (iii) detection of differential gene expression. In order to assure that the intensities had similar distributions across arrays, VSN normalization (Huber *et al.*, 2002) was applied to the intensity values as a method for between-array normalization. To estimate the average group values for each gene and assess differential gene expression, a simple linear model was fit to the data, and group-value averages and standard deviations for each gene were obtained. To find genes with significant expression changes between groups, empirical

Bayes statistics were applied to the data by moderating the standard errors of the estimated values (Smyth, 2004). P-values were obtained from the moderated t-statistic and corrected for multiple testing with Benjamini-Hochberg method (Benjamini and Hochberg, 1995). For each gene, the null hypothesis, that there is no differential expression between degradation levels, was rejected when its adjusted p-value was lower than 0.05.

Chromatin Immunoprecipitation (ChIP)

Chromatin immunoprecipitation was performed using the Low Cell ChIP Kit from Diagenode (Liege, Belgium). 11 μ l of magnetic beads were mixed together with 22 μ l of Buffer A into each tube. The tubes were then placed on a magnetic rack and were left there for 1 min. After that the supernatant was removed and 22 μ l of Buffer A were added. The tubes were again left on a magnetic rack for 1 min and the supernatant was removed. The beads were then resuspended in 11 μ l Buffer A. After that 90 μ l of Buffer A was aliquoted into 0.2 ml tubes (Nerbe Plus, Winsen/Luhe, Germany), and 10 μ l of bead + Buffer A solution and 4 μ l of antibody were added into each tube. The tubes were then placed in a 15 ml falcon tube (Greiner Bio-One, Solingen, Germany) and incubated on a rotating wheel (Barloworld Scientific, Staffordshire, UK) at 4°C for 2 h at 20 rpm. In the meantime the tissue was homogenized 500 μ l distilled Dulbecco's PBS (DPBS henceforth; Pan Biotech, Aidenbach, Germany) + Protease Inhibitor (Roche Applied Science, Mannheim, Germany) on ice. After that 13.5 μ l of 37% Formaldehyde were added to the mixture and the tubes were incubated on a thermomixer (Eppendorf, Hamburg, Germany) at 22°C for 15 min at 500 rpm. Later 57 μ l of 1.25 M Glycine were added and the tubes were incubated for an additional 5 min on the thermomixer at above-mentioned conditions. After that the tubes were centrifuged for 10 min at 3000 rpm at 4°C, the supernatant was removed and the pellet was washed twice with 500 μ l DPBS+PI – i.e. centrifuged for 10 min at 3000 rpm. Then the pellet was resuspended in 130 μ l Buffer B + PI solution (200V Buffer B : 1V PI) and left on ice for 5 min. After that the samples were subjected to sonication in Bioruptor (Diagenode, Liege, Belgium) – 30 sec ON/OFF, HIGH, 2x10 min + 3x5 min. After shearing, 870 μ l of Buffer A + PI (200V

Buffer A : 1V PI) solution was added to the sheared chromatin. After that 100 μ l of the chromatin samples were added to the bead + Buffer A + antibody solutions (200 μ l) that had been incubated for 2 h at 4°C on a rotating wheel. The samples were then incubated again on the rotating wheel under the same conditions overnight. At the same time additional 100 μ l of each chromatin sample were kept as input at 4°C. The next day the beads were washed thrice with Buffer A – placed on magnetic rack for 1 min, supernatant removed and the pellet (beads) resuspended in 100 μ l of Buffer A. After these washes the beads were also washed once with Buffer C in the same way. Then the beads were resuspended in DNA Isolation Buffer (DIB) + Proteinase K (100 μ l DIB + 1 μ l Proteinase K). The beads were transferred into 1.5 ml tubes and 1 μ l of each input sample was transferred to 99 μ l DIB + Proteinase K solution into a 1.5 ml tube as well. From then on the input samples were treated in parallel with chromatin samples. The samples were incubated on the thermomixer at 55°C for 15 min and then at 99°C for an additional 15 min. Finally, the samples were centrifuged at 14000 rpm for 5 min at 4°C and the supernatant was collected.

qPCR analysis was performed using SYBR Green dye as described above (see “RNA Isolation and Quantitative Real Time PCR (qPCR)”). Here are the primers used:

| Primer | Sequence (5'- 3') | Tm° |
|-------------------------|--------------------------|------------|
| Acot4 Promoter Forward | gctgtagcatccaggggaagt | 63.2 |
| Acot4 Promoter Reverse | ccacgtggtgttgtaaagt | 63.6 |
| Adcy5 Promoter Forward | gaggctctgttcgccttcc | 64.0 |
| Adcy5 Promoter Reverse | cctgccagcattattttctt | 60.7 |
| Ap1s3 Promoter Forward | gcgaggtgtaagcactg | 63.4 |
| Ap1s3 Promoter Reverse | tcctggcttctctcaattgt | 63.5 |
| Car4 Promoter Forward | catcttgccccaaatcaagt | 63.8 |
| Car4 Promoter Reverse | cagggcttagaagcggagta | 63.3 |
| Ckap4 Promoter Forward | ttcaaagctttgcgaggat | 63.6 |
| Ckap4 Promoter Reverse | ctctccacagctccagttc | 64.1 |
| Dusp2 Promoter Forward | tagactccaggccgacactt | 63.8 |
| Dusp2 Promoter Reverse | cgggtcaccatacaagggtta | 64.5 |
| E2f1 Promoter Forward | ggctctggctacgaaagaaa | 63.3 |
| E2f1 Promoter Reverse | cttcaggctcacctccaaag | 63.9 |
| Gabrg3 Promoter Forward | cgtgttaattggggaaactcc | 64.1 |
| Gabrg3 Promoter Reverse | gctctcgggagcgatcag | 66.5 |
| Gkap1 Promoter Forward | agttttaaaaatgtaatgccaatg | 60.8 |
| Gkap1 Promoter Reverse | gggttgaggacagaggag | 63.8 |

| | | |
|-------------------------|------------------------|------|
| Nkapl Promoter Forward | gctcaaggtggggaatgtaa | 63.8 |
| Nkapl Promoter Reverse | cgcaggcgactagagac | 65.1 |
| Prkra Promoter Forward | tgactactgcaggcgaaaga | 63.7 |
| Prkra Promoter Reverse | ggcattgtcctacgtcacia | 63.6 |
| Ptgr1 Promoter Forward | ggcctcacgacaggaagtag | 63.8 |
| Ptgr1 Promoter Reverse | gaggggtgtgtgtgtgtt | 63.6 |
| Rab38 Promoter Forward | cagcttgacaggcagtagca | 64.4 |
| Rab38 Promoter Reverse | ccttctactctgaggactc | 62.9 |
| Stxbp2 Promoter Forward | gtccgttcgagtcctgtc | 64.9 |
| Stxbp2 Promoter Reverse | cgtggctctacgcgtcat | 65.2 |
| Sypl2 Promoter Forward | gttctagtggggaccatcc | 64.4 |
| Sypl2 Promoter Reverse | gtcaacaatctggcggact | 64.0 |
| Tpm4 Promoter Forward | aaaggctcccaggtaagtgc | 64.3 |
| Tpm4 Promoter Reverse | ctgtcaggcccatgaggt | 64.0 |
| Adcy5 Exon Forward | tttctcctctgcagctgt | 64.0 |
| Adcy5 Exon Reverse | ggctcctcttactcacctg | 64.1 |
| Dusp2 Exon Forward | gacctcttgctaatacacc | 62.5 |
| Dusp2 Exon Reverse | tgcacaatgaagacaacaattc | 63.7 |
| Tpm4 Exon Forward | tctgactttccaggctgaag | 62.2 |
| Tpm4 Exon Reverse | accctgtcttccacaggtc | 65.4 |

Protein Isolation and Western Blotting

Subcellular Fractionation: Nuclear proteins were isolated with the Subcellular Fractionation Kit (Proteo Extract) from Calbiochem. The samples were dissolved in 1 ml Extraction Buffer I with 5 μ l Protease Inhibitor Mix and were incubated at 4°C for 10 min and after that centrifuged at 4°C at 1000g. The collected supernatant was the cytosolic fraction. The pellet was dissolved in 1 ml Extraction Buffer II with 5 μ l Protease Inhibitor Mix. The samples were incubated at 4°C for 30 min and later were centrifuged at the same temperature at 6000g for 10 min. The collected supernatant was protein/organelle extract. The remaining pellet was dissolved in 500 μ l Protein Extraction Buffer III with 5 μ l Protease Inhibitor Mix and 1.5 μ l Benzoase. The resulting solution was incubated at 4°C for 10 min and after that centrifuged at 10000g. The collected supernatant was nuclear protein extract. The remaining pellet was dissolved in 500 μ l Extraction Buffer with 5 μ l Protease Inhibitor Mix and was the cytoskeletal fraction.

Protein Isolation with TRIZOL: For protein isolation using TRIZOL Reagent the organic phenol-chloroform phase was mixed with 120 (300) μl of 100% ethanol and centrifuged at 2000g at 4°C for 5 min. The resulting supernatant was mixed with 240 (600) μl of isopropanol and incubated at RT for 10 min. Then the samples were centrifuged at 12000g at 4°C for 10 min. 400 (1000) μl of 0.3 M guanidine hydrochloride/95% EtOH was added to the pellet. Then the samples were incubated at RT for 20 min and were centrifuged for 5 min at 7600g at 4°C. The supernatant was discarded and 1.5 ml of 100% EtOH was added to the pellet. The samples were incubated on a rocking shaker for 30 min. Then they were centrifuged again for 5 min at 7600g at 4°C, and were left to dry at RT for 10 min. Finally, the pellet was dissolved in 300 μl of 9.5 M Urea/1% SDS.

Protein Isolation with TX Buffer: Alternatively, proteins were also isolated by TX extraction buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1mM EDTA; 1% Nonidet P40 (IPTGAL); 0.05% SDS). A protease inhibitor tablet (Roche) was added to the extraction buffer before use and dissolved by vortexing. The tissue was homogenized in 400 μl of extraction buffer and subjected to sonication in Bioruptor (Diagenode, Liege, Belgium) (15 min, HIGH, 30 sec ON/OFF). The samples were incubated at 4°C for 5 min with gentle agitation. Afterwards, the samples were centrifuged at 4°C for 10 min at 14000 rpm. The supernatant was collected and used for further experiments.

Determination of Protein Concentration: Protein concentration was determined by Bradford assay (Bradford, 1976). Roti-Quant reagent (Carl Roth GmbH, Karlsruhe, Germany) was diluted 5-fold in 0.01 M PBS pH 7.0 (called Bradford Reagent henceforth). Bovine serum albumin (Sigma, Deisenhofen, Germany) was chosen to prepare the standard concentrations to generate a standard curve representing protein concentrations versus optical density. 60 μl solutions of the following dilutions were prepared: 1.0, 2.0, 4.0, 8.0, 16.0, 32.0 and 64.0 $\mu\text{g}/\mu\text{l}$. 2 μl of the protein lysate and 50 μl of each standard dilution were dissolved in Bradford Reagent to a total volume of 1 ml in a glass spectrophotometer cuvette (10 mm x 4 mm x 45 mm, Sarstedt, Nuembrecht, Germany). The Bradford Reagent was used as a blank reference. Optical density, and

through that the protein concentration, was measured using a BioPhotometer (Eppendorf, Hamburg, Germany) at a wavelength of 595 nm.

Alternatively, since Bradford method is not reliable at high detergent concentrations, the concentration of the protein samples isolated by TRIZOL protocol was measured by NanoDrop Spectrophotometer (PeqLab Biotechnologie GmbH, Erlangen, Germany) at a wavelength of 280 nm.

Preparation of Protein Lysates for SDS-PAGE and Immunoblot: Protein lysates were diluted to 3 $\mu\text{g}/\mu\text{l}$ in TX Buffer and SDS loading dye. Afterwards, the samples were incubated on a thermomixer (Eppendorf, Hamburg, Germany) at 95°C for 5 min at 1000 rpm, and spun down shortly. The protein solution was stored at 4°C.

SDS-Page: Proteins were resolved by SDS-PAGE (BIO-RAD Laboratories, Munich, Germany). The polymerization unit consisted of two glass plates, a spacer plate (1.5 mm) on the outer side and a plane plate on the inner side. The acrylamide gel consisted of a separating gel (12%) and a stacking gel (5%). The preparation of the gels is outlined in the table below. After the gel polymerized, the unit was put into an electrophoresis chamber which was after that filled with SDS electrophoresis buffer (25 mM Tris, 250 mM Glycine and 0.1% SDS in ddH₂O). 30 μg of proteins were pipetted into the wells and 5 μl PageRuler™ Prestained Protein Ladder was loaded into one of the wells. The proteins were resolved through a current at 60V for 40 min and then at 120V until bromophenol blue front reached the bottom of the gel.

| Components | 12% Separating Gel | 6% Separating Gel | 5% Stacking Gel |
|--------------------|--------------------|-------------------|-----------------|
| ddH ₂ O | 3.3 ml | 5.3 ml | 2.1 ml |
| 30% Acrylamide | 4.0 ml | 2.0 ml | 0.5 ml |
| 1.5 M Tris pH 8.8 | 2.5 ml | 2.5 ml | - |
| 1.0 M Tris pH 6.8 | - | - | 0.38 ml |
| 10% SDS | 0.1 ml | 0.1 ml | 0.03 ml |
| 10% APS | 0.1 ml | 0.1 ml | 0.03 ml |
| TEMED | 0.004 ml | 0.008 ml | 0.003 ml |

Protein Transfer: After electrophoresis, the proteins were transferred to a nitrocellulose membrane. Transfer-Blot Cell tank transfer system (BIO-RAD Laboratories, Munich, Germany) was filled with transfer buffer (192 mM Glycine, 25 mM Tris-HCl pH 8.3,

20% Methanol) and the transfer chamber (BIO-RAD Laboratories, Munich, Germany) with the gel and the membrane attached to each other was put into it.

Immunoblot: Following transfer, the membranes were incubated in 3% milk in 0.01 M PBS with 0.1% Triton X-100 at RT for 1h to block non-specific binding sites. Primary antibodies were dissolved in 0.3% milk in 0.01 M PBS with 0.1% Triton X-100. The membranes were incubated with the antibodies overnight at 4°C. Then, the membranes were washed thrice with 0.01 M PBS with 0.1% Triton X-100 and after that were incubated in fluorescent secondary antibody dissolved in 0.3% milk in 0.01 M PBS with 0.1% Triton X-100 for 2 h at RT in the dark. After that the membranes were washed twice with 0.01 M PBS with 0.1% Triton X-100 and then transferred into 0.01 M PBS and were kept in it until and throughout scanning. The membranes were scanned and the results quantified using the Odyssey Imager (Licor). The antibodies are listed in the table below:

| Primary Antibody | Manufacturer | Dilution |
|---------------------------|---------------------|-----------------|
| Anti-Ac-H3K9 | Millipore | 1:1000 |
| Anti-Ac-H3K14 | Millipore | 1:1000 |
| Anti-Ac-H4K5 | Millipore | 1:1000 |
| Anti-Ac-H4K8 | Millipore | 1:1000 |
| Anti-Ac-H4K12 | Millipore | 1:1000 |
| Anti-H3K4me1 | Millipore | 1:1000 |
| Anti-H3K4me2 | Millipore | 1:1000 |
| Anti-H3K4me3 | Millipore | 1:1000 |
| Anti-β-Actin | Santa-Cruz | 1:1000 |
| | | |
| Secondary Antibody | Manufacturer | Dilution |
| Anti-rabbit IgG 680CW | Li-Cor | 1:15000 |
| Anti-rabbit IgG 800CW | Li-Cor | 1:15000 |
| Anti-mouse IgG 680CW | Li-Cor | 1:15000 |
| Anti-mouse IgG 800CW | Li-Cor | 1:15000 |

Immunostaining

Immunostaining was done on frozen sections. Brain tissue was fixed in 4% PFA and was cut using a cryostat into 40 μm sections that were stored in PBS with

Penicillin/Streptomycin (1:100) at 4°C. Sections were washed in 0.01 M PBS containing 0.1% Triton X-100. Blocking of non-specific binding sites was achieved through incubating the sections in blocking buffer (5% goat serum + 0.3% Triton X-100 in 0.01 M PBS) for 90 min at room temperature (RT). Immunostaining was performed by incubation in primary antibody diluted in blocking buffer at 4°C overnight. After that the sections were washed in wash buffer (1% goat serum + 0.2% Triton X-100 in 0.01 M PBS) thrice for 10 min each at RT. The sections were incubated in secondary antibody diluted in blocking buffer at RT in the dark for 2h. After that the sections were washed thrice for 10 min each in 0.01 M PBS, and then incubated in 4',6-diamidino-2-phenylindole (DAPI, 10 µg/ml) for 30 min. Finally the sections were washed twice in 0.01 M PBS at RT. The sections were then mounted on SuperFrost glass slides and dried in the dark at RT. Finally, the sections were covered with glass cover slips by adding Mowiol anti-fade medium. The sections were stored at 4°C in the dark. The antibodies used for immunostaining are listed in the table below:

| Primary Antibody | Manufacturer | Dilution |
|---------------------------|------------------------|-----------------|
| Anti-NeuN | Chemicon | 1:1000 |
| Anti-MAP2 | Synaptic Systems | 1:1000 |
| Anti-Synaptophysin | Sigma-Aldrich | 1:1000 |
| | | |
| Secondary Antibody | Manufacturer | Dilution |
| Cy3 anti-rabbit IgG | Jackson Immunoresearch | 1:500 |
| Alexa488 anti-rabbit IgG | Jackson Immunoresearch | 1:500 |
| Cy3 anti-mouse IgG | Jackson Immunoresearch | 1:500 |
| Alexa488 anti-mouse IgG | Jackson Immunoresearch | 1:500 |

Confocal Microscope: Immunostained mouse brain sections were imaged on Leica AOBS SP2 confocal microscope (Leica Microsystems, Wetzlar, Germany). Alexa488 and Cy3 conjugated secondary antibodies were excited using 488 nm and 561 nm lasers respectively.

Statistical Analysis

Data were analysed by unpaired Student's t-test and two-way ANOVA (Analysis of Variance) when appropriate. Errors are displayed as standard error of mean (SEM). Graphs were generated using GraphPad Prism.

RESULTS:

PART 1: CHARACATERIZATION OF FOREBRAIN SPECIFIC *MLL2* CONDITIONAL KNOC-OUT MICE

***Mll2* Expression in Different Brain Regions**

First, I analyzed the endogenous levels of *Mll2* expression in different regions of the brain with qRT-PCR. Interestingly, I observed that *Mll2* expression is slightly higher in dentate gyrus (DG) compared to CA region and prefrontal cortex (PFC) (Figure 1.1). Though, the difference failed to reach significance.

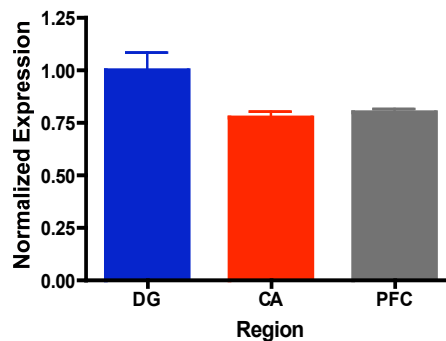


Figure 1.1: *Mll2* mRNA expression in different brain regions (n = 4).

Creating forebrain specific *Mll2* conditional knock-out mice

The mice heterozygous for the floxed exon 2 of *Mll2* were a kind gift of the group of Francis Stewart and Andrea Kranz (Biotechnology Centre – Technical University Dresden). They were crossed to the mice bearing a Cre transgene under a CamKII promoter in order to confer forebrain specificity for Cre expression. The heterozygous mice bearing the Cre transgene that originated from this cross were then crossed to each other in order to give rise to homozygous floxed mice without the Cre transgene (F/F) and homozygous floxed mice with the Cre transgene (F/F CKII) which were the conditional knock-out mice. The F/F mice were used as controls in all the experiments

that followed. In the homozygous mice bearing the Cre transgene under CamKII promoter 2nd exon of *Mll2* was recombined. The presence of FRT sequence between the 1st and 2nd exon ensured that this recombination resulted in a premature stop codon through frameshift mutation. The scheme used for obtaining the conditional knock-out is outlined in the figure below.

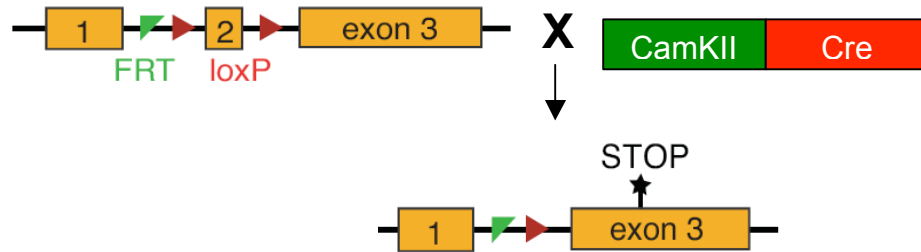


Figure 1.2: The exon 2 of *Mll2* gene is contains loxP sites at its borders. The removal of exon 2 through Cre recombination results in a frameshift mutation which induces a premature stop codon.

The expression of the full-length transcript of *Mll2* is decreased in the forebrain of F/F CKII mice

Next, I compared the expression of the full-length transcript of *Mll2* in DG, CA, prefrontal cortex and cerebellum in F/F and F/F CKII mice. For this purpose I used a primer pair targeting exon 1 and exon 2 of *Mll2* gene. As expected, I saw a downregulation of the full-length transcript of *Mll2* in the hippocampus, prefrontal cortex but not in the cerebellum of the CKII mice (Figure 1.3A).

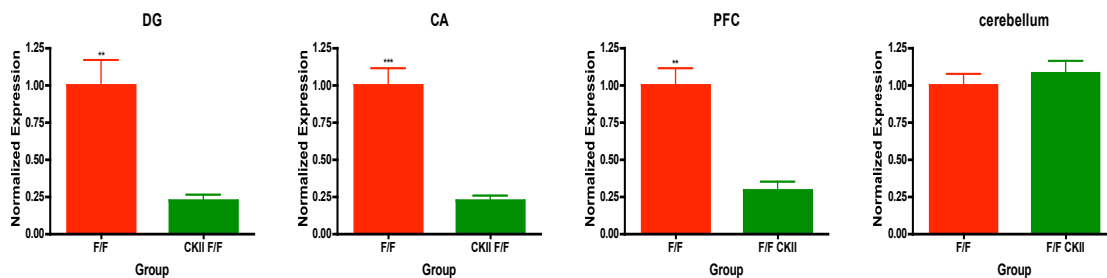


Figure 1.3: Recombination of exon 2 in the *Mll2* gene occurs in hippocampus (DG and CA) and prefrontal cortex but not in cerebellum. (n = 9, ** $p < 0.01$, *** $p < 0.001$).

Moreover, the knock-down of Mll2 in the forebrain does not lead to any change in Mll1 and Mll3 – its close homologs (Figure 1.4).

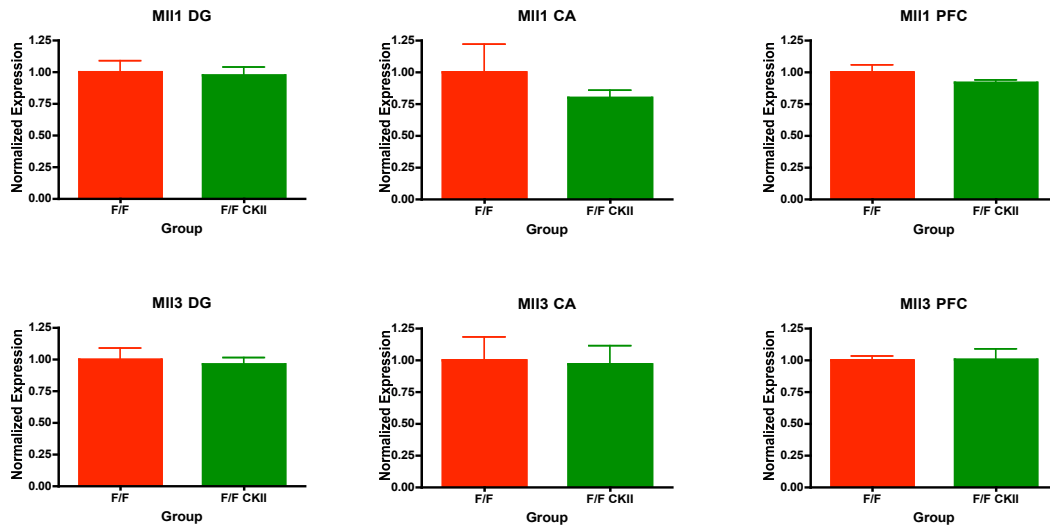


Figure 1.4: There is no up or down regulation of Mll1 or Mll3 in response to Mll2 knock-down (n = 5).

Brain morphology and body size are normal in F/F CKII mice

Brain and body size of F/F CKII mice were comparable to those of the controls (Figure 1.5). Although female F/F CKII mice showed a slightly increased body weight compared to their control counterparts (Figure 1.5).

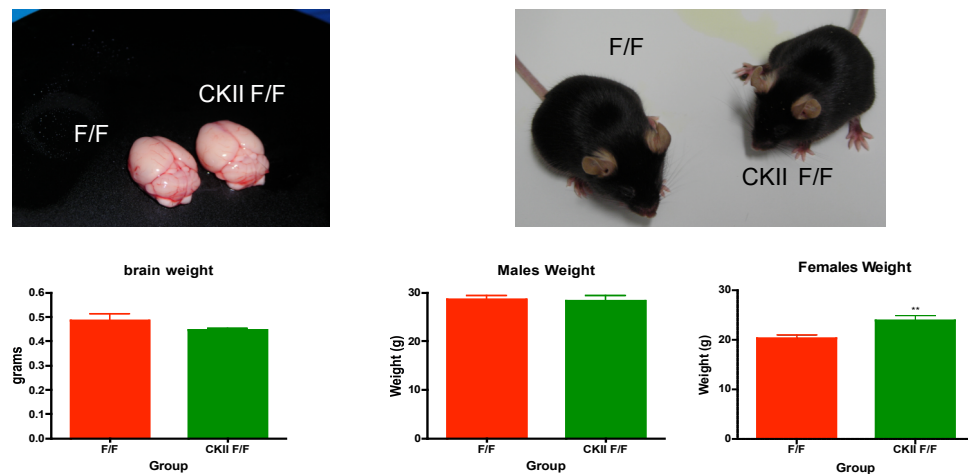


Figure 1.5: The brain (n = 8) and body weight (n = 10) of F/F CKII mice do not differ from those of the controls. Although female knock-out mice show a slightly increased body weight ($p < 0.01$).

Next, the neuronal and synaptic integrity was tested in F/F CKII mice. Staining for MAP2 indicates that there is no difference in dendrite density between F/F and CKII

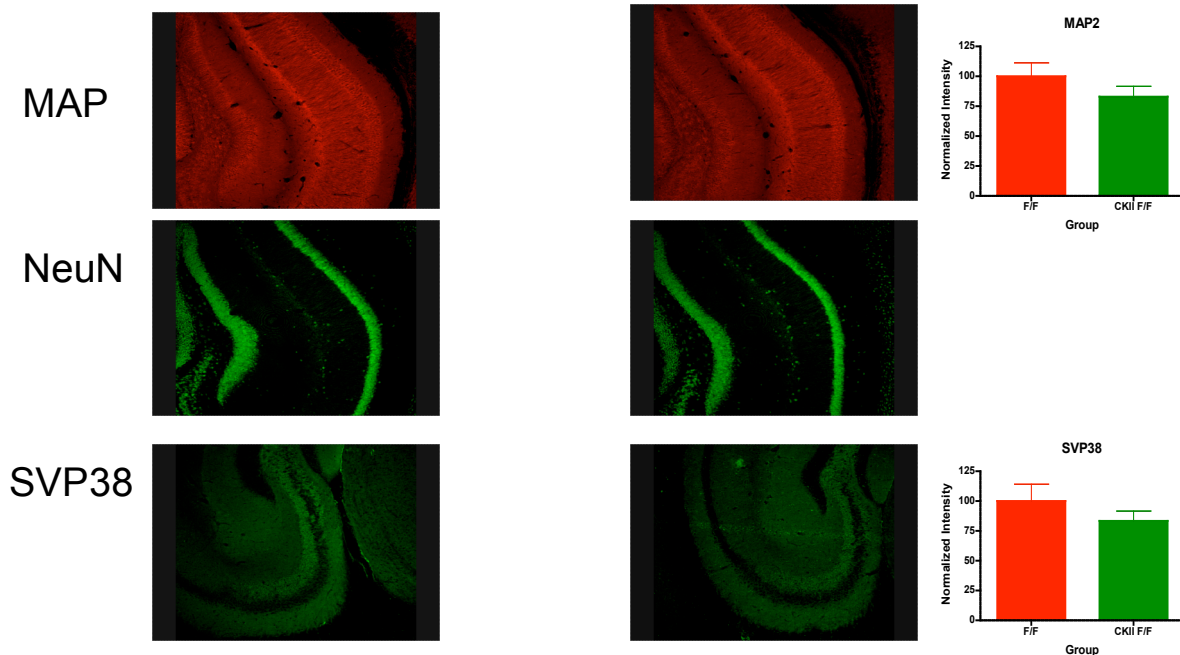


Figure 1.6: F/F CKII mice have a normal neuronal and synaptic morphology as observed by MAP2, NeuN and Synaptophysin staining (n = 4).

F/F mice. Synaptophysin staining showed an uncompromised integrity of the presynaptic terminals in F/F CKII mice. And staining for NeuN did not reveal any obvious differences between groups (Figure 1.6).

Knock-down of MII2 in the forebrain leads to increased anxiety and learning deficits

It has previously been shown that MII1 heterozygous knock-out mice show impaired synaptic plasticity and memory impairment in fear conditioning (Kim *et al.*, 2007; Gupta *et al.*, 2010). Therefore I set out to study the effect of MII2 knock-out in the forebrain on behaviour in mice.

Increased Anxiety: Firstly, I observed that F/F CKII mice show increased anxiety compared to the controls. This manifested itself in significantly decreased time spent in the center of an open field and in the open arms of an elevated plus maze (Figure 1.7A). However, total distance travelled in the open field and total object exploration did not differ between F/F and F/F CKII mice (Figure 1.7B).

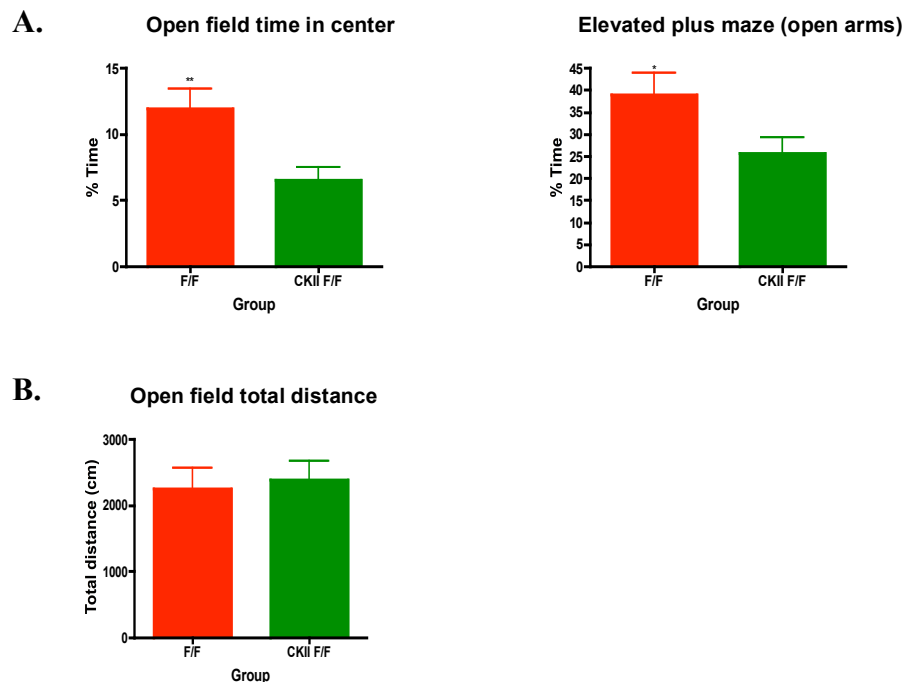


Figure 1.7: (A) Mll2 knock-down in the forebrain leads to increased anxiety as measured by Open Field (F/F: n = 30; F/F CKII: n = 31) and Elevated Plus Maze paradigms (F/F: n = 19; F/F CKII n = 26) (* $p < 0.05$, ** $p < 0.01$). (B) Mll2 knock-down does not lead to a change in total activity (F/F: n = 24; F/F CKII: n = 26).

Learning Impairment: F/F CKII mice also showed impaired performance in diverse learning paradigms – i.e. novel object recognition (NOR), Pavlovian fear conditioning and Morris water maze (Figure 1.8). This reflected itself in no preference for the new object above 50% chance level in novel object recognition paradigm, both 5 min and 24 h after exposure to similar objects (Figure 1.8A); in significantly lower freezing upon reexposure to the same context 24 h after receiving the footshock (Figure 1.8B); and in no preference to the target quadrant above chance level in the probe test of Morris water maze task (Figure 1.8C). Interestingly however, when mice were subjected to a more

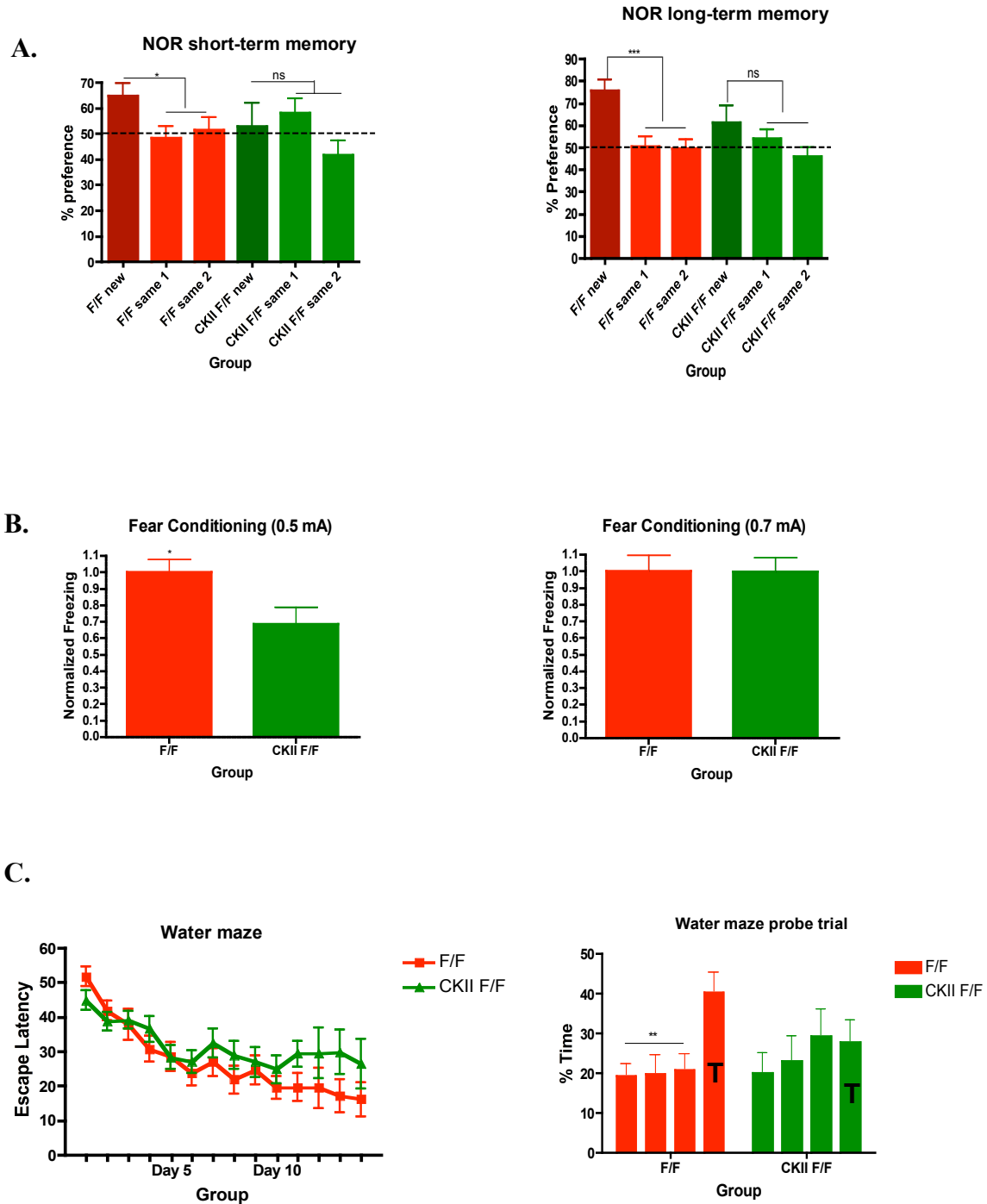


Figure 1.8: (A) Ml12 knock-down leads to a deficiency in short-term and long-term object memory manifested in F/F CKII mice showing no preference to a new object over the old one (F/F: $n = 14$; F/F CKII: $n = 10$; * $p < 0.05$, *** $p < 0.001$).

(B) Ml12 F/F CKII mice display a lower freezing level upon reexposure to the context 24 h after the 0.5 mA footshock (F/F: $n = 20$; F/F CKII: $n = 15$), however their freezing level is comparable to that of controls if 0.7 mA footshock is applied (F/F: $n = 22$; F/F CKII: $n = 25$; * $p < 0.05$).

(C) Ml12 F/F CKII mice show an impaired spatial memory in Morris Water Maze task with no preference to the target quadrant during probe trial (F/F: $n = 19$; F/F CKII: $n = 23$; ** $p < 0.01$).

stringent protocol of the fear conditioning paradigm (0.7 mA footshock) the F/F CKII mice did not show any impairment.

In all of the behavioural graphs shown (except for novel object recognition test) males and females were grouped together. No sex specific effects and no sex-genotype interaction with respect to behavioural performance were revealed by two-way ANOVA. Only male mice were included in the graphs showing the performance in novel object recognition task; the reason for this was that females of both groups barely approached the objects during the training session. There was however no difference between the genotypes in total object exploration time during testing (data not shown).

These results clearly indicate that *Mll2* functioning is crucial for a wide range of memory types (object recognition memory, associative memory, spatial memory) and anxiety behaviour.

Confirmation of differences in dorso-ventral gene expression pattern in hippocampus

There exists a dorso-ventral specificity of function within hippocampus, with dorsal hippocampus responsible for learning & memory (esp. spatial memory) and ventral hippocampus involved in anxiety (Moser and Moser, 1998). And since *Mll2* F/F CKII mice show increased anxiety together with learning impairment it was important to study dorsal and ventral parts of hippocampus separately in molecular terms in order to dissociate involvement of *Mll2* in anxiety phenotype from its involvement in learning & memory.

I decided to analyse gene expression in dorsal and ventral hippocampus individually. In order to confirm that our dissection method is reliable I selected some genes, which have previously been shown to have dorso-ventral specificity in hippocampus (Thompson *et al.*, 2008; Dong *et al.*, 2009; Fanselow and Dong, 2010), and compared their relative expression in dorsal and ventral hippocampus by qRT-PCR. I observed that genes, which were shown to be dorsal specific, were expressed at a higher level in dorsal hippocampal samples, and those known to be ventral specific had a higher expression in ventral hippocampal samples (Figure 1.9).

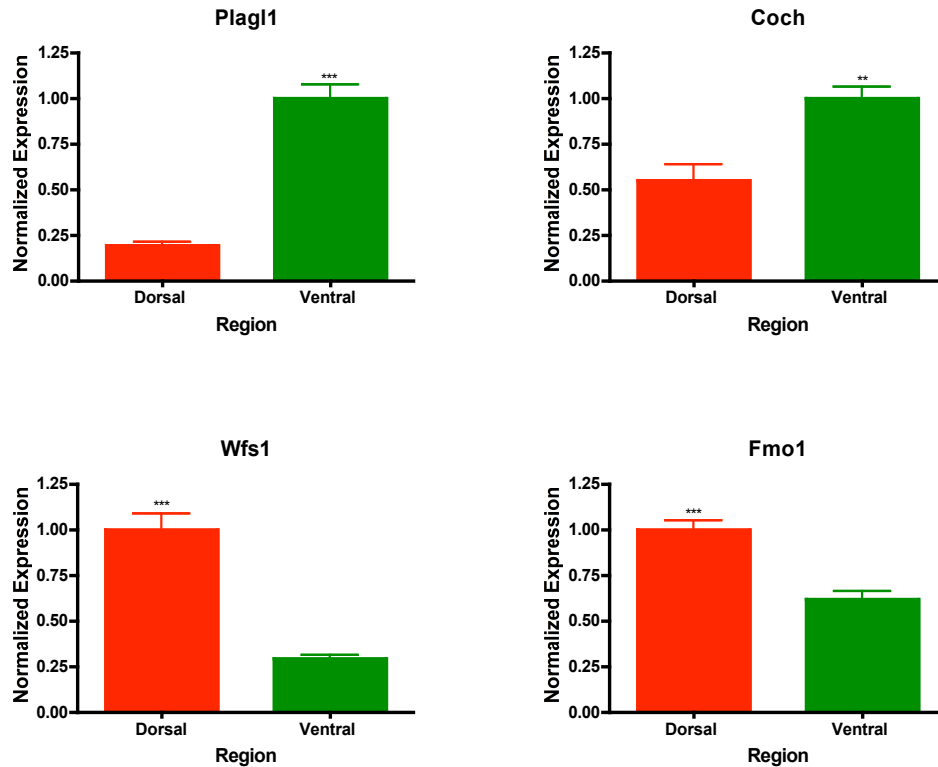


Figure 1.9: Differential expression of genes showing dorso-ventral specificity was confirmed by qRT-PCR in ventral (A) and in dorsal (B) hippocampus (n = 4, ** $p < 0.01$, *** $p < 0.001$).

Knock-down of Mll2 leads to deregulation of gene expression in dentate gyrus

The next thing I wondered was whether Mll2 knock-down leads to changes in gene expression profile that could lead to the observed learning impairment. For this purpose a DNA microarray analysis was carried out from dorsal dentate gyrus and dorsal CA regions of F/F (n=3) and F/F CKII (n=5) mice. It was observed that knock-down of Mll2 lead to a significant reduction in expression levels of 153 genes (fold change = 1.5; FDR = 0.05) in the dorsal dentate gyrus (Figure 1.10) which is consistent with the fact that Mll2, as a histone 3 methyltransferase, is a transcriptional activator (Demers *et al.*, 2007; Glaser *et al.*, 2009). Moreover, there were also 10 genes, which were upregulated the dorsal dentate gyrus of F/F CKII mice, which may be attributed to secondary effects of Mll2 knock-down (see the full list of differentially regulated genes in Appendix 1). 16 of the downregulated genes were chosen for further qPCR confirmation (Figure 1.11).

Interestingly however, such a drastic downregulation of gene expression was not observed in the dorsal CA region (Figure 1.10).

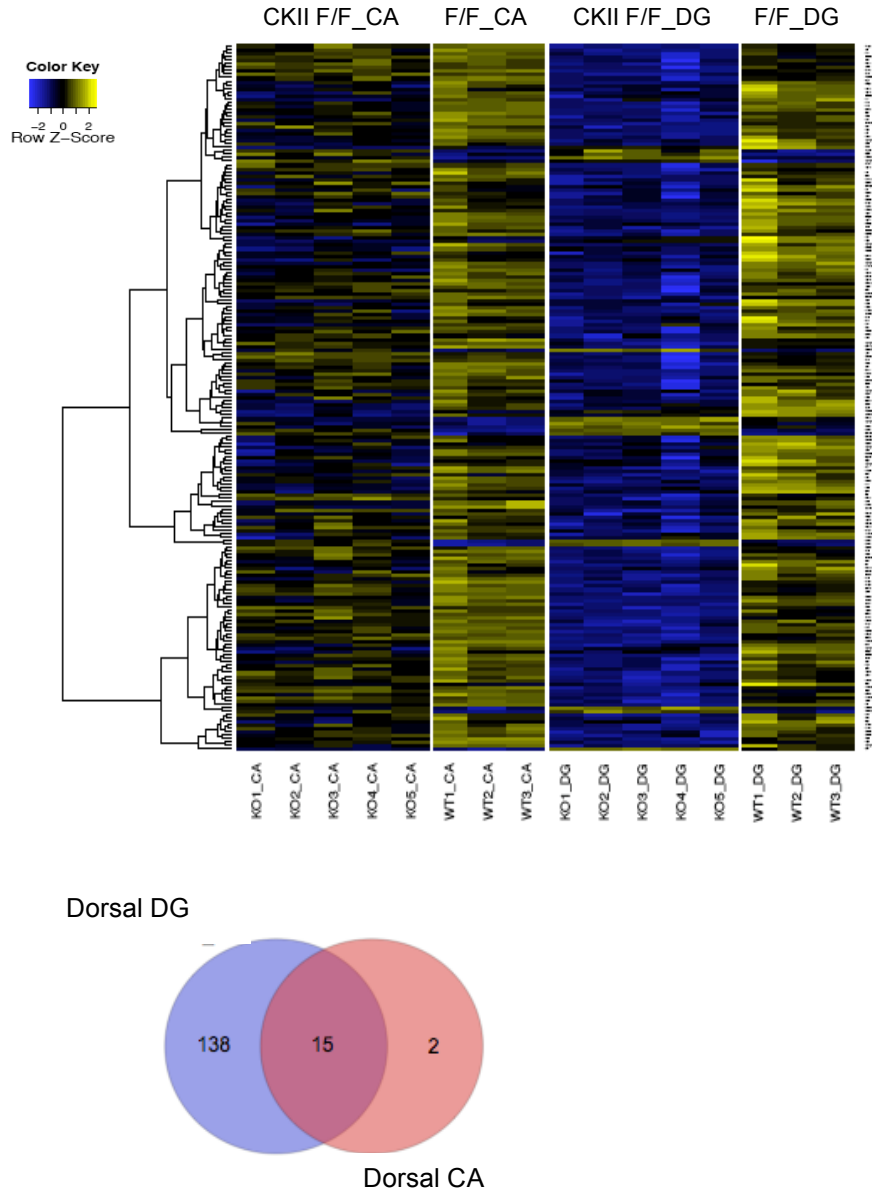


Figure 1.10: Mll2 knock-down leads to extensive downregulation of gene expression in dorsal dentate gyrus but not in dorsal CA (F/F: n = 3; F/F CKII: n = 5). The Venn diagram shows the number of downregulated genes in DG and CA regions of Mll2 F/F CKII mice.

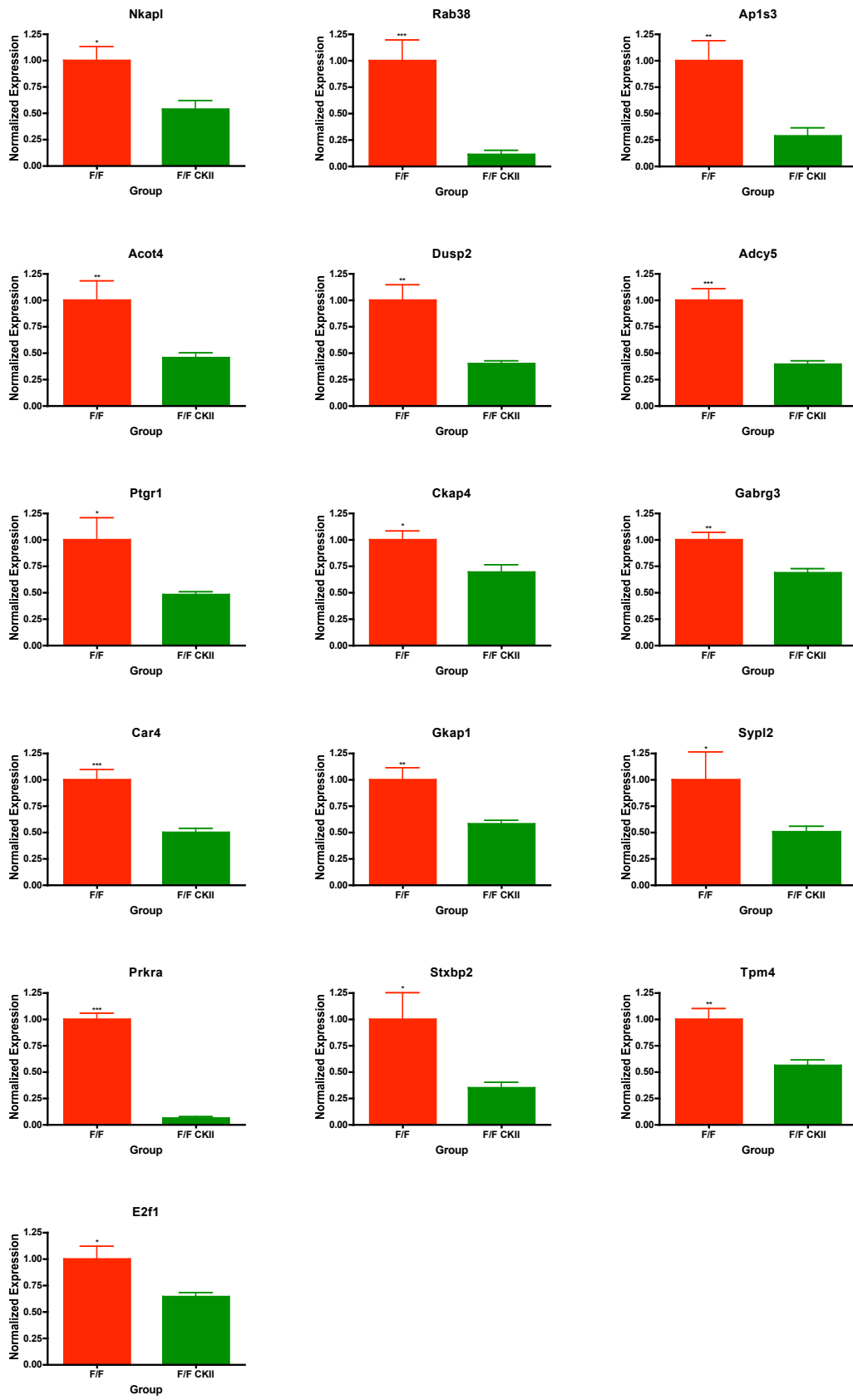


Figure 1.11: 16 genes that were found to be downregulated in dorsal dentate gyrus of F/F CKII mice were chosen for qRT-PCR confirmation. All of them were confirmed (F/F: n = 3; F/F CKII: n = 5; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

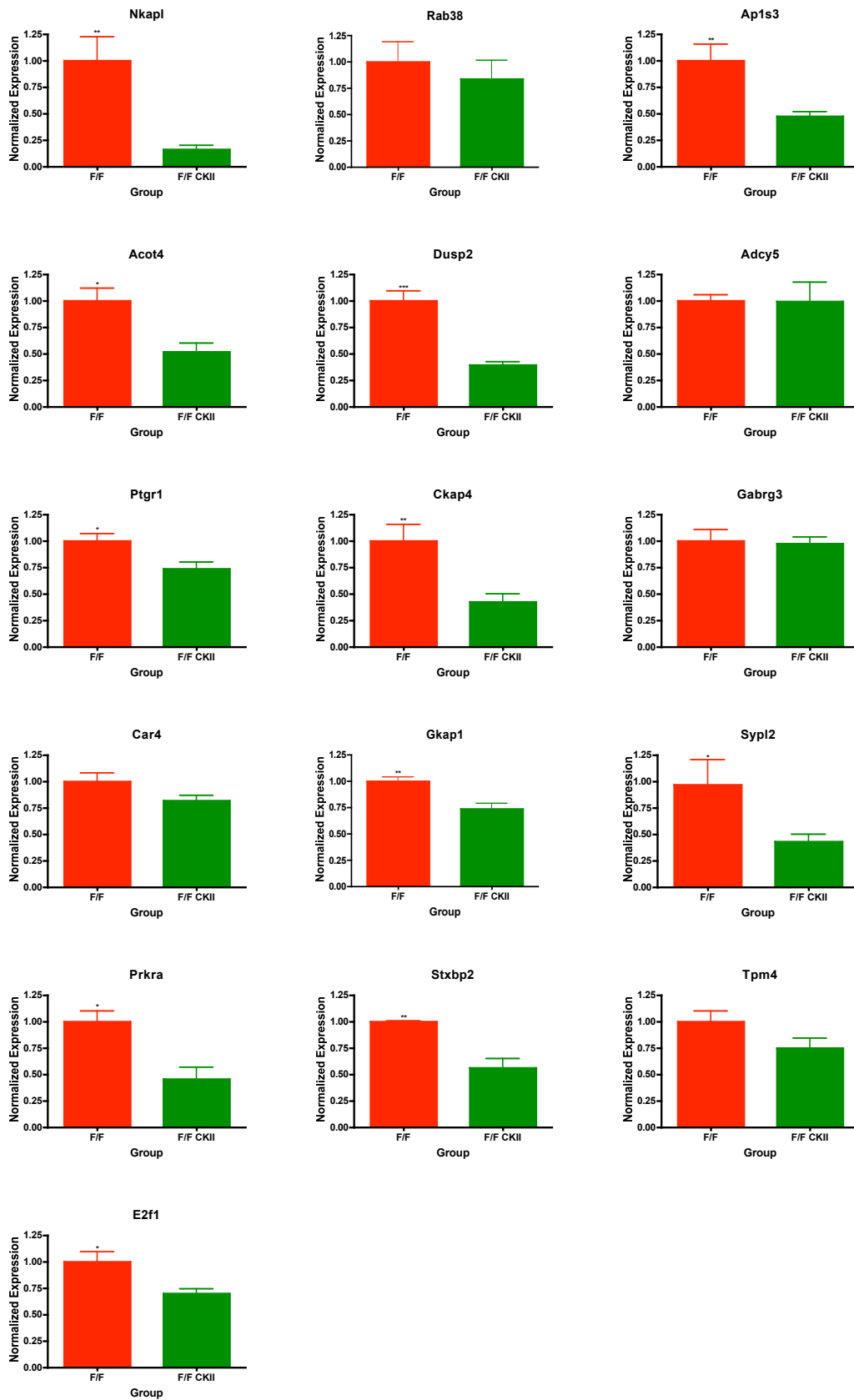


Figure 1.12: Most of the genes that were downregulated in dorsal dentate gyrus in F/F CKII mice are also downregulated in the ventral dentate gyrus. However, in spite of being downregulated in dorsal dentate gyrus, *Rab38*, *Adcy5*, *Gabrg3*, *Car4* and *Tpm4* do not show downregulation in ventral part (F/F: n = 3; F/F CKII: n = 5; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

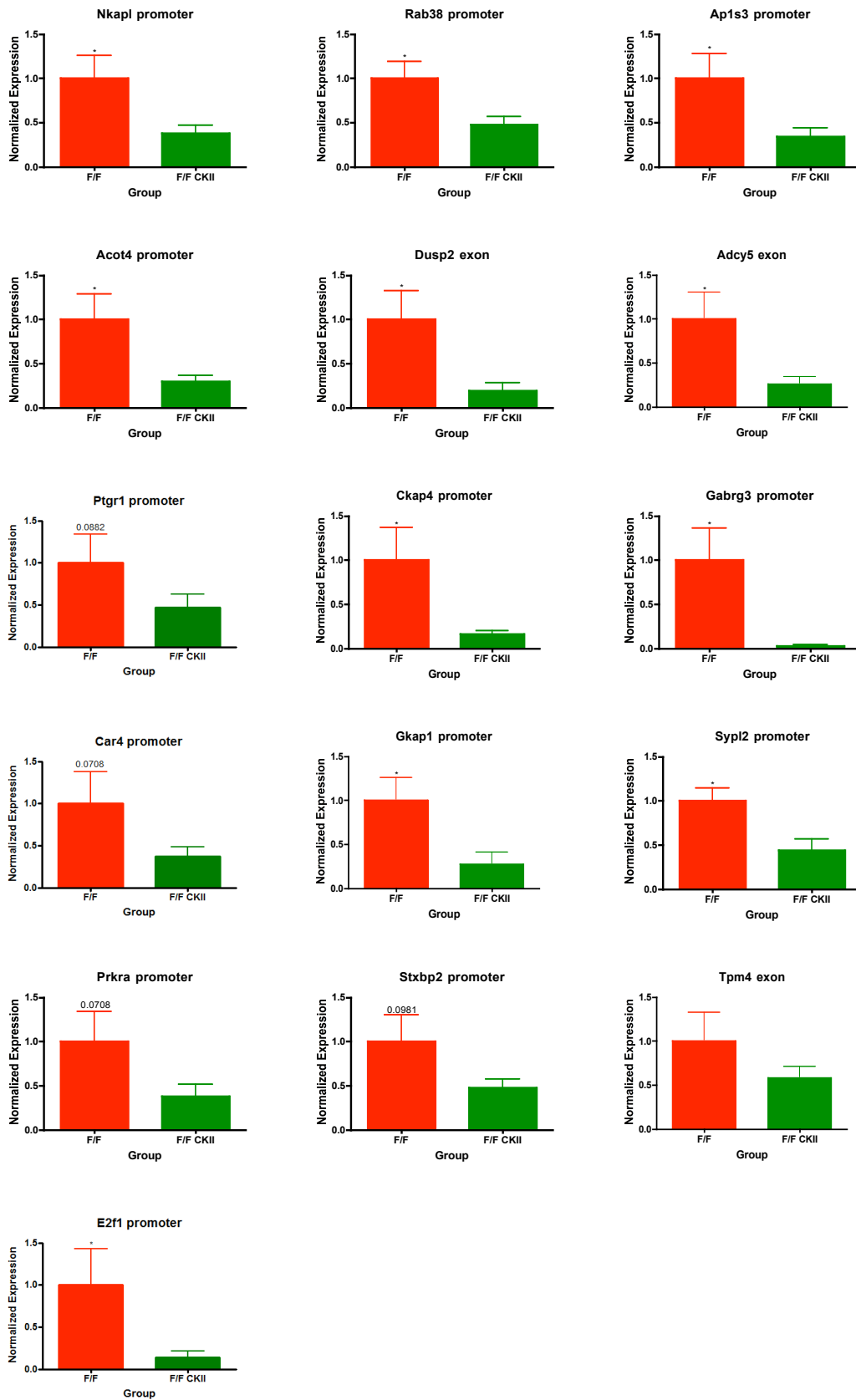


Figure 1.13: The genes that are downregulated in the dorsal dentate gyrus of F/F CKII mice also show decreased level of H3K4 trimethylation at their promoters ($n = 5$; $p < 0.05$).

The genes chosen for qPCR confirmation were also reconfirmed in a separate batch of mice, which were not used for the microarray. Interestingly, 4 of those genes (*Rab38*, *Adcy5*, *Gabrg3*, *Car4*, *Tpm4*), which were downregulated in the dorsal dentate gyrus, were not downregulated in the ventral dentate gyrus of the knock-out mice (Figure 12), which indicates that there is some dorso-ventral specificity of Mll2 action in dentate gyrus.

Mll2 knock-down leads to decreased histone 3 Lysine 4 methylation (H3K4me) at gene promoters

Since there was an extensive downregulation of gene expression in dentate gyrus as a result of Mll2 knock-down, my next step was to study the level of H3K4 methylation at the promoters of those genes. In line with the findings from the DNA microarray ChIP analysis revealed downregulation in H3K4me3 (Figure 1.13) and H3K4me2 (Figure 1.14), but not in H3K4me1 levels (Figure 1.15) at the gene promoters (primers for the promoters of *Dusp2*, *Adcy5* and *Tpm4* did not lead to an amplification, therefore primers targeting the coding region were used for these genes). At some gene promoters (i.e. *Ptgr1*, *Car4*, *Prkra*, *Stxbp2*) H3K4me3 levels showed a strong tendency towards downregulation in the F/F CKII mice, which nevertheless failed to reach significance. These results further confirm that histone methyltransferase activity of Mll2 plays an important role in regulating gene expression in the dentate gyrus, and through this in affecting the cognitive and behavioural phenotype in mice.

Injection of HDAC inhibitor SAHA does not change the expression of the genes neither in F/F nor in F/F CKII mice

It has been indicated in a previous study that the Histone 3 Lysine 4 methyl mark serves as a prerequisite for the binding of histone acetyltransferases (HATs) at the promoter regions (Wang *et al.*, 2009); in other words Histone 3 Lysine 4 methylation serves a key to enable histone acetylation at the same promoter. It has already shown in our lab that

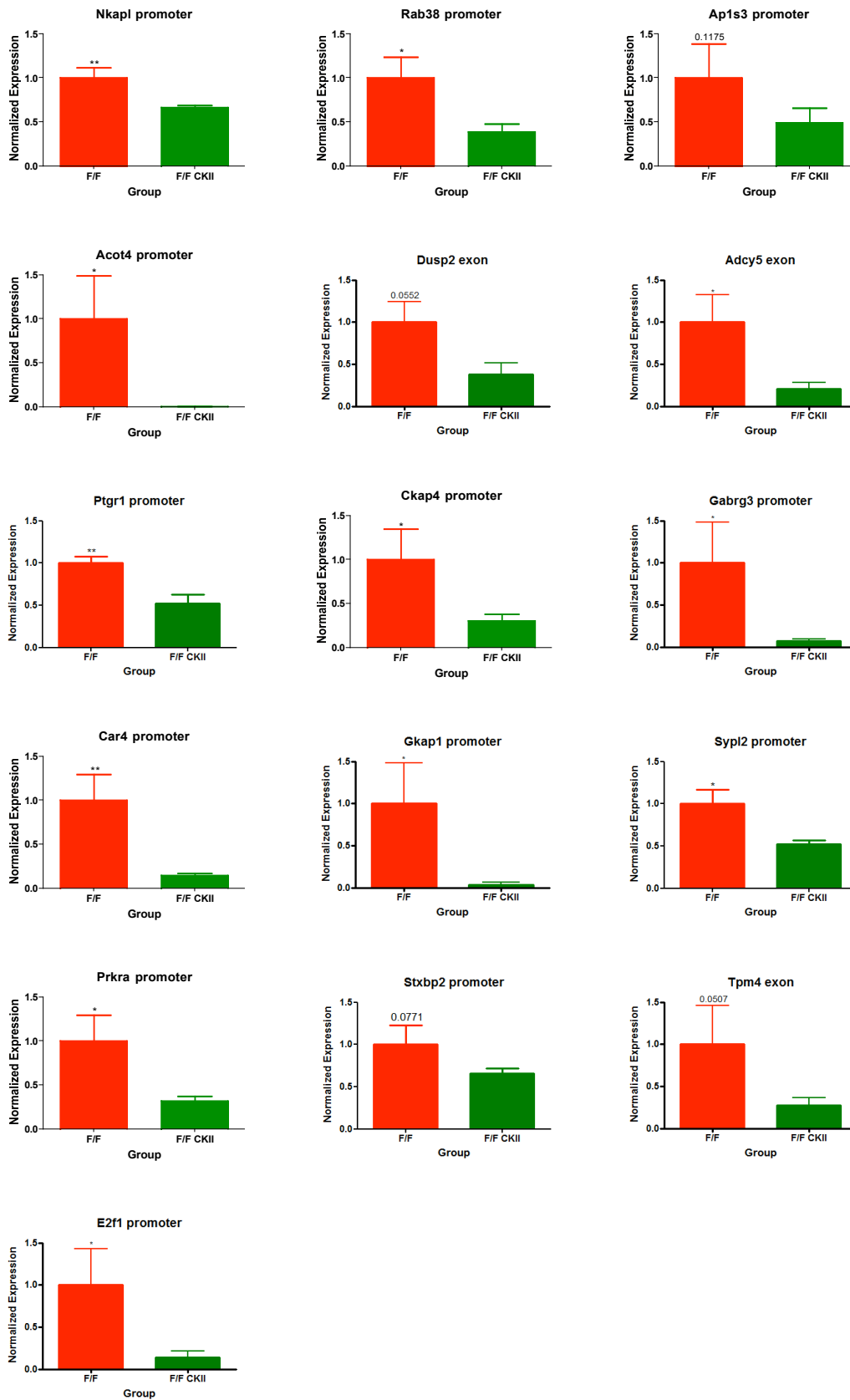


Figure 1.14: The genes that are downregulated in the dorsal dentate gyrus of F/F CKII mice also show decreased level of H3K4 dimethylation at their promoters (n = 5; * $p < 0.05$, ** $p < 0.01$).

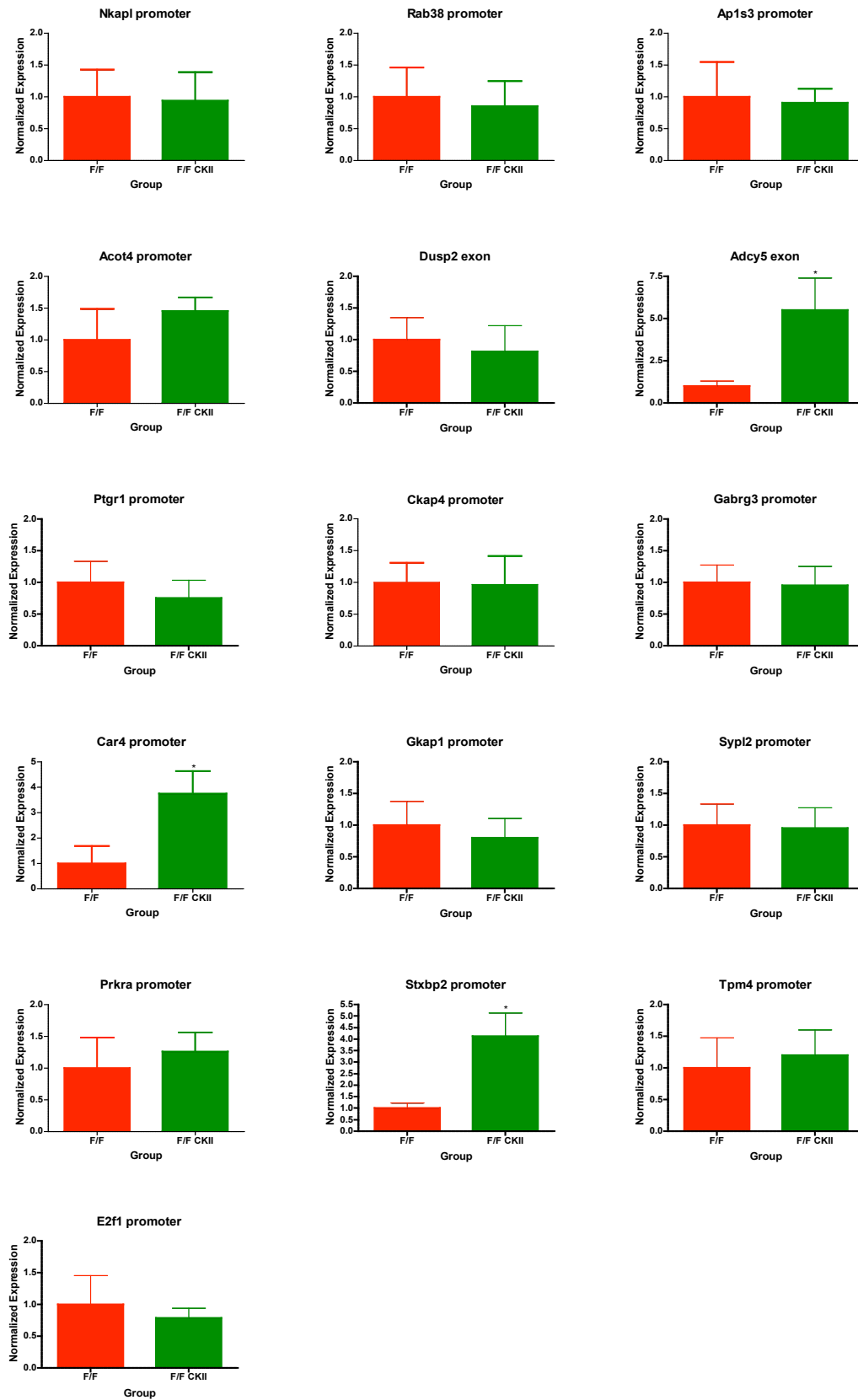


Figure 1.15: The genes that are downregulated in the dorsal dentate gyrus of F/F CKII mice do not show a decrease in the level of H3K4 monomethylation at their promoters ($n = 5$; $* p < 0.05$).

acute intraperitoneal injection of SAHA leads to an increase in bulk histone acetylation in hippocampus of wild type B6J mice, which peaks at 1 hour after injection. Therefore, before proceeding with the analysis of gene expression I tested the effect of SAHA in hippocampus of F/F and F/F CKII mice 1 hour after injection. Both F/F and F/F CKII mice had an increase in bulk histone acetylation 1 hour after SAHA treatment (Figure 1.16). Next, I set out to investigate whether treatment of mice with an HDAC inhibitor would increase the expression of the genes, which were downregulated in F/F CKII mice. For this purpose another group of F/F and CKII F/F mice was injected intraperitoneally either with SAHA or vehicle. The expression of selected genes was tested in the dorsal dentate gyrus of F/F and F/F CKII mice 1h after they received the intraperitoneal SAHA injection. Interestingly however, it was observed that SAHA injection did not lead to an increase in the expression of the genes previously shown to be downregulated as a result of Mll2 knock-down, neither in F/F nor in F/F CKII mice (Figure 1.17).

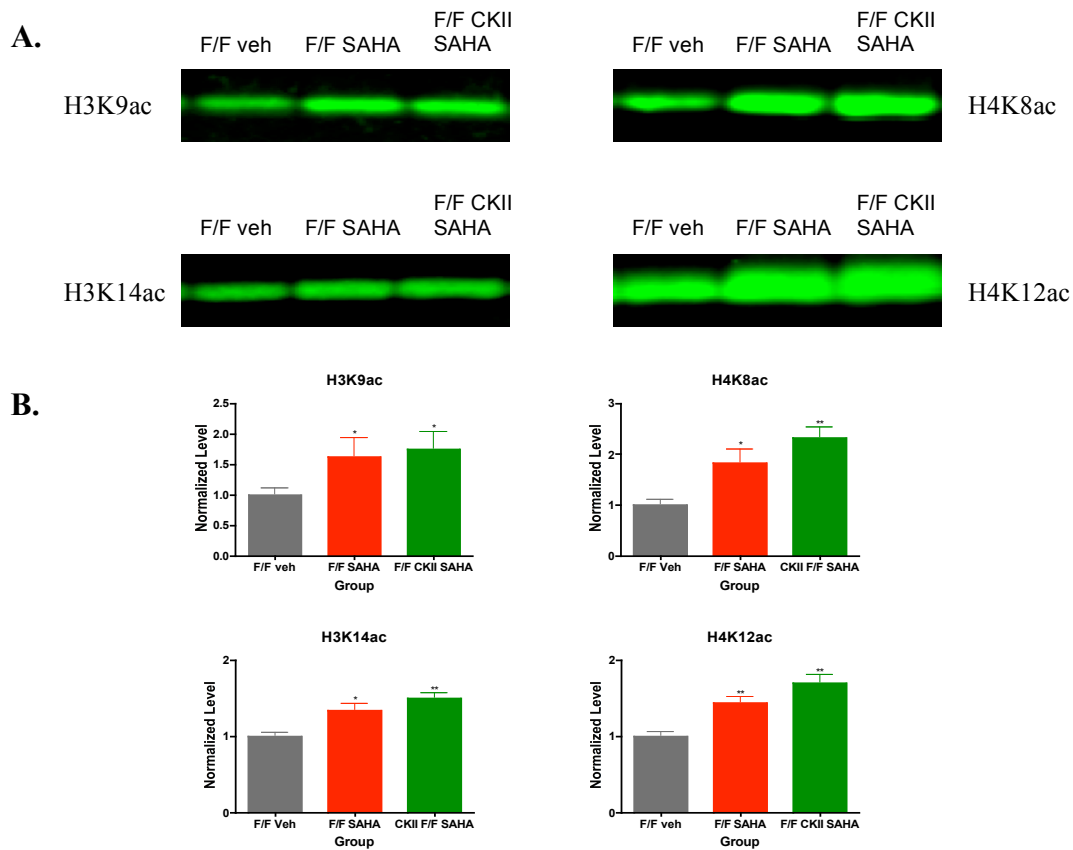


Figure 1.16: (A) Acute SAHA injection increases bulk histone acetylation in hippocampus of F/F and F/F CKII mice 1 hour after injection.

(B) Quantification of the immunoblots in (A) (F/F veh: n = 4; F/F SAHA: n = 5; F/F CKII SAHA: n = 5; * $p < 0.05$, ** $p < 0.01$).

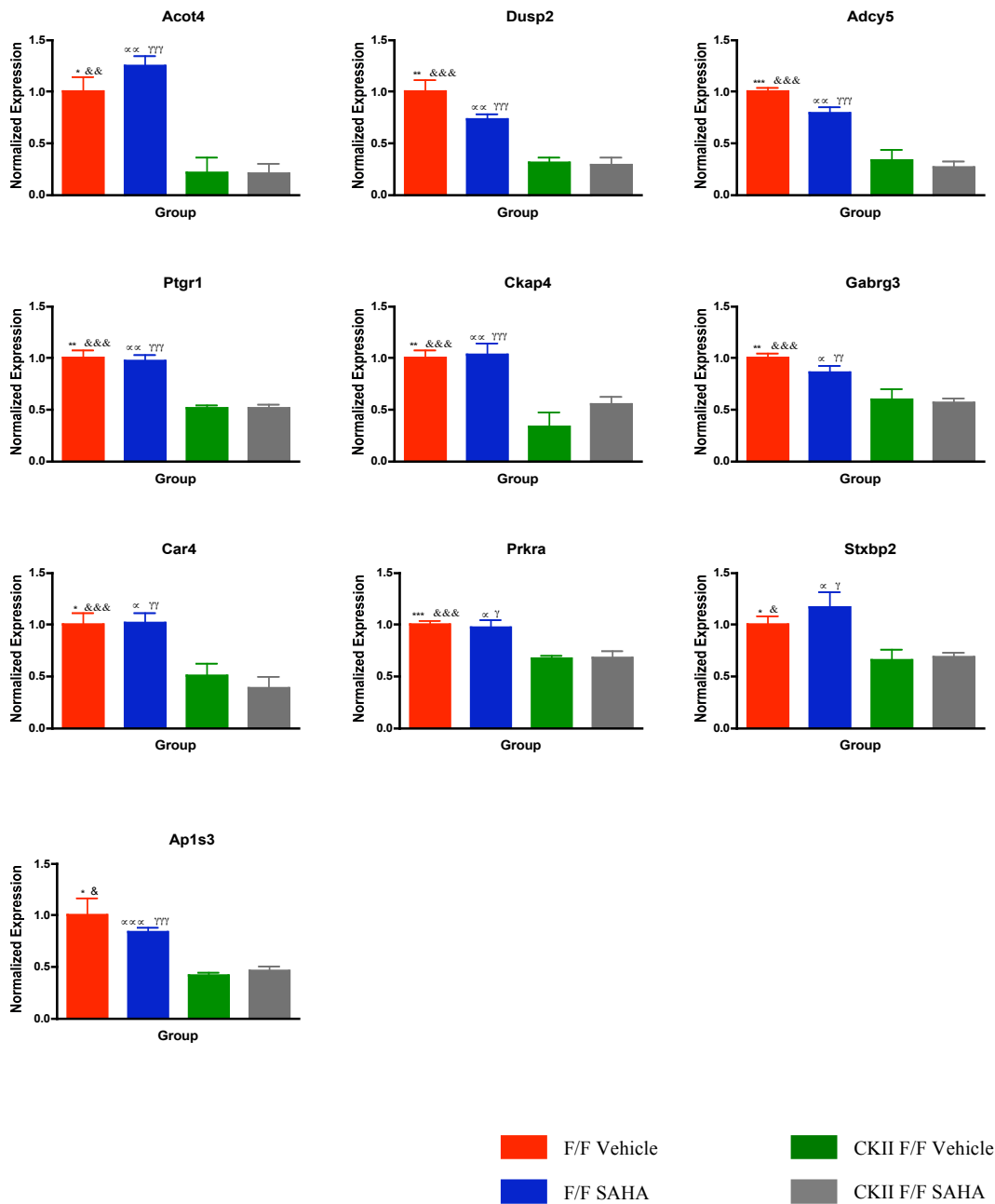


Figure 1.17: The genes found to be downregulated after M12 knock-down do not show any change in expression after SAHA injection in either group (* = F/F Vehicle vs CKII F/F Vehicle; & = F/F Vehicle vs CKII F/F SAHA; α = F/F SAHA vs CKII F/F Vehicle; γ = F/F SAHA vs CKII F/F SAHA; *, &, α , γ $p < 0.05$; **, &&, $\alpha\alpha$, $\gamma\gamma$ $p < 0.01$; ***, &&&, $\alpha\alpha\alpha$, $\gamma\gamma\gamma$ $p < 0.001$).

PART 2: EFFECTS OF DIFFERENT DURATIONS OF ENVIRONMENTAL ENRICHMENT ON LEARNING AND MEMORY

2 weeks of EE are enough to facilitate learning whereas 1 week of EE is not

My first aim was to establish the minimum amount of EE in our experimental setting, which would be enough to facilitate learning. Up to this day there is no standardized environmental enrichment protocol. That is why before proceeding with further experiments it was necessary to establish what amount of EE is enough to facilitate learning in our laboratory. I observed that 2 weeks of EE were enough to facilitate learning in Pavlovian fear conditioning paradigm, whereas 1 week of EE was not (Figure 2.1). This finding obviously does not exclude the possibility that environmental enrichment performed for an amount of time between 1 – 2 weeks will also be enough to facilitate learning, however for the sake simplicity in all further experiments I performed environmental enrichment for at least 2 weeks. And for the sake of simplicity in presentation and discussion of results I will treat 2 weeks of EE as the minimum amount of EE required to facilitate learning henceforth.

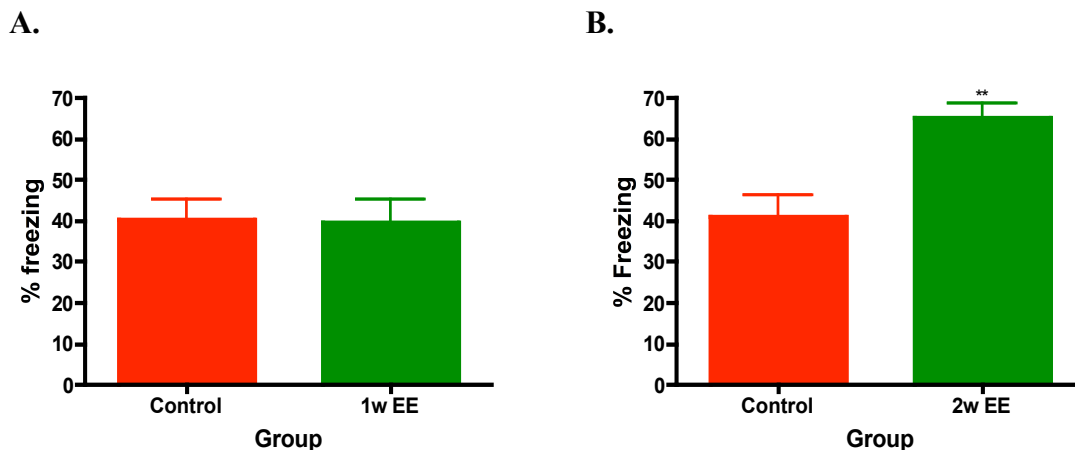


Figure 2.1: Environmental enrichment of a duration of 1 week is not enough to facilitate learning (A) in wild-type B6J mice, whereas it does facilitate learning when performed for 2 weeks (B) ($n = 8$; $p < 0.01$).

Long-term EE has persistent effects on learning whereas short-term EE does not

Once that I established the minimal amount of EE required to exert positive effects on learning and memory, my next aim was to find out whether that effect is persistent and if yes to what extent; i.e. will the mice who were enriched for 2 weeks still perform better on learning tasks after enrichment is terminated and they are housed in standard home cages? To investigate this, two different groups of mice were subjected to Morris water maze and Pavlovian fear conditioning paradigms; (i) mice that were kept in standard home cages (HC) and (ii) mice that were enriched for 2 weeks and were left in standard home cages for 1 week before being subjected to learning experiments (2w EE – 1w HC). It turned out that the effects of 2 weeks of EE were only partly persistent; i.e. the mice in

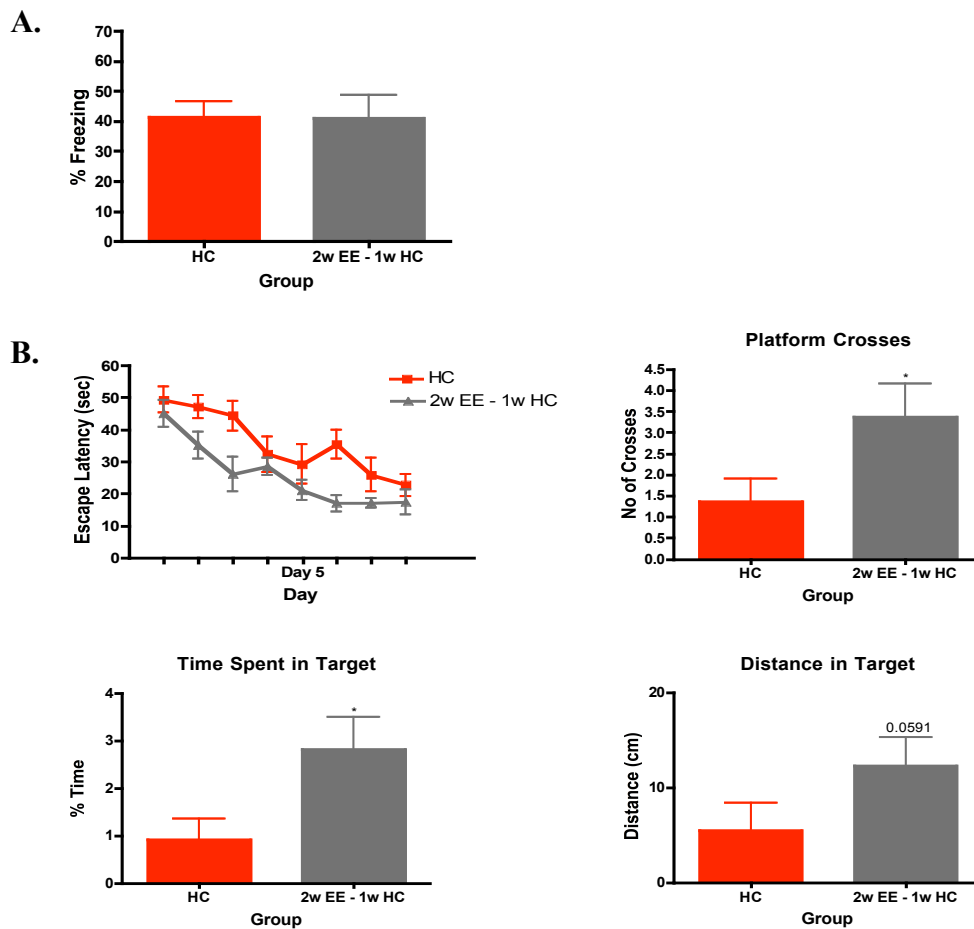


Figure 2.2: (A) 2w of EE do not have persistent effects on learning in Pavlovian fear conditioning ($n = 8$).

(B) Mice that undergo 2w of EE continue to have facilitated memory even after being kept in home cage for 1 week ($n = 8$, $p < 0.05$).

the “2w EE – 1w HC” group performed better than the home caged mice in the Morris water maze (Figure 2.2B), however unlike the mice which were enriched for 2 weeks and subjected to learning experiments right after that, the mice in the “2w EE – 1w HC” group did not have increased memory in Pavlovian fear conditioning anymore (Figure 2.2A).

These findings made me wonder whether 2 weeks of EE would still have its positive effects on learning & memory in the water maze paradigm if the retention period would be extended far beyond 1 week. For this reason, the same mice that were used in previous experiment were still kept in home cages for up to 2 months, and then subjected to a novel water maze experiment. However, in this case it was necessary to ensure that the mice are really subjected to a completely novel task without any possible influence from the memory acquired in the previous water maze learning. Therefore, for this experiment the spatial cues were rearranged and the platform was put to another location. It was observed that when kept in home cage for 2 months after 2 weeks of EE the mice did not have enhanced memory anymore (Figure 2.3). Along with the result obtained from

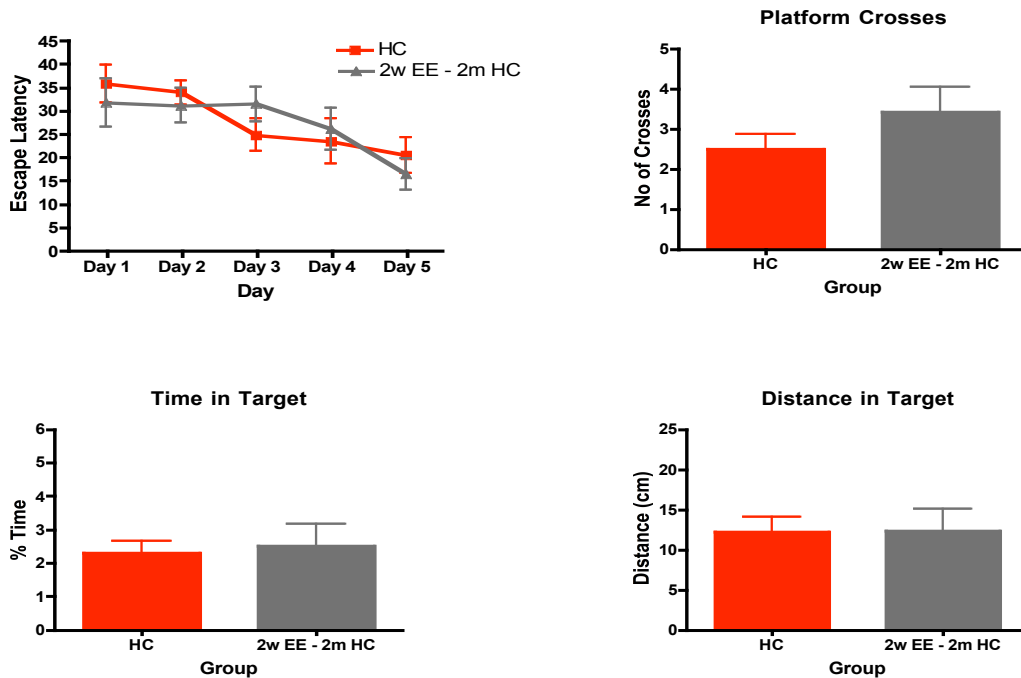
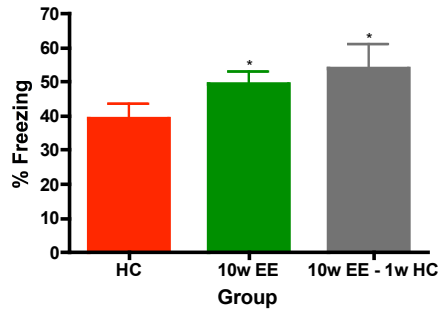


Figure 2.3: The positive effects of 2 weeks of EE on learning fade away when the retention period becomes much longer – i.e. 2 months (n = 8).

Pavlovian fear conditioning this finding once again indicates that 2 weeks of environmental enrichment, although being enough to facilitate learning, do not have long-lasting effects on cognition.

A.



B.

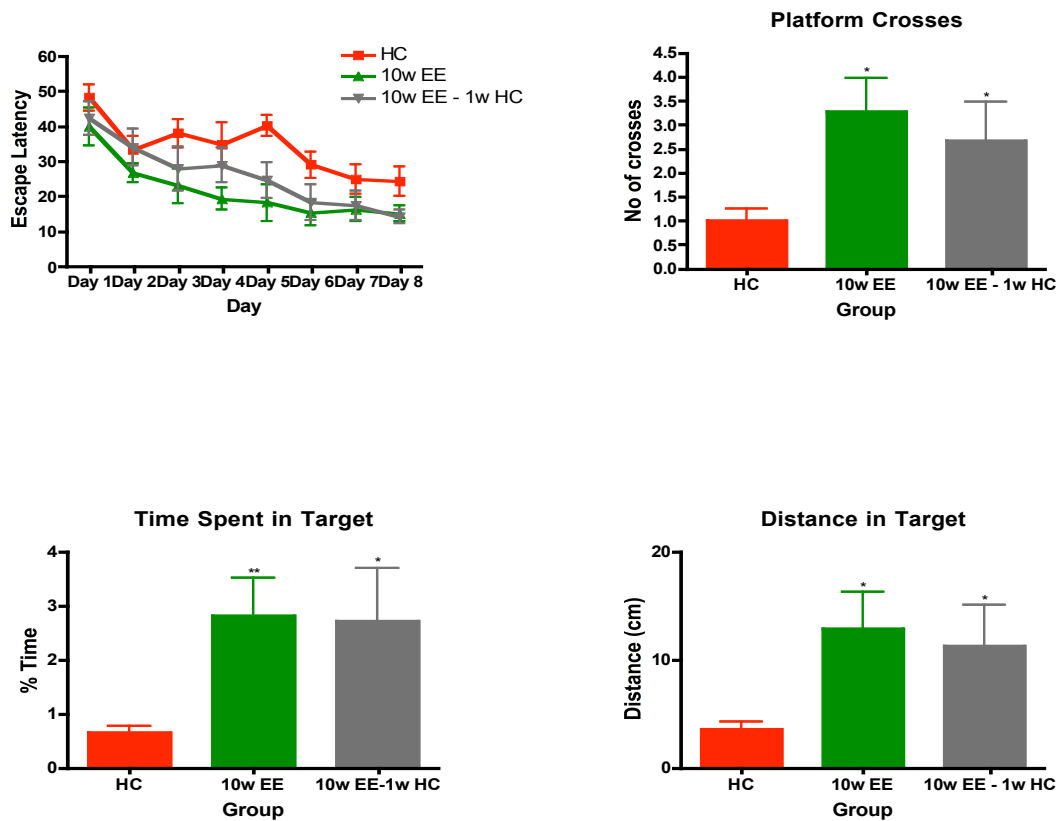


Figure 2.4: (A) 10 weeks of EE retain their positive effects on learning in fear conditioning task even after 1 week of retention ($n = 8, p < 0.05$).

(B) 10 weeks of EE have persistent effects on learning in water maze test as well ($n = 8, p < 0.05$).

Next, I wondered whether a longer period of enrichment could exert fully long-lasting effects on cognition. For this purpose a group of mice was enriched for 10 weeks and then kept at home cage 1 week before being subjected to learning experiments. Another group was housed in enriched environment for 10 weeks and subjected to learning tests straight after that. A control group that was housed in standard home cages throughout the experiments was included as well. The first interesting observation was that the mice that underwent 10 weeks of EE and were kept in home cage for 1 week afterwards performed significantly better in the fear conditioning (Figure 2.4A) and water maze tasks (Figure 2.4B) compared to the home cage controls.

These observations already provided the first hint that in contrast to 2 weeks of EE, 10 weeks of EE should have long-lasting effects on cognition. Therefore, the same mice that were used for the experiments mentioned above were kept in home cages for up to two months. For this particular experiment the “10w EE” and “10w EE – 1w HC” groups were grouped together into one group, which was named “10w EE – 2m HC”. The mice were then subjected to the modified water maze experiment mentioned above (see Figure 2.3). Interestingly, it was observed that even after being in the home cage for 2 months, the mice that underwent EE for 10 weeks still displayed increased learning (Figure 2.5). This indicates that long-term environmental enrichment exerts lasting effects on learning performance.

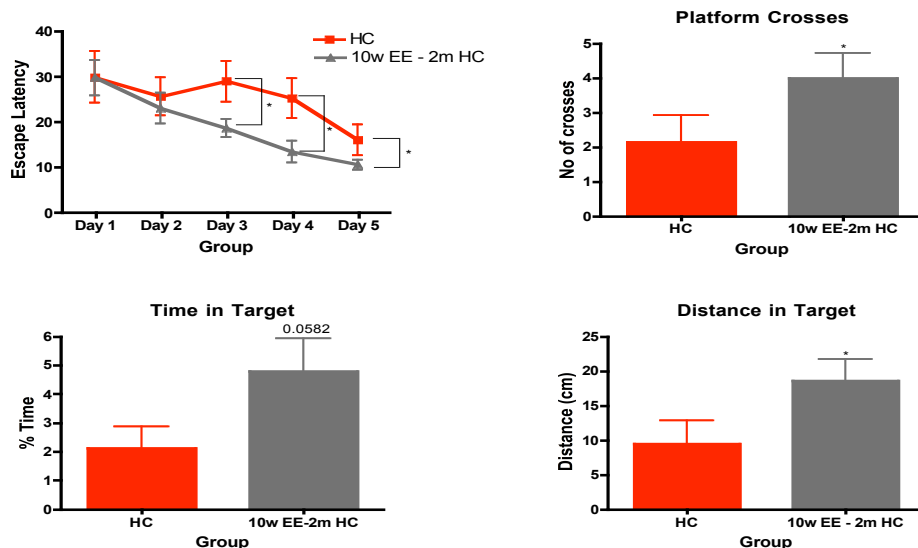


Figure 2.5: The positive effects of 10 weeks of EE on learning persist at least up to 2 months (HC: $n = 8$; 10w – 2m HC: $n = 16$; $p < 0.05$).

Long-term EE induces a substantial change in gene expression in hippocampus:

The next question was whether long-term and short-term enrichment elicit their effects through different mechanisms, such that the effects of the former are long lasting and the effects of the latter are not. In order to dwell into this question gene expression in hippocampus of mice enriched for 10 weeks was compared to those housed in standard home cages, by DNA microarray. It was observed that 10 weeks of EE induced an extensive change in gene expression pattern in hippocampal tissue (Figure 2.6). There were 62 genes that were upregulated and 69 genes that were downregulated as a result of 10 weeks of EE (FDR: 0.05, fold change: 1.5). 16 genes were chosen for qPCR confirmation (Figure 2.7); for the qPCR experiment also another group of mice, the ones that underwent 10 weeks of EE and then were kept in home cage for 2 months, was included. Surprisingly, it was observed that among those 16 genes tested only one of them (*Ccl8*) retained its expression level induced by enrichment for up to 2 months after the cessation of EE (Figure 2.7).

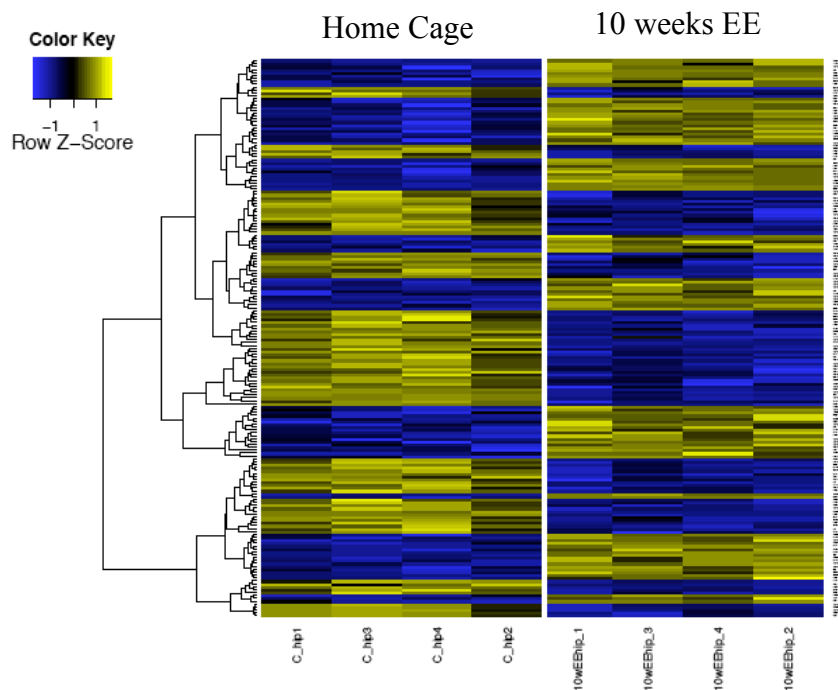


Figure 2.6: 10 weeks of EE induce upregulation in 62 and downregulation in 69 genes (FDR = 0.05; fold change = 1.5; n = 4).

Short-term enrichment induces only a subtle change in gene expression:

The genes that were confirmed to be differentially regulated in response to 10 weeks of EE were also tested in the hippocampus of mice that underwent only 2 weeks of EE. It was observed that the effect on 2 weeks of EE on gene expression was very mild. Only 3 out of those 16 genes had a different expression level in hippocampus after 2 weeks of EE when compared to the control levels - *Ptbd1*, *Pde6h* and *Klf1* (Figure 2.8).

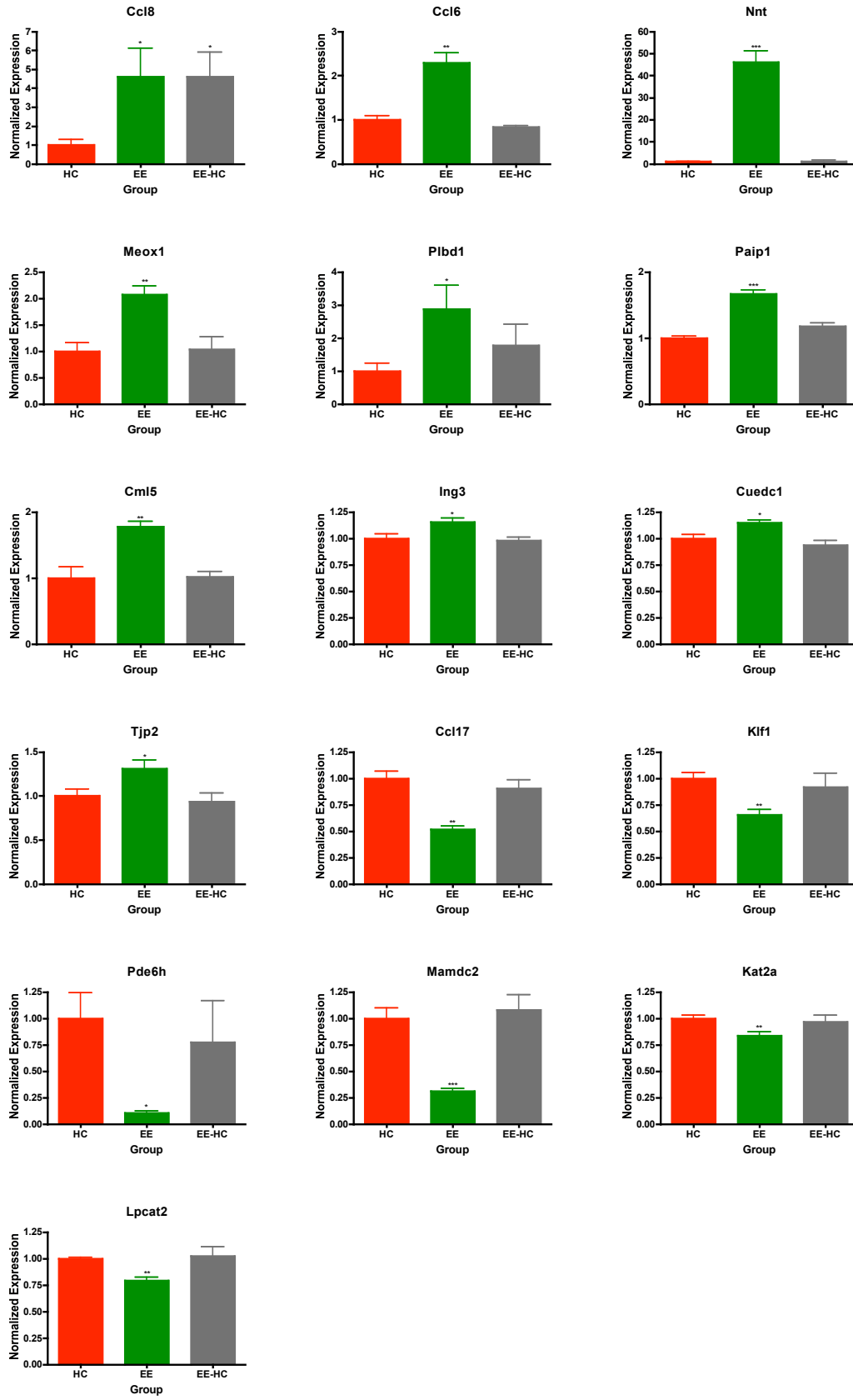


Figure 2.7: 16 of the genes that were differentially regulated in hippocampus after 10 weeks of enrichment (“EE” group) were chosen for qPCR confirmation. All of them except one (Ccl8) returned to basal levels after the mice were transferred to home cages (“EE – HC” group) ($n = 4$, * $p < 0.05$, ** $p < 0.01$).

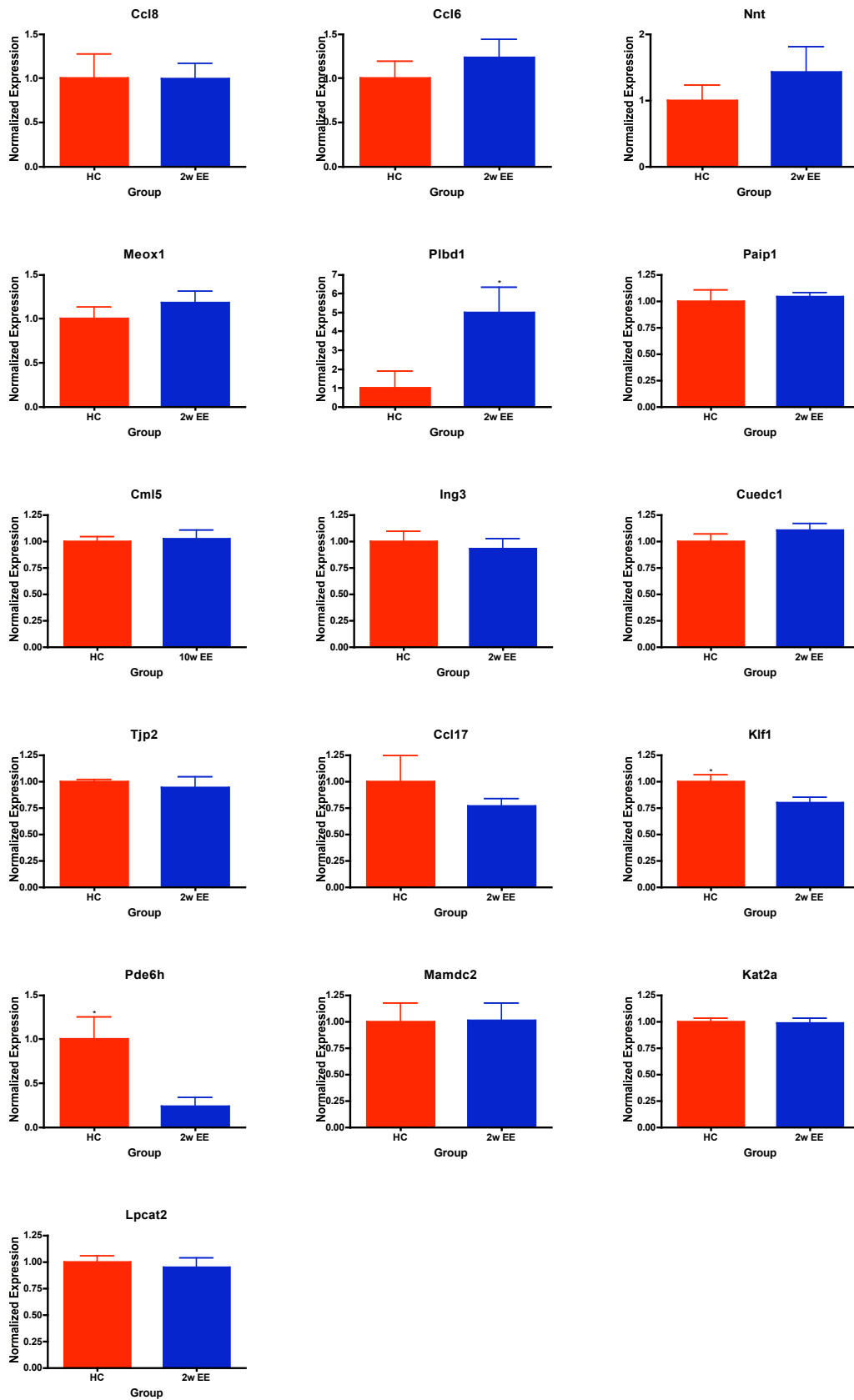


Figure 2.8: 15 of the genes that were differentially regulated in hippocampus after 10 weeks of enrichment (“EE” group) were chosen for qPCR confirmation. All of them except one (*Ccl8*) returned to basal levels after the mice were transferred to home cages (“EE – HC” group) ($n = 8$, * $p < 0.05$).

DISCUSSION

My thesis work consisted of two major directions. In one of them I analyzed the effects of knock-down of a histone methyltransferases (Mll2, in this case) in the forebrain on behavioural performance in mice and the molecular mechanisms underlying this. With this project I aimed to shed more light on the role of histone methylation in behaviour and specifically on learning & memory.

In my other project I investigated the role of environmental enrichment in improving learning & memory, with an emphasis on investigating to what extent its effects remain persistent after mice are removed from enriched environment.

Both of the projects yielded interesting results and venues for further research in the quest for deciphering the molecular mechanisms underlying learning & memory.

PART 1: CHARACTERIZATION OF FOREBRAIN SPECIFIC MLL2 CONDITIONAL KNOCK-OUT MICE

Mll2 knock-down in the forebrain and general characterization

Knock-down of Mll2 was achieved through Cre – loxP recombination of the 2nd exon of the gene. The exon2 of *Mll2* gene was flanked by loxP sites and in addition an FRT sequence was inserted into the first intronic region (F/F). Recombination of the “floxed” 2nd exon through the action of Cre protein lead to a frameshift mutation which in turn gave rise to a premature stop codon on the 3rd exon and hence prevented the translation of the mRNA into a functional protein. The occurrence of the frameshift mutation was in turn rendered possible by the presence of the above-mentioned FRT sequence. However, my aim was to induce the knock-down of Mll2 in the adult stage and specifically in the forebrain, and then to study the consequences of this manipulation on behaviour and molecular mechanisms underlying it. There are several reasons for this strategy rather than studying behavioural phenotype in mice with straight Mll2 knock-out. Firstly and most importantly, complete Mll2 knock-down was shown to be lethal after embryonic day 11.5 (E11.5) (Glaser *et al.*, 2009). Secondly, having the gene of interest knocked down in the whole body would provide less information and even raise further questions

with regards to its role in the observed behavioural phenotypes which are subserved only by certain brain parts (see ‘Introduction’). Therefore the best way to study the effect of a gene, or of any manipulation for that matter, on cognition and behaviour is to restrict the knock-down of that gene to regions directly responsible for the cognitive and/or behavioural task under investigation. To achieve this aim conditional knock-out strategy has long been employed in various studies (Orban *et al.*, 1992; Tsien *et al.*, 1996; Zörner *et al.*, 2003; Valor *et al.*, 2011; McQuown *et al.*, 2011).

In this case the conditional knock-down of *Mll2* was achieved by crossing the mouse line having the exon2 of *Mll2* gene “floxed” to the mouse line expressing Cre recombinase under CamKII promoter. CamKII promoter in turn is only active in the forebrain glutamatergic (i.e. excitatory neurons) after postnatal day 19 (P19) (Mayford *et al.*, 1996a; Mayford *et al.*, 1996b; Tsien *et al.*, 1996; Minichiello *et al.*, 1999). As a result, loxP recombination of exon2 of *Mll2* gene (i.e. *Mll2* knock-down) is achieved only in forebrain excitatory neurons at the end of the third week after birth (F/F CKII). This strategy has previously been used successfully in our lab (Kuczera *et al.*, 2010).

Before proceeding with the knock-out mice I first set out to determine the relative expression level of *Mll2* mRNA in different brain regions; relative mRNA levels were analyzed in three main forebrain regions important for learning & memory – dentate gyrus, CA and prefrontal cortex. The mRNA analysis showed that the expression of *Mll2* is slightly lower in the CA region and prefrontal cortex compared to the dentate gyrus. This may indicate that *Mll2* might play a more important function in the dentate gyrus compared to the other two brain regions. However, the difference in expression was not so high and did not reach significance to rule out the importance of *Mll2* function for CA and prefrontal cortex as well at this stage. Next, I set to confirm the loxP recombination of *Mll2* and also the knock-down of the protein. For the confirmation of recombination a qRT-PCR was performed with one of the primers targeting the 2nd exon (the “floxed” one) and the other one targeting the 1st. The qRT-PCR results showed a 4 to 5 fold downregulation of the wild type transcript of *Mll2* in dentate gyrus, CA and prefrontal cortex but not in cerebellum in F/F CKII mice.

Mll2 protein has a very big size (400 kDa) and hence it has been difficult to find an antibody which would recognize it in brain samples. Therefore, the staining for *Mll2*

protein – both in wild-type samples comparing its level in different brain regions and also in knock-out samples to confirm the knock-down of the protein – in different brain regions has not been performed up to now. However, as of now it is in progress and the staining protocol is being optimized by Dr. Andrea Kranz from Technical University of Dresden. Indeed, the specific antibody against Mll2, which is currently being optimized for immunoblot in hippocampal, prefrontal cortical and cerebellar samples from F/F and F/F CKII mice, has been used successfully before by the same group (Glaser *et al.*, 2006; Glaser *et al.*, 2009). However in those studies the staining was done in embryonic stem (ES) cells, testis and *whole* brain lysates. The reason why Mll2 staining with the same antibody was observed in testis and whole brain lysates but not in separate brain regions could simply be that the former two are of much bigger size compared to the latter and hence simply contain more proteins in the first place. And the reason for observing Mll2 staining in ES cells could be that endogenous Mll2 expression is higher in ES cells than in separate brain regions.

One thing that needs to be mentioned is that there was no complete knock-down of Mll2 in neither of the forebrain regions examined. One reason for it is that the knock-down of Mll2 was achieved only in excitatory neurons, which means the inhibitory neurons and glial cells were spared. Another reason could be that this knock-out technology does not achieve the knock-down of the gene of interest in all the excitatory neurons (Valor *et al.*, 2011). Nevertheless, the extent of downregulation was still large enough in order to observe a behavioural and molecular phenotype.

Next, it was necessary to see whether the knock-down of Mll2 induces some compensatory changes in expression of its close homologues *Mll1* and *Mll3*. However, there was no such change in neither of the regions examined (dentate gyrus, CA and prefrontal cortex). These results strongly suggest that the behavioural and/or molecular phenotype that will be observed in these mice will not be due to interference from the function of Mll1 and/or Mll3.

The brain size and weight of the F/F CKII mice were comparable to those of controls. This is expected in view of the fact that in F/F CKII mice knock-out of Mll2 occurs not earlier than the 3rd week after birth. Although in rodents development of certain brain parts continues for a certain amount of time after birth (Akazawa *et al.*, 1995; Clinton *et*

al., 2000; Zhang *et al.*, 2005), nothing of a potentially gross effect on whole brain morphology occurs from the 3rd week after birth. Consistent with this observation there were also no gross abnormalities in hippocampal morphology observed by NeuN staining. There was however a slight decrease in MAP2 staining in stratum radiatum and synaptophysin staining in stratum lucidum of F/F CKII mice which nevertheless failed to reach significance. MAP2 (microtubule associated 2) is a cytoskeletal protein present in the dendrites, and playing a role in stabilizing dendritic shape during synapse development (Caceres *et al.*, 1992; Johnson and Jope, 1992; Gamblin *et al.*, 1996; Lim *et al.*, 2000). Synaptophysin in turn is an integral membrane protein of synaptic vesicles (Jahn *et al.*, 1985; Wiedenmann and Franke, 1985; Südhof *et al.*, 1987; McMahon *et al.*, 1996). These observations could therefore indicate that although they do not have any gross defects in brain morphology, synapse development and/or fine-tuning of synaptic connections may be slightly impaired in F/F CKII mice.

The body weight of the male F/F CKII mice was comparable to that of their control counterparts. However, female knock-out mice showed a slightly but nevertheless significantly increased body weight.

MII2 knock-down in the forebrain leads to increased anxiety and learning deficits

Once the knock-down of MII2 in the forebrain was confirmed the next step was to investigate the effect of this on behaviour. The mice were subjected to a behavioural battery that consisted of paradigms testing for anxiety and learning & memory. The *MII2* F/F CKII mice were observed to spend significantly less time in the centre of an open field arena compared to F/F mice. However, the total distance travelled by those two groups during a 5 min exploration was similar. Open field test is a widely used paradigm to test anxiety. The rationale behind the test is that the centre of the open field appears less safe compared to the periphery; and therefore a relatively anxious mouse will tend to spend more time in the centre compared to a less anxious one. In other words, the readout for anxiety in this test is the relative amount of time spent in the centre of the open field arena – the more anxious the mouse the less time it will spend in the centre. In view of this it becomes apparent that the knock-down of MII2 in the forebrain leads to elevated

anxiety in mice without affecting general explorative behaviour. The same phenotype was observed in male and female mice separately and there was no sex specific effect or sex-genotype interaction. In order to confirm this finding, the mice underwent another test for anxiety namely elevated plus maze test. The rationale of this test is similar to the one employed in the previous one – utilizing the aversion of mice from open and less protected spaces. The elevated plus maze test is performed on a plus maze with four arms which are elevated above the ground. Two of the arms are surrounded by walls and two of the arms are open. And the readout for anxiety in this case is the relative amount of time spent in the open arms. Since the open arms are less safe a relatively anxious mouse will tend to spend less time in the open arms of an elevated plus maze compared to a less anxious one. And in accordance with the previous finding from the open field test the F/F CKII mice spend relatively less time in the open arms of the elevated plus maze compared to their control counterparts during a 5 min exploration. And in this case as well, males and females showed the same trend when examined separately and there was no sex specific effect or sex-genotype interaction.

Next, I set out to study the effect of selective Mll2 knock-down in the forebrain on learning & memory. The first test that I used for this purpose was novel object recognition (NOR). This paradigm utilizes the natural proclivity of rodents to explore objects that are not familiar to them – i.e. the objects that they have not encountered before. During the training phase mice are put into an open field arena and are introduced to two similar objects (i.e. of the same size, shape and colour). They are left to explore them for a certain amount of time (it was 5 min in this study) and then are put back into the home cages, retained there and then are put back into the open field with the objects for the actual memory test. But this time one of the objects is exchanged to a new one; and the readout for memory is the relative preference that the mouse shows to exploring the new object over the old one (i.e. the one encountered during the training phase) (see ‘Materials and Methods’ for details). The amount of time that the mouse is retained in the home cage between the training phase and the memory test depends on the type of memory being investigated (see ‘Introduction’). It has been observed that when the mice are introduced to the novel object after 5 min of retention the F/F CKII mice fail to show a preference to the novel object over the old one. This clearly indicates that knock-down

of *Mll2* leads to impairment in memory which does not depend on gene transcription but rather utilizes already existing synapses and signalling machinery (e.g. pre and post-synaptic proteins, signalling molecules etc.). This is supported by my previous observation that F/F CKII mice show a decrease in MAP2 and Syp staining in stratum radiatum and stratum lucidum respectively compared to their control counterparts. 24 hours later the mice were put back into the same open field arena and this time presented with the same old object and with another novel object different from the previous one. And in this case as well, the mice having their *Mll2* gene knocked-down in the forebrain glutamatergic neurons do not show preference to the novel object over the old one. This indicates that *Mll2* knock-down leads to impairment in long-term memory which is dependent on novel protein synthesis and gene expression. As a result it is tempting to speculate that *Mll2* plays an important role in activity-induced transcription as well. However, a simpler and more straightforward explanation can also be provided to this phenomenon. Since there already seems to be an impairment in pre and/or post-synaptic signalling mechanisms and since synaptic signalling constitutes the initial stage of the whole signalling machinery which eventually converges onto the nucleus and mediates gene expression in response to a learning stimulus, it should come as no surprise that having the upstream portion of the machinery already impaired would lead to the impairment of the machinery as a whole. In this case the role of *Mll2* in gene expression dependent memory would be only secondary. However, this explanation still does not exclude the possibility of *Mll2* also having a role in additional gene expression induced by learning-related stimuli. A possible future strategy to dissociate the role of *Mll2* in constitutive expression of genes involved in synaptic and neuronal function from its role in mediating learning-related stimulus dependent transcription would be to subject F/F mice to training and short-term memory test but shortly after that to induce *Mll2* knock-down through Cre-recombination by injecting adenovirus bearing CamKII-Cre construct, and then 24 hours later test the mice for long-term memory. However one caveat with this approach would be that it is quite difficult to find an adenovirus which would be expressed and have functional consequences 24 hours after injection.

The next learning test the mice were subjected to was Pavlovian fear conditioning. This paradigm tests for associative memory. Mice are put into a chamber with the floor

consisting of metal grids, and are allowed to explore it for 3 min. After that they receive a mild foot-shock (see ‘Materials and Methods’ for details). And when they are put back into the same chamber 24 hours later without receiving any electric foot-shock this time, they associate the context with the unpleasant experience that they had in this same context 24 hours ago (i.e. the foot-shock) and as a result they show freezing which is the sign of fear. Freezing behaviour is an inborn behaviour which rodents manifest when they are confronted with a potentially dangerous situation. Therefore, in the Pavlovian fear conditioning paradigm the readout for memory strength is the level of freezing that rodents show when they are subjected to the same context. Simply put, the stronger the memory the more the freezing. In view of this fact it becomes obvious that F/F CKII mice have a slight impairment in associative memory, since they show significantly lower freezing when introduced into the same context 24 hours later after receiving the a 0.5 mA foot-shock. Again the same phenotype is observed in males and females separately. One interesting observation is that when they were subjected to a stronger foot-shock (0.7 mA) the memory impairment was absent in F/F CKII mice. This indicates that *Mll2* knock-down in the forebrain leads to an impairment in associative memory only when the task becomes more challenging. Another explanation for the mild phenotype that was observed in F/F CKII mice in fear conditioning may come from the observation that they also manifest anxiety (see above). The freezing level in the fear conditioning paradigm is not dependent only on the strength of associative memory but also on the level of anxiety (Sadler *et al.*, 2011; Sartori *et al.*, 2011); with increased anxiety there will be an increase in the freezing level. Therefore it is quite possible that the results from the fear conditioning experiment underestimate the level of memory impairment in F/F CKII mice. There are two interesting experiments that could be performed in the future. One is to test the short-term memory in *Mll2* F/F CKII mice in contextual fear conditioning – i.e. to test their freezing level when they are reintroduced into the same context 1 hour later. Another one is to test their associative memory in cued fear conditioning paradigm. In this test rodents are again allowed to explore the fear conditioning chamber for 3 minutes under the accompaniment of a sound, and after that they receive a foot-shock. During the memory test they are put into a different context but provided with the same sound that was present during the training phase, and the level of

freezing that they manifest shows their associative memory towards the cue (i.e. the sound). Cued fear conditioning and contextual fear conditioning are subserved by different brain regions – the former by amygdala and the latter by hippocampus.

The last memory test to which the mice were subjected was Morris water maze. It tests the ability of rodents to find a platform hidden beneath opaque water using the spatial cues in the room. It tests for spatial memory which is highly dependent on hippocampus. At the end of the training, which in this study lasted for 2 weeks, the mice are subjected to the probe test in which their persistence to swim in the area where the platform was previously located indicates the strength of the memory (see ‘Materials and Methods’ for details). During the training phase of 2 weeks F/F CKII mice already showed a higher escape latency to reach the platform compared to the F/F mice, which especially became obvious during the last days of training. And during the probe test F/F CKII mice failed to show a preference for the quadrant where the platform was previously located (i.e. target quadrant designated as “T”). This indicates that apart from inducing anxiety, impairing object memory and associative memory, Mll2 knock-down also impairs spatial memory.

Stress and anxiety are mediated by an intricate circuitry utilizing hippocampus (ventral hippocampus in particular as will be discussed below), amygdala and medial prefrontal cortex (mPFC) (Campeau and Davis, 1995; Müller *et al.*, 1997; Oddie and Bland, 1998; Coutureau *et al.*, 2000; Gray and McNaughton, 2000; LeDoux, 2000; Petrovich *et al.*, 2001; Bannermann *et al.*, 2004; Degroot and Treit, 2004; Bertoglio *et al.*, 2006; Pentkowski *et al.*, 2006; Nascimento Häckl and Carobrez, 2007; Etkin *et al.*, 2011). All of those three structures are connected to each other through bilateral connections (Maren and Fanselow, 1995; Gray and McNaughton, 2000; Vertes, 2006; LeDoux, 2000; Price, 2007; Canteras *et al.*, 2010), and each of them in turn projects to hypothalamus the main structure of the brain involved in neuroendocrine functions mediating defensive behaviour, which comprises anxiety behaviour as well (Risold and Swanson, 1996; Canteras *et al.*, 1997; Risold *et al.*, 1997; LeDoux, 2000; Dielenberg *et al.*, 2001; Canteras, 2002; Blanchard *et al.*, 2003; Markham *et al.*, 2004; Blanchard *et al.*, 2005; Gabbott *et al.*, 2005). Several studies have shown that lesion and/or inactivation of

ventral hippocampus or amygdala leads to a decrease in anxiety (Henke, 1990; Campeau and Davis, 1995; Müller *et al.*, 1997; LeDoux, 2000; Kjelstrup *et al.*, 2002; Gale *et al.*, 2004; Pentkowski *et al.*, 2006; Nascimento Häckl and Carobrez, 2007). In other words, this indicates that hippocampal and amygdalar activity facilitates anxiety behaviour and therefore inhibition of these structures reduces anxiety. Apart from lesion studies also studies on knock-out mice which have impairment in hippocampal function showed repeatedly that they have decreased anxiety (Abeliovich *et al.*, 1993; Izquierdo and Medina, 1995; Masugi *et al.*, 1999; Contarino *et al.*, 1999; Wall and Messier, 2000; Bowers *et al.*, 2000; Otto *et al.*, 2001a; Otto *et al.*, 2001b; Bontekoe *et al.*, 2002; D'Adamo *et al.*, 2004; Greco and Carli, 2006). One thing that needs to be borne in mind is that decreased hippocampal dysfunction also leads to decreased learning and memory, and therefore it has until lately been perceived as a conceived wisdom that learning impairment should correlate with decreased anxiety (Izquierdo and Medina, 1995) which would contradict my findings where *Mil2* F/F CKII mice have *increased* anxiety together with *decreased* learning. However, things did not turn out so simple; prior to my findings contradicting this conceived wisdom several other studies showed that transgenic mice having impairments in learning and memory had *increased* anxiety (Müller *et al.*, 1994; Parks *et al.*, 1998; Ross *et al.*, 2000; Sarnyai *et al.*, 2000; Sibille *et al.*, 2000; Dirks *et al.*, 2001; Guadano-Ferraz *et al.*, 2003; Bannerman *et al.*, 2004; Venero *et al.*, 2005). In other words, memory and anxiety phenotypes do not always follow a linear pattern but rather co-occur in a more complex manner (Ribeiro *et al.*, 1999; El Hage *et al.*, 2004; Bierman *et al.*, 2005; El Hage *et al.*, 2006; Kalueff, 2007). Moreover, things are exactly the opposite with regards to the involvement of medial prefrontal cortex (mPFC) in anxiety behaviour. *Inhibition* of mPFC function was shown to *increase* anxiety and exposure to anxiogenic stimuli (e.g. elevated plus maze, open field) was shown to elicit and increase in *Fos* expression in that region (Sewards and Sewards, 2002; Bishop, 2007; Canteras *et al.*, 2010). Therefore the reason for increased anxiety in *Mil2* F/F CKII mice could lie in dysfunction of mPFC. For this reason a further venue of research on the way of discerning the molecular mechanisms underlying the increased anxiety in *Mil2* F/F CKII mice would be analysing changes in gene expression in mPFC by DNA microarray or by

RNA-Seq. Of special interest would be genes that would be downregulated specifically in mPFC of F/F CKII mice but *not* in dentate gyrus.

Mll2 activates transcription of genes important for neuronal function in dentate gyrus

Although ventral part of hippocampus is involved in anxiety behaviour, the dorsal part of hippocampus is involved in learning & memory (esp. spatial memory) (Henke, 1990; Jung *et al.*, 1994; Moser *et al.*, 1995; Moser and Moser, 1998; Pothuizen *et al.*, 2004). The afferent and efferent connections that these two parts of hippocampus make with other brain regions are distinct from each other (Swanson and Cowan, 1977; Fanselow and Dong, 2010). Moreover, apart from differences in anatomical connections hippocampus also manifests differences in gene expression along dorso-ventral axis (Thompson *et al.*, 2008; Dong *et al.*, 2009; Fanselow and Dong, 2010). Since Mll2 knock-down leads at the same time to learning and anxiety phenotypes it was important to dissociate dorsal and ventral parts of hippocampus when proceeding with molecular analysis. Surprisingly, dissection of dorsal and ventral parts of mouse hippocampus has never been performed by hand; the data that exist concerning the differences in gene expression along dorso-ventral axis come from *in situ* hybridization (Thompson *et al.*, 2008; Dong *et al.*, 2009; Fanselow and Dong, 2010). That is why before proceeding further it was important for me to test whether we can faithfully isolate dorsal and ventral parts of hippocampus by dissection by hand. For this purpose I selected some genes which have been shown before to show dorso-ventral specificity in hippocampus (Thompson *et al.*, 2008; Dong *et al.*, 2009; Fanselow and Dong, 2010) and compared their expression levels in dorsal versus ventral hippocampal samples by qRT-PCR. I managed to confirm the dorsal and ventral specific genes in the tissue samples, which indicated that our way of dissecting dorsal and ventral hippocampus is reliable.

Then, I decided to concentrate on learning impairment caused by Mll2 knock-down and therefore proceeded with dorsal part of dentate gyrus and CA. DNA microarray was performed from dorsal dentate gyrus and dorsal CA of F/F and F/F CKII mice in order to see whether knock-down of Mll2 lead to changes in gene expression in those regions.

Quite intriguingly, it was observed that *Mll2* knock-down lead to an extensive downregulation of gene expression in dorsal dentate gyrus which is expected in view of the fact that *Mll2* mediates Histone 3 Lysine 4 methylation which is a mark activating gene expression; however there was no such drastic downregulation in dorsal CA region. This can partly be explained by slightly higher expression of *Mll2* in the dentate gyrus, however still cannot account for such a drastic difference in the number of downregulated genes. This finding indicates that *Mll2* might play a special role confined to dentate gyrus, whereas not being that important for regulation of gene expression in CA region. There were also some upregulated genes in dorsal dentate gyrus of F/F CKII mice which can be accounted for by the secondary effects resulting from *Mll2* knock-down.

Among downregulated genes there were many which are involved in molecular processes and/or pathways known to be involved in synaptic signalling and synaptic plasticity. For example, there were many genes (*Nkapl*, *Adcy5*, *Dusp2*, *Prkra*) that code for proteins that are involved in G-protein – cAMP and MAPK/ERK pathways, which have many times been shown to be crucial for synaptic plasticity in hippocampus and learning & memory (Frey *et al.*, 1993; English and Sweatt, 1996; English and Sweatt, 1997; Atkins *et al.*, 1998; Impey *et al.*, 1998; Roberson *et al.*, 1999; Sweatt, 2001; Levenson *et al.*, 2004; Chwang *et al.*, 2006; Wood *et al.*, 2006; Reissner *et al.*, 2006; Sindreu *et al.*, 2007; Brami-Cherrier *et al.*, 2007; Ehninger *et al.*, 2008; Brami-Cherrier *et al.*, 2009; Reul *et al.*, 2009). Two genes that deserve special attention here is NF- κ B activating protein like (*Nkapl*) and adenylate cyclase 5 (*Adcy5*). NF- κ B signalling has repeatedly been shown to be crucial for synaptic plasticity and learning & memory (Meberg *et al.*, 1996; Albensi and Mattson, 2000; Yeh *et al.*, 2002; Freudenthal *et al.*, 2004; Lubin and Sweatt, 2007; Ahn *et al.*, 2008), and adenylate cyclases are the enzymes involved in the synthesis of cAMP whose role in synaptic plasticity and learning & memory is also well established (see above and ‘Introduction’). An interesting observation here is that dual specificity phosphatase 2 (*Dusp2*) is among the genes that are downregulated in F/F CKII mice. Dual specificity phosphatases dephosphorylate MAP kinases and function to inactivate MAP/ERK signalling pathway (Charbonneau and Tonks, 1992). Indeed, it has been shown that another dual specificity phosphatase – MCF-1, also known as *Dusp1* – is rapidly transcribed both in fibroblasts after stimulation with serum growth factor (Lau

and Nathans, 1985; Sun *et al.*, 1993; Charles *et al.*, 1993), and also in hippocampus or specifically in granule cells of dentate gyrus after induction of LTP (Qian *et al.*, 1993; Qian *et al.*, 1994; Davis *et al.*, 2000). At first it may come as a surprise that firstly something that actually inactivates a pathway involved in synaptic plasticity is induced after neuronal stimulation, and secondly that something else of a similar type is *downregulated* in the dentate gyrus of mice that have learning impairment. However, the fact that a dual specificity phosphatase is rapidly transcribed upon induction in order to inactivate MAP/ERK signalling may indicate that it is important in regulating the feedback loop which in turn renders the pathway responsive to the next stimulus. Indeed, it has been suggested that activity of phosphatases confers flexibility to the MAPK/ERK signalling pathway making it respond to a stimulus acutely (Bhalla and Iyengar, 1999; Bhalla *et al.*, 2002). Seen in this light it makes perfect sense that activity of MAPK phosphatases are important for the proper functioning of MAPK/ERK pathway and for occurrence of phenomena induced by it (e.g. long-term potentiation). It is highly likely that dephosphorylating the MAPK is necessary to make it ready to respond to the next stimulus and hence a failure in the molecular mechanisms responsible for MAPK dephosphorylation may lead to its unresponsiveness and lack of flexibility and may result in deficits in synaptic plasticity. Therefore induction of expression of gene(s) coding for MAPK dephosphatase(s) after LTP induction might serve to revert MAPK to the original state ready to respond to later stimuli. However, this might be achieved not only through online induction but also through constitutive expression of MAPK dephosphatases, a decrease in expression of which might as well lead to deficits in synaptic plasticity and learning & memory. And this is what we observe in the case of Mll2 F/F CKII mice; downregulation of *Dusp2* expression in dentate gyrus is highly likely to leave certain MAP kinase(s) hyperphosphorylated already at the basal level hence rendering them incapable of responding to upcoming stimuli.

Another set of genes that were downregulated in dorsal dentate gyrus as a result of Mll2 knock-down code for proteins involved in synaptic vesicle transport and fusion (e.g. *Stxbp2*, *Sypl2*, *Ap1s3* and *Ckap4*). Others code for transcription factors involved in transcriptional initiation (e.g. *E2f1*) and transcriptional elongation (e.g. *Tceal1*). Different transcriptional activators (e.g. CREB, Fos, Egr1, Egr2, Jun, Arc) have previously been

shown to be involved in synaptic plasticity and LTP induction (Impey *et al.*, 1996; Deisseroth *et al.*, 1996; Impey *et al.*, 1998; Taubenfeld *et al.*, 1999; Tischmeyer and Grimm, 1999; Albenisi and Mattson, 2000; Lee *et al.*, 2004; Frankland *et al.*, 2004; Thomas and Huganir, 2004; Ramanan *et al.*, 2005).

Another set of downregulated genes previously shown to facilitate synaptic plasticity and learning & memory (Sun and Alkon, 2001) comprises carbonic anhydrases (*Car4* and *Car10*). What is more interesting in the findings of Sun and Alkon is that presence of carbonic anhydrase activators facilitated spatial learning by inducing a switch in GABAergic responses from inhibitory to excitatory. Indeed it has previously been shown that GABAergic synaptic responses can be switched from inhibitory to excitatory (Alkon *et al.*, 1992; Collin *et al.*, 1995; Kaila *et al.*, 1997; Sun *et al.*, 2000; Sun *et al.*, 2001). All these become quite intriguing given the fact that the gene coding for GABA receptor subunit $\gamma 3$ (*Gabrg3*) is also downregulated in dorsal dentate gyrus of F/F CKII mice. It is highly likely that decrease in expression of carbonic anhydrases and also the GABA receptor lead to the inability of F/F CKII mice to utilize GABAergic inputs for learning purposes – i.e. there most probably is a decrease in GABAergic signalling in the first place but also the remaining GABAergic signals are most probably not being converted to excitatory ones hence leading to deficits in learning & memory. Moreover, neither *Car4* nor *Gabrg3* is downregulated in the ventral part of dentate gyrus in F/F CKII mice (see below), which makes perfect sense in light of findings indicating that GABAergic switch is important for spatial memory (see above). Therefore involvement of *Mll2* in hippocampus dependent memory (esp. spatial memory) can at least partly be attributed to its regulating the expression of *Car4* and *Gabrg3* specifically in dorsal part of dentate gyrus.

There were also genes coding for proteins involved in lipid metabolism – *Ptgr1*, *Ptgr2*, *Acot1* and *Acot4*. Another gene that deserves attention is *Oxr1* which is involved in protecting cells against oxidative stress (Volkert *et al.*, 2000; Elliott and Volkert, 2004; Durand *et al.*, 2007). Moreover, in contrast to many others this gene was downregulated in dorsal CA as well. Increased oxidation has been shown to impair neuronal function and synaptic plasticity and in contrast reducing agents have been shown to facilitate LTP (Gozlan *et al.*, 1995; Cai *et al.*, 2008; Yang *et al.*, 2010). Moreover, a recent study has

implicated *Oxr1* in protecting neuronal cells from oxidative stress and neurodegeneration (Oliver *et al.*, 2011). That is why an interesting venue for future research would be to test whether *Mll2* F/F CKII mice show increased neurodegeneration as they age compared to the controls. Moreover, since *Mll2* apparently plays a more important role in dentate gyrus and appears to be dispensable for CA, it is tempting to speculate that *Mll2* may also play a role in adult neurogenesis. One piece of evidence pointing to this possibility is that *E2f1*, which is downregulated in dorsal dentate gyrus of F/F CKII mice, has been shown to be involved in neurogenesis in adult mice (Cooper-Kuhn *et al.*, 2002).

Another interesting observation was that among 16 genes confirmed to be downregulated in dorsal dentate gyrus by qRT-PCR most *but not all* were downregulated in the ventral part. The genes that were not downregulated in ventral dentate gyrus of F/F CKII mice are *Adcy5*, *Gabrg3*, *Car4*, *Rab38* and *Tpm4*. This in turn points to the possibility that apart from its specificity for dentate gyrus, *Mll2* also may show some specificity of function along dorso-ventral axis of hippocampus.

Histone 3 Lysine 4 methylation is decreased at promoters and coding regions of genes downregulated in dentate gyrus as a result of *Mll2* knock-down

Since *Mll2* methylates Histone 3 at Lysine 4 residue the next obvious step was to see whether there is a decrease in H3K4 methylation at the promoters of the genes downregulated in dorsal dentate gyrus in F/F CKII mice. And as expected I observed a decrease in H3K4 di and trimethylation, which are markers for active transcription, at the promoters of those genes. SET1 domain histone methyltransferases have repeatedly been shown to be responsible for H3K4 tri and dimethylation inside the cell (Miller *et al.*, 2001; Santos-Rosa *et al.*, 2002; Krogan *et al.*, 2003; Ng *et al.*, 2003; Bannister and Kouzarides, 2004; Hughes *et al.*, 2004; Schneider *et al.*, 2005; Tenney and Shilatifard, 2005; Dou *et al.*, 2006; Sims and Reinberg, 2006; Bhaumik *et al.*, 2007; Wood *et al.*, 2007; Malik and Bhaumik, 2010; Ansari and Mandal, 2010). An interesting phenomenon observed previously is that promoters and coding regions of constitutively active genes are marked both by H3K4me3 and H3K4me2 (Santos-Rosa *et al.*, 2002). In other words, it follows that both H3K4 tri and dimethylation marks are responsible for mediating

transcription of genes at the “naïve” state. And my findings regarding the effect of knock-down of Mll2 on gene expression changes and H3K4 methylation in the dentate gyrus are perfectly consistent with this notion. The mice, which were used for DNA microarray and chromatin immunoprecipitation analyses were not subjected to any stimulus prior to being sacrificed. Therefore the genes downregulated in Mll2 F/F CKII mice are expected to have at least an appreciable level of transcription at the basal (i.e. “naïve”) state. And since H3K4 tri and dimethylation are responsible for mediating the transcription of constitutively active genes (Santos-Rosa *et al.*, 2002), it is absolutely expected that genes downregulated in “naïve” mice as a result of Mll2 knock-down will have reduced trimethyl and dimethyl marks at their promoters and/or coding regions. However, another interesting finding from the study of Santos-Rosa *et al.* was that only H3K4 trimethylation *but not dimethylation* was responsible for transcriptional activation of genes whose activity is regulated in response to a stimulus (Santos-Rosa *et al.*, 2002). In light of this observation it would be interesting to compare the transcription profile of control and Mll2 F/F CKII mice in response to a learning stimulus. However, in this case the analysis would be more complicated since F/F CKII mice show downregulation in many genes, which are actually involved in mediating transcriptional activation in response to a stimulus – either directly like in the case of transcription initiation/elongation factors (e.g. *E2f1* and *Tceal1*) or through being part of signalling cascades that lead to changes in gene expression (e.g. *Nkapl*, *Adcy5*, *Dusp2*, *Prkra*). That is why the differences in regulated gene expression that could possibly be observed between control and Mll2 F/F CKII mice may not be only due to impaired H3K4 trimethylation but also due to secondary effects resulting from already existing differences in gene regulation machinery. One possible way to disentangle this caveat could be to analyze H3K4me3 level throughout the genome after a learning stimulus by ChIP-Seq analysis, and compare this in control and F/F CKII mice.

Another interesting finding was that in contrast to decrease in H3K4 trimethyl and dimethyl marks there was no change in monomethyl mark at gene promoters in F/F CKII mice, and for some genes (*Car4*, *Adcy5*, *Stxbp2* and *Acot4*) it was even *increased* or tended to increase in the knock-outs. In fact it was shown by Andreu-Vieyra *et al.* that Mll2 knock-down is required for bulk H3K4 trimethylation and dimethylation, but not

monomethylation in oocytes (Andreu-Vieyra *et al.*, 2010). My findings from dorsal dentate gyrus are in line with the results obtained from that study. These all indicate that either in the mammals H3K4 monomethylation is carried out by some other histone methyltransferase(s) and that Mll2 is not responsible for that at all, or that a possible monomethylase activity of Mll2, but not dimethylase and trimethylase activity, can be compensated by other enzymes inside the cell.

Injection of Histone Deacetylase (HDAC) inhibitor does not increase expression of genes downregulated as a result of Mll2 knock-down

It has previously been shown that H3K4 methylation serves as a mark that facilitates the recruitment of Histone Acetyltransferases (HATs) at gene promoters (Wang *et al.*, 2009). Therefore it is likely that impairment of H3K4 methylation may lead to impairment in mediating histone acetylation as well. For this purpose I decided to compare the response of F/F and F/F CKII mice to injection of Suberoylanilide hydroxamic acid (SAHA), a potent inhibitor of histone deacetylases. It has been shown previously in our lab that intraperitoneal SAHA injection leads to an increase in bulk histone acetylation levels in hippocampus, with the peak of this increase being at 1 hour after injection (our unpublished observations). Firstly, I confirmed this observation in the Mll2-Cre mouse line, and observed that bulk histone acetylation increases in hippocampus 1 hour after SAHA injection both in F/F and also in F/F CKII mice. This indicates that F/F CKII mice at least do not have impairment in regulating bulk histone acetylation, and that 1 hour after injection is the best time point to further study the effects of SAHA in more detail. Still, the fact that Mll2 knock-down does not lead to impairment in increasing bulk levels of histone acetylation after SAHA treatment does not mean that there will not be any impairment in regulation of expression of genes already shown to be downregulated in dentate gyrus of F/F CKII mice. Therefore, I set out to investigate whether SAHA injection leads to an increase in expression of those genes in dorsal dentate gyrus in F/F and F/F CKII mice. Interestingly, it turned out that those genes are not upregulated in dorsal dentate gyrus 1 hour after SAHA injection neither in F/F CKII nor in F/F mice. This was quite an unexpected finding; the expectation was that those genes will be

upregulated at least in the control mice after SAHA injection but not in F/F CKII mice. At this stage this finding may indicate two things, not necessarily mutually exclusive. Firstly, it is possible that in the brain genes regulated by histone methylation are not regulated by an additional increase in histone acetylation. However, one more crucial experiment is necessary in order to definitely prove this hypothesis; it should be tested by chromatin immunoprecipitation (ChIP) whether acute SAHA injection increases histone acetylation at the promoters of those genes in the first place. Another interesting possibility, which would be more intriguing, is that by itself acute SAHA injection and the resulting increase in histone acetylation do not lead to any changes in gene expression in the brain at all, but rather only create a permissive state at the chromatin so that genes are transcribed faster and/or at a higher level only when a necessary stimulus arrives. Interestingly, all the previous studies demonstrating the effect of acute injection of HDAC inhibitors on gene expression in hippocampus always coupled it to another stimulus – e.g. fear conditioning (Vecsey *et al.*, 2007; Peleg *et al.*, 2010). The only obvious way to shed light on this hypothesis is to compare hippocampal transcriptome of mice treated with vehicle to those acutely treated with SAHA, either by DNA microarray or by RNA-Seq.

Summary and Future Directions

In the first part of my work I characterized the transgenic mouse line that had a gene coding for histone methyltransferase, *Mll2*, knocked-out in forebrain excitatory neurons, in order to gain a closer understanding of the role of histone methylation in behaviour and cognition. The knock-out mice had increased anxiety and impaired learning assessed by several paradigms. This was accompanied by extensive downregulation of gene expression in dorsal dentate gyrus but not in dorsal CA. Many of downregulated genes code for proteins that play role in pathways/mechanisms involved in synaptic signalling and plasticity. Downregulation of gene expression as a result of *Mll2* knock-down was also accompanied by a decrease in Histone 3 Lysine 4 tri and dimethylation, but not monomethylation, at gene promoters. Together with several other recent studies (Schaefer *et al.*, 2009; Balemans *et al.*, 2010; Jiang *et al.*, 2010; Gupta *et al.*, 2010)

findings of this study will make a profound contribution to understanding the role of histone methylation in behaviour and learning & memory in particular. One of the further experiments that is currently being performed on Mll2 forebrain-specific conditional knock-out mice are analysing gene expression in ventral dentate gyrus and CA by RNA-Seq in order to elucidate any specific effect that Mll2 knock-down may have on ventral hippocampus differently from the dorsal part of it. Another experiment that will soon be performed is analysing if acute SAHA injection leads to an increase in histone acetylation at gene promoters, downregulated as a result of Mll2 knock-down, in control and in F/F CKII mice (see ‘Discussion’).

Mll2 is not the only H3K4 methyltransferase in mammalian cells (see ‘Introduction’). Therefore in order to gain a deeper and more complete understanding of the role of histone methylation in learning and memory the involvement of other methyltransferases in learning and memory should be investigated. And one further step that I am currently making in this direction is characterizing Mll1 F/F CKII mice. These mice also show learning impairment in fear conditioning and water maze paradigms. However, differently from Mll2 F/F CKII mice they do not show impairment in novel object recognition, and in contrast to Mll2 conditional knock-out mice they tend to show a *decrease* in anxiety behaviour. The molecular analysis of Mll1 F/F CKII mice is currently underway.

PART 2: EFFECTS OF DIFFERENT DURATIONS OF ENVIRONMENTAL ENRICHMENT ON LEARNING AND MEMORY

A certain minimum duration of environmental enrichment is required to facilitate learning

Although a plethora of studies exist documenting the beneficial effect of environmental enrichment on learning & memory, yet there exists no standardized protocol by which mice can be provided an enriched environment in different laboratories. As a result enriched environments provided to mice in different laboratories could differ from each other in their level of stimulation. Therefore before proceeding further with enrichment experiments it is always necessary to find out the duration that is enough to facilitate learning in given laboratory conditions. I found out that in our conditions 1 week of environmental enrichment was not yet enough to facilitate learning whereas 2 weeks of EE already were. This indicates that providing a novel and stimulating environment *per se* is not enough to facilitate learning. It has to be provided continuously for a certain amount of time. In the future it would be interesting also to find out how different components of enrichment affect the outcome. For example, in this study the mice were provided new toys everyday, and also everyday the existing toys were rearranged inside the cage (see ‘Materials and Methods’). It would therefore be interesting to see to what extent changing and rearranging the toys contributes to the positive effects of enrichment on cognitive function. Would 2 weeks of EE still be enough to facilitate learning if during this time period the toys were not changed at all? Or changed only once, twice? And so on... Or, will environmental enrichment be effective at all without providing the novelty? Another interesting question that can be addressed is what roles physical and cognitive components of enrichment play on memory enhancement. Would providing mice only with toys without running wheels attenuate the beneficial effect of enrichment? These are all interesting questions that can be addressed in future studies.

The effects of long-term enrichment are persistent whereas those of short-term enrichment fade away soon after enrichment ceases

Although, as mentioned above, a lot of studies exist documenting the beneficial role of environmental enrichment on cognition the number of studies investigating the persistence of its effects once enriched environment does not exist anymore are very scarce – almost non-existent. The only study known so far investigated the persistence of effect of EE on habituation to the open field (Amaral *et al.*, 2008), where the authors showed that when enriched environment is presented for 8 weeks starting from weaning its effects persist up to at least 2 months after cessation of enrichment. However, this does not happen when mice are enriched for 4 weeks starting from weaning or from the tenth week of age (P70). Nevertheless, in my mind this study was incomplete since they did not test the persistence of effects of 8 weeks of EE starting from P70. Moreover, what interests me more is the effect of environmental enrichment on learning & memory, which has not been investigated in that study. Furthermore, it would be highly beneficial for therapeutical purposes if the beneficial effects of environmental enrichment on cognition were persistent even when it is provided at adulthood. It would also be highly interesting for purely theoretical purposes to find out whether a living being, be it mouse or human, remains smart even after the enriched environment to which it was previously subjected to is removed. Therefore I set out to investigate this.

My first finding was that although 2 weeks of EE are enough to facilitate cognition, the effect of barely lasts up to 1 week. Mice that were enriched for 2 weeks and then kept in standard home cages for an additional 1 week did not have improved memory in fear conditioning test anymore compared to their control counterparts. However, in water maze test the learning-facilitating effects of EE persisted at least up to 1 week after cessation of enrichment – i.e. the mice that underwent 2 weeks of EE and then were kept in standard home cages for an additional 1 week still managed to perform better in the water maze task compared to the ones which were kept in standard housing all the time. Next, I wondered to what extent the facilitated learning performance in the water maze task will persist in mice enriched for 2 weeks – i.e. whether the mice will remain smart for a much longer period of time after cessation of enrichment. In order to figure that out

I kept the same mice in home cages for an additional 2 months. After that they were subjected to a modified water maze test, in which the platform was removed to another place and the visual cues around the water maze were rearranged. These were done in order to ensure that the mice are subjected to a completely novel learning test without any possible interference from the memory acquired in the original water maze test. On the first training day mice that were previously enriched and the ones always kept in home cages had similar escape latencies, from which it can be concluded that the enhanced memory that the enriched mice acquired in the original water maze task did not interfere with the current performance. It was observed that when kept in home cages for a much longer time (i.e. 2 months), mice that were enriched for 2 weeks do not perform better in the memory task than their control counterparts anymore. This indicates that the effects of short-term enrichment are not really persistent and fade away soon after enrichment ceases.

The next question to be addressed was whether the effects of enrichment on learning & memory could be persistent if it is applied for a much longer duration. In other words, is environmental enrichment at all able to exert long-lasting effects on cognition? To find this out a group of mice was enriched for 10 weeks and then kept in standard home cages before being subjected to fear conditioning and water maze experiments. Two other groups included mice that were kept in home cages all the time and another one where mice were subjected to learning experiments immediately after 10 weeks of EE. First of all, differently from mice that underwent only 2 weeks of EE, mice that underwent 10 weeks of EE had increased memory in the fear conditioning task even after being kept in home cages for an additional 1 week. And as expected they also performed better than the ones continuously kept home cages in the water maze task as well. This indicates that when exerted for a much longer period, environmental enrichment is able to exert more persistent effects of cognition. However, still the crucial question was whether 10 weeks of EE would continue to exert its positive effects on learning & memory for much longer time. Therefore, as in the experiment with mice enriched for 2 weeks, mice were kept in home cages for additional 2 months and then subjected to the modified water maze experiment explained above. Interestingly, they still had improved memory compared to the ones that were kept in home cages continuously. This indicates that environmental

enrichment, when performed for a sufficiently long period of time *does* exert persistent effects on learning, which continue to last long after its cessation. This indeed is in line with findings of Amaral *et al.*, where they demonstrated that mice enriched for 8 weeks starting from weaning continue to show facilitated habituation to the open field even 6 months after being kept in home cages. It is still likely that due to their more plastic brain juvenile mice may have a more facilitated response to environmental enrichment than the adult ones, and as a result enrichment performed at juvenile stage may have a stronger and probably more long-lasting effect which could last up to 6 months. However, given my findings that the effects of long-term enrichment in adult mice last at least up to 2 months it would still be interesting to see whether the adult mice enriched for 2-3 months would still have facilitated learning performance when tested even 6 months after being put back into standard home cages.

These findings strongly argue that there are qualitatively three levels of environmental enrichment; (i) the first level at which the amount of enrichment is still not enough to facilitate learning at all, (ii) the second level where the amount of enrichment is enough to facilitate learning but its effects fade away soon after its cessation, and (iii) the third level at which the beneficial effects of enrichment on learning last for a long-time after its cessation. This hierarchy strongly resembles the hierarchy that exists in synaptic plasticity; with simple synaptic transmission not being able to lead to any changes at the synapse, with stimulation of a certain strength leading to short-term changes at the synapse that account for early LTP which fades away quite soon after being initiated, and finally with stronger stimulation exerting a qualitatively different effect where changes at synapses undergo late LTP which lasts for much longer time (see 'Introduction' for details). It is interesting to note that the most important aspect that differentiates late LTP from early LTP and simple synaptic transmission is the involvement of novel gene expression in the former, and its absence in the latter two. Therefore, it was very tempting to speculate that the differences in the effects of different durations of environmental enrichment may lie in their differential ability to exert changes in gene expression – with enrichment of longer duration leading to changes in gene expression whereas enrichment of shorter duration not.

Long-term EE leads to substantial changes in gene expression whereas such changes are absent after short-term EE

Hippocampal gene expression profile of mice kept in standard cages was compared to those that underwent 10 weeks of EE, by DNA microarray. It has been observed that 10 weeks of EE induced changes in expression of many genes (see the complete list in Appendix 2). Quite a lot of them are involved in transcriptional activation, especially through histone acetylation. Some of them were upregulated (*Cml5*, *Meox1*, *Ing3*) and some were downregulated (*Klf1*, *Kat2a*). This may indicate that environmental enrichment mobilizes some additional transcriptional machinery, which has been latent before, when responding to novel stimuli. The increase in the level of some transcription factors may not in turn lead to an additional wave of changes in gene expression after enrichment as long as the mice remain in the “naïve” state. However, those novel transcription factors may come into play and make a difference when the mice are subjected to a learning stimulus. Therefore, it would be interesting to compare gene expression changes after a learning stimulus (e.g. fear conditioning) in home caged mice and mice enriched for 10 weeks.

Another group of genes differentially regulated after enrichment and which also deserve special attention are chemokine ligands (*Ccl8*, *Ccl6* and *Ccl17*). Chemokine ligands are secretory molecules that are secreted from glial cells and act on neurons through binding to chemokine receptors. The involvement of chemokine signalling in synaptic plasticity, and in neuronal function and survival has been repeatedly documented (Vlkolinsky *et al.*, 2004; Ben Achour and Pascual, 2010; Nicolai *et al.*, 2010; Nelson *et al.*, 2011). An interesting observation was that among the genes that were confirmed by qRT-PCR only the expression of *Ccl8* persisted at the same level up to 2 months after cessation of enrichment. The expression of all the other genes went back to the basal (i.e. home cage) levels when the mice were kept in home cages for two months after 10 weeks of enrichment. This is a very intriguing finding, which deserves further study. It might be possible that persistently elevated levels of *Ccl8* in the hippocampus may serve to facilitate synaptic plasticity even after the enriched environment is not provided anymore. The mechanism ensuring the persistence in the expression level of *Ccl8* may in turn act

through changes in DNA methylation at its promoter. DNA methylation is known to be a more persistent epigenetic mark than histone modifications (Day and Sweatt, 2010), and has also previously been shown to be responsible for long-term changes in behaviour even throughout generations (Miller and Sweatt, 2007; Lubin *et al.*, 2008; Miller *et al.*, 2008; Feng *et al.*, 2010; Miller *et al.*, 2010; Franklin *et al.*, 2010; Franklin and Mansuy, 2010). Moreover, the fact that environmental enrichment induces an increase also in the expression of genes coding for proteins secreted from glial cells indicates that EE affects not only neurons but glial cells as well, and that the role of glial cells in learning & memory improvement should not be disregarded.

Another group of genes differentially regulated after 10 weeks of EE are involved in cGMP signalling. The one which is upregulated is *Tjp2*, and it possesses guanylate kinase activity which in turn facilitates cGMP signalling pathway. Another one, which is downregulated, is *Pde6h* and codes for phosphodiesterase. Phosphodiesterases catalyze cGMP catabolism thereby inhibiting cGMP-signalling pathway. Indeed, application of phosphodiesterase inhibitors has been shown to facilitate LTP and improve memory (Barad *et al.*, 1998; Navakkode *et al.*, 2004), hence it is quite expected that environmental enrichment would facilitate learning by removing a brake on synaptic plasticity.

Two more genes that deserve attention are *Paip1* and *Plbd1*. Both of them are upregulated after 10 weeks of EE. The protein encoded by *Paip1* facilitates translational initiation. Apart from regulation of transcription, regulation of mRNA translation has also been shown to be involved in synaptic plasticity and LTP induction (Kelleher *et al.*, 2004a; Kelleher *et al.*, 2004b). Especially, regulation of mRNA translation at the dendrites and spines is known to be a crucial factor in synaptic plasticity (Kelleher *et al.*, 2004a; Kelleher *et al.*, 2004b). It is therefore highly likely that environmental enrichment facilitates learning not only through mediating changes in gene expression but also through regulating mRNA translation. The other gene is *Plbd1* and codes for a phospholipase. Phospholipases promote synaptic plasticity through cleavage of membrane phospholipids, and the cleavage products (i.e. lipid messengers) that occur as a result of phospholipase action act as second messengers in intracellular signalling

cascades involved in LTP induction (Dumuis *et al.*, 1988; Pontzer *et al.*, 1990; Sanfeliu *et al.*, 1990; Wolf *et al.*, 1995; Hawthorne, 1996; Gottschalk *et al.*, 1999; Yang *et al.*, 2001).

However, the most intriguing finding was that 2 weeks of enrichment induced only mild changes in gene expression. Among 16 genes which have been confirmed in hippocampus of mice that underwent 10 weeks of EE only three were regulated in the same way after 2 weeks of EE. These were *Pde6h*, *Plbd1* and *Klfl*. However, in order to reach more general conclusions a DNA microarray study comparing hippocampal transcription profile of home caged mice and mice enriched for 2 weeks has to be performed. Nevertheless, even at this stage it can be fairly argued that one of the crucial differences between short-term and long-term enrichment is in the extent of gene expression changes that they can mediate. And this differential ability to induce changes in gene expression is most probably the main cause behind the qualitatively different outcomes that different durations of enrichment lead to. This in turn acts in favour of the hypothesis that induction of synaptic plasticity and the actions of environmental enrichment are exact phenocopies of each other, being governed by comparable processes.

Summary and Future Directions

In the second part of my PhD work I studied the effect of environmental enrichment (EE) on learning & memory and different effects that different durations of EE exert. It turned out that the process of environmental enrichment can be broadly classified into three different stages: (i) duration of EE that is not enough to facilitate learning at all (e.g. 1 week), (ii) short-term EE that is enough to lead to learning enhancement but whose effects fade away soon after enrichment ceases, and (iii) long-term EE which is both enough to facilitate learning and also exert long-lasting effects which persist for a long time after its cessation. One mechanism underlying this difference in the level of persistence between short-term and long-term EE is the differential ability to lead to changes in gene expression – long-term EE leading to changes in expression of many

genes whereas short-term EE resulting only in limited change in gene expression. One more interesting observation was that among the genes confirmed by qRT-PCR (16 genes) only *Ccl8* was shown to retain its level of expression induced by EE even after cessation of the latter. Further analyses need to be performed in order to elucidate the mechanism(s) mediating the persistent expression of *Ccl8* gene – DNA methylation being the strongest candidate.

One further interesting question that may be addressed is whether the memory enhancement mediated by short-term and long-term enrichment depends on the same mechanisms in the first place. In other words, given the obvious difference in the level of persistence of the effects of those two kinds of enrichment it would be wise to ask whether the learning enhancement itself mediated by short-term enrichment is mechanistically different from that mediated by long-term enrichment – are these two “learning enhancements” of a different type in the first place. Therefore, an interesting experiment would be to compare the changes in hippocampal gene expression in “Home Cage”, “2 weeks EE” and “10 weeks EE” mice after fear conditioning. It has previously been shown that fear conditioning leads to profound changes in expression of many genes involved in synaptic plasticity (Peleg *et al.*, 2010). Hence it would be interesting to see whether short-term and long-term enrichment would lead to a different response to fear conditioning in terms gene expression – different from home caged mice and also different from each other.

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Appendix 1

Table 1: List of differentially regulated genes in dorsal dentate gyrus of F/F CKII mice (the genes in red were confirmed by qRT-PCR)

| Symbol | Full Name | log (fold change) | Adjusted <i>p</i> -value |
|--------------|--|-------------------|--------------------------|
| Vsig8 | V-set and immunoglobulin domain containing 8 | -2,88 | 5,42E-07 |
| Hfm1 | HFM1, ATP-dependent DNA helicase homolog (S. cerevisiae) | -2,62 | 1,32E-07 |
| Efhc2 | EF-hand domain (C-terminal) containing 2 | -2,57 | 9,75E-05 |
| Nkapl | NFKB activating protein-like | -2,52 | 1,04E-05 |
| Dazl | deleted in azoospermia-like | -2,50 | 1,98E-06 |
| Igsf1 | immunoglobulin superfamily, member 1 | -2,33 | 1,89E-06 |
| Lor | loricrin | -2,33 | 5,94E-11 |
| Hdx | highly divergent homeobox | -2,17 | 8,19E-08 |
| Fbx17 | F-box and leucine-rich repeat protein 7 | -2,13 | 1,02E-05 |
| Wtip | WT1-interacting protein | -2,05 | 1,21E-07 |
| Prss16 | protease, serine, 16 (thymus) | -2,02 | 2,40E-04 |
| Zfp459 | zinc finger protein 459 | -1,96 | 6,67E-06 |
| Wdr17 | WD repeat domain 17 | -1,92 | 1,63E-08 |
| Rab38 | RAB38, member of RAS oncogene family | -1,90 | 1,31E-04 |
| Tmem22 | transmembrane protein 22 | -1,89 | 3,44E-07 |
| Lrrn4 | leucine rich repeat neuronal 4 | -1,89 | 4,97E-06 |
| Prr15 | proline rich 15 | -1,89 | 3,49E-06 |
| Ap1s3 | adaptor-related protein complex AP-1, sigma 3 | -1,84 | 5,45E-07 |
| Gprc5c | G protein-coupled receptor, family C, group 5, member C | -1,81 | 8,53E-05 |
| Nmnat3 | nicotinamide nucleotide adenylyltransferase 3 | -1,77 | 6,13E-07 |
| Gm9934 | predicted gene 9934 | -1,74 | 5,01E-05 |
| Acot4 | acyl-CoA thioesterase 4 | -1,74 | 4,45E-05 |
| Tceal1 | transcription elongation factor A (SII)-like 1 | -1,72 | 1,74E-07 |
| Cd55 | CD55 antigen | -1,71 | 2,54E-04 |
| BB557941 | expressed sequence BB557941 | -1,70 | 1,79E-06 |
| Rimklb | ribosomal modification protein rimK-like family member B | -1,69 | 1,62E-06 |
| Gprc5c | G protein-coupled receptor, family C, group 5, member C | -1,69 | 1,29E-04 |
| Dusp2 | dual specificity phosphatase 2 | -1,67 | 8,38E-07 |
| Tcf15 | transcription factor 15 | -1,66 | 1,16E-06 |

| | | | |
|-----------|--|-------|----------|
| Tmem159 | transmembrane protein 159 | -1,66 | 1,72E-05 |
| Artn | artemin | -1,61 | 5,81E-05 |
| Naaladl2 | N-acetylated alpha-linked acidic dipeptidase-like 2 | -1,55 | 1,68E-07 |
| Ltk | leukocyte tyrosine kinase | -1,55 | 3,62E-05 |
| Zfp184 | zinc finger protein 184 (Kruppel-like) | -1,55 | 2,88E-05 |
| Zfp647 | zinc finger protein 647 | -1,53 | 3,82E-08 |
| Gm2960 | predicted gene 2960 | -1,53 | 4,08E-05 |
| Mfsd9 | major facilitator superfamily domain containing 9 | -1,52 | 2,48E-07 |
| Zfp51 | zinc finger protein 51 | -1,48 | 3,01E-07 |
| Six4 | sine oculis-related homeobox 4 homolog (Drosophila) | -1,46 | 7,90E-05 |
| Rad51ap2 | RAD51 associated protein 2 | -1,46 | 2,69E-05 |
| Mir17hg | MIR17 host gene 1 (non-protein coding) | -1,45 | 1,54E-04 |
| Ccdc160 | coiled-coil domain containing 160 | -1,45 | 3,64E-04 |
| Tusc1 | tumor suppressor candidate 1 | -1,44 | 1,47E-06 |
| Nipsnap3b | nipsnap homolog 3B (C. elegans) | -1,43 | 1,14E-05 |
| Haus4 | HAUS augmin-like complex, subunit 4 | -1,42 | 1,47E-06 |
| Nipsnap3b | nipsnap homolog 3B (C. elegans) | -1,38 | 6,12E-07 |
| Gm11818 | predicted gene 11818 | -1,36 | 9,85E-05 |
| Zfp760 | zinc finger protein 760 | -1,35 | 3,55E-05 |
| Wdsub1 | WD repeat, SAM and U-box domain containing 1 | -1,35 | 2,46E-06 |
| Adcy5 | adenylate cyclase 5 | -1,34 | 9,63E-06 |
| Fam117a | family with sequence similarity 117, memberA | -1,32 | 6,59E-06 |
| Ccdc91 | coiled-coil domain containing 91 | -1,31 | 8,47E-09 |
| Foxq1 | forkhead box Q1 | -1,31 | 2,02E-05 |
| Metrn1 | meteorin, glial cell differentiation regulator-like | -1,29 | 4,01E-07 |
| Adcy5 | adenylate cyclase 5 | -1,27 | 9,03E-07 |
| Zfp641 | zinc finger protein 641 | -1,27 | 8,36E-06 |
| Ccdc111 | coiled-coil domain containing 111 | -1,26 | 1,91E-04 |
| Ptgr1 | prostaglandin reductase 1 | -1,25 | 1,89E-04 |
| Neil2 | nei like 2 (E. coli) | -1,25 | 4,00E-04 |
| Car10 | carbonic anhydrase 10 | -1,24 | 4,18E-05 |
| Ikzf2 | IKAROS family zinc finger 2 | -1,24 | 2,23E-04 |
| Prkra | protein kinase, interferon inducible double stranded RNA dependent activator | -1,22 | 7,15E-07 |
| Immtp2l | IMP2 inner mitochondrial membrane peptidase-like (S. | -1,22 | 2,31E-07 |

| | | | |
|---------------|---|-------|----------|
| | cerevisiae) | | |
| Gabrg3 | gamma-aminobutyric acid (GABA) A receptor, subunit gamma 3 | -1,22 | 3,64E-04 |
| Ikzf2 | IKAROS family zinc finger 2 | -1,21 | 2,94E-05 |
| Zfp429 | zinc finger protein 429 | -1,20 | 1,71E-05 |
| Xkrx | X Kell blood group precursor related X linked | -1,17 | 3,53E-05 |
| Adcy5 | adenylate cyclase 5 | -1,16 | 2,80E-05 |
| Glyctk | glycerate kinase | -1,14 | 2,41E-04 |
| Marveld1 | MARVEL (membrane-associating) domain containing 1 | -1,13 | 2,11E-04 |
| Nt5dc1 | 5'-nucleotidase domain containing 1 | -1,13 | 5,42E-05 |
| Syde2 | synapse defective 1, Rho GTPase, homolog 2 (C. elegans) | -1,13 | 2,31E-05 |
| Ppic | peptidylprolyl isomerase C | -1,12 | 1,79E-04 |
| Pole4 | polymerase (DNA-directed), epsilon 4 (p12 subunit) | -1,12 | 1,42E-05 |
| Car4 | carbonic anhydrase 4 | -1,11 | 1,30E-04 |
| Tox3 | TOX high mobility group box family member 3 | -1,11 | 3,61E-04 |
| Larp1b | La ribonucleoprotein domain family, member 1B | -1,10 | 1,27E-05 |
| Myom3 | myomesin family, member 3 | -1,10 | 1,56E-05 |
| Sord | sorbitol dehydrogenase | -1,08 | 4,44E-07 |
| Oxr1 | oxidation resistance 1 | -1,08 | 5,91E-07 |
| Fah | fumarylacetoacetate hydrolase | -1,07 | 1,11E-06 |
| Ckap4 | cytoskeleton-associated protein 4 | -1,07 | 6,12E-07 |
| Paox | polyamine oxidase (exo-N4-amino) | -1,07 | 2,84E-07 |
| Zfp612 | zinc finger protein 612 | -1,07 | 1,12E-05 |
| Fbxl21 | F-box and leucine-rich repeat protein 21 | -1,07 | 4,96E-05 |
| Stxbp2 | syntaxin binding protein 2 | -1,06 | 3,16E-04 |
| Btg3 | B-cell translocation gene 3 | -1,06 | 4,53E-05 |
| Kdelc2 | KDEL (Lys-Asp-Glu-Leu) containing 2 | -1,05 | 3,71E-06 |
| Rimbp3 | RIMS binding protein 3 | -1,02 | 1,60E-04 |
| Paox | polyamine oxidase (exo-N4-amino) | -1,02 | 1,11E-06 |
| Gm347 | predicted gene 347 | -1,00 | 6,61E-05 |
| Irf1 | interferon regulatory factor 1 | -0,99 | 1,29E-06 |
| B3gnt6 | UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 6 (core 3 synthase) | -0,98 | 3,05E-04 |
| Gpx7 | glutathione peroxidase 7 | -0,97 | 2,62E-05 |

| | | | |
|----------|---|-------|----------|
| Stbd1 | starch binding domain 1 | -0,97 | 5,00E-05 |
| Rabepk | Rab9 effector protein with kelch motifs | -0,97 | 1,13E-05 |
| Bag2 | BCL2-associated athanogene 2 | -0,97 | 5,25E-07 |
| Ptgr2 | prostaglandin reductase 2 | -0,97 | 6,91E-06 |
| Gm11110 | predicted gene 11110 | -0,96 | 1,70E-04 |
| Parp11 | poly (ADP-ribose) polymerase family, member 11 | -0,94 | 8,40E-07 |
| Gk5 | glycerol kinase 5 (putative) | -0,92 | 6,62E-05 |
| Pole | polymerase (DNA directed), epsilon | -0,92 | 7,68E-05 |
| Chst7 | carbohydrate (N-acetylglucosamino) sulfotransferase 7 | -0,92 | 1,05E-04 |
| Echdc2 | enoyl Coenzyme A hydratase domain containing 2 | -0,92 | 6,50E-06 |
| Bves | blood vessel epicardial substance | -0,91 | 2,06E-05 |
| Sypl2 | synaptophysin-like 2 | -0,90 | 7,01E-05 |
| Neu3 | neuraminidase 3 | -0,90 | 7,09E-06 |
| Acot1 | acyl-CoA thioesterase 1 | -0,89 | 1,02E-05 |
| Jag2 | jagged 2 | -0,89 | 1,84E-04 |
| Cntln | centlein, centrosomal protein | -0,89 | 1,70E-05 |
| Rab42-ps | RAB42, member RAS oncogene family, pseudogene | -0,89 | 2,84E-04 |
| Nudt14 | nudix (nucleoside diphosphate linked moiety X)-type motif 14 | -0,87 | 3,32E-05 |
| Lck | lymphocyte protein tyrosine kinase | -0,84 | 2,01E-04 |
| Rel1 | RELT-like 1 | -0,84 | 7,22E-06 |
| Slc1a6 | solute carrier family 1 (high affinity aspartate/glutamate transporter), member 6 | -0,84 | 1,88E-04 |
| Echdc2 | enoyl Coenzyme A hydratase domain containing 2 | -0,83 | 9,43E-05 |
| Ccdc138 | coiled-coil domain containing 138 | -0,83 | 7,65E-06 |
| Galnt12 | UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 12 | -0,83 | 8,22E-06 |
| Mina | myc induced nuclear antigen | -0,83 | 5,11E-05 |
| Slc35f5 | solute carrier family 35, member F5 | -0,83 | 1,22E-06 |
| Reep6 | receptor accessory protein 6 | -0,82 | 3,96E-06 |
| Gkap1 | G kinase anchoring protein 1 | -0,82 | 4,27E-05 |
| Pparg | peroxisome proliferator activated receptor gamma | -0,82 | 6,38E-06 |
| Gkap1 | G kinase anchoring protein 1 | -0,81 | 1,72E-05 |
| Gprc5c | G protein-coupled receptor, family C, group 5, member C | -0,78 | 1,79E-04 |

| | | | |
|-------------|--|-------|----------|
| Asl | argininosuccinate lyase | -0,78 | 6,34E-05 |
| Churc1 | churchill domain containing 1 | -0,77 | 2,91E-04 |
| Gpre5c | G protein-coupled receptor, family C, group 5, member C | -0,77 | 3,15E-04 |
| Peci | peroxisomal delta3, delta2-enoyl-Coenzyme A isomerase | -0,76 | 7,54E-06 |
| Prorsd1 | prolyl-tRNA synthetase domain containing 1 | -0,76 | 1,74E-04 |
| Gm2954 | predicted gene 2954 | -0,75 | 1,40E-04 |
| BC016495 | cDNA sequence BC016495 | -0,75 | 8,10E-05 |
| Eda | ectodysplasin-A | -0,75 | 1,50E-04 |
| Kctd15 | potassium channel tetramerisation domain containing 15 | -0,75 | 3,52E-04 |
| Crlf2 | cytokine receptor-like factor 2 | -0,74 | 1,70E-04 |
| Ano10 | anoctamin 10 | -0,74 | 1,72E-04 |
| Sfxn4 | sideroflexin 4 | -0,74 | 1,49E-05 |
| Cdyl2 | chromodomain protein, Y chromosome-like 2 | -0,72 | 3,17E-05 |
| Smo | smoothened homolog (Drosophila) | -0,71 | 4,38E-06 |
| Tmem53 | transmembrane protein 53 | -0,71 | 1,66E-04 |
| Slc38a6 | solute carrier family 38, member 6 | -0,71 | 2,77E-04 |
| Zfp105 | zinc finger protein 105 | -0,71 | 1,40E-04 |
| Ebf4 | early B-cell factor 4 | -0,70 | 1,53E-04 |
| Cdca7 | cell division cycle associated 7 | -0,70 | 1,66E-05 |
| Acadm | acyl-Coenzyme A dehydrogenase, medium chain | -0,69 | 1,31E-05 |
| Tpm4 | tropomyosin 4 | -0,69 | 2,17E-04 |
| Sh3bgrl2 | SH3 domain binding glutamic acid-rich protein like 2 | -0,67 | 2,91E-04 |
| Cntln | centleln, centrosomal protein | -0,67 | 2,99E-05 |
| Cnga4 | cyclic nucleotide gated channel alpha 4 | -0,66 | 2,98E-04 |
| Hddc2 | HD domain containing 2 | -0,66 | 5,03E-06 |
| Bend3 | BEN domain containing 3 | -0,65 | 9,88E-06 |
| Gpr125 | G protein-coupled receptor 125 | -0,65 | 2,30E-05 |
| Galk1 | galactokinase 1 | -0,64 | 2,41E-04 |
| Pttg1 | pituitary tumor-transforming gene 1 | -0,64 | 4,79E-05 |
| Accs | 1-aminocyclopropane-1-carboxylate synthase homolog (Arabidopsis)(non-functional) | -0,64 | 1,70E-04 |
| Ebpl | emopamil binding protein-like | -0,63 | 1,75E-05 |
| Trmt2b | TRM2 tRNA methyltransferase 2 homolog B (S. cerevisiae) | -0,63 | 1,23E-05 |
| Zfp948 | zinc finger protein 948 | -0,63 | 2,86E-04 |
| Zfp945 | zinc finger protein 945 | -0,63 | 4,59E-05 |
| Ebi3 | Epstein-Barr virus induced | -0,62 | 2,14E-04 |

| | | | |
|----------|---|-------|----------|
| | gene 3 | | |
| Tmsb15b1 | thymosin beta 15b1 | -0,62 | 8,01E-05 |
| Rangrf | RAN guanine nucleotide release factor | -0,61 | 8,53E-05 |
| Gusb | glucuronidase, beta | -0,61 | 2,18E-04 |
| Trpv2 | transient receptor potential cation channel, subfamily V, member 2 | -0,61 | 4,01E-05 |
| Gm4535 | predicted gene 4535 | -0,60 | 2,12E-04 |
| Churc1 | churchill domain containing 1 | -0,60 | 1,05E-04 |
| Galnt12 | UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 12 | -0,59 | 2,78E-04 |
| E2f1 | E2F transcription factor 1 | -0,59 | 9,37E-05 |
| Cabyr | calcium-binding tyrosine-(Y)-phosphorylation regulated (fibrousheathin 2) | 0,64 | 2,26E-04 |
| Ccdc88b | coiled-coil domain containing 88B | 0,66 | 1,01E-04 |
| Angpt1 | angiopoietin 1 | 0,70 | 3,75E-04 |
| Pcdhb22 | protocadherin beta 22 | 0,80 | 3,74E-07 |
| Ccdc88b | coiled-coil domain containing 88B | 0,95 | 1,46E-05 |
| Pcdhb21 | protocadherin beta 21 | 1,01 | 9,00E-06 |
| Krt9 | keratin 9 | 1,01 | 6,28E-06 |
| Chrdl2 | chordin-like 2 | 1,06 | 1,79E-05 |
| Megf6 | multiple EGF-like-domains 6 | 1,22 | 8,98E-05 |
| Dach2 | dachshund 2 (Drosophila) | 3,43 | 2,90E-08 |

Table 2: List of genes differentially regulated in CA region of F/F CKII mice

| Symbol | Name | log (fold change) | Adjusted <i>p</i> -value |
|--------|--|-------------------|--------------------------|
| Lor | loricrin | -1,52 | 1,51E-08 |
| Ccdc91 | coiled-coil domain containing 91 | -1,02 | 1,75E-07 |
| Dazl | deleted in azoospermia-like | -2,71 | 8,00E-07 |
| Prss16 | protease, serine, 16 (thymus) | -3,41 | 9,51E-07 |
| Zfp647 | zinc finger protein 647 | -1,12 | 1,59E-06 |
| Oxr1 | oxidation resistance 1 | -0,92 | 3,42E-06 |
| Parp11 | poly (ADP-ribose) polymerase family, member 11 | -0,83 | 3,57E-06 |
| Reep6 | receptor accessory protein 6 | -0,82 | 4,26E-06 |

| | | | |
|---------------|--|-------|----------|
| Hdx | highly divergent homeobox | -1,50 | 6,37E-06 |
| Immp2l | IMP2 inner mitochondrial membrane peptidase-like (S. cerevisiae) | -0,92 | 6,60E-06 |
| Hfm1 | HFM1, ATP-dependent DNA helicase homolog (S. cerevisiae) | -1,78 | 1,14E-05 |
| Wdr17 | WD repeat domain 17 | -1,11 | 1,12E-05 |
| Rimbp3 | RIMS binding protein 3 | -1,22 | 2,76E-05 |
| Zbtb8a | zinc finger and BTB domain containing 8a | -1,29 | 3,06E-05 |
| Ap1s3 | adaptor-related protein complex AP-1, sigma 3 | -1,26 | 3,69E-05 |
| Rabepk | Rab9 effector protein with kelch motifs | -0,87 | 3,64E-05 |
| Cmya5 | cardiomyopathy associated 5 | -0,89 | 4,09E-05 |
| Epb4.9 | erythrocyte protein band 4.9 | -0,44 | 2,79E-05 |
| 4930403L05Rik | protein phosphatase 1 regulatory subunit 2 pseudogene | 1,92 | 2,07E-05 |
| Dach2 | dachshund 2 (Drosophila) | 3,04 | 1,26E-07 |

Appendix 2:

Table 3: List of differentially regulated genes in hippocampus of wild-type mice after 10 weeks of enrichment (the genes in red were confirmed by qRT-PCR)

| Symbol | Name | log (fold change) | Adjusted <i>p</i> -value |
|---------------|--|-------------------|--------------------------|
| Lrriq4 | leucine-rich repeats and IQ motif containing 4 | 3,71 | 1,46E-04 |
| Cuedc1 | CUE domain containing 1 | 3,31 | 2,75E-08 |
| Slfn13 | schlafen family member 13 | 3,12 | 2,74E-10 |
| Pdxdc1 | pyridoxal-dependent decarboxylase domain containing 1 | 2,74 | 3,03E-07 |
| Gm3219 | B-cell CLL/lymphoma 7C pseudogene | 2,68 | 6,39E-06 |
| Pde5a | phosphodiesterase 5A, cGMP-specific | 2,36 | 1,98E-04 |
| Pcdhb2 | protocadherin beta 2 | 2,07 | 1,74E-06 |
| Slfn10-ps | schlafen 10, pseudogene | 2,02 | 1,16E-06 |
| Plbd1 | phospholipase B domain containing 1 | 1,63 | 1,99E-04 |
| Tjp2 | tight junction protein 2 | 1,62 | 6,03E-08 |
| Tmco6 | transmembrane and coiled-coil domains 6 | 1,59 | 4,17E-06 |
| Ccl6 | chemokine (C-C motif) ligand 6 | 1,40 | 5,34E-07 |
| Paip1 | polyadenylate binding protein-interacting protein 1 | 1,39 | 1,10E-05 |
| Nnt | nicotinamide nucleotide transhydrogenase | 1,24 | 2,30E-04 |
| Kank1 | KN motif and ankyrin repeat domains 1 | 1,15 | 4,84E-08 |
| Cml5 | camello-like 5 | 1,14 | 7,19E-05 |
| Mfi2 | antigen p97 (melanoma associated) identified by monoclonal antibodies 133.2 and 96.5 | 1,13 | 4,59E-07 |
| Dap3 | death associated protein 3 | 1,09 | 3,92E-04 |
| Slc26a10 | solute carrier family 26, member 10 | 1,05 | 4,02E-04 |
| Pdzd2 | PDZ domain containing 2 | 1,05 | 2,77E-04 |
| Ccl8 | chemokine (C-C motif) ligand 8 | 1,05 | 4,51E-04 |
| Med1 | mediator complex subunit 1 | 1,03 | 2,67E-06 |
| Pdxdc1 | pyridoxal-dependent decarboxylase domain containing 1 | 1,01 | 8,62E-05 |
| Sec24d | Sec24 related gene family, member D (<i>S. cerevisiae</i>) | 1,00 | 1,83E-04 |
| Ptar1 | protein prenyltransferase alpha | 0,98 | 4,19E-05 |

| | | | |
|---------------|---|------|----------|
| | subunit repeat containing 1 | | |
| Slc15a2 | solute carrier family 15 (H ⁺ /peptide transporter), member 2 | 0,97 | 1,23E-06 |
| Cdkl5 | cyclin-dependent kinase-like 5 | 0,95 | 1,04E-04 |
| Pgm5 | phosphoglucomutase 5 | 0,94 | 3,24E-06 |
| Syce2 | synaptonemal complex central element protein 2 | 0,84 | 1,38E-04 |
| Impg2 | interphotoreceptor matrix proteoglycan 2 | 0,81 | 3,08E-04 |
| Acvr1c | activin A receptor, type IC | 0,80 | 1,87E-04 |
| Dcpp3 | demilune cell and parotid protein 3 | 0,80 | 2,30E-04 |
| Nkd1 | naked cuticle 1 homolog (Drosophila) | 0,78 | 3,14E-04 |
| Nsa2 | NSA2 ribosome biogenesis homolog (S. cerevisiae) | 0,78 | 1,63E-06 |
| Rnls | renalase, FAD-dependent amine oxidase | 0,78 | 3,08E-04 |
| Cbwd1 | COBW domain containing 1 | 0,77 | 3,75E-06 |
| Camk4 | calcium/calmodulin-dependent protein kinase IV | 0,76 | 6,80E-05 |
| Mfsd6 | major facilitator superfamily domain containing 6 | 0,75 | 5,31E-04 |
| Jakmip3 | janus kinase and microtubule interacting protein 3 | 0,73 | 3,99E-04 |
| Fastk | Fas-activated serine/threonine kinase | 0,73 | 3,28E-04 |
| Galnt11 | UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 11 | 0,72 | 8,70E-05 |
| Ing3 | inhibitor of growth family, member 3 | 0,72 | 4,94E-05 |
| Tmem2 | transmembrane protein 2 | 0,72 | 1,81E-04 |
| Otos | otospiralin | 0,70 | 3,62E-05 |
| Stard4 | StAR-related lipid transfer (START) domain containing 4 | 0,70 | 2,33E-05 |
| Robo1 | roundabout homolog 1 (Drosophila) | 0,69 | 7,24E-05 |
| Ptgs2 | prostaglandin-endoperoxide synthase 2 | 0,67 | 4,11E-05 |
| Gm16500 | predicted gene 16500 | 0,67 | 1,02E-04 |
| A930018M24Rik | RIKEN cDNA A930018M24 gene | 0,67 | 3,58E-04 |
| LOC100504473 | RNA-binding protein Musashi homolog 2-like | 0,66 | 1,64E-04 |
| Smek2 | SMEK homolog 2, suppressor of mek1 (Dictyostelium) | 0,66 | 2,89E-04 |
| Gabra2 | gamma-aminobutyric acid (GABA) A receptor, subunit alpha 2 | 0,64 | 1,01E-04 |
| Xbp1 | X-box binding protein 1 | 0,62 | 3,88E-04 |

| | | | |
|---------------|---|-------|----------|
| Eif2c4 | eukaryotic translation initiation factor 2C, 4 | 0,62 | 1,96E-04 |
| A730020M07Rik | RIKEN cDNA A730020M07 gene | 0,61 | 3,74E-04 |
| Mrgpra2b | MAS-related GPR, member A2B | 0,61 | 1,98E-04 |
| Fam150b | family with sequence similarity 150, member B | 0,61 | 2,40E-04 |
| Meox1 | mesenchyme homeobox 1 | 0,60 | 4,08E-04 |
| Crebl2 | cAMP responsive element binding protein-like 2 | 0,59 | 2,60E-04 |
| Hist1h2bc | histone cluster 1, H2bc | 0,59 | 8,72E-05 |
| Tctex1d2 | Tctex1 domain containing 2 | 0,59 | 8,76E-06 |
| Kat2a | K(lysine) acetyltransferase 2A | -0,58 | 5,78E-04 |
| Pisd-ps3 | phosphatidylserine decarboxylase, pseudogene 3 | -0,58 | 1,11E-04 |
| Rab5b | RAB5B, member RAS oncogene family | -0,58 | 3,46E-05 |
| Prr12 | proline rich 12 | -0,59 | 1,06E-04 |
| 38961 | septin 6 | -0,59 | 6,60E-04 |
| Gm4072 | predicted gene 4072 | -0,60 | 5,36E-04 |
| Cnksr1 | connector enhancer of kinase suppressor of Ras 1 | -0,60 | 3,82E-04 |
| Pik3r2 | phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 2 (p85 beta) | -0,61 | 2,16E-05 |
| Sncb | synuclein, beta | -0,61 | 6,02E-04 |
| Gm7109 | predicted gene 7109 | -0,63 | 9,87E-05 |
| Prepl | prolyl endopeptidase-like | -0,63 | 2,10E-04 |
| Grin2b | glutamate receptor, ionotropic, NMDA2B (epsilon 2) | -0,63 | 2,30E-04 |
| Smarcd1 | SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily d, member 1 | -0,63 | 1,65E-04 |
| Mlycd | malonyl-CoA decarboxylase | -0,65 | 4,48E-04 |
| Kif1c | kinesin family member 1C | -0,65 | 1,64E-04 |
| Glod4 | glyoxalase domain containing 4 | -0,67 | 2,94E-04 |
| Chchd10 | coiled-coil-helix-coiled-coil-helix domain containing 10 | -0,67 | 7,06E-05 |
| Ssx2ip | synovial sarcoma, X breakpoint 2 interacting protein | -0,68 | 3,33E-04 |
| Rps6kb1 | ribosomal protein S6 kinase, polypeptide 1 | -0,68 | 1,81E-06 |
| ErbB3 | v-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian) | -0,69 | 5,70E-04 |
| Gm3652 | predicted gene 3652 | -0,69 | 4,37E-04 |
| Dgke | diacylglycerol kinase, epsilon | -0,69 | 1,74E-05 |
| Pcdhb4 | protocadherin beta 4 | -0,70 | 5,94E-05 |
| Asna1 | arsA arsenite transporter, ATP- | -0,71 | 1,83E-04 |

| | | | |
|-------------|---|-------|----------|
| | binding, homolog 1 (bacterial) | | |
| Lipc | lipase, hepatic | -0,71 | 2,73E-04 |
| Gm5151 | predicted gene 5151 | -0,73 | 3,94E-04 |
| Olfm1 | olfactomedin 1 | -0,73 | 1,06E-04 |
| Ssbp4 | single stranded DNA binding protein 4 | -0,73 | 1,31E-04 |
| Nsg2 | neuron specific gene family member 2 | -0,75 | 8,67E-05 |
| Golt1a | golgi transport 1 homolog A (S. cerevisiae) | -0,75 | 5,08E-04 |
| D8Erttd738e | DNA segment, Chr 8, ERATO Doi 738, expressed | -0,77 | 2,36E-04 |
| Mcm9 | minichromosome maintenance complex component 9 | -0,78 | 3,08E-04 |
| Cdc23 | CDC23 (cell division cycle 23, yeast, homolog) | -0,78 | 1,08E-04 |
| Hdgf | hepatoma-derived growth factor | -0,78 | 1,09E-04 |
| Smarce1 | SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily e, member 1 | -0,78 | 2,13E-05 |
| Zfp961 | zinc finger protein 961 | -0,79 | 9,50E-05 |
| Tom1 | target of myb1 homolog (chicken) | -0,79 | 4,97E-04 |
| Accn3 | amiloride-sensitive cation channel 3 | -0,81 | 2,66E-04 |
| Pcgf2 | polycomb group ring finger 2 | -0,82 | 6,46E-04 |
| Gfap | glial fibrillary acidic protein | -0,92 | 1,93E-04 |
| Pi4kb | phosphatidylinositol 4-kinase, catalytic, beta polypeptide | -0,92 | 7,58E-05 |
| Gadd45gip1 | growth arrest and DNA-damage-inducible, gamma interacting protein 1 | -0,93 | 2,69E-04 |
| Lpl | lipoprotein lipase | -0,94 | 4,21E-04 |
| Olfir678 | olfactory receptor 678 | -0,96 | 5,57E-04 |
| Hapln4 | hyaluronan and proteoglycan link protein 4 | -0,98 | 2,25E-04 |
| Plvap | plasmalemma vesicle associated protein | -1,00 | 4,69E-06 |
| Apold1 | apolipoprotein L domain containing 1 | -1,03 | 1,67E-05 |
| Hdgf | hepatoma-derived growth factor | -1,05 | 1,84E-05 |
| Fam32a | family with sequence similarity 32, member A | -1,05 | 1,64E-04 |
| Plvap | plasmalemma vesicle associated protein | -1,10 | 8,01E-05 |
| Stac2 | SH3 and cysteine rich domain 2 | -1,10 | 1,18E-05 |
| Lpcat2 | lysophosphatidylcholine acyltransferase 2 | -1,15 | 5,49E-04 |
| Vmn1r90 | vomer nasal 1 receptor 90 | -1,15 | 2,72E-04 |

| | | | |
|---------|--|-------|----------|
| Lpl | lipoprotein lipase | -1,17 | 2,32E-05 |
| Il12rb1 | interleukin 12 receptor, beta 1 | -1,18 | 2,77E-04 |
| Camk4 | calcium/calmodulin-dependent protein kinase IV | -1,22 | 2,07E-05 |
| Klf1 | Kruppel-like factor 1 (erythroid) | -1,34 | 9,11E-05 |
| Ccl17 | chemokine (C-C motif) ligand 17 | -1,36 | 1,07E-05 |
| Slfn2 | schlafen 2 | -1,49 | 6,23E-06 |
| Zfp963 | zinc finger protein 963 | -1,52 | 9,23E-06 |
| Def8 | differentially expressed in FDCP 8 | -1,52 | 1,13E-04 |
| Mamdc2 | MAM domain containing 2 | -1,96 | 8,76E-07 |
| Capns2 | calpain, small subunit 2 | -2,07 | 1,43E-04 |
| Fastk | Fas-activated serine/threonine kinase | -2,10 | 8,34E-09 |
| Pde6h | phosphodiesterase 6H, cGMP-specific, cone, gamma | -2,29 | 2,33E-05 |
| Ocell | occludin/ELL domain containing 1 | -2,92 | 9,23E-05 |
| Tbx4 | T-box 4 | -3,46 | 1,44E-08 |
| Rheb | Ras homolog enriched in brain | -4,79 | 6,27E-10 |
| Krt12 | keratin 12 | -6,60 | 4,66E-08 |

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Curriculum Vitae

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Name: Cemil Kerimoglu

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EDUCATION

- Oct. 2008 – current PhD study in the Laboratory for Aging and Cognitive Diseases, Dr. Andre Fischer in European Neuroscience Institute (ENI), Göttingen / GERMANY
- 2007 - 2008 MSc student in IMPRS MSc/PhD/MD-PhD Program in Neurosciences, Göttingen / GERMANY
- 2003 - 2007 BSc in Middle East Technical University (METU) Department of Molecular Biology and Genetics, Ankara / TURKEY

SCHOLARSHIPS

- 2007 - 2008:** Stipend of the Excellence Foundation for the Promotion of the Max Planck Society
- Summer 2006:** British Genetics Society Summer Studentship, UK

RESEARCH

- Oct. 2008 – current Doctoral project: Role of Histone Methylation in Cognitive Function and Molecular Mechanisms Mediating the Effect of Environmental Enrichment on Learning & Memory

PUBLICATIONS

Bolender J., Erdeniz B., Kerimoglu C.; "Hominid Uniqueness, Cognition by Description and Procedural Memory"; *Biolinguistics*, 2008

Baharijavan S., Maddalena A.*, Kerimoglu C.*, Wittnam J., Held T., Bähr M., Burkhardt S., Delalle I., Kügler S., Fischer A. and Sananbenesi F.; "HDAC1 Regulates Fear Extinction in Mice" (accepted for publication from *Journal of Neuroscience*)

* equal contribution

Agis-Balboa R.C., Pavelka Z., Kerimoglu C., Sananbenesi F., Fischer A.; "Targeting Hdac5 in the Alzheimer's disease animal model APPPS1-21 is detrimental for cognition" (submitted to *Journal of Alzheimer's Disease*)

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Kerimoglu C., Stilling R., Rao P., Agis-Balboa R.C., Fischer A. "Persistence of the effects of environmental enrichment depends on its duration" (in preparation)

Kerimoglu C., Baharijavan S., Fischer A. "Environmental enrichment dampens the response to a novel stimulus" (in preparation)