Epigenomic Imaging of Neuropsychiatric Diseases:

The Role of Chromatin Plasticity in Schizophrenia and Anxiety Diseases

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Declaration

Hereby I declare that the PhD thesis entitled:

"Epigenomic Imaging of Neuropsychiatric Diseases: The Role of Chromatin Plasticity in Schizophrenia and Anxiety Diseases"

has been written independently and with no other sources and aids than quoted.

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Meiner Mutter

“ Im Leben kommt es nicht darauf an, ein gutes Blatt in der Hand zu haben, sondern mit schlechten Karten gut zu spielen”.

Robert LB. Stevenson (1850- 1984)

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1. Introduction

1.1 Genome x Environment interactions: Uncovering the etiology of neuropsychiatric disorders

Complex neuropsychiatric diseases such as schizophrenia are multifactorial and cannot be solely explained by genetic risk. Thus, it is now clear that the pathogenesis of such diseases arise on the background of genome-environment interactions (GxE). The precise mechanisms involved are however poorly defined and a better understanding of such mechanisms holds great potential to eventually develop surrogate- and biomarker for neuropsychiatric diseases as well as novel therapeutic approaches. The determinants that define an individual's state of health and disease have been investigated for a long time by epidemiologists. One of the first epidemiologists was Hippocrates who established a relation between the occurrence of a disease and environmental influences [1,2]. The importance of genetics in human diseases for epidemiological studies was recognized over 30 years ago, creating a new discipline termed Genetic Epidemiology ***"…. that focuses on joint effects of genes and the environment and incorporates disease biology into conceptual models…"*** [3,4]. With the completion of the Human Genome Project [5] and the HapMap project [6] as well as the rapid advances in Next-Generation-Sequencing technologies [7], genetic epidemiological studies increased and revealed hundreds of genetic loci for traits and diseases with public health significance [3,8]. Hence, genetic epidemiological studies identified genetic differences among individuals of a population that may be linked to disease susceptibility [3,9,10]. Yet genetics alone cannot explain the onset of neuropsychiatric disease on an individual basis. As mentioned above, mental disorders such as depression, addiction, schizophrenia and anxiety disorders are complex multifactorial neurological syndromes with genetic heterogeneity and high heritability with non-mendelian pattern of inheritance. Genetic studies on concordance among monozygotic twins suggest that genetics account for ~ 50% of the disease risk [11–16].

Thus, Genome Wide Association Studies (GWAS), studies investigating Single Nucleotide Polymorphisms (SNPs) and Copy Number Variations (CNVs) did not fulfill the hope to identify and validate key susceptibility genes. Such data clearly

indicated the involvement of non-genetic processes in disease pathogenesis [17]. In line with this assumption, traditional epidemiological studies revealed several environmental risk factors for neuropsychiatric disorders, such as early life stress, parental age, maternal infection during pregnancy, nutritional deficiency and migration [17–25]. However, these environmental risk factors are, as in the case for genetics, considered to be only contributory, since exposure to those factors does not lead necessarily to disease onset [17]. Hence, epidemiological and medical genetic studies together have provided mounting evidence that genome and environmental interactions (GxE) play a key role in the etiology of neuropsychiatric disorders [26,27]. Research of last decades identified molecular mechanisms that mediate GxE interactions, among which the most prominent are so-called epigenetic mechanisms. Moreover, deregulation of epigenetic mechanisms has been implicated with the pathogenesis of neuropsychiatric disorders [28–30].

1.1.1 Epigenetics

The developmental biologist Conrad H. Waddington coined the term "Epigenetics" and defined it as "***the branch of biology which studies the casual interactions between genes and their products which bring the phenotype into being***" [31,32].
In more detail, Waddington´s definition referred to an "epigenetic landscape" to describe the process of cellular decision-making during development in a multicellular organism [33]. Here, each cell shares an identical genome but differentiate into specific cell types by activation or repression of particular gene-networks, resulting into a distinct gene-expression profile [34,35].

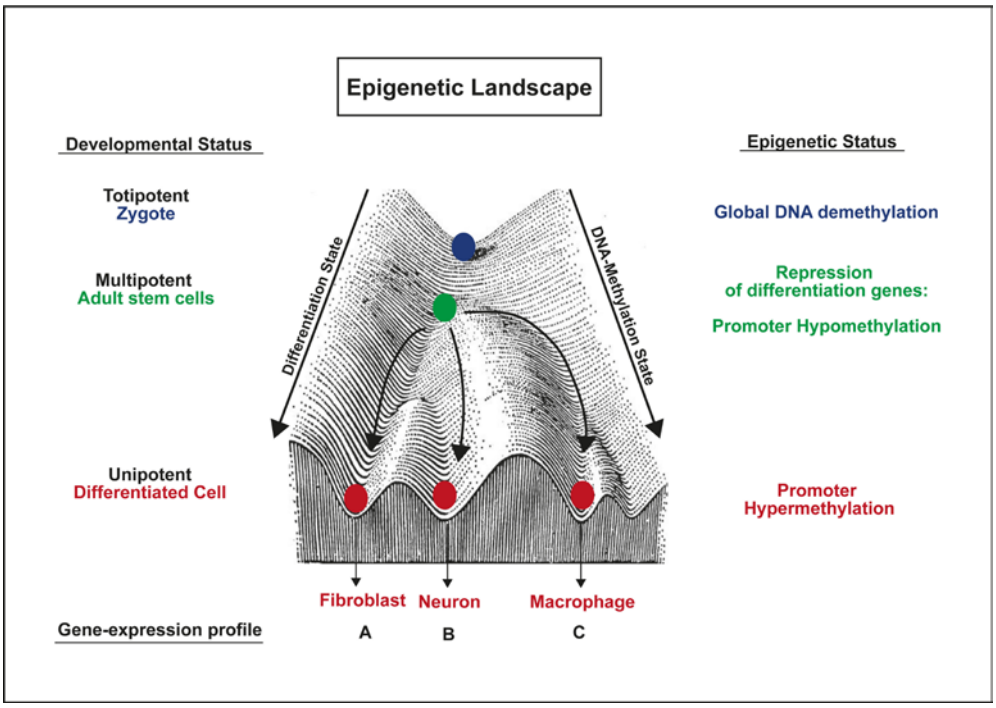

Figure 1 Differentiation and epigenetic states in "Waddington´s Epigenetic Landscape".

A model of Waddington´s epigenetic landscape showing cell populations at different developmental states (left) and their corresponding epigenetic states (right). The model is representing cellular decision-making during development. The cell (presented by a ball) rolls down the landscape into one of several valleys that represent cell fates with distinct gene-expression profiles. Adapted and modified from [36].

Such distinct gene-expression profiles reflect cell-type specific population identities that are transmitted to daughter cells and maintained during many cell divisions, generating a so-called cellular memory [37,38]. An evolved and more up-to-date definition of epigenetics these days is, ***"…the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence."*** [39]. Thus, in order to be defined as an epigenetic trait or epigenetic mechanism, the conventional definition requires heritability of these traits. However, the necessity of heritability for a mechanism to be accepted as epigenetics is debated [40,41]. Epigenetic research in the last decades consisted of examining the classical epigenetic mechanisms that regulate gene-expression (without affecting DNA-sequence) by analyzing higher-order structure chromatin, DNA and histone-tail modifications, transcriptional effects of RNA interference and how extracellular stimuli affect gene-expression through chromatin signaling, leading to long-lasting changes in phenotypes that are not necessarily transmitted to the next generation [40,42–49].

Thus, the concept of cellular memory and epigenetics has emerged as a new research field in molecular and behavioral neurobiology, creating the field of neuroepigenetics that promised to further elucidate learning and memory processes in the brain under physiological and pathological situations.

1.1.2 Epigenetic mechanisms

Local chromatin structure plays a crucial role in regulating gene-expression.
Two of the most described epigenetic mechanisms implicated in the organization of chromatin structure are covalent methylation of DNA and post-translational modifications of histone-tails.

1.1.3 DNA-Methylation

Methylation of DNA in eukaryotes was first described in 1948 by Hotchkiss [50] and was associated about 20 years later with transcriptional regulation [43,51,52]. In mammals, DNA methylation is catalyzed by enzymes called DNA methyltransferases (DNMTs) that transfer methyl-groups to cytosine residues specifically at the 5-position of the pyrimidine ring (5mC), a process that always occurs on CG dinucleotides. Almost 70-80% of CG dinucleotids are methylated in mammalian genome [53]. Remaining non-methylated CG dinucleotides are most often found in or closed to gene promoters possessing CG dinucleotide clusters called CpG islands (sequence of at least 200bp) [54,55]. Since promoters of silenced genes have been shown to exhibit more methylated cytosines compared to actively transcribed genes, methylation of DNA is in general associated with transcriptional repression [56,57]. However, recent reports indicated that DNA methylation at gene-bodies correlates with increased gene activity [58–61]. DNMTs are categorized in *de novo* DNMTs (DNMT3a and DNMT3b) that establish DNA methylation in early embryogenesis during implantation [62–64] and in maintenance DNMTs (DNMT1) that show affinity towards hemi-metyhlated DNA that restore and preserve the fully methylated state during DNA replication [65].

The role of DNA-methylation was not subject of this work and is therefore not going to be further explained.

1.1.4 Modification of Histone-tails

In eukaryotic cells, DNA is packed into Chromatin, a highly organized, functional and fundamental structure of the genome [66–68]. The basic repeating subunit of chromatin is the nucleosome, consisting of approximately 146 bp of DNA wrapped around a core of histone proteins, the so- called histone-octamer. Each histone-octamer contains two copies of the core histones H2A, H2B, H3 and H4. Each nucleosome is connected through linker DNA with the linker Histone H1, allowing the formation of higher order structure chromatin [67,69]. Histone-tail modifications affect global gene-expression profiles through two general mechanisms: 1.Alteration of chromatin structure to influence binding accessibility and recruitment of the transcriptional machinery and 2.Recruitment of ATP- dependent chromatin remodeling complexes [70]. Thus, histone-marks function as a signal platform to recruit different proteins, such as chromatin readers and modifiers that recognize and bind specific histone tails and direct downstream biological processes [71].

There are at least eight distinct types of histone-tail modifications (acetylation, methylation, phosphorylation, ubiquitination, sumoylation, ADP ribosylaion, deamination, proline isomerization) [72]. The specific combination and timing of histone tail modifications is finely orchestrated in response to different stimuli and build up the histone code – a combinatorial pattern of modifications – priming transcriptional programs [73].

Table 1. Different Classes of Histone-tail Modifications Identified

Histone-tail Modification	Residue Modified	Functions Regulated
Acetylation	**K**-ac	Transcription, Repair, Replication, Condensation
Lysine Methylation	**K**-me1, **K**-me2, **K**-me3	Transcription, Repair
Arginine Methylation	**R**-me1, **R**-me2, **R**-me3	Transcription
Phosphorylation	**S**-ph, **T**-ph	Transcription, Repair, Condensation
Ubiquitylation	**K**-ub	Transcription, Repair
Sumoylation	**K**-su	Transcription
ADP Ribosylation	**E**-ar	Transcription
Deimniation	**R> Cit**	Transcription
Proline Isomerization	**P-cis > P-trans**	Transcription

K : Lysine; R: Arginine; S: Serine; E: Glutamate; Reproduced and modified from Kouzarides et al., 2007

In general, the overall function of histone-tail modifications is the orchestration of DNA based biological tasks such as transcription, DNA replication, DNA repair and establishment of global chromosome environments such as silent heterochromatin or active euchromatin [72].

1.1.4.1 Histone-acetylation

Acetylation of histone-tails is mediated by enzymes called histone-acetyltransferases (HATs) that transfer an acetyl group from acetyl-coenzyme A to the ε-NH^+-group of a lysine residue [74]. Increased acetylation of histone-tails is predominantly associated with transcriptional activation of genes by neutralizing the positively charged lysine-residues [75]. The resulting decreased electrostatic binding between lysine-residues and negatively charged phosphate-groups along the DNA backbone leads to locally less condensed chromatin accessible for transcriptional machinery [68,76–79].

The process of histone-acetylation is reversible and catalyzed by histone-deacetylases (HDACs) that remove acetyl from lysine residues. De-acetylation of lysine-residues is mainly correlated with transcriptional repression of gene-expression [80–82]. Nevertheless, the concept of histone-deacetylases acting solely as transcriptional co-

repressors and hypoacetylation of histones being associated with transcriptional repression is an oversimplified view [83]. Thus, several studies reported the contribution of histone-deacetylase activity with transcriptional activation of specific complicating the interpretation of histone acetylation -mediated signal transduction genes [84–88].

1.2 Histone-deacetylases: Classification, localization, mechanisms of action and tissue distribution

In mammals, the two protein families that possess histone-deacetylase activity are the Histone-deacetylases (HDACs) and the Silent information regulator 2 proteins (Sirtuins). They belong to an ancient protein superfamily found in plants, fungi and prokaryotic organisms such as the archaea and eubacteria, indicating HDACs to have additional physiologically important substrates next to histones [89–91].

Members of the mammalian histone-deacetylase families are grouped into Class I-IV HDACs based on subcellular localization, mechanism of action and DNA-sequence homologies to their respective yeast histone-deacetylase orthologous Rpd3, HdaI and Sir2 [91–94] .Class I, II and IV HDACs are referred to as classical HDACs and comprises in total 11 family members (HDAC1-HDAC11) [95]. Sirtuins are grouped into class III HDACs consisting of seven family members (Sirt1-Sirt7) and are structurally unrelated to the classical HDACs [96–98]. Classical HDACs differ from Sirtuins in their mechanism of catalytic action. Classical HDACs require a Zn^{2+}-Ion as an essential cofactor for their catalytic activity, while Sirtuins are NAD^{+}- dependent enzymes. Zinc-chelating compounds such as hydroxamic acids inhibit the enzymatic activity of HDACs [99].

The remaining of this section will focus on Class I HDACs as they were analyzed during the course of this work.

1.2.1 Class I HDACs

The first human HDAC, HDAC1, was isolated and cloned in 1996 by the Schreiber laboratory [100]. Class I HDACs consists of HDAC1, HDAC2, HDAC3 and HDAC8. These HDACs share high DNA-Sequence homology with the global transcriptional repressor Rpd3p in yeast *Saccharomyces Cervisiea* [101]. Members of the class I HDAC lack a DNA-binding motif and require the interaction with different multi-protein complexes targeting chromatin to help HDACs to exert their catalytic function. HDAC1, HDAC2 and HDAC3 associate with different so-called transcriptional co-repressor complexes such as Sin3, Mi-2/NuRD, REST/ CoREST and NCoR/SMRT [102–108]. The exact composition of these HDAC complexes depends on the cellular context and may differ at specific developmental stages [106,109]. To date, HDAC8 was not found to be part of any co-repressor complex and is the only class I HDAC that can perform its enzymatic function in isolation[90,102]. Class I HDACs are predominantly localized in the nucleus where their main substrates, the histones, are found. Nuclear localization is ensured via a nuclear localization signal (NLS) or through co-localization with proteins of the transcriptional repressor complexes. Under physiological conditions, HDAC1 and HDAC2 are particularly nuclear proteins due to the lack of a nuclear export signal (NES) [95]. HDAC3 carries a NLS and a NES and can therefore shuttle between the nucleus and cytoplasm [90,110]. Localization of HDAC8 occurs to be cell type dependent and can be therefore found in the nucleus as well as in the cytoplasm [111,112]. In mammals, class I HDACs are expressed ubiquitously in all tissue types with different expression levels, based on the Serial Analysis of Gene Expression database (SAGE) and the Human Transcriptome Map [95].

HDAC1 and **HDAC2** share approximately 82% overall amino acid sequence homology and originated from a common ancestor via gene duplication [90]. Their N-terminus comprises a dimerization domain and the conserved catalytic domain with 92% sequence identity. The C-terminal tail contains the nuclear localization domain and two casein kinase-2 (CK2) phosphorylation sites with 72% identity. Both proteins are strongly involved in development, especially in cellular proliferation, cell cycle and apoptosis [113]. Besides high structural similarity, HDAC1 and HDAC2 are usually

co-expressed and posses redundant functions [114]. For instance, conditional deletion of either HDAC1 or HDAC2 in different organ systems such as heart, epidermis, adipose tissue or hematopoietic system did not lead to an overt phenotype [115–118]. Further, targeted depletion of HDAC1 or HDAC2 have shown a compensatory up-regulation of HDAC1 or HDAC2, respectively [119–121]. Importantly, simultaneous knock-out of HDAC1 and HDAC2 has drastic effects on proliferation, differentiation and cell survival [122,123]. Nevertheless, while HDAC1 and HDAC2 share many functions in various biological processes, they also have distinct roles. Global germline deletion of HDAC1 in mice lead to severe proliferation defects and retardation in development, causing embryonic lethality before E9.5 that cannot be compensated by concomitant up-regulation of HDAC2 or HDAC3, suggesting HDAC1 having a special role in embryonic stem cell regulation [114,119,124]. HDAC2-deficient mice are partially viable but die mostly due to cardiac defects in the perinatal period [115,121,125]. The molecular rational of when and why HDAC1 and HDAC2 have overlapping versus specific functions is still unclear but supposed to rely most likely on the cell or tissue type in which different signaling pathways trigger and regulate the activity of HDAC1 or HDAC2 [113]. HDAC1 and HDAC2 enzyme activity are regulated by post-translational modifications (PTMs) such as phosphorylation, acetylation, ubiquitination, SUMOylation, nitrosylation and carbonylation [113,126–133]. Interestingly, the majority of PTMs occurs in the less conserved C-terminal domains on serine-lysine sites and were shown to appear in response to extracellular signals such as hypoxia and hormone stimulation [134,135]. Moreover, the C-terminal part is speculated to be pivotal for fine-tuning and differential regulation of these two highly related proteins [113]. The highly conserved catalytic domain is exposed to PTMs on tyrosine-cysteine sites undergoing mostly nitrosylations and carbonylations in response to stress stimuli such as cigarette smoke and oxidative stress [113,131,132]. Thus, whereas HDAC1 and HDAC2 have similar and even redundant functions, evidence indicates that they have biological relevant and context dependent non-overlapping functions.

HDAC3 exhibit 68% sequence identity with the catalytic domain of HDAC1 and HDAC2 and posses its catalytic domain in the less conserved C-terminal part [136,137]. HDAC3 associates together with members of class II HDACs, HDAC4, 5 and 7 through complex-formation with the co-repressors N-CoR and SMRT [95,138,139].

HDAC3 is thought to be essential for DNA replication, DNA damage control, proper cell cycle progression and apoptosis [114,140–142]. HDAC3-KO mice die before E9.5 due to gastrulation defects during early embryonic development [143,144]. Cardiac specific deletion of HDAC3 is lethal within 3-4 months after birth. Liver-specific loss of HDAC3 causes aberrant lipid and cholesterol biosynthesis shortly after birth [142]. Serine-phosphorylation (Ser24) on HDAC3 by CK2 increases its enzymatic activity and dephosphorylation on the same residue by protein phosphatase 4 complex ($PP4_C$) reduces HDAC3 deacetylase activity [137].

HDAC8 was the last class I HDAC member to be discovered and is most similar to HDAC3 with 34% overall sequence identity. In contrast to the other class I HDACs, HDAC8 carries no CK2 phosphorylation site but a conserved motif for protein kinase A (PKA) phosphorylation. Phosphorylation of HDAC8 by PKA inhibits its enzymatic activity [145]. HDAC8 function has been strongly involved in tumorigenesis, telomerase activity and skull morphogenesis [146]. HDAC8-KO mice are viable but show craniofacial defects due to repression of transcriptional factors in cranial neural crest cells [122].

1.3 HDAC inhibitors (HDACi)

Given the central role of histone acetylation-dependent signaling in cellular function and the deregulation under pathological conditions, the development of drugs that could alter the fine balance of histone acetylation and deacetylation soon became a priority. Hence, so-called HDAC inhibitors (HDACi) were developed, their potential first being discovered as a result of their ability to induce cellular differentiation [147,148]. HDACi are grouped into four major classes, based on their chemical structure: hydroxamates (e.g. TSA and SAHA), benzamides (MS-275), cyclic peptides (e.g. Depsipeptide) and aliphatic acids (e.g. Valproic acid and Phenylbutyrate) [149–151].

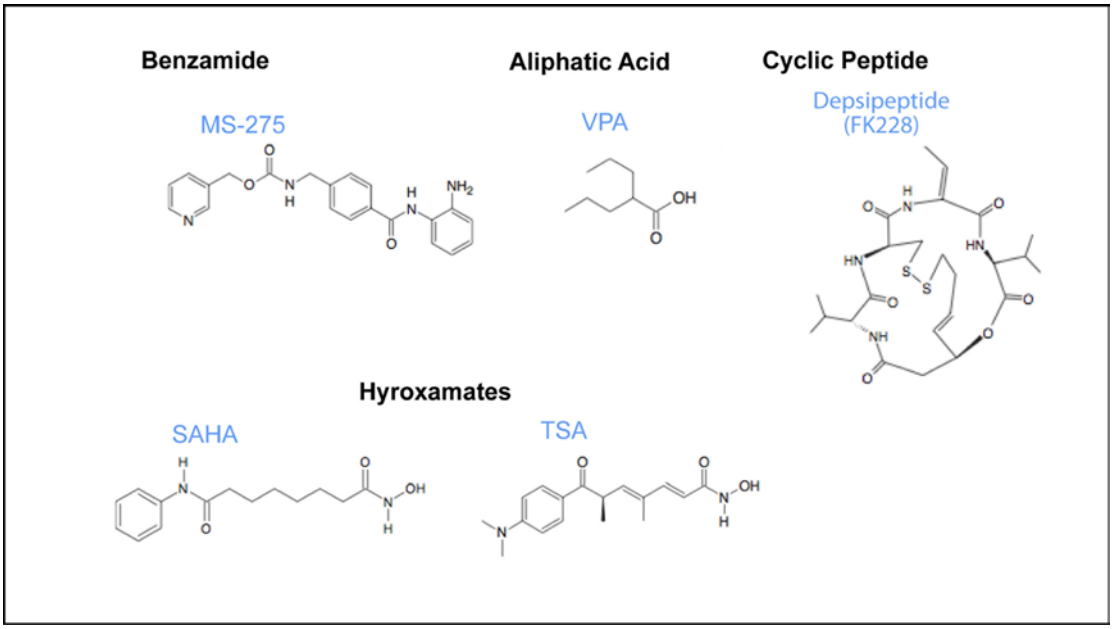

Figure 2 Structure of HDAC inhibitors of the four major classes.

Structures adapted from Grayson et al., 2010

All HDACi contain a zinc-binding group and achieve HDAC inhibition by chelating the zinc ion in the HDAC catalytic pocket structure [152–155]. As previously stated, these Zn^{2+}-chelating HDACi do not inhibit the activity of the NAD^{+}- dependent Sirtuins [95]. HDACi alter gene expression by inhibiting the classical HDACs resulting in increased histone-acetylation that affect the transcriptional regulatory network, via chromatin structure relaxation [156–158]. Aberrant histone-acetylation levels and gene-expression of HDACs themselves have been frequently reported in various cancer types. Thus, HDACs are considered to be promising targets in drug development for cancer therapy and HDACi have been shown to reverse aberrant epigenetic regulation of gene-expression associated with cancers [159–161]. HDACi were shown to induce cell growth arrest, terminal differentiation, apoptosis and inhibition of angiogenesis in cancer cells [162]. Thus, HDAC inhibitors such as Vorinostat are already approved for the treatment of distinct types of cancer [163]. To date, the majority information obtained about HDACi is from clinical trials of cancer therapy where they increase the expression of genes involved in growth arrest and promote apoptosis of cancer cells [157,164].

Table 2. Examples of HDACi in clinical trials

Class	Compound	HDAC Target	Potency in vitro	Phase	Company	Ref.
Hydroxamate	SAHA	Classes I, II	µM	FDA CTCL	Merck	Marcks 2007
Cyclic peptide	FK228	HDAC1, 2	nM	Phase II	Novartis	Bhalla 2005
Benzamide	MS-275	HDAC1, 2, 3	µM	Phase II	Schering AG	Bolden 2006
Aliphatic acid	VPA	Classes I, IIa	mM	Phase II	NCI	Bolden 2006

Partially reproduced from [162] Abbrevations: FDA, Food and Drug Administration; CTCL, cutaneous T-cell lymphoma; HDACi, Histone deacetylase inhibitor; NCI, National Cancer Institute; SAHA, suberoyl anilide hydroxamic acid

However, recent animal studies implicated that HDACi have great therapeutic potential for the treatment of various neurodegenerative and neuropsychiatric disorders and cognition per se.

For instance, administration of the HDACi TSA, SAHA and VPA enhanced cognitive function in aging mice and various mouse models of neurodegenerative disorders [165–168]. Thus, HDACi have been shown to have neuroprotective effects in animal models of Alzheimer´s disease, Huntington´s disease, spinal muscular atrophy, ischemia, Parkinson´s disease and amyotrophic lateral sclerosis [169–175]. In Cognition, the HDAC inhibition induced increase in hippocampal histone-acetylation has been demonstrated to be required for proper memory formation in normal mice [176]. Moreover, SAHA and MS-275 has been shown to induce elevated histone-acetylation and consequently increase gene-expression of neuron-specific genes, promoting neural differentiation [177].

VPA is already employed in psychiatry as an antimanic and mood-stabilizing drug for the treatment of bipolar disorder and acute depression [178–180]. Moreover, in a mouse model for schizophrenia, VPA was shown to potentiate the antipsychotic effects of Clozapine, a drug commonly used in the treatment of schizophrenia [181]. However, VPA has only recently discovered to have HDAC inhibition effects [180,182,183].

The following sections will focus on the hydroxamate derived HDACi SAHA (Suberoylanilide Hydroxamic Acid) and the benzamide-based HDACi MS-275, which were used in this study.

1.3.1 Hydroxamates

Hydroxamate-based HDACi are the most widely analyzed group of HDACi [148]. Trichostatin A (TSA) was the first natural hydroxamate discovered to inhibit HDACs [184]. In 2006, Suberoylanilide Hydroxamic Acid (SAHA; commercial name Vorinostat or Zolinza) was the first HDACi approved by the Food and Drug Administration (FDA) for the treatment of cancer patients with subcutaneous T-cell lymphoma [185]. TSA and SAHA are considered to be class I and II pan-HDAC inhibitors [186]. However, it was currently suggested that SAHA has only a weak inhibitory effect on class IIa HDACs and act rather as a class I HDAC inhibitor with some activity towards HDAC6 [148,172,187]. Nearly all clinical trials conducted with SAHA are directed to cancer therapy (www.clinicaltrials.gov). In addition to its approved action towards T-cell lymphoma, SAHA is currently under investigation in phase I and II clinical trials for other types of cancers such as solid tumors and hematologic malignancy [188–193]. Although SAHA was in general well tolerated in humans in such studies when used as monotherapy, SAHA showed either modest activity or no efficacy and is therefore suggested to be employed as combination therapy for such types of cancers [188,191,193]. The maximal tolerated dose (MTD) for SAHA given orally is 400 mg per day. The most common drug-related adverse reactions are diarrhea, fatigue, hyperglycemia, hepatic impairments, nausea, thrombocytopenia, anorexia and dysgeusia [185,193].

In animal models for neurodegenerative disorders, SAHA have been shown to be neuroprotective and to ameliorate impairments learning and memory [165,168,194–197]. Moreover, SAHA have been shown to have anti-depressant effects in a mouse model for depression and to reduce side-effects of atypical antipsychotic drugs such as clozapine in mice [178,198].

1.3.2 Benzamides

MS-275 (commercial name: Entionstat) is a benzamide derivative that has been used for the treatment of leukemia, lymphomas or solid tumors and is currently investigated under phase I and II clinical trials showing potent anti-proliferative activity [193,199–202]. MS-275 inhibits preferentially class I HDACs with highest

specificity against HDAC1 at nanomolar range with a 100-fold lower EC_{50} (half maximal effective concentration) towards HDAC2/HDAC3 and shows no activity towards the other HDACs [160,203,204]. Applied as single agent, MS-275 had limited effects on acute leukemias [205]. However, in myeloid malignancies, combination therapy with MS-275 and the FDA approved DNMT inhibitor 5´AZA revealed greater clinical response [148,206,207]. Depending on the cancer types treated in clinical trials, the MTD of MS-275 was between 8-10 mg /m^2 with a biological half-life of 30-50 hours. Side effects of MS-275 are mostly gastrointestinal side effects or fatigue [199,200,205]. Many different Phase II clinical trials with MS-275 in cancer therapy are still ongoing.

To date, the effect of MS-275 on cognition, neurodegenerative and neuropsychiatric disorders is poorly investigated. Moreover, MS-275 has been shown to exhibit poor blood-brain-barrier penetration [208]. However, MS-275 was reported to be a potent and brain-region selective inhibitor of HDAC1 and to reverse transcriptional repression of schizophrenia susceptibility genes in mice [209,210]. Further on, MS-275 reset the gene-expression profile observed in an animal model for depression similar to the antidepressant drug fluoxetine [178].

1.4 Schizophrenia: Nomenclature, clinical Symptoms, criteria of Diagnosis and Etiology?

Schizophrenia is a severe brain disorder with high phenotypic complexity that affects approximately 1% of the world population [211]. Patients with Schizophrenia may suffer from various symptoms ranging from features of psychosis (e.g. delusions and hallucinations), alterations in neurocognition (e.g. deficits in attention and working memory) and affective dysregulation (e.g. affective flattening, lack of motivation) [212]. Resulting difficulties in perception and emotional stability hinder diseased patients from proper functioning of every day life tasks, affecting their social interactions. Schizophrenia affects men and woman equally in all ethnic groups around the world [213].

The age of onset of the disease is typically between late adolescence and early adulthood (app. 16 – 25), whereas women develop symptoms several years later. The

later disease onset in women is thought to be due to protective effects of estrogen and hormone replacement therapy with estrogen is discussed as possible treatment for women with schizophrenia [214,215]. Interestingly, the risk to develop schizophrenia over the age of 45 is extremely low [216,217]. Childhood onset schizophrenia (under the age of 18) is observed very rarely, especially in children below 7-8 years old [213]. The sooner onset of schizophrenia has been associated with more sever-impairments [218]. Moreover, schizophrenia is marked by high mortality rate due to suicide of patients [219]. Schizophrenia itself does not present one defined clinical picture .It rather reflects and consists of a variety of appearances that are summarized as the group of Schizophrenias (e.g. paranoid Schizophrenia, catatonic Schizophrenia). Hence, Schizophrenia comprises a heterogeneous group of patients with diverse symptoms and none of the symptoms are unique to Schizophrenia [220–222]. This hampers the process of reliable diagnosis and development of optimized and functional therapeutic treatments of patients [223,224]. While the etiology of Schizophrenia remains elusive, disrupted brain region connectivity, deregulated neurotransmission, non-mendelian genetic heritability and various environment factors such as drug abuse, prenatal infections and early life stress are thought to contribute to the pathogenesis of Schizophrenia [19,225–227] .

1.4.1 Symptom based diagnosis of Schizophrenia

In clinics, Schizophrenia is currently diagnosed based on the 4th Diagnostic and Statistical Manual of Mental Disorders IV (DSM-IV and DSMIV-TR) and the 10th International Classification of Diseases (ICD-10) (American Psychiatric Association, 1994; World Health Organisation, 1992) (see Table 3.) [228,229]. It is important to mention, that the concept of Schizophrenia has changed from the first to the current revised editions of DSM and ICD, reflecting the difficulties in understanding and defining the clinical picture of Schizophrenia (American Psychiatric Association 1959,2000; World Health organisation 1949,1992) [220,221,230,231]. Depending on the predominant symptomology, DSM-IV defined subtypes of Schizophrenia (e.g. paranoid, disorganized, catatonic, undifferentiated and residual Type). ICD-10 defined two additional subtypes, namely Post-schizophrenic depression and Simple schizophrenia.

Table 3. Diagnostic Criteria for Schizophrenia according to DSM-IV and ICD-10

Diagnostic Criteria DSM-IV	Diagnostic Criteria ICD-10
A. Characteristic symptoms Delusions, hallucinations, disorganized speech, catatonic behavior, negative symptoms	**Characteristic symptoms** **At least on of**: Thought echo, thought insertion/withdrawl, passivity, delusional perception, third person auditory hallucination, running commentary, persistent bizarre delusions
B. Social/occupational dysfunction For a significant portion of the time since the onset of the disturbance, one or more major areas of functioning such as work, interpersonal relations, or self-care, are markedly below the level achieved prior to the onset.	**Two or more of:** Persistent hallucinations, thought disorder, catatonic behavior, negative symptoms, significant behavior change
Duration: Continuous signs of the disturbance persist for at least six months. This six-month period must include at least one month of symptoms (or less, if symptoms remitted with treatment).	**Duration:** More than 1 month
	Exclusion criteria: Mood disorder, schizoaffective disorder, Overt brain disease, drug intoxication or withdrawal

Modified from DSM-IV and ICD-10

The above-mentioned table shows, that diagnosis of schizophrenia is to date only based on symptom profiles. One major resultant problematic is that those symptoms are shared by other disorders and do not present discrete entities with natural boundaries that separate them from other syndromes [220,232]. Thus, both classificatory systems are discussed to lack reliability and fail to be validated [222,230,233]. General criticism of both diagnostic systems concerns therefore the shortage of neurobiological markers and thus demands a more etiopathological based diagnostic system [220,234]. However at present, there are no robust available biological marker or psychological tests for diagnosis, classifying and subtyping the Schizophrenia Syndrome [235].

1.4.2 Nomenclature

The current classification systems of Schizophrenia are in general formulated on the basis of historical definitions of Schizophrenia from Emil Kraepelin, Eugen Bleuler and Kurt Schneider [221,236,237]. However, while the term *Schizophrenia* coined originally by Dr. Eugen Beuler in 1908 means “split-mind” (schizein = σχίζειν = "to split" and phrēn = φρήν = "mind"), Schizophrenia does not represent a multiple personality disorder [238]. Consequently, the term Schizophrenia has been problematic. The ambivalence of the term and the common misunderstanding of the disorder in the public, causing a stigma, lead the Japanese Society of Psychiatry and Neurology to replace the Japanese term for Schizophrenia “Seishin Bunretsu Byo” (i.e. mind-split-disease) by “Togo Shitco Sho” (i.e. integration disorder),[239]. Additionally, due to poor diagnostic validity of Schizophrenia, it has been stated that the current definition of Schizophrenia is based on approximate assumptions. Thus, a polydiagnostic approach for classification of Schizophrenia has been proposed [240,241]. The polydiagnostic approach aims to use alternative definitions of Schizophrenia and to apply different sets of criteria for a given diagnostic category to the same group of patients [230,242]. Nowadays a debate exists, whether the term Schizophrenia should be further retained in the DSM-V and ICD-11.

1.4.3 Neuropathology and Etiology

The fact that Schizophrenia is a brain disorder was primarily demonstrated by noninvasive structural neuroimaging techniques (e.g. computerized tomography (CT)) that showed brain abnormalities such as ventricular enlargement in schizophrenia patients [243,244]. Magnetic resonance imaging studies (MRI) revealed reduction in gray matter volume in the temporal lobe and in medial lobe structures (hippocampus, parahippocampal gyrus, amygdala) [245–248]. These structural abnormalities seem to be consistent findings in schizophrenia, as shown by family studies and twin studies, in which the affected twin has larger ventricles and smaller cortical and hippocampal size [249–253]. Further structural abnormalities have been reported as well in the thalamus and cerebellum [254,255]. Young adults and adolescents yet not diseases buts at high risk

for developing schizophrenia based on their family history, display the observed brain abnormalities suggesting that the neuropathology precedes the onset of symptoms [248,256,257]. Interestingly, progression of brain pathology was not observed, excluding a neurodegenerative process. This is supported by the findings that no overall neuronal loss and gliosis could be observed in post-mortem tissue [257–260]. Positron emission tomography (PET) and functional magnetic resonance imaging (fMRI) studies in schizophrenia showed derangements in interaction-patterns among brain regions with abnormal connectivity especially within the neocortical- limbic neuronal network and altered distributed activity [261–265]. Furthermore, cytoarchitectural abnormalities have been investigated in post-mortem tissue of schizophrenia patients, however, whereas most of the findings were not clearly reproduced, smaller neuronal cell bodies, decreased presynaptic and dendritic markers, reduced dendritic spine density, reduction in cortical thickness and cell migration in the hippocampus and dorsolateral prefrontal cortex (DLPFC) have been more consistent [257,266–270].

The above-mentioned neuropathological findings and the observation that symptoms of schizophrenia generally appear during late adolescence lead and supported the prevailing neurodevelopmental hypothesis of schizophrenia [257,260,261,271,272]. In general, the neurodevelopmental hypothesis suggests that disruption in early brain development, caused by environmental and genetic factors, would increase the risk of later developing schizophrenia. Thus, neurodevelopmental abnormalities remain relatively unapparent in early life until they manifest themselves in diagnostically recognizable symptoms later in life, when complete brain maturation, involving synaptic pruning, a process that eliminates superfluous synapses and connections and is finished for example in the prefrontal cortex with approximately 16, is reached [273–276].

According to a large body of epidemiological studies, environmental factors frequently found in schizophrenia causing neurodevelopmental abnormalities are obstetric complications [24,256,277]. Most significant obstetric complications found are complications of pregnancy (e.g. bleeding, pre-clampsia, diabetes, rhesus incompatibility), abnormal fetal growth and development (e.g. low birth weight, congenital malformations), complications of delivery (e.g. asphyxia, hypoxia, uterine atony, Cesarean section) and viral infections during pregnancy [278–282]. While obstetric complications affect early developmental stages, later development is as well susceptible to environmental factors, such as migration, urban environment, substance

abuse, urban environment, severe emotional stress (e.g. early childhood trauma) that contribute to the development of schizophrenia [283–286]. In addition, linkage and association studies have identified chromosomal abnormalities (e.g. chromosome q42, 11q43, 22q11) and candidate genes (e.g. BDNF, COMT, DTNBP1, NRG1, RGS4, DISC1, G72, GAD1) associated with schizophrenia [287–290] . Some of these genes were found to be regulated by hypoxia, NRG1, COMT, RELN, RGS4 and GAD1 [291]. These susceptibility genes have been implicated with specific developmental correlates and show brain region specific expression pattern at different neurodevelopmental stages. However, none of the identified susceptibility genes have been replicated in every study [282,292].

Taken together, it has been suggested that the onset of schizophrenia is triggered when individuals with genetic susceptibility are exposed to specific environmental risk factors, forming the basis of the two-hit hypothesis [226,276,293]. The first hit represent a dysfunctional gene as predisposition that are involved in abnormal brain development and the second hit comprises environmental factors occurring later in life that modulate the function of susceptibility genes and/or developmental processes resulting into vulnerability of schizophrenia [293,294].

For many years, researches suggested that the etiology of schizophrenia involves deregulated neurotransmission, in particular alterations in dopaminergic, glutamatergic, serotnonergic and GABAergic systems. But although abnormalities in these neurotransmittion systems have been observed and reported, there is still no consensus whether the neurochemical findings are causative or reflect rather secondary pathology as a result of compensatory mechanisms and are not going to be reviewed further in this chapter [257,295–297].

1.4.4 Cognitive Dysfunction in Schizophrenia: Core feature and Endophenotype

"My whole mental power has disappeared, I have sunk intellectually below the level of a beast"

(Schizophrenia patient quoted by Dr. Emil Kraepelin [298])

An organism's ability to acquire new knowledge, skills and behaviors is defined as learning. As such, organisms can alter and adapt their behavior to their environmental context. Memory is an organism's ability, to store information and refers to the process of recalling acquired information. Cognition defines a collection of ways in which organisms perceive, reason, understand, diagnose and solve problems, thereby relying on learning and memory processes. Cognition represent to this end an umbrella term for all higher mental processes [299]. Symptoms of Schizophrenia, especially negative symptoms are suggested to be based on cognitive impairments observed in patients [300–302].

Emil Kraepelin named Schizophrenia originally as *"Dementia praecox"*, reflecting cognitive abnormalities in schizophrenia patients, and stated that ***"…patients are distracted, inattentive…they can not keep the thought in mind. "*** [303]. Cognitive dysfunctions such as deficits in attention, global verbal memory, working memory, episodic memory, language function, inhibition and sensory processing, and executive function (e.g. reasoning, problem solving, organizational flexibility) have been reported frequently in schizophrenia patients. Thus, cognitive dysfunction became a core feature and focus of investigation for therapeutic treatments in schizophrenia [304,305]. Importantly, cognitive impairments often predate the illness onset and are also present during periods of remission [306]. Thus, neurocognition functioning in schizophrenia is suggested to be a robust predictor for the long-term functional outcome of the disease [302]. Unfortunately, existing antipsychotics do not ameliorate cognitive deficits in schizophrenia [307,308].

While in classical Mendelian diseases causative genes are in direct connection to the disease phenotype, in the case of schizophrenia, that harbors genetic heterogeneity and vulnerability to environmental factors, a linear relation between genotype and

phenotype cannot be drawn. This is especially hampered due the shared symptomology between neuropsychiatric disorders. Furthermore, symptoms may only represent compensatory behaviors and change during the course of illness [309].

Thus another approach in psychiatry aims to reduce the complex symptomology observed in schizophrenia into different components such as neuroanatomical, biochemical or cognitive units to investigate the genetic basis of the disease and better understanding of its etiology [310]. This approach refers to the endophenotype concept [311,312]. In order to be defined as an endophenotype, following criteria have to be fulfilled:

1. Association with the illness in a population
2. Heritable
3. State-independent and thus visible in an individual whether illness is active or not
4. Cosegregates within families
5. Found at higher rates in unaffected relatives than in the general population.

Cognitive dysfunctions that are based on brain abnormalities are thought to be more stable markers that can be genetically examined.

Among all cognitive dysfunctions observed in schizophrenia, deficits in sensorimotor gating function and impairments in working memory performance are suggested to be the most promising endophenotypes.

1.4.5 Working memory impairments

In general, working memory refers to the concept of a flexible and dynamic memory, that actively maintain, temporarily store and manipulate limited amount of information during the performance of cognitive tasks in order to guide thought processes or sequences of behavior [235,313,314]. We use our working memory in every day life for example when remembering a phone number between the time of hearing and dialing it, solving a math calculation in mind or executing driving directions [315,316]. Thus, working memory can be understood as a temporary workspace such as the “random-access memory” (RAM), the working memory of a computer that

accesses and stores information while it is working with it [315,317]. The strength of working memory capacity is strongly associated with cognitive skills needed in efficient and effective performance in professional live and impairments in working memory are often responsible for learning disabilities [318].

Today´s concept of working memory is based mainly on the multicomponent model of working memory introduced by Baddeley and Hitch in 1974 [319]. It comprises a control system, the so-called central executive, which is assisted by two subsidiary short-term storage components (slave systems), the so-called phonological loop ("verbal-auditory memory") that is based on sound and language and the visuospatial sketchpad ("visual-spatial working memory"). The central executive has a limited attentional capacity, is flexible and manipulates the information stored in the slave systems and coordinates between them. It is responsible for directing attention to relevant information and suppressing irrelevant information. Baddeley extended the system with a third slave system, the episodic buffer [320]. The episodic buffer serves as an interface between the phonological loop and visuospatial sketchpad in order to integrate visual, spatial and verbal information in a time-dependent manner and is regarded as a crucial feature of working memory capacity [320]. It is known that working memory capacity increases gradually during childhood and declines gradually during aging [321–323].

Working memory deficits have been highlighted in schizophrenia patients. For instance, several studies reported impaired performance of schizophrenia patients in a variety of working memory tasks [324,325]. In addition, working memory deficits have been reported in un-affected relatives of schizophrenia patients and are thought to be heritable [326,327]. Impaired working memory has been shown to be associated with the negative symptoms and contribute to many cognitive deficits observed in Schizophrenia [328,329]. It was suggested that, schizophrenia patients have difficulties with processes attributed to the central executive component of working memory [330]. Thus, profound working memory deficits reflects in impaired behavioral flexibility, strategy shifting and response to environmental feedback. The most widely used tasks analyzing working memory function in humans showing behavioral flexibility, and strategy shifting are the Wisconsin Card Sorting Test, the Category Test and the Tower of Hanoi Task [331–333].

The dorsolateral prefrontal cortex circuitry (DLPFC) mediates working memory processes and shows altered activation in schizophrenia patients during performance of working memory tasks [334,335]. As such, dysfunction specifically in the DLPFC has been shown to be associated with working memory deficits in schizophrenia patients [336]. Interestingly, prefrontal cortex dysfunction was shown to be specific to schizophrenia patients with first episode psychosis, never medicated, and not present in individuals with other psychotic disorders [235,337].

Additionally, the prefrontal cortex plays a key role in decision-making, executive functions, emotional perception, memory encoding and retrieval that have been reported to be to be affected in schizophrenia patients [261,338]. Animal studies have shown as well the importance of the hippocampus for working memory performance [339]. Especially the interplay between the prefrontal cortex and the hippocampus is suggested to be essential for normal working memory function [340–343]. Disconnection lesions in the hippocampal-prefrontal cortex pathway by transient inactivation of the ventral hippocampus and the contralateral prelimbic cortex resulted into impairments in working memory performance [339,344]. Thus numerous studies in individuals with schizophrenia as well as in animal models of schizophrenia have demonstrated altered hippocampal-prefrontal connectivity during the working memory performance [345–347].

However, while the exact neural circuitry of hippocampal-prefrontal cortex interaction underlying working memory is still under investigation, it is hypothesized that the involvement of the hippocampus during working memory performance is its maintenance rather then its encoding [343]. The most widely used tasks analyzing working memory function in humans is the Wisconsin Card Sorting Test (WCST), the Category Test, Delayed Response Task and the Rey Verbal Learning Test [324,332,333,348,349].

1.4.6 Sensorimotor gating function

Sensorimotor gating function is a fundamental component for information processing in the brain. It refers to the ability, to filter out or “gate” un-necessary information from all possible environmental stimuli in order to prevent sensory overload in the brain [350–352]. Thus, impairments in sensorimotor gating function leads to sensory

overload and is thought to result in cognitive fragmentation and disorganization as observed in schizophrenia [353–356]. Impairments in sensorimotor gating function have been frequently reported in individuals with schizophrenia and unaffected relatives [355,357,358]. An operational measure of sensorimotor gating function that is homologous in humans and rodents is the prepulse inhibition of the acoustic startle response (PPI) [355]. PPI refers to a neurobiological paradigm in which a weak stimulus ("Prepulse") presented typically 30 to 500 milliseconds before a stronger startling stimulus ("Pulse") suppresses or inhibits the startle reflex.

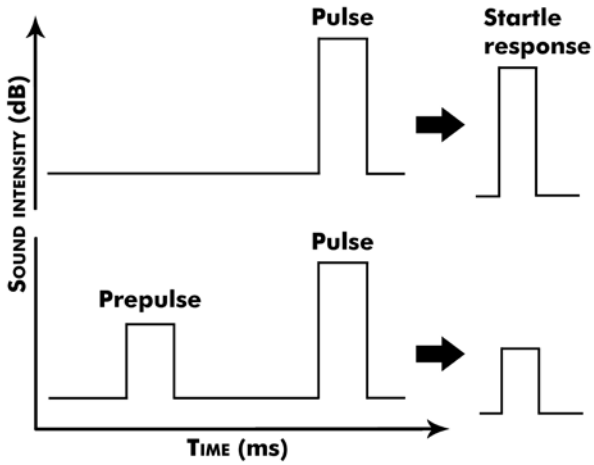

Figure 3 Scheme of Prepulse inhibition of acoustic startle response.

Top: A startling stimulus ("Puls"), typically at 120db sound intensities, elicits a startle reflex. Bottom: A weaker stimulus ("Prepulse") presented shortly before the startling stimulus inhibits startle reflex. Adapted and modified from http://en.wikipedia.org/wiki/File:Prepulse_Inhibition_schematically.png

Disrupted sensorimotor gating in schizophrenia is presented in reduced PPI in comparison to normal controls [355,359,360]. Similarly, reduced PPI is as well observed in various animal models of schizophrenia [361–363]. Neuroimaging studies in humans with schizophrenia and rodents reported that deficits in PPI were associated with abnormalities in the hippocampus, prefrontal cortex, amygdala, nucleus accumbens or ventral tegmental area [364–368]. Thus, the neural circuitry of PPI is thought to rely on limbic structures.

1.5. Neurobiology of learning and memory

Neuronal plasticity is the ability of the brain to undergo reversible morphological and functional changes in response to activity, in order to adapt to the environment, and thus to mediate proper cognition processes [369,370]. These activity-dependent morphological changes of the nervous system represent structural plasticity of synapses, changing the strength and number of synaptic connections resulting in altered synaptic transmission [371–373]. This phenomenon is referred as synaptic plasticity and its key implication is to alter synaptic connectivity within a neuronal circuit to form memory. Thus, synaptic plasticity reflects the cellular correlate of memory formation. The concept that memory results from activity-dependent changes in synapses was established by Donald O. Hebb. Hebb postulated that if two neurons in close proximity are repeatedly activated "… some growth process or metabolic change takes place in one or both cells…" so that at a later time point the activation of one neuron leads to the activity of another neuron [374]. His theory is generalized with "Cells that fire together, wire together" [375] and referred as the Hebbian plasticity.

Memory represents a multicomponent process and is divided into short-term memory and long-term memory based on the duration in which information is stored and accessed to recall. Short-term memory can last from seconds to hours whereas long-term memory can persist from hours to days, even to long-lasting memory over lifetime [376–378].

In order for new acquired memories to become persistent, long-term, memory needs to be 'fixed' or become resistant to disruption [379,380]. Thus, consolidation describes the process in which short-term memory is stabilized into long-term memory after initial acquisition [376,377,381]. The retention of memory is brain region specific as lesion studies in rodents and humans demonstrated that new memories are encoded in the hippocampus and transferred to distal cortical regions for permanent [379,382–384]. Hippocampal lesions in patients caused severe short-term memory deficits and inhibited the transfer of the new acquired memory to the neocortex, avoiding the formation of hippocampus dependent long-term memories [383,385,386]. On the molecular level, separation of memory into short-term and long-term memory originates from studies investigating the role of transcription and translation processes. Hence, animal studies showed that injection of protein synthesis inhibitors (e.g. anisomycin,

puromycin) in the hippocampus immediately after training in behavioral learning tasks disrupted the formation of long-term memories but did not interfere with the acquisition or retention of short-term memory [387–390]. Next, application of RNA synthesis inhibitors (e.g actinomycin D, dichloro-1-β-$_D$- ribofuranosylbenzimidazole) impaired memory formation when injected around the time or 3-6 hours after training, but has no effect when injected after 24 hours [389,391]. Thus, while the short-term phase of the memory is transcriptional and translational independent, the long-term phase of memory is sensitive to inhibition of transcription and translation processes. As synaptic plasticity is underlying the formation of new memories, neurobiologists established two cellular artificial models for long-term memory, namely long-term potentiation (LTP) and long-term depression (LTD) [371,372]. As memory is divided into two phases with short-term and long-term memory, LTP consists as well of two phases based on its persistence: the early phase LTP (E-LTP; ranging from seconds to hours) and the late phase LTP (L-LTP; ranging from hours to days and months) [392,393]. On the cellular and molecular level, E-LTP and L-LTP represent short-term and long-term memory, respectively. It was shown, that E-LTP and short-term memory depend on post-translational modification of post-synaptic proteins, whereas L-LTP and long-term memory being mediated by *de novo* transcription of specific genes and translation of respective proteins [394–397]. However, in general, short-term and long-term synaptic plasticity share the same initial signaling cascades, but while the signal required to cause short-term memory is bordered at the synapse vicinity, for long-term memory to occur, the signal needs to be transferred from the synapse to the nucleus in order to induce transcription and translation processes [398].

In summary, it is well established that the formation of long-term memory, unlike short-term memory, requires gene-expression and *de novo* protein synthesis [388,390,399,400].

In this aspect, the importance of epigenetic mechanisms was brought onto focus regarding the control of gene-expression and accumulative data demonstrated that epigenetic changes are involved in the successful transcription of genes required for long-term memory, mediating cognitive processes. In particular, histone-acetylation has been shown to be essential for memory formation and the application of histone-deacetylase inhibitors (HDACi) enhanced long-term memory processes. Moreover,

deregulation of histone-acetylation is implicated in various neurodegenerative and neuropsychiatric disorders. The involvement of epigenetic mechanisms in memory formation is going to be described in following chapter.

1.5.1 Epigenetics in learning and memory

Several studies indicated the contribution of deregulated epigenetic gene-expression to the etiopathogenesis of neurodegenerative disorders such as Rubinstein-Taybi Syndrome, Rett Syndrome, Friedrich´s ataxia, Chorea Huntington and Alzheimer´s disease. The common feature of all these neurodegenerative disorders is cognitive decline with severe memory loss [29,59,401–403].

Epigenetic mechanisms regulate gene-expression during mammalian development forming cellular memories [31,38]. As it is known that the formation of long-term memories requires gene-expression, the idea that epigenetic mechanisms are involved in learning and memory is not surprising [404]. Histone-acetylation is among all histone-tail modification sites the best-studied modification to be involved in learning and memory. Various behavioral learning tests in rodents demonstrated the importance of epigenetic mechanisms in memory formation [165,168,175,176,405]. For instance, associative learning that is assessed in rodents using contextual fear conditioning, is specifically accompanied with an increase of acetylation at multiple Histone H3 and Histone H4 lysine sites in the hippocampus of mice and remain unchanged in the absence of learning [168,176]. Moreover, interference with the NMDA-receptor-dependent synaptic transmission and the ERK/MAPK signaling, that is required for the formation of long-term memory, inhibits the induction of memory-associated increase in histone-acetylation [176]. Administration of global HDACi such as TSA or sodium butyrate was shown to up-regulate histone-acetylation and to increase LTP and enhance long-term memory. Age-associated memory decline in elderly mice were found to be caused by specific deregulation of H4K12 acetylation, failing to up-regulate its acetylation during fear conditioning in order induce gene-expression necessary for memory consolidation. Treatment of aged-mice with the HDACi SAHA reinstated H4K12 acetylation and learning-induced gene-expression, resulting into improved associative memory [168]. In rodents, environmental enrichment that refers to special housing

conditions providing additional sensory, feeding and social stimuli, have been shown to facilitate learning and memory [406–408]. Fischer and colleagues demonstrated that environmental enrichment in a mouse model for neurodegenerative diseases reinstated and reestablished the access to long-term memory that was correlated with increased histone-acetylation in the hippocampus [166]. Furthermore, administration of HDACi restored the capacity to form long-term memories and induced increased histone-acetylation in these mice, mimicking the effect of environment enrichment [166]. Several studies have displayed the potential of HDACi enhancing memory formation by increasing histone-acetylation [166,168,176,409–414]. Thus, consolidation of long-term memory correlates with increased histone-acetylation. The importance of learning-associated increase in histone-acetylation was as well demonstrated in animal models with disrupted histone-acetylase (HAT) activity. For example, knockdown or mutations in the CREB binding protein (CBP), a transcriptional co-activator that possesses endogenous HAT activity, lead to long-term memory impairments in mice tested in different behavioral experiments such as cued fear conditioning, novel object recognition and Morris water maze [415–417]. Notably, these memory deficits correlated with severe impairments in hippocampal histone-acetylation [165,194,412]. Other HATs, including P300 and p300/CBP-associated factor have been suggested to play an important role in memory consolidation [418,419]. Attempts have been made to identify which HDACs are specifically important for the formation of long-term memories. It was discovered that mice overexpressing HDAC2 exhibited impaired learning and memory in the fear conditioning and morris water maze test, whereas knockout of HDAC2 improved memory formation [196]). Likewise, hippocampus specific deletion of HDAC3 resulted into facilitated long-term memory formation in mice during the object location test [420]. Recently published data demonstrated that endogenous knockdown of HDAC6 restored memory deficits observed in a mouse model for Alzheimer´s disease [421]. Additionally, inhibition of HDAC6 mediated neuroprotection- and regeneration [422]. Thus it has been suggested that targeting HDAC2, HDAC3 and HDAC6 might be suitable drug targets to treat cognitive decline [196,405,421–423].

Taken together, these findings highlight the importance of epigenetic mechanisms such as histone-acetylation in learning and memory and the potential of epigenetic therapy with HDACi for the treatment of neurodegenerative disorders.

1.6 Epigenetics of Neuropsychiatric disorders

Neuropsychiatric disorders such as schizophrenia are characterized by multifactorial mode of inheritance and influenced by environmental risk factors such as early life stress. Nevertheless, current genetic approaches failed to identify the diseases underlying mechanism and environmental factors independently are not sufficient to cause psychosis [30,424,425]. To date, the etiology of major psychiatric disorders is hypothesized to rely on the interplay between genetic and environmental risk factors [28,29,426,427]. Epigenetic mechanisms mediate GxE interactions and deregulated epigenetic gene-expression have been frequently reported in post-mortem tissue samples of patients and in animal models of major psychiatric disorders such as fear-related anxiety disorders, schizophrenia, depression and drug-addiction [176,181,183,428–438].

In rodents, the most representative study demonstrating how epigenetic mechanisms mediate GxE interactions underlying psychosis stems from Michael Meaney and colleagues. Thus it was demonstrated in rats, that low maternal care measured by licking and grooming of the offspring, caused exaggerated anxiety later in life [429]. The poor maternal care of pubs induced greater activity of the hypothalamic-piutiary-adrenal (HPA) axis and decreased expression of hippocampal glucocorticoid receptors (GRs). The activation of HPA axis in response to stress and the function of GRs as central mediators of stress is a validated paradigm in rodents and humans [439,440]. As such, individuals with depression that were subjected to abuse during childhood displayed increased methylation, down-regulation of hippocampal GRs and long-term vulnerability to stress [30,432]. Likewise, decreased hippocampal GRs expression in rats with less maternal care was mediated by increased DNA methylation and decreased H3 acetylation at the GR1 promoter. Strikingly, treatment with the global HDACi TSA reversed the anxiety phenotype [429].

Additionally, application of HDACi showed to facilitate fear extinction, paradigm used in rodents to treat anxiety diseases similar to exposure therapy [410,414,441].

1.6.1 DNA Methylation in Schizophrenia

To date, the majority of studies focused on the role of DNA methylation in schizophrenia. The hypothesis that deregulated DNA methylation is involved in the disease´ etiology exists since clinical studies from Osmond and Smythies in 1952 [442]. Chronic treatment of schizophrenia patients with L-methionine, a precursor of S-adenosylmethionine (SAM) biosynthesis that serves as a methyl donor for DNMT´s, resulted into worsening of symptoms [443,444]. Almost 40 years later, the methionine-induced exacerbated symptoms in schizophrenia patients were reproduced in mice [183,445–447]. Thus, methionine treated mice exhibited endophenotypes of schizophrenia such as deficits in prepulse inhibition and impaired attention [183,446,447]. It was observed, that increased levels of SAM by L-methionine treatment caused an increase in DNA methylation and down-regulation of schizophrenia GABAergic susceptibility genes such as *Reelin* and *GAD67* [210,446]. Interestingly, administration of the global HDACi Valproate (VPA) diminished methionine-induced schizophrenia like symptoms in mice and increased *GAD67* gene expression [448]. Moreover, treatment of mice with VPA and the atypical antipsychotic drug Clozapine decreased promoter methylation of GABAergic candidate genes accompanied with improved symptoms [181]. Importantly, increased SAM levels and promoter hypermethylation of GABAergic genes have been reported in the prefrontal cortex of patients with schizophrenia [449–452]. Subsequently, hypermethylation was associated with reduced GABAergic cortical gene-expression [449,450,453]. Consistently, high levels of DNMT1 and DNMT3a mRNA expression have been found in schizophrenia post-mortem cortical GABAergic neurons [454–460]. Moreover, monozygotic twins discordant of schizophrenia display significant difference in methylation pattern [461]. Fascinatingly, it has been shown that the schizophrenia-affected twin shared higher epigenetic marking with a non-related individual with schizophrenia than to his own un-affected sibling [30,461].

Taken together, preclinical animal studies and post-mortem tissue analysis suggested that deregulated DNA methylation leading to aberrant cortical gene-expression might underlie the etiopathogenesis of schizophrenia.

1.6.2 Histone-modifications in Schizophrenia

Deregulation of histone-tail modifications resulting into aberrant gene-expression has been associated with the etiopathogenesis in schizophrenia. For instance, tri-methylation of histone 3 lysine 4 (H3K4me3) that serves as a marker for active transcription was found to be reduced at the promoter of cortical GABAergic genes in schizophrenia patients mediating transcriptional repression. Consistently, the transcriptional repressor mark, H3K27me3, was increased at the promoter of repressed GABAergic genes [462]. Furthermore, higher levels of the repressive chromatin mark H3K9me2 in lymphocytes of living patients have been correlated with earlier disease onset [463,464].

Additionally, transcriptional repression of schizophrenia candidate genes has been associated with reduced acetylation of H3K14 [465]. Further post-mortem studies identified aberrant gene-expression of HDACs in schizophrenia patients. Thus, HDAC1, 3 and 4 mRNA levels have been reported to be over- expressed in schizophrenia patients, correlating with decreased expression of GABAergic genes [433,435]. Interestingly, inhibition of HDAC1 enzyme activity by MS-275 induced transcriptional activation of the schizophrenia susceptibility genes *Reelin* in *GAD67* in mice. This gene-expression activating effect of MS-275 on *Reelin* and *GAD67* was demonstrated to be more efficient than with VPA [209,446]. In striatal neurons, the antipsychotic drugs haloperidol and raclopride that act as dopamine D2 receptor antagonists induce Histone H3 phosyphoacetylation [466]. Interestingly, the antipsychotic effect of another drug were potentiated if co-administered with VPA [181]. Additionally, clozapine induced increased Histone H3 acetylation in the frontal cortex of mice at the promoter of GABAergic genes [181].

Thus, the present data indicate the importance of deregulated histone-modification in the etiopathogenesis of schizophrenia and that HDACi might have beneficial effect in the treatment of patients.

However, the function of histone-acetylation and HDACs in the etiopathogenesis of schizophrenia remains elusive.

2. Objectives

The etiology of neuropsychiatric disorders such as schizophrenia is thought to rely on genome and environmental (GxE) interactions. Epigenetic mechanisms such as histone-acetylation regulate gene-expression in response to environmental stimuli and are considered as key processes that mediate GxE interactions. The role of histone-acetylation and histone-deacetylases (HDACs) in the etiopathogenesis of schizophrenia are to date only poorly investigated. Recent human post-mortem studies have reported elevated HDAC1 expression in the hippocampus and prefrontal cortex of individuals with schizophrenia [433,435]. Moreover, HDAC1 mRNA expression was upregulated in neurons exposed to hypoxia, an identified environmental risk factor for schizophrenia, associated with obstetric complications [467]. Additionally, HDAC1 has been shown to regulate the expression of the schizophrenia susceptibility genes *Gad67* and *Reelin*, mediating transcriptional repression and inhibition of HDAC1 by MS-275 reversed HDAC1 induced down-regulation of these genes [210].

Thus, the aim of my work was to use mice as model organism in order to elucidate the role of HDAC1 in cognitive function and to the etiopathogenesis of schizophrenia.

Mores specifically I asked the following questions:

1. Does HDAC1 contribute to the etiopathogenesis of schizophrenia?

In order to address this question, I designed a gain-of-function model using a viral-system to overexpress neuronal HDAC1 specifically in the dorsal hippocampus and medial prefrontal cortex of mice. This analysis was combined with loss-of-function models using siRNA-mediated knockdown of HDAC1 or pharmacological inhibition. The corresponding animals were subjected to molecular and behavioral analysis.

2. Do environmental risk factors for schizophrenia influence the expression of HDAC1?

The present data suggested that up-regulation of HDAC1 in schizophrenia patients is unlikely due to genetic effects. Thus, I planed to investigate the expression of HDAC1 and the associated molecular and behavioral underpinnings in response to environmental risk factors for schizophrenia. Here I to subjected mice to early life stress by maternal separation and social isolation, as they are well-documented environmental risk factors of schizophrenia.

3. Materials and Methods

3.1 Technical Equipment

Benchtop centrifuge	Eppendorf
Benchmark Sterotaxic Intrument	Myneurolab
Bilateral guide cannulae	Bilaney Consultants
Bioanalyzer 2100	Agilent
Biorupter Standard Sonication Device	Diagenode
Computerized fear conditioning system	TSE
Confocal microscope	Leica
Cryotome	Leica
Digital Stereotaxic Control Panel	Myneurolab
End-over-end shaker	Stuart
Eppendorf Pipettes	Eppendorf
Glass Capillaries	World Precision Instruments
Hamilton Syringes	Hamilton Company
LightCycler 480 Real-time PCR system	Roche Applied Science
Magnetic stirrer	Heidolph
Mastercycler ep gradient	Eppendorf
Microsyringe pump	World Precision Instruments
Microcentrifuge	Eppendorf
Mini Trans-Blot Cell	BIO-RAD
Nanoliter 2000 Injector	World Precision Instruments
NanoDrop 1000	Thermo Scientfic
Odyssey infrared imaging system	LI-COR Biosciences
Peristaltic perfusion pump	Heidolph
Programmable Multipipetter Puller	World Precision Instruments
Power supply	Sigma-Aldrich
Spectra-Por MWC 1000 membranes	Spectrumlabs
Startle Response System	TSE
VideoMot2 tracking system	TSE
Vortex mixer	Eppendorf
Water bath	Lauda
Weighing machine	Sartorius

3.2 Reagents

Acetic acid	Roth
Acrylamide	Roth
Agarose	Roth
Ammonium persulfate	Roth
Ampicillin	Applichem
Ampuwa	Fresenius AG
BSA	Roth
Bromophenol blue	Roth
Chloroform	Applichem
DAPI	Vectarshield
DEPC	Sigma
DNA Marker	Fermentas
DTT	Roth
EDTA	VWR
Ethanol	Roth
Ethidium bromide	Roth
Formaldehyde	Applichem
Glycerol	Roth
Glycine	Applichem
Guanidine hydrochloride	Roth
Isoamylalcohol	Applichem
β-Mercaptoethanol	Roth
Methanol	Roth
Milk powder	Roth
NaOH	Roth
NaCl	Applichem
Proteinase K	Roth
Paraformaldehyde	Roth
PBS	Roth
Penicillin/Streptomycin	Roth
Phenol	Applichem
Sucrose	Applichem
TEMED	Roth
Tris	Applichem
Triton-X-100	Roth
TRI-reagent	Sigma
Tween-20	Roth
Yeast extract	Applichem

3.3 Kits

Transscriptor First Strand cDNA Synthesis Kit	Roche
One Day ChIP Kit	Diagenode
Shearing Optimization Kit	Diagenode

QIAquick Gel Extraction Kit	Quiagen
QIAquick PCR Purification Kit	Quiagen
ProteoExtract Subcellular Fractionation Kit	Calbiochem

3.4 Buffers and Solutions

Gel-electrophoresis buffers

APS solution (10%): Ammonium persulfate in H2O

Laemmli Buffer: 312.50 mM Tris-HCl pH 6.8 150 mM DTT

Running buffer (5x) for 5 L: 75.5 g Tris/HCl (pH 8.3), 360.0 ml Glycine, 25.0 g SDS pH 8.3

Sample buffer (2x): 0.5 M Tris/HCl (pH 6.8), 20% Glycerol, 10% β-Mercaptoethanol, 4% SDS

Separating gel (12%) per ml: 1.6 µl H2O, 1.3 µl of 1.5 M Tris/HCl (pH 8.3), 0.05 µl of 10% SDS and 10% APS, 2.0 µl of 30% acrylamide mix and 0.002 µl of TEMED

Stacking gel (5%) per ml: 0.68 µl H2O, 0.13 µl of 1.0 M Tris/HCl (pH 6.8), 0.01 µl of 10% SDS and 10% APS, 0.17 µl of 30% acrylamide mix and 0.001 µl of TEMED

50 X Tris-Acetate-EDTA (TAE): 242 g Tris base, 57.1 mL Acetic acid, 100 mL 0.5 M EDTA (before use shake vigorously). Add H2O to 1 L and adjust pH to 8.5 using KOH

Transfer buffer: 5.8 g Tris-HCl, 2.92 g Glycine, 20% Methanol, 3.7 ml 10% SDS, ddH2O to 1000 ml

For Immunohistochemistry

Blocking Buffer: 5% goat serum 0.3 % Tx-100 0.01 M PBS

4% Fixation Buffer: 4 g PFA in PBS dissolved with few drops of 10 M NaOH, pH 7.4, PBS to 100 ml

Mounting Medium: 0.1 % gelatine 10 % Ethanol 27

30% Sucrose: 30 g of Sucrose dissolved in 100 ml of PBS, pH 7.4

Washing Buffer: 1 % goat serum 0.1 % Tween 0.01 M PBS

Other Buffers and Solution

PBS buffer: 8 mM Na2HPO4, 140 mM NaCl, 2 mM KH2PO4, 2.7 mM KCl, pH 7.4

TBST (10x): 12.11g Tris, 87.66g NaCl, 5ml Tween 20, 1g NaN3, Water up to 1L

3M Sodium Acetate (80ml): 19.69 g anhydrous sodium acetate, pH 5.2

3. 5 Animal Surgery and Injections

3.5.1 Animals

All animals used in this study were male C57 /B6J mice and group-housed in individually ventilated cages (IVC; 365 x 207 x 140 mm) until behavioral experiments. Mice were housed under a 12 hours light/dark cycle with food and water ad libitum. All animal surgery and behavioral test were performed in accordance to the national German law protection of animals and approved animal experimental protocols Az: 10/0186 and Az: G63.39630.

3.5.2 Transcardial perfusion of mice

Rapid fixation of biological tissue is essential for anatomical, histological and molecular studies. Transcardial perfusion with aldehyde-based fixatives is a common method in order to preserve the morphology and to interrupt biochemical processes that would lead to autolysis of the cells. Fixation of mice was performed by perfusion of 4 % paraformaldehyde (PFA) in PBS through the vascular system, using a peristaltic pump. Mice were anesthetized with an intraperitoneal injection of 0.1 mg /kg Temgesic. After opening the abdominal skin by a longitudinal incision and its removal, the thoracic diaphragm was cut laterally on both sides across the ribs with a sharp scissor. The heart was exposed and a perfusion needle was inserted into left ventricle of the heart in the ascending aorta. Shortly after inserting the needle, a small cut in the right auricle allowed the outflow of return circulation of the heart. Perfusion

was first performed with ice-cold sterile PBS for 3 min in order to wash out blood from the circulatory system. Subsequently, fixation was performed with perfusion of ice-cold 4% PFA in PBS for 6-8 min until optimal perfusion was indicated by the twitching of the tail and blood-less, white appearing liver. After decapitation and opening of the skull, the brain was isolated and post-fixed in 4 % PFA in PBS for 24 hours at 4 °C under gentle agitation. Subsequently, brains were stored for 2-3 days in 30 % sucrose in PBS at 4 °C for dehydration. Finally, brains were frozen over liquid nitrogen and stored at – 80 °C for further processing.

3.5.3 Brain sectioning

PFA-fixed brains were embedded in cryomatrix and placed in a LEICA cryotome for 25- 40 µm thick coronal and sagittal sections and collected as free floating sections in PBS with Stretpomycin/Penicilin and stored at 4 °C.

3.5.4 Stereotaxic implantation of bilateral-guide microcannulae

Three days before stereotaxic surgery, mice received drinking water containing 3ml/L of Sodium metamizol for advanced pain treatment. On the day of stereotaxic surgery, mice were anaesthetized with a mixture of 120 mg/kg ketamine and 8 mg/kg of xylazine. The eyes of the mice were covered with bepanthen to protect eyes against drying-out. After fixing the head of mice in a Benchmark stereotaxic instrument, the head of mice was disinfected with 70 % Ethanol and the skin and connective tissue above the skull carefully removed and cleaned with PBS.. The coordinates for drilling holes were set according to the region desired for implantation. Using an electrical drill with a 0.5 mm driller, two holes were drilled bilaterally on the skull. For intrahippocampal injections in the dentate gyrus of the dorsal hippocampus, bilateral-guide cannulae were inserted at following coordinates: anterior/posterior -1.70 mm relative to bregma; medial/lateral ± 1.00 mm and dorsoventral – 2.00 mm. After insertion, cannulae were mounted and fixed on the skull by dental cement.

Afterwards, mice were place on a warming plate at 37 °C and kept until they woke up and returned to their cages. To minimize pain, 10 mg/kg of rymadil were injected subcutaneously. Sodium metamizol containing drinking was further administered for additional 4 days. Microcannulae implanted mice were subjected to behavioral test one week after recovery from stereotaxic surgery.

3.5.5 Bilateral injections of MS-275, SAHA and siRNA

MS-275was prepared as a 10 mg/ml stock solution in DMSO. For intra- hippocampal injections, MS-275 was diluted to a concentration of 750 ng/µl, and a total amount of 375 ng injected per hemisphere. Controls were injected with DMSO mixed with artificial cerebrospinal fluid (aCSF). Intrahippocampal injections with SAHA were performed as described before [168]. Briefly, a stock solution of SAHA was prepared in DMSO. Before the experiment SAHA (40µg/µl) was dissolved in aCSF and 5 µg/µl bilaterally injected in the dorsal hippocampus in order to have a total amount of 10 µg. Controls were injected with aCSF. For siRNA injections, siSTABLE Control siRNA from Thermo Scientific Dharmacon targeted against HDAC1 was diluted in PBS and prepared with the HiPerfect transfection reagent from Qiagen to a final concentration of 500 µM according to a protocol previously described [468]. siSTABLE Non-targeting siRNA was used as a negative control (scramble).

3.5.6 Stereotaxic injection of AAV using glass capillaries

For stereotaxic injections of AAV particles in the prefrontal cortex and dorsal hippocampi of mice, treatment with painkillers and anesthesia was performed similar to the stereotaxic implantation of microcannulae. After fixation of the mouse in the stereotaxic instrument, the head of mice was cleaned and disinfected with 70 % Ethanol. A longitudinal cut was made on the head from between the eyes and until between the ears of mice, exposing the bregma and lambda of the skull. After removal of the skin and connective tissue on the skull, two holes were drilled according to the desired coordinates. In the case of the prefrontal cortex, the two holes were drilled according to the following coordinates: anterior /posterior + 1.40 mm respective to bregma; medial/lateral ± 0.45 mm. In the case of dorsal hippocampus:

anterior/posterior 1.70 mm relative to bregma; medial/lateral ± 1.00 mm. Glass capillaries filled with mineral oil on the top, a small air bubble in the middle and respective AAV particles in the bottom were placed on a nanoliter 2000 microinjector. The microinjector was connected to an ultra microsyringe pump in order to control the speed and volume of injection. For injections with HDAC1-GFP-AAV or GFP-AAV 1 µl with 1.0* 10^8 transducing units were injected per hemisphere. After injections, the skin of mice was joined together using histoacryl. Mice were kept on warming pads at 37 °C until they woke up and returned to their home-cages. To minimize pain, 10 mg/kg of rymadil were injected subcutaneously and received for additional 4 days sodium metamizol containing drinking water.

3.5.7 Intraperitoneal Injections of MS-275

For intraperitoneal injections with MS-275, a protocol previously described in Engmann et al., was applied [469]. MS-275 stock-solution (100 µg/µl) was diluted 1:80 in DMSO and PBS in order to have a 1.25 µg/µl working-solution. Mice were then injected intraperitoneally with 12.5 mg/kg of MS-275 for 10 days. Control mice received a PBS- DMSO mixture.

3.6 Behavioral experiments

3.6.1 Open field test (OF)

General exploration activity, locomotion and basal anxiety of mice were analysed in the Open field test. Each mouse was placed to the centre of a square open arena (length 1 m; width 1 m; side walls 20 cm height) and behavioural activity was recorded for 5 min using the VideoMot2 tracking system (TSE). The total distance travelled and relative time spent in the centre of the open field was quantified to address explorative activity.

3.6.2 Novel-object-recognition-test (NOR)

Recognition memory of mice was addressed by using the novel-object recognition (NOR) test. It was first proposed by Ennaceur and Delacour [470] and is based on the spontaneous behaviour of rodents to spend more time in exploring a novel object than a familiar object. The test is usually conducted subsequent to the open field (OF) test and contains in general three phases: habituation, training and testing. During the training phase, mice are introduced to explore two identical objects whereas in the testing phase, a novel one replaces one of the two objects. However, depending on the time of delay in which the novel object is presented to asses recognition memory, the testing phase involves two trials of the object recognition task: 1. Working memory with presenting the novel object at the same day of training and 2. Long-term memory with a 24 hours delay before presenting the novel object to the mice. After the OF test, mice were habituated to the open arena for 5 min on two consecutive days. Twenty-four hours after this additional habituation period, mice were exposed for 5 min to the familiar arena with two identical objects placed at an equal distance (18 cm from the sidewalls). Habituation to the two identical objects was carried out for two or three days until anxiety levels of mice in approaching the objects were reduced. Twenty- four hours after, mice were subjected to the object recognition task for the working memory test involving the introduction of two new identical objects in the training phase and the exchange of one of the two identical objects to a new object in the testing phase. To this end, mice were allowed to explore the open arena in the presence of two new identical objects for 5 min in the training phase, followed by a retention period in the home cage for 5 min. Meanwhile, one of the two identical objects was replaced with a new object. During the testing phase, mice were exposed to this situation and working memory assessed by scoring exploration of the new object on the basis of sniffing and direct contact with the object. Twenty- four hours after the working memory test, the new object was replaced again by another new object to test long-term memory. The index for object preference was calculated as per cent of time spent with novel object using following formula: (time spent with novel object/ time spent with both objects)*100%.

6.3.3 Cross-maze exploration test (X-maze)

The Cross-maze (X-maze) exploration test originates from the same principle as the Y-maze and was performed in order to analyse the spatial working memory in mice. The X-maze protocol used here was based on previously published protocol from Jawahr et al [471] with minor modifications. The X-maze was built from light grey plastic material consisting of four arms arranged in 90°C position (arm sizes: 30 cm length, 8 cm width, and 15cm height) rising from a central square measuring 8 x 8 cm. The walls of each arm contained a slot in which paperboard with different geometrical patterns were inserted to facilitate discrimination and recognition of each arm for the mice. During a 10 min. test session, each mouse was placed in the central square of the maze and allowed to move freely through the maze. Arm entries were recorded using the VideoMot2 tracking system (TSE Systems). Percentage of arm-entry alternation was used as readout for memory strength. One successful alternation was defined as sequential entries into the four arms in overlapping quadruple sets (e.g. 2,4,3,1 or 1,3,2,4 but not 1,2,3,1 or 3,4,2,4). The alternation percentage was calculated as the percentage of actual alternations to the possible number of arm entries.

3.6.4 Elevated Plus-Maze (EPM)

The elevated plus-maze was conducted as an additional test to analyze basal anxiety. The maze consisted of a plus-shaped arena with two open and two enclosed arms with an open roof. The plus-shaped arena itself was elevated in a height of 53 cm from the ground. Each arm measured 45 x 10 x 30 cm and a central field was a square with 10 cm. Each mouse was placed in the central square and allowed to explore the maze for 5 min and the activity recorded using the VideoMot2 tracking system (TSE Systems). Percentage of time spent in close and open arms was taken as a measure of basal anxiety level. Increased time spent in the open arms of the plus-maze indicated heightened anxiety levels.

3.6.5 Porsolt forced swim test (FST)

The Porsolt forced swim test was carried out in order to analyze depressive-lile behavior in mice. Mice were placed in a vertical plastic cylinder (20 cm diameter) filled with room temperature warm water. Swimming activity was grouped into periods of immobility and time of floating measure for 5 min with a stop-watch. Floating time in seconds was used as an index to show depressive-like behavior.

3.6.6 Prepulse inhibition of startle response (PPI)

Sensorimotor gating function was measured by the prepulse inhibition of startle response (PPI) on the basis of a previously described protocol from Radyushkin et al., using an apparatus from TSA [472]. Mice were placed individually in a small cylindrical cage with an integrated stainless floor grid (80 x40 x 45 cm) to restrict major movements that was placed on a sensitive transducer platform in a sound-attenuating cabinet. Acoustic stimuli were delivered through loudspeakers above the cage and startle response signals presented by the TSA startle response software. During one test session, mice were habituated first for 3 min to 65 db background noise followed by a 2 min baseline recording. After baseline recording, mice were exposed to six pulse-alone trials with each consisting of 120 db startle stimuli intensity for 40 ms duration in order to decrease the influence of within-session habituation and to scale down the initial startle response to a stable plateau. Startle reaction to acoustic stimuli was recorded with the presentation of the startle stimuli for a time window of 100 milliseconds. PPI of startle activity was conducted by trials presenting startle stimuli of 120 db for 40 ms alone or pre-ceeding non-startling prepulses of 5, 10, 15, 20 and 25 db above the 65 db background noise (70 db, 75 db, 80 db, 85, 90 db). An interval of 100 ms with background nose was introduced between each prepulse and pulse-alone stimulus. Each trial (startle pulse alone, pulse preceded by 70, 75, or 80 dB, or no stimulus) was presented in a pseudorandom order with intertrial intervals ranging from 8 to 22 s. The startle response amplitude was defined as the average of the maximum force (Max G) detected during a reaction to a 120 db startle stimulus. Percentage of prepulse inhibition of startle response was calculated using the

following formula: (%)= 100 - [(startle amplitude after prepulse and pulse)/(startle amplitude after pulse only)° *100].

3.6.7 Morris Water Maze (MWM)

The Morris water maze test was carried out in order to analyze spatial learning and memory of mice. The MWM test was conducted in a circular tank (diameter 1.2 m) filled with opaque water and with a platform hidden below the surface. The swimming path of mice was recorded using the VideoMot2 tracking system.

One training session consisted of four trials in which mice were put into the water maze subsequently from different locations presented by different spatial cues and allowed to swim for maximal 60 seconds. If the mouse did not find the hidden platform within 60 s, it was gently guided to it and placed on it for 10 seconds. The MWM training consisted of approximately 10 sessions. Twenty-four hours after the last training session, mice were subjected to the probe test that represents a memory test in which the platform was removed and each mouse was left in the maze for 1 min. The relative time spent in the quadrant where the platform was previously located and the number of crosses through the region outlining the former location of the platform were used as a read-out for spatial memory strength.

3.6.8 Contextual Fear Conditioning and Extinction (FC-Ext)

Mice were subjected first to contextual FC in order to analyze associative learning and memory. The fear conditioning test consisted of a training session and the memory test. The test was carried out in an automated fear conditioning system of TSE in which a computer was connected to a control unit including a shock and a tone generator, and a white plastic box [58 cm (length) × 30 cm (width) × 27 cm (height)] with a 12 V light at the ceiling, inside which the training chamber was located. Training was performed in a plexiglass chamber [35 cm (length) × 20 cm (width) × 20 cm (height)] placed on a removable metallic grid made of stainless steel

rods (diameter 4 mm; bars spaced 0.9 cm apart), through which an electrical shock could be conducted. During contextual fear conditioning, a neutral conditioning stimulus (CS) presented by the training chamber, is associated with an aversive unconditional stimulus (US) that is presented by an electrical foot shock. Thus during the training session, animals were placed into the training chamber, that represented the context, and were allowed to explore the chamber for 3 minutes, followed by an mild electrical foot shock for 2s with 0.7 mA. Twenty-four hours after the training session, mice were subjected to the memory test and associative memory measured as context-dependent freezing, defined as the absence of movements other than those required for breathing. Associative memory was expressed by calculating the number of measurements when the mouse showed freezing behavior, divided by the total number of measurements. (% Freezing = Freezing counts/18 *100). If 24 hours later followed by repeated exposure to the same context (chamber), the memory test was called as extinction day 1 (E1). Thus, extinction of contextual fear memory was performed on consecutive days consisting of reexposure to the context for 3 min until freezing behavior of mice significantly declined compared to E1.

3.7 Molecular biology and biochemical techniques

3.7.1 Isolation of total RNA and Proteins from brain tissue

Total RNA isolation of brain tissue was conducted using the TRI-reagent from Sigma-Aldrich and adjusted the manufacture´s protocol to our purposes. The TRI-reagent is a mixture of phenol and guanidine-isothiocyanate that enables the researcher to isolate RNA, DNA and protein simultaneously from one sample [473]. Depending on the brain region, volumes of used TRI-reagent varied between 200-500 µl. Frist, frozen or fresh isolated brain tissue was homogenized in TRI- reagent using sterile micropstiles or an electric homogenizer. After complete homogenization, additional TRI-reagent was added and incubated for 5 min at room temperature (RT). Afterwards, 200-400 µl of chloroform was added to the suspension, shortly vortexted (3 sec) and incubated for additional 5 min at RT. The mixture was centrifuged for 5 min at 4°C at 12000 g for 15 min in order to accelerate the full separation of the

organic, aqueous and inter-phase. The aqueous phase, containing the RNA was transferred to a new tube, while the inter- and organic phase containing DNA and proteins respectively were stored at – 80 °C for later processing. Isopropanol was then added to the RNA-containing aqueous phase, mixed vigorously and incubated at -20 °C for 30 min or over night for precipitation of the RNA. After incubation at -20 °C, mixture was centrifuged and at 12000 g for 30 min at 4°C. The supernatant was then carefully discarded by decantation and the RNA pellet washed twice with 75 % Ethanol and dissolved in 20-50 µl of DEPC-H_2O. In order to isolate proteins, 100% Ethanol was added to the inter-phase containing DNA, incubated for 5 min on a rotation-wheel and centrifuged for 5 min at 2000 g at 4°C. The supernatant containing proteins were transferred to a Spectra-Por MWC 1000 membrane for dialysis against 0.1 % SDS in H_2O in a big beaker under agitation over night. The next day, a globular white mass containing proteins were then transferred to a 1.5 ml eppendorf tube and resuspended in 4 M Urea/0.5 % SDS and warmed up at 70 °C under agitation to resolve the protein pellet completely.

3.7.2 Subcellular Fractionation

Subcellular fractionation was performed by using the Proteo Extract® Subcellular Proteome Extraction Kit from Calbiochem according to the manufacture´s instructions in order to isolate cytosolic, membrane and nucleus protein fractions. Brain samples were homogenized in 500 µL of ice-cold Extraction buffer I with 2,5 µL Protease Inhibitor Cocktail and incubated for 10 min at 4 °C with gentle agitation on a rotation-wheel. After centrifugation for 10 min at 4°C at 750 g, supernatant containing the cytosolic fraction (Fraction I) were removed and stored on ice. Pellet was resuspended again with 500 µl ice cold Extraction Buffer II and 2,5 µL Protease Inhibitor Cocktail. After incubation at 4°C for 30 min by gentle agitation, insoluble material were pelleted at 5500 rcf for 10 min. Supernatant containing the membrane fraction (Fraction II) were removed and stored on ice. Nuclear pellet were resuspended with 250 µL Extraction Buffer III and 2,5 µL Benzonase® Nuclease by gentle flicking of the tube and incubated for 10 min at 4 °C by gentle agitation. After centrifugation at 4 °C for 10 min at 7500 g, the supernatant containing the nuclear

fraction (Fraction III) were removed and stored on ice. Subcellular fractions were analyzed via immunoblot.

3.7.3 Determination of Nucleic Acid and Protein Concentrations

Concentrations and quality of DNA, RNA was determined using a NanoDrop 1000 spectrophotometer by measuring the absorption of DNA and RNA samples at A260 nm and proteins at A280 nm according the manufacture´s instructions. The sample purity was determined by the 260/280 ratio.

3.7.4 *In silico* design and synthesis of Oligonucleotides

In order to design oligonucleotides for polymerase chain reaction (PCR), gene-region specific sequences were selected using the Gene database of the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/) and oligonucleotides sequences *in silico* designed using the Primer3 online tool (http://frodo.wi.mit.edu/). Oligonucleotides were then ordered from Sigma-Aldrich. Lyophilised oligonucelotides were then reconstituted in sterile H_2O to a stock concentration of 100 µM and further diluted to a concentration of 10 µM for PCR reactions.

3.7.5 Semi-quantitative Polymerase Chain Reaction (PCR)

The amplification of specific DNA sequences for cloning was carried out by using semi-quantitative PCR. A standard PCR reaction mixture was prepared as follows:

Table 4. Standard PCR-reaction mixture

10 x Dream Taq Buffer (Green)	2.5 µl
dNTP Mix (2.5 mM)	2.0 µl
Forward Primer (10 µM)	1.0 µl
Reverse Primer (10 µM)	1.0 µl
Dream Taq Polymerase	0.2 µl

Template DNA	1.0 µl
PCR-grade H_2O	17.3 µl

PCR reaction was performed in a Masertcycler ep gradient from Eppendorf with following general program:

Table 5. PCR-Program

Step	Temperature	Duration	
Initialization	95 °C	5 min	
Denaturation	95°C	30 sec	⎫ 30x
Annealing	55-70 °C	30 sec	⎭
Elongation	72 °C	1 min	
Final Elongation	72 °C	7 min	
Final Hold	4 °C	∞	

After PCR, 5 µl of amplification product tested by agarose gel electrophoresis and remaining PCR reaction mixture purified using the PCR purification Kit from Quiagen.

3.7.6 DNA agarose gel electrophoresis

Agarose gel electrophoresis was conducted in order to analyze the quality and size of DNA fragments after enzymatic digestion of plasmid DNA or of PCR amplification products. In general during gel electrophoresis, macromolecules such as DNA, RNA and protein are separated in an electrical field according to their size and charge resulting into different migration of macromolecules. Depending of the size of the expected DNA fragments, different quantities of agarose was dissolved in 1x TAE buffer and boiled in the microwave to dissolve agarose. After cooling down of the agarose solution to approximately 50°C, 1 µl of ethidium bromide per 50 ml of solution was added and the mixture pored into a horizontal gel trey containing a comb for polymerization at room temperature. Polymerized gel was placed into an electrophoresis chamber filled with 1x TAE buffer as running buffer and DNA samples mixed with 5 x loading dye loaded into the wells of the gel. After loading a DNA marker, electrophoresis was carried out for 45-60 min at 80- 120 V. After gel

electrophoresis, agarose gel was photographed using a UV-light gel documentation system.

3.7.7 Isolation and purification of DNA fragments from Agarose gel

The isolation and purification of DNA fragments such enzymatically digested and linearized DNA fragments were conducted after DNA agarose gel electrophoresis using the QIAquick gel extraction kit from Quiagen. The DNA band of interest was cut out of an agarose gel and transferred to a 1.5 ml eppendorf tube and weighted. Three volumes of QG buffer was added to the gel slice and incubated at 50 °C until the gel slice was melted (approximately 5-10 min.).
Subsequently, one volume of isopropanol and 1/10 volume of 3 M sodium acetate was added, mixed and transferred to a QIAquick spin column placed in a 2 ml collection tube. After centrifugation of 1 min at 13000 rpm, the outflow was discarded and 0.75 ml of PE buffer added and centrifuged again at 13000 rpm. The column was transferred to a 1.5 ml eppendorf tube and sterile water added in order to elute bound DNA. The quality and concentration of eluted DNA was analysed with the NanoDrop spectrometer.

3.7.8 Purification of PCR Products

Purification of PCR products were conducted using the QIAquick PCR purification kit from Quiagen according to the manufactures instructions. Five volumes of buffer BP was added to one volume of PCR product and mixture transferred to a QIAquick column placed into a collection tube and centrifuged shortly for 30-60 seconds. After discarding the flow-through, QIAquick column was placed into a new collection tube and 20-50 µl H_2O added to the center of the QIAquick without touching the membrane with the tip of the pipette and incubated for 1 minute. Purified PCR product was then subjected to enzymatic digestion for cloning purposes.

3.7.9 Enzymatic restriction of DNA

Plasmid DNA or PCR products were digested using restriction Enzymes for 1 hour to over night at 37°C in order to ensure complete digestion. The required units of restriction enzymes for proper digestion were calculated according following formula: Y= X µg of DNA * 6. For cloning purposes, 2-4 µg of DNA was mixed with 10x restriction buffer and restriction enzyme and brought to a volume of 50 µl sterile H_2O. For control digestion, 500 ng of DNA was mixed with 10x restriction buffer and restriction enzyme and brought to a volume of 30 µl sterile H_2O. The reaction was loaded on a agarose gel for determining the success of restriction and the desired target DNA fragment excised and subjected to purification using the QIAquick gel extraction kit from Quiagen.

3.7.10 DNA Ligation

During a ligation reaction, a T4 DNA Ligase joins two DNA fragments with compatible ends by catalyzing a phosphodiester bond, forming a circular DNA. Ligation reaction mixture was set up in a volume of 20 µl with a 3:1 ratio of Insert DNA and vector DNA respectively, 10 x ligation buffer and 400 units T4 DNA ligase. Ligation reaction mixture were then incubated over night at 16 °C in a water-bath. As a negative control, a ligation reaction mixture without adding the Insert DNA was used.

3.7.11 Transformation of *Escherichia coli (E.coli)* by electroporation

Transformation of *E.coli* was conducted by electroporation of competent SURE or DH5α cells. Electrocompetent DH5α or SURE cells were thawed on ice and 1 -10 µl of DNA ligation mixture was added. After incubation of 5 min, cell-mixture was transferred to a pre-cooled electroporation cuvette. Electroporation was carried out using the electroporator 2510 device (1800V). Immediately after electroporation, 500

µl of warm LB medium without antibiotics were added to the cells and 50 µl, 100 µl, and 200 µl of the suspension were plated on an agar plate containing the appropriate antibiotic. Next, agar plates were incubated for 10-15 hours at 37°C.

7.4.12 Isolation of plasmid DNA from *E.coli*

Isolation of plasmid DNA was performed by an alkaline lysis method [474] using P1,P2 and P3 buffers from Quiagen. Single bacteria colonies were inoculated in 5 ml LB medium containing the appropriate antibiotic and incubated over night at 37°C. Next, *E.coli* cells were centrifuged at 7000 x g for 7 minutes at RT in order to harvest the cells. The bacterial pellet was resuspended in 300 µl buffer P1 and transferred to a new 1.5 ml Eppendorf tube (Eppendorf AG, Germany). After adding 300 µl of buffer P2 to the mixture and incubation for 5 min at RT, cells were mixed with additional 300 µl of P3 buffer and centrifuged for 15 min at 16000 rpm at 4°C. The supernatant were transferred to a new tube and incubated for 5 min at 50°C in order to remove the RNA. DNA was then additionally purified by phenol/chloroform extraction by adding phenol:chloroform:isoamylalcohol (25: 24:1) in a 1:1 ratio. Mixture were transferred to a PhaseLock Gel Heavy tube (Eppendorf, Germany) and centrifuged for phase separation at 16000 rpm for 1 min at RT. The upper phase was transferred in a new 1.5 ml tube and mixed with an equal volume of chloroform. After 1 min centrifugation, the upper phase was collected and DNA precipitated with 0.7 volume isopropanol. After centrifugation for 15 min at 16000 rpm at 4°C, the DNA pellet was resuspended in 30 µl of PCR-grade H_2O.

3.7.13 Adeno-associated-Virus (AAV) production

The AAV infectious particles used in this study were produced by a Viral Vector platform in Dr. Sebastian Kügler´s laboratory. Recombinant AAV vectors were prepared according to standard protocols. Serotype 2 and 6 vectors were propagated in HEK293 cells, purified by iodixanol step gradient ultracentrifugation and heparin affinity FPLC, followed by extensive dialysis against PBS. Genome copies were

determined by quantitative real time PCR and purity > 99% by SDS gel electrophoresis and silver staining. Virus production was conducted by Dr. Sebastian Kügler at the University Medicine Göttingen, Department of Neurology..

3.7.14 Reverse Transcription PCR (RT-PCR)

Complementary DNA (cDNA) was synthesized from messenger RNA (mRNA) using the Transscriptor First Strand cDNA Synthesis Kit from Roche according to the manufacture´s protocol. A reverse transcription reaction mixture consisted of 1 µg of RNA, random hexamer primers (600 pmol/µl) and PCR-grade H_2O in a total volume of 13 µl. For denaturation of RNA secondary structure, the mixture was incubated at 65 °C for 10 min in a Mastercycler ep gradient. Afterwards, 20 U/µl reverse transcriptase, deoxynucelotide mix (10 mM each), 5 x transcription buffer and 40 U/µl of protector RNase transcriptase was added to the mixture. The final mixture was incubated for 10 min at 25 °C followed by incubation at 55 °C for 30 minutes. The reverse transcriptase was inactivated by incubating the mixture for 5 min at 85 °C. The generated cDNA was stored at -20 °C or further processed for quantitative real time PCR.

3.7.15 Quantitative real time PCR (qPCR)

Quantitative real time PCR (qPCR) was conducted in order to investigate differential gene-expression or analysis of chromatin immunoprecipitated (ChIP) DNA fragments using a Roche 480 light cycler. For gene-expression analysis, gene specific primers and respective universal library probes were mixed with cDNA and a light cycler pcr mix. Data was normalized to the housekeeping gene hypoxanthine-gianine phosphoribosyltransferase (*Hprt*). For ChIP analysis, gene-region specific primers were mixed with ChIPed DNA and SYBR Green Master Mix from Roche. For ChIP data analysis, data was normalized to the corresponding input.

3.7.16 Chromatin Immunoprecipitation (ChIP)

In order to analyze protein-DNA interactions, we applied Chromatin Immunoprecipitation (ChIP), a technique that allows localization of proteins such as transcription factors or histone-tail modifications to a specific genomic region. Two methodologies exist for carrying out ChIP. While XChIP protocols involve fixation of chromatin in which protein-DNA complexes are cross-linked using formaldehyde and fragmentation by sonication, NChIP relies on native chromatin prepared by enzymatic digestion using micrococcal nuclease. In general, XChIP is more suitable when studying DNA binding of non-histone proteins or not direct DNA interacting proteins. However, XChIP includes the risk of creating artificial protein-DNA interactions [475]. This risk is higher when working with human post mortem tissue, which is in generally not fresh [476]. In my experimental settings, I worked with freshly isolated hippocampal tissue that were directly subjected to XChIP-experiments, using a standardized protocol from Diagenode (Diagenode, Belgium).

For preparing sheared chromatin followed by immunoprecipitation, the DNA-shearing and One-Day ChIP Kit from Diagenode (Diagenode, Belgium) was used according to the manufactures protocol with minor modifications. After homogenization of tissue using polypropylene pestles (Bioquote, UK) in PBS containing a mixture of protease inhibitors, cells were fixed using formaldehyde with a final concentration of 1% for 10 min on a rotation wheel. Fixation was stopped using 1.25M Glycine and homogenates were further processed with Buffer A, B and C from the DNA-shearing Kit. For chromatin fragmentation, shearing was conducted in Buffer D and samples were placed in a Bioruptor (Diagenode, Belgium) connected to a water cooler, in order to keep the temperature constantly at 4°C. To obtain chromatin fragment size between 200-500 bp, Bioruptor work settings were used as followed: 20 cycles, power setting high, 30 sec ON, 30 sec OFF. For immunoselection, sheared chromatin was incubated with 3-10 µg of antibody for 1h in an ultrasonic cleaner to increase antibody-binding kinetics [477]. Sheared chromatin incubated with non-immune IgG served as negative control. Immunoprecipitation were conducted by incubating the chromatin-antibody complex with pre-blocked protein A agarose-beads for 60 min on a rotating wheel at 4°C. Chromatin-antibody-bead complex was washed with ChIP buffer and after Proteinase K digestion for 1h at

55°C, DNA was isolated using DNA purifying slurry. Precipitated DNA was analyzed in a Bioanalyzer (Agilent, Germany) and amplified by quantitative real-time PCR using SYBR Green and gene-region specific primers.

3.7.17 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was conducted in order to separate proteins electrophoretically according to their molecular weights in the presence of denaturating SDS detergent using the gel electrophoresis system from BIO-RAD. Depending on the protein size later desired to analyze by immunoblotting, 8 %, 10% and 12 % resolving gels with pH 8.8 were polymerized. Stacking gels with pH 6.8 were polymerized over the resolving gel to collect proteins of any given size to move through the gel at the same rate. Protein lysates were mixed with 5x Laemmli buffer and denaturated for 5 min at 95 °C. Then 20-40 µg of protein samples were loaded on the gel. Electrophoresis carried out at 60 V for 30-35 min through the stacking gel and 120 V for 2 h in the resolving gel.

6.4.18 Immunoblotting

After gel electrophoresis, proteins were transferred (blottet) to a nitrocellulose membrane using the transfer system from BIO-RAD filled with transfer-buffer. Transfer of proteins were performed by applying a current at constant voltage of 45 V for maximal 18 hours at 4 °C. After blotting, nitrocellulose membranes were blocked in 3 % milk in 1x PBS for 1 hour at RT. Afterwards, membranes were incubated with primary antibodies for over night at 4 °C and washed subsequently three times for 10-15 min with 1x PBS containing 0.1 % Tween. Protein bands were detected with secondary Alexa800- 30 labeled goat anti-rabbit or anti-mouse antibody using the Odyssey Infrared Imaging System (Licor). Levels of detected proteins were normalized to the total amounts of their corresponding loading controls.

3.7.19 Immunohistochemistry

For immunohistochemistry (IHC), brain sections were washed three times with TBS for 5 min, permeabilized and blocked against non-specific binding sites by incubation for 2 hours with blocking buffer at RT. Immunostaining was performed by incubating brain sections in primary antibody diluted in blocking buffer at 4 °C overnight. Afterwards, brain sections were washed three times for 10-15 min at RT with washing buffer before incubating with secondary anti-rabbit Cy3- labeled or anti-mouse Alexa488-labeled antibody for 2 hours at RT. Finally, the sections were incubated with 4',6-diamidino-2-phenylindole (DAPI) for nuclear staining and then mounted with Mowiol and coverslips.

3.7.20 Confocal microscopy

Confocal microscopy was carried out using the Leica SP2 AOBS confocal microscope and Leica confocal Software (LCS). Alexa488 and Cy3 conjugated secondary antibodies were excited using 488 nm and 561 nm lasers respectively

3.8 Statistical Analysis

All data was analyzed with the Prism software. Significance was evaluated using unpaired student´s t-test, Mann-Whitney test or one-or two-way analysis of variance (ANOVA) including repeated measures when appropriate. Bonferroni post-hoc test was performed after ANOVA analysis when applicable. Significance was set at $p \leq 0.05$ for all tests. Errors are shown as average ± SEM in figures and text if not otherwise stated.

4. Results

4.1 HDAC1 mediates fear extinction learning

4.1.1 HDAC1 expression in the adult mouse hippocampus and different brain regions in schizophrenia

In order to study the role of HDAC1 in the etiopathogenesis of schizophrenia, I chose the mouse as a model organism and decided first to analyze HDAC1 expression in different brain regions in the adult mouse brain, namely the Hippocampus (Hip), Hypothalamus (Hy), Septum (Sep), Prefrontal Cortex (PFC) and Striatum (Str). Immunoblot analysis showed robust HDAC1 expression in the selected brain regions (Fig. 4A). Subcellular fractionation and immunohistochemical analysis of the hippocampus displayed prominent HDAC1 localization in neuronal nuclei compared to the cytosolic compartment (Fig. 4B, C). The robust expression and primarily nuclear localization of HDAC1 is in line with previous data [478].

Next, I wondered whether HDAC1 expression would show a similar pattern in the human brain. Immunoblot analysis of post-mortem tissue obtained from individuals that did not suffer from any neuropsychiatric disorder revealed that HDAC1 protein expression was present in all brain regions selected with higher levels in the hippocampus and prefrontal cortex (Fig. 4D).

HDAC1 mRNA was reported to be elevated in the hippocampus and prefrontal cortex of schizophrenia patients [433,435]However, since the analysis of post-mortem tissue often result into deviant observed results in different laboratories, I analyzed HDAC1 mRNA expression in hippocampal and prefrontal cortical post-mortem tissue available in our laboratory. Quantitative real-time PCR (qPCR) revealed significantly increased HDAC1 expression in the hippocampus and prefrontal cortex of schizophrenia patients compared to age-matched control samples (Fig 4E). Thus, I was able to reproduce the finding of Benes and Sharma showing elevated HDAC1 expression levels in the hippocampus and prefrontal cortex of individuals with schizophrenia.

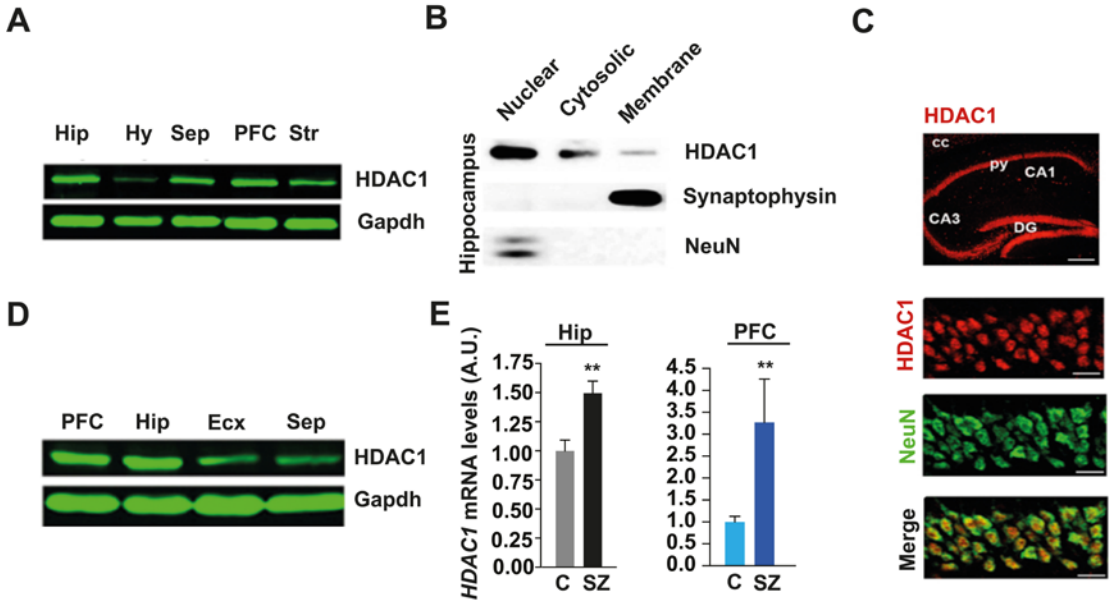

Figure 4: Expression of HDAC1 in the adult mouse and human brain.

A. Robust HDAC1 expression in the adult mouse brain. Representative immunoblot pictures showed HDAC1 protein and the loading control Gapdh in the hippocampus (Hip), hypothalamus (Hy), septum (Sep), prefrontal cortex (PFC) and striatum (Str). **B.** Representative immunoblot analysis of hippocampal subcellular fractionation showed enriched HDAC1 in the nuclear fraction. Synaptophysin and NeuN (Neuronal Nuclei) served as loading controls for the membrane and nuclear fraction, respectively. **C.** Representative confocal images showed HDAC1 protein in the entire mouse hippocampus and in NeuN-positive neurons of the dentate gyrus. . Scale bars for confocal images: Top, 200 µm; bottom 20 µm; hippocampal subfields CA1, CA2,CA3; corpus callosum (cc); dentate gyrus (DG); pyramidal cell layer (Py). **D.** Representative immunoblot analysis showed HDAC1 protein in the prefrontal cortex, hippocampus, entorhinal cortex (Ecx) and septum of postmortem human brain tissue of patients that did not suffer from any neuropsychiatric disorder. The image depicts the representative pattern observed in 4 individuals**. E.** Elevated *Hdac1* mRNA expression levels in the hippocampus and prefrontal cortex of post-mortem human brain tissue of individuals with schizophrenia (n= 8/group). Control C, Schizophrenia SZ (**A, B, D)**. 30 µg of protein were subjected to SDS-PAGE); **p≤ 0.01; students two-tailed t-test. Error bars represent ± SEM.

4.1.2 Adeno-associated virus (AAV) mediated neuronal overexpression of HDAC1 in the adult mouse hippocampus

I could reproduce elevated HDAC1 mRNA levels in the hippocampus and prefrontal cortex of individuals with schizophrenia. However since over-expression of HDAC1 from early developmental stages did not cause any overt phenotype in mice [196]) I hypothesized that increased HDAC1 levels might not reflect a developmental but rather a genome-environment effect during the pathogenesis of schizophrenia. Thus, acute overexpression of HDAC1 in the adult mouse brain would help to better

understand the role of increased HDAC1 levels observed in the diseased brain, simulating cognitive endophenotypes of schizophrenia.

To this end, I generated an Adeno-associated virus (AAV) that expressed HDAC1 fused with GFP (HDAC1-GFP AAV) under the neuron-specific synapsin 1 promoter in order to allow neuron-specific expression of HDAC1 [479]. AAVs expressing GFP alone (GFP-AAV) served as controls. To address the question whether elevated HDAC1 levels would lead to cognitive endophenotypes of schizophrenia, I first focused on the hippocampus, a brain region affected in schizophrenia and important for cognitive processes.

AAV particles expressing HDAC1-GFP or GFP alone were injected into the dorsal hippocampus of mice and AAV the corresponding expression was tested by qPCR and immunoblot analysis after 6 or 14 days of injection (Fig. 5A). qPCR analysis revealed a six-fold increased expression of HDAC1 after 14 days of injection compared to mice injected with GFP. This six-fold increase expression of HDAC1 resulted in a two-fold increase of hippocampal HDAC1 protein compared to endogenous HDAC1 protein when measured 14 days after injection by immunoblot analysis (Fig. 5 A, D). No significant differences in HDAC1 mRNA expression were observed after 6 days of injection (Fig. 5B). Additionally, confocal microscopy of injected hippocampal sections could not detect HDAC1-GFP expression in the dorsal hippocampus after 6 days of injection (Fig. 5D). After 14 days of injection, HDAC1-GFP expression was localized to neuronal nuclei within the dorsal hippocampus (Fig. 5D). Moreover, injection of the dorsal hippocampus did not affect the ventral hippocampus as shown by the lack of GFP expression (Fig. 5D). Also other brain regions such as cortex, cerebellum and cortex were not affected (data not shown).

Importantly, significant overexpression of HDAC1 did not alter mRNA and protein expression levels of other Class I HDAC members (Fig. 5C; Fig. 6C).

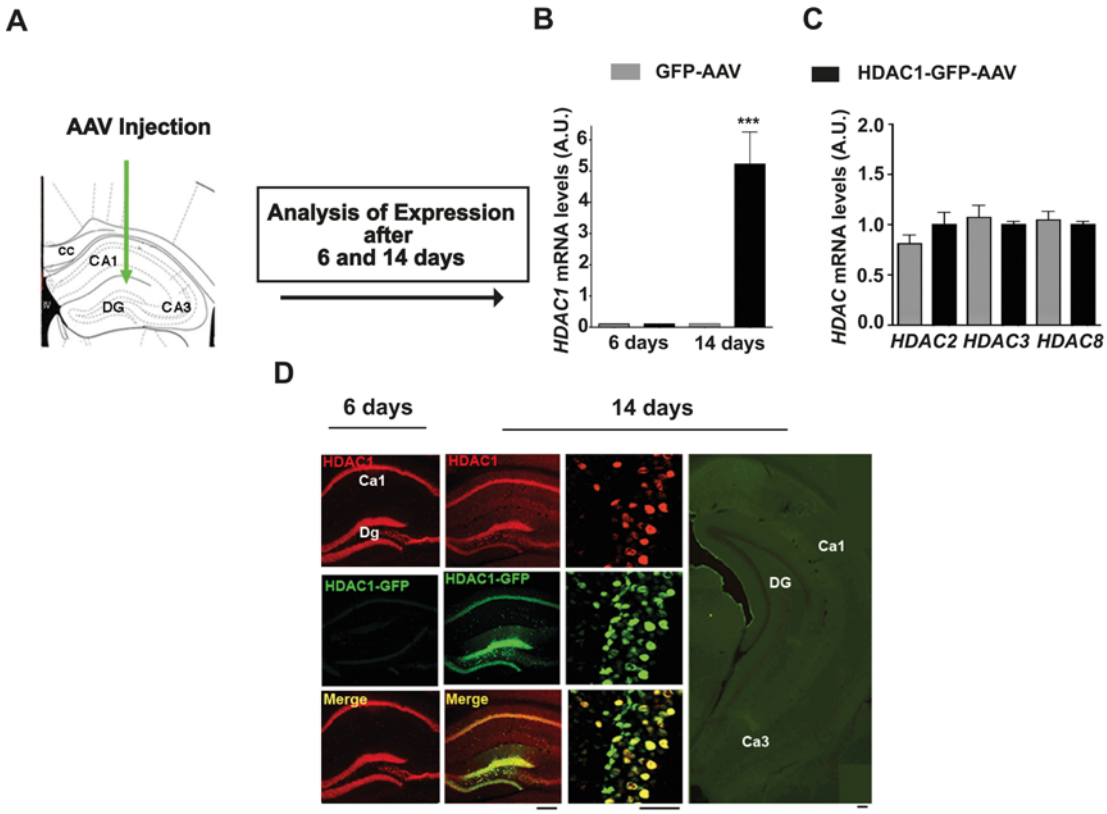

Figure 5: AAV-mediated overexpression of HDAC1 in the dorsal hippocampus of mice.

A. Experimental Design. AAV particles were injected in the dorsal hippocampus of mice and HDAC1-GFP expression analyzed 6 and 14 days after injection. **B.** Quantitative real-time PCR analysis showed a six-fold increase in *Hdac1* mRNA levels after 14 days of injection in HDAC1-GFP-AAV mice (n= 3/group). **C.** The expression of other Class I HDACs after 14 days of AAV injection was unchanged. **D.** Representative confocal imaging of hippocampal sections confirmed that nuclear HDAC1-GFP was not detectable in mice 6 days after injection but after 14 days. No GFP fluorescence was visible in the ventral hippocampus of mice after 14 days of HDAC1-GFPAAV (n=4 /group). At 14 days left panel: Colocalization of endogenous HDAC1 with HDAC1-GFP in the dorsal hippocampus. Scale bar 200 µm. Middle panel: high-magnification images of the dorsal dentate gyrus of HDAC1-GFP AAV injected mice. Scale bar: µm. At 14 days right panel: Ventral hippocampus of HDAC1-GFP AAV injected mice. Error bars represent ± SEM

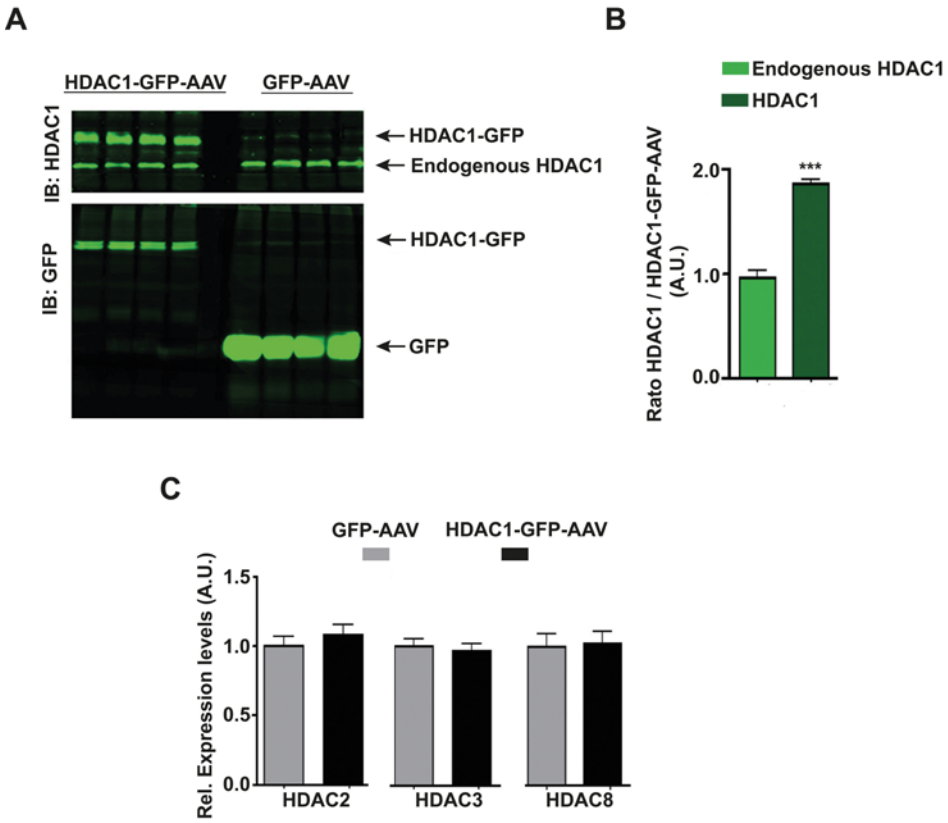

Figure 6: HDAC1 protein expression in HDAC1-GFP-AAV mice.

A. Immunoblot analysis of hippocampal protein lysates after 14 days of AAV injection. Proteins lysates were isolated from the same samples in which qPCR analysis was performed using TRI-reagent. **B**. Quantification of immunoblot analysis in **A**. Mice injected with HDAC1-GFP AAV showed a two-fold increase in HDAC1 protein levels when compared to endogenous HDAC1.**C**. Quantification of immunoblot analysis of same lysates described in **A** were used to analyze protein levels of HDAC2, 3 and 8. No alteration in other Class I HDAC proteins levels was observed among groups. 30 µg of protein was used for immunoblot analysis n= 4/ group. Error bars indicate ± SEM.

Taken together, we conclude that using the AAV expression system was suitable to achieve spatially restricted and time-dependent HDAC1 overexpression in the adult mouse brain in order to study its impact on cognitive function in mice.

4.1.3 Cognitive function in mice overexpressing hippocampal neuronal HDAC1

In order to investigate whether elevated hippocampal HDAC1 levels in the adult mouse brain would lead to cognitive impairments seen in schizophrenia patients, I injected mice with HDAC1-GFP or GFP alone viral particles in the dorsal

hippocampus and subjected them to behavioral test addressing explorative and depressive-like behavior, spatial, associative and working memory as well as sensorimotor gating function.

When subjected to the open field test, HDAC1-GFP-AAV and GFP-AAV mice spent similar time in the center of the open field and showed no alter in their explorative activity indicating that basal anxiety was not affected (Fig. 7A). The same mice were subjected to the Porsolt Forced Swim test, a commonly used paradigm to address depressive-like behavior in rodents. However, no differences in the floating time of mice were observed (Fig. 7B). During working memory performances using the cross-maze and novel object recognition test, HDAC1-GFP and GFP-AAV mice again did not show significant differences (Fig. 7C, D). The consolidation of long-term memories was analyzed by subjecting mice to the long-term memory test of the novel object recognition test, the morris water maze and contextual fear conditioning that all depend on proper functioning of the hippocampus. However, no significant differences in the consolidation of long-term memories were observed among groups (Fig. 7E, F, G). Additional mice were exposed to the prepulse inhibition of startle response test, a paradigm that is used to address sensorimotor gating function that is impaired in schizophrenia patients and animal models of schizophrenia [480,481]. As no overall effect of virus expression was revealed in the two-way analysis of variance (ANOVA), HDAC1-GFP-AAV mice did not show altered prepulse inhibition (PPI). However, student t-test of PPI at 70 db revealed significant increased PPI in HDAC1-GFP-AAV mice (Fig. 8A). Baseline startle response of HDAC1-GFP and GFP-AAV mice was similar (Fig. 8B).

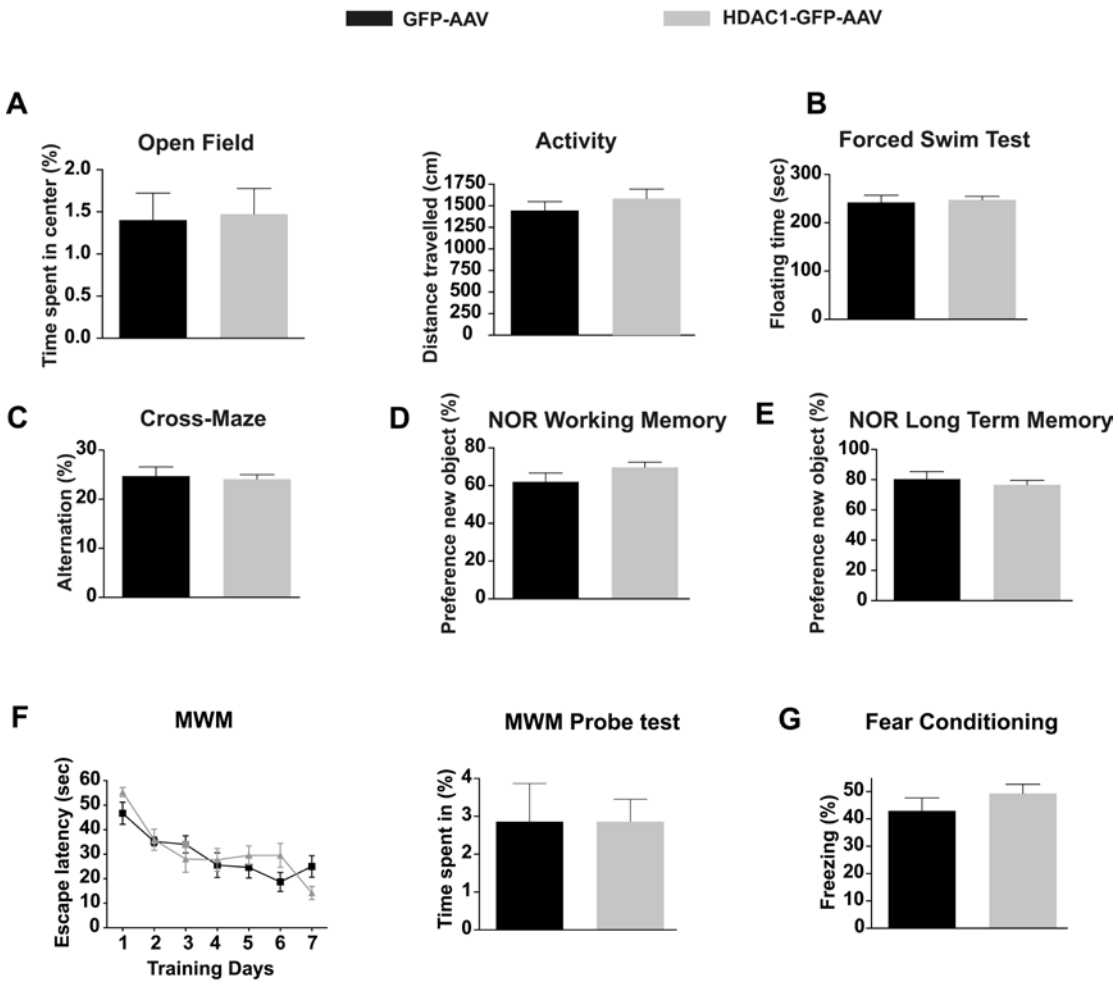

Figure 7: Behavioral characterization of mice overexpressing neuronal HDAC1 14 days after injection.

A. Open field analysis of basal anxiety. No differences were observed in the time spent in the center of the open field or exploration activity among groups. **B.** Analysis of depressive-like behavior during the forced swim test. No differences in floating time were observed among groups. **C.** Working memory performance of mice during the cross-maze test was not affected among groups. **D.** Working memory performance of mice during the novel object recognition test. Mice showed similar object preference. **E.** Long-term memory performance during the novel object recognition test. No differences in long-term memory were observed among groups. **F.** Spatial memory of mice during the morris water maze (MWM) test. Left panel: No significant differences in escape latency were observed during MWM training among groups. Right panel: Probe test 24 hours after the last training session with no differences among groups. **G.** Associative memory during contextual fear conditioning test. n= 9-10 /group. Error bars represent ± SEM.

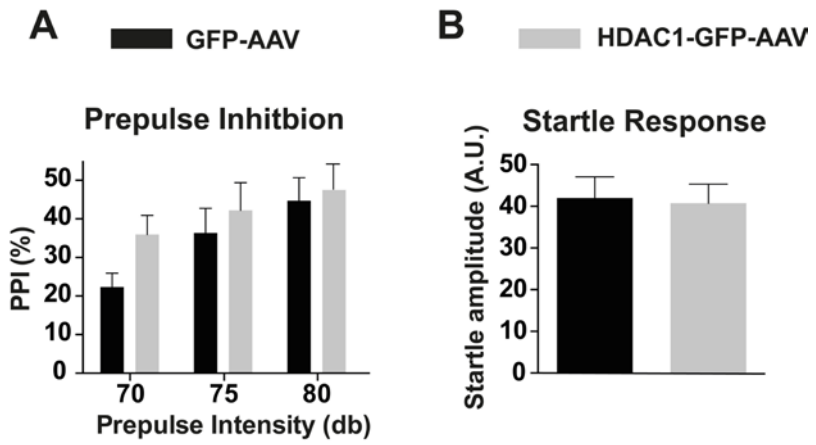

Figure 8: Sensorimotor gating function in mice overexpressing neuronal HDAC1 in the dorsal hippocampus.

A. Overexpression of HDAC1 in the dorsal hippocampus did not affect Prepulse inhibition of startle response. Two-way ANOVA of repeated measures revealed no significant effect of virus expression [F (1, 16)= 1.018, p = 0.03280] while prepulse intensity had still a significant effect [F (2, 32)= 1.391, p = 0.2635]. However, students two-tailed t-test revealed a mild significant difference between HDAC1-GFP and GFP-AAV mice at 70 db of prepulse intensity (p= 0,0403). **B.** The acoustic startle response to 120 db sound did not differ between GFP-AAV and HDAC1-GFP-AAV mice (p = 0,7137, Mann-Whitney test). Error bars represent ± SEM; = 12 GFP-AAV/ 17 HDAC1-GFP. n= 9 GFP-AAV/ 10 HDAC1-GFP AAV.

In summary, AAV-mediated neuronal overexpression of HDAC1 in the adult hippocampus of adult mice did not result into schizophrenia-like symptoms such as increased anxiety, depressive-like behavior, memory impairments or disrupted sensorimotor gating function.

4.1.4 AAV-mediated neuronal overexpression of HDAC1 in the adult mouse hippocampus facilitates fear extinction learning

The finding that AAV mediated over-expression of HDAC1 in the hippocampus of adult mice did not cause cognitive endophenotypes of schizophrenia or lead to obvious detrimental behaviors was surprising but in line with previous results from Guan et al [196], in which overall overexpression of HDAC1 in transgenic mice did not affect memory formation in the morris water maze and conditional fear conditioning test.

Nevertheless, as a nuclear protein deacetylating histones and member of different co-repressor complexes mediating transcriptional repression, HDAC1 is suggested to be a master regulator of gene-expression [482]. Moreover, HDAC1 have been shown to be essential in neuroprotection and neuronal development [483,484]. On this basis, I assumed that HDAC1 might have a specialized function in memory formation. Differential regulation of hippocampal histone acetylation is known to be required for memory formation and studies investigating the impact of HDACi in the fear extinction indicated that histone-acetylation may also play an important role in the extinction of fear memories [410,414,485].

Fear extinction is a specific form of learning, a so-called inhibitory learning and emotional memory that represents a decline in excessive fear response by repeated exposure to the fear-triggering stimulus in the absence of the aversive event. The paradigm of fear extinction as exposure therapy is typically employed in the treatment of anxiety disorders such as post-traumatic stress disorder (PTSD) [486,487]. Notably, fear extinction has been reported to be affected in individuals affected by schizophrenia [488].

In rodents, hippocampus-dependent fear extinction is assessed on the basis of contextual fear conditioning following repeated re-exposure to the context. In the contextual fear conditioning test, rodents are exposed to a novel context followed by an electric foot shock eliciting the consolidation of fear memory 24 hours after receiving the foot shock, measured as the amount of freezing, a behavior that rodents express upon threatening situations. During fear extinction training, rodents are re-exposed to the conditioned fear without receiving the foot shock again (extinction trial, E). Repeated re-exposure to the context is conducted until freezing response of mice decline in order to achieve successful fear extinction [441,489,490].

In order to investigate the impact of AAV mediated hippocampal overexpression of HDAC1 during contextual fear extinction of mice, it is from utmost importance not to interfere with the acquisition and consolidation of fear memories. Thus, the AAV-mediated overexpression of HDAC1 represented a suitable approach to design an experiment in which HDAC1-GFP was specifically overexpressed during fear extinction without affecting the process of memory consolidation.

Mice were subjected to contextual fear conditioning 4 days after HDAC1-GFP-AAV injection (Fig. 9A), a time-point with no detectable HDAC1-GFP expression in the

dorsal hippocampus as outlined in Figure 2. Control mice were injected with GFP-AAV and treated in similar manner. Twenty-four hours after fear conditioning training, mice were subjected to the memory test by re-exposure to the context that is referred here to as extinction day 1 (E1) (Fig. 9A). Thus, the acquisition and consolidation of fear memories occurred in the absence of HDAC1 overexpression. Eight days after E1, when HDAC1-GFP is strongly overexpressed, mice were subjected to extinction training (E2-E5) until the freezing response was significantly reduced (Fig. 9A). Interestingly, HDAC1-GFP-AAV mice showed significantly facilitated extinction when compared to GFP-AAV injected mice (Fig. 9A).

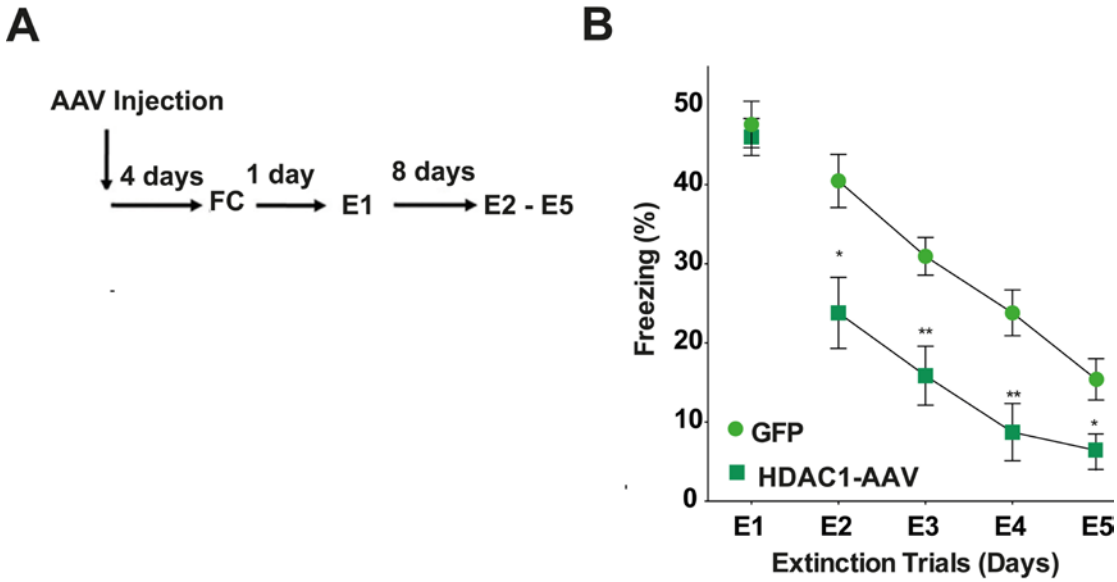

Figure 9: AAV-mediated overexpression of HDAC1 accelerates fear extinction learning.

A. Experimental design. Mice were subjected to contextual fear conditioning (FC) training 4 days after AAV injections in the dorsal hippocampus and 24 hours later to E1 (Extinction day 1) that corresponds to the memory test of FC. After exposure to E1, mice were kept for 8 days in their home cages and subjected to fear extinction training from E2-E5 when HDAC1-GFP was robustly overexpressed in the dorsal hippocampus of mice. **B.** Contextual fear extinction in HDAC1-GFP AAV and GFP-AAV mice. While mice showed similar freezing behavior at E1, HDAC1-GFP AAV mice showed significantly enhanced fear extinction learning displayed by accelerated reduction in freezing behavior compared to GFP-AAV mice. Two-way ANOVA of repeated measures: significant main effect of virus [F (1, 12) = 8.635, p = 0.0124], significant main effect of fear extinction training [F (4, 48) = 132.7, p < 0.0001]; significant virus x fear extinction interaction [F (4, 48) = 6.507, p = 0.0003];(n=10/group). ; *p≤ 0.05; **p≤0.001; ***p≤0.0001; students t-test; Error bars represent ± SEM.

Thus, AAV-mediated neuronal overexpression of HDAC1 in the hippocampus facilitated fear extinction indicating that HDAC1 may regulate the extinction of hippocampus-dependent fear memories.

4.1.5 Inhibition and knock-down of hippocampal HDAC1 impairs fear extinction in mice

While the AAV-mediated overexpression of HDAC1 represents a gain-of function model, I wanted to apply a loss-of function model in order to further investigate the involvement of hippocampal HDAC1 during fear extinction. To this end, I used two approaches: 1. Administration of the HDAC inhibitor (HDACi) MS-275 that inhibits HDAC1 enzyme activity at nanomolar concentrations [203,204,491] and 2. Injection of siRNA targeting HDAC1 in order to knock-down hippocampal HDAC1.

After stereotaxic implantation of bilateral cannulae in the dorsal hippocampus and one week recovery of surgery, mice were subjected to fear conditioning followed by extinction training (Fig. 10A). Immediately after each extinction trial, mice received intra-hippocampal injections of MS-275 (Fig. 10A). The control group received a vehicle solution and was treated in the same manner as the MS-275 injected group.

Notably, freezing response of MS-275 injected mice did not decline during extinction training, while freezing behavior of the vehicle treated group was significantly reduced (E1 vs. E5). Thus, inhibition of hippocampal HDAC1 activity by MS-275 blocked the extinction of fear memories. I also tested the effect of MS-275 on memory acquisition. Therefore mice were injected with MS-275 immediately after contextual fear conditioning. Inhibition of hippocampal HDAC1 activity did not affect freezing during the memory test suggesting that the acquisition of fear memories is independent of HDAC1 (Fig. 10B).

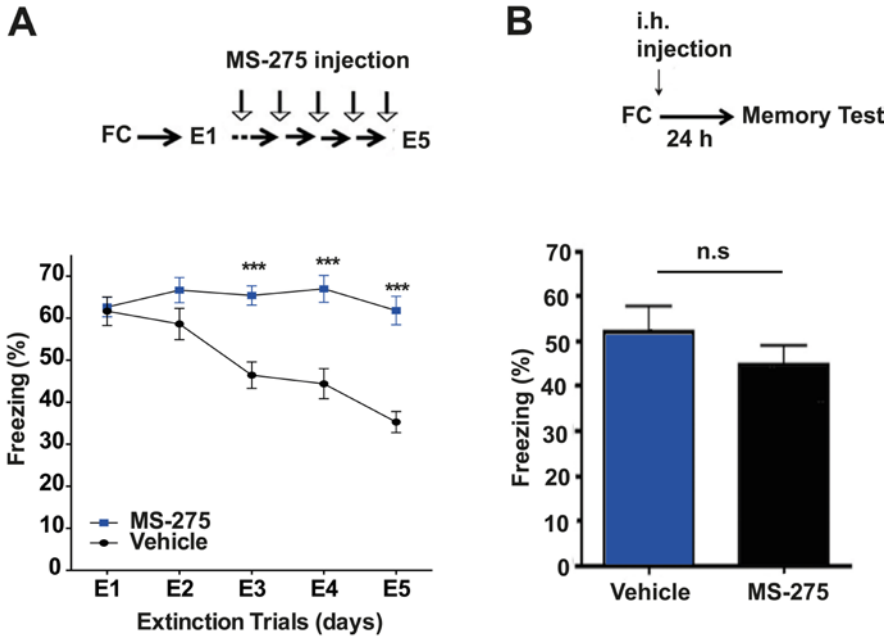

Figure 10: Inhibition of HDAC1 activity by MS-275 impairs fear extinction without affecting the acquisition of fear memories

A. Top panel: Wild-type mice implanted with bilateral guide microcannulae in their dorsal hippocampus were subjected to FC and received intrahippocamapl injections of MS-275 immediately after each extinction trial. Bottom: MS-275-treated mice displayed significantly impaired fear extinction compared to vehicle treated mice. **B.** Top panel: Wild-type mice received immediately after FC training a single intrahippocampal injection of MS-275 and were subjected to the memory test 24 hours after. Bottom: Freezing behavior during the memory test was similar between MS-275 and vehicle injected-mice. ; *p≤ 0.05; **p≤0.001; ***p≤0.0001; students t-test; Error bars represent ± SEM.

While MS-275 has highest affinity towards HDAC1 showing a 100-fold higher EC_{50} (half maximal effective concentration) compared to HDAC2 and HDAC3, the possibility that the inhibition of HDAC2 and HDAC3 activity is affecting fear extinction cannot be excluded. I therefore chose the siRNA approach using previous validated siRNA targeting HDAC1 to affect hippocampal HDAC1 levels and exclude the involvement of HDAC2 and HDAC3 during fear extinction [468,492]. Mice received intra-hippocampal siRNA injections starting immediately after E1. Within 48 hours, mice were injected with HDAC1 siRNA 4 times every 12 hours before exposure to E2 and continued after each extinction trial until E5 performed on consecutive days (Fig. 11A). Mice injected with scrambled siRNA served as a control group. Mice injected with siRNA targeting HDAC1 demonstrated significantly impaired fear extinction compared to the control group. Furthermore, injection of HDAC1 siRNA resulted into significantly reduced hippocampal HDAC1 mRNA and protein levels compared to the control group correlating with impaired fear extinction in HDAC1 siRNA mice (Fig. 11B, C, D).

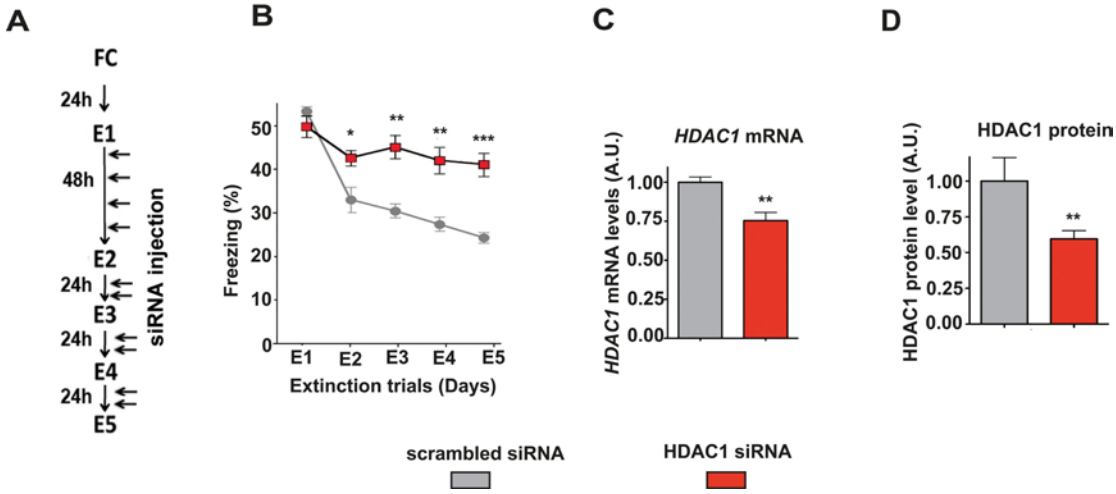

Figure 11: siRNA-mediated knockdown of HDAC1 impairs fear extinction learning.

A. Experimental design. Immediately after fear conditioning training, wild-type mice received intrahippocampal injection of siRNA and subjected to E1 24 hours later. After E1, mice received siRNA injections every 12 hours and were subjected to E2 48 hours after E1. Extinction training was continued on subsequent day every 12 hours and siRNA injections were continued until E5 every 12 hours. **B.** Fear extinction learning. Intrahippocampal injections of HDAC1 siRNA impaired fear extinction learning when compared to the control group that received scrambled siRNA. n= 6 /group. **C.** qPCR analysis showed significantly reduced hippocampal *Hdac1* mRNA levels in HDAC1 siRNA injected mice when compared to the control group. n= 6/ group. **D.** Quantitative immunoblot analysis showed significantly reduced HDAC1 protein levels in HDAC1 siRNA injected mice when compared to the control group injected with scrambled siRNA. n= 6/ group; *p≤ 0.05; **p≤0.001; ***p≤0.0001; students t-test; Error bars represent ± SEM.

In sum, inhibition of hippocampal HDAC1 activity by MS-275 and knockdown of HDAC1 using HDAC1 specific siRNA impaired fear extinction learning, while neuronal overexpression of HDAC1 in the hippocampus enhanced fear extinction. Taken together, these results indicated a specific role of HDAC1 in the process of hippocampus-dependent fear extinction.

4.1.6 Recruitment of HDAC1 to *c-Fos* promoter and decreased *c-Fos* expression during fear extinction

Since HDAC1 activity is strongly associated with transcriptional repression of neuronal genes [90,163,493,494], I wondered whether HDAC1 affects hippocampal-dependent fear extinction by transcriptional repression of learning induced genes.

The expression of the immediate early gene (IEG) *c-Fos* is known to be transiently upregulated after contextual fear conditioning and respective *c-Fos* protein levels are

reduced during fear extinction learning [168,490,495–497]. Moreover, HDAC1 activity has been implicated in the regulation of *c-Fos* gene-expression [498–500].
Thus, I decided to monitor transcriptional regulation of hippocampal *c-Fos* during fear extinction in order to explore the mechanism by which endogenous HDAC1 mediates the extinction of fear memories under physiological conditions.

To this end, I subjected wild-type mice to our fear extinction paradigm. After successful fear extinction learning, in which each mice showed reduced fear response throughout extinction training (Fig. 12A), hippocampi were isolated 1 h after each extinction trial from randomly selected mice in order to carry out molecular analysis. Hippocampi from one hemisphere were subjected to qPCR and immunoblot analysis while hippocampi of the second hemisphere were chosen to perform chromatin immunoprecipitation (ChIP) followed by qPCR analysis. As such, I was able to monitor transcriptional changes and HDAC1 binding on the *c-Fos* gene simultaneously in the same mice. Naïve animals that did not undergo contextual fear extinction served as control group and were treated in the same way. All data revealed from qPCR, immunoblot and ChIP analysis were normalized to naïve mice.
Analysis of *c-Fos* mRNA levels using qPCR revealed significantly increased *c-Fos* expression after exposure to E1 but progressively declined throughout fear extinction training from E1-E5, being significantly reduced at E5 compared to E1 going back to baseline levels observed in naïve mice (Fig. 12B). Thus, decreased *c-Fos* mRNA levels throughout extinction training correlates with previous finding reporting decreased *c-Fos* protein levels after fear extinction learning [490].

To further investigate whether transcriptional repression of *c-Fos* during extinction is mediated by HDAC1, I performed ChIP analysis in the hippocampi of the same mice with greater focus on E1, E3 and E5. For HDAC1 binding analysis on the *c-Fos* promoter, I preferably selected the cAMP responsive element (cre) binding region of the promoter (Appendix Fig. 1), a well analyzed region that is known to be bound by various transcription factors such as CREB (cAMP responsive element binding protein) resulting into transcriptional activation of *c-Fos* [501]
Strikingly, ChIP analysis showed significantly increased HDAC1 binding at the *c-Fos* promoter 1h after E5 compared with E1 or E3, a time-point of decreased *c-Fos* expression (Fig. 12C).

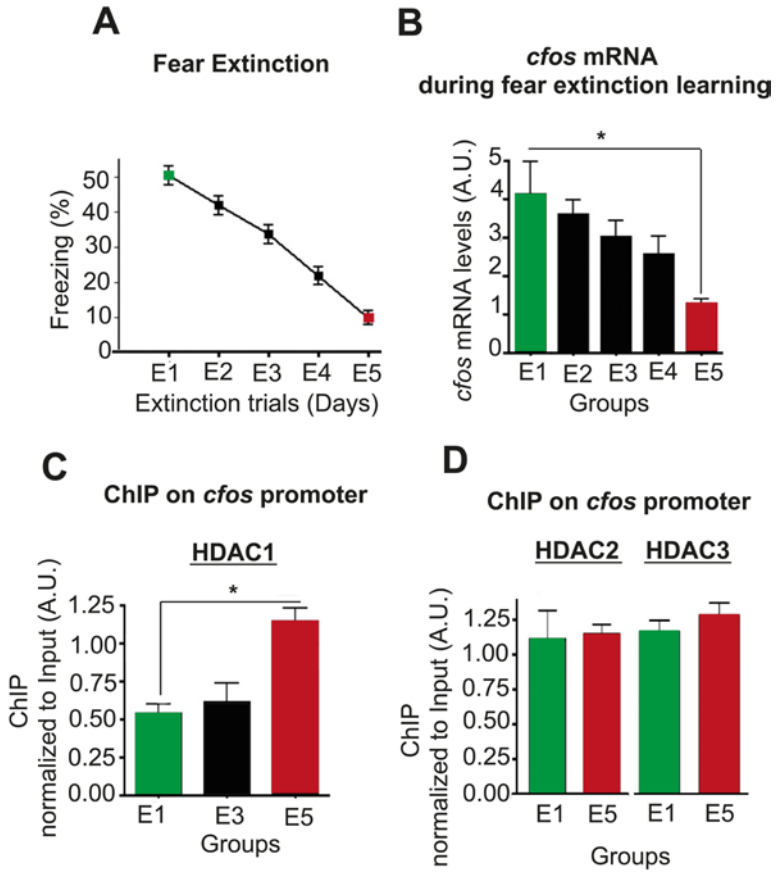

Figure 12: Recruitment of HDAC1 to the promoter of *c-Fos* and transcriptional repression during contextual fear extinction.

A. Freezing behavior of wilt-type mice during contextual fear extinction (n= 45) for molecular analysis (n= 5/ group). **B.** qPCR analysis of hippocampal tissue isolated 1 hour after each extinction trial. Data was normalized to naïve control mice. *c-Fos* expression levels decreased transiently during fear extinction training and was significantly reduced at E5 compared to E1. **C.** HDAC1 ChIP on *c-Fos* promoter 1hour after E1, E3 and E5 showed significantly increased HDAC1 binding at E5 compared to E1. **D.** HDAC2 and HDAC3 ChIP showed no changes in the binding to the *c-Fos* promoter between E1 and E5. *p≤ 0.05; students t-test; Error bars indicate ± SEM

However, increased HDAC1 protein binding at the *c-Fos* promoter was not due to altered HDAC1 mRNA or protein levels when compared hippocampal lysates 1h after E1 and E5 (Fig. 13A, B). Moreover, the other class I HDACs, HDAC2 and HDAC3 that are also act as transcriptional repressors did not show altered binding on the *c-Fos* promoter (Fig. 12D) or altered hippocampal mRNA expression during fear extinction (Fig. 13C).

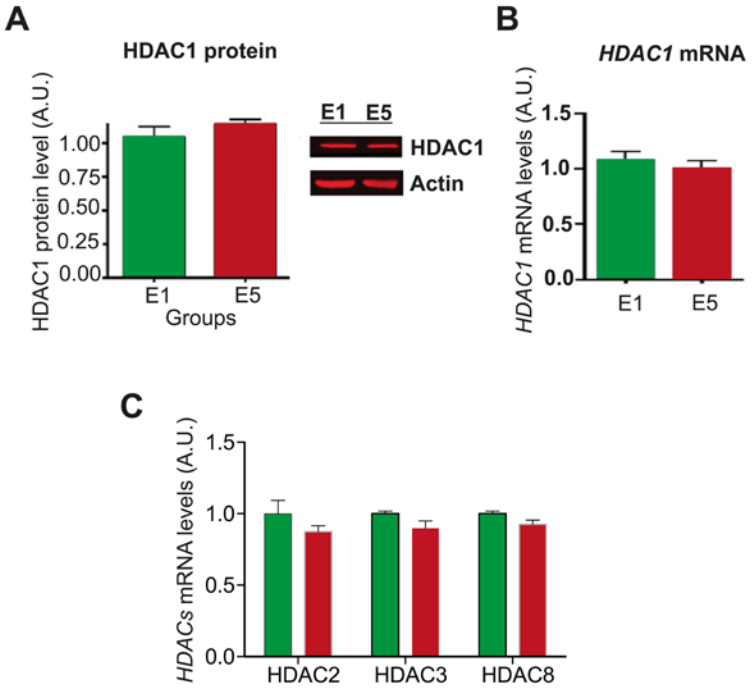

Figure 13: HDAC1 expression during fear extinction.

A. Immunoblot analysis of HDAC1 at extinction day E1 and E5. Left panel: Quantitative immunoblot analysis showed no differences in HDAC1 protein levels during fear extinction learning at E1 and E5. Right panel: Representative immunoblot images showing HDAC1 immunoreactivity at E1 and E5. Actin served as a loading control. **B.** qPCR analysis of *Hdac1* mRNA levels showed no differences between E1 and E5. **C.** qPCR analysis of other class I HDACs *Hdac2, Hdac3* and *Hdac8* showed no differences in expression levels between E1 and E5. 30 µg of hippocampal protein was loaded per lane. Data was normalized to naïve control mice. Error bars indicate ± SEM.

However, the question remained whether the decrease of *c-Fos* expression and HDAC1 recruitment to the corresponding promoter might be due to the passing of time. In order to address this question, I performed a control experiment (Fig. 14A) consisting of three experimental groups. One group of mice were subjected to contextual fear conditioning and sacrificed one hour after the memory test at E1. The second group consisted of mice that were exposed to extinction day E2 3 days after E1 exposure (E1-3 days group) and were thus not exposed to extinction training, being kept at their home cages in the meantime. The third experimental group consisted of mice that were subjected to extinction training on 4 consecutive days (E1-E5 group). Interestingly, while the E1-E5 group showed successful fear extinction learning displaying significantly reduced fear response compared to E1, the E1-3days group did not show altered freezing behavior compared to the E1, remaining at significantly high freezing levels in contrast to the E1-E5 group. Analysis of hippocampal *c-Fos* expression revealed that while *c-Fos* expression levels declined in the E1-E5 group when compared with E1, *c-Fos* mRNA expression was

not altered in the E1-3 days group (Fig. 14B,C). Thus, *c-Fos* expression levels corresponded to freezing levels observed in mice (Fig. 14B,C). Similarly, HDAC1 *c-Fos* promoter binding did not show differential binding levels between the E1 and E1-3days group but were significantly increased after E5 in the E1-E5 group (Fig. 14D).

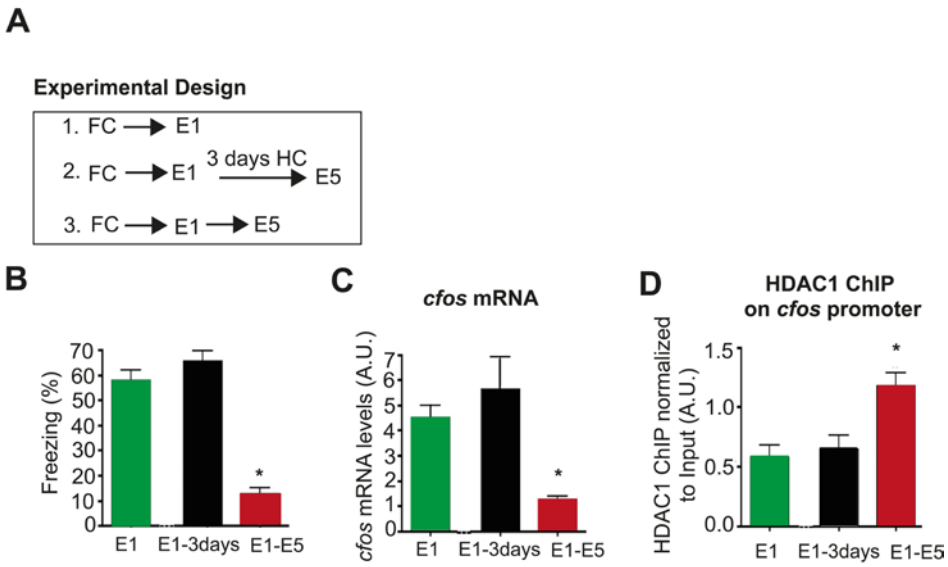

Figure 14: Fear extinction training-dependent recruitment of HDAC1 to the *c-Fos* promoter.

A. Experimental design showing three different experimental groups. One group of mice was subjected to contextual fear conditioning (FC) followed by exposure to E1 (E1 group). The second group of mice were subjected to FC followed by E1 but were kept on the subsequent 3 days in their home cages before exposure to E2 that corresponds on the basis of the number of days to E5 (E1-3days group). The third group was subjected to E1 and underwent fear extinction training on subsequent days from E1-E5 (E1-E5 group). **B.** Freezing behavior of mice. The E1-3 days group that did not undergo extinction training on subsequent days from E1-E5, showed significantly higher freezing behavior when compared to E1-E5 (n=5/group). **C.** qPCR analysis showed significantly higher *c-Fos* expression levels in the E1-3 days group compared to mice of the E1-E5 group (n=5/group). **D.** HDAC1 ChIP on the *c-Fos* promoter showed increased binding in the E1-E5 group (n=5/group). All data was normalized to naïve control mice. *p≤ 0.05; students t-test; Error bars indicate ± SEM.

Taken together, these results suggested that the downregulation of *c-Fos* and recruitment of HDAC1 to the corresponding promoter during fear extinction reflects an active process.

4.1.7 HDAC1-mediated transcriptional repression during fear extinction learning

To further address the hypothesis that HDAC1 mediates transcriptional repression during fear extinction, we compared hippocampal histone modifications at the c-fos promoter 1 h after E1 and E5. Thus, the same samples obtained in the previous experiment (Fig. 12) were subjected to ChIP analysis in order to monitor changes of hippocampal histone-modifications. However, in order to ensure that the region selected on the *c-Fos* promoter is suitable to investigate transcriptional repression of *c-Fos* during fear extinction, I performed a control ChIPs analysis for the binding of CREB phosphorylated at Serine 133 (pCREB(Ser133)) and phosphorylation of Histone3-Serine10 (H3S10P).

Phosphorylation of CREB at Serine 133 is activity dependent, involved in the formation of long-term memories and known to be required for CREB induced transcriptional activation of *c-Fos* [502–505] Phosphorylation of H3S10P has been shown to be important in transcriptional activation of genes and has been associated as well with the transcriptional activation of *c-Fos* and other IEG´s [45,506]. Moreover, phosphorylation of H3S10P at the *c-Fos* promoter was shown to depend on the presence of pCREB(Ser133) binding at the cre-element of the *c-Fos* promoter [507].

ChIP analysis of pCREB(Ser133) binding and H3S10P levels at the *c-Fos* promoter revealed significantly reduced phospho-CREB and phosphorylated H3S10 levels at E5 when compared to E1, correlating with decreased *c-Fos* mRNA expression at E5 (Fig. 15A). Moreover, elevated H3S10P and increased binding of pCREB(Ser133) at E1 is in line with previously reported data that contextual fear conditioning induces phosphorylation of CREB and increases phosphorylation of H3S10 in the hippocampus [87,508].

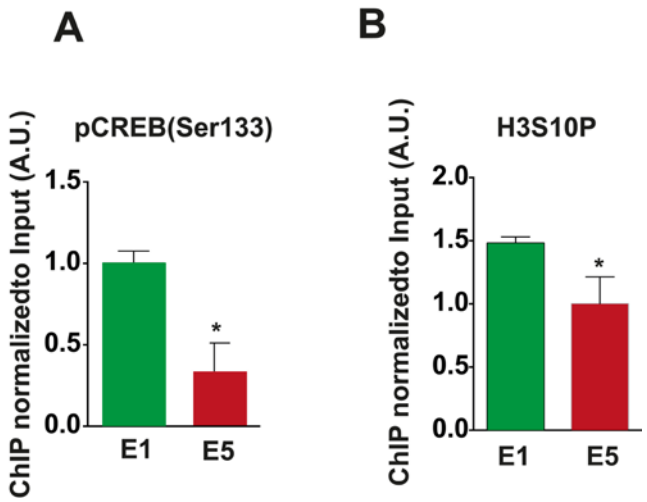

Figure 15: Phospho-CREB and phospho-H3S10 during fear extinction.

A. ChIP analysis of hippocampi in wild-type mice showed significantly decreased phospho-CREB at Serine 133 (pCREB (Ser133)) and **B.** phosphorylated H3S10 (H3S10P) at the *c-Fos* cre-element promoter region at E5 when compared to E1. *p≤ 0.05; students t-test; Error bars represent ± SEM.

Since HDAC1 is a histone-deacetylase, I focused on changes in histone-acetylation sites. Among all histone-acetylation sites analyzed (H3K9ac, H3K14ac, H4K5ac), acetylation of H3K9, a histone-modification associated with active gene expression and HDAC1 activity [163,168] was significantly decreased at the *c-Fos* promoter in the E5 group when compared to the E1 group (Fig. 17C).

Deacetylation of H3K9 is known to serve as a prerequisite for H3K9 trimetyhlation (H3K9me3) [509], a histone-modification enriched at heterochromatin regions marking transcriptional repression [510], I analyzed levels of H3K9me3 on the *c-Fos* promoter at E1 and E5. Notably, levels of H3K9me3 were elevated at E5 at the *c-Fos* promoter (Fig.17C).

As an additional control experiment, I analyzed levels of H3K9 acetylation and trimethylation at a region more upstream to the TSS of the *c-Fos* promoter, hereafter termed the “no promoter region” based on sequence homology analysis using the ECRbase (database for evolutionary conserved regions, promoters, and transcription factor binding sites in the vertebrate genome) [511] revealing a non- conserved region in the *c-Fos* gene among species (Appendix Fig.1).

Importantly, no differences in H3K9 modifications were observed between the E1 and E5 groups (Fig.16).

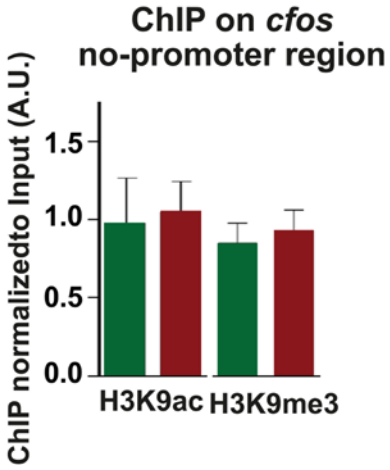

Figure 16: ChIP-analysis of H3K9 acetylation and trimethylation at *c-Fos* "no-promoter" region.

ChIP analysis of H3K9 acetylation and trimethylation revealed no differences during fear extinction in binding of the *c-Fos* promoter in a less-conserved region approximately 1200bp upstream of the transcriptional start site (TSS), defined here as "no-promoter" region. Error bars represent ± SEM.

One of the key enzymes that regulate H3K9 trimethylation is the histone methyl-transferase suppressor of variegation 3-9 homolog (SUV30H1) [509]. In turn, SUV39H1 is activated via deacetylation by SIRT1 (silent mating type information regulation 2 homolog), a Sirtuin belonging to Class III histone deacetylases, thereby regulating heterochromatin formation [509]. Additionally, SUV39H1 is known to interact with HDAC1 and can act together in concert with the HDAC1/mSIN3b co-repressor complex to mediate transcriptional repression of genes [106,500,512]. Taking these findings into account, I decided to investigate the levels of mSIN3b, SUV39H1 and SIRT1 at the *c-Fos* promoter during fear extinction. Remarkably, mSIN3b, SUV39H1 and SIRT1 showed significantly increased binding similar to HDAC1 at the *c-Fos* promoter at E5 compared to E1 (Fig. 17D, B).

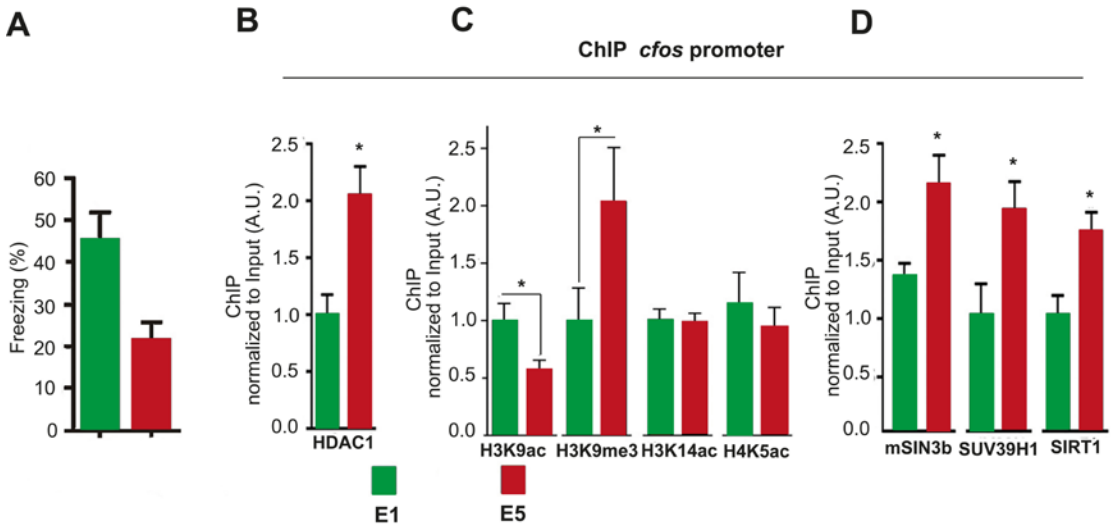

Figure 17: HDAC1 mediated chromatin remodeling at the *c-Fos* promoter during fear extinction.

A. Wild-type mice were subjected to fear extinction training and hippocampal tissue isolated 1 hour after E1 and E5 and processed in ChIP experiments. **B.** HDAC1 ChIP showed significantly increased HDAC1 binding on the *c-Fos* promoter at E5 compared to E1. **C.** ChIP analysis of different histone-modification sites. H3K9 acetylation was significantly decreased at E5 and H3K9 trimethylation significantly increased at E5 on the *c-Fos* promoter when compared to E1. **D.** Increased binding of transcriptional repressor proteins mSIN3b, SUV39H1 and SIRT1 at E5 compared to E1. n= 5/group; *p≤ 0.05; students t-test; Error bars represent ± SEM.

In conclusion, these data suggested that transcriptional repression of *c-Fos* observed at E5 of fear extinction is mediated via a mechanisms including deacetylation of H3K9 by HDAC1 in concert with the co-repressor protein mSIN3b and subsequent trimethylation of H3K9 via SUV39H1 and SIRT1 at the *c-Fos* promoter (Fig. 14 and Fig.17).

In order to test directly whether HDAC1 regulate specifically H3K9 acetylation and indirectly trimethylation of H3K9me3 at the *c-Fos* promoter and *c-Fos* expression during fear extinction, I further assessed chromatin modifications and *c-Fos* expression levels during fear extinction in our gain-of –function model overexpressing HDAC1-GFP and in the loss-of function systems in which mice either received intrahippocampal injections of MS-275 or HDAC1 siRNA.

To investigate chromatin modifications in HDAC1-GFP-AAV mice during fear extinction, I similarly conducted the experiment as described (Fig. 18A, 10, 11). However this time, I chose to sacrifice and isolate hippocampal tissue of HDAC1-GFP and GFP-AAV injected mice already at E3 rather than E5, since freezing levels

of HDAC1-GFP-AAV mice were already significantly reduced at this time point when compared to GFP-AAV mice (Fig. 18B).

Quantitative real-time PCR of *c-Fos* mRNA levels at E3 showed significantly reduced *c-Fos* expression in HDAC1-GFP-AAV injected mice compared to the GFP-AAV group of mice (Fig. 18C). Notably, the levels of H3K9 acetylation at the *c-Fos* promoter were significantly reduced in HDAC1-GFP mice when compared to the GFP-control group (Fig. 18C). Conversely, trimethylation of H3K9 levels were strongly increased (Fig. 18D). In agreement with the recruitment of HDAC1, mSIN3b, SUV39H1 and SIRT1 to the promoter of *c-Fos* at E5 when mice display successful fear extinction learning (Fig. 9,7), I detected increased levels of HDAC1, mSIN3b, SUV39H1 and SIRT1 on the *c-Fos* promoter at E3 in the HDAC1-GFP-AAV group (Fig. 18D).

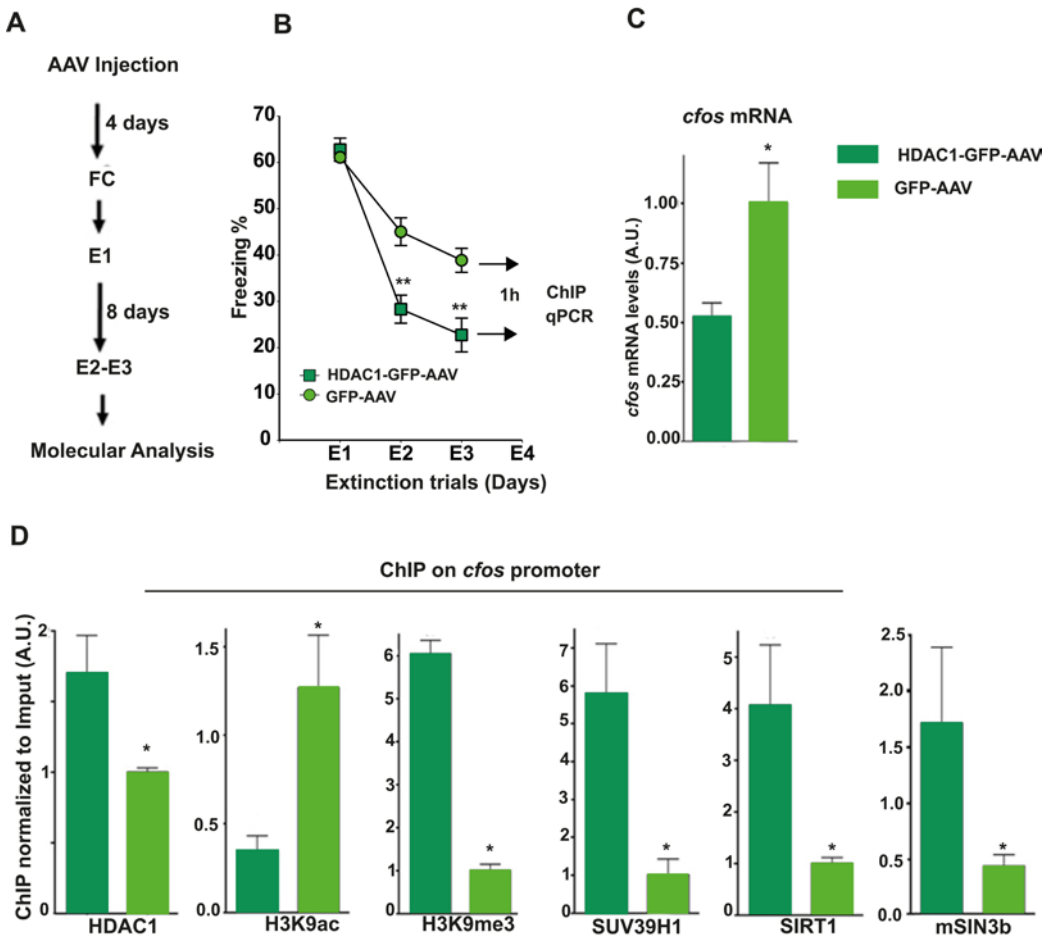

Figure 18: HDAC1 mediated regulation of H3K9 modifications and *c-Fos* expression during fear extinction.

A. Experimental design for B, C and D. **B.** Fear extinction performance of HDAC1-GFP and GFP-AAV mice. While freezing levels of HDAC1-GFP and GFP-AAV mice were similar at E1, HDAC-

GFP AAV mice display significantly reduced fear extinction upon fear extinction at upon E2-E3. **C.** qPCR showed significantly reduced hippocampal *c-Fos* expression levels in HDAC1-GFP AAV mice at E3 compared to GFP-AAV mice. **D.** ChIP analysis of the *c-Fos* promoter with HDAC1, H3K9 acetylation (H3K9ac), H3K9 trimethylation (H3K9me3), SUV39H1, SIRT1 and mSIN3b. n= 10/ group; *p≤ 0.05; **p≤0.001; students t-test; Error bars represent ± SEM.

Next, I analyzed these chromatin modifications in the two loss-of-function models and conducted the experiments similarly as previously described (Fig. 18B, 19). In line with my previous described results, inhibition of hippocampal HDAC1 enzyme activity by MS-275 and knockdown of hippocampal HDAC1 protein by administration of HDAC1 siRNA impaired fear extinction learning (Fig. 19, 10A, 11B). Notably, *c-Fos* mRNA expression at E5 in both experiments was consistently increased in MS-275 and HDAC1 siRNA injected mice compared to *c-Fos* expression in the corresponding control groups at E5 (Fig. 19B, D). Using ChIP analysis, I observed significantly increased H3K9 acetylation and decreased H3K9 trimethylation levels at E5 in the loss-of gain models, correlating with increased hippocampal *c-Fos* mRNA expression in MS-275 and HDAC1 siRNA treated mice at E5 when compared to the corresponding control groups (Fig. 19 B,C,D,E). Furthermore, HDAC1 *c-Fos*-promoter binding was significantly reduced when analyzed in HDAC1 siRNA injected mice compared to the control group (Fig. 19E).

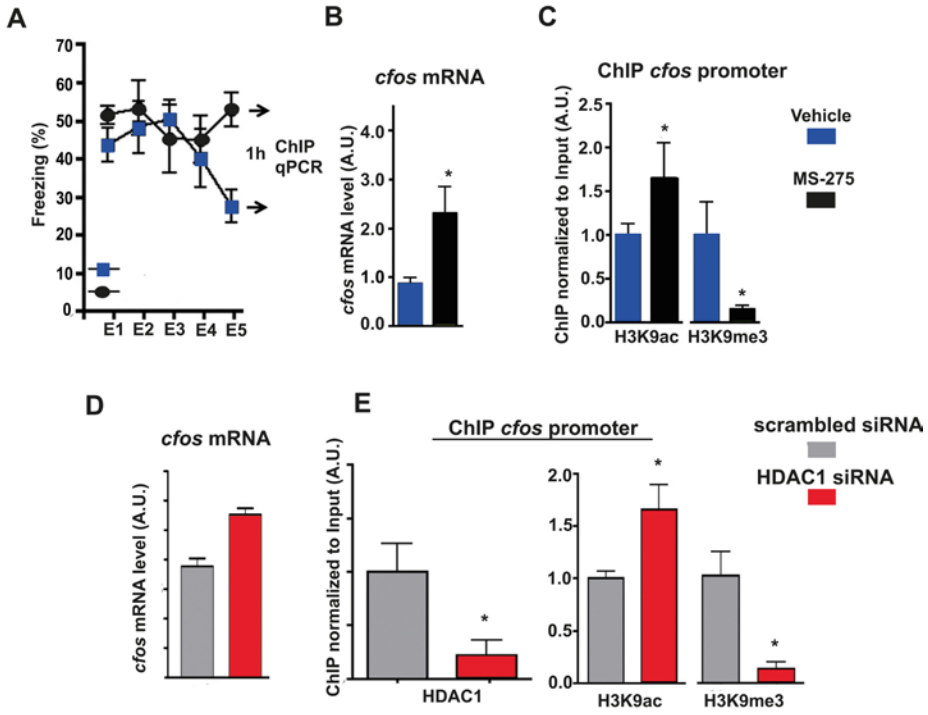

Figure 19: Inhibition of HDAC1 prevents transcriptional repression of *c-Fos* and deacetylation of H3K9 at the *c-Fos* promoter.

A. Mice that received intrahippocampal injections of MS-275 after each extinction trial showed impaired fear extinction when compared with vehicle-treated mice. **B.** qPCR analysis showed increased hippocampal *c-Fos* expression levels at E5 in MS-275-treated mice when compared to the vehicle group. **C.** ChIP analysis on the *c-Fos* promoter revealed increased H3K9 acetylation and reduced H3K9 trimethylation in MS-275-injected mice when compared with the vehicle group at E5. **D.** qPCR analysis in mice that received intrahippocampal injections of HDAC1 siRNA showed significant reduction in hippocampal *c-Fos* expression when compared to the control group injected with scrambled siRNA. **E.** HDAC1 siRNA-treated mice showed reduced HDAC1 levels and increased H3K9 acetylation and decreased H3K9 trimethylation at the *c-Fos* promoter when compared to scrambled siRNA-treated mice. n= 5/ group; *$p \leq 0.05$; students t-test; Error bars represent ± SEM.

In conclusion, while AAV-mediated overexpression of neuronal HDAC1 in the hippocampus of adult mice after 14 days did not led to an overt detrimental phenotype (Fig. 7, 8), hippocampal HDAC1 was shown to be involved in hippocampus-dependent fear extinction learning since elevated hippocampal HDAC1 facilitated extinction of fear memories and inhibition of HDAC1 using the HDACi MS-275 and HDAC1 specific siRNA impaired fear extinction. Furthermore, qPCR and ChIP analysis suggested that HDAC1-dependent transcriptional repression of gene-expression is required for the extinction of fear memories by the deacetylation and subsequent trimethylation of target genes.

4.2 Elevated HDAC1 in the prefrontal cortex of mice induces schizophrenia-like symptoms and cognitive endophenotypes

4.2.1 AAV-mediated neuronal overexpression of HDAC1 in the Prefrontal Cortex of adult mice results in cognitive schizophrenia endophenotypes

Two studies have reported elevated HDAC1 mRNA expression in post-mortem brain tissue of individuals diagnosed with schizophrenia. The first identified elevated HDAC1 expression in microdissected GABAergic neurons of the CA2/CA3 region in the hippocampus of schizophrenic subjects [433], and the second reported increased HDAC1 expression in the prefrontal cortex of individuals with schizophrenia [435].
However in in the first part of my study, when HDAC1 was overexpressed in the hippocampus of adult mice, no cognitive endophenotypes of schizophrenia were observed. Based on the study from Sharma et al, reporting elevated HDAC1 levels in the prefrontal cortex, the possibility remained that virus-mediated overexpression of HDAC1 in the prefrontal cortex of adult mice would result into schizophrenia-like symptoms and cognitive endophenotypes.

To test this possibility, I used the same AAV generated and applied in the first part of my thesis and injected it in the prefrontal cortex of adult mice. Mice expressing GFP alone served as controls. HDAC1-GFP AAV and GFP AAV-injected mice were then subjected again to behavioral tests that assess anxiety and depressive-like behavior (OF, FST), sensorimotor gating function (PPI), working memory and formation of long-term memories (NOR, FC-Ext) (Fig. 20A). Behavioral tests were again conducted after 14 days of injection, since we defined this time-point for optimal virus expression as detected by molecular analysis (Fig. 5,6).
Interestingly, HDAC1 overexpression in the prefrontal cortex caused schizophrenia-like behaviors and cognitive schizophrenia endophenotypes (Fig. 20,21,22,23). Thus, mice injected with HDAC1-GFP AAV in the prefrontal cortex showed increased basal anxiety in the open field test (OF) when compared to control mice, while

explorative activity was not affected (Fig. 20B). In the forced swim test (FST), HDAC1-GFP-AAV mice showed increased depressive-like behavior as the floating time of these mice was significantly less than the control group (Fig 20C). Working memory performance in the novel object recognition test (NOR), which is impaired in patients with schizophrenia and unaffected relatives, and constitutes as a well-defined cognitive endophenotype of schizophrenia, was significantly impaired in HDAC1-GFP mice when compared to GFP-AAV mice (Fig. 20D). Moreover, working memory performance was below the 50 % chance level for new object preference in the HDAC1-GFP-AAV group, indicating that HDAC1-GFP mice could not discriminate between the new object presented and the old object. Additionally, in the long-term memory test of the novel object recognition test, HDAC1-GFP-AAV subjects also performed below 50% chance level, though it did not reach significance vs. GFP AAV controls (Fig. 20E). Importantly, GFP-AAV mice showed higher than 50% preference for the new object in both short- and long-term memory tests, indicating that they could properly learn the test and discriminate the old from the new object (Fig. 20D, E).

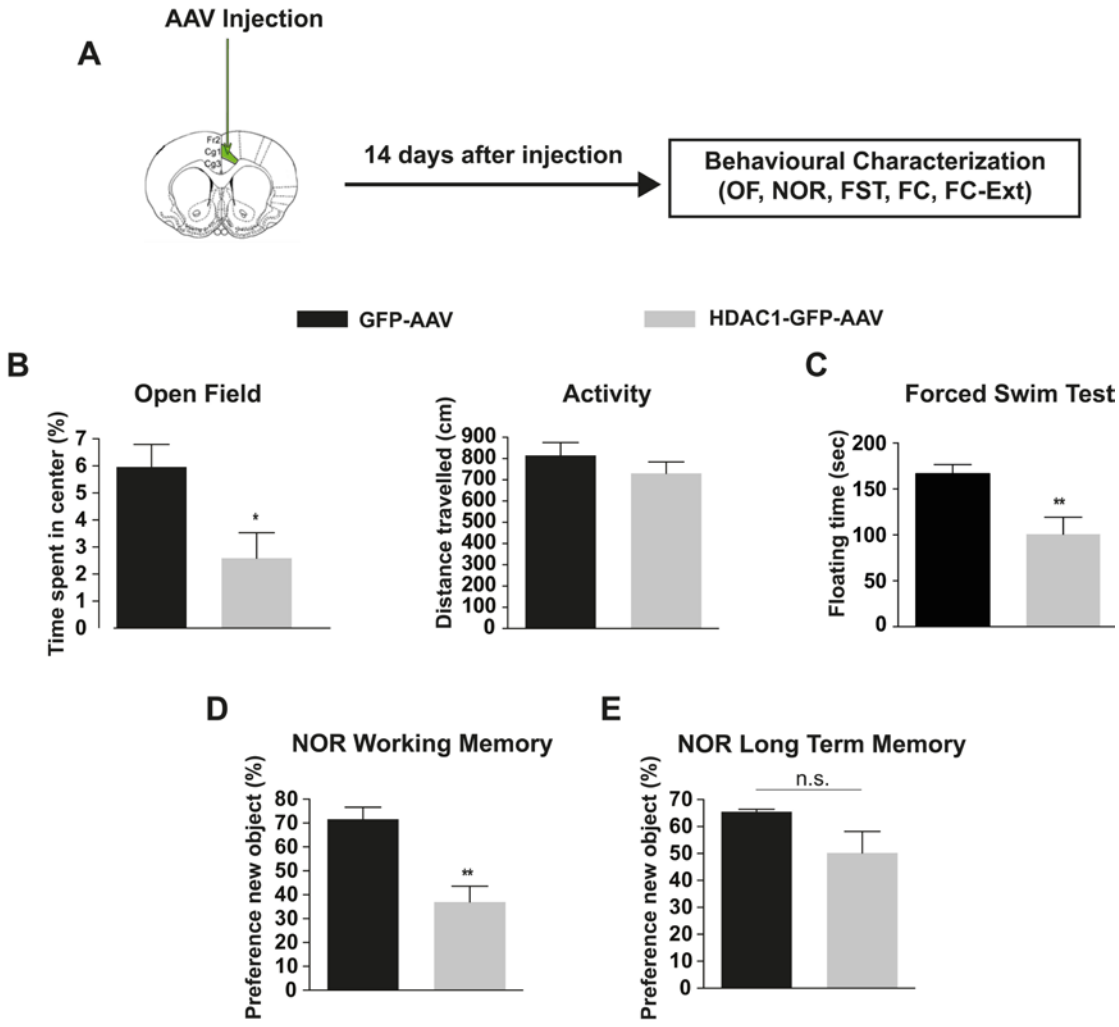

Figure 20: Neuronal over-expression of HDAC1 in the PFC induces anxious and depressive-like behavior and working memory impairment in adult mice.

A. HDAC1-GFP AAV were injected in the medial prefrontal cortex of 10 weeks old C57/B6J mice and subjected to behavioral characterization 14 days after injection, a time-point where HDAC1-GFP-AAV is strongly expressed. **B.** Basal anxiety, locomotor and explorative activity were tested using the Open Field test. Left panel: HDAC1-GFP mice spent significantly less time in the center of the OF and showed increased basal anxiety compared to control GFP-AAV mice. Right panel: HDAC1-GFP and GFP-AAV mice show similar locomotor and exploratory activity in the OF. **C.** Increased depressive-like behavior of HDAC1-GFP-AAV mice assessed in the Porsolt-Forced-Swim Test (FST). HDAC1-GFP-AAV mice stayed significantly longer in an immobile state compared to GFP-AAV. **D.** HDAC1-GFP-AAV mice showed significantly less preference for the novel object in the working memory test compared to GFP-AAV mice. **E.** Long-term memory in the NOR was not significant different among groups. *$p \leq 0.05$, ** $p \leq 0.01$; students two-tailed t-test vs. GFP-AAV mice. Error bars represent ± SEM; n= 12 GFP-AAV/ 17 HDAC1-GFP.

In another group of mice injected with HDAC1-GFP-AAV and GFP-AAV I tested sensorimotor gating function using the prepulse inhibition of startle response test (PPI). HDAC1-GFP-AAV mice displayed reduced PPI compared to GFP-AAV mice, which is also observed in schizophrenia patients, indicating disrupted sensorimotor

gating function (Fig. 21A). No significant difference was observed in the startle response of mice (Fig. 21B).

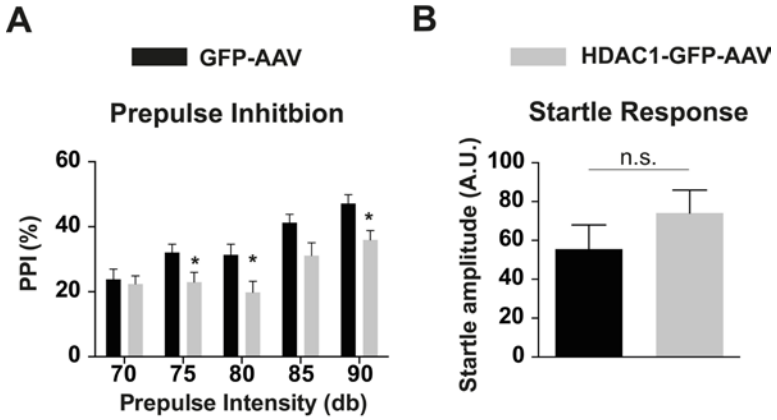

Figure 21: Impaired sensorimotor gating of the acoustic startle response in mice over-expressing neuronal HDAC1 in the PFC of adult mice.

A. Percentage of PPI in GFP-AAV and HDAC1-GFP-AAV mice is shown for each prepulse intensity. HDAC1-GFP-AAV mice are impaired in the prepulse inhibition test, showing decreased PPI at 75, 80 and 90 db. Two-way ANOVA of repeated measures showed a significant effect of Virus expression [F (4, 27)= 5.640, p = 0.0249] and significant effect of Prepulse Intensity [F (4, 108)= 2.123, p = 0.0829]. Bonferroni post-hoc test revealed significant impaired PPI in HDAC1-GFP mice at 75 db, (p=0,0432507), 80 db (p=0,010581), 85 db (p= 0,0242066), 90 db (p= 0,0242066). **B.** The acoustic startle response to 120 db sound did not differ between GFP-AAV and HDAC1-GFP-AAV mice (p = 0.3093, Mann-Whitney test). Error bars represent ± SEM; = 12 GFP-AAV/ 17 HDAC1-GFP.

Notably, the same group of mice was subjected again to the PPI test 9 months later (at 12 months of age) and showed still impaired PPI compared to GFP-AAV mice (Fig 22).

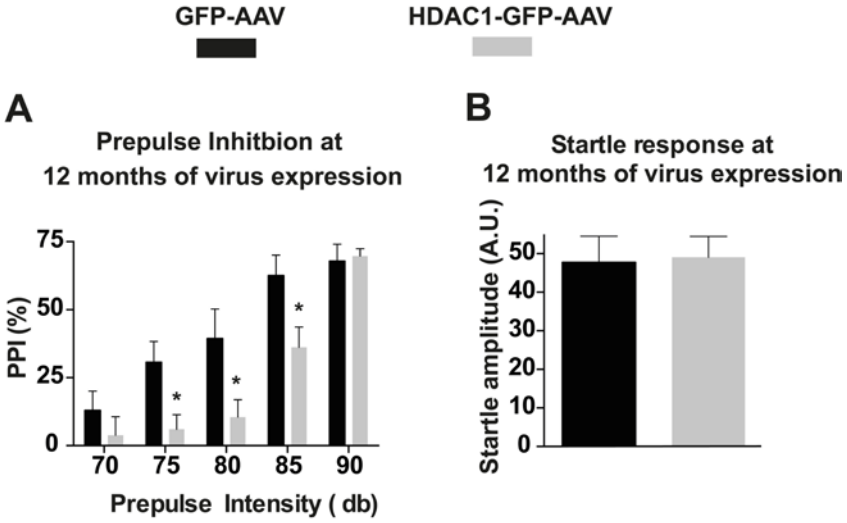

Figure 22: Sensorimotor gating function is still impaired in mice overexpressing HDAC1 in the prefrontal cortex of mice at 12 months of age,

A. HDAC1-GFP AAV mice at 12 months of age show significantly reduced PPI when compared to the control group. Two-way ANOVA of repeated measures showed a significant effect of Virus expression [F (1, 12)= 6.192, p = 0.0285], significant effect of Prepulse Intensity [F (4, 48)= 37.35,$p\leq$ 0.0001] and

significant virus x prepulse intensity interaction [F (4, 48)= 2.706, p = 0.0411]. Bonferroni post-hoc test revealed significant impaired PPI in HDAC1-GFP mice at 75 db, (p=0,369385), 80 db (p=0,00564202), 85 db (p= 0,0110542). **B.** The acoustic startle response to 120 db sound did not differ between GFP-AAV and HDAC1-GFP-AAV mice (p = 0,9083, Mann-Whitney test). Error bars represent ± SEM; n = 7 GFP-AAV/ 6 HDAC1-GFP. Error bars represent ± SEM.

AAV-injected mice that were previously subjected to OF, NOR, and FST were later subjected to fear conditioning (FC) followed by fear extinction training. HDAC1-GFP-AAV mice showed impaired associative memory in the FC test, showing significantly reduced freezing response to the context (Fig. 23A). Subsequently, during fear extinction training, fear response of HDAC1-GFP AAV mice did not decline over the course of extinction training and remained close to that displayed at E1 (Fig. 23B). On the contrary, GFP-AAV mice underwent successful fear extinction learning as their freezing response at E5 was significantly reduced compared to E1 (Fig. 23B right panel). Thus, HDAC1-GFP-AAV mice showed significantly higher freezing levels at E5 compared to GFP-AAV mice at E1 (Fig. 23B right panel).

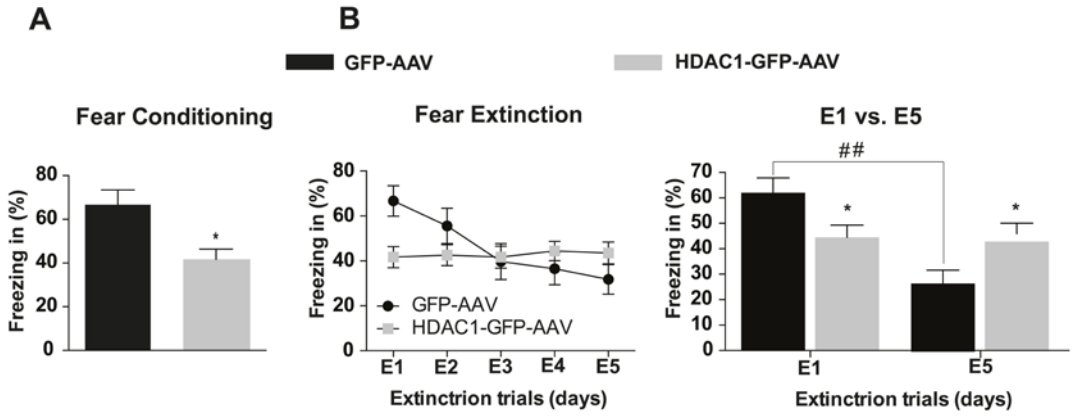

Figure 23; Impaired associative memory and fear extinction in mice over-expressing neuronal HDAC1 in the PFC.

A. Impaired associative memory of HDAC1-GFP-AAV mice in the contextual fear-conditioning test. HDAC1-GFP-AAV mice display significantly lower freezing level upon re-exposure to the context 24 hours after receiving an electrical foot-shock of 0.7 mA compared to GFP-AAV mice. **B.** Fear extinction learning performance of HDAC1-GFP-AAV and GFP-AAV mice. Left panel: Freezing of control GFP- AAV mice declines during the course of fear extinction training, while freezing levels of HDAC1-GFP-AAV mice remain nearly unchanged from E1 to E5. Right panel: HDAC1-GFP-mice display significantly lower freezing levels at E1 and E5 compared to GFP-AAV mice at E1 and E5. GFP-AAV mice undergo successful fear conditioning learning with significantly decreased freezing levels from E1 to E5. *p≤; students two-tailed t-test vs. GFP-AVV. Error bars represent ± SEM; n = 8 GFP-AAV/ 7 HDAC1-GFP.

In summary, AAV-mediated neuronal overexpression of HDAC1 in the prefrontal cortex of mice resulted in schizophrenia-like symptoms, such as increased anxiety and

depressive-like behavior and impaired fear extinction and cognitive endophenotypes such as impaired working memory performance and deficits in sensorimotor gating function. Thus, elevated HDAC1 in the prefrontal cortex might contribute to the pathogenesis of schizophrenia.

4.2.2 Early life stress and social isolation rearing in mice mediate cognitive endophenotypes of schizophrenia and regulate expression of HDAC1 in the prefrontal cortex

Considering my findings that elevated HDAC1 levels in the prefrontal cortex mimic schizophrenia-like behaviors in mice and the fact that HDAc1 is overexpressed in the prefrontal cortex of schizophrenia patients, I wondered about the cause of elevated HDAC1 levels in patients.

Exposure to adverse events early in life and chronic psychological stress have been identified by epidemiological studies as environmental risk factors and suggested as "second hits" in the development of neuropsychiatric disorders. According to the neurodevelopmental hypothesis of schizophrenia from Weinberger, certain environmental factors early in life influence developmental processes in the brain that develop into a phenotype once the brain reaches full maturation [513,514].

Thus, such events are thought to negatively affect neurodevelopmental processes and interact with genetic predisposition factors, triggering the outcome of neuropsychiatric disorders such as schizophrenia. The prefrontal cortex reaches anatomical and functional maturity in early adulthood, and abnormalities in the prefrontal cortex are typically implicated in schizophrenia [334,515,516].

Among most of the early life stress procedures applied in rodents in laboratories, maternal deprivation by separating the mother from the offspring and social isolation rearing are the most studied. Thus, maternal separation and social isolation rearing are considered to be valuable paradigms in order to investigate pathophysiological changes observed in schizophrenia and I decided to test the hypothesis that early life stress induced by maternal separation could be one cause that mediates elevated HDAC1 levels [517–519].

However, while it is impossible to model the whole disease, including positive and negative symptoms of schizophrenia, some cognitive deficits such as working

memory and sensorimotor gating function deficits are tractable in rodents. As such, maternal separation and social isolation rearing have been shown to disrupt prepulse inhibition, causing working memory deficits and increased anxiety- and depressive-like behavior [361,520–523]. Nevertheless, the majority of these studies have been carried out in the rat and to date, there have been few investigations of the effects of maternal separation and social isolation rearing in the mouse. Moreover, most of the maternal separation protocols applied in mice resulted in anxiety and depressive-like behavior but not in deficits in PPI [524–526].

However, it has been recently suggested that combining maternal separation with other stressors, such as social isolation rearing and modifications in the period and extension in which maternal separation takes place, could contribute to more robust, long-lasting changes that further resemble schizophrenia-like symptoms [527–529].

Thus, recent published data from Niwa et al, combining maternal separation during the third postnatal week (postnatal day 15-21) together with social isolation rearing in mice, demonstrated schizophrenia-like symptoms such as increased anxiety, depressive-like behavior and deficits in working memory performance and prepulse inhibition of startle response [362].

Therefore, in order to further investigate the effect of environmental risk factors of schizophrenia on the expression of HDAC1, I decided to subject mice to the protocol applied in the study of Niwa et al., which I named hereafter as “Social Disturbance”. In agreement with previous published data of Niwa et al [362], mice subjected to social disturbance showed increased basal anxiety in the open field test, as they spent significantly less time in the center of the open field when compared to control mice (Fig. 24B). Explorative activity, measured by the distance travelled in the open field arena was not affected between the groups (Fig. 24B, left panel). When subjected to the elevated-plus-maze (EPM), another paradigm to address anxious behavior in rodents, SD mice also showed increased anxiety compared to control mice (Fig. 24C). Furthermore, SD mice displayed depressive-like behavior in FST, floating significantly less time then control mice (Fig. 24D). Moreover, working memory performance of SD mice was significantly impaired in the NOR test when compared to control mice. However, SD mice performed significantly over the 50% chance level, indicating that SD mice learned to discriminate between the old and the new

object presented (Fig. 24E). No significant differences were observed among groups when tested for long-term memory of the NOR test (Fig. 24F).

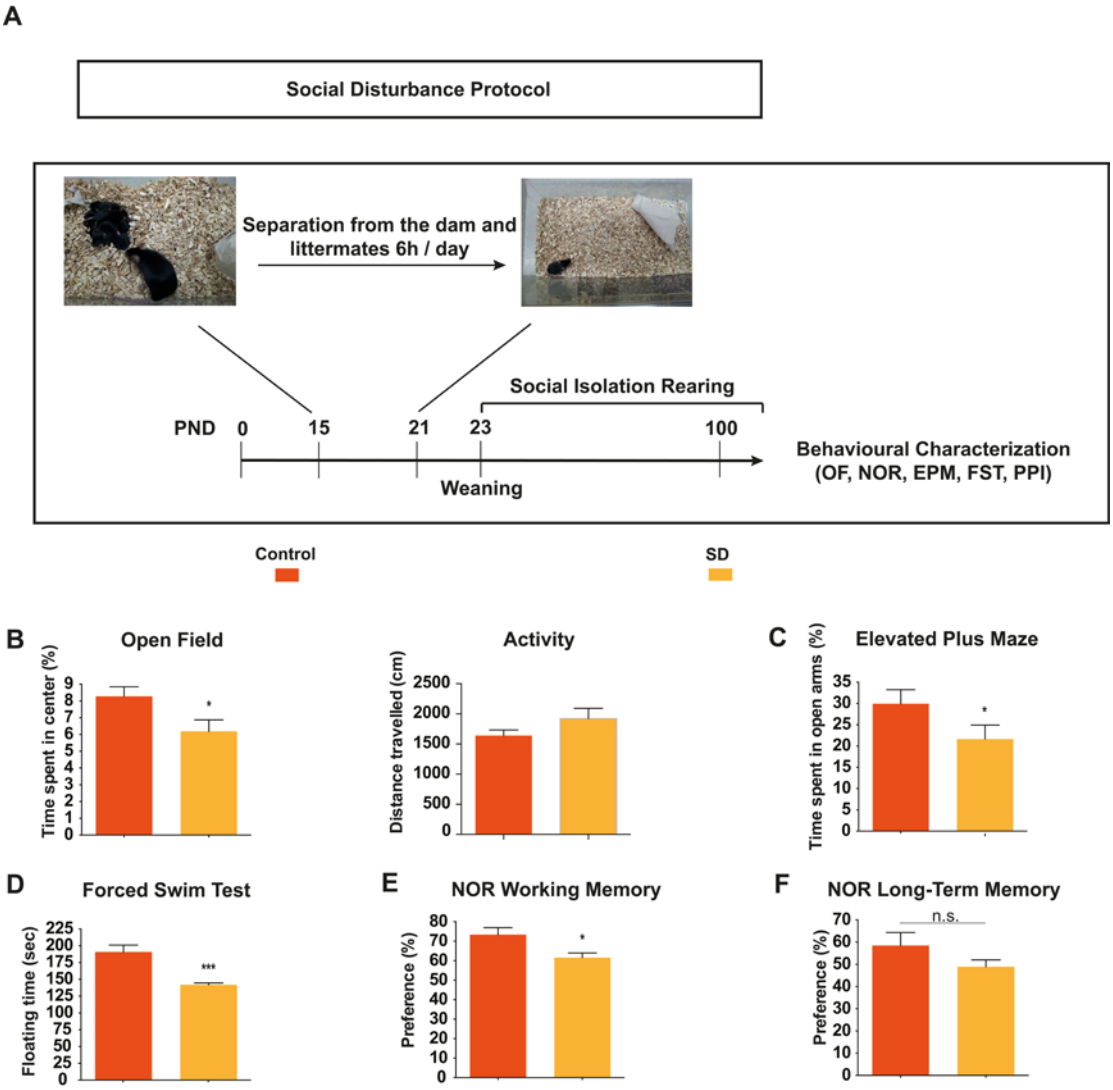

Figure 24: Mice subjected to social disturbance show anxious-and depressive-like behavior and working memory impairments.

A. Experimental design of the social disturbance protocol acquired and modified from Niwa M et al., 2011, followed by behavioral characterization. From postnatal day (PND) 15, pubs were removed from their mothers and home cages and kept isolated from littermates into individual cages. After 6 hours, pubs were returned together with littermates to their mothers. Social disturbance (SD) protocol was performed between PND 15 and 21. On PND 23, pubs were weaned and kept isolated until sampling after behavioral characterization with. Control mice were grouped housed and separated from their mothers for 10 min each day (handling). **B.** Left panel: Mice subjected to social disturbance show increased basal anxiety in the open field test. Right panel: Mice show no difference in distance travelled. **C.** Percent time spent the in open arms of the elevated plus maze (EPM). SD mice show increased anxiety spending significant less time in the open arms of the EPM compared to control mice .**D.** Performance of mice in the FST with decreased active floating of SD mice showing depressive-like behavior. **E.** Working memory performance of mice in the NOR test. SD mice show significant less preference to the new object compared to control mice. **F.** Object recognition of mice in the long-term memory test of the NOR experiment. No significant difference between control and SD mice was displayed during the long-term memory test of NOR. *p≤ 0.05, ** p≤ 0.01, *** p≤ 0.001; students two-tailed t-test vs. controls. Error bars represent ± SEM; n= 15/ group.

Further on, social disturbance in mice significantly reduced PPI without affecting the baseline startle amplitude (Fig. 25 A, B).

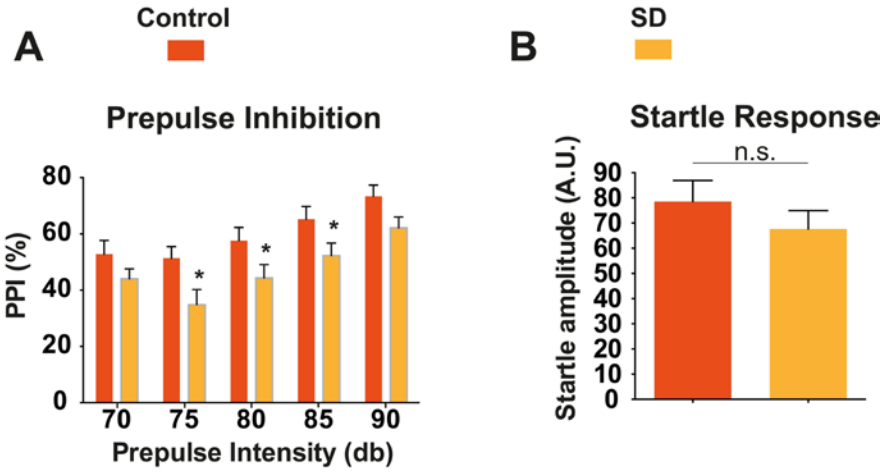

Figure 25: Sensorimotor gating deficits in mice subjected to social disturbance

A. Social disturbance in mice significantly reduced PPI. Two-way ANOVA of RM showed a significant effect of SD [F (4, 41)= 5.341, * p = 0.0259] and significant effect of Prepulse Intensity [F (4, 164)= 2.123, ***p < 0.0001]. Bonferroni post-hoc test revealed significant impaired PPI at 75 db (p= 0.0089), 80 db (p=0.0356) and 85 db (p= 0.0389). **B.** Startle response of mice. Social disturbance in mice did not affect baseline startle amplitude (p= 0.3682; Mann-Whitney-test). Error bars represent ± SEM; n= 21/group.

Taken together, I was able to reproduce the phenotype induced by the protocol applied in the study of Niwa et al in mice of our laboratory, showing cognitive endophenotypes and schizophrenia-like symptoms with increased anxiety and depressive-like behavior (OF, EPM, FST), impaired working memory (NOR) and reduced sensorimotor gating function (PPI).

Based on the results presented above, I decided to apply the protocol of Niwa et al, as a valuable paradigm to model cognitive deficits of schizophrenia in mice and to further investigate if the expression of HDAC1 is influenced by environmental risk factors of schizophrenia.

Interestingly, mice subjected to SD had significantly increased HDAC1 mRNA expression levels in the prefrontal cortex compared to control mice, similar to the elevated HDAC1 mRNA levels observed in post-mortem prefrontal cortex samples of schizophrenia subjects from our laboratory and Sharma et al. (Fig. 26B, D).

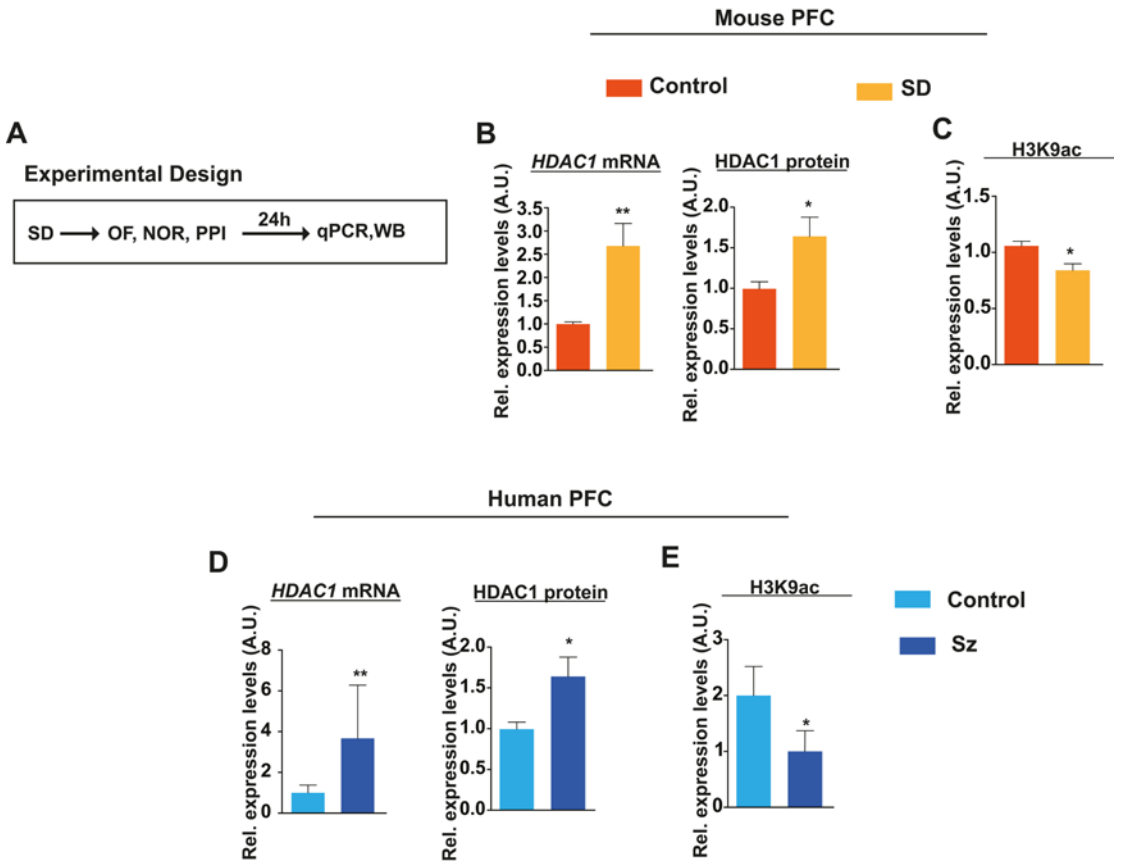

Figure 26: HDAC1 expression in human PFC post-mortem tissue and in PFC of mice subjected to social disturbance.

A. Experimental design. Social disturbance mice underwent behavioral characterization and hippocampal and prefrontal cortical tissue isolated 24 hours after the last behavioral test for molecular analysis with qPCR and WB. **B.** qPCR (right panel) and quantitative immunoblot analysis (left panel) revealed significantly increased HDAC1 expression in the prefrontal cortex of SD mice when compared to the control group (n= 5/group). **C.** Quantitative immunoblot analysis of H3K9 acetylation showed a significant reduction in mice subjected to SD compared to control mice (n= 5/group). **D.** Left panel: qPCR analysis in human post-mortem prefrontal cortex samples showed elevated *Hdac1* mRNA expression in schizophrenia compared to control subjects. Right panel: Quantitative immunoblot analysis revealed increased HDAC1 protein levels in individuals with schizophrenia when compared to age-matched controls (n=6/group). **E.** Significantly reduced bulk changes in H3K9 acetylation in the prefrontal cortex of schizophrenia compared to control subjects (n=6/group). *p≤ 0.05, ** p≤ 0.01; students two-tailed t-test vs. controls. Error bars represent ± SEM.

Interestingly, SD did not significantly affect the expression levels of other class I HDACs, in line with the observations inhuman post-mortem samples (Fig. 27).

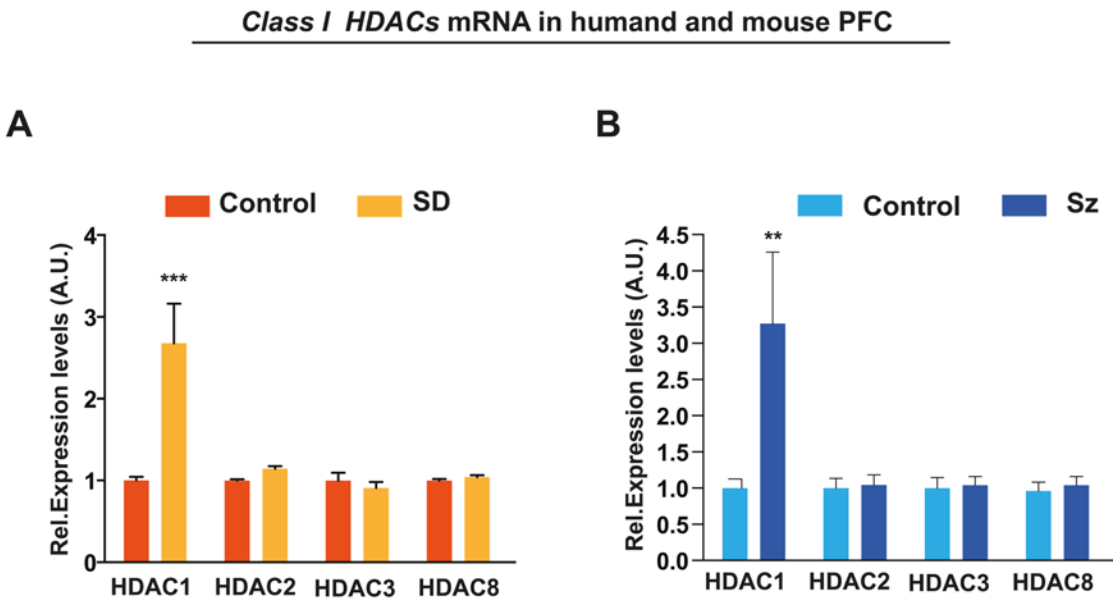

Figure 27: Class I *Hdac* expression in the prefrontal cortex of mice subjected to social disturbance and individuals with schizophrenia.

A. qPCR analysis of class I *Hdacs* in the prefrontal cortex of mice showed significant increased *Hdac1* mRNA levels while the other class I HDACs *Hdac2, HDAC3* and *HDAC8* revealed no differences (n=5/group). **B.** qPCR analysis displayed elevated *Hdac1* mRNA levels in the prefrontal cortex of human post-mortem samples (n=6/group). ** p≤ 0.01, *** p≤ 0.001; students two -tailed t-test vs. controls. Error bars represent ± SEM.

Moreover, the increase in HDAC1 mRNA expression was specific to the prefrontal cortex of mice, since no significant change was observed for HDAC1 expression in the hippocampus of SD mice (Fig. 28A).

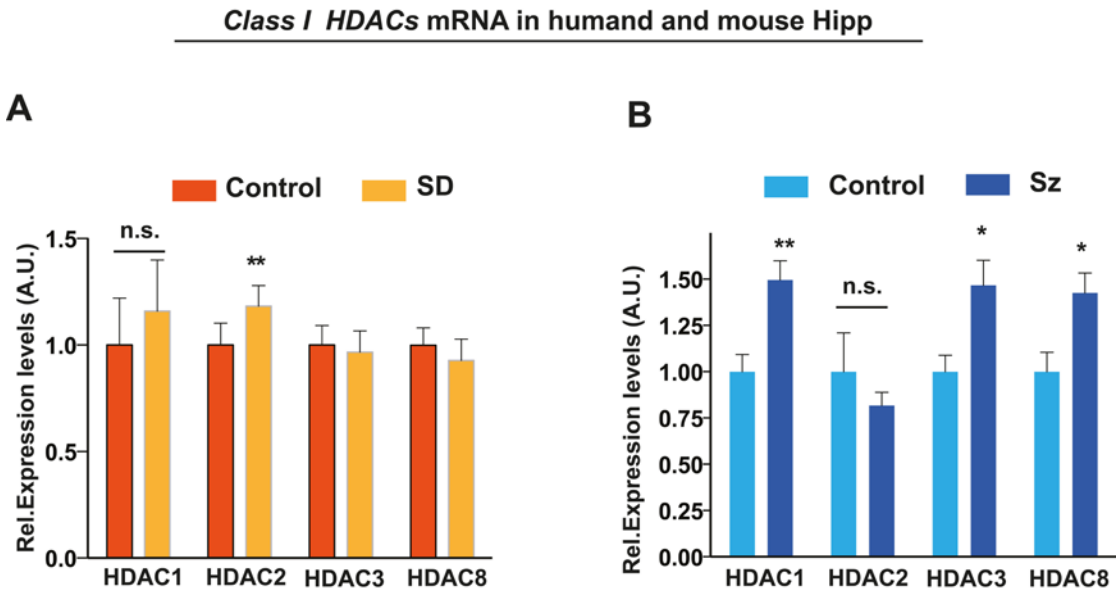

Figure 28: Class I *Hdac* expression in the hippocampus of mice subjected to social disturbance and individuals with schizophrenia.

A. qPCR analysis of class I *Hdacs* in the hippocampus showed no differences in expression levels (n=5/group). **B.** qPCR analysis displayed elevated *Hdac1*, *Hdac3* and *Hdac8* mRNA levels in the prefrontal cortex of human post-mortem samples (n=6/group). *p≤ 0.05; ** p≤ 0.01, students two-tailed t-test vs. controls. Error bars represent ± SEM.

The increase of elevated HDAC1 mRNA expression was as well observed on the protein level, showing increased HDAC1 protein expression in the prefrontal cortex of schizophrenia subjects and mice subjected to SD (Fig. 26 B, D right panel). HDAC1 activity has been previously linked to the regulation of H3K9ac [168,209,530] and shown to mediate transcriptional repression of the susceptibility genes *Gad67* and *Reelin* through binding to the corresponding promoters [210]. Furthermore, ChIP-Seq analysis in neurons and stem cells has revealed a specific enrichment of H3K9 at gene promoters and is thought to be a marker of active gene expression [168,531]. In light of the results of from the first part of my thesis showing HDAC1 deacetylating H3K9 through the course of fear extinction (Fig. 17C; 19C, E), I wondered whether there would be changes in H3K9 acetylation in the prefrontal cortex of SD mice and schizophrenia subjects. Interestingly, immunoblot analysis revealed a significant reduction in the bulk levels of H3K9ac in the prefrontal cortex of SD mice and human schizophrenia samples (Fig. 26C, E). Thus, the increase in HDAC1 protein expression correlated with reduced H3K9 acetylation in the prefrontal cortex of SD mice and schizophrenia subjects (Fig. 26B, C, D, E).

In conclusion, exposing mice to social disturbance as an early environmental risk factor induced cognitive endophenotypes of schizophrenia and mediated the up-regulation of prefrontal cortical HDAC1 mRNA and protein levels. This data mimics the situation observed in post-mortem brain samples of schizophrenia patients.

4.2.3 Chronic administration of the HDAC inhibitor MS-275 rescues deficits in prepulse inhibition of startle response in mice subjected to social disturbance

Recently, a study from Engmann et al showed that chronic administration of MS-275 alleviated schizophrenia-like symptoms in transgenic mice lacking the protein p35, a neuron-specific activator of CDK5 (cyclin-dependent kinase 5) [532]. In detail, intraperitoneal injections of MS-275 over 10 days rescued deficits in PPI observed in p35 knockout mice [532] Additionally, subcutaneous injections of MS-275 in naïve mice induced a brain region-specific increase in H3 acetylation [209]. As such, low doses (15 μmol/kg) of MS-275 induced maximal H3 acetylation in the frontal cortex, whereas higher doses (60 μmol/kg) were required in order to induce H3 acetylation in the hippocampus. No induction was observed in the striatum. Importantly, the MS-275-induced increase in histone-acetylation was specific to H3 and did not affect Histone H4 acetylation (Simonini et al., 2006). The reported MS-275 specificity is supported by another study from our laboratory, showing that intrahippocampal injections of MS-275 increased H3K9 but not H4K12 acetylation [168]

Based on these findings and my results, showing increased HDAC1 expression levels and decreased H3K9 acetylation in the prefrontal cortex of mice subjected to social disturbance, I decided to treat SD mice with intraperitoneal injections of MS-275 to see if this could induce functional recovery. As an initial characterization, I designed a pilot experiment in which I injected naïve mice intraperitoneally with MS-275 for 10 days according to the protocol described in the study of Engmann et al (Fig. 29A), in order to test whether HDAC1 inhibition in naïve mice would alter PPI. This experiment would also serve to test whether I would observe changes in H3 acetylation levels specifically in the prefrontal cortex of mice, as reported by Simonini et al [209].

Intraperitoneal administration of MS-275 in mice did not affect PPI or startle response of mice, as two-way ANOVA did not reveal a significant overall effect of drug injection (Fig. 29B, C). Interestingly and in line with the data reported by Simonini et al, administration of MS-275 induced an increase in H3K9 acetylation in the prefrontal cortex of mice, while no significant change was observed in H3 acetylation in the hippocampus (Fig. 29 D,E).

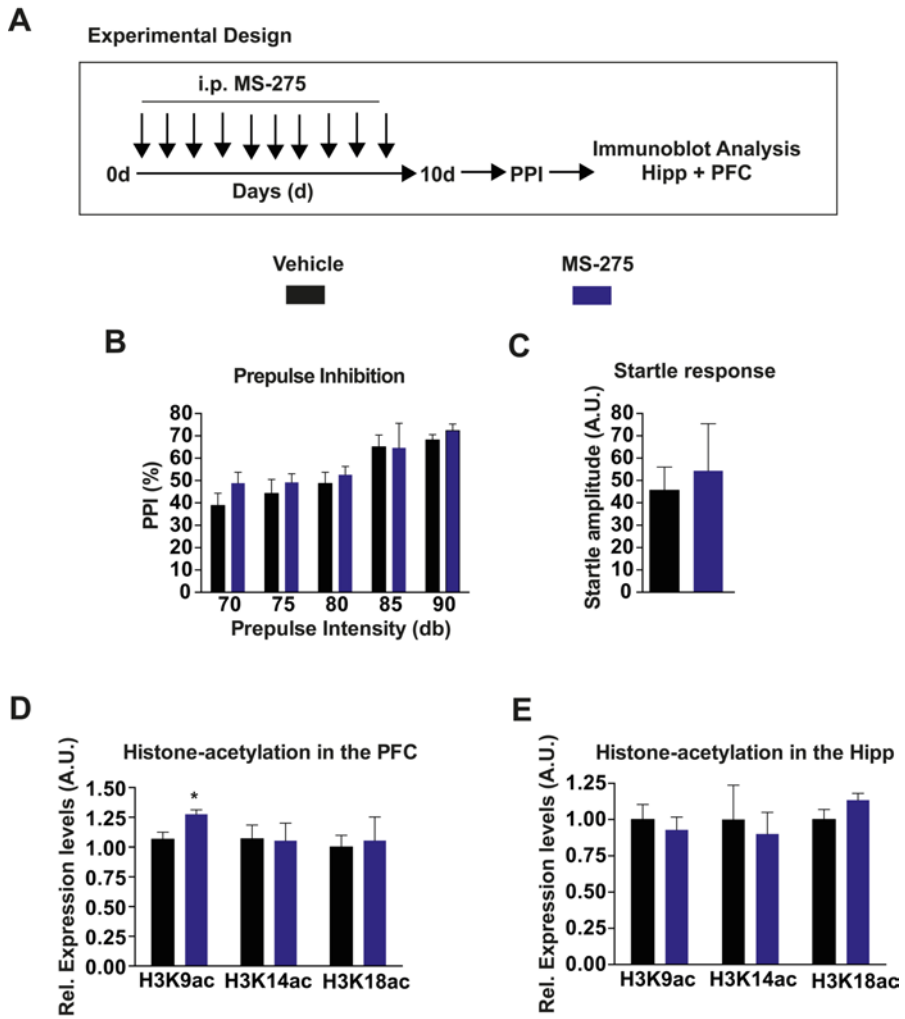

Figure 29: Prepulse inhibition of startle response in wild-type mice subjected to chronic MS-275 treatment.

A. Experimental design. Wild-type mice received intraperitoneal injections of MS-275 for 10 days and subjected to PPI immediately after the last injection. Following PPI, mice were sacrificed and hippocampal and prefrontal cortex tissue isolated for immunoblot analysis. **B.** Mice treated with MS-275 or Vehicle for 10 days did not show differences in PPI. **C.** Baseline startle response was not altered among groups. **D.** Quantitative immunoblot analysis showed significant increase in H3K9 acetylation in the prefrontal cortex of MS-275 treated mice. **E.** No differences in bulk acetylation levels of H3K9, H3K14 and H3K18 were detected in hippocampi of Vehicle and MS-275 treated mice. n=6/group; *p≤ 0.05; students two-tailed t-test vs. controls. Error bars represent ± SEM.

Thus, I went on and subjected mice that previously underwent the social disturbance protocol and were characterized for cognitive endophenotypes in the OF, NOR and PPI, to intraperitoneal injections of MS-275 for 10 days (Fig.30A). Immediately after the last injection, mice were subjected to PPI. Four hours after PPI, mice were sacrificed and prefrontal cortices isolated for molecular analysis using qPCR and ChIP. While SD mice treated with vehicle still displayed significantly reduced PPI compared to control mice treated with Vehicle (Fig. 30B), SD mice injected with MS-275 showed significantly increased PPI when compared to SD mice treated only with vehicle (Fig. 30C). No significant changes in startle response were observed among experimental groups (Fig. 30E).

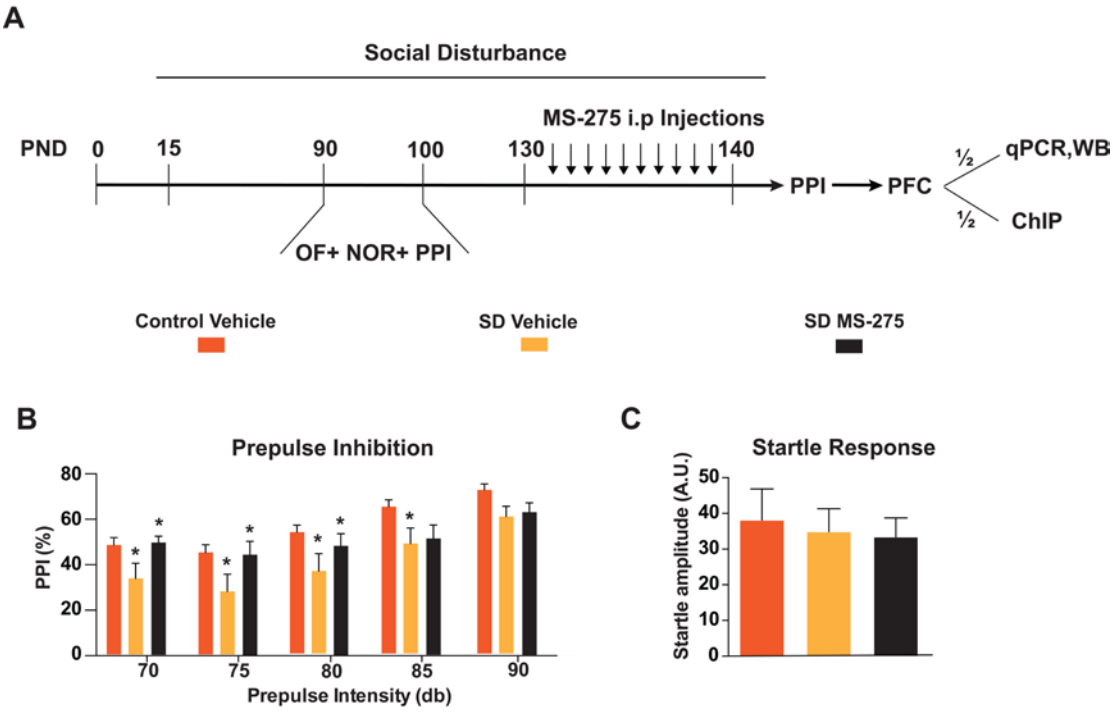

Figure 30: Chronic administration of MS-275 rescues sensorimotor gating deficits in social disrupted mice.

A. Experimental Design. Mice subjected to SD underwent first behavioural characterization with OF, NOR and PPI in order to assess cognitive endophenotypes before MS-275 treatment. 30 days after behavioural characterization, one group of SD and control mice were injected intraperitonealy for 10 days with MS-275 and a second group of SD and control mice with Vehicle. 24 hours after the last injection, mice were subjected to PPI and sacrificed afterwards for molecular analysis with ChIP and qPCR. **B.** SD subjected mice treated with vehicle showed reduced PPI compared to control vehicle treated mice demonstrating still impaired sensorimotor gating function. Two-way ANOVA of RM revealed significant effect of SD [F (1, 66)= 5.265, * p = 0.0250] and of Prepulse Intensity [F (4, 264)= 6014, *** p < 0.0001]. Bonferroni post-hoc test revealed significant reduced PPI for 70 db (p= 0.0442), 75 db (p= 0.0189), 80 db (p= 0.0191) and 85db (p= 0.0267). Chronic administration of MS-275 in SD subjected mice ameliorates PPI deficits compared to SD mice treated with Vehicle. Two-was ANOVA demonstrated a significant effect of drug treatment [F (1, 66)= 5.265, * p = 0.0250] and of Prepulse Intensity [F (4, 264)= 6014, *** p < 0.0001] as well as a significant drug x prepulse intensity interaction [F (4, 84)= 3.322, * p = 0.0141]. Bonferroni post-hoc test showed significant reduced PPI

of SD vehicle treated mice at 70 db (p = 0.0078), 75 db (p= 0.0031), 80 db (p= 0.0365) compared to SD MS-275 treated mice. **B.** Startle response among all experimental groups was similar [F (3, 84)= 0.1454, p = 0.9321; One-way ANOVA]. Error bars represent ± SEM. n= 15-20/group.

In summary, intraperitoneal injections of MS-275 rescued PPI deficits in mice subjected to social disturbance.

Thus in agreement with previously published data [209,210,469], this data suggest that a selective HDAC1 inhibitor should be a suitable therapeutic strategy to treat negative symptoms of schizophrenia.

5. Discussion

The aim of the study was to explore the role of HDAC1 in the etiopathogenesis of Schizophrenia. Two studies have reported elevated HDAC1 expression in brain regions with higher vulnerability in schizophrenia, namely the hippocampus and the prefrontal cortex [433,435]. Using post-mortem tissue samples of the hippocampus and prefrontal cortex from schizophrenia patients and age-matched controls I was able to reproduce these findings. I observed a significant up-regulation of HDAC1 mRNA in the hippocampus. The 3-fold up-regulation of HDAC1 mRNA in the prefrontal cortex of schizophrenia patients was however much more robust. In order to better understand the role of elevated HDAC1 in these brain regions, I employed a gain-of-function model in which I overexpressed HDAC1 in a spatial and temporal-restricted manner using an adeno-associated viral system and a loss of function approach by using a selective HDAC1 inhibitor and HDAC1 siRNA. Interestingly, manipulating HDAC1 specifically in the dorsal hippocampus or in the medial prefrontal cortex resulted into distinct phenotypes, indicating brain-region specific functions of HDAC1 in mediating memory formation and emotional behavior.

5.1 Role of elevated HDAC1 in the hippocampus of mice

5.1.1 AAV-mediated overexpression of neuronal HDAC1 in the dorsal hippocampus of adult mice

In order to better understand the role of elevated HDAC1 expression in the hippocampus of individuals with schizophrenia, I decided to design a gain-of-function model in which HDAC1 would be overexpressed in a spatial and temporal-specific manner.

To this end, I generated an adeno-associated virus (AAV) expressing a fusion protein of HDAC1-GFP (HDAC1-GFP-AAV) under the neuron-specific human synapsin 1 promoter in order to ensure neural-specific expression [479]. HDAC1-GFP-AAV particles were then injected in the dorsal hippocampus of adult mice and functionality of the virus was tested using immunoblot, immunohistochemistry and qPCR analysis.

Thus, injection of HDAC1-GFP-AAV in the dorsal hippocampus showed robust overexpression after 14 days of injection, resulting into a 6 –fold increase of HDAC1 mRNA expression and a 2-fold increase in the corresponding HDAC1 protein. Importantly, overexpression of HDAC1 by HDAC1-GFP-AAV did not affect mRNA or protein levels of other Class I HDACs, HDAC2, 3 and 8. It was important to address that other class I HDACs would not be affected by HDAC1-GFP-AAV overexpression, since HDAC2 and HDAC3 has been shown to act in concert together with HDAC1 in various transcriptional co-repressor complexes [104–106,533]. Additionally, overexpression of HDAC1 in the dorsal hippocampus did not affect the ventral hippocampus as no GFP fluorescence could be detected by confocal microscopy. This could be due to the serotype of the AAV used in this study, AAV6. AAV6 is known to have poor transduction efficiency over a greater volume of tissue and thus our virus might not been able to transduce the cells of the ventral hippocampus [534]. Moreover, not all neurons that showed endogenous HDAC1 expression was transduced with HDAC1-GFP-AAV, as observed in the dentate gyurs of the dorsal hippocampus. No HDAC1-GFP expression was detected when analyzed 6 days after injection. Thus, our virus-system allowed us to specifically investigate the role of elevated HDAC1 in the hippocampus of adult mice. Although my approach allowed me to express HDAC1 specifically in neurons, one potential drawback is the fact that overexpression is achieved in all neuronal subtypes. To circumvent this problem a future approach should employ mice that express CRE recombinase either in excitatory or inhibitory neurons. Injection of such transgenic mice with a HDAC1-GFP virus in which HDAC1 expression is controlled by floxed STOP codon will allow cell type specific overexpression.

5.1.2 Cognition in mice with neuronal overexpression of HDAC1 in the dorsal hippocampus

Overexpression of neuronal HDAC1 in the dorsal hippocampus did not led to an overt phenotype. Thus, mice did not show significant alterations in basal anxiety and explorative behavior, depressive-like behavior, working memory performance, long-term memory or sensorimotor gating function. The finding that neuronal overexpression of hippocampal HDAC1 did not alter the formation of long-term

memories is in line with previously reported data of Guan et al., in which forebrain specific neuronal overexpression of HDAC1 from developmental stages on did not affect long-term memories of contextual fear conditioning or morris water maze [196]. Additionally, conditional deletion of HDAC1 in other organ systems was not reported to result into an obvious detrimental phenotype [115–117,535]. This observation was suggested to result from compensatory mechanisms, in which HDAC2 was shown to be upregulated when HDAC1 was conditionally depleted. Although it is known that HDAC1 and HDAC2 share many redundant functions in various biological processes [119–121], no compensatory down-regulation of HDAC2 was observed in my experiments. Taken into account that altered hippocampal-dependent consolidation of memories is observed in mice in which HDAC2 was conditionally overexpressed or deleted in neurons of the forebrain and that such phenotypes could not be compensated by HDAC1 [196], my findings further suggest distinct roles of HDAC1 and HDAC2 in hippocampal-dependent memory formation. In fact, hippocampal HDAC1 seems to be expendable for the consolidation of memories. Another class I HDAC that has been shown to facilitate memory formation was HDAC3. Specific deletion of HDAC3 in the dorsal hippocampus of mice using AAV-Cre recombinase led to facilitated long-term memory formation in the novel object recognition task [405].

Thus, while inhibition of HDAC2 and HDAC3 facilitates hippocampal memory formation and are thought therefore to be negative regulators of memory formation, my results indicate that hippocampal HDAC1 may regulate the fine-tuning of cognitive function but is not essential for memory formation per se. However it remains elusive, if temporal-extended AAV-mediated HDAC1 overexpression in the dorsal hippocampus of mice, for instance after 10 months, would lead to memory impairments. In this case, overexpression of dorsal hippocampal HDAC1 could have a detrimental effect when combined with the well-known aging-associated memory impairments in mice. However, aging did not alter HDAC1 protein levels in the rat hippocampi, arguing against an age-related effect of HDAC1 on memory formation [536]. Moreover, altered HDAC1 mRNA or protein levels have not been reported to date in human hippocampal post-mortem tissue of aged individuals and were not changed in hippocampi of Alzheimer patients [423]. On the contrary, HDAC2 protein levels were elevated in aged rat hippocampi and post-mortem tissue of Alzheimer patients, being in agreement with impaired memory formation in mice overexpressing HDAC2 [196,536].

In conclusion, the current experimental evidence suggests that hippocampal HDAC1 is not essential for memory consolidation. However, the possibility remains that HDAC1 affects hippocampal plasticity in a subregion specific manner. In my experiments, HDAC1 was over-expressed in the dorsal hippocampus. The absence of altered emotional behavior such as anxiety and depressive-like behavior in mice overexpressing HDAC1 in the dorsal hippocampus could be due to the reason that the ventral hippocampus is more susceptible in the modulation of emotional behavior such as anxiety and depressive-like behavior. The dorsal and ventral hippocampus has been shown to be anatomically and functionally distinct areas [537]. In rodents, lesions of the ventral hippocampus but not that from the dorsal hippocampus were shown to reduce anxiety in behavioral tests [538]. Thus it is in general suggested, that the dorsal hippocampus (ventral in humans) is implicated in the regulation of learning and memory, while the ventral hippocampus (dorsal in humans) is involved in the regulation of stress response and anxiety [539–541]. In rodents, the ventral hippocampus directly projects to the medial prefrontal cortex, amygdala, nucleus accumbens shell and hypothalamus [542–544]. Thus, regulation of stress and anxiety response by the ventral hippocampus has been shown to arise from projections to hypothalamic and limbic forebrain structures [545]. The hypothalamus in turn plays a central role in controlling stress and anxiety in humans and rodents by regulating the neuroendocrine system, especially in regulating glucocorticoids, through the hypothalamic-pituitary-adrenal (HPA) axis [440,546,547] The hippocampus contains a high density of glucocorticoid receptors (GRs) and is thus part of the feedback system that modulates the activation of the HPA axis [548,549]. Interestingly, HDAC1 has been reported to bind to the promoter of the GR resulting surprisingly in transcriptional activation of GR [550,551]. In general, stressful events induce the release of glucocorticoids and increased GR expression is associated with stress and anxiety [552].

My data showed that overexpression of HDAC1 in the dorsal hippocampus of mice did not lead to general impairments in sensorimotor gating function, which is often reduced in schizophrenia patients. This could as well be due to the reason, that hippocampal modulation of sensorimotor gating function during prepulse inhibition of startle response is thought to rely more on the ventral hippocampus than the dorsal hippocampus [363,368]. Additionally, lesions in the ventral hippocampus disrupt the reciprocal interaction of the hippocampal-prefrontal cortex circuitry and were shown to impair working memory [225,553–555].

Thus in conclusion, one can speculate that overexpression of HDAC1 in the ventral hippocampus would more likely result into schizophrenia-like symptoms and cognitive endophenotypes. Moreover, while it is not obvious if Benes et al., examined the dorsal or ventral hippocampi of schizophrenia subjects, elevated HDAC1 expression was found specifically in GABAergic neurons of the stratum oriens of the CA2/CA3 region of the hippocampi of schizophrenia. Thus furthermore, cell-type specific overexpression of HDAC1, namely in GABAergic neurons of the ventral hippocampus in mice, might lead to the phenotypes observed in schizophrenia. Importantly, elevated hippocampal HDAC1 levels in the study from Benes et al., were observed only in schizophrenia subjects but not in hippocampi of individuals with bipolar disorder, indicating possible HDAC1 specificity to schizophrenia [433].

Further on, the involvement of other HDACs cannot be excluded and need further investigations. For instance, qPCR analysis of post-mortem hippocampal tissue available in our laboratory revealed significant elevated HDAC3 and HDAC8 levels in schizophrenia compared to control subjects. However, additional genome-wide association studies with bigger sample size as well as samples from different populations will be necessary in order to reveal greater significance for the involvement of HDACs in schizophrenia.

Further studies investigating HDACs in a brain-region and cell-type specific manner, combined with Next-Generation-Sequencing, will identify target genes of HDAC1 and contribute to a better understanding of the molecular underpinnings of neuropsychiatric disorders such as schizophrenia and anxiety disease.

To date, none of the HDACs have been reported in genome-wide association studies for psychiatric diseases. This might also indicate that de-regulation of HDACs does not compromise a genetic but rather en environmental risk, potentially as a initial compensatory response that becomes eventually detrimental. Similar observations have been made in the case of neurodegenerative diseases where the compensatory induction of plasticity mechanisms eventually drives neurodegenerative processes when chronically activated [556,557].

5.1.3 Neuronal overexpression of HDAC1 in the dorsal hippocampus regulates fear extinction in mice

While the overexpression of HDAC1 in the dorsal hippocampus did not affect the consolidation of memories or emotional behavior, the possibility that HDAC1 would have a specific role in cognition under physiological conditions was not ruled out. Fear extinction is a specific form of emotional memory, reflected in the reduction of fear response and was interestingly shown to be impaired in schizophrenia [488]. The extinction of fear memories represent an active learning process, a so-called inhibitory form of learning allowing the adaptive control of conditioned fear responses rather than an erasure of the original fear memory [558]. In general, fear extinction is highly context dependent [486,559]. Neuronal networks engaged in the process of extinction of contextual fear involve the amygdala, hippocampus and prefrontal cortex [559]. Although in general the amygdala is the thought to be mainly involved in the conditioning of fear responses, the hippocampus mediates the encoding of contextual fear conditioning [489,560]. Hippocampal lesions were shown to severely impair the extinction of fear memories [561]. While the ventral hippocampus is more susceptible to anxiety and fear, pharmacological interventions in the dorsal hippocampus have been shown to be as well important for contextual fear extinction [441,562,563]. Moreover, dorsal intrahippocampal injections of HDAC inhibitors such as TSA and VPA were repeatedly reported to facilitate the extinction of fear memory, demonstrating the implication of HDACs in epigenetic control of gene-expression during the process of fear extinction [410,414,485].

Indeed, in my experimental settings I was able to show that dorsal hippocampal HDAC1 regulates fear extinction, using our gain-of-function model overexpressing HDAC1 via AAV and by loss-of-function models, in which I specifically knocked-down HDAC1 protein with siRNA or inhibited its enzyme activity by intrahippocampal injections of MS-275. Thus mice that overexpressed HDAC1 displayed enhanced fear extinction, while inhibition of HDAC1 by HDAC1 targeted siRNA and MS-275 impaired the extinction of contextual fear memories. Importantly, in my experimental paradigms I paid great attention not to interfere with the acquisition and consolidation of memories in order to analyze the role of HDAC1 in the extinction of contextual fear memories. As such, our viral-system enabled me to generate an acute HDAC1 overexpression after the consolidation of fear memories

occurred. Interestingly, intrahippocampal injections of MS-275 after fear conditioning training did not affect associative memory. Overexpression of HDAC1 in the dorsal hippocampus before fear conditioning training similarly did not affect associative memory in contextual fear conditioning. The fact that inhibition or overexpression of HDAC1 in the dorsal hippocampus does not affect acquisition and consolidation of contextual fear is in line with another study reporting that intraperitoneal injections of MS-275 even for 10 days did not alter fear conditioning [564]. Interestingly in this study, the effect of MS-275 on associative learning in the contextual fear conditioning was concentration dependent. As such, mice treated with 25 mg/kg MS-275 over 10 days reduced freezing behavior indicating impaired associative memory, while 12.5 mg/kg did not affect associative memory as mentioned above [564]. The different effects of other HDAC inhibitors on fear extinction in contrast to MS-275 could be explained by the fact that they are rather unselective, inhibiting more or less all HDAC proteins [414,485]. On the other hand, the beneficial effect of the pan HDAC inhibitor TSA on fear extinction could be partially explained based on the observation that administration of TSA on neuronal cells increased the expression of HDAC1 [565]. Interestingly, when I injected the unselective Class I and II HDAC inhibitor SAHA in the dorsal hippocampi of mice, extinction of contextual fear was significantly improved when compared to vehicle treated mice (Appendix Figure 3). The beneficial effect of SAHA on memory formation is shown as well in another study in which intrahippocampal injections of SAHA improved contextual fear conditioning and impaired associative memory in aged mice [168]. Additionally, administration of SAHA in a mouse model for Huntingtons´disease decreased HDAC2 protein levels in the cortex, improving motor impairments in those mice [197]. These data support the idea of specific roles of individual HDACs during memory formation. As such it is currently suggested, that HDAC2 and HDAC3 act as negative constraints of memory formation, since the deletion of the respective proteins were shown to improve memory formation [196,566,567]. In conclusion, these data indicate that HDAC1 plays a specific role in memory formation, namely in the extinction of fear memories, while the other class I HDACs HDAC2 and 3 are involved in the acquisition and consolidation of fear memories. However, further investigations are needed to address the role of other HDACs during memory formation.

5.1.4 Transcriptional repression during fear extinction

HDAC1 activity is in general associated with transcriptional repression of genes. Several studies, especially studies involving HDAC inhibitors, have indicated the importance of transcriptional repression of specific subset of genes during contextual fear extinction [410,414,490,563,568]. In line with previous observations [490,569,570] I found that during the course of contextual fear extinction, hippocampal expression of *c-Fos* gradually declined when measured 1 h after exposure to each extinction trial. Importantly, the decline in *c-Fos* expression represented an active was not simply due to the passing of time but was specific for extinction training. Thus, transcriptional repression of *c-Fos* at E5 was regulated through the recruitment of HDAC1 together with its co-repressor mSIN3b to the promoter of *c-Fos*, leading into reduced acetylation of H3K9, while no other histone-acetylation sites such as H3K14 and H4K5 were altered. Furthermore I observed an increase of H3K9 trimethylation through the H3K9 specific histone-metyhltransferase SUV39H1 and its activator SIRT1 accompanied the decrease of H3K9 acetylation at E5. The parallel increase in H3K9 trimethylation is in full agreement with the general observation, that deacetylation of H3K9 serves as prerequisite for the trimetyhlation of H3K9 and that H3K9me3 is known to be enriched at heterochromatin regions marking, transcriptional repression [509,510]. Thus, my data showed the recruitment of HDAC1 together with other repressor proteins to the *c-Fos* promoter to mediate transcriptional repression of *c-Fos* in order to achieve successful extinction of contextual fear memories, which was inhibited by intrahippocampal injections of MS-275 and HDAC1 specific siRNA. Thus, inhibition of HDAC1 during fear extinction impaired the process of transcriptional repression of *c-fos* mediated by HDAC1, whereas overexpression of HDAC1 enhanced transcriptional repression of *c-fos* during fear extinction. In order to further confirm the formation of a chromatin–silencing complex during successful fear extinction learning mediated by HDAC1, co-immunoprecipitation experiments during the course of extinction would reveal additional support. Furthermore, HDAC1 regulated transcriptional repression required for successful fear extinction was as well monitored on another immediate early gene, *egr-2*. Thus, HDAC1 was shown to mediate as well transcriptional repression of *egr-2* during the course of fear extinction (Appendix Fig. 2). Transcriptional repression of

c-fos and *egr-2* during successful fear extinction learning was supported by another study from our lab, in which gene array analysis of hippocampi 1 hour after E1 and after fear extinction training displayed reduced *c-fos* and *egr-2* expression [563]. Interestingly, in our experimental settings as well as in other studies, the expression levels of the immediate early genes *c-fos* and *egr-2* were both upregulated after the contextual fear conditioning or respectively E1 or in more general, when fear response was high [490,563,568,571,572]. Thus immediate early genes such as *c-Fos* and *Egr-2* are transcriptionally activated upon fear conditioning [573–575]. Furthermore it is in general accepted, that the consolidation of fear memories requires brain region specific transcriptional activation and protein synthesis [490,576,577].

Thus one could speculate, that successful fear extinction learning requires transcriptional repression of genes that are activated after contextual fear conditioning, presenting different molecular pathways. HDAC1 could be therefore involved in transcriptional repression of genes that are especially upregulated after contextual fear conditioning. ChIP-Sequencing analysis of hippocampal HDAC1 after E1 and E5 could reveal further inside for this hypothesis.

Taken together, my data suggest that HDAC1 is critical for the extinction of contextual fear memories and may provide a novel therapeutic avenue to treat anxiety diseases. However, it has been suggested that successful therapy in anxiety disorders such as PTSD would rather require the erasure of fear memories, since the extinction of fear memories were shown to be labile due to the spontaneous re-occurrence of fear [578,579]. The renewal of fear memories was shown to rely on the interactions between the ventral hippocampus the amygdala and prefrontal cortex. Thus, disconnections from the ventral hippocampus from either the amygdala or prefrontal cortex eliminated the renewal of fear memories [572,579].

Therefore it would be interesting to investigate, whether pharmacological or genetic modulations of HDAC1, and thus HDAC1 mediated regulation of gene-expression specifically in the ventral hippocampus would be involved in the process of renewal of fear memories and thus extinction of fear memories in order to present an therapeutic target for the treatment of anxiety disorders.

5.2 Role of elevated HDAC1 in the prefrontal cortex of mice

5.2.1 Cognitive endophenotypes and schizophrenia-like symptoms in mice with elevated HDAC1 levels in the prefrontal cortex

The AAV-mediated overexpression of HDAC1 in the dorsal hippocampus of mice did not result in cognitive endophenotypes of schizophrenia. However, in agreement with previously reported data, we found elevated HDAC1 levels in the prefrontal cortex in schizophrenia subjects. Moreover, immunoblot analysis of the same samples revealed a 1.7-fold increase in the corresponding protein levels. Interestingly in our samples, no alterations in the expression of other class I HDACs were observed. Notably, overexpression of HDAC1 in the prefrontal cortex of mice resulted into elevated anxiety and depressive-like behavior, working memory deficits, disrupted sensorimotor gating and impaired associative fear memory with subsequent deficits in extinction of fear memories, similar to the phenotypes observed in schizophrenia patients.

Deficits in sensorimotor gating and working memory performance have been repeatedly reported in schizophrenia patients and in animal models of schizophrenia [24,326,327,580–582]. Impaired sensorimotor gating and working memory deficits are as well present in unaffected relatives of individuals of schizophrenia and defined therefore as the most promising cognitive endophenotypes of schizophrenia. However it is important to mention, that these deficits are not only unique to schizophrenia patients. For instance, deficits in sensorimotor gating assessed by PPI is as well present in patients with Huntington´s disease, Parkinson´s disease, Tourette Syndrome and Alzheimer´s disease [583,584]. It is therefore interesting to note that all these disorders can be accompanied by psychosis. Thus one can speculate, that HDAC1 function in the prefrontal cortex might in general be involved in the process of working memory and sensorimotor gating. Altered prefrontal cortical circuits are strongly associated with cognitive impairments that are nowadays recognized as the core-feature [330,585–587]. Thus the prefrontal cortex is found to be the key mediator of working memory performance and sensorimotor gating [353,368,588,589]. Altered prefrontal cortex circuitry is associated with abnormalities in neurotransmission signaling such as for example the dopaminergic, glutamatergic, serotonergic and GABAergic signaling pathways

[590–594]. Regarding the role of HDAC1, the GABAergic signaling pathways seems to provide the most promising targets for HDAC1 mediated regulation of gene-expression underlying schizophrenia. As such, elevated HDAC1 expression correlates with the down-regulation of GABAergic genes in the prefrontal cortex [209,210,595]. Furthermore, ChIP experiments in a mouse model for schizophrenia have shown that HDAC1 regulates transcriptional repression of the schizophrenia GABAergic genes *Gad67* and *Reelin* through increased binding at the respective promoters which was reversed by administration of the HDAC1 selective HDAC inhibitor MS-275 [210].

Taken these data into consideration, cognitive endophenotypes in mice overexpressing HDAC1 in the prefrontal cortex might be due the downregulation of GABAergic genes mediated by HDAC1. Besides *Gad67* and *Reelin*, Calbindin and Parvalbumin would possibly represent additional target genes, since they were reported to be as well downregulated in schizophrenia [297,453,596].

5.2.2 Elevated prefrontal cortical HDAC1 expression in response to environmental risk factors of schizophrenia

Various studies in humans and animals have demonstrated stress-induced prefrontal cortical impairments, resulting into working memory deficits and sensorimotor gating disruption [597–600]. Furthermore, severe and chronic stressful events are considered to be environmental risk factors, contributing to the development of neuropsychiatric disorders. Therefore, since environmental risk factors are known induced dysfunction in prefrontal cortex circuitries associated with severe memory impairments, I wondered whether these factors would influence the expression of HDAC1 in the prefrontal cortex.

To this end, I first subjected mice to maternal separation and social isolation rearing which I classified as social disturbance mice (SD). In line with previous data, SD mice displayed increased anxiety-and depressive like behavior, working memory deficits and impaired prepulse inhibition. Noteworthy, this stress procedure induced elevated HDAC1 mRNA and protein levels in the prefrontal cortex of SD mice in

comparison to the control group, while HDAC1 levels in the hippocampus where not significantly altered. Moreover, the stress-induced increase in HDAC1 expression was specific to the prefrontal cortex, as it was not observed in the hippocampi of the same mice. However, based on our previous assumptions and the knowledge that the ventral hippocampus in rodents is highly involved in the modulation of prepulse inhibition and working memory through its efferent projections to the prefrontal cortex, it would be interesting to investigate whether social disturbance would influence HDAC1 expression levels specifically in the ventral hippocampus of mice. The possibility remains namely, that altered HDAC1 levels in the ventral hippocampus would not be accessible for detection or result into insignificance due to a dilutionary effect of the dorsal hippocampus. Nevertheless, SD in mice simulated cognitive endophenotypes and elevated HDAC1 mRNA and protein levels in the prefrontal cortex of mice as similarly observed in individuals with schizophrenia.

It was previously reported, that early life stress does not induce deficits in prepulse inhibition of startle response before early adulthood in rodents, resembling the time course of schizophrenic symptomatology showing appearance after puberty, when the prefrontal cortex reaches maturation. Thus for example in rats subjected to maternal deprivation, deficits in PPI only started at post natal day (pnd) 69 but were not present at pnd 34 [361,520]. Similarly, I was able to reproduce this observation in mice in which no significant alterations of PPI were visible when tested at pnd 34. Thus, two-way ANOVA analysis revealed that there was no significant main effect of the social disturbance protocol on mice at pnd 34 during PPI, in contrast to PPI conducted in SD mice during adulthood (3 months old or approximately pnd 96). Thus it would be interesting to investigate HDAC1 expression levels in the prefrontal cortex (and ventral hippocampus) of SD mice at pnd 34, when no deficits in sensorimotor gating function is visible. If this would be the case, elevated prefrontal cortical HDAC1 would present a sort of molecular endophenotype reflecting sensorimotor gating functions.

When subjected SD mice to intraperitoneal injections with MS-275 for 10 days, deficits in sensorimotor gating function were ameliorated in SD mice. Thus while SD mice treated only with vehicle displayed reduced PPI, SD mice treated with MS-275 showed PPI responses that were similar to control mice not subjected to SD. This data

indicates that MS-275 injections improved sensorimotor gating deficits. The beneficial effect of MS-275 in sensorimotor gating function in our mouse model is in agreement with previously reported data, in which 10 days of intraperitoneal injections of MS-275 in heterozygous knock-out mice improved earlier observed PPI deficits in these mice [469]. Importantly, the effect of MS-275 on cognition was shown to be concentration dependent [564].

Early life stress or chronic stress in rodents and humans induces altered activity of the HPA axis and many of the behavioral consequence such as depression or cognitive impairments are mediated through HPA-axis induced increase in glucocorticoid secretion and subsequent activation of glucocorticoid receptors, resulting into altered gene-expression [437,549,601–603]. Altered HPA axis has been observed in schizophrenia patients [604]. Interestingly, activation of glucocorticoid receptor expression was shown to be mediated by HDAC1 promoter binding [550,551] and glucocorticoid receptors in the prefrontal cortex were shown to regulate stress-induced alterations in dopaminergic neurotransmission. Analysis of HDAC1 mediated transcriptional activation of glucocorticoid receptors in the prefrontal cortex, leading to cognitive impairments, would be an additional interesting hypothesis. Additionally, HDAC1 has been shown to act together in concert with DNA methyltransferases DNMT1, 3a and 3b in order to form a repressor complex to downregulate GABAergic susceptibility genes [210]. DNMT1 and 3 a have been shown to be upregulated in GABAergic neurons of schizophrenia patients [457]. Moreover, DNA methylation has been shown to play an important function in response to early life stress and HPA-axis regulating the expression of glucocorticoid receptors in rodents [429,437,605,606]. Thus the involvement of DNA methylation involved in prefrontal cortex induced cognitive endophenotypes would present an additional interesting case.

Interestingly, genome-wide mapping of HDACs and HATs using ChIP experiments followed by next-generation sequencing revealed that both, HDACs and HATs were preferentially found at the promoters of active genes by phosphorylated RNA polymerase II [607]. It was suggested, that the in general known transcriptional repression activity of HDACs takes mostly place in order to reset the chromatin state by deacetylating histones at active genes [566,607].

Since social disturbance mediated the HDAC1 expression in the prefrontal cortex of mice, it would be interesting to see, whether beneficial environmental factors such as

“good” maternal care or environmental enrichment in mice result as well in altered HDAC1 expression in the prefrontal cortex. Furthermore, it would be interesting to investigate through which upstream molecular pathways social disturbance regulates HDAC1 levels in the prefrontal cortex of mice. Since long-lasting changes in gene-expression are often linked to altered DNA-methylation which affects the binding of transcription factors [608], one future experiment is to study the promoter and enhancer regions linked to HDAC1 gene-expression with respect to the DNA-methylation status.

6. Summary

Schizophrenia is a severe neuropsychiatric disorder with high phenotypic complexity and multifactorial inheritance. Cognitive dysfunctions have been identified as the core feature of the disease and they are resistant to treatment with available antipsychotics. Impaired working memory and disrupted sensorimotor gating, which refers to the improper filtering or "gating" between irrelevant and relevant information leading to sensory overload, are the cognitive hallmarks of schizophrenia. Both of these cognitive dysfunctions are defined as cognitive endophenotypes that present a biomarker and guidepost for identification of the cause and course of schizophrenia. The etiopathogenesis of schizophrenia is thought to rely on genome and environment (GxE) interactions. Epigenetic enzymes such as histone-deacetylases (HDACs) are key mediators of GxE interactions. HDACs remove acetyl-groups of histone-proteins in response to environment stimuli, thereby changing the chromatin structure resulting into differential gene-expression important for cognition. Deregulated histone-acetylation leads to impairments in learning and memory. Two independent human post-mortem studies have reported elevated HDAC1 levels in the hippocampus and prefrontal cortex of individuals with schizophrenia, with both brain regions being important for the regulation of cognitive endophenotypes of schizophrenia.

The goal of this study was to investigate the role of HDAC1 in cognitive function and its contribution to the etiopathogenesis of schizophrenia in mice using both gain- and loss-of-function models. In the gain-of-function model, HDAC1 was specifically overexpressed in the dorsal hippocampus and prefrontal cortex of mice using an adeno-associated-viral (AAV)-system. The loss-of-function model consisted of pharmacological inhibition of HDAC1 using the HDAC1-specific inhibitor MS-275 and siRNA-mediated knockdown of HDAC1. Moreover, the effect of early life stress, an environmental risk factor for schizophrenia on HDAC1 expression was also examined.

My results showed, that overexpression of neuronal HDAC1 in the prefrontal cortex of adult mice resulted in schizophrenia-like symptoms such as increased anxiety,

depressive-like behavior, impaired fear extinction and cognitive endophenotypes such as impaired working memory performance and deficits in sensorimotor gating function. Inhibition of HDAC1 ameliorated such phenotypes. Moreover, environmental risk factors for schizophrenia such as early life stress induced cognitive endophenotypes of schizophrenia and mediated the up-regulation of prefrontal cortical HDAC1, simulating the situation observed in the post-mortem prefrontal cortex tissue of individuals with schizophrenia. A role of HDAC1 in early life stress-induced schizophrenia-like behavior was suggested by the finding that pharmacological inhibition of HDAC1 ameliorated such phenotypes.

Interestingly, while manipulating neuronal HDAC1 levels in the prefrontal cortex of mice caused schizophrenia-like phenotypes, affecting neuronal HDAC1 levels in the dorsal hippocampus had no impact on such behaviors.

Instead I demonstrated that under physiological conditions, HDAC1 in the dorsal hippocampus regulated the extinction of fear memories in mice. Using a combination of molecular and behavioral technologies, I found that HDAC1 regulated fear extinction via a mechanism that involved H3K9 deacetylation and subsequent trimethylation on the promoter of the immediate early genes (IEG´s) *c-fos* and *egr-2*, resulting into transcriptional repression. Inhibition of HDAC1 by MS-275 or siRNA impaired fear extinction and inhibited transcriptional repression of IEG´s during fear extinction.

In conclusion, these data indicate a brain-region specific function of HDAC1 in cognition and emotional behavior and provide important knowledge on the role of HDAC1 in the adult brain. Especially, the role of HDAC1 in the prefrontal cortex is of particular interest and suggests that a selective HDAC1 inhibitor might be suitable to treat cognitive endophenotypes in schizophrenia patients. Part of this results have been published recently in the *Journal of Neuroscience* and another manuscript describing the results related to the role of HDAC1 in the prefrontal cortex is currently under preparation.

7. References

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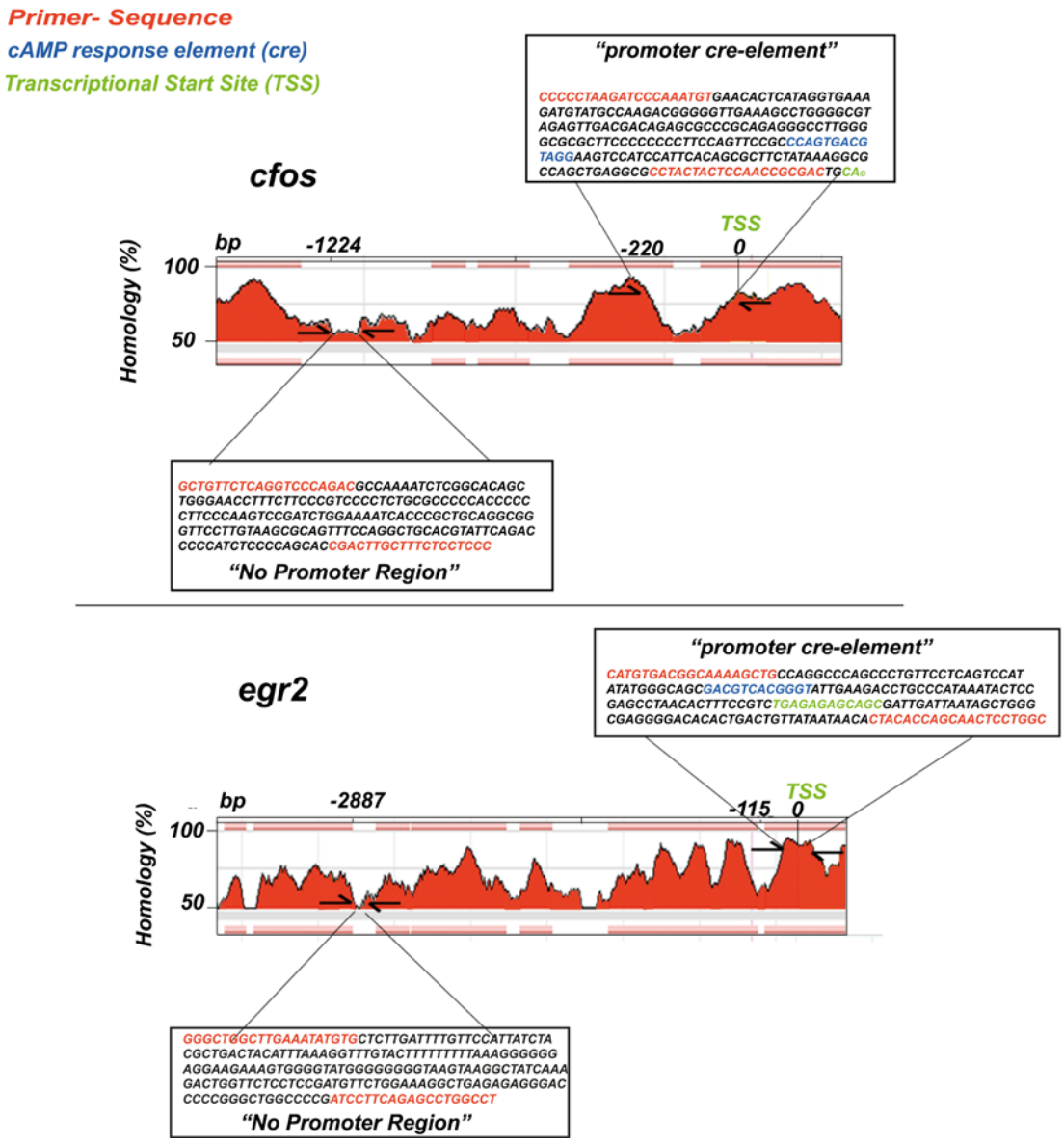
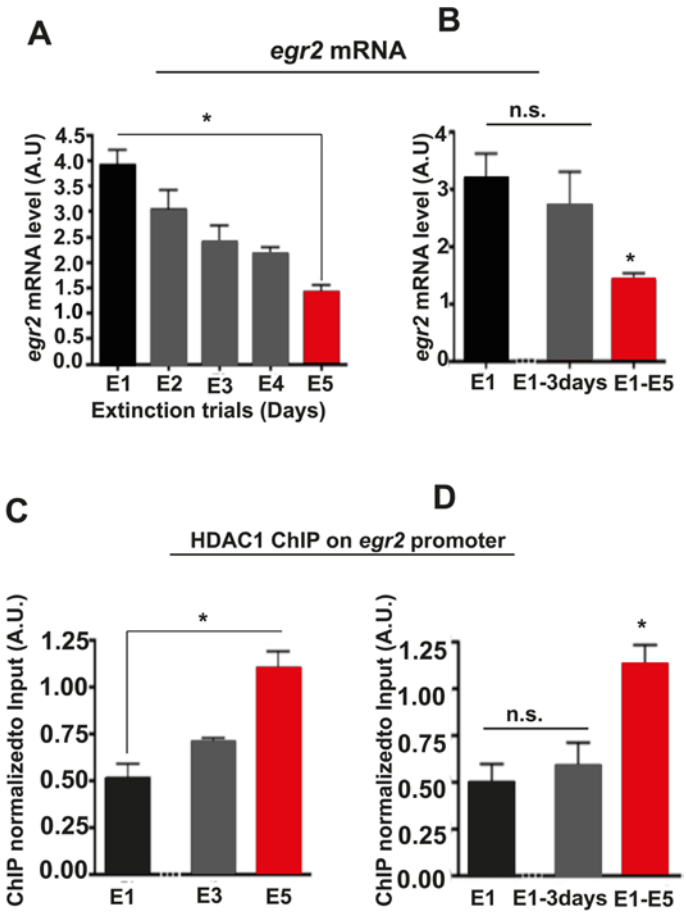
8. Appendix

8.1 Appendix Figures



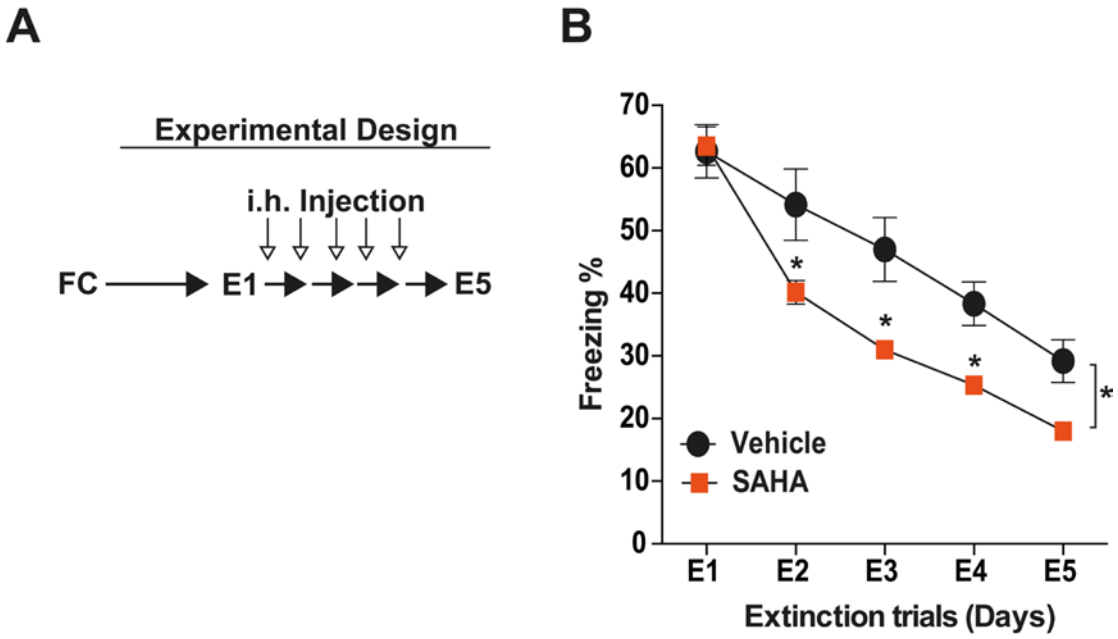
Appendix Figure 1:Analysis of the *c-Fos* and *Egr-2* promoter.

The genomic region of the *c-Fos* (Top) and *Egr-2* (Bottom) promoter was analyzed for homology between mouse and human using the Evolutionary Conservation of Genomes (ECR) browser tool (http://ecrbrowser.dcode.org/). High homology in the promoter regions indicates conserved regions most likely implicated with gene-regulation. The primers used to analyze epigenetic modifications of the *c-Fos* and *Egr-2* promoter span the conserved cAMP-response element (cre) that is required for *c-Fos* and *Egr-2* expression. The schematic drawing also indicate the genomic region and the corresponding primer pairs we used to analyze "no promoter regions" which were selected on the basis of low homology between species.



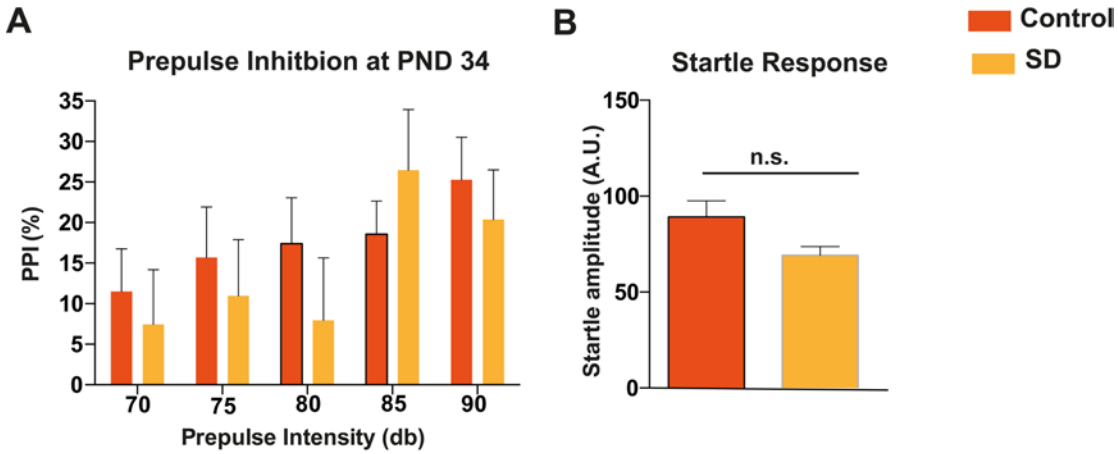
Appendix Figure 2: Fear extinction-dependent recruitment of HDAC1 to the *Egr-2* promoter

Fear extinction training (see Fig. 13, 15) was performed in the mice (n= 40) that were used for the molecular analysis in ***A*** and ***B***. ***A***, *Egr-2* expression was analyzed via qPCR in hippocampal tissue isolated 1 h after exposure to extinction trials. The data are normalized to tissue obtained from a naive control group. ***B***, HDAC1 ChIP was performed from hippocampal tissue 1 h after exposure to E1, E3, and E5. Note that the downregulation of *Egr-2* correlates with recruitment of HDAC1 to the *Egr-2* promoter. ***C***, We used the same samples described in Figure 5*D* to analyze *Egr-2* expression and HDAC1 recruitment to the *Egr-2* promoter in the E1, the E1–3 d, and the E1–E5 group. We would like to reiterate that freezing behavior in the E1–3 d group was significantly higher when compared with the E1–E5 group. *Egr-2* expression was measured 1 h after extinc- tion trials. *Egr-2* levels were significantly higher in the E1–3 d group when compared with E1–E5 group. (*p □ 0.05 vs E1 and E1–3 d). ***D***, HDAC1 ChIP was performed from hippocampal tissue 1 h after exposure to extinction trial in the E1, E1–3 d, and E1–E5 groups. Note that the increased *Egr-2* expression in the E1–3 d group correlates with reduced HDAC1 level at the *Egr-2* promoter. *p □ 0.05 vs E1 and E1–3 d. Error bars indicate SEM.



Appendix Figure 3: Intrahippocampal injections of the pan-HDAC inhibitor SAHA facilitate fear extinction learning.

A. Experimental design. Intrahippocampal injection of the pan-HDAC inhibitor SAHA immediately after each extinction trial significantly facilitated fear extinction when compared to control mice on individual extinction trials (*P<0.05 vs. vehicle) or by repeated measurements (p<0.05; F= 6.345).



Appendix Figure 4: Effect of social disturbance on sensorimotor gating in mice at postnatal day (PND) 34.

A. Social disturbance did not alter PPI in mice when tested at PND 34. No significant effects was observed among groups. Two-way ANOVA or repeated measures revealed no significant main effect of social disturbance at PND 34 [F (1, 21)= 0.1711, p = 0.6834] while the main effect of prepulse intensity remained significant, indicating functional sensorimotor gating [F (4, 84)= 4.702, p = 0.0018].

B. Startle response did not show differences among groups. n= 11/group. Error bars represent ± SEM

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10. Curriculum Vitae

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10thInternational Congress of the Polish Neuroscience Society, Lodz, Poland

07/2012 **Poster:** "HDAC1 Regulates Fear Extinction in Mice"
FENS Forum 2012, Barcelona, Spain

TEACHING

09/2010 **Advanced Method Course for GGNB: "**Chromatin-Immunoprecipitation and epigenomic gene-profiling in the adult brain"

2010-2013 **Supervision of five lab rotations** (two months each)

PUBLICATIONS

1. **Bahari-Javan S**, Benito-Garagorri E, Halder R, Burkhardt S, Schmitt A, Falkai P, Sananbenesi F & Fischer A. **HDAC1 induced cognitive endophenotypes of schizophrenia in mice.** (Manuscript in preparation)

2. Kerimoglu C, Agis-Balboa RC*, Kranz A*, Stilling R*, **Bahari-Javan S**, Benito-Garagorri E, Halder R, Burkhardt S, Stewart FC, Fischer A. **Histone-methyltransferase MLL2 (KMT2B) is required for memory formation in mice**. **J Neurosci.** 2013 Feb 20;33(8):3452-64. doi: 10.1523/JNEUROSCI.3356-12.2013.

3. **Bahari-Javan S**, Maddalena A*, Kerimoglu C*, Wittnam J, Held T, Bähr M, Burkhardt S, Delalle I, Ku□gler S, Fischer A & Sananbenesi F. **HDAC1 Regulates Fear Extinction in mice. J Neurosci**. 2012 Apr 11;32(15):5062-73. doi: 10.1523/JNEUROSCI.0079-12.2012

4. Zovoilis A*, Agbemenyah HY*, Agis-Balboa RC, Stilling RM, Edbauer D, Rao P, Farinelli L, Delalle I, Schmitt A, Falkai P, **Bahari-Javan S**, Burkhardt S, Sananbenesi F, Fischer A. **microRNA-34c is a novel target to treat dementias. EMBO J**. 2011 Sep 23;30(20):4299-308. doi: 10.1038/emboj.2011.327.

5. Kuczera T, Stilling RM, Hsia HE**, Bahari-Javan S**, Irniger S, Nasmyth K,
   Sananbenesi F, Fischer A. **The anaphase promoting complex is required for memory function in mice. Learn Mem. 2010 Dec 22**;18(1):49-57. doi: 10.1101/lm.1998411.

6. Peleg S*, Sananbenesi F*, Zovoilis A*, Burkhardt S, **Bahari-Javan S**, Agis-Balboa RC, Cota P, Wittnam JL, Gogol-Doering A, Opitz L, Salinas-Riester G, Dettenhofer M, Kang H, Farinelli L, Chen W, Fischer A. **Altered histone acetylation is associated with age-dependent memory impairment in mice. Science. 2010 Jun 25**;328(5986):1634

   *Equal Contributions