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COGNITIVE DEFICITS TRIGGERED BY EARLY LIFE STRESS: THE ROLE OF HISTONE DEACETYLASE 1

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Abstract

Studies showed that histone deacetylase (HDAC) inhibitors can reverse cognitive deficits found in neurodegenerative disorders and age-related memory decline. However, the role of HDACs in stress-induced cognitive deficits has not been investigated. In the stress-susceptible mouse strain Balb/c, early life stress triggers a persistent decrease in HDAC expression in the forebrain neocortex, including reduced expression of class I HDACs. The same mice show pronounced cognitive deficits in adulthood, namely deficits in working memory and attention set-shifting. Here we show that these mice also exhibit reduced association of HDAC1 with promotor III of the brain-derived neurotrophic factor (*Bdnf*) gene, and that cognitive testing leads to abnormally increased *Bdnf* mRNA expression. A pharmacological reduction of *Bdnf*-tropomyosine kinase B receptor signaling effectively reverses the cognitive deficits, indicating that enhanced transcriptional activation of the *Bdnf* gene contributes to their emergence. In contrast to Balb/c mice, C57Bl/6 mice only develop attention set-shifting deficits when raised by Balb/c foster mothers during the time the pups are exposed to early life stress. HDAC1 levels at *Bdnf* promotor III are unaltered in such C57Bl/6 mice, although they exhibit decreased levels of HDAC1 at the promotor of the early-growth response gene 2 (*Egr2*) and abnormally increased *Egr2* mRNA expression after cognitive testing. Hence, contrary to the beneficial effects of HDAC inhibition in neurodegenerative diseases, the reduced HDAC1 levels at promoters of distinct plasticity-associated genes predispose animals exposed to early life stress to enhanced expression of these genes upon cognitive challenge, an effect that negatively influences cognitive task performance.

INTRODUCTION

Deficits in executive cognitive functions occur in 20 to 30 percent of patients with major depression (McIntyre et al., 2013; Millan et al., 2012) as well as in a large subset of patients with unipolar depression (Trivedi and Greer, 2014). Such deficits include widely replicated

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CONFLICT OF INTEREST:

The authors declare no competing financial interest.

findings of deficits in working memory (WM) and attention, i.e., deficits that are principal mediators of psychosocial impairment, including impaired workforce performance (McIntyre et al., 2013). The observations that patients in remission from depression still exhibit cognitive deficits, and that the presence of such deficits contributes to relapse, indicates that the cognitive deficits are core deficits rather than an epiphenomenon of depression (Millan et al., 2012; McIntyre et al., 2013). The causes of these cognitive deficits are not known and, at present, no mechanisms has been identified that could explain their emergence.

One prominent risk factor for depression is early life stress (Pechtel and Pizzagalli, 2011), and numerous studies on animal models of early life stress demonstrated persistent changes in emotional behavior that are also found in human depression. For example, in the inbred mouse strain Balb/c a widely employed rodent paradigm of early life stress, infant maternal separation (IMS), elicits not only increased anxiety and depression-like behavior in adulthood but also pronounced deficits in spatial WM and attention set-shifting (Mehta and Schmauss, 2011). Moreover, several of the gene expression changes found in IMS Balb/c mice are found in the forebrain neocortex, and they are attributable to a histone-based epigenetic response to IMS exposure, namely increased acetylation of histone H4 protein that is due to decreased activity of several class I/II HDACs, including class I HDACs 1 and 3 (Levine et al., 2012). This epigenetic phenotype emerges during adolescent development and persists into adulthood. While it ameliorates the severity of the emotional phenotype in adulthood (Levine et al., 2012) and increases responsiveness to antidepressant treatment (Schmauss, 2015), only a pharmacological treatment that activated class I HDACs 1 and 3 during adolescent development of IMS Balb/c mice was able to abolish their cognitive deficits (Schmauss et al., 2014). This suggests that reduced HDAC1 and/or HDAC3 activity contributes to the emergence of cognitive deficits, a hypothesis tested in the present study.

Contrary to the pronounced deficits in WM and attention set-shifting found in IMS Balb/c mice, these cognitive functions are unaffected after IMS exposure of the more resilient strain C57Bl/6 (Mehta and Schmauss, 2011). However, C57Bl/6 foster pups raised by Balb/c mothers during IMS exposure also develop deficits in attention set-shifting, but no deficits in WM (Schmauss et al., 2014). This raises the question whether or not the comparatively milder cognitive deficits of foster IMS C57Bl/6 mice are also functionally related to reduced HDAC1 and/or HDAC3 activity, a question also addressed in the present study.

Here we show that both IMS Balb/c mice and foster IMS C57Bl/6 mice exhibit reduced HDAC1 association with promoters of distinct plasticity-associated genes, and that cognitive test exposure triggers an abnormally increased expression of these genes. However, the affected genes differed between the two strains: In IMS Balb/c mice, reduced HDAC1 levels are found on promoter III of the *Bdnf* gene, and increased expression *Bdnf* and hence, increased *Bdnf*-tropomyosine kinase B (TrkB) receptor signaling can explain the cognitive phenotype of IMS Balb/c mice. Moreover, a pharmacological reduction of HDAC1 activity during adolescent development of non-stressed Balb/c mice precisely recapitulates this effect. In contrast, in IMS C57Bl/6 mice fostered by Balb/c mothers, reduced HDAC1 levels were detected at the promoter of the early growth response gene 2 (*Egr2*), and cognitive task exposure also enhanced the expression of the *Egr2* gene above normal levels. Hence, while

reduced HDAC1 association at promoters of distinct plasticity-associated genes is a common denominator contributing to early-life stress-triggered cognitive deficits, the genes affected are determined by the genetic backgrounds.

MATERIALS AND METHODS

Animals

Balb/cJ and C57Bl/6J mice were housed in a temperature- and light-controlled barrier facility with free access to food and water. All experiments involving the animals were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committees at Columbia University and the New York State Psychiatric Institute.

Infant Maternal Separation (IMS)

In these experiments, Balb/c mothers either raised their own pups or fostered Balb/c or C57Bl/6 pups from other litters from the day of birth. All pups exposed to the IMS paradigm were separated from their mothers daily for three hours (from 1:00 to 4:00 PM), starting at postnatal age P2 and ending at P15. They were weaned at P28 and group-housed by sex (see Fig. 1A). Other pups of Balb/c or C57Bl/6 mothers were left undisturbed with their mothers and were also weaned at P28. These mice are referred to as standard-facility reared (SFR). Housing and husbandry conditions were identical for SFR and IMS mice.

Drug treatments

Drugs were administered to mice (males and females) starting at P35 and ending at P59 (see Fig. 1B). All drugs were administered via the drinking water. Drug intake was monitored daily and drugs were replenished every 48 hours (MS-275; Sigma Aldrich, St. Louis, MO) or 24 hours (Ana-12; Calbiochem; Billerica, MA). IMS Balb/c mice that received Ana-12 consumed 1mg/kg/day, and SFR mice that received MS-275 consumed 15 μ M/day. We previously showed that neither treatment altered the emotional behavior of these mice when tested at the end of this treatment (Schmauss, 2015). However, at the dose administered, Ana-12 effectively blocked the antidepressant effects of fluoxetine in IMS Balb/c mice (Schmauss, 2015) and, in SFR Balb/c mice, MS-275 increased the levels of acH4K12 and Pol II at *Bdnf* promoter III only when co-administered with fluoxetine (Schmauss, 2015).

Cognitive tests

To avoid litter effects, one male and one female mouse of a total of at least 4 litters per treatment group were assigned to cognitive testing. Similar to our previous study (Mehta and Schmauss, 2011), no significant sex differences in cognitive task performance of SFR and IMS mice were found. Hence, males and females are equally represented in the groups of mice studied here.

Starting at postnatal age P60, mice were food restricted such that they gradually (over the period of 4 to 5 days) lost 10–15% of their free-feeding body weights. Prior to ASST testing, mice learned to dig for food buried deeply in unscented terra cotta pots filled with familiar bedding medium. Then, they went through four consecutive test phases of the ASST,

beginning with a simple discrimination (SD) between odor (scented terra cotta pots) or texture (different digging media), and followed by a compound discrimination (CD) in which another stimulus property (a second odor or texture) was introduced that was not a reliable predictor of food reward. The third phase required an intradimensional shift of attention (IDS), i.e., both relevant and irrelevant stimulus properties were changed, but the relevant stimulus dimension used in the SD and CD (odor or texture) remained the same. Finally, the formerly irrelevant stimulus dimension became relevant and required an extradimensional shift of attention (EDS). In all test phases, animals had to reach a criterion of 6 consecutive correct trials, and the number of trials to criterion was referred to as response accuracy.

Some of the Balb/c mice that completed the ASST were further trained for alternate arm entries in the T-maze until they reached more than 70% correct arm entries (in 10 trials per day) on 2 consecutive days with 5 sec inter-trial delay periods. Then, mice performed the test with 2 longer inter-trial delays (15 and 20 sec, each tested on 2 consecutive days) to test for their working memory. (All groups of mice did not maintain spatial WM at 30 sec inter-trial delay). Only correct arm entries were rewarded with food, and the percentage of correct arm entries in the total number of 10 trials per delay period was taken as a measure of response accuracy.

RNA extraction and real-time PCR

One hour after completion of either the ASST or the WM test, forebrain neocortical tissue was dissected from male and female mice using the mesodiencephalic junction as the anatomic landmark for the caudal border of the forebrain. ASST-tested mice were 66 days old (see Fig. 1C), and their non-tested controls were matched for age and sex. The age of WM-tested mice and their non-tested controls (also derived from at least 4 different litters per group) ranged from P81 to P84 (see Fig. 1C). Total RNA was extracted using guanidine/cesium chloride ultracentrifugation. First-strand cDNA was synthesized using Murine Moloney Leukemia Virus reverse transcriptase (USB, Cleveland, OH) in conjunction with oligo dT₁₅ primers. Real time PCR was performed using the iQ Real Time PCR detection System (Bio-Rad, Hercules, CA) and SYBR Green (Bio-Rad). Bdnf mRNA was amplified using the primers targeting Bdnf transcript variant III reported by Tsankova et al. (2006). Egr2 mRNA was amplified using the primer pair 5'-ATGAACGGAGTGGCGGGA-3'/5'-AGTAGAGGTGGTCCAGTT-3', and c-Fos mRNA was amplified using the primer pair 5'-ATGATGTTCTCGGGTTTGAA-3'/5'-CACCGTGGGGATAAAGTTGG-3'. Cycle thresholds (Ct) of amplification (normalized to those obtain for β -actin) were expressed as $1/2^{-Ct}$ values so that higher numbers reflect higher expression.

Chromatin Immunoprecipitations (ChIP)

ChIP was performed on forebrain neocortical tissue of groups of male and female mice (selected from different litters as described above) either at P60 (baseline) or after cognitive testing with and without drug treatment (see Fig. 1). Dissected tissue was fixed with 1% paraformaldehyde, dounce-homogenized and sonicated using the Microson ultrasonic cell disrupter (Misonix, Farmingdale, NY) to an average DNA length of 200 to 400 base pairs. Samples were centrifuged at 15,000xg, and 50 μ l aliquots of the supernatant were

immunoprecipitated overnight with 20 μ l protein A magnetic beads (Millipore, Temecula, CA) and ChIP-grade antibodies directed either against HDAC1 (Millipore), HDAC3 (Abcam Inc., Cambridge, MA), RNA Polymerase II CTD repeat YSPTSPS (Abcam), trimethylhistone H3K9 (Millipore), or acetyl-histone H3K9 (Millipore). Beads were treated according to the instruction of the manufacturer. Immunoprecipitated DNA and a serial dilution of 1% input were analyzed by SYBR-Green real-time PCR using primers targeting *Bdnf* promoter III (original nomenclature) adopted from Tsankova et al. (2006) and the promoters of the *Egr2* and *Fos* genes adopted from Bahari-Javan et al. (2012).

Statistical Analyses

Repeated Measures ANOVA and one-way ANOVA were performed using GraphPad Software (InStat; La Jolla, CA). Two-way ANOVA was performed using the VassarStats Statistical Computation website (American Library Association, February 27, 2012).

RESULTS

Reduced levels of HDAC1 at *Bdnf* promoter III and increased *Bdnf* mRNA expression in IMS Balb/c mice exposed to cognitive tests

The finding that activation of HDAC1 and HDAC3 during adolescent development abolished the WM and attention set-shifting deficits of IMS Balb/c mice (Schmauss et al., 2014) suggests that reduced activity of these HDACs is functionally linked to the emergence of these deficits. To test this, we first performed chromatin immunoprecipitation (ChIP) experiments to compare the levels of HDAC1 and HDAC3 at promoters of selected plasticity-associated genes between standard facility-reared (SFR) Balb/c controls and IMS Balb/c mice at postnatal age P60 (see Fig. 1A). To this end, we focused on the promoters of the *Bdnf* gene because of the proposed role of *Bdnf* expression in modulating synaptic plasticity in the prefrontal cortex (PFC), the anatomic regions that governs the cognitive functions affected by early life stress exposure (Lewis et al., 2005). We further targeted the promoters of two immediate-early genes that encode transcription factors known to participate in the modulation of synaptic plasticity in response to cognitive challenges. One of the targeted genes is *Egr2*. We previously showed with immunocytochemical studies on C57Bl/6 mice performing an attention-set-shifting task (ASST) that *Egr2* immunoreactivity was specifically increased in the ventrolateral orbital frontal cortex and the pre- and infralimbic subregions of the medial prefrontal cortex (mPFC) during the associative learning phases of the ASST, and that infralimbic *Egr2* expression further increased when mice performed the set-shifting phases of the ASST (DeSteno and Schmauss, 2008). In contrast to ASST-tested mice, mice that performed a spatial WM test did not exhibit induced *Egr2* gene expression (DeSteno and Schmauss, 2008). This differs from the other gene targeted in this study, namely the *Fos* gene. We previously showed that stimulation-induced levels of *Fos* gene expression in the anterior cingulate and prelimbic subregions of the mPFC positively correlate with the performance of mice in the spatial WM test. Moreover, exposure to the ASST leads to induction of *Fos* gene expression in all subregions of the mPFC, and the magnitude of this induction is critical for optimal ASST performance (Glickstein et al., 2002; Glickstein et al., 2005). In contrast to *Egr2* gene induction, ASST-tested mice also exhibit *Fos* gene induction in other brain regions, including orbital, motor

and somatosensory cortices, hippocampus, dorsal striatum, nucleus accumbens, thalamus, and hypothalamus (Glickstein et al., 2005).

We used forebrain neocortical tissue that includes the entire mPFC to determine the levels of HDAC1 and HDAC3 on promoters of the *Bdnf*, *Egr2*, and *Fos* genes. As shown in Fig. 2A, in SFR Balb/c mice the highest levels of HDAC1 were found at *Bdnf* promoter III, and HDAC1 association with the same promoter was significantly reduced in IMS Balb/c mice. In contrast, the levels of HDAC1 at *Bdnf* promoter IV as well as the HDAC1 levels at the promoters of the *Egr2* and *Fos* genes were indistinguishable between SFR and IMS mice. Moreover, no significant differences were found for HDAC3 associations with any of the promoters examined.

The finding of reduced HDAC1 association with *Bdnf* promoter III in IMS mice suggests increased acetylation of histone marks of open chromatin and facilitated gene transcription, thus allowing increased gene transcription from this promoter either at baseline or in response to stimulation. The later possibility is supported by our previous study showing, for example, that enhanced serotonergic stimulation led to increased enrichment of acetylated histone H4K12 (acH4K12) and RNA Polymerase II (Pol II) at *Bdnf* promoter III along with increased expression of *Bdnf* mRNA transcribed from this promoter in IMS Balb/c mice (Schmauss, 2015). Here we compared the expression of *Bdnf* transcript variant III mRNA in non-tested and WM-tested SFR and IMS Balb/c mice. In this study, tested mice had completed the WM test at 20 sec delay one hour prior to brain collection (see Fig. 1B). Age- and sex matched non-tested SFR and IMS mice served as controls. The results of real-time RT-PCR experiments are shown in Fig. 2B. A 2-way ANOVA revealed a significant main effect of rearing groups (SFR and IMS; $F(1,20)=21.9$; $p<0.0001$), a significant main effect of treatment (tested versus non-tested; $F(1,20)=30.4$; $p=0.0001$), and a significant interaction between these main effects ($F(1,20)=18.5$, $p=0.0004$). *Post hoc* Tukey HSD tests resolved these differences for WM-tested IMS Balb/c mice that expressed significantly more *Bdnf* mRNA compared with non-tested IMS mice as well as tested and non-tested SFR mice. This enhanced transcriptional activation of *Bdnf* promoter III is consistent with results of ChIP experiments demonstrating significantly increased density of the actively elongating form of RNA polymerase II (Pol II) at *Bdnf* promoter III in WM-tested compared with non-tested IMS Balb/c mice (Fig. 2C). The levels of HDAC1 at *Bdnf* promoter III of WM-tested mice did not differ from the baseline levels determined for IMS mice at P60 ($1/2$ Ct: 2.7 ± 1.1 (IMS at P60) and 2.5 ± 0.9 (WM-tested IMS at P84)).

In contrast to the large increase in *Bdnf* mRNA expression in WM-tested IMS Balb/c mice, *Egr2* mRNA levels did not significantly differ between WM-tested and non-tested SFR and IMS Balb/c mice (Fig. 2D), and WM test exposure did also not lead to increased recruitment of Pol II to the *Egr2* promoter (not shown). Finally, both SFR and IMS mice exhibited a similar test-induced induction of *c-Fos* mRNA expression (not shown).

Reducing *Bdnf*-TrkB signaling during adolescent development improves the cognitive functions of IMS Balb/c

Next we asked whether overexpression of the *Bdnf* gene contributes to the cognitive deficits of IMS Balb/c mice. Since *Bdnf* protein mediates its effect through TrkB receptors (Kaplan

and Miller, 2000), we took a pharmacological approach to reduce Bdnf-TrkB-receptor activation using the low-molecular weight TrkB antagonist Ana-12. Ana-12 that has been shown to penetrate the blood-brain barrier, to bind selectively to TrkB receptor, and to prevent TrkB-receptor activation by Bdnf with high potency. Ana-12 does not affect TrkA and TrkC receptor function (Cazorla et al., 2011).

In the study shown in Fig. 3, we treated both SFR and IMS Balb/c mice with Ana-12. Treatment was initiated at postnatal day 35 (P35; the developmental time point at which reduced HDAC levels were firmly established in the forebrain neocortex of IMS mice (Levine et al., 2012)), and it continued until P59 (see Fig. 1B). At the end of this treatment, SFR and IMS mice and their non-treated IMS and SFR controls were tested in an attention set-shifting test (ASST), followed by training and testing in the spatial WM test.

Fig. 3A summarizes results obtained from the ASST. In this test, mice proceed through 4 consecutive test phases, beginning with a simple discrimination (SD) between two different odors or textures to find a food reward, and followed by a compound discrimination (CD) in which an additional stimulus property (one new odor or texture) is added that does not guide correct response selection. Then, a completely new set of odors and textures is presented, but the stimulus property (odor or texture) that associated with correct response selection in the CD still guides correct response selection. This is the intradimensional set-shifting phase (IDS) of the ASST. Finally, another set of new odors and textures is presented, but in this extradimensional set-shifting task (EDS), the previously irrelevant stimulus dimension (odor or texture) is now guiding correct response selection. For all groups of mice, Repeated Measures ANOVA and *post hoc* Tukey-Kramer multiple comparisons showed that the number of trials to criterion was significantly higher for the EDS phase compared with all other test phases, including the IDS (SFR: $p < 0.001$; SFR-Ana12: $p < 0.001$; IMS: $p < 0.001$; IMS-Ana12: $p < 0.01$). Moreover, 2-way ANOVA revealed a significant main effect of treatment ($F(3,108)=7.2$, $p=0.0002$), a significant main effect of test phase ($F(3,108)=75.8$; $p < 0.0001$), and a significant interaction between these main effects ($F(9,108)=6$, $p < 0.0001$). *Post hoc* Tukey HSD tests resolved these differences for the EDS phase of the ASST: Consistent with our previous findings (Mehta and Schmauss, 2011), compared to non-treated SFR mice, non-treated IMS Balb/c mice required significantly more trials to criterion to complete the EDS phase (Fig. 3A). This deficit, however, was not detected in IMS Balb/c mice that were treated with the TrkB antagonist Ana-12 during adolescence (Fig. 3A). Strikingly, Ana-12 treatment of SFR mice significantly impaired their performance in the EDS phase. These findings indicate that both hypo- and hyperactivation of TrkB-receptor can cause deficits in this test.

A similar result was found in a delayed alternation spatial WM test (T maze). Here, 2-way ANOVA revealed a significant main effect of treatment ($F(3,67)=3.4$, $p=0.02$), a significant main effect of delay periods ($F(3, 67)=19.4$, $p < 0.0001$), and a significant interaction between these main effects ($F(6,67)=2.4$, $p=0.03$). *Post hoc* Tukey HSD tests resolved these differences for the groups of mice tested at 20 s inter-trial delay. At this delay, non-treated IMS mice had a significantly lower percentage of correct arms entries compared to non-treated SFR mice, but Ana-12 treated IMS mice did not differ from non-treated SFR mice (Fig. 3B). Moreover, similar to the effect of Ana-12 treatment of SFR mice on the

performance in the EDS phase of the ASST, Ana-12 treatment of SFR mice also impaired significantly their WM performance when tested at 20 sec delay, again indicating that both hypo- and hyperactivation of the Bdnf-TrkB signaling pathway can cause cognitive deficits.

Reducing HDAC 1 activity during adolescent development of SFR Balb/c mice also leads to cognitive deficits

The results shown above indicate that, in IMS Balb/c mice, enhanced Bdnf-TrkB signaling plays a significant role in the emergence of their executive cognitive deficits. To further test the role of reduced activity of HDAC1 in this process, we conducted additional studies on SFR Balb/c mice that were treated during adolescence (P35 to P59) with MS-275, an inhibitor of class I HDACs with nanomolar affinity to HDAC1, a 100-fold lower affinity to HDAC3, and no affinity to other HDACs (Hu et al., 2003; Kahn et al., 2008). As shown in Fig. 4A, compared to non-treated SFR mice, MS-275-treated SFR mice exhibit deficits in the EDS phase of the ASST that are similar to those found in IMS mice. Moreover, they exhibit a significant deficit in the spatial WM test at 20 s inter-trial delay (Fig. 4B).

Next we compared the levels of mRNA transcribed from *Bdnf* promotor III and the *Egr2* promotor between SFR Balb/c mice and non-tested and WM-tested SFR Balb/c mice that were treated with MS-275 using real-time RT-PCR. The tested MS-275-treated mice completed the spatial WM test (20s delay) one hour prior to brain collection (see Fig. 1B). Fig. 4C illustrates that, although the levels of Bdnf mRNA did not differ between SFR and non-tested MS-275-treated Balb/c mice, tested MS-275-treated Balb/c mice expressed significantly more Bdnf transcript variant III mRNA. In contrast, *Egr2* mRNA levels did not significantly differ between the three groups of mice (Fig. 4D). Hence, MS-275 treatment during adolescent development of SFR mice leads to the same cognitive deficits detected in IMS Balb/c mice and the same test-induced increase of Bdnf transcript variant III mRNA expression.

Cognitive deficits of C57Bl/6 mice fostered by Balb/c mothers during IMS exposure

We previously showed that C57Bl/6 mice raised by Balb/c mothers and exposed to the IMS paradigm do not develop WM deficits but strikingly, they do exhibit deficits in the EDS phase of the ASST (Schmauss et al., 2014). We replicated this finding in the present study in which C57Bl/6 mothers raised either their own pups with and without IMS exposure, and Balb/c mothers fostered C57Bl/6 pups during IMS exposure (see Fig. 1C). For the ASST performance of SFR and IMS C57Bl/6 mice raised by their biological mothers, Repeated Measures ANOVA and *post hoc* Tukey-Kramer multiple comparisons revealed that their numbers of trials to criterion were significantly higher for the EDS phase compared to all other test phases, including the IDS phases ($p < 0.01$) (Fig. 5A). The EDS performance of foster IMS C57Bl/6 mice raised by Balb/c mothers also differed significantly from all other test phases, including the IDS ($p < 0.001$) (Fig. 5A). A 2-way ANOVA further revealed a significant main effect of rearing groups ($F(2, 76) = 10.46$, $p = 0.0001$), a significant main effect of test phase ($F(3, 76) = 34.31$, $p < 0.0001$), and a significant interaction between these main effects ($F(6, 76) = 5.0$, $p = 0.0002$). *Post hoc* Tukey HSD tests resolved these differences for foster IMS C57Bl/6 mice that required a significantly higher number of trials to complete the EDS phase of the ASST (Fig. 5A).

In order to test whether foster IMS C57Bl/6 mice also exhibit decreased HDAC1 association with promoters of any of the three plasticity associated genes studied here at baseline (P60; see Fig. 1A), we performed HDAC1 and HDAC3 ChIPs on forebrain neocortical tissues of SFR and IMS C57Bl/6 mice raised by their biological mothers and on foster IMS C57Bl/6 mice that were raised by Balb/c mothers. No significant differences were found between the three groups for the levels of HDAC1 and HDAC3 at *Bdnf* promoters III and IV and the *Fos* promoter (Fig. 5B). A different result was obtained for the HDAC1 ChIP targeting the *Egr2* promoter: Compared with SFR and IMS C57Bl/6 mice, the levels of HDAC1 (but not HDAC3) were significantly lower in foster IMS C57Bl/6 mice (Fig. 5B).

We further measured *Bdnf* transcript variant III and *Egr2* mRNA in ASST-tested and non-tested foster IMS C57Bl/6 mice by real-time RT-PCR and compared these results to non-tested and ASST-tested IMS Balb/c mice. In these experiments, all pups raised by Balb/c mothers were foster pups, and brains of tested animals were collected 1 hour after completion of the EDS phase of the ASST (see Fig. 1C). For *Bdnf* mRNA expression, 2-way ANOVA revealed a significant main effect of test exposure ($F(1,16)=4.1$, $p=0.05$) and a significant interaction between strain and test exposure ($F(1,16)=4.1$, $p=0.05$). *Post hoc* Tukey HSD tests resolved these differences for ASST-tested foster IMS Balb/c mice that exhibited significantly increased *Bdnf* mRNA expression compared to all other groups of mice (Fig. 6A). (The magnitude of this increase, however, is only ~2.5-fold compared to the ~10-fold increase detected in WM-tested IMS Balb/c mice (see Fig. 2B)).

For *Egr2* mRNA expression, 2-way ANOVA also revealed significant main effects of test exposure ($F(1,18)=6.55$, $p=0.02$) and strain ($F(1,18)=4.4$, $p=0.05$), and a significant interaction between them ($F=4.36$, $p=0.05$). *Post hoc* Tukey HSD tests showed that *Egr2* mRNA levels were significantly elevated in tested foster IMS C57Bl/6 mice compared with all other groups of mice (Fig. 6B), a finding consistent with the increased levels of Pol II at the *Egr2* promoter in ASST-tested foster IMS C57Bl/6 mice relative to non-tested foster IMS C57Bl/6 mice (Fig. 6C). The HDAC1 levels at the *Egr2* promoter of ASST-tested foster IMS C57Bl/6 mice, however, did not differ from corresponding baseline levels measured at P60 ($1/2$ Ct: 0.42 ± 0.09 (foster IMS C57Bl/6 at P60) and 0.37 ± 0.03 (ASST-tested foster IMS C57Bl/6 at P66)). In summary, foster IMS C57Bl/6 mice that have only deficits in the ASST differ from IMS Balb/c mice by exhibiting reduced HDAC1 levels at the *Egr2* promoter rather than promoter III of the *Bdnf* gene, and they exhibit increased *Egr2* mRNA expression rather than increased *Bdnf* mRNA expression after ASST testing.

Finally, there are also differences in histone modifications associated with *Bdnf* promoter III and the *Egr2* promoter between IMS Balb/c and foster IMS C57Bl/6 mice. Whereas IMS Balb/c mice have increased acH4K12 levels associated with *Bdnf* promoter III compared with SFR Balb/c mice (Schmauss, 2015), the levels of acH4K12 at this promoter did not differ between SFR C57Bl/6 controls and foster IMS C57Bl/6 mice ($1/2$ Ct: 3.01 ± 1.03 (SFR C57Bl/6) and 3.18 ± 0.9 (foster IMS C57Bl/6) ($p=0.9$)). For the *Egr2* promoter, however, we observed a significant reduction of the levels of a repressive mark of gene transcription, namely H3K9me3, in foster IMS C57Bl/6 mice. Since reduced levels of acH3K9me3 were not found in IMS Balb/c mice, we conducted additional ChIPs targeting the *Egr2* promoter using an antibody directed against acH3K9, a marker of active gene

transcription, and we expressed these results as the ratio of acH3K9/H3K9me3 levels associated with the *Egr2* promoter. As shown in Fig. 6D, foster IMS C57Bl/6 mice (raised by Balb/c mothers) had a significantly higher acH3K9/H3K9me3 ratio compared to SFR and IMS C57Bl/6 and Balb/c mice (raised by their biological mothers). Hence, although reduced promoter-associated levels of HDAC1 are a common denominator for Balb/c and C57Bl/6 mice raised by Balb/c mothers during IMS exposure, the genes affected and the histone modification profiles at the promoters of the affected genes are also influenced by the genetic backgrounds of the pups.

DISCUSSION

Optimal cognitive task performance depends upon a finely-tuned balance between neuronal activation and inhibition, a balance that is maintained by tightly regulated gene induction and repression (Rao and Goldman-Rakic, 2000; Millan et al., 2012; Pezze et al., 2014). Since HDACs are in a strong position to modulate the expression of plasticity-related genes implicated in the control of cognitive functions, the present study asked whether the cognitive deficits found in mice exposed to early life stress can be explained by reduced expression of class I HDACs 1 and/or 3 that we previously detected in IMS Balb/c mice (Levine et al., 2012). We found that IMS Balb/c mice exhibit reduced association of HDAC1 (but not HDAC3) specifically with *Bdnf* promoter III, and that cognitive task exposure leads to increased density of the transcriptionally active form of Pol II at this promoter, a finding consistent with the large increase in *Bdnf* transcript variant III expression found after cognitive testing. Moreover, when non-stressed Balb/c mice were treated during adolescence with the HDAC1-preferring inhibitor MS-275, they exhibit the same cognitive deficits found in IMS mice as well as the same enhancement of test-induced *Bdnf* gene expression. The results of these pharmacological studies indicate clearly that the cognitive deficits and the associated change in *Bdnf* gene expression are due to reduced HDAC1 activity.

Our finding that blunting the *Bdnf*-TrkB receptor signaling during adolescence abolishes both the WM and attention set-shifting deficits in IMS Balb/c mice indicates that overexpression of the *Bdnf* gene contributes substantially to the emergence of these deficits. However, we found that SFR mice treated with the TrkB receptor antagonist Ana-12 also exhibit deficits in spatial WM and the EDS phase of the ASST. While this result was unexpected, it is consistent with the prevalent view that both hypo- and hyperactivation can cause cognitive deficits (Rao and Goldman-Rakic, 2000; Pezze et al., 2014), i.e., both *reduced* and *enhanced* *Bdnf*-TrkB receptor signaling can have detrimental effects on cognitive functions.

Strikingly, in IMS Balb/c mice, the same HDAC1-dependent increase in *Bdnf*-TrkB signaling has been shown to enhance the antidepressant efficacy of adolescent treatment with fluoxetine (Schmauss, 2015). However, the present finding can explain why adolescent treatment of IMS Balb/c mice with fluoxetine, although effectively improving their emotional phenotype, does not improve their cognitive deficits (Schmauss et al., 2014). This raises the intriguing question whether antidepressant drug treatments that enhance the *Bdnf*/TrkB-signaling effects benefit those patients that also have cognitive deficits, a question particularly germane to findings that patients in remission from depression that continue to

have cognitive deficits are more likely to relapse (Millan et al., 2012; McIntyre et al., 2013; Trivedi and Greer, 2014).

Although studies illustrated that the effect of IMS exposure on pups is due to the IMS-triggered alteration in maternal care (Huot et al., 2004; Schmauss et al., 2014), we found that IMS-triggered cognitive deficits as well as the reduced HDAC1 association with promoters of distinct plasticity-related genes are influenced by the genetic backgrounds of the pups. IMS C57Bl/6 mice raised by Balb/c mothers during IMS exposure exhibit deficits in attention set-shifting, but no WM deficits. Moreover, while their levels of HDAC1 at *Bdnf* promoter III are unaltered, they exhibit decreased HDAC1 levels at the promoter of the *Egr2* gene, a gene selectively induced in the ventrolateral orbital frontal cortex and the pre- and infralimbic subregions of the mPFC during ASST (but not WM) exposure (DeSteno and Schmauss, 2008). Indeed, only foster IMS C57Bl/6 mice exhibit a robust (~ 5 fold) increase of transcription of the *Egr2* gene upon ASST exposure. Since we previously showed that mice with enhanced ASST performance expressed lower levels of *Egr2* than those with diminished ASST performance (DeSteno and Schmauss, 2008), the present finding lends further support for the conclusion that overexpression of the *Egr2* gene associates specifically with ASST-selective cognitive deficits.

Compared with IMS Balb/c mice, foster IMS C57Bl/6 mice also exhibit a different histone modification phenotype at the *Egr2* promoter that is characterized by an increased ratio of acetylated H3K9 (a histone mark of open chromatin and active gene transcription) over H3K9me3 (a marker of gene repression). While IMS Balb/c mice exhibit increased acetylation of histone H4 protein, especially histone H4K12 acetylation at *Bdnf* promoter III (Schmauss, 2015), increased acetylation of histone H3 protein was not detected in these mice (Levine et al., 2012). Hence, IMS Balb/c and foster IMS C57Bl/6 mice do not only exhibit reduced HDAC1 levels at promoters of different genes, the promoters of the affected genes also differ in their histone modification profiles. Nevertheless, despite the cognitive task-induced enhanced transcription of different plasticity-related genes in both strains of mice, reduced HDAC1 association with the respective promoters is a common epigenetic phenotype. Importantly, just as ongoing cognitive experience has been shown to regulate the molecular consequences of HDAC inhibition (Sewal et al., 2015), the present study shows that the effect of reduced promoter-associated HDAC1 levels on gene expression is potentially unmasked by cognitive challenge. Moreover, in mice exposed to early life stress, reduced HDAC1 levels are not found on promoters of all plasticity-associated genes examined here, and cognitive challenge exerts different, gene-specific effects in different genetic backgrounds. This finding points to a sensitive interaction between genomic sequence variations (acting in *cis*) and the stress-modulated epigenome, an interaction unlikely to be mimicked with studies on genetically modified mice harboring a conditional HDAC1 deficiency.

Contrary to the present study that uncovered a role of reduced HDAC1 activity in the emergence of early-life stress-triggered cognitive deficits that are largely governed by the PFC, several other studies have demonstrated that HDAC inhibitors can ameliorate age-related cognitive decline (Peleg et al., 2010) or memory impairments in neurodegenerative disorders, i.e., functions that are primarily governed by the hippocampus (Fischer et al.,

2007; Kilgore et al., 2009; Gräff et al., 2012). For example, in an animal model of inducible severe neurodegeneration and synapse loss, the non-selective HDAC inhibitor sodium butyrate increased histone H3 and H4 acetylation in the hippocampus, promoted associative and spatial learning, and increased expression of synaptic proteins in remaining neurons (Fischer et al., 2007). Similarly, in an animal model of Alzheimer's Disease with early onset contextual memory impairment, three non-selective HDAC inhibitors (sodium butyrate, sodium valproate, and suberoylanilide hydroxamic acid (SAHA)) rescued memory deficits and led to stably maintained consolidated memory for two weeks (Kilgore et al., 2010). Yet, the study of Kilgore et al. (2010) also showed with biochemical assays that all three HDAC inhibitors share in common a potent inhibition of class I HDACs1-3, and other studies pointed to a specific role of HDAC2 in the modulation of distinct cognitive functions. For example, studies on HDAC1 and HDAC2 overexpressing mice revealed that, although both lines of mice exhibit decreased acetylation of histone H4K12 in the hippocampus, only HDAC2 overexpressing mice have deficits in associative spatial learning (a hippocampal function) and WM (a PFC function) (Guan et al. 2009). Conversely, HDAC2 knockout mice with increased histone H4 and H2B acetylation in the hippocampus exhibit increased associative learning and improved WM, and they have increased hippocampal spine density and synaptic terminals (Guan et al., 2009). HDAC2 deficient mice also exhibit increased acetylation of histone H3 and H4 at *Bdnf* promoter I/II, *Egr2*, and *Fos*, and they are refractory to the effect of SAHA on synaptic plasticity and learning (Guan et al., 2009).

A specific role of HDAC2 in memory impairment is further supported by findings of increased HDAC2 expression in the hippocampal CA1 subfield and the PFC in an animal model of severe neurodegeneration. In this model, shRNA-mediated HDAC2 knockdown lead to increased acetylation of histone H4K12 at promoters of plasticity-associated genes along with increased expression of these genes, re-instated morphological and synaptic plasticity of surviving neurons, and improved associative and spatial memory (Gräff et al., 2012).

Finally, studies also identified specific roles for HDAC1 and HDAC3 in distinct hippocampal-dependent cognitive processes. One study showed that virus-mediated overexpression of HDAC1 in the hippocampus did not affect WM and short- and long-term memory. However, it facilitated the extinction of fear memories and, conversely, hippocampal injection of MS-275 or knockdown of HDAC1 with siRNA significantly impaired fear extinction. The authors linked these effects to binding of HDAC1 to *Fos* and *Egr2* promoters where it affects histone H3K9 acetylation (Bahari-Javan et al., 2012). Another study on mice with a focal deletion of dorsal hippocampal HDAC3 and increased histone H4K8 acetylation (that correlated with increased *Fos* and *Nr4a2* gene expression) revealed a cognitive phenotype of facilitated long-term memory (McQuown et al., 2011).

It is evident from the studies summarized above that class I HDACs1-3 play distinct roles in several cognitive functions. However, while reducing the activity of hippocampal HDACs1-3 (and possibly also prefrontal cortical HDAC2) can exert a plethora of pro-cognitive effects, the present study revealed that early-life stress-triggered cognitive deficits arise primarily from reduced HDAC1 association with promoters of distinct plasticity-associated genes. Whether reduced HDAC1 activity in mice exposed to early life stress only affects executive

cognitive function governed by the PFC, or whether hippocampal-related functions are also affected by reduced HDAC1 (or HDAC3 activity) remains to be investigated. However, since the HDAC-dependent epigenetic response of IMS Balb/c mice is only firmly established by mid-adolescence (P35) (Levine et al., 2012) it is conceivable that it predominantly affects functions governed by late-maturing brain regions such as the PFC (Pechtel and Pizzagalli, 2011).

Finally, while the present study focused on three genes with either known roles in the cognitive processes tested here or known to be induced by them, it is entirely possible that also other plasticity-associated genes (such as *Arc*, *Egr1*, and *Npas4*; see Saha et al., 2011) are differentially affected by reduced promotor-associated HDAC1 levels in early-life stress-exposed mice with different genetic backgrounds. Whether this is the case, and whether an enhanced transcription of such additional genes also contribute to cognitive deficits remains to be investigated.

CONCLUSIONS

This study demonstrates for the first time that an epigenetic response to early life stress exposure contributes the emergence of working memory and attention set-shifting deficits, i.e. deficits that often accompany mental disorders and that are largely resistant to treatment. The results show that reduced histone deacetylase 1 levels at promoters of distinct plasticity-related genes allow for abnormally increased expression of these genes upon cognitive testing, an effect that influences cognitive task performance negatively. The genes affected by reduced HDAC1 activity differ in genetically different strains of mice, indicating that genomic sequence variations and their differential interaction with the stress-modulated epigenome determine the outcome of early-life stress-triggered cognitive impairment.

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Research Highlights

- Early life stress triggers persistent executive cognitive deficits
- Early life stress leads to a reduced histone deacetylase 1 (HDAC1) levels at promoters of distinct plasticity-associated genes
- In different strains of mice, the genes affected by reduced promotor-associated HDAC1 levels are determined by their genetic background
- Reduced HDAC1 activity predisposes to enhanced expression of the affected genes in response to cognitive task exposure
- Enhanced expression of these genes negatively influences cognitive task performance

Figure 1. Experimental design and timeline

(A) Mice exclusively used for HDAC1 and HDAC3 ChIPs were exposed to the IMS from P2 and P15, weaned at P28 and raised to adulthood (P60). At P60, their brains were removed. SFR mice raised in parallel served as controls. (B) Other groups of mice were chronically treated with either Ana-12 (IMS Balb/c mice) or with MS-275 (SFR Balb/c mice) from postnatal age P35 until P59. At the end of treatment, mice were tested in the ASST followed by training and testing in the WM test. One hour post completion of the WM test at 20 sec delay (typically around P84), brains were collected for real-time RT-PCR and ChIP

experiments. Age- and sex-matched vehicle-treated mice served as controls. (C) Forster IMS C57Bl/6 or foster IMS Balb/c mice were raised by Balb/c mothers during IMS exposure, weaned at P28, and tested in the ASST in adulthood (at P66). One hour post completion of the EDS phase of the ASST, brain were collected for real-time PCR and ChIP experiments. Age- and sex-matched non-tested mice served as controls.

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Figure 2. Reduced HDAC1 levels at promotor III of the *Bdnf* gene in IMS Balb/c mice

(A) Comparison of the levels of HDAC1 and HDAC3 at promoters III and IV of the *Bdnf* gene and the promoters of the *Egr2* and *Fos* genes in SFR and IMS Balb/c mice. Data are mean $\pm$ sem (n=8/group for the HDAC3 *Bdnf* PIII ChIP (4 males and 4 females) and n=6/group for all other groups (3 males and 3 females)) and were compared by Student's *t*-test. SFR and IMS Balb/c mice differ significantly in their levels of HDAC1 associated with *Bdnf* promotor III. (B) *Bdnf* transcript variant III expression in non-tested and WM-tested (20 s delay) SFR and IMS Balb/c mice. Data (mean $\pm$ sem; n=6/group) were compared by 2-way ANOVA (see Results) and statistical differences were resolved *post hoc* (Tukey HSD test) as indicated. (C) Comparison of the levels of Pol II association at *Bdnf* promotor III between non-tested and WM-tested IMS Balb/c mice. Data ((mean $\pm$ sem; n=6/group) were compared using two-tailed Student's *t* test. (D) *Egr2* mRNA expression levels in non-tested and WM-tested SFR and IMS Balb/c mice. Data (mean $\pm$ sem; n=6/group) were compared by 2-way ANOVA that revealed no significant differences.

Figure 3. The effects of adolescent Ana-12 treatment of IMS and SFR Balb/c mice on cognitive task performance in adulthood

(A). Performance of SFR, IMS and Ana-12-treated SFR and IMS Balb/c mice in the ASST. Data are mean $\pm$ sem of 8 animals per group (4 males and 4 females) and were compared by 2-way ANOVA (see Results). Statistical differences were resolved *post hoc* (Tukey HSD tests) as indicated. Compared to SFR mice, IMS Balb/c mice exhibited significant deficits in the EDS phase of the ASST, a deficits that is abolished in Ana-12-treated IMS mice. In contrast, Ana-12 treatment of SFR Balb/c mice led to the same deficits found in IMS mice.

(B) Performance of the same groups of mice in the WM test. Data were compared by 2-way ANOVA (see Results) and significant differences resolved *post hoc* (Tukey HSD tests) are indicated. When tested at 20 s inter-trial delays, the percentages of correct arm entries of IMS Balb/c and Ana-12-treated SFR mice were significantly lower compared to non-treated SFR and Ana-12-treated IMS mice.

Figure 4. Effect of adolescent MS-275 treatment of SFR Balb/c mice on cognitive task performance and expression of Bdnf and Egr2 mRNA

(A) Performance of SFR Balb/c mice treated with MS-275 in the ASST. Data are mean $\pm$ sem (n=8/group). Significant differences between non-treated and MS-275-treated SFR mice were found only for the EDS phase of the ASST (Student's *t* test). (B) Performance of the same group of mice shown in (A) in the spatial WM test. At 20 s delay, significant differences were found between the two groups (Student's *t* test). (C) Real-time RT-PCR measures of Bdnf transcript variant III expression. Data are mean $\pm$ sem (n=6/group). Significant differences revealed by one-way ANOVA ($F(2,16)=4.56$; $p=0.02$) were resolved *post hoc* (Tukey-Kramer multiple comparisons test) only for tested MS-275-treated mice that exhibited significantly increased expression of Bdnf mRNA. (D) For the same group of mice shown in (C), real-time RT-PCR measures of Egr2 mRNA revealed no significant differences between the three groups (ANOVA, $F(2,16)=0.5$; $p=0.62$).

Figure 5. Foster IMS C57Bl/6 mice have deficits in the ASST and reduced HDAC1 levels at the promoter of the *Egr2* gene

(A) ASST performance of SFR and IMS C57Bl/6 mice raised by their biological mothers and foster IMS C57Bl/6 mice raised by Balb/c mothers. Data are mean $\pm$ sem ($n=7$ /group) and were compared by 2-way ANOVA (see Results). Statistical differences were resolved *post hoc* (Tukey HSD tests) as indicated. (B) Comparison of the levels of HDAC1 and HDAC3 at promoters III and IV of the *Bdnf* gene and the promoters of the *Egr2* and *Fos* genes in SFR and IMS C57Bl/6 mice raised by their biological mothers and foster IMS C57Bl/6 mice raised by Balb/c mothers. Data are mean $\pm$ sem ($n=6$ /group). One-way ANOVA revealed significant differences between groups only for the levels of HDAC1 at the *Egr2* gene promoter ($F(2,14)=9.12$; $p=0.005$). *Post hoc* Tukey-Kramer multiple comparisons resolved these differences for foster IMS C57Bl/6 mice that had significantly lower levels of HDAC1 compared to SFR and IMS C57Bl/6 mice.

Figure 6. Foster IMS C57Bl/6 mice exhibit increased *Egr2* mRNA expression after ASST testing and increased histone H3 acetylation at the *Egr2* promotor

(A) Comparison of Bdnf transcript variant III and (B) *Egr2* mRNA levels between non-tested and ASST-tested foster IMS C57Bl/6 and Balb/c mice. Data (mean $\pm$ sem; $n=6$ /group) were compared by 2-way ANOVA (see Results). Significant differences resolved *post hoc* (Tukey HSD tests) are indicated. Whereas foster IMS Balb/c mice exhibited increased Bdnf mRNA expression after ASST testing, ASST-tested foster IMS C57Bl/6 mice expressed significantly higher levels of *Egr2* mRNA. * $p<0.05$ in (A) and $p<0.01$ in (B). (C) Comparison of the levels of Pol II association with the *Egr2* promotor between non-tested and ASST-tested foster IMS C57Bl/6 mice. Data ((mean $\pm$ sem; $n=6$ /group) were compared using two-tailed Student's *t* test. (D) Ratio of acH3K9 over H3K9me3 levels associated with the *Egr2* promotor of SFR and IMS C57Bl/6 and Balb/c mice compared to foster IMS C57Bl/6 mice. Data are mean $\pm$ sem ($n=5$ /group). One-way ANOVA revealed significant differences between groups ($F(4,19)=3.9$; $p=0.02$)) that were resolved *post hoc* for foster IMS C57Bl/6 mice that exhibited a significantly higher acH3K9/H3K9me3 ratio. * $p<0.05$ compared to all other groups.