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Altered sirtuin deacetylase gene expression in patients with a mood disorder

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ABSTRACT

Sirtuins are a family of NAD+-dependent enzymes that regulate cellular functions through deacetylation of various proteins. Although recent reports have suggested an important role of deacetylases (i.e., histone deacetylases) in mood disorders and antidepressant action, the involvement of sirtuins in the pathophysiology of mood disorders is largely unknown. In this study, we aimed to determine whether there are alterations in sirtuin mRNA expression in peripheral white blood cells of patients with a mood disorder. Also, to examine whether the altered sirtuin mRNA expression is state- or trait-dependent, mood disorder patients who were in a remissive state were assessed. We used quantitative real-time polymerase chain reaction to measure the mRNA levels of seven sirtuin isoforms (SIRT1–7) in peripheral white blood cells of patients with major depressive disorder (MDD) or bipolar disorder (BPD) during depressive and remissive states and in normal healthy subjects. The SIRT1, 2 and 6 mRNA levels in MDD and BPD patients decreased significantly in those who were in a depressive state compared to healthy controls, whereas the expression of those mRNAs in both MDD and BPD of patients in a remissive state were comparable to those in healthy controls. Thus, our data suggest that altered SIRT1, 2 and 6 expression is state-dependent and might be associated with the pathogenesis and/or pathophysiology of mood disorders.

1. Introduction

Recent evidence suggests that aberrant gene regulation in the brain are associated with the pathophysiology of many neuropsychiatric disorders, including mood disorders (**Mill and Petronis, 2007; Tsankova et al., 2007**). Changes in chromatin structure play a role in the regulation of gene expression attributable to covalent histone modification via acetylation and deacetylation (**Grunstein, 1997; Hsieh and Gage, 2005**). Histone deacetylases (HDACs) are major enzymes involved in chromatin remodeling (**Grunstein, 1997; Hsieh and Gage, 2005**). The epigenetic gene regulation that occurs through HDACs has been suggested to contribute to behavioral responses to chronic stress and antidepressants in rodents (**Tsankova et al., 2006; Renthal et al., 2007; Covington et al., 2009; Bredy et al., 2010; Uchida et al., 2011**).

The human HDAC family consists of four classes based on their homology to yeast HDACs (**Chuang et al., 2009**). Among them, class I (HDAC1–3 and 8) and class II (HDAC4–7, 9, and 10) HDACs have been most extensively investigated for their roles in the central nervous system. Recent evidence suggests the involvement of HDAC2 and HDAC5 in the behavioral response to stress and antidepressant actions in rodents (**Tsankova et al., 2006; Renthal et al., 2007; Covington et al., 2009**). Furthermore, altered expression of HDAC2, 4, 6, or 8 have been observed in mood disorder patients (**Covington et al., 2009; Hobara et al., 2010**). Thus, dysfunction of class I and II HDACs may play important roles in the pathophysiology of mood disorders and antidepressant responses.

In contrast, sirtuins (SIRTs) are class III HDACs, and are a family of nicotinamide adenine dinucleotide (NAD+)-dependent enzymes that regulate cellular functions through deacetylation of various protein targets (**Whittle et al., 2007; Bao and Sack, 2010**). The mammalian sirtuins, SIRT1–7, are implicated in a variety of cellular functions, such as gene silencing, cell cycle control, apoptosis, general metabolism, energy homeostasis, and aging (**Michan and Sinclair, 2007**). However, the involvement of SIRTs in the pathophysiology of mood disorders is largely unknown.

Patients with a mood disorder and individuals who are chronically stressed exhibit altered gene expression in the central nervous system and peripheral blood cells (**Matsubara et al., 2006; Anitha et al., 2008; Miller et al., 2008; Otsuki et al., 2008**). However, biochemical and molecular biological studies using brain biopsies of living psychiatric patients are often unrealistic. A study using static measures such as postmortem brain samples makes it

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difficult to investigate a change that depends on the state of a mental illness. In contrast, peripheral blood samples may be convenient and accessible. Thus, the search for peripheral biomarkers in psychiatric disorders is worthy (**Iga et al., 2006; Gavin and Sharma, 2010**). It should be noted that lymphocytes may be exploited as a neural and possible genetic probe in psychiatric research studies (**Gladkevich et al., 2004**). In the present study, we aimed to determine whether there were alterations in the mRNA expression of SIRT isoforms (SIRT1–7) in peripheral white blood cells of patients with mood disorders. In addition, to investigate whether the altered SIRT mRNA expression was state- or trait-dependent, patients with a mood disorder who were in a remissive state were also assessed.

2. Methods and materials

2.1. Subjects

Major depressive disorder (MDD) and bipolar disorder (BPD) were diagnosed according to the criteria in the Diagnostic and Statistical Manual of Mental Disorders, fourth edition (**American Psychiatric Association, 1994**). These included both outpatients and inpatients of the Division of Neuropsychiatry at Yamaguchi University Hospital. The extent of the depressive state was assessed by the 21-item Hamilton Depression Rating Scale (HDRS). Subjects were classified as under a current depressive state when they showed a score of more than 18 on HDRS and met the DSM-IV criteria for a major depressive episode. Subjects were classified as being in remission when they showed a score of less than 6 on HDRS and did not show any symptoms of a major depressive episode based on the DSM-IV criteria for more than two months. Individuals were excluded if they had an abnormal physical examination or abnormal results of routine medical laboratory tests such as a complete blood count or renal, liver, or thyroid function. Female subjects who were pregnant or took oral contraceptives were also excluded. All healthy controls subjects were genetically unrelated residents living in Japan without any history of mental illness. Controls and patients were of Japanese ethnicity, and no significant population stratification was observed, as reported by several groups (**Kakiuchi et al., 2003; Yamada et al., 2004**). This protocol was approved by the Institutional Review Board of Yamaguchi University Hospital. Informed written consent was obtained from all subjects.

2.2. Blood sample preparation, RNA isolation, and cDNA synthesis

Blood sample preparation, total RNA isolation, and cDNA synthesis were performed as previously described (**Otsuki et al., 2010**). Briefly, blood was obtained by venipuncture between 10:00 and 11:00 and processed for total RNA purification from peripheral blood cells using the QIAamp RNA Blood Mini Kit (Qiagen, Chatsworth, California), according to the manufacturer's manual. RNA quality was determined based on the A_{260}/A_{280} ratio, which was 1.8–2.0 for all RNA preparations.

2.3. Quantitative real-time polymerase chain reaction (Q-PCR)

Q-PCR was performed in the Applied Biosystems 7300 Fast Real-Time PCR system with SYBR green PCR Master Mix (Applied Biosystems, Foster City, California), as previously reported (**Hobara et al., 2010; Uchida et al., 2010**). The list of primer sequences used is shown in **Table 1**. All measurements were performed in duplicate, and at least two independent experiments per primer set were conducted. Levels of *Gapdh* mRNA were used to normalize the relative expression levels of target mRNA. Methods and results of the quantitative analysis of glucocorticoid receptor α (GRα) mRNA levels were described in detail in our previous report (**Matsubara et al., 2006**).

Table 1
List of primer sequences used.

Gene	Forward (5′–3′)	Reverse (5′–3′)
SIRT1	TCAGTGGCTGGAACAGTGAG	AGCGCCATGGAAAATGTAAC
SIRT2	GAACAGGAGGACTTGGTGGA	GGCGTCACCTCAGAGAAGAT
SIRT3	GGGCAGGTGAAACCAGAATA	TGGCCCTGACTGTAAACACA
SIRT4	CTTGGCGTGTCTGAAACTGA	TGAAAGTCCCTGTTCCAGGT
SIRT5	GCTCGCCCACTGTGATTTAT	TCCGTGTTAAATTCAGCCACT
SIRT6	AGGATGTCGGTGAATTACGC	TGGAAGACTGCCAGACCAG
SIRT7	CCCTGAAGCTACATGGGAAG	AGTCGCCAGTGAGAAAATGC
GAPDH	CAGCCTCAAGATCATCAGCA	TGTGGTCATGAGTCCTTCCA

2.4. Serum cortisol determination

Serum cortisol concentrations were measured by radioimmunoassay (SRL Corp., Tokyo, Japan).

2.5. Dexamethasone (Dex)/corticotrophin-releasing hormone (CRH) test

The Dex/CRH test was performed as previously reported (**Matsubara et al., 2006**). We defined non-suppressors as those individuals whose post-Dex serum cortisol levels were greater than 5 μg/dl.

2.6. Statistical analyses

Commercial software (SPSS version 16.0; SPSS Inc., Illinois, Chicago) was used to perform the data analyses. All data are expressed as the mean ± standard error of the mean. Multivariable analysis was conducted using the mRNA level of each SIRT as a dependent variable with age, gender, and antidepressant as independent variables. Gender distribution was analyzed by the χ^2 test. The mRNA levels of SIRTs were subjected to a one-way analysis of variance followed by a *post-hoc* Dunnett's test. Two-group comparisons in the same patients with mood disorders before and after remission and for the SIRT mRNA levels in the suppressors and non-suppressors of the Dex/CRH test were performed using the paired *t*-test and unpaired *t*-test, respectively. Pearson's correlations were calculated to assess the correlation between data. In all cases, *p*-values were two-tailed, and comparisons were considered to be statistically significant at $p < 0.05$.

3. Results

3.1. SIRT mRNA levels in patients with a mood disorder

Table 2 shows the demographic and clinical characteristics of the subjects. The majority of patients were on medication. Mean ages were not significantly different among the MDD or BPD patients and healthy control subjects ($F(4,126) = 1.178$, $p > 0.05$). BPD patients showed a significantly larger ratio of females to males ($\chi^2 = 15.36$, $p < 0.001$).

In multivariable analyses ($n = 103$), the mRNA levels of all SIRTs examined were not associated with gender or medication (antidepressant) (data not shown). However, multivariable analyses indicated that the mRNA levels of SIRT1 and 6 were significantly associated with age.

We examined the mRNA levels of SIRTs (SIRT1–7) in MDD and BPD patients in a current depressive state (**Fig. 1**A) and in

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Table 2
Demographic and clinical characteristics of subjects.

	Control	Patient			
		MDD		BPD	
		Depressed	Remission	Depressed	Remission
	$n = 28$	$n = 20$	$n = 39$	$n = 12$	$n = 32$
Age (years)	50.0 ± 1.8	52.3 ± 3.5	57 ± 2.2	54.8 ± 3.9	52.4 ± 2.6
Gender (Male/Female)	15/13	10/10	15/24	2/10	7/25
HDRS		25.8 ± 1.9	3.3 ± 0.2	24.5 ± 1.1	2.8 ± 0.6
Serum Cortisol (mg/dl)	8.6 ± 0.8	10.2 ± 1.4	11.9 ± 1.1	11.2 ± 3.0	10.3 ± 0.2
Medication					
No medication	28	3	4	1	0
SSRI	0	5	23	3	5
SNRI	0	5	13	4	3
TCA	0	8	6	1	3
Tetracyclic Antidepressant	0	11	15	3	9
Sulpiride	0	4	7	7	1
Li	0	0	0	3	17
VPA	0	0	0	7	14
CBZ	0	0	0	2	8

MDD, major depressive disorder; BPD, bipolar disorder; HDRS, Hamilton Depression Rating Scale; SSRI, selective serotonin reuptake inhibitor; SNRI, serotonin norepinephrine reuptake inhibitor; TCA, tricyclic antidepressant; Li, lithium; VPA, valproic; CBZ, carbamazepine.

a remissive state (Fig. 1B). The Q-PCR results revealed that the expression of SIRT1, 2, and 6 mRNA decreased significantly in MDD patients in a current depressive state (SIRT1, $F(2,57) = 9.358$, $p < 0.001$, *post-hoc*, $p < 0.01$; SIRT2, $F(2,57) = 6.751$, $p < 0.01$, *post-hoc*, $p < 0.05$; SIRT6, $F(2,57) = 10.746$, $p < 0.001$, *post-hoc*, $p < 0.001$) and in patients with BPD (SIRT1, *post-hoc*, $p < 0.01$; SIRT2, *post-hoc*, $p < 0.01$; SIRT6, *post-hoc*, $p < 0.01$) compared to healthy control subjects (Fig. 1A). In contrast, the expression of those mRNAs in patients with MDD and BPD who were in remission were comparable to those of healthy control subjects (SIRT1, $F(2,96) = 4.016$, $p > 0.05$; SIRT2, $F(2,96) = 1.462$, $p > 0.05$; SIRT6, $F(2,96) = .883$, $p > 0.05$) (Fig. 1B).

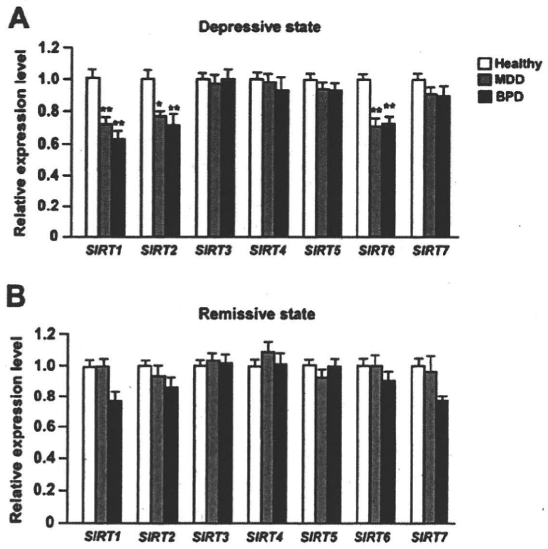

Fig. 1. mRNA levels of sirtuin (SIRTs) in patients with major depressive (MDD) or bipolar disorder (BPD) in a depressive and remissive state. (A) Quantitative real-time PCR analysis of SIRTs mRNA levels in patients with MDD ($n = 20$) or BPD ($n = 12$) who were in a current depressive state and healthy control subjects ($n = 28$). SIRT1, 2, and 6 mRNA expression decreased significantly in patients with either mood disorder compared to healthy control subjects. (B) Quantitative real-time PCR analysis of SIRT mRNA levels in patients with MDD ($n = 39$) or BPD ($n = 32$) who were in a remissive state and healthy control subjects ($n = 28$). SIRT1, 2 and 6 mRNA expression in patients with a mood disorder was comparable to those in healthy control subjects. Values are mean ± standard error. $^*p < 0.05$; $^{**}p < 0.01$ versus controls.

We next examined the mRNA levels of SIRTs in the same MDD ($n = 15$) and BPD ($n = 10$) patients before and after remission. In patients with MDD, the mRNA levels of SIRT1, 2, and 6 increased significantly in those in a remissive state compared with those in a depressive state (SIRT1, $t = 6.242$, $p < 0.01$; SIRT2, $t = 7.574$, $p < 0.001$; SIRT6, $t = 6.825$, $p < 0.01$) (Fig. 2A–C). In patients with BPD, SIRT1 mRNA expression levels increased significantly in those in a remissive state compared with those in a depressive state (SIRT1, $t = 7.258$, $p < 0.05$) (Fig. 2D). No significant difference was observed in SIRT2 and 6 mRNA expression (SIRT2, $t = 6.686$, $p > 0.05$; SIRT6, $t = 6.483$, $p > 0.05$) (Fig. 2E and F), as the expression of those SIRTs decreased in a depressive but not in a remissive state in patients with bipolar disorder compared to healthy controls (Fig. 1A and B). These results suggest that decreased SIRT1, 2, and 6 mRNA expression was state-dependent in MDD and BPD patients.

3.2. SIRT expression and hypothalamic-pituitary-adrenal (HPA) axis function

Depressed patients often exhibit a dysregulation of the HPA axis on the Dex/CRH test (Holsboer, 2000; de Kloet et al., 2005; Anacker et al., 2010). To investigate the association between SIRT mRNA levels and HPA axis activity, the mRNA levels of SIRTs in patients with a mood disorder who were currently in a depressive state were compared between suppressors ($n = 10$; seven patients with MDD and three with BPD) and non-suppressors ($n = 9$; three patients with MDD and six with BPD) on the Dex/CRH test. No significant differences in the expression levels of any SIRTs were found between suppressors and non-suppressors (Fig. 3A). In addition, no significant correlation was observed between the mRNA levels of SIRTs and serum cortisol concentrations in patients with MDD and BPD and healthy controls (data not shown). GR plays an important role in the regulation of brain functions, including neuroendocrine function, immune function, and stress responses (Holsboer et al., 1994; McEwen, 1997; de Kloet et al., 2005). We previously reported decreased expression of GRα mRNA in the peripheral blood cells of patients with a mood disorder (Matsubara et al., 2006). We next investigated the correlation between GRα mRNA expression and SIRT mRNA expression in MDD and BPD patients who were in a depressive state. We found a significant correlation between SIRT2 mRNA levels and GRα in healthy control subjects ($r = 0.427$, $p < 0.05$), whereas such a correlation was not observed in patients with MDD ($r = 0.078$, $p > 0.05$) or BPD

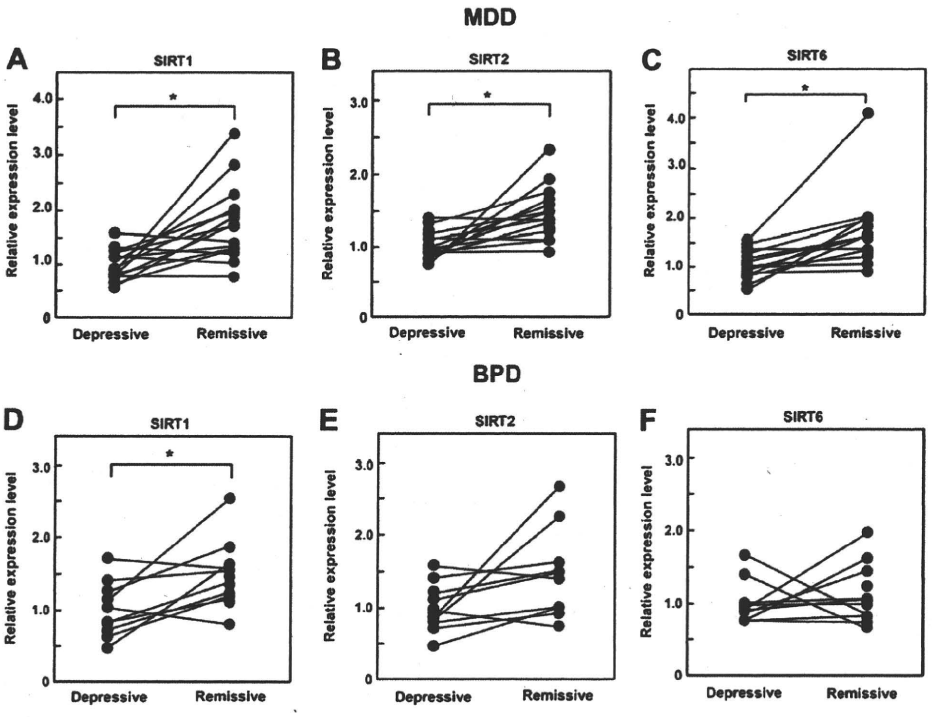

Fig. 2. mRNA levels of SIRTs in patients with MDD or BPD before and after remission. (A–C) Quantitative real-time PCR analysis of SIRT mRNA levels in patients with MDD before and after remission ($n = 15$). SIRT1, 2 and 6 mRNA expression levels increased significantly in patients in a remissive state compared with those in a depressive state. (D–F) Quantitative real-time PCR analysis of SIRT mRNA levels in patients with BPD before and after remission ($n = 10$). SIRT1 mRNA expression levels increased significantly in patients in a remissive state compared with those in a depressive state. Values are mean ± standard error. *$p < 0.05$ versus controls.

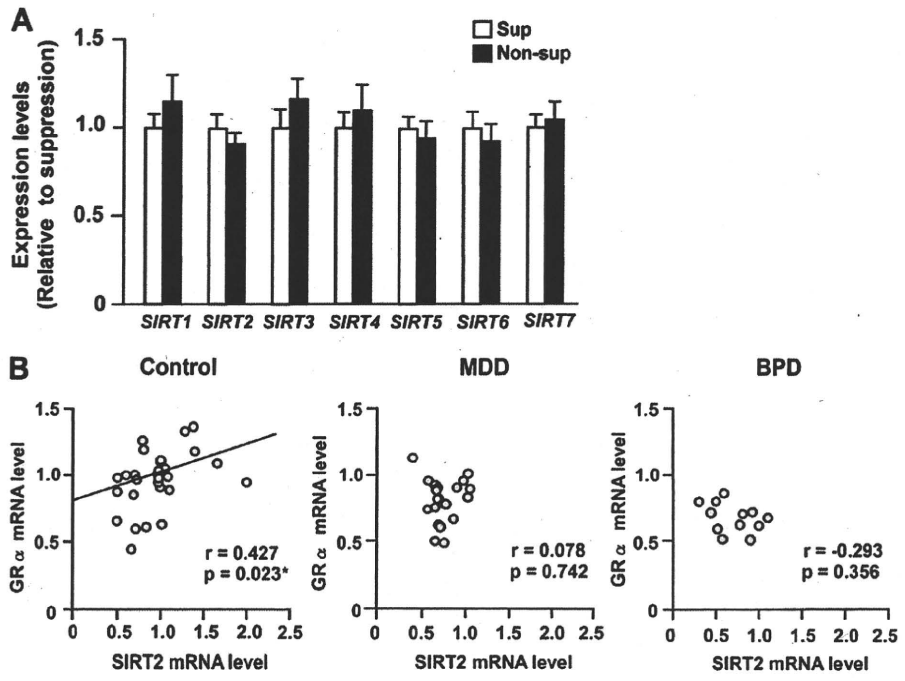

Fig. 3. Comparison of the expression levels of SIRTs mRNA between suppressors and non-suppressors on the dexamethasone (Dex)/corticotropin releasing hormone (CRH) test and the correlation of SIRT mRNA expression with glucocorticoid receptor (GR) α mRNA expression. (A) Comparison of the expression levels of SIRT mRNA between suppressors ($n = 10$; seven patients with major depressive disorder and three with bipolar disorder) and non-suppressors ($n = 9$; three patients with major depressive disorder and six with bipolar disorder) of Dex/CRH test. No significant difference was observed in the expression levels of any of the SIRT mRNAs between suppressors and non-suppressors. Sup, suppressors; Non-Sup, non-suppressors. Values are mean ± standard error. (B) A significant correlation was found between SIRT2 mRNA expression and GRα mRNA expression in healthy control subjects, but not in patients with major depressive disorder (MDD) or bipolar disorder (BPD).

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($r = -0.293$, $p > 0.05$) (Fig. 3B). There was no significant correlation between SIRT1 and SIRT6 mRNA level and GRα in healthy controls, MDD, and BPD (data not shown).

3.3. Effect of antidepressants on SIRT mRNA expression

As shown in Table 2, almost all patients with a mood disorder who participated in this study were on medication, so we considered the effect of medications on SIRT mRNA expression, and to examine whether SIRTs expressions are influenced by the types of medication, we analyzed SIRTs levels in mood disorder patients receiving any type of antidepressants (tricyclics, tetracyclics, selective serotonin reuptake inhibitor, serotonin norepinephrine reuptake inhibitor) and sulpiride, as well as three types of mood stabilizers (lithium, valproate, carbamazepine) with one-way ANOVA. There were no significant differences in SIRT mRNA expression were found among patients with MDD or BPD who were receiving any type of antidepressants (SIRT1, $F(4,40) = .810$, $p = 0.526$; SIRT2, $F(4,40) = 0.962$, $p = 0.439$; SIRT3, $F(4,40) = 1.610$, $p = 0.191$; SIRT4, $F(4,40) = 1.597$, $p = 0.194$; SIRT5, $F(4,40) = .497$, $p = 0.738$; SIRT6, $F(4,40) = .264$, $p = 0.899$; SIRT7, $F(4,40) = .661$, $p = 0.623$) (Fig. 4), as well as mood stabilizers (SIRT1, $F(2,9) = .028$, $p = 0.973$; SIRT2, $F(2,9) = 2.292$, $p = 0.157$; SIRT3, $F(2,9) = .483$, $p = 0.632$; SIRT4, $F(2,9) = 2.601$, $p = 0.128$; SIRT5, $F(2,9) = .477$, $p = 0.635$; SIRT6, $F(2,9) = 1.367$, $p = 0.303$; SIRT7, $F(2,9) = .001$, $p = 0.999$). In addition, no significant difference was found in the medication (imipramine equivalent) of MDD or BPD patients between those in a current depressive and those in a remissive state (data not shown). Moreover, no correlation was observed between the expression level of each SIRT mRNA and medication (imipramine equivalent) in MDD or BPD patients (data not shown) in both a depressive and remissive state.

4. Discussion

We found reduced SIRT1, 2, and 6 mRNA expression in peripheral blood cells of MDD and BPD patients who were in a current depressive state. Furthermore, these alterations in SIRT expression were not observed in patients in a remissive state. Thus, our data suggest that the reduced expression of SIRT1, 2 and 6 mRNA is state-dependent in patients with MDD and BPD.

SIRTs function as the primary site for oxidative metabolism and play crucial roles in apoptosis (Michan and Sinclair, 2007; Bao and Sack, 2010; Chung et al., 2010). It is important to note that extensive studies suggest the involvement of apoptosis in the pathophysiology of mood disorders (Harlan et al., 2006).

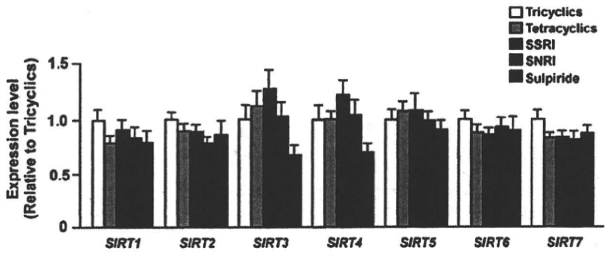

Fig. 4. Effects of antidepressants and sulpiride on the SIRTs mRNA levels in patients with MDD or BPD who were in a depressive state. No significant differences in the SIRT mRNA levels were found among the patients receiving any type of antidepressants or sulpiride who were in a depressive state (tricyclics, $n = 9$; tetracyclics, $n = 15$; selective serotonin reuptake inhibitor [SSRI], $n = 8$; serotonin norepinephrine reuptake inhibitor [SNRI], $n = 9$; sulpiride, $n = 5$). Values are mean ± standard error.

Interestingly, increased levels of apoptosis have been observed in peripheral blood cells of patients with mood disorders who were in a depressive state (Eilat et al., 1999; Ivanova et al., 2007). Furthermore, the mRNA expression of glyoxalase I, an antioxidant enzyme, is downregulated in the peripheral blood cells of patients with MDD and BPD (Fujimoto et al., 2008). Thus, altered expression of SIRTs may affect apoptosis via oxidative metabolism in peripheral blood cells, and this may be one of the mechanisms for the immune inadequacy observed in patients with a mood disorder (Miller et al., 2009).

Patients with mood disorders often exhibit abnormalities in circadian rhythms and some endocrine–metabolic parameters (McClung, 2007; Germain and Kupfer, 2008; Takahashi et al., 2008). The functions of SIRT are involved in the regulation of circadian rhythms and energy metabolism (Bao and Sack, 2010; Chung et al., 2010). Indeed, a transcription factor of the CLOCK gene is significantly associated with bipolar disorder in a gene-wide test corrected for the number of single-nucleotide polymorphisms analyzed in some circadian genes (Soria et al., 2010). CLOCK and BMAL1 play a critical role in the regulation of circadian rhythms (Green et al., 2008; Gekakis et al., 1998; Antoch et al., 2005), and SIRT1 is necessary for the circadian expression of circadian clock genes; SIRT1 binds to the heterodimeric transcription factor complex of CLOCK–BMAL1 in a circadian manner (Asher et al., 2008; Bao and Sack, 2010). Notably, a significant genetic association has been found in the SIRT1 gene of Japanese patients with MDD (Kishi et al., 2010). Taken together, aberrant expression and/or function of SIRTs may be involved in the pathophysiology of mood disorders through the dysregulation of circadian and metabolic systems.

GR dysfunction is also implicated in stress-related disorders, including depression, and also affects emotional behavior, mood, learning, and memory (Holsboer et al., 1994; de Kloet et al., 2005; McEwen, 2008). To our knowledge, no reports have indicated the involvement of SIRTs in the regulation of GR expression. We found a significant correlation between SIRT2 levels and GRα levels in healthy control subjects (Fig. 3B). Interestingly, this correlation was not observed in patients with MDD or BPD. Thus, we speculate that the reduced expression of SIRT2 may be a causal mechanism of GRα downregulation in patients with MDD or BPD.

The principal limitation of this study was that majority of the patients were on medication, and hence, the effect of therapeutic agents on the SIRT mRNA levels should be considered. The influence of medications on class II HDACs has been reported. For example, downregulation of HDAC4 and 5 by the antidepressant imipramine derepresses brain-derived neurotrophic factor expression and suppresses depressive-like behavior in mice (Tsankova et al., 2006). However, no reports have examined the influence of antidepressants on SIRTs. In this study, we did not find any differences related to medications (imipramine equivalent) in our patients between those in a depressive state and those in a remissive state. Furthermore, SIRT1–7 expression was not affected by any of the therapeutic agents used (Fig. 4). These results suggest that the observed alterations, at least, in the expression of SIRT1, 2 and 6 mRNAs in MDD or BPD patients were unlikely due to the effects of the medication. However, the types of medications seem to be changed in patients before and after remission (Table 2). In addition, it is still unclear whether the imipramine equivalent can be applicable to our peripheral data. Thus, we cannot exclude completely the possibility that the therapeutic agents which have an antidepressant activity alter SIRTs expression. Further study using medication-free subject is needed to dissolve this issue.

SIRTs are NAD-dependent deacetylase. Mammals predominantly use nicotinamide, a form of vitamin B3, as a precursor for NAD biosynthesis (Magni et al., 1999; Rongvaux et al., 2003). The

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intimate connection between NAD biosynthesis and sirtuin activity suggests that promoting NAD biosynthesis by nutriceutical nicotinamide mononucleotide (NMN) application could effectively enhance sirtuin activity at a systemic level (**Imai, 2010**). Nicotinamide is directly converted to NMN by nicotinamide phosphoribosyltransferase (Nampt). It has been reported that the level of intracellular Nampt in the brain is very little (**Revollo et al., 2007**), and NAD has been synthesized depending on extracellular biosynthesis of NMN (**Imai, 2010**). There is a possibility that the altered levels of extracellular nicotinamide, NMN, and NAD can affect SIRTs activity in the brain and subsequent neural functions. To our knowledge however, there is no direct evidence demonstrating the altered levels of NAD and NMN in the plasma of mood disorder patients, and further studies are needed.

Recently, SIRT1 has been suggested to be involved in the functional relevance of normal brain physiology and neurological disorders. The cognitive deficits in SIRT1 knockout mice or mutant mice lacking SIRT1 catalytic activity are associated with defects in synaptic plasticity in the hippocampus (**Gao et al., 2010; Michàn et al., 2010**). Also, SIRT1 knockout mice exhibit a decrease in dendritic branching, branch length, and complexity of neuronal dendritic arbors (**Michàn et al., 2010**). Moreover, SIRT1 knockout mice show altered hippocampal gene expression, which plays important roles in synaptic and structural functions (**Michàn et al., 2010**). These results indicate that SIRT1 regulates synapse formation and synaptic plasticity. Although this evidence demonstrates a functional role for SIRTs in the brain, their role in peripheral blood cells is still unclear. Also, no evidence indicates that SIRT expression in peripheral blood cells is correlated with that in the brain. Further studies are needed to clarify the functional consequence of aberrant SIRT expression in the peripheral blood cells of patients with a mood disorder.

In conclusion, our data suggest that altered SIRT1, 2, and 6 mRNA expression in peripheral blood cells may be a useful biological marker for mood disorders. In addition, altered SIRT expression may be associated with the pathophysiology of depression. Further clinical and experimental studies are needed to clarify the role of SIRTs in the pathophysiology of mood disorders.

Contributors

N. Abe, S. Uchida and Y. Watanabe designated the research. N. Abe, K. Otsuki, T. Hobara, H. Yamagata, F. Higuchi, T. Shibata performed the experiments. The manuscript was written by N. Abe, S. Uchida and Y. Watanabe. All authors discussed results and commented on the manuscript.

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Conflict of interest

There are no conflicts of interest including any financial, personal, or other relationships with people for any of the coauthors related to the work described in the article.

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Article

Epigenetic Status of *Gdnf* in the Ventral Striatum Determines Susceptibility and Adaptation to Daily Stressful Events

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SUMMARY

Stressful events during adulthood are potent adverse environmental factors that can predispose individuals to psychiatric disorders, including depression; however, many individuals exposed to stressful events can adapt and function normally. While stress vulnerability may influence depression, the molecular mechanisms underlying the susceptibility and adaptation to chronic stress within the brain are poorly understood. In this study, two genetically distinct mouse strains that exhibit different behavioral responses to chronic stress were used to demonstrate how the differential epigenetic status of the glial cell-derived neurotrophic factor (*Gdnf*) gene in the ventral striatum modulates susceptibility and adaptation to chronic stress. Our results suggest that the histone modifications and DNA methylation of the *Gdnf* promoter have crucial roles in the control of behavioral responses to chronic stress. Our data provide insights into these mechanisms, suggesting that epigenetic modifications of *Gdnf*, along with genetic and environmental factors, contribute to behavioral responses to stress.

INTRODUCTION

Major depressive disorder is one of the most common and serious health problems in societies worldwide. While the etiology of this disorder is multifactorial and poorly understood, both genetic and environmental factors may be involved in the precipitation of depression (Charney and Manji, 2004; Krishnan and Nestler, 2008; Feder et al., 2009). Chronic stressful life events during adulthood are potent adverse environmental factors that can activate or amplify the expression of depression symptoms (Leonardo and Hen, 2008). Many individuals exposed to stressful events do not show signs or symptoms of depression; however, some individuals exposed to psychological stress are predisposed to major depression (Charney, 2004). Thus far, the molecular mechanisms underlying the susceptibility and adaptation to chronic stress within the brain are poorly understood.

Genetically distinct mouse strains that exhibit substantial differences in anxiety and stress reactivity have been used as animal models for investigating the influence of genetic and environmental factors on brain functions and behaviors (Francis et al., 2003; Hovatta et al., 2005; Mozhui et al., 2010). In particular, the inbred BALB/c (BALB) mouse strain demonstrates unique responses to stress. Compared to the C57BL/6 (B6) stress-resilient strain, BALB mice show maladaptive responses to stressful stimuli (Francis et al., 2003; Hovatta et al., 2005; Bhansali et al., 2007; Palumbo et al., 2009). Therefore, BALB mice are considered a stress-vulnerable strain, and comparing the stress responses of BALB and B6 mice may provide useful information regarding the mechanisms of susceptibility and adaptation to stressful stimuli in brain function and behavior, such as those associated with depression.

Neuronal activity regulates a complex program of gene expression that is involved in the structural and functional plasticity of the brain (Flavell and Greenberg, 2008). There is also increasing evidence indicating that aberrant transcription regulation is one of the key components in the pathophysiology of depression (Tsankova et al., 2007; Krishnan and Nestler, 2008; Feder et al., 2009). Recent reports have suggested that the epigenetic regulation of genes, such as DNA methylation and histone modification, can trigger the development of stress vulnerability and contribute to the behavioral responses to chronic stress and antidepressants (Weaver et al., 2004; Tsankova et al., 2006; Fyffe et al., 2008; Jakobsson et al., 2008; LaPlant et al., 2010). However, the role of environmental factors along with genetic factors in the epigenetic regulation of the pathogenesis of depression is largely unknown.

The aim of the present study was to clarify the molecular mechanisms underlying the susceptibility and adaptation to chronic stress using stress-vulnerable BALB and stress-resilient B6 mice strains. Our results show that the differential epigenetic status of the glial cell-derived neurotrophic factor (*Gdnf*) gene in the nucleus accumbens (NAc) influences differential behavioral responses to stress. Therefore, we propose that epigenetic regulation of *Gdnf* by environmental factors, along with genetic factors, contributes to the level of susceptibility and adaptation ability of individuals to chronic stressful life events.

Table 1. Summary of Behavioral Characterizations of B6 and BALB Mice Subjected to CUMS

Behavioral Assay	Phenotype Tested	Stressed B6 Versus Nonstressed B6	Stessed BALB Versus Nonstressed BALB	Stressed BALB with IMI	Nonstressed BALB with IMI
Forced swim test	Immobility time	↔	↑	↔	↓
	Latency to immobility	↔	↓	↔	↑
	First immobility time	↔	↑	↔	↔
Sucrose preference test	Preference ratio	↔	↓	↔	↔
	Total (water + sucrose) intake	↔	↔	↔	↔
Social interaction test	Interaction time	↑	↓	↔	↔
	Total number of interactions	↔	↓	↔	↔
Novelty-suppressed feeding test	Latency to feed	↓	↑	↔	↓
	Food consumption	↔	↔	↔	↔
	Body weight loss	↔	↔	↔	↔

This table shows the behavioral differences between B6 and BALB mice subjected to CUMS conditions for 6 weeks. Also shown are the effects of 3 weeks of antidepressant treatment in stressed and non-stressed BALB mice. ↔, no change; ↑, significantly greater changes; ↓, significantly fewer changes. IMI; imipramine.

RESULTS

Complete statistical summaries of behavior, gene expression by quantitative real-time PCR (Q-PCR) and Western blotting, and chromatin immunoprecipitation (ChIP) data are provided in Tables S1, S2, and S3 (available online), respectively.

Differential Behavioral Responses to Chronic Stress in B6 and BALB Mice

We first investigated the behavioral consequences of 6 weeks of chronic ultra-mild stress (CUMS) exposure, a procedure based solely on environmental and social stressors that do not include food or water deprivation (Lanfumey et al., 1999; Rangon et al., 2007), in BALB and B6 mice. The experimental design is shown in Figure S1A, and the results are summarized in Table 1. Anhedonia, diminished interest or pleasure, is one of the core symptoms of major depression (Wong and Licinio, 2001). Therefore, we examined whether this trait was present in stressed BALB mice using a sucrose preference test (Figures S2A and S2B). CUMS significantly decreased sucrose preference, and this effect was reversed by continuous treatment (via drinking water) with imipramine (IMI, 18 mg/kg/day), a tricyclic antidepressant (Figure S2A). Total fluid intake was not affected by either treatment (Figure S2B). We then subjected BALB mice to the acute forced swim test, which uses increased immobility time as an index of behavioral despair (Porsolt et al., 1977). CUMS significantly increased immobility times (Figure S2C) and the duration of the first immobility episode (Figure S2D) and reduced the latency to the first immobility episode (Figure S2E). These behavioral effects were reversed with continuous IMI treatment (Figures S2C–S2E).

Anxiety is frequently comorbid in patients with major depression. To examine the effects of CUMS on anxiety behavior, we performed the novelty-suppressed feeding test. The latency to begin eating in a novel environment has been used as an index of anxiety behavior (Richardson-Jones et al., 2010). Stressed BALB mice showed significantly longer latency periods to feeding (Figure S2F), with no significant differences in weight loss induced by food deprivation (Figure S2G) or feeding activities (Figure S2H). Furthermore, the increased latency to feed induced by CUMS was reversed with continuous IMI treatment (Figure S2F). Anxiety behavior was also examined using the elevated zero maze test. The amount of time spent in the open section and frequency of rearing were not affected by CUMS (data not shown). Social interaction time also provides an index of anxiety and depression-like behavior. More anxious and depressed rodents spend less time in social interactions (File and Seth, 2003; Berton et al., 2006). Stressed BALB mice spent significantly less time engaged in social interactions and had fewer interactions than the nonstressed controls. This effect was also reversed with continuous IMI treatment (Figures S2I and S2J). Taken together, these results indicate an increase in depression- and anxiety-related behaviors in stressed BALB mice.

In contrast with the BALB mice, B6 mice subjected to CUMS did not show any behavioral changes in the sucrose preference test (Figures S3A and S3B) or forced swim test (Figures S3C and S3D), but they did demonstrate a reduced latency to feed in the novelty-suppressed feeding test (Figure S3E) and increased interaction times in the social interaction test (Figure S3G), suggesting a decrease in anxiety-related behaviors in stressed B6 mice. In addition to behavioral characterization, we also examined the plasma corticosterone (CORT) levels of mice to investigate how CUMS influences neuroendocrine function. We found increased plasma CORT levels 60 min after the initiation of a stressor in both BALB and B6 mice on day 3 of the CUMS session (Figures S4A and S4B). In contrast, on day 38 of the CUMS session, B6 mice showed a reduction in plasma CORT levels 60 min after the initiation of the stressor (Figure S4B). This effect was not observed in BALB mice (Figure S4A). Thus, BALB mice responded to CUMS with an increase in depression-like phenotypes, whereas the B6 mice responded to the same stress conditions with a decrease in anxiety-related behaviors. These behavioral and neuroendocrine data indicate

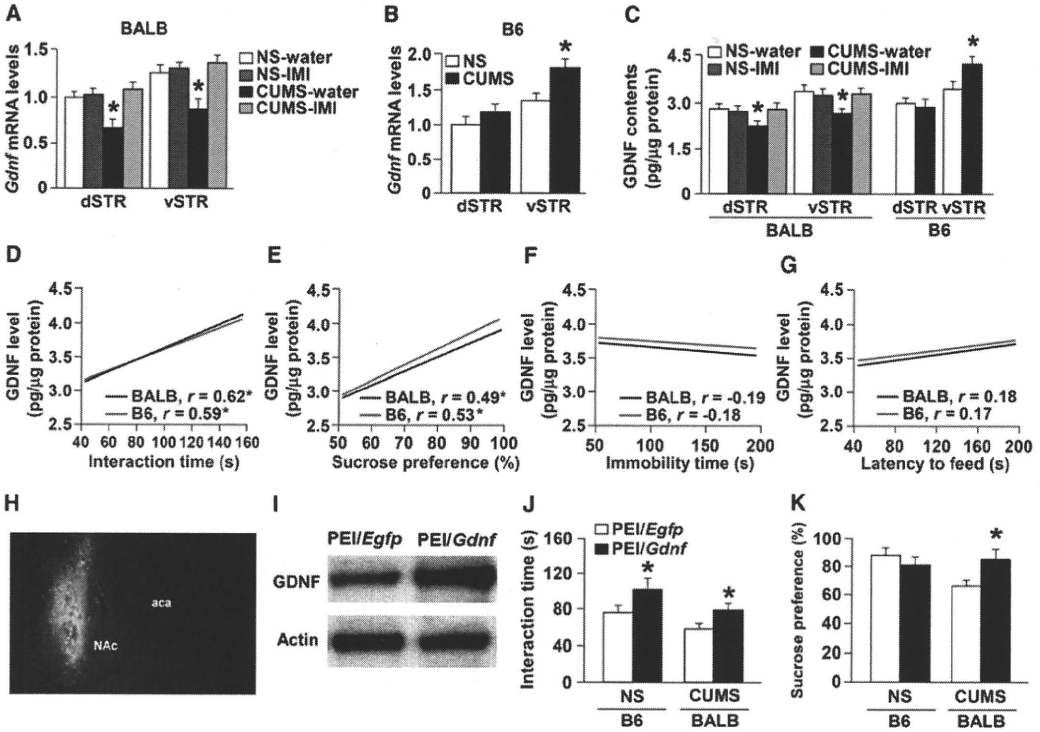

Figure 1. Differential Regulation of *Gdnf* Expression in Stress-Susceptible and Stress-Adaptive Mice Strains

(A) mRNA expression of *Gdnf* in the dSTR and vSTR of BALB mice subjected to CUMS or nonstress (NS) conditions with or without continuous IMI (18 mg/kg/day) treatment (n = 6 per group; *p < 0.05 versus NS mice receiving vehicle (normal water) in corresponding brain regions).

(B) mRNA expression of *Gdnf* in the dSTR and vSTR of B6 mice subjected to CUMS or NS conditions (n = 6 per group; *p < 0.05 versus NS in corresponding brain regions).

(C) Levels of GDNF proteins in the dSTR and vSTR of BALB and B6 mice subjected to CUMS or NS conditions with or without continuous IMI treatment (n = 8–12 per group; *p < 0.05 versus NS mice receiving water in corresponding brain regions).

(D–G) Correlation analyses of GDNF levels in the vSTR of nonstressed BALB (black line) and B6 (red line) mice and (D) the social interaction times (BALB; n = 21, B6; n = 12), (E) the sucrose preferences (BALB; n = 28, B6; n = 16), (F) the immobility times in the forced swim test (BALB; n = 28, B6; n = 16), and (G) the latency to feed in the novelty-suppressed feeding test (BALB; n = 28, B6; n = 16) (*p < 0.05).

(H and I) Successful transductions of EGFP (H) and GDNF (I) into the NAc using the PEI gene delivery system are shown.

(J and K) Effects of GDNF overexpression in the NAc of nonstressed B6 and stressed BALB mice (n = 14–19 per group) on social interaction times (J) and sucrose preference (K) (*p < 0.05 versus PEI/*Egfp* in corresponding strains). Data are presented as mean ± SEM.

that BALB and B6 mice develop "passive" and "active" responses to stress, suggesting that these strains of mice are susceptible and adaptive strains to CUMS, respectively.

Expression Analyses of a Variety of Neurotrophic Factors in a Mouse Model of Depression

Neurotrophic factors play important roles in the regulation of synaptic and structural plasticity in the brain and may be involved in depression (Nestler et al., 2002; Duman and Monteggia, 2006). To investigate the contribution of neurotrophic factors to the behavioral abnormalities of stressed BALB mice, the mRNA levels of multiple neurotrophic factors were examined, including *Bdnf*, *Gdnf*, *Vegf*, *Nt-3*, *Nt-4/5*, *Cdnf*, *Ngf*, *Fgf2*, and *Igf1*, in regions of the brain associated with stress, such as the hippocampus (HP), prefrontal cortex, amygdala, striatum (STR), and hypothalamus, of BALB mice subjected to 6 weeks of CUMS either with or without continuous IMI treatment. Q-PCR revealed that the expression levels of *Bdnf*, *Vegf*, and *Igf1* mRNA were significantly increased by continuous IMI treatment, but were not affected by CUMS (Figures S5B, S5D, and S5H). Interestingly, the mRNA levels of *Gdnf* and *Nt-3* in the STR and HP, respectively, were significantly decreased by CUMS, and these effects were reversed by continuous IMI treatment (Figures S5A and S5E). In addition, the mRNA expression level of *Gdnf* in stressed BALB mice was significantly decreased in both the dorsal STR (dSTR) and the ventral STR (vSTR) (Figure 1A). On the contrary, the mRNA expression level of *Gdnf* in stressed B6 mice was significantly increased in the vSTR but not in the dSTR (Figure 1B). These changes in GDNF expression were confirmed at the protein level using an ELISA assay (Figure 1C). These results suggest that the transcriptional regulation of *Gdnf* in the vSTR is differentially regulated in the two mouse strains and may contribute to the observed behavioral responses to CUMS.

Role of GDNF in the NAc in Depression-like Behaviors

We next investigated whether a correlation exists between *Gdnf* expression in the vSTR and behavioral performances in mice. We found that GDNF protein levels in the vSTR of nonstressed BALB and B6 mice were significantly correlated with social interaction time (Figure 1D) and sucrose preferences (Figure 1E), but not with immobility times in the forced swim test (Figure 1F) or the latency to feed in the novelty-suppressed feeding test (Figure 1G). These data suggest an important role for GDNF in the vSTR for determining certain types of depression-like behaviors.

To directly investigate the role of GDNF in depression-like behaviors, GDNF was overexpressed in the NAc of mice using the polyethylenimine (PEI) gene delivery system. The experimental design is shown in Figure S1B. The successful transduction of EGFP (Figure 1H) and GDNF (Figure 1I) into the NAc of mice using this system was confirmed. We first assessed social interaction time and sucrose preference for nonstressed B6 mice 2 weeks after the injections of PEI/*Gdnf* or PEI/*Egfp* complexes. We found that GDNF overexpression increased the social interaction time (Figure 1J), but not the sucrose preference (Figure 1K). We next investigated the effect of GDNF overexpression in stressful conditions. BALB mice were subjected to 4 weeks of CUMS and injected bilaterally into the NAc with either PEI/*Gdnf* or PEI/*Egfp* complexes on day 14 of the CUMS session. After the CUMS session, we performed behavioral assays. We found that the social interaction time (Figure 1J) and sucrose preference (Figure 1K) of the stressed BALB mice that received PEI/*Gdnf* complexes were significantly greater than those of the mice receiving PEI/*Egfp* complexes. These results suggest a crucial role for GDNF in social interactions and sucrose preference. The transcriptional regulation of *Gdnf* in the NAc may also be involved in the development of susceptibility and adaptation to CUMS.

Regulation of Histone Modifications by CUMS and Continuous IMI Treatment

To explore the molecular mechanisms by which CUMS alters *Gdnf* mRNA levels, resequence analysis of the *Gdnf* promoter (4000 base pairs) was performed on BALB and B6 mice. No differences were observed between the two mice strains (data not shown), suggesting that epigenetic regulations may account for altered *Gdnf* expression in stressed mice. Next, we measured the levels of several posttranslational histone modifications to the *Gdnf* promoter in vSTR tissues using a ChIP assay. We found several differences in the histone modifications of both BALB and B6 mice after CUMS and/or continuous IMI treatment. Q-PCR measurements indicated that *Gdnf* promoter-containing DNA fragments were significantly less common in the acetylated histone 3 (H3ac) immunoprecipitates prepared from stressed BALB mice. This effect was reversed by continuous IMI treatment (Figure 2A). Acetylated histone 4 (H4ac) levels at the *Gdnf* promoter were not affected by either CUMS or continuous IMI treatment (Figure 2B). In stressed B6 mice, H3ac levels at the *Gdnf* promoter, but not H4ac levels, were significantly increased by CUMS (Figures 2A and 2B). We also examined the effects of CUMS on the level of trimethylated histone 3 at lysine 27 (H3K27me3) and trimethylated histone 3 at lysine 4 (H3K4me3), which are the respective repressive and activating markers of transcription, at the *Gdnf* promoter. The levels of H3K27me3 were not affected by CUMS and IMI in BALB mice, but they were significantly reduced in B6 mice by CUMS (Figure 2C). The levels of H3K4me3 were significantly reduced by CUMS in both strains, and this reduction was reversed by IMI in stressed BALB mice (Figure 2D). These data suggest that histone modifications to the *Gdnf* promoter in response to CUMS are differentially regulated in each mouse strain.

Next, we investigated the mechanisms underlying the changes in the histone acetylation of the *Gdnf* promoter. We hypothesized that the altered expression of histone deacetylases (HDACs) could account for the altered level of histone acetylation. The levels of mRNA for HDACs (HDAC 1–11) were measured in the vSTR of BALB mice using Q-PCR. Several significant changes in *Hdacs* expression were observed following CUMS and/or continuous IMI treatment (Figure 2E). Of particular note, the mRNA level of *Hdac2* in stressed mice increased approximately two-fold compared with that of nonstressed controls. This enhancement was reversed by continuous IMI treatment. Changes at the protein level were also determined using Western blot analysis (Figure 2F). However, in the HP of BALB mice (Figure 2G) and the vSTR of B6 mice (Figure 2H), there were no significant effects of CUMS or IMI treatment on HDAC2 expression. Thus, these results suggest that HDAC2 may be an important regulator of the epigenetic repression of *Gdnf* expression in the vSTR of stressed BALB mice.

To determine whether CUMS influences the binding of HDAC2 to the *Gdnf* promoter, we performed a ChIP assay with vSTR DNA. Q-PCR measurements indicated that *Gdnf* promoter-containing DNA fragments are enriched in HDAC2 immunoprecipitates prepared from stressed BALB mice, and this effect was reversed by continuous IMI treatment (Figure 2I). No changes were observed at the *Bdnf* promoter II region (Figure S6A), whose transcript (*Bdnf* exon II) was not altered by either CUMS or IMI treatment (Figure S6B). This finding validates the specificity of the ChIP assay used in this study. In contrast to BALB mice, there was no significant effect of CUMS on HDAC2 binding to the *Gdnf* promoter in B6 mice (Figure 2J).

Rapid Antidepressant Effects of SAHA on CUMS-Induced Behavioral Deficits

Our data indicate that CUMS increases HDAC2 expression in the vSTR of BALB mice but not in B6 mice. This observation led to the hypothesis that this effect may be important for the transcriptional repression of *Gdnf* and the behavioral susceptibility to CUMS. To test the functional role of altered H3ac levels at the *Gdnf* promoter and HDAC2 expression in stressed BALB mice, suberoylanilide hydroxamic acid (SAHA), an HDAC inhibitor, was systemically administered (25 mg/kg/day) for the last 5 days of each 6-week CUMS sessions and during behavioral testing. In addition, to evaluate the possible antidepressant effects of SAHA, either IMI or fluoxetine (FLX), a selective serotonin reuptake inhibitor, was administered (25 mg/kg/day). The experimental design is shown in Figure S1C. The mice that received subchronic SAHA but not subchronic IMI or FLX

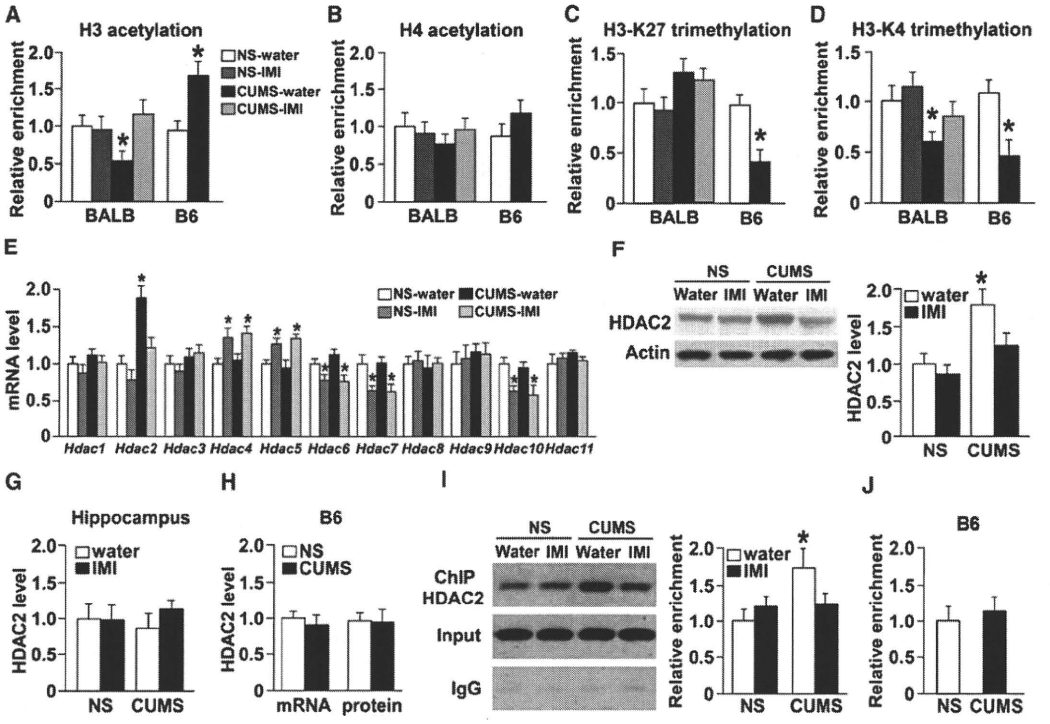

Figure 2. Differential Regulations of Histone Modifications in Stress-Susceptible and Stress-Adaptive Mice Strains

(A–D) Levels of posttranslational histone modifications in the *Gdnf* promoter of the vSTR of BALB and B6 mice subjected to CUMS or nonstressed (NS) conditions with or without IMI treatment were measured using ChIP assays with antibodies for acetylated histone 3 (A), acetylated histone 4 (B), and histone 3 trimethylated on lysine 27 (H3-K27 trimethylation) (C) or lysine 4 (H3-K4 trimethylation) (D) (n = 6 per group; *p < 0.05 versus NS mice receiving vehicle (normal water) in corresponding strains).

(E) mRNA levels of *Hdacs* in the vSTR of BALB mice subjected to CUMS or NS conditions with or without IMI treatment (n = 6 per group; *p < 0.05 versus NS mice receiving water).

(F and G) Western blot analysis of HDAC2 protein levels in the vSTR (F) and HP (G) of BALB mice subjected to CUMS or NS conditions with or without IMI treatment (n = 7–8 per group; *p < 0.05 versus NS mice receiving water).

(H) mRNA and protein levels of HDAC2 in the vSTR of stressed B6 mice (n = 6 per group).

(I and J) HDAC2 levels at the *Gdnf* promoter in the vSTR of BALB (I) and B6 (J) mice were measured using ChIP assays with a specific antibody for HDAC2 (n = 7–8 per group; *p < 0.05 versus NS mice receiving water). Data are presented as mean ± SEM.

exhibited increased social interaction times compared with vehicle-treated mice in stressed conditions (Figure 3A). Similarly, the sucrose preference of mice receiving SAHA, but not IMI or FLX, was significantly increased compared to that of mice receiving vehicle in stressed conditions (Figure 3B). In the novelty-suppressed feeding test, SAHA reduced the latency to feed in mice from both the nonstressed and the stressed conditions, whereas subchronic IMI and FLX treatments did not affect the latency to feed (Figure 3C). In addition, the immobility times during the forced swim test were significantly decreased for mice receiving SAHA, but not IMI or FLX, compared to vehicle-treated mice from both the nonstressed and the stressed conditions (Figure 3D). Furthermore, subchronic SAHA treatment, but not IMI or FLX treatments, increased the mRNA levels of *Gdnf* in the vSTR of stressed mice (Figure 3E). These data suggest that HDAC inhibition can reverse both the increased depression-like behaviors and the reduction of *Gdnf* expression by CUMS. Our results also imply that SAHA has a more rapid antidepressant effect than IMI and FLX.

Role of HDAC2 in Behavioral Responses to CUMS

To test the direct contribution of HDAC2 in the NAc to CUMS-induced depression-like behaviors, dominant-negative HDAC2 (dnHDAC2; HDAC2 H141A) was overexpressed in the NAc of BALB mice using adeno-associated virus (AAV)-mediated gene transfer. Replacing His141 with Ala in the catalytic domain of HDAC2 reduces deacetylase activity by 75% (Humphrey et al., 2008). The experimental design is shown in Figure S1D. The successful transduction of AAV-mediated dnHDAC2 and control EGFP was first confirmed: EGFP fluorescence was observed in the NAc (Figure 3F), and Western blot analysis showed that dnHDAC2 was overexpressed in the vSTR region (Figure 3G). The NAc was then bilaterally infected with AAV-dnHDAC2 or AAV-EGFP. Seven days after the injection of AAV, mice were subjected to CUMS for 4 weeks, followed by the social interaction and sucrose preference tests. Mice that received AAV-dnHDAC2 exhibited increased social interaction times (Figure 3H) and sucrose preferences (Figure 3I) compared with the mice that received AAV-EGFP. Furthermore, the mRNA levels

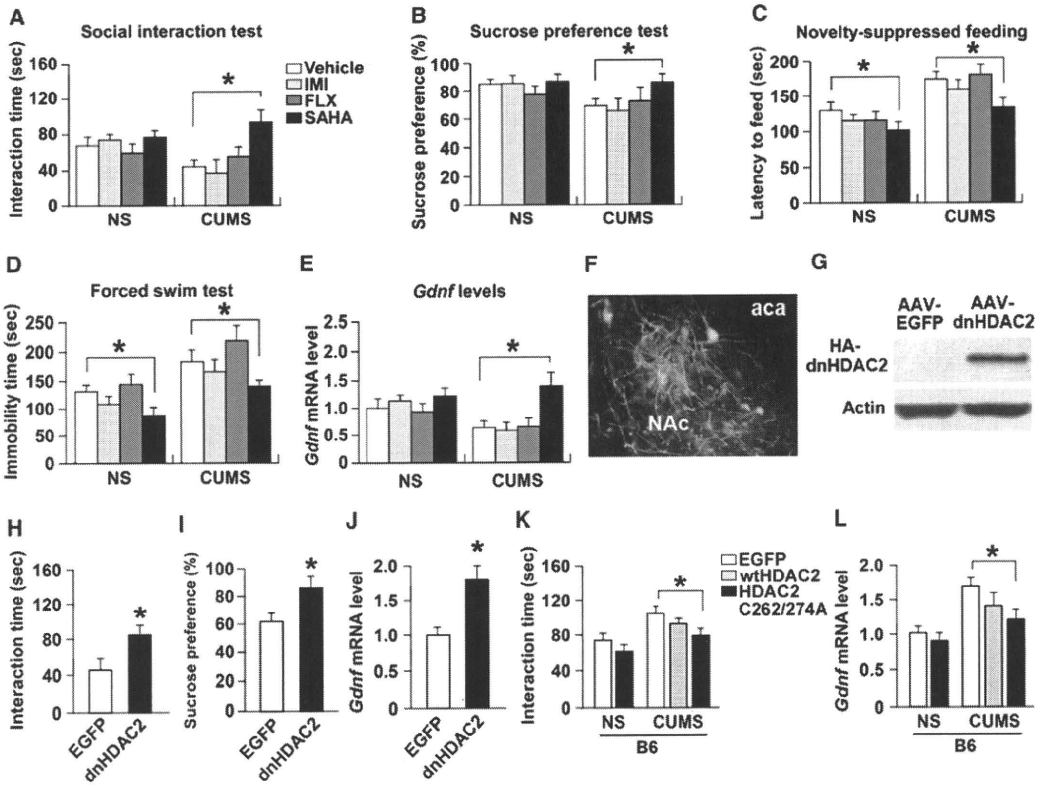

Figure 3. Inhibition of HDAC2 Function Leads to a Stress-Resilient Phenotype

(A–E) Either SAHA, IMI, FLX, or saline were intraperitoneally administered (25 mg/kg of body weight for all drugs) on the last 5 days of each 6-week period of CUMS or nonstressed (NS) conditions and during behavioral testing. The social interaction times (n = 15–19 per group), (B) sucrose preferences (n = 18-20 per group), (C) latencies to feed (n = 18–20 per group), (D) immobility times (n = 18–20 per group), and (E) mRNA expression levels of *Gdnf* in the vSTR (n = 8 per group) are shown (*$p < 0.05$).

(F and G) Successful transductions of EGFP (F) and dominant-negative HDAC2 (dnHDAC2; G) using AAV-mediated gene transfer are shown.

(H–J) Effects of dnHDAC2 overexpression induced by AAV-mediated gene transfer in the NAc of BALB mice subjected to CUMS on (H) the social interaction time test (n = 14–15 per group), (I) the sucrose preference test (n = 17–19 per group), and (J) the *Gdnf* mRNA levels (n = 8 per group; *$p < 0.05$).

(K and L) Effects of the overexpression of wild-type HDAC2 (wtHDAC2) or the HDAC2 C262/274A mutant induced by AAV-mediated gene transfer in the NAc of B6 mice on the (K) social interaction time (n = 14–15 per group) and (L) mRNA levels of *Gdnf* (n = 8 per group; *$p < 0.05$). Data are presented as mean ± SEM.

of *Gdnf* in the vSTR of stressed mice that received AAV-dnHDAC2 were significantly increased compared to those of stressed mice injected with AAV-EGFP (Figure 3J). These results strongly suggest that the CUMS-induced activation of HDAC2 represses *Gdnf* transcription in the NAc, which results in aberrant behavioral responses in BALB mice.

To investigate the influence of HDAC2 on adaptive responses to CUMS in B6 mice, we overexpressed wild-type HDAC2 in the NAc of B6 mice and examined social interaction time and *Gdnf* expression. Stressed mice injected with AAV-HDAC2 did not show a reduction in social interaction time (Figure 3K) or *Gdnf* expression (Figure 3L) when compared with stressed mice injected with AAV-EGFP. A recent report showed that the nitrosylation of HDAC2 induces its release from chromatin, which promotes transcription. In the HDAC2 C262/274A mutant, which lacks S-nitrosylation sites, HDAC2 strongly associates with chromatin, thus repressing transcription (Nott et al., 2008). We investigated the effects of HDAC2 C262/274A overexpression in the NAc of stressed B6 mice on social interaction and *Gdnf* expression. We found that stressed mice injected with AAV-HDAC2 C262/274A showed a reduction in social interaction time (Figure 3K) and *Gdnf* expression (Figure 3L) compared with stressed mice injected with AAV-EGFP. These results indicate that the gain of function of HDAC2 in B6 mice leads to a lack of active response to CUMS.

In contrast, the overexpression of the HDAC2 C262/274A mutant in nonstressed B6 mice did not affect the social interaction time or *Gdnf* expression (Figures 3K and 3L). Similar effects were also observed in nonstressed BALB mice receiving bilateral injections of either AAV-HDAC2 or AAV-HDAC2 C262/274A into the NAc (Figure S7). These manipulations did not alter the social interaction time (Figure S7B), sucrose preference (Figure S7C), or *Gdnf* expression (Figure S7D). These data suggest that other molecular mechanisms modulated by CUMS may also be involved in the HDAC2-mediated *Gdnf* repression and subsequent behavioral alterations.

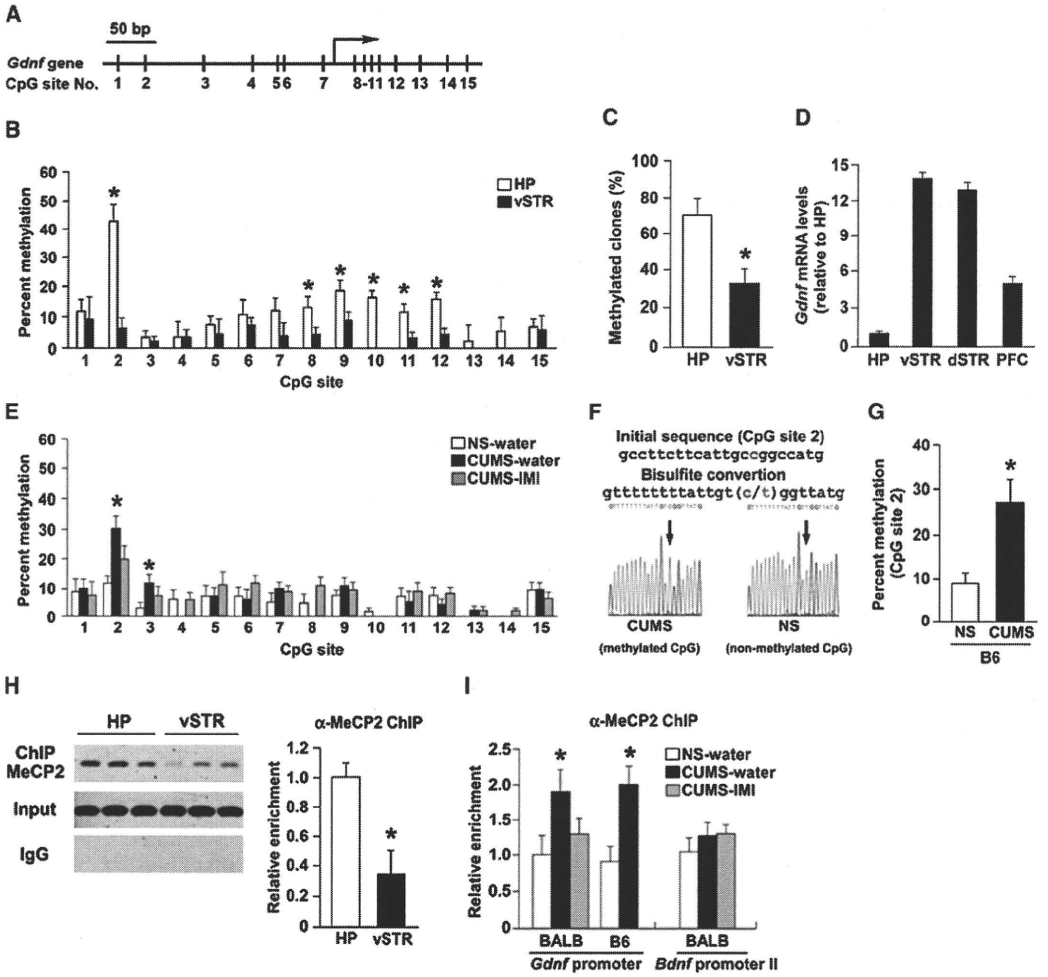

Figure 4. CUMS Induces Hypermethylation of the *Gdnf* Promoter and Increases MeCP2 Binding to Its Promoter in Both Mice Strains

(A) Position of the CpG sites within the mouse *Gdnf* promoter.

(B) Methylation of the *Gdnf* promoter showing the frequency of methylation observed at each CpG site for the HP and vSTR (n = 8 per group; *p < 0.05).

(C) Mean percentages of the methylated clones for the HP and vSTR. The methylation percentage was calculated as the number of clones with at least one methylated CpG site divided by the total number of clones (n = 8 per group; *p < 0.05).

(D) mRNA levels of *Gdnf* in the HP, vSTR, dSTR, and prefrontal cortex (PFC) are shown (n = 6).

(E) CpG methylation profiles in the vSTR of stressed (CUMS) BALB mice with or without continuous IMI treatment, and nonstressed (NS) mice (n = 7–8 per group; *p < 0.05 versus NS mice receiving vehicle [normal water]).

(F) Samples of the sequence fluorograms obtained using bisulfite sequencing of DNA isolated from the vSTR of CUMS- and NS-BALB mice are shown. Arrows indicate methylated and nonmethylated sequences of CpG site 2.

(G) Mean percent of the methylation of CpG site 2 at the *Gdnf* promoter in stressed B6 mice (n = 7–8 per group; *p < 0.05).

(H) MeCP2 occupancy at the *Gdnf* promoter in the HP and vSTR were measured using ChIP analysis with antibodies specific to MeCP2 (n = 6 per group; *p < 0.05).

(I) MeCP2 levels at the *Gdnf* promoter and *Bdnf* promoter II were measured by ChIP analysis of vSTR DNA from mice subjected to CUMS (n = 7–8 per group; *p < 0.05 versus NS mice receiving water in the corresponding strain). Data are presented as mean ± SEM.

CUMS Increases DNA Methylation at the *Gdnf* Promoter in Both Strains

Previous reports have suggested that histone methylation can affect DNA methylation at specific promoter regions (Lachner and Jenuwein, 2002). To investigate whether CUMS and/or IMI-induced alterations in the levels of H3K27me3 and H3K4me3 at the *Gdnf* promoter (Figures 2C and 2D) correlate with an increase in DNA methylation, DNA methylation assays were performed. Cytosine methylation is a highly stable epigenetic process that regulates gene expression through its effects on transcription factor binding (Bird, 2001). Computational analysis (Takai and Jones, 2003) predicted that the *Gdnf* promoter has CpG islands adjacent to the transcription start site (CG > 60%, observed CpG/expected CpG > 0.81, and length > 300 bp; Figure 4A). Furthermore, these CpG islands are highly conserved in mice, rats, and humans (data not shown). First, to

examine whether CpG sites within the *Gdnf* promoter are truly methylated in vivo, the methylation levels of each CpG site were measured within the *Gdnf* promoter and a portion of the first exon. We used sodium bisulfite mapping to examine the methylation status of individual CpG sites within *Gdnf*. This method can detect both 5-methylcytosine and 5-hydroxy-methylcytosine. Sequence analysis of the bisulfite-converted DNA isolated from the HP and vSTR of BALB mice revealed less methylation at CpG sites 2 and 8–12 in the vSTR compared with congruent CpG sites in the HP (Figure 4B). In addition, sodium bisulfite mapping revealed a significantly lower percentage of methylated clones in the vSTR compared with the HP (Figure 4C). Concomitantly, the mRNA level of *Gdnf* in the vSTR was approximately 13-fold higher than that of the HP (Figure 4D), suggesting an association between the CpG methylation level and *Gdnf* mRNA expression in vivo. Therefore, the effects of 6 weeks of CUMS and continuous IMI treatment on CpG methylation were analyzed with bisulfite-converted DNA isolated from the vSTR of BALB mice. As indicated in Figures 4E and 4F, CUMS significantly increased methylation levels at CpG sites 2 and 3, but these hypermethylations were reversed by IMI treatment. Unexpectedly, the level of methylation at CpG site 2, but not at site 3, was also increased by CUMS in the vSTR of B6 mice (Figure 4G and data not shown).

CUMS Increases the Binding of MeCP2 at the *Gdnf* Promoter in Both Strains

The binding of methyl-CpG binding proteins (MBDs; MBD1, MBD2, MBD3, MBD4, and MeCP2) to the target gene promoter is a precise mechanism of gene transcription. Among MBDs, MeCP2 is most abundantly expressed as a chromosomal protein and requires a single methylated CpG site for preferential binding to DNA (Nan et al., 1997; Jones et al., 1998). Therefore, the binding of MeCP2 to the *Gdnf* promoter was directly assessed using the ChIP assay. First, to determine whether there is a difference in binding of MeCP2 to this promoter in the HP and vSTR of naive adult BALB mice, Q-PCR analysis of recovered DNA was performed using *Gdnf* promoter-specific primers. *Gdnf* promoter-containing DNA fragments were significantly less common in MeCP2 immunoprecipitates prepared from the vSTR compared with those from the HP (Figure 4H). Q-PCR analysis of the same immunoprecipitates was performed with a specific primer for *Gdnf* exon 3, which has no CpG island, and the immunoprecipitated DNA fragments were less common or undetectable (data not shown), validating the specificity of the ChIP protocol used. Next, the effect of 6 weeks of CUMS and continuous IMI treatment on the binding of MeCP2 to the *Gdnf* promoter was analyzed in the vSTR (Figure 4I). ChIP analysis revealed that CUMS significantly increased MeCP2 binding to the *Gdnf* promoter in both BALB and B6 mice, and continuous IMI treatment reversed this effect in stressed BALB mice. There was no significant difference in the binding of MeCP2 to the *Bdnf* promoter II region, which was assessed as a control. These results indicate that CUMS enhances the binding of MeCP2 to the *Gdnf* promoter in both mouse strains.

We next investigated the functional role of methylated CpG site 2 on *Gdnf* expression in Neuro2a cells. Treatment of these cells with 5-aza-2′-deoxycytidine, an inhibitor of DNA methylation, reduced the methylation level at the *Gdnf* promoter (Figure S8A) and concomitantly increased *Gdnf* mRNA expression (Figure S8B). Next, the promoter activity of a CpG site 2-specific methylated *Gdnf* luciferase reporter gene was investigated. We found that CpG site 2-specific methylation resulted in an approximately 68% decrease in reporter activity when MeCP2 and HDAC2 were cotransfected into Neuro2a cells (Figure S8C). Previous reports have indicated that the high-affinity binding of MeCP2 to methylated DNA requires a run of four or more A/T bases adjacent to the methylated CpG site (Klose et al., 2005). We found two runs of A/T motifs located downstream of CpG site 2 (Figure S8D). To test the role of these motifs on *Gdnf* promoter activity, wild-type and mutant reporters were constructed for the A/T motifs in CpG site 2 (m1, m2, and m3; Figure S8D). Then, the promoter activity of the CpG site 2-specific methylated and nonmethylated luciferase reporters was measured using cotransfection experiments with MeCP2 and HDAC2 in Neuro2a cells (Figure S8E). We found that in nonmethylated conditions, there was no mutation effect on reporter activity by cotransfection with MeCP2 and HDAC2, whereas in the specific methylation of CpG site 2, the reporter activities of wild-type and m1 and m2 mutants, but not m3 mutant, were affected by HDAC2 and MeCP2 overexpresson. These results suggest that the A/T motifs adjacent to CpG site 2 are critically involved in the MeCP2-HDAC2-mediated silencing of *Gdnf* transcription. Furthermore, we found that among the MBDs, MeCP2 was the most potent repressor of the CpG site 2-specific methylated reporter vector (Figure S8F). Together with the results observed in vivo, these findings suggest that the methylation of CpG site 2 is important for the epigenetic repression of *Gdnf* expression.

CUMS Increases the Binding of MeCP2-HDAC2 to the *Gdnf* Promoter in BALB Mice

The decreased expression level of *Gdnf* after CUMS in BALB mice was investigated to determine if it is triggered by the binding of MeCP2-HDAC2 complexes to the methylated CpG site of the *Gdnf* promoter. This hypothesis was supported, in part, by the finding that MeCP2 and HDAC2 are colocalized in the NAc (Figure 5A). The interactions of MeCP2 and HDAC2 were assessed using IP-Western blot analysis of vSTR proteins. We found that CUMS increased the formation of MeCP2-HDAC2 complexes in stressed BALB mice. This effect was reversed by continuous IMI treatment (Figure 5B). Next, to investigate the effect of CUMS on the binding of MeCP2-HDAC2 complexes at the *Gdnf* promoter, we performed re-ChIP assays using an antibody for HDAC2 on the vSTR samples that were initially immunoprecipitated with an antibody for MeCP2. The re-ChIP assays indicated that the *Gdnf* promoter-containing DNA fragments of stressed BALB mice, but not B6 mice, were significantly enriched compared with those of nonstressed mice, and this effect was reversed by continuous IMI treatment (Figure 5C). These results suggest that the CUMS-induced binding of MeCP2-HDAC2 complexes to the *Gdnf* promoter silences its transcription.

To investigate the role of DNA methylation in the CUMS-induced suppression of *Gdnf* expression and on depression-like behaviors, zebularine (ZEB), a DNA methyltransferase inhibitor, was continuously delivered into the NAc of BALB mice by an osmotic pump. The experimental design is shown in

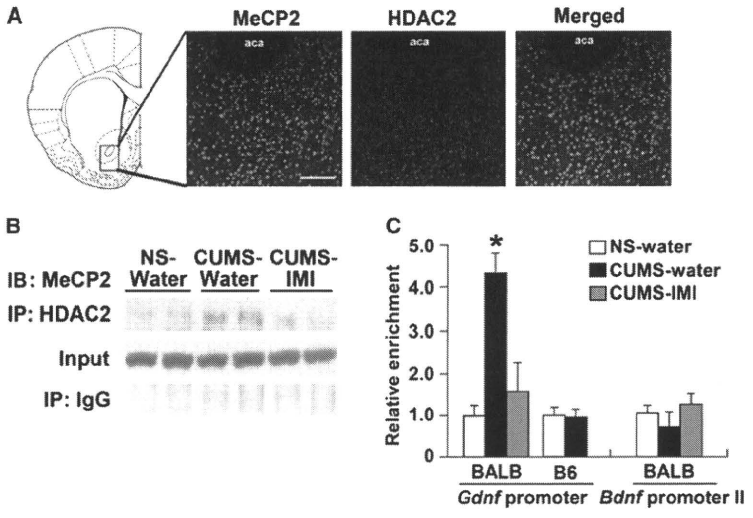

Figure 5. Increased MeCP2-HDAC2 Occupancy at the *Gdnf* Promoter in Stressed BALB Mice

(A) Immunohistochemistry for HDAC2 and MeCP2 demonstrated strong nuclear staining in the NAc. Scale bar, 100 μm.

(B) Nuclear extracts prepared from the vSTR of stressed (CUMS) or nonstressed (NS) BALB mice with or without IMI treatment were immunoprecipitated to evaluate the association of HDAC2 with MeCP2.

(C) Q-PCR assays of the reimmunoprecipitates of HDAC2 antibodies of ChIP samples treated with MeCP2 antibodies (re-ChIP assay) showed that *Gdnf* promoter-containing DNA fragments were enriched in stressed BALB mice (n = 6–8 per group; *p < 0.05 versus NS mice receiving vehicle). Data are presented as mean ± SEM.

Figure S1E. Five days after surgery, mice were subjected to 4 weeks of CUMS, followed by behavioral and expression analyses. We found that the social interaction times and sucrose preferences of stressed mice receiving ZEB (100 μM) were significantly higher compared with those times and preferences of vehicle-treated mice (Figures 6A and B). In the novelty-suppressed feeding test, the latency to feed was significantly decreased in stressed mice receiving ZEB compared with vehicle-treated controls (Figure 6C). In the forced swim test, the immobility times were significantly shorter in stressed and nonstressed mice receiving ZEB compared with the times of vehicle-treated mice (Figure 6D). Furthermore, the mRNA levels of *Gdnf* in ZEB-treated mice were greater than the levels in vehicle-treated mice (Figure 6E) in stressed conditions. These findings confirm that there is less DNA methylation of CpG site 2 at the *Gdnf* promoter in stressed mice treated with ZEB compared with vehicle-treated mice (Figure 6F). We also tested whether intra-NAc delivery of RG108, a potent, nonnucleoside inhibitor of DNA methylation, could reverse the increased depression-like behaviors in BALB mice. Similar to the effects of ZEB, continuous delivery of RG108 (100 μM) directly into the NAc increased the social interaction time (Figure 6G) and sucrose preference (Figure 6H) of mice in the stressed condition. Furthermore, we found that CUMS increased the mRNA expressions for DNA methyltransferase 1 (DNMT1) and DNMT3a, but not DNMT3b, in the vSTR of stressed mice. This effect was reversed by continuous intra-NAc delivery of ZEB and RG108 (Figure 6I). These results suggest that DNA methylation is critical for the CUMS-induced *Gdnf* repression and subsequent depression-like behaviors in BALB mice. Our data also suggest that the continuous intra-NAc delivery of DNMT inhibitors represses the expression of Dnmts at the transcription level in postmitotic neurons.

CUMS Increases Binding of MeCP2-CREB to the *Gdnf* Promoter in B6 Mice

Although DNA methylation is generally thought to be associated with transcriptional repression of the target genes, a recent study suggested that the binding of a complex of MeCP2 and cyclic AMP response element (CRE)-binding protein (CREB) to the methylated CpG site can activate transcription (Chahrour et al., 2008). Interestingly, the putative CRE site is adjacent to CpG site 2 of the *Gdnf* gene (Figure 7A). In addition, we found that MeCP2 and CREB are colocalized in the NAc (Figure 7B). These facts led us to speculate that the binding of the MeCP2-CREB complex to the *Gdnf* promoter may be a causal mechanism of the increased *Gdnf* expression in stressed B6 mice. To test this possibility, we assessed the interactions of MeCP2 and CREB in vSTR proteins of B6 and BALB mice. IP-Western blot analysis showed that there is no apparent difference in the formation of MeCP2-CREB complexes between stressed and nonstressed mice in both strains (Figure 7C). Next, to investigate the binding of MeCP2-CREB complexes at the *Gdnf* promoter, we performed re-ChIP assays using an antibody for CREB on vSTR samples that had been initially immunoprecipitated with an antibody for MeCP2. Consistent with a previous report (Chahrour et al., 2008), CREB-MeCP2 complexes on the *somatostatin* promoter were enriched, whereas they were reduced on the *myocyte enhancer factor 2c* promoter (data not shown), validating the specificity of the re-ChIP used. We found that the *Gdnf* promoter-containing DNA fragments of stressed B6 mice were significantly enriched in the reimmunoprecipitates of samples treated with CREB antibodies compared with those of nonstressed mice. This effect was not seen in stressed BALB mice (Figure 7D). These results suggest that the CUMS-induced binding of MeCP2-CREB complexes to the *Gdnf* promoter leads to the activation of its transcription.

DISCUSSION

This study used genetically distinct inbred mouse strains to describe one of the molecular mechanisms underlying susceptibility and adaptation responses to chronic stress. The proposed mechanisms underlying stress susceptibility and adaptation are described in Figure 7E. Our results suggest that CUMS increases DNA methylation at CpG site 2, and this is associated with increased MeCP2 binding. MeCP2 associated with CpG site 2 interacts with HDAC2, which in turn decreases the level

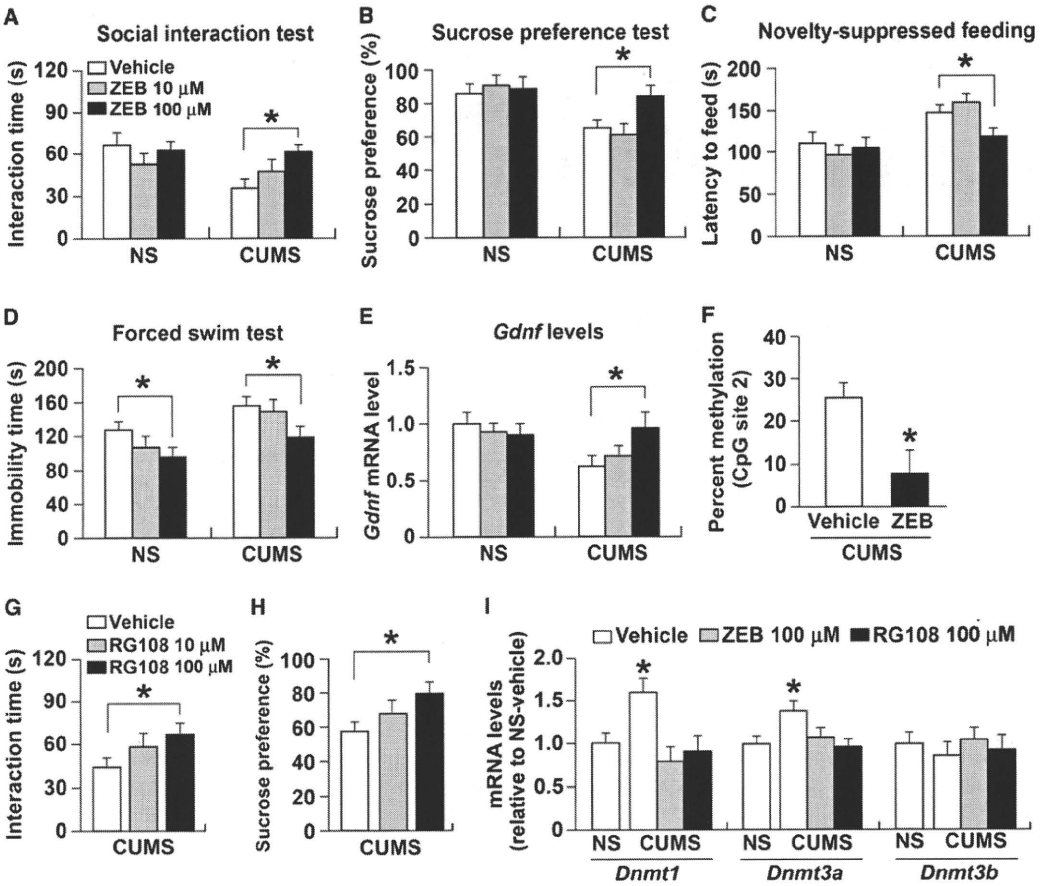

Figure 6. Effects of a DNA Methyltransferase Inhibitor on CUMS-Induced Depression-Like Behaviors and *Gdnf* mRNA Expression

Either ZEB (10 μM or 100 μM) or a vehicle control was continuously and bilaterally delivered into the NAc of BALB mice. After each 4-week CUMS session, the depression-like behaviors of mice were analyzed.

(A–D) Social interaction times (n = 9–12 per group), (B) sucrose preferences (n = 10–12 per group), (C) latencies to feed (n = 10–12 per group), and (D) immobility times (n = 10–12 per group) are shown (*$p < 0.05$).

(E) The mRNA levels of *Gdnf* in the vSTR were measured by Q-PCR (n = 6–8 per group; *$p < 0.05$).

(F) Mean percent methylation of CpG site 2 at the *Gdnf* promoter in stressed BALB mice receiving ZEB (100 μM) or vehicle (n = 6 per group; *$p < 0.05$).

(G and H) RG108 (10 μM or 100 μM) or vehicle was continuously and bilaterally delivered into the NAc of stressed BALB mice. After each 4-week CUMS session, the (G) social interaction time (n = 9–12 per group) and (H) sucrose preference (n = 11–15 per group) of the subjects were analyzed (*$p < 0.05$).

(I) The mRNA levels of *Dnmt1*, *Dnmt3a*, and *Dnmt3b* in the vSTR of mice receiving ZEB or RG108 (100 μM) were measured by Q-PCR (n = 6–8 per group; *$p < 0.05$ versus NS mice receiving vehicle). Data are presented as mean ± SEM.

of H3 acetylation and concomitantly represses *Gdnf* transcription, leading to the formation of a more depression-susceptible phenotype in BALB mice. Continuous IMI treatment relieves MeCP2 occupancy and reverses HDAC2 levels, which leads to normal levels of H3 acetylation and subsequent *Gdnf* transcription, resulting in normal emotional behaviors. Although increased DNA methylation at CpG site 2 and increased MeCP2 occupancy were also observed after CUMS exposure in B6 mice, the acetylation levels of H3 and *Gdnf* expression were greater. Importantly, we found evidence for the binding of the MeCP2-CREB complex to the methylated CpG site on the *Gdnf* promoter in stressed B6 mice. This may be a causal mechanism for the induction of *Gdnf* expression in stressed B6 mice. Thus, our data provide evidence that differential epigenetic marks in the NAc, along with environmental and genetic factors, may influence either the susceptibility or adaptation responses of an organism to chronic daily stressful events.

Role of GDNF in Stress Responses

NAc has been implicated in the development of depression-like behaviors and has an influence on the action of antidepressants (Charney and Manji, 2004; Krishnan and Nestler, 2008; Feder et al., 2009). The data presented here indicate that differential histone modifications at the *Gdnf* promoter between stressed BALB and B6 mice result in differential levels of *Gdnf* expression. Overexpression of GDNF in the NAc increased social interaction times and sucrose preference in the stressed and/or the non-stressed conditions. Conditional GDNF knockout mice showed

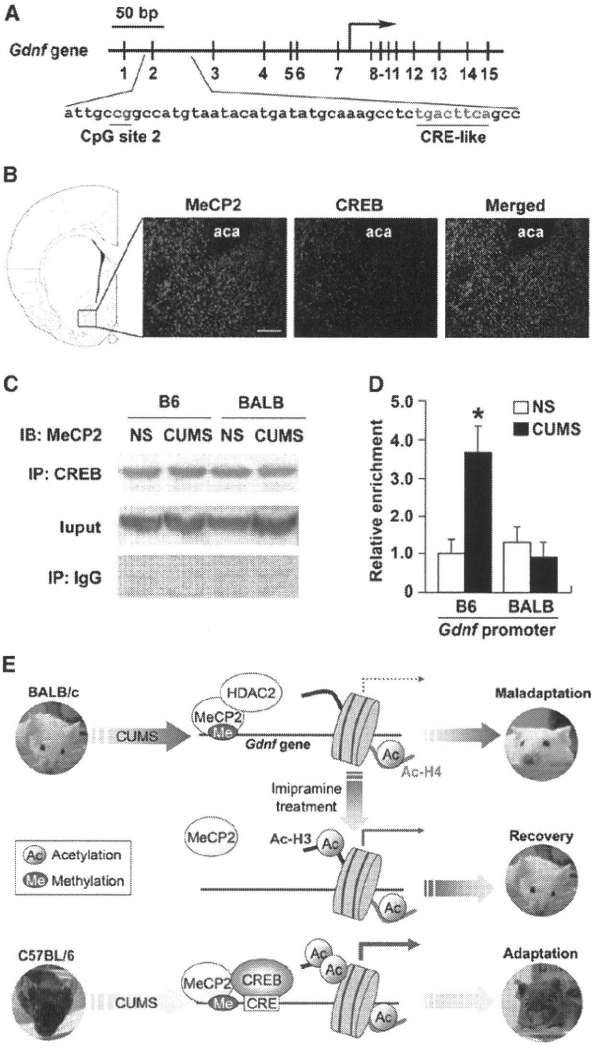

Figure 7. Increased MeCP2-CREB Occupancy at the *Gdnf* Promoter in Stressed B6 Mice

(A) Positions of the CpG site 2 and putative CREB-binding site within the *Gdnf* promoter.

(B) Immunohistochemistry for MeCP2 and CREB demonstrated strong nuclear staining in the NAc. Scale bar, 100 μm.

(C) Nuclear extracts prepared from the vSTR of stressed (CUMS) or non-stressed (NS) B6 mice were immunoprecipitated to evaluate the association of MeCP2 with CREB.

(D) Q-PCR analyses of reimmunoprecipitates for CREB antibodies of ChIP samples treated with MeCP2 antibodies (re-ChIP assay) showed that *Gdnf* promoter-containing DNA fragments were enriched in stressed B6 mice (n = 4–5 per group; *$p < 0.05$ versus NS mice). Data are presented as mean ± SEM.

(E) Proposed mechanisms detailing how the chromatin microenvironment at the *Gdnf* promoter regulates its expression after CUMS exposure with or without IMI treatment in BALB and B6 mice. This study proposes that dynamic epigenetic changes in the *Gdnf* promoter may serve either as a repressive or activating marker of transcription in the NAc, and these changes may serve as causal mechanisms of the different behavioral responses to stress in BALB and B6 mice (refer to the Discussion for more details).

reduced spontaneous activity in the open field test (Pascual et al., 2008). In addition, mice that are not susceptible to social defeat stress show increased *Gdnf* expression in the ventral tegmental area (VTA) (Krishnan et al., 2007). The VTA-NAc network of the mesolimbic dopamine system may be involved in susceptibility and resistance responses to chronic stress (Nestler and Carlezon, 2006; Krishnan et al., 2007). GDNF promotes the survival and maintenance of midbrain dopamine-containing neurons, and GDNF protects neurons in the dopamine system from various toxic stimuli (Lin et al., 1993; Bespalov and Saarma, 2007; Pascual et al., 2008). Thus, the data presented here support the hypothesis that the mesolimbic dopamine system is involved in the formation of susceptibility and resistance responses to chronic stress.

In our experiments, continuous IMI treatment rescued the reduced GDNF expression in the vSTR of stressed BALB mice, suggesting that GDNF is also involved in the behavioral responses to antidepressants. The rescue of GDNF expression in stressed BALB mice returned behavioral performances back to control levels. However, it is still unclear whether the IMI-mediated upregulation of GDNF expression is critically involved in the antidepressant responses. IMI treatment also enhanced the mRNA expressions for other neurotrophic factors, including BDNF and VEGF, in multiple brain regions of BALB mice, and these molecules are thought to be associated with the behavioral responses to antidepressants (Warner-Schmidt and Duman, 2007; Krishnan and Nestler, 2008). Thus, we cannot exclude the possibility that molecules other than GDNF are important for the behavioral effects of antidepressant in the animal models used this study. Further experiments are needed to clarify the role of GDNF in the behavioral responses to antidepressants.

CUMS and Antidepressants Affect Histone Modifications in the *Gdnf* Promoter

Persistent depressive symptoms suggest the involvement of stable changes in gene expression in brain, which may reflect a degree of chromatin remodeling, such as histone acetylation (Krishnan and Nestler, 2008; Tsankova et al., 2007). Recent reports have suggested that modulations of histone acetylation by HDAC2 and HDAC5 are also involved in the actions of antidepressants (Tsankova et al., 2006; Covington et al., 2009). In addition, subchronic administration of SAHA directly into the NAc of mice reverses the reduced social interaction time caused by social defeat stress (Covington et al., 2009). Similarly, this study demonstrated that the increased depression-like behaviors caused by CUMS were reversed by the subchronic administration of SAHA and the overexpression of dnHDAC2. However, nonstressed mice that received subchronic SAHA treatment did not exhibit any observable effects in their social interaction times, sucrose preferences, or expression levels of *Gdnf* mRNA. Taken together, these findings suggest that the hyperactive HDACs are involved in the reduction of *Gdnf* expression and subsequent depression-like behaviors induced by CUMS. In addition, we found that the overexpression of the HDAC2 C262/274A mutant, but not wild-type HDAC2, in the NAc of stressed B6 mice decreased social interaction time and *Gdnf* expression, suggesting a possible contribution of the *S*-nitrosylation of HDAC2 to the stress responses. We also found

that CUMS reduced the levels of H3K4me3 at the *Gdnf* promoter in both BALB and B6 mice, whereas the levels of H3K27me3 at its promoter were decreased only in B6 mice. These findings seem to be inconsistent with regard to the levels of *Gdnf* expression. The reduced H3K4me3 level at the *Gdnf* promoter in the NAc may be a common mechanism for responses to CUMS, and the reduced H3K27me3 level may be one of the important mechanisms modulating the chromatin microenvironment that primes adaptation responses to CUMS.

DNA Methylation at the *Gdnf* Gene Promoter Is Required for Both Susceptible and Adaptive Responses to CUMS

In addition to histone acetylation, the data presented here suggest an important role for DNA methylation in *Gdnf* expression and the subsequent behavioral responses to chronic stress. The epigenetic molecular mechanisms of DNA methylation in the brain may play important roles in the regulation of synaptic plasticity, memory formation, and stress responses (Weaver et al., 2004; Levenson and Sweatt, 2005; Krishnan and Nestler, 2008; Feder et al., 2009). Our data indicate that CUMS enhances DNA methylation at particular CpG sites on the *Gdnf* promoter in BALB mice. Importantly, our work indicates that the CUMS-induced depression-like behaviors and reduced *Gdnf* expression were reversed by the intra-NAc delivery of DNA methyltransferase inhibitors, a result that has been replicated in a recent report (LaPlant et al., 2010). Unexpectedly, the increased DNA methylation and MeCP2 binding also occurred in stress-resilient B6 mice. In general, DNA methylation is primarily associated with the repression of gene transcription. However, a recent study indicated that MeCP2-CREB complexes have assumed the role of inducing target gene expression (Chahrour et al., 2008). In addition, *Gdnf* expression may be regulated by CREB (Cen et al., 2006). Together with these findings, this study suggests that the binding of different MeCP2 complexes (i.e., MeCP2-CREB and MeCP2-HDAC2) to the methylated CpG site on the *Gdnf* promoter may be a causal mechanism for the induction and repression of *Gdnf* expression in the NAc of B6 and BALB mice.

Conclusion

This study provides insights into the role that genetic factors, in combination with environmental factors, may play in the epigenetic regulation of *Gdnf*. Dynamic epigenetic regulations of the *Gdnf* promoter in the NAc play important roles in determining both the susceptibility and the adaptation responses to chronic stressful events. Elucidation of the mechanisms underlying the modulations of HDAC2 expression, histone modifications, and DNA methylation influenced by CUMS could lead to novel approaches for the treatment of depression.

EXPERIMENTAL PROCEDURES

Details can be found in the Supplemental Experimental Procedures.

Animals

Adult male C57BL/6J and BALB/c mice (Charles River Japan) were maintained on a 12 hr/12 hr light/dark cycle with mouse chow and water ad libitum. Four mice were housed in each cage. Eight- or nine-week-old mice were used at the start of experiments (i.e., CUMS, stereotaxic surgery). All experimental procedures were performed according to the Guidelines for Animal Care and Use at Yamaguchi University Graduate School of Medicine.

CUMS Procedure

The CUMS procedure has been previously described in detail (Lanfumey et al., 1999; Rangon et al., 2007) and was conducted here with minor modifications. This procedure was based solely on environmental and social stressors, which did not include food/water deprivation. A total of three stressors were used in this study. For the first stressor, two of the following five ultra-mild diurnal stressors were delivered randomly over a period of 1–2 hr with a 2 hr stress-free time period between the two stressors: a period of cage tilt (30°), confinement to a small cage (11 × 8 × 8 cm), paired housing, soiled cage (50 ml water per 1 l of sawdust bedding), and odor (10% acetic acid), The second stressor consisted of four ultra-mild nocturnal stressors, including one overnight period with difficult access to food, one overnight period with permanent light, one overnight period with a 30° cage tilt, and one overnight period in a soiled cage. For the third stressor, a reversed light/dark cycle was used from Friday evening to Monday morning. This procedure was scheduled over a 1-week period and repeated four or six times, but the reversed light/dark cycle was omitted during the weekend of the last week (either the fourth or sixth week) of the session. Nonstressed mice were handled everyday for weighing purposes.

Behavioral Procedures

Behavioral tests were performed during the light phase (9 a.m. to 2 p.m.) with minor modifications, as reported previously (Uchida et al., 2008; 2010). All behavioral tests were conducted by experimenters who were blind to the treatment condition of the animal. Details can be found in the Supplemental Experimental Procedures.

Drugs

IMI, FLX, and 5-aza-2′-deoxycytidine were purchased from Sigma. ZEB and RG108 were purchased from Calbiochem. SAHA was synthesized as described previously (Suzuki et al., 2009). Details can be found in the Supplemental Experimental Procedures.

PEI-Mediated Gene Delivery

PEI-mediated gene delivery was performed as previously reported (Uchida et al., 2010). Plasmid DNA/PEI complexes were prepared according to the manufacturer's instructions (in vivo-jet PEI; PolyPlus Transfection). Seven days after bilateral canulae implantation into the NAc (+ 1.5 mm AP, ± 1.0 mm ML, −4.0 mm DV), mice were subjected to a 4-week CUMS session. PEI/plasmid complexes (0.5 μl/hemisphere) were injected on day 14 of the CUMS session. Details can be found in the Supplemental Experimental Procedures.

AAV-Mediated Gene Transfer

AAV-mediated gene transfer was performed as previously reported (Uchida et al., 2010). The genomic titer of each virus was determined using Q-PCR. The titers of AAV-EGFP, AAV-HA-HDAC2, AAV-HA-dnHDAC2, and AAV-HA-HDAC2 C262/274A were measured as 5.6×10^{12} viral genomes (vg)/ml, 3.1×10^{12} vg/ml, 3.5×10^{12} vg/ml, and 2.1×10^{12} vg/ml, respectively. For virus injections, the AAV vector (0.5 μl) was injected bilaterally into the NAc (+ 1.5 mm AP, ± 1.0 mm ML, −4.5 mm DV) at a rate of 0.1 μl/min. Mice were allowed to recover for 1 week after surgery. Details can be found in the Supplemental Experimental Procedures.

Statistical Analysis

Analyses of the data were performed using an appropriate analysis of variance. Significant effects were followed up with Bonferroni's post hoc tests. Unpaired t tests were used for two-group comparisons. Pearson correlations were calculated to assess correlations between data. In all cases, p values were two-tailed, and the comparisons were considered statistically significant when $p < 0.05$. Data are presented as the mean ± SEM.

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SUPPLEMENTAL INFORMATION

Supplemental Information includes eight figures, four tables, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.neuron.2010.12.023.

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