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Research report

Long-term naturalistic follow-up of lithium augmentation: Relevance to bipolarity

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ABSTRACT

Background: Whether bipolarity (unrecognized bipolar disorder) is related to the treatment response to lithium augmentation in antidepressant-refractory depression remains unclear. This study of responders and non-responders to lithium augmentation of 29 antidepressant-refractory patients with major depression, whom we had studied during 1995–1997, compared the bipolar diagnosis at the follow-up based on diagnostic confirmation after long-term follow-up.

Methods: Before being classified as stage 2 treatment-resistant depression, these patients had been treated adequately with at least two tricyclic or heterocyclic antidepressants from different pharmacological classes (a minimum of the equivalent of 150 mg of imipramine for 4 weeks). During 1995–1997, 29 patients received lithium augmentation. Their treatment responses were recorded. Mean follow-up was 8.0 years (range, 1–13 years). Bipolar conversion and full remission were evaluated.

Results: After the long-term follow-up, diagnoses were changed to bipolar depression in 3 of 4 lithium responders and 3 of 25 lithium non-responders; lithium augmentation was more effective for unrecognized bipolar patients. Only the family history of bipolar disorder predicted subsequent bipolar conversion.

Limitations: Treatment was not controlled in this naturalistic study, which had a small sample size.

Conclusions: Results of this long-term follow-up study suggest that bipolarity is related to a positive response to lithium augmentation in stage 2 treatment-resistant major depression. The family history of bipolar disorder suggests false unipolar depression, and therefore indicates lithium responders.

1. Introduction

The most important issue in the treatment of major depression is treatment-resistant depression, which is generally defined as the persistence of significant or moderate depressive symptoms despite at least two treatment trials with antidepressants from different pharmacological classes (Bauer et al., 2002; Lam et al., 2009; Thase and Rush, 1995). It is classified as stage 2 major depression according to the staging of depression based on prior treatment response proposed by Thase and Rush (1995). It is estimated to occur in 5–10% of major depression cases (Inoue et al., 2002). Lithium, thyroid hormones, and atypical antipsychotic drugs are recommended in various treatment guidelines as augmentation for antidepressant therapies (Bauer et al., 2002; Lam et al., 2009). Nevertheless, little evidence has been reported for stage 2 major depression except for that related to atypical antipsychotic drugs (DeBattista and Hawkins, 2009; Stimpson et al., 2002). Lithium augmentation, a representative

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augmentation therapy for treatment-resistant depression, has been studied in stage 1 major depression (nonresponse to an adequate trial of one medication) (Thase and Rush, 1995), but it was demonstrated as ineffective in stage 2 major depression in a study with a notably small sample size (Nierenberg et al., 2003).

Lithium augmentation is effective not only for unipolar depression, but also for bipolar depression (Goodwin and Jamison, 2007; Nelson and Mazure, 1986). Several clinical studies – including a meta-analysis – of lithium augmentation have included unipolar and bipolar patients (Bauer and Döpfmer, 1999); such subject selection has been criticized on methodological grounds (Stimpson et al., 2002). On the other hand, lithium alone is effective for bipolar depression and is recommended as a first-line treatment (Yatham et al., 2009). Consequently, although one might expect that depressed bipolar patients will respond to lithium augmentation better than unipolar patients will, this notion has not been investigated using randomized–controlled trials, although it has been suggested by results of a retrospective study (Goodwin and Jamison, 2007; Nelson and Mazure, 1986). Furthermore, a favorable response to lithium augmentation in bipolar depressed patients might simply represent a response to lithium rather than a response to the combination of agents.

Bipolar disorder is a common reason for stage 2 treatment-resistant major depression (Inoue et al., 2006; Parker et al., 2005; Sharma et al., 2005). Unrecognized bipolarity or misdiagnosis of major depression among patients with bipolar disorder might account for a considerable share of all treatment-resistant major depression because antidepressant monotherapy is inappropriate for treatment of bipolar depression (Goodwin and Jamison, 2007). False unipolar depression (Goodwin and Jamison, 2007), which is depression classified as unipolar that subsequently experiences a manic or hypomanic episode, must respond to lithium treatment. Our previous study examined the efficacy of lithium augmentation in stage 2 major depression, i.e., treatment-resistant major depression (Abekawa et al., 1998; Inoue et al., 1996). We have since treated and followed up them for a long period (1–13 years). To elucidate the relation between lithium augmentation for stage 2 major depression and bipolarity, the final follow-up diagnosis was compared in responders and non-responders to results of lithium augmentation published to date (Abekawa et al., 1998; Inoue et al., 1996).

2. Methods

2.1. Subjects

This study was a naturalistic follow-up study of 29 adult patients with antidepressant-refractory major depressive disorder (DSM-III-R) who received lithium augmentation. During 1995–1997, we investigated their demographic characteristics, symptoms, and treatment responses to a lithium augmentation therapy (Abekawa et al., 1998; Inoue et al., 1996). Inclusion criteria were moderate depressed symptoms after adequate treatment with two or more tricyclic and tetracyclic antidepressants from different pharmacological classes (a minimum of the equivalent of 150 mg of imipramine for 4 weeks); they were stage 2 major depression. Depressed patients with brain MRI or EEG evidence of organic brain disease were excluded from this study. Patients with concurrent severe medical problems were also excluded from this study.

2.2. Assessment

According to the Clinical Global Impressions (CGI) scale (National Institute of Mental Health, 1985), treatment efficacies of lithium augmentation were evaluated as worse, no change, minimally improved, much improved, or very much improved. Patients rated very much improved or much improved were regarded as responders. Following the completion of this study, these patients continued to attend our department and receive treatment. Treatment, symptoms, and social functioning were recorded prospectively for 13 years during 1995–2008. The authors investigated the final diagnosis, severity of symptoms, social functioning (employment, etc.), scores of Global Assessment of Functioning (GAF) scale, whether the patients had experienced full remission during the 13 years, and the prevalence of bipolar spectrum disorder at the start of lithium augmentation (Ghaemi et al., 2001). A score of 80 or higher on the GAF scale is a good and straightforward indicator of full remission (Inoue et al., 2006).

2.3. Data analyses

Continuous data are presented as means with standard deviations (SD). For dichotomous variables, Fisher's exact test was used to calculate the p values. For all other continuous variables, a t-test with or without Welch's correction was used. Differences were considered significant at $p<0.05$.

3. Results

3.1. Initial treatment effect of lithium augmentation and clinical and demographic data

Of 29 patients with treatment-resistant (stage 2) major depressive disorder, only 4 patients were responders (response rate = 13.8%). Clinical and demographic parameters at the start of lithium augmentation (gender, age, age at onset, number of previous depressive episodes, duration of index episode, comorbidity, prevalence of bipolar spectrum disorder, marital status, employment status, education and family history of bipolar disorder) were not statistically different between responders ($n=4$) and non-responders ($n=25$) (Table 1).

3.2. Final diagnosis at follow-up and outcome

After the mean follow-up period of 8.0 years (range, 1–13 years), among the 29 patients with major depression, 6 patients were diagnosed with bipolar disorder (1 bipolar I and 5 bipolar II). At follow-up, 3 of 4 responders and 3 of 25 non-responders were bipolar patients (Table 1). Bipolar conversion, i.e. prevalence of unrecognized bipolar disorder or false unipolar depression, was significantly higher in lithium responders than in non-responders ($p=0.02$, Fisher's

Table 1
Comparison of lithium-responders and non-responders.

	Responders ($n=4$)	Non-responders ($n=25$)	p values
Gender (male:female)	2:2	6:19	0.300
Age at follow-up, years	50.5 ± 9.3	51.8 ± 16.2	0.882
Length of follow-up interval, years	6.3 ± 3.3	8.3 ± 5.2	0.455
Age at start of lithium augmentation, years	44.3 ± 9.5	43.4 ± 15.7	0.922
Age at onset of mood disorder, years	36.5 ± 6.6	34.7 ± 16.0	0.826
Number of prior depressive episodes*	2.5 ± 3.0	3.8 ± 11.5	0.642
Duration of index episode, years*	3.5 ± 3.8	4.9 ± 3.9	0.513
Refractoriness of onset episode*	3 (75%)	19 (76%)	1.000
Comorbidity*	1 (25%)	2 (8%)	0.371
BSD*	0 (0%)	4 (16%)	1.000
Number of positive items of BSD*	1.0 ± 0.8	2.0 ± 1.4	0.188
Marital Status (married:single)*	3:1	15:10	1.000
Living alone*	0 (0%)	4 (16%)	1.000
Employment status (employed:unemployed)*	1:3	5:20	1.000
Homemakers*	1	11	
Education, mean ± SD (years)	14.5 ± 1.9	13.0 ± 2.3	0.213
1st-degree relative with bipolar disorder	1 (25%)	1 (4%)	0.261
Diagnosis at follow-up (unipolar:bipolar)	1:3	22:3	0.020
GAF score at follow-up	90.0 ± 8.2	73.8 ± 12.4	0.018
Full remission rate (%) at follow-up	100%	52%	0.121

Data presented as means ± SD or numbers (percentages). BSD = Bipolar Spectrum Disorder, GAF = Global Assessment of Functioning, *at start of lithium augmentation. For dichotomous variables, Fisher's exact test was used to calculate the p values; for all other variables, the t-test was used with or without Welch's correction.

exact test). At the final observation, lithium responders had significantly higher GAF scores than non-responders did ($p=0.018$, t-test), but the remission rates were not significantly different between the two groups.

Only one clinical parameter was significantly different between bipolar and unipolar patients at the start of lithium augmentation: a family history of bipolar disorder in a first degree relative was reported in 2 of 6 bipolar patients and in 0 of 23 unipolar patients ($p=0.0369$, Fisher's exact test), but other parameters were not different between the two groups (data not shown). One of two patients with a positive family history was a lithium responder.

4. Discussion

The main finding of this study is that 75% of lithium responders in Stage 2 treatment-resistant major depression showed false unipolar depression, i.e. unrecognized bipolar disorder. Several lines of evidence show that the family history of bipolar disorder in a first-degree relative is a reliable predictor of bipolarity (Goodwin and Jamison, 2007). Consistent with this finding, only a family history of bipolar disorder in a first-degree relative predicted false unipolar depression in the present study. If the reliability of the prediction of false unipolar depression is sufficiently high, then we should study the effect of lithium monotherapy rather than lithium augmentation in such patients as the next step, but it would be difficult.

Nelson and Mazure (1986) reported that bipolar stage 1 psychotic depression cases responded to lithium augmentation better than unipolar ones did. Another study showed equal efficacies of lithium augmentation in unipolar and bipolar stage 1 depression (Price et al., 1986). More recent reports described that severe symptoms and lower comorbidity of personality disorders might be predictors of lithium augmentation (Alvarez et al., 1997; Bschor et al., 2001), but no study of the relevance of lithium augmentation to bipolarity has been reported in the literature.

Nierenberg et al. (1990) followed up stage 1 depression with lithium augmentation therapy for an average 29 months and reported that the positive response predicted a good subsequent course. Similarly, our study showed higher GAF scores, i.e. better function, at the follow-up in lithium responders. However, Adli et al. (2009) described a negative result in their long-term follow-up study of lithium augmentation. Prior long-term follow-up studies of lithium augmentation did not examine bipolar conversion in lithium responders (Adli et al., 2009; Nierenberg et al., 1990; Shergill et al., 1999). Two reports described a few converters from unipolar to bipolar—3.8–4.3% of the total samples; another report made no mention of this conversion. Staging of treatment-resistant depression might affect the conversion rate from unipolar to bipolar because bipolarity is putatively relevant to stage 2 treatment resistance (Inoue et al., 2006; Parker et al., 2005; Sharma et al., 2005).

The low response rate (13.8%) of lithium augmentation in stage 2 major depression found in the present study contrasts with the average 50% of response rates in stage 1 depression reported for other studies (Bauer and Döpfmer, 1999). A similar low response rate (12.5%) of lithium augmentation was reported in stage 2 major depression by Nierenberg et al. (2003). This result caused the very small sample size of lithium responders in the present study, which is one limitation of the present study. A larger sample must be studied to elucidate the relevance of bipolarity to lithium augmentation.

In conclusion, results of this long-term follow-up study suggest that bipolarity might be partly related to a positive response to lithium augmentation in stage 2 treatment-resistant major depression. A family history of bipolar disorder suggests the possibility of false unipolar depression, and therefore indicates lithium responders.

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There is no funding for this study.

Conflict of interest
All authors declare that they have no conflicts of interest.

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SSR504734, a glycine transporter-1 inhibitor, attenuates acquisition and expression of contextual conditioned fear in rats

Hiroyuki Nishikawa[a], Takeshi Inoue[a], Takeshi Izumi[b], Shin Nakagawa[a] and Tsukasa Koyama[a]

Conditioned stress-induced freezing has been used as an indicator of anxiety in rodents to evaluate the anxiolytic effects of various compounds. However, the role of glycinergic neurotransmission in fear conditioning is not well understood. In this study, we investigated the effects of a selective glycine transporter-1 inhibitor, SSR504734, on contextual fear conditioning. In a fear acquisition experiment, rats were administered SSR504734 (3–30 mg/kg, intraperitoneal) 1 h before fear conditioning (i.e. inescapable footshock). Twenty-four hours after fear conditioning, the rats were placed in the experimental chamber without footshock, and freezing behavior was observed. SSR504734 (30 mg/kg) significantly inhibited contextual conditioned freezing. In a fear expression experiment, rats were administered SSR504734 (3–30 mg/kg, intraperitoneal) 23 h after fear conditioning and were tested 1 h after injection. SSR504734 (30 mg/kg) significantly inhibited contextual conditioned freezing. These findings indicate that SSR504734 attenuates both the acquisition and expression of contextual conditioned fear, and suggest that glycinergic neurotransmission may play an important role in conditioned fear. *Behavioural Pharmacology* 21:576–579 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Glycine is an important neurotransmitter at both excitatory and inhibitory synapses in the central nervous system of vertebrates. Extracellular levels of glycine are controlled by two types of specific transporter, that is, glycine transporter-1 (GlyT1) and glycine transporter-2 (Legendre, 2001). GlyT1 is predominantly localized on glial cells, but is also present on neurons in the brainstem, spinal cord, and cerebellum. GlyT1 is also found, though at somewhat lower levels, in regions of the forebrain, especially hippocampus and cerebral cortex (Adams *et al.*, 1995; Zafra *et al.*, 1995; Jursky and Nelson, 1996; Cubelos *et al.*, 2005), both of which play crucial roles in Pavlovian fear conditioning (LeDoux, 2000). Glycine transporter-2, in contrast, is found only in the central nervous system and is localized on glycinergic neurons in the spinal cord and brainstem (Zafra *et al.*, 1995; Jursky and Nelson, 1996).

Several studies have shown that glutamate and dopamine facilitate the acquisition and expression of conditioned fear (Maren *et al.*, 1996; Guarraci *et al.*, 1999; Li *et al.*, 2004) while γ-amino butyric acid and serotonin inhibit its acquisition and expression (Hashimoto *et al.*, 1996; Muller *et al.*, 1997; Harris and Westbrook, 1998; Inoue *et al.*, 2004; Li *et al.*, 2006). However, the role of glycinergic neurotransmission in fear conditioning is uncertain. Recently, the piperidinebenzamide derivative, SSR504734 [2-chloro-*N*-[(S)-phenyl[(2S)-piperidin-2-yl] methyl]-3-trifluoromethyl benzamide, monohydrochloride] was developed as a selective and reversible GlyT1 inhibitor, which increases extracellular concentrations of endogenous glycine in the brain (Depoortère *et al.*, 2005). In this study, we investigated the effects of a GlyT1 inhibitor, SSR504734, on fear acquisition and expression using the rat contextual conditioned fear model.

Methods

Subjects

Male Sprague–Dawley rats (250–350 g) obtained from Shizuoka Laboratory Animal Center (Shizuoka, Japan) were housed in groups of four in a temperature-controlled environment (22 ± 1°C) with free access to food and water. The subjects were maintained on a 12 h light/dark cycle (light phase, 06 : 30–18 : 30 h) and tested during the light phase after a 1-week acclimatization period. The rats were tested between 08 : 30 and 11 : 30 h.

All experiments were approved by the Hokkaido University School of Medicine Animal Care and Use Committee, and were in compliance with the Guide for the Care and Use of Laboratory Animals.

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Procedures

The rats were individually subjected to a total of 150 s of inescapable electric footshock (five 2.5 mA scrambled footshocks, pulse wave, 30 s duration) that were delivered at intershock intervals of 35–80 s (mean 60 s) in a shock chamber with a grid floor (19 × 22 × 20 cm, Medical Agent Co., Kyoto, Japan). Electric shocks were produced by a Shock Generator (Model SGS-02D, Medical Agent Co.). Twenty-four hours after the footshock, the rats were again placed in the shock chamber without footshock and observed for 5 min. The behavior was videotaped and scored later by human observation. During the observation period (i.e. testing), the duration of the freezing behavior was recorded using a modified time-sampling procedure, as described earlier (Inoue *et al.*, 2004). Every 10 s, the behavior in which the subject was currently engaged was classified as either 'freezing' or 'activity'. Freezing was defined as the absence of all observable movements of the skeleton and the vibrissae, except those related to respiration. All other behaviors were scored as activity. The subject was classified as either freezing or active according to its behavior throughout the entire 10-s period: if a rat showed any activity during the 10-s sampling period (including freezing for up to 9 s) we considered this period as activity. We observed rats for successive 10-s periods during 5 min (i.e. 30 successive sampling periods). Percentage freezing score [freezing (%)] was computed as the proportion of 10-s periods during which the subject remained frozen all the time.

In a fear acquisition experiment, the rats received a single intraperitoneal injection of SSR504734 (3–30 mg/kg) 1 h before footshock (i.e. 25 h before testing). In a fear expression experiment, the rats received a single intraperitoneal injection of SSR504734 (3–30 mg/kg) 23 h after footshock (i.e. 1 h before testing).

Motor activity of unshocked rats was measured after administration of SSR504734 (30 mg/kg, intraperitoneal). The rats were in their home cages habituated to the testing room for a day. SSR504734 was administered 1 h before testing. Rats were individually placed in a testing cage, and motor activity was automatically recorded for 5 min as described by Ohmori *et al.* (1994) using an infrared sensor that detected thermal radiation. Horizontal movement was digitized and uploaded to a computer. Locomotion was responsible for most of the count, though other body movements also contributed when they included a substantial horizontal component.

Drug

SSR504734, a gift from Sanofi-Aventis, Chilly Mazarin, France, was suspended in 10 ml of distilled water with two drops (approximately 40 µl) of Tween 80. SSR504734 was intraperitoneally administered to rats in a volume of 5 ml/kg. Doses are expressed as the weight of the salt.

Data analysis

All data are presented as the mean ± SEM. Statistical analyses of freezing behavior data were performed with one-way analysis of variance followed by Dunnett's test for parametric multiple comparisons. Statistical analysis of motor activity data was performed with Welch's *t*-test.

Results

SSR504734 (3–30 mg/kg, intraperitoneal) given 1 h before footshock dose-dependently attenuated conditioned freezing [$F(3,44) = 4.69$, $P < 0.01$] in rats tested 24 h after footshock (Fig. 1). Post-hoc analysis revealed a significant inhibitory effect of SSR504734 (30 mg/kg, intraperitoneal) on conditioned freezing compared with vehicle ($P < 0.01$, Dunnett's test).

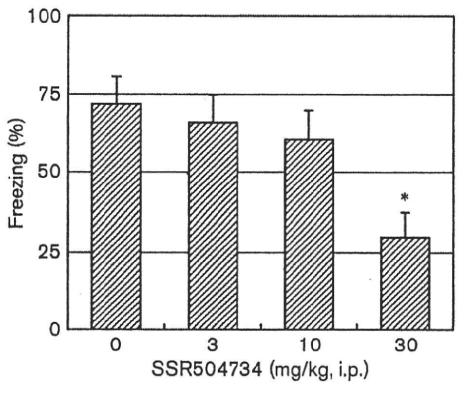
SSR504734 (3–30 mg/kg, intraperitoneal) given 1 h before testing dose-dependently attenuated conditioned freezing [$F(3,44) = 3.37$, $P < 0.05$] in rats tested 24 h after footshock (Fig. 2). Post-hoc analysis revealed a significant inhibitory effect of SSR504734 (30 mg/kg, intraperitoneal) on conditioned freezing compared with vehicle ($P < 0.05$, Dunnett's test).

SSR504734 (30 mg/kg, intraperitoneal) significantly reduced motor activity of unshocked rats during the 5-min testing period compared with vehicle [vehicle, 559.9 ± 131.9 counts; SSR504734, 136.8 ± 26.0 counts; $P < 0.01$, Welch's *t*-test].

Discussion

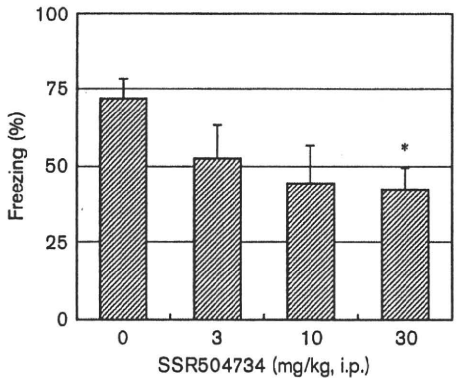
In this study, we investigated the effects of acute administration of SSR504734 on the acquisition and expression of contextual conditioned fear in rats. We found that acute administration of SSR504734 dose-dependently attenuated conditioned freezing in both the

Fig. 1



Effects of SSR504734 on acquisition of conditioned fear in rats. SSR504734 was injected 1 h before footshock. Freezing behavior was observed 24 h after footshock. *$P < 0.01$ versus vehicle (Dunnett's test) $n = 12$. i.p., intraperitoneal.

Fig. 2



Effects of SSR504734 on expression of conditioned fear in rats. SSR504734 was injected 1 h before testing. Freezing behavior was observed 24 h after footshock. *$P<0.05$ versus vehicle (Dunnett's test) $n=8-16$. i.p., intraperitoneal.

fear acquisition and expression experiments. This is the first study to show the inhibitory effect of a GlyT1 inhibitor on conditioned fear. The inhibitory effects of SSR504734 on freezing behavior in both the fear acquisition and expression experiments suggest that GlyT1 inhibitors have clinical potential to ameliorate conditioned fear responses.

Consistent with our results, it has been reported that acute administration of SSR504734 reduces ultrasonic distress calls in rat pups separated from their mother, as observed with several anxiolytics, such as fluoxetine, benzodiazepines, and 5-hydroxytryptamine1A receptor agonists (Depoortère *et al.*, 2005). Moreover, chronic administration of SSR504734 has been shown to prevent physical degradation in mice subjected to chronic mild stress, an effect shared with other established or putative antidepressant/anxiolytic compounds (Depoortère *et al.*, 2005).

Motor activity was not increased, but was decreased by 30 mg/kg of SSR504734. Hence, this motor effect cannot explain the inhibition of the conditioned freezing response, because a sedative effect would increase freezing rather than decreasing it. According to our observations, SSR504734 did not cause any other abnormal movement in rats. Moreover, although the half-life of SSR504734 has not been reported, its inhibitory effect on ex-vivo uptake of glycine was observed until 7 h, but was diminished at 16 and 24 h after SSR504734 administration (Depoortère *et al.*, 2005). Thus, it is unlikely that SSR504734 administered 25 h before testing remained in the brain and affected behavior in the acquisition experiment.

Depoortère *et al.* (2005) have reported that SSR504734 increases extracellular levels of glycine in the rat prefrontal cortex, resulting in enhanced glutamatergic neurotransmission. Indeed, SSR504734 potentiates *N*-methyl-D-aspartic acid (NMDA)-mediated excitatory postsynaptic current in rat hippocampal slices (Depoortère *et al.*, 2005). Thus, the inhibitory effects of SSR504734 on fear acquisition and expression in this study might be mediated by activation of glutamatergic neurotransmission through increased extracellular levels of glycine, which acts as an agonist at the strychnine-insensitive glycine recognition site (glycine B site) of the NMDA receptor complex. In contrast, there is another possibility that the increase in glycine induced by SSR504734 acts on inhibitory glycine receptors (glycine A site), as glycine receptors exist not only in the spinal cord and brainstem, but also in the cerebral cortex and hippocampus (Legendre, 2001).

To elucidate the neurochemical mechanism of action of SSR504734, we examined the glycine/NMDA receptor antagonist L-701 324 (3 mg/kg intraperitoneal) on the effect of SSR504734 on the expression of conditioned freezing (our unpublished data). However, L-701 324 did not alter the effect of SSR504734, but caused marked ataxia in rats, which made it difficult to interpret these results. An earlier study reported that D-cycloserine accelerated extinction of cue-conditioned fear with light, and inhibited expression of contextual conditioned fear (Ledgerwood *et al.*, 2003). However, this effect of D-cycloserine may be attributed to its partial agonist property, which is less effective than endogenous ligands, such as D-serine and glycine (Davis *et al.*, 2006). In contrast, it might be possible that the inhibitory effect of SSR504734 on conditioned fear is related to its effect on the NMDA/glycine receptor (glycine B site) on inhibitory γ-amino butyric acid neurons of the amygdala, but there are contradictory findings, as the facilitation of glutamate enhances conditioned fear, as mentioned in the introduction. Thus, it may be more likely that the effect of SSR504734 on conditioned fear is mediated by its inhibitory effect on neurotransmission through the glycine receptor although this remains uncertain. In addition, there is no selective glycine receptor antagonist available for in-vivo experiments. Altogether, we cannot conclude whether potentiation of the NMDA receptor (excitatory) or the glycine receptor (inhibitory) is involved in the effect of SSR504734 until more suitable agents are discovered.

In conclusion, the findings of this study show that SSR504734, a GlyT1 inhibitor, attenuates the acquisition and expression of contextual conditioned fear, as shown by reduced conditioned freezing, and that endogenous glycine may play an important role in conditioned fear in rats.

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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. 1A) 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).

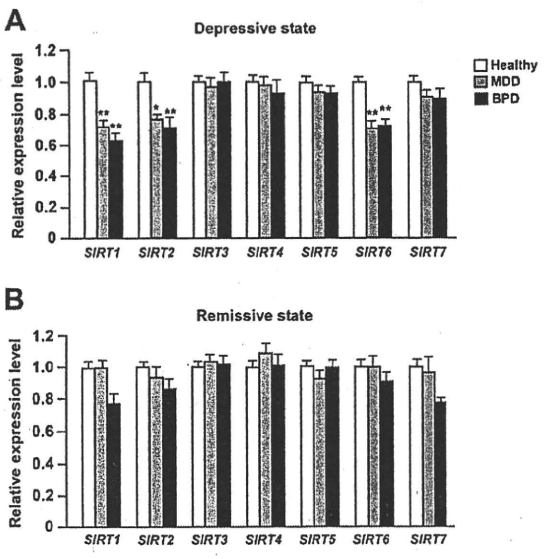

Fig. 1. mRNA levels of sirtuin (SIRTs) in patients with major depressive (MDD) or bipolar disorder (BPD) in a depressive and remissive state. (A) Quantitative real-time PCR analysis of SIRTs mRNA levels in patients with MDD ($n = 20$) or BPD ($n = 12$) who were in a current depressive state and healthy 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

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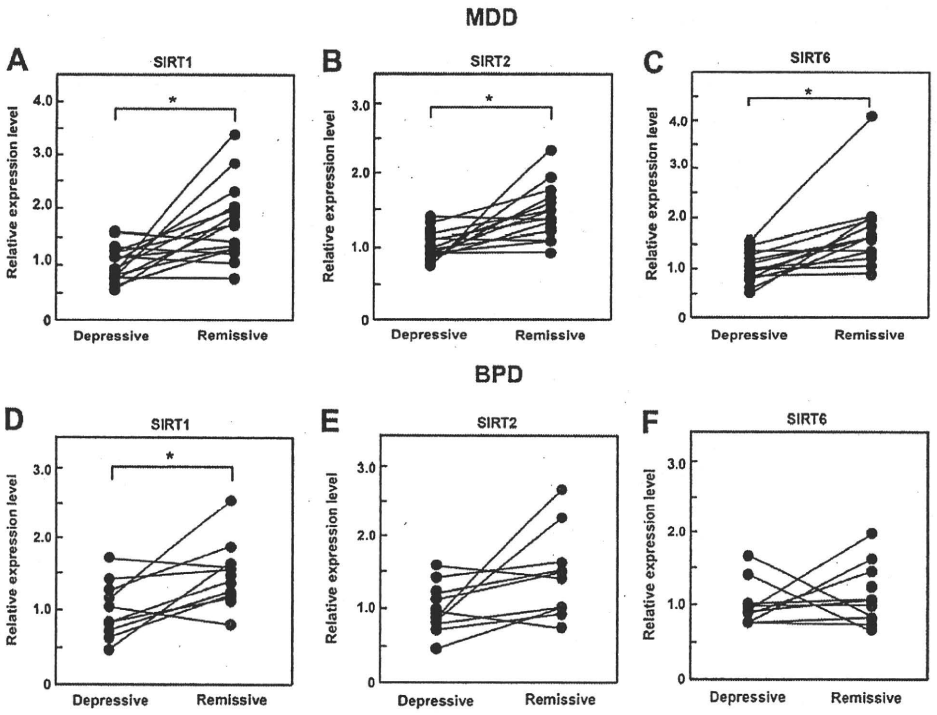

Fig. 2. mRNA levels of SIRTs in patients with MDD or BPD before and after remission. (A–C) Quantitative real-time PCR analysis of SIRT mRNA levels in patients with MDD before and after remission ($n = 15$). SIRT1, 2 and 6 mRNA expression levels increased significantly in patients in a remissive state 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.

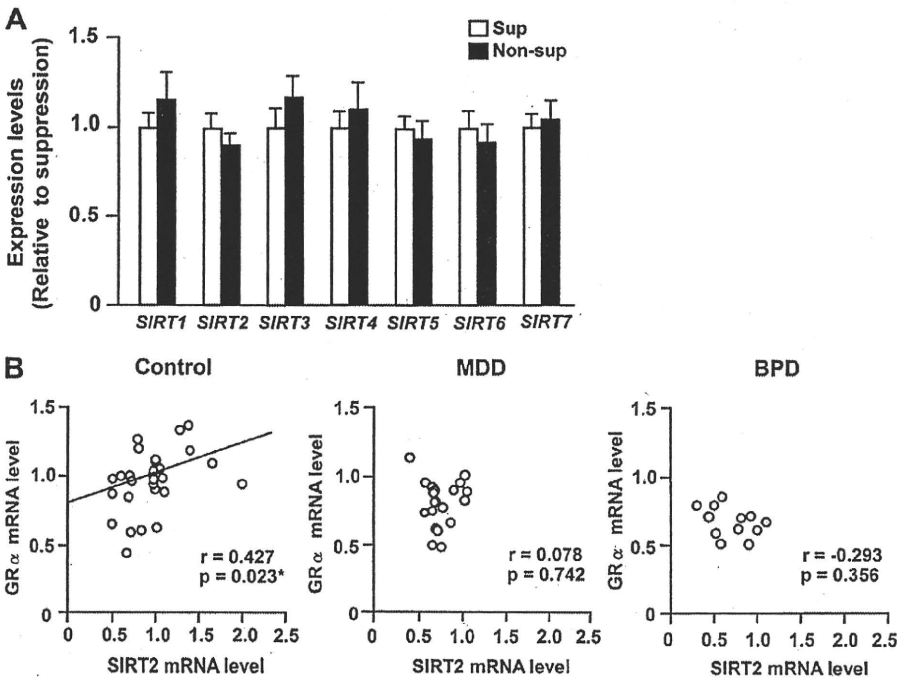

Fig. 3. Comparison of the expression levels of SIRTs mRNA between suppressors and non-suppressors on the dexamethasone (Dex)/corticotropin releasing hormone (CRH) test and the correlation of SIRT mRNA expression with glucocorticoid receptor (GR) α mRNA expression. (A) Comparison of the expression levels 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). 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).

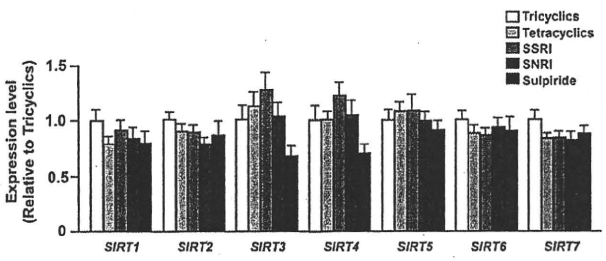

Fig. 4. Effects of antidepressants and sulpiride on the SIRTs mRNA levels in patients with MDD or BPD who were in a depressive state. No significant differences in the SIRT mRNA levels were found among the patients receiving any type of antidepressants or sulpiride who were in a depressive state (tricyclics, $n = 9$; 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.

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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Neuron
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	Stressed 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

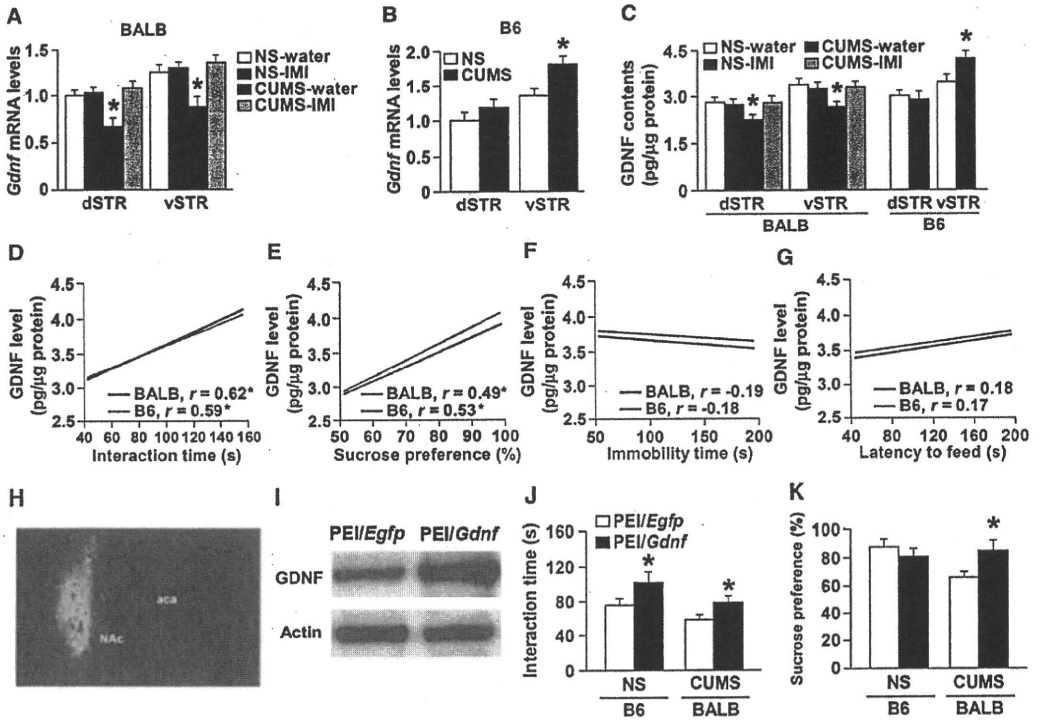

Figure 1. Differential Regulation of *Gdnf* Expression in Stress-Susceptible and Stress-Adaptive Mice Strains

(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

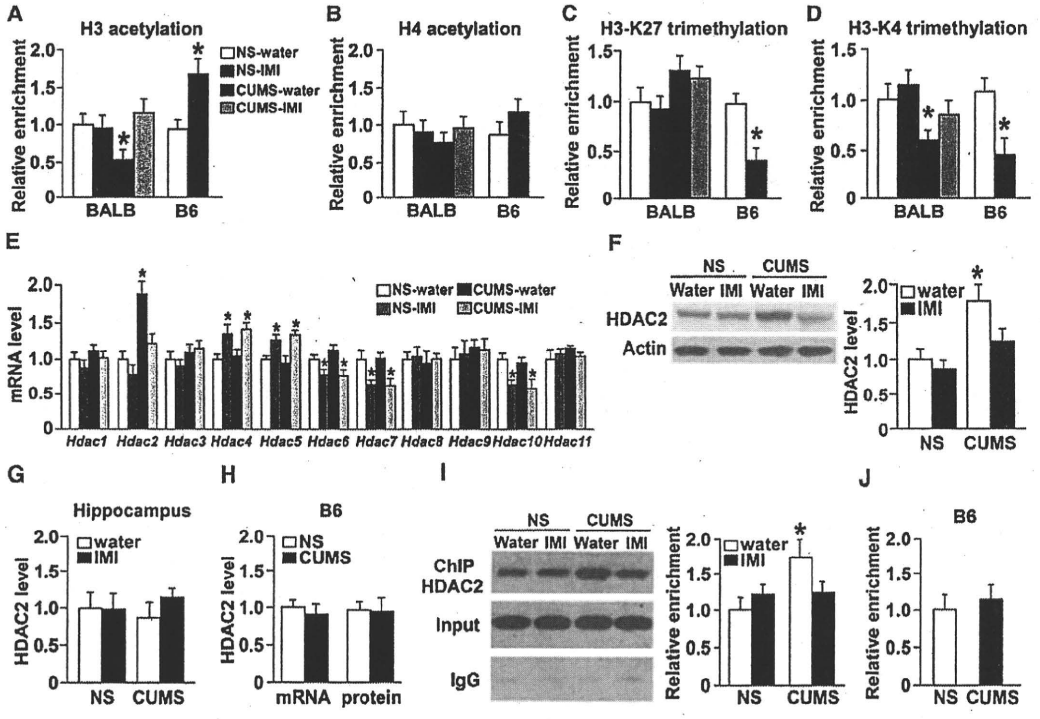

Figure 2. Differential Regulations of Histone Modifications in Stress-Susceptible and Stress-Adaptive Mice Strains

(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