Aripiprazole was a gift from Otsuka Pharmaceuticals. Haloperidol and methamphetamine were gifts from Dainippon Sumitomo Pharma Co. Ltd., Japan.

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Effects of mood stabilizers on adult dentate gyrus-derived neural precursor cells

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ABSTRACT

Neurogenesis in the adult dentate gyrus (DG) is considered to be partly involved in the action of mood stabilizers. However, it remains unclear how mood stabilizers affect neural precursor cells in adult DG. We have established a culture system of adult rat DG-derived neural precursor cells (ADP) and have shown that lithium, a mood stabilizer, and dexamethasone, an agonist of glucocorticoid receptor, reciprocally regulate ADP proliferation. Neurogenesis constitutes not only proliferation of neural precursor cells but also apoptosis and differentiation. To develop further understanding of mood stabilizer effects on neural precursor cells in adult DG, we investigated and compared the effects of four common mood stabilizers—lithium, valproate, carbamazepine, and lamotrigine—on ADP proliferation, apoptosis, and differentiation. ADP proliferation, decreased by dexamethasone, was examined using Alamar Blue assay. Using TUNEL assay, ADP apoptosis induced by staurosporine was examined. The differentiated ADP induced by retinoic acid was characterized by immunostaining with anti-GFAP or anti-Tuj1 antibody. Lithium and valproate, but not carbamazepine and lamotrigine, recovered ADP proliferation decreased by dexamethasone. All four mood stabilizers decreased ADP apoptosis. Retinoic acid differentiated ADP into both neurons and astrocytes. Lithium and carbamazepine increased the ratio of neurons and decreased that of astrocytes. However, valproate and lamotrigine increased the ratio of astrocytes and decreased that of neurons. Therefore, these four stabilizers exhibited both common and differential effects on ADP proliferation, apoptosis, and differentiation.

1. Introduction

Drugs of a group including lithium (Li), valproate (VPA), carbamazepine (CBZ), and lamotrigine (LTG), known as mood stabilizers, are commonly used to treat bipolar disorder (Goodwin, 2003). Although the biochemical effects of mood stabilizers have been investigated extensively (Schloesser et al., 2007; Schloesser et al., 2008), the essence of their mood-stabilizing effects remains unclear.

Recently, neurogenesis has been confirmed to occur in the adult hippocampus (Kempermann, 2006; Gage et al., 2008). Stem cells (Type-1 cells) are located in the subgranular zone between the granular cell layer and hilus in the dentate gyrus (DG), and differentiate to amplify progenitor cells (Type-2a and Type-2b cells). Thereafter, numerous newborn cells die, presumably by apoptosis (Kempermann et al., 2003). New surviving neurons mature morphologically and electrophysiologically. They ultimately integrate into active neural circuits. Although the function of these newborn cells remains unclear, they are reportedly involved in the therapeutic action of antidepressants (Santarelli et al., 2003; Surget et al., 2008; David et al., 2009). Moreover, recent reports have described that Li and VPA affect neurogenesis through increasing cell proliferation and/or promotion of neuronal differentiation of neural precursor cells (Chen et al., 2000; Son et al., 2003; Hao et al., 2004; Hsieh et al., 2004; Kim et al., 2004b; Laeng et al., 2004; Wexler et al., 2008) and that Li blocks the effects of stress on depression-like behaviors through increasing hippocampal neurogenesis in adult rodent models (Silva et al., 2008). Results of these studies suggest that adult hippocampal neurogenesis plays an important role in the therapeutic action of mood stabilizers as well.

We have already established the culture system of adult DG-derived neural precursor cell (ADP), which approximately corresponds to Type-2a amplifying progenitor cells (Boku et al., 2009). Li reported a lack of an effect on ADP proliferation but recovered ADP proliferation decreased by dexamethasone (DEX), a specific agonist of glucocorticoid receptor (Boku et al., 2009). To expand knowledge about the effect of mood stabilizers on adult neurogenesis, we

Abbreviations: Li, lithium; VPA, valproate; CBZ, carbamazepine; LTG, lamotrigine; DG, dentate gyrus; ADP, adult rat DG-derived neural precursor cell; DEX, dexamethasone; STS, staurosporine; RA, retinoic acid; PHT, phenytoin; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; DAPI, 4′·6-diamino-2-phenylindole.

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examined and compared the effects of four mood stabilizers—Li, VPA, CBZ, and LTG—on ADP proliferation, apoptosis and differentiation (cell-fate determination). Although these four stabilizers had varied effects on ADP proliferation and differentiation, all commonly decreased ADP apoptosis.

2. Materials and methods

2.1. Drugs

Dexamethasone (DEX) was purchased from Sigma Chemical Co. (St. Louis, MO). Retinoic acid (RA) was purchased from Invitrogen Corp. (Carlsbad, CA). Staurosporine (STS) was kindly donated by Asahi-Kasei Medical Co. Ltd. (Shizuoka, Japan). Lithium chloride (Li) was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Sodium valproate (VPA) was kindly donated by Kyowa Hakko Kogyo Co. Ltd. (Tokyo, Japan). Carbamazepine (CBZ) was kindly donated by Nihon Ciba-Geigy K.K. (Tokyo, Japan). Lamotrigine was kindly donated by Glaxo SmithKline plc. (London, UK). Phenytoin (PHT) was kindly donated by Dainippon Sumitomo Pharma Co. Ltd. (Osaka, Japan).

2.2. Isolation and culture of ADP

ADP was isolated from the dentate gyrus of adult male Sprague–Dawley rats (8 weeks old), as described in a previous report (Boku et al., 2009). The ADPs were maintained with Neurobasal (Invitrogen Corp., Carlsbad, CA)/B27 supplement minus vitamin A (Invitrogen Corp.)/1 mM L-glutamine (Invitrogen Corp.)/20 ng/ml bFGF (Invitrogen Corp.) (proliferation medium) at 37 °C on laminin (Invitrogen Corp.)-ornithine (Sigma)-coated dishes and fed with new medium every 2 or 3 days by replacing 50% of the medium. When cell confluency reached 80–90%, cells were passaged by trypsinization, and the cell density for plating was approximately 1×10^4 cells/cm^2.

2.3. Cell counting

Alamar Blue assay is a rapid and simple non-radioactive assay used to estimate the number of living cells (Ahmed et al., 1994). Alamar Blue dye is a fluorogenic redox indicator and is converted from the oxidized form to the reduced form in cells. The reduced form of Alamar Blue dye is highly fluorescent; fluorescence in Alamar Blue assay reflects the number of cells. Although BrdU-based assays are often used for cell counting, BrdU-positiveness reflects the duplication of DNA, but not the number of cells. Additionally, we confirmed that fluorescence in Alamar Blue assay is proportional to the simply counted number of ADPs (data not shown). Therefore, we used Alamar Blue assay to estimate the effects of drugs on the number of cells. First, 1×10^4 cells/well were put in laminin–ornithine coated 96-well plates in 100 μl/well of proliferation medium. After overnight incubation, cells were treated with each drug at each concentration. After 3 days, 10 μl/well of Alamar Blue solution (Invitrogen Corp.) was added to medium, and cells were incubated at 37 °C for 3 h. Subsequently, 50 μl of medium was dispensed into plates and the fluorescence of samples were measured and calculated as described in the manufacturer's manual. Statistical analysis was performed using one-way ANOVA and Dunnet's post hoc test. Significance was inferred for $p<0.05$. Data are expressed as means ± SEM.

2.4. TUNEL assay

First, 2×10^4 cells/well were put in laminin–ornithine coated 8-chamber slides (Lab-Tek II; Nalge Nunc International, Naperville, IL) with proliferation medium. After overnight incubation, cells were treated in proliferation medium with STS, PHT, Li, VPA, CBZ, and/or LTG. After 2 days, cells were fixed in 4% paraformaldehyde for 15 min. Permeabilization was performed with PBS containing 0.2% Triton X-100 for 30 min. Subsequently, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed with a DeadEnd Fluorometric TUNEL System (Promega Corp., Madison, WI), as described in the manufacturer's manual. Fluorescent signals were detected using a fluorescence microscope system (IX-71; Olympus Corp.). The quantities of both TUNEL and 4′·6-diamino-2-phenylindole (DAPI; Vector Laboratories Inc., Burlingame, CA) signals were counted in four randomly selected fields/well. Then the ratio of TUNEL signals/DAPI signals was calculated. Statistical analysis was performed using Student's *t*-test. Significance was defined as $p<0.05$. Data are expressed as means ± SEM.

2.5. Immunocytochemistry

First, 2×10^4 cells/well were put in laminin–ornithine coated Lab-Tek II eight-chamber slides with proliferation medium without bFGF (differentiation medium). After overnight incubation, cells were treated in differentiation medium with 1 μM RA/0.5% fetal bovine serum (Invitrogen) and Li, VPA, CBZ, or LTG. After 7 days, cells were fixed 4% paraformaldehyde for 15 min. Permeabilization was performed with PBS containing 0.2% Triton X-100 for 30 min. Subsequently, samples were blocked in PBS containing 3% goat serum for 20 min at room temperature (RT), incubated in PBS containing 3% goat serum containing primary antibodies at 4 °C overnight, and incubated in PBS containing secondary antibodies for 1 h at RT. Samples were coverslipped with Vectashield containing DAPI. Fluorescent signals were detected using the IX-71 fluorescent microscope system described above. Primary antibodies were used at the following concentrations: mouse anti-nestin (1:2000; BD Biosciences, Franklin Lakes, NJ), rabbit anti-glial fibrillary acidic protein (GFAP) (1:2000; Dako, Glostrup, Denmark) and mouse anti-Tuj1 (1:5000; Covance Inc., Princeton, NJ). Secondary antibodies were used at the following concentrations: FITC-conjugated goat anti-mouse IgG antibody (1:100; Jackson Immuno Research Laboratories, Inc., West Grove, PA) and Cy3-conjugated goat anti-rabbit IgG antibody (1:100; Jackson Immuno Research Laboratories, Inc.). The quantities of signals of each marker gene and DAPI were counted in four randomly selected fields/well. Then the ratio of each marker gene-derived signals/DAPI signals was calculated. Statistical analysis was performed using Student's *t*-test. Significance was defined as $p<0.05$. Data are expressed as means ± SEM.

3. Results

3.1. Effects of mood stabilizers on ADP proliferation

Adult neurogenesis in DG is decreased in rodent models for stress-related disorders (Malberg and Duman, 2003; Jayatissa et al., 2006; Silva et al., 2008). Although it remains unclear how adult neurogenesis in DG is decreased in these models, reports of some studies have suggested that glucocorticoids are involved in them (Cameron and McKay, 1999; Kim et al., 2004a). We have already shown that Li has no effect on ADP proliferation but recovers ADP proliferation decreased by dexamethasone (DEX), a specific agonist of glucocorticoid receptor (Boku et al., 2009). Following our previous study, we used Alamar Blue assay to examine the effects of Li, VPA, CBZ and LTG on ADP proliferation in the absence or presence of 5 μM DEX. Results showed that ADP proliferation decreased significantly with 5 μM DEX. Furthermore, 0.3–3 mM Li and 30–1000 μM VPA showed no effect on ADP proliferation in the absence of DEX but 1–3 mM Li and 100–300 μM VPA recovered ADP proliferation decreased by 5 μM DEX in a dose-dependent manner (Fig. 1A and B). However, 3–30 μM CBZ and 30–300 nM LTG had no effect on ADP proliferation, either in the absence or presence of 5 μM DEX (Fig. 1C and D). The ADP

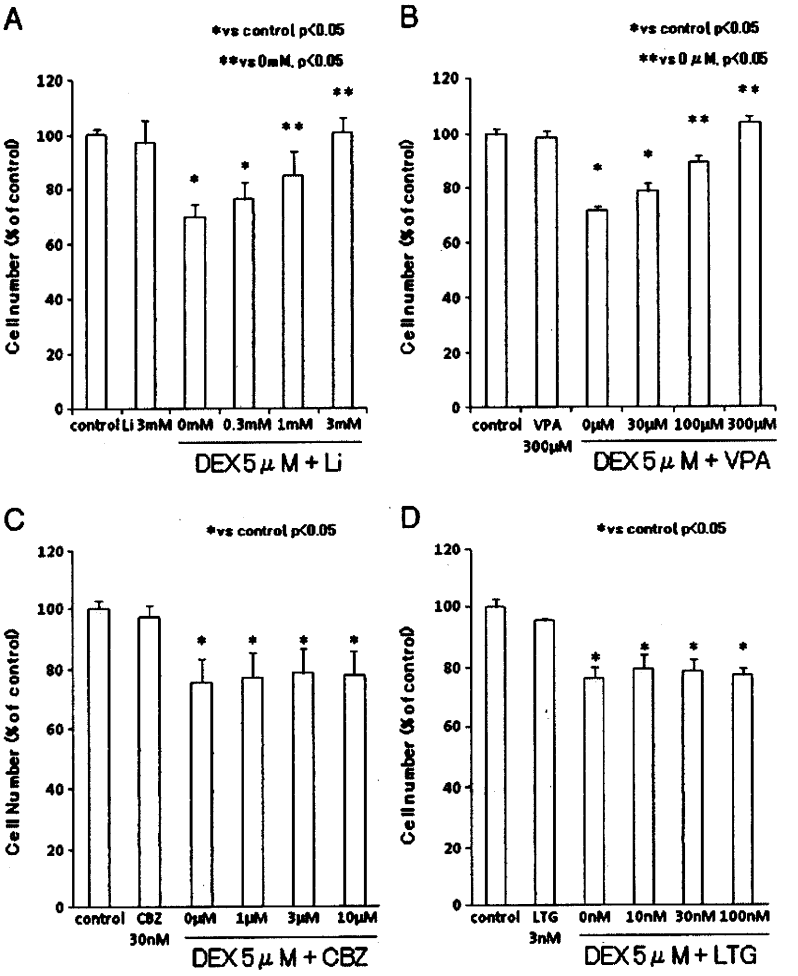

Fig. 1. Effects of mood stabilizers on ADP proliferation Li (A) and VPA (B) recovered ADP proliferation decreased by DEX, but not CBZ (C) and LTG (D). Alamar Blue assay was performed 3 days after drug treatments for four independent cultures. Data are shown as means ± SEM. *$p<0.05$, compared with control; **$p<0.05$, compared with 0 mM (A: Li), 0 μM (B: VPA).

proliferation was decreased remarkably more than 30 μM CBZ and 300 nM LTG (data not shown).

3.2. Effects of mood stabilizers on ADP apoptosis

Staurosporine (STS) is a common inhibitor of Protein Kinase C that is often used to induce apoptosis on culture cells (Sanchez et al., 1992). Apoptosis has two pathways: the internal pathway via mitochondria and external pathways via death receptors (Adams, 2003); STS is well known as an inducer of internal pathway (Ferrari et al., 1998). All four of these stabilizers increase the expression of Bcl-2, a key regulator of the internal pathway (Chen et al., 1999b; Chang et al., 2008). In addition, the internal pathway, but not the external pathway, is involved in the apoptosis of neural progenitor cells (Ekdahl et al., 2003; Ceccatelli et al., 2004). Our preliminary data showed that Tumor Necrosis Factor-α, a ligand of death receptors and an inducer of external pathway, did not induce apoptosis on ADP (data not shown). Therefore, we specifically examined the internal pathway in the present study. First, the effect of STS on ADP apoptosis was examined using TUNEL assay at 0, 100, 300 nM, and 1 μM. Only a few TUNEL signals were found at 0 and 100 nM STS. Most of the cells were removed from the bottom of the eight-well chamber at 1 μM STS (data not shown). However, around 70% of cells were TUNEL-positive with 300 nM STS (Fig. 2). Therefore, we investigated the effects of Li, VPA, CBZ, and LTG on ADP apoptosis induced by 300 nM STS. Next, the effects of Li (1, 3 and 10 mM), VPA (100, 300 μM and 1 mM), CBZ (1, 3, 10 and 30 μM), and LTG (10, 30, 100 and 300 nM) on ADP apoptosis induced by 300 nM STS were examined using TUNEL assay. All of 1 mM Li, 1 mM VPA, 3 μM CBZ, and 30 nM LTG decreased the ratio of TUNEL-positive cells to around 30% (Fig. 2A–D). Nevertheless, none of these four mood stabilizers had any effect on the ratio of TUNEL-positive cells or peeled off many cells from the bottom of the eight-well chamber at other doses (data not shown). We also examined the effect of 5 μM phenytoin (PHT), an antiepileptic drug that is not used as a mood stabilizer, on ADP apoptosis induced by 300 nM STS. It is noteworthy that 5 μM PHT had no effect on the ratio of TUNEL-positive ADPs (Fig. 2E).

3.3. Effects of mood stabilizers on ADP differentiation

Retinoic acid (RA) is widely used as a potent inducer of neural differentiation by multipotent cells of various types, such as neural stem cells, embryonal carcinoma cells, and embryonic stem cells *in vitro* (Takahashi et al., 1999; Soprano et al., 2007). Recent findings have shown that endogenous RA is involved in neural differentiation in adult hippocampus *in vivo* (Jacobs et al., 2006; McCaffery et al., 2006). We have already shown that RA induces ADP to both neuron and astrocyte (Boku et al., 2009). Moreover, ADP did not differentiate at all by only the depletion of bFGF, and all of Li, VPA, CBZ, and LTG had no effect on ADP differentiation without RA (data not shown).

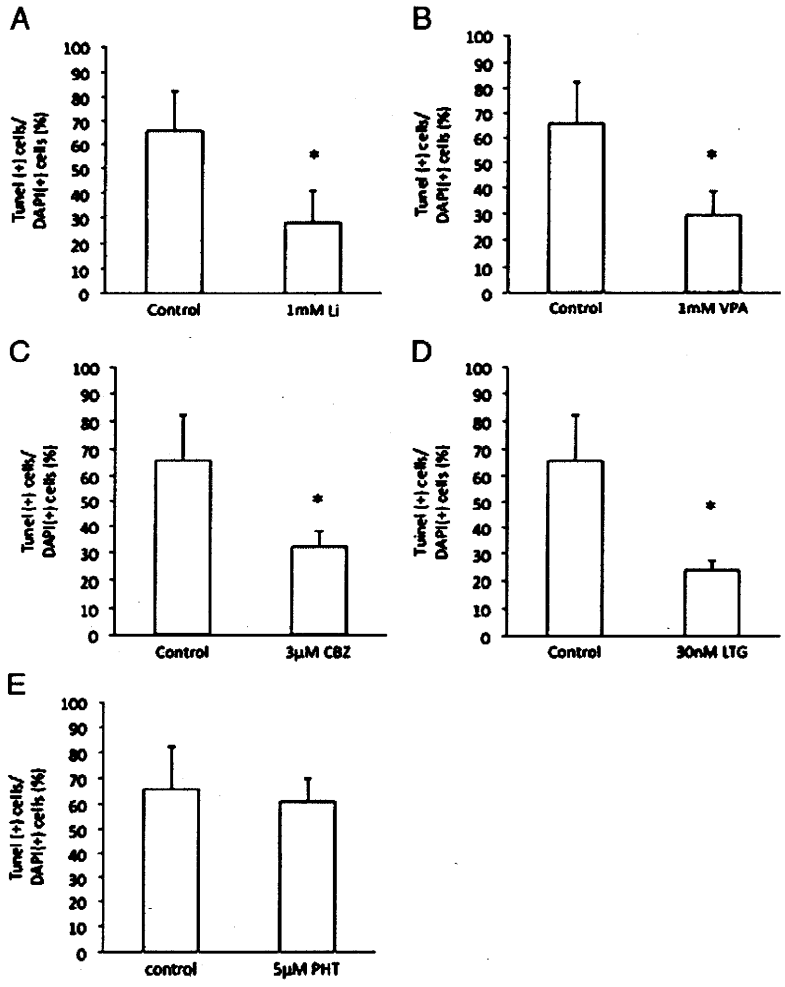

Fig. 2. Effects of mood stabilizers on ADP apoptosis. All four mood stabilizers decreased ADP apoptosis induced by staurosporine (STS) (A–D). Phenytoin (PHT), an anticonvulsant drug, did not affect staurosporine-induced apoptosis (E). TUNEL assay was performed 2 days after drug treatments for four independent cultures. The percentage of TUNEL (+)/DAPI (+) ADPs with STS is expressed as control. Data are shown as means ± SEM. $^{*}p<0.05$, compared with control.

Therefore, we examined the effects of Li (1, 3 and 10 mM), VPA (100, 300 μM and 1 mM), CBZ (1, 3, 10 and 30 μM), and LTG (10, 30, 100 and 300 nM) on ADP differentiation induced by RA. Results show that 1 μM RA differentiated around 50% of ADP into a neuron-like cell (Tuj1-positive cell) and around 12% of ADP into an astrocyte-like cell, which is GFAP-positive and which has larger size and a more spread shaped than that of ADP (Fig. 3A–C). Both 1 mM Li and 3 μM CBZ increased the ratio of neuron-like cells to around 70% and decreased the ratio of into astrocyte-like cells to around 5% (Fig. 3B and C). Both 1 mM VPA and 30 nM LTG decreased the ratio of neuron-like cells to around 10% and increased the ratio of astrocyte-like cell to around 25% (Fig. 3B and C). None of Li, VPA, CBZ, or LTG had any effect on the number of ADP in these doses (data not shown). None of these four mood stabilizers showed any effect on the ratio of Tuj1 or GFAP-positive cells or peeled off many cells from the bottom of the eight-well chamber at other doses (data not shown).

4. Discussion

Results show that four commonly used mood stabilizers—Li, VPA, CBZ, and LTG—have varying effects on ADPs (Table 1). Only four comparative studies have examined cells of other types, such as tumor-derived cell lines or primary neuron cultures. In addition, each study specifically addressed only a single phenomenon: neuronal differentiation or apoptosis (Li et al., 2002; Mai et al., 2002; Williams et al., 2002; Daniel et al., 2005). Therefore, the present report describes is the first comparative study of the effects of four mood stabilizers in adult DG-derived neural precursor cells on three phenomena which constitute neurogenesis: proliferation, apoptosis and differentiation.

Our present results differ from past studies in many points. In the case of proliferation, some reports have described that Li and VPA increased the proliferation of neural precursor cells (Kim et al., 2004b; Laeng et al., 2004; Wexler et al., 2008). In the case of differentiation, some reports have described that Li and VPA, but not CBZ, promote the differentiation of neural precursor cells into neurons (Hao et al., 2004; Hsieh et al., 2004; Kim et al., 2004b; Laeng et al., 2004). In these studies, neural precursor cells are derived from entire adult hippocampi, partly including the subventricular zone, and embryos. In contrast, our ADP is derived from dissected DG from adult hippocampi. Moreover, the proliferation potency of neural stem cells decreases according to age (Molofsky et al., 2006). Although neural stem cells in the early developmental stage tend to be differentiated into neurons by LIF, those in the late developmental stage tend to be differentiated into astrocytes (Takizawa et al., 2001). Moreover, in contrast to ADP-derived dissected DG, adult

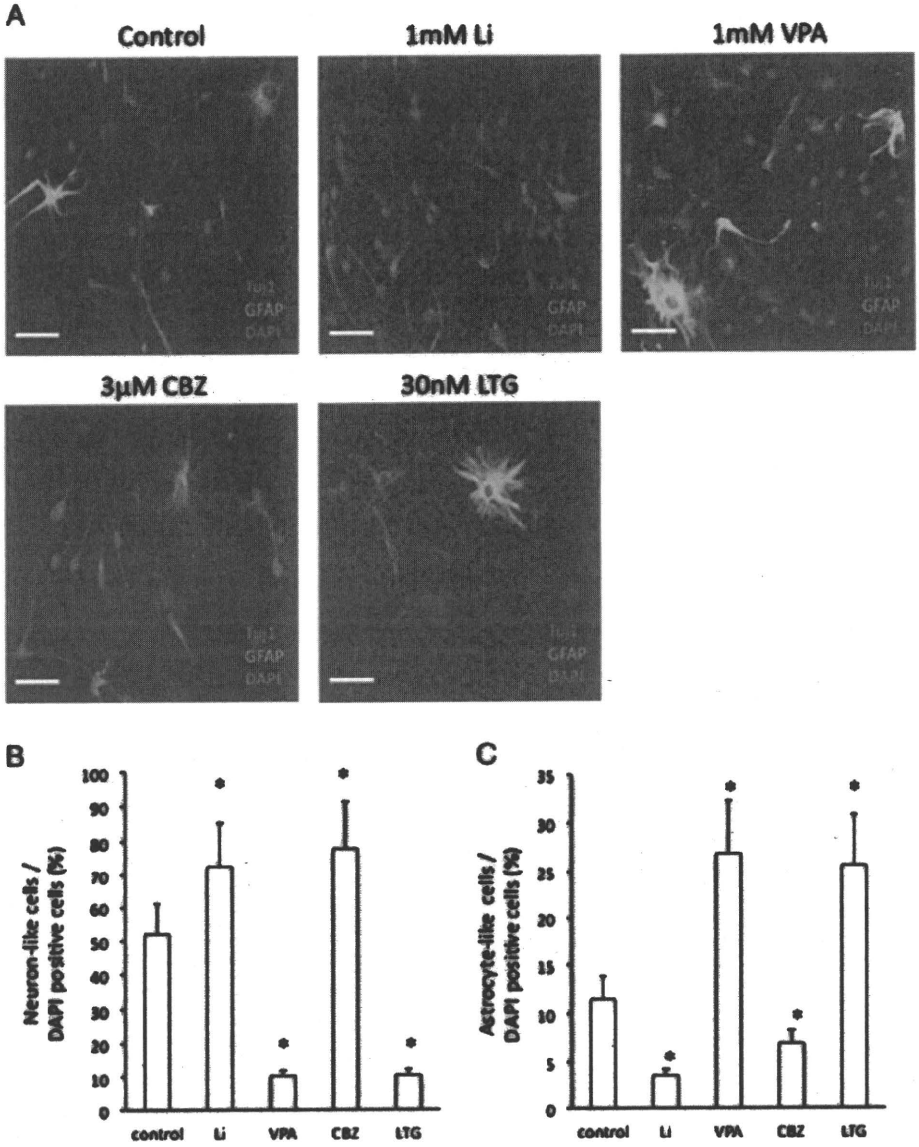

Fig. 3. Effects of mood stabilizers on ADP differentiation Li and CBZ increased the ratio of neuron-like cell induced by retinoic acid (RA). However, VPA and LTG decreased it and increased the ratio of astrocyte-like cell. Immunocytochemistry was performed 7 days after drug treatment for four independent cultures. A: Scale bar = 120 μm. B, C: Concentrations of mood stabilizers are as follows: Li, 1 mM; VPA, 1 mM; CBZ, 3 μM; LTG, 30 nM. Data are shown as means ± SEM. $^{*}p<0.05$, compared with control.

hippocampal neural progenitors in past studies are derived from entire adult hippocampi. The culture condition of ADP also differs from that of adult hippocampal progenitors. Therefore, the reactivity to drugs of ADP might differ from those of embryonic neural stem cells and adult hippocampal neural progenitors. To confirm that point, some comparison is needed of the effects of mood stabilizers on proliferation, apoptosis and differentiation between ADP and neural precursor cells derived from other sources.

That GSK-3β and β-catenin/TCF pathways regulate cell proliferation is well known (Takahashi-Yanaga and Sasaguri, 2009). Regarding the effect of Li on ADP proliferation decreased by DEX, we have already shown part of its mechanism: DEX decreases ADP proliferation through activation of GSK-3β and following inhibition of β-catenin/TCF pathway; and Li reverses the inhibitory effect of DEX on ADP proliferation through inhibiting activated GSK-3β and following activation of β-catenin/TCF pathway (Boku et al., 2009). In the present

Table 1
The summary of the effects of mood stabilizers on ADPs.

	Lithium	Valproate	Carbamazepine	Lamotrigine
DEX-decreased proliferation	Recover	Recover	No effect	No effect
STS-induced apoptosis	Recover	Recover	Recover	Recover
RA-induced differentiation	Neuron↑ astrocyte↓	Neuron↓ astocyte↑	Neuron↑ astrocyte↓	Neuron↓ astrocyte↑

study, we showed that VPA recovered ADP proliferation decreased by DEX as in the case of Li. We also showed that the recovery effect of VPA on ADP proliferation decreased by DEX is reversed by quercetin, an inhibitor of β-catenin/TCF pathway, as in the case of Li (our unpublished data). In addition, some reports have described that VPA promotes β-catenin/TCF pathway through inhibition of GSK-3β (Chen et al., 1999a; Kim et al., 2005). These findings suggest that VPA also regulates ADP proliferation through GSK-3β and the β-catenin/TCF pathway. However, the other reports have described that the activity of GSK-3β is not inhibited by VPA (Williams et al., 2002; Kozlovsky et al., 2003; Ryves et al., 2005). Additionally, it has been shown that VPA actions are partly mediated by histone deacetylase (HDAC) inhibition (Phiel et al., 2001). Further investigation is necessary to elucidate how VPA recovers ADP proliferation decreased by DEX.

Our results of the effects of mood stabilizers on ADP differentiation induced by RA have shown that Li and CBZ increase neuronal differentiation and decrease astroglial differentiation and that VPA and LTG are vice versa. The opposite effects of Li/CBZ and VPA/LTG on ADP differentiation are so interesting that the investigation of the mechanism underlying these opposing effects might be expected to engender new aspects of the action mechanism of mood stabilizers. RA and brain-derived neurotrophic factor (BDNF) synergistically promote neuronal differentiation of neural precursor cells (Takahashi et al., 1999). In addition, RA and leukemia inhibitory factor (LIF) synergistically promotes astroglial differentiation of neural precursor cells (Asano et al., 2009). Therefore, Li/CBZ might affect BDNF pathway and VPA/LTG might affect the LIF pathway. This speculation is apparently interesting but has some problems. For example, the pathways cannot be merely separated from each other because some reports have described the existence of cross talk between BDNF and LIF pathways (Rajan et al., 1998; Lund et al., 2008; Yasuda et al., 2009). Although it has been shown that Li induces BDNF expression (Fukumoto et al., 2001), the effect of CBZ on BDNF pathway and the effects of VPA and LTG on LIF pathway remain poorly understood. However, our speculation might be worth further consideration. To confirm results of the effects of mood stabilizers on ADP differentiation induced by RA and to validate our speculation of the mechanism underlying them, further investigation using other physiological inducers of neural and glial differentiation, such as BDNF and LIF, is needed.

Although the effects of mood stabilizers on ADP proliferation decreased by DEX and ADP differentiation induced by RA differ among them, all of these four mood stabilizers decreased ADP apoptosis induced by STS (Table 1). In addition, phenytoin, an anticonvulsant but not mood stabilizer, had no effect on it. Therefore, their anti-apoptotic effects on neural precursor cell might be involved in a part of common mood-stabilizing effects. Results of some studies have suggested that internal pathway-related factors (e.g., GSK-3β, Bcl-2 and HSP70) might be candidates of the common anti-apoptotic effects of mood stabilizers (Chen et al., 1999b; Li et al., 2002; Jope and Bijur, 2002; Ren et al., 2003; Pan et al., 2005). Furthermore, Williams et al. showed that prolyl oligopeptidase is a candidate factor of the common effects of mood stabilizers on the collapse of sensory neuron growth cones (Williams et al., 2002); prolyl oligopeptidase is reportedly involved in apoptosis (Odaka et al., 2002). Therefore, we are going to investigate the mood-stabilizing effects of these molecules in ADP, which might engender elucidating the molecular mechanism of mood-stabilizing effects. However, staurosporine is not a physiological inducer of apoptosis. Therefore, further investigation using more physiological inducers of apoptosis, such as mitogen or insulin withdrawal and glucose deprivation, is necessary to confirm our results.

The differentiation stages of proliferative neural precursor cells have been proposed for the adult rodent hippocampus *in vivo* (Kempermann, 2006; Gage et al., 2008): the first stage (Type-1 cell), the second stage (Type-2a cell), and the third stage (Type-2b cell). Type-1 cell corresponds to stem cells. Type-2a cell and Type-2b cell correspond to amplifying progenitor cells. In these developmental stages, our ADP might correspond to Type-2a cell (Boku et al., 2009). Fluoxetine, an antidepressant, increases Type-2a-like cells in adult DG (Encinas et al., 2006); electroconvulsive seizure mainly increases Type-1-like cells in adult DG (Segi-Nishida et al., 2008). Our present study has elucidated the effects of mood stabilizers on Type-2a cell, but not on Type-1 cells and Type-2b cells. Therefore, it might be important to investigate the effects of specific drugs on precursor cells of other types.

Finally, results show that the four common mood stabilizers—Li, VPA, CBZ, and LTG—exert various effects on the actions of ADP, Type-2a like neural precursor cell in adult DG. However, *in vivo* functional significance of these results and mechanisms underlying them remain unclear. To investigate them might be beneficial to further the understanding of action mechanisms of mood stabilizers and the pathophysiology of mood disorders, which might engender the development of new therapeutic targets of mood disorder.

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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

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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).

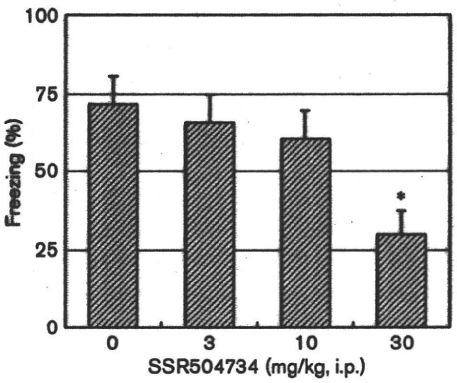
SSR504734 (3–30 mg/kg, intraperitoneal) given 1 h before testing dose-dependently attenuated conditioned freezing [$F(3,44) = 3.37$, $P < 0.05$] in rats tested 24 h after footshock (Fig. 2). Post-hoc analysis revealed a significant inhibitory effect of SSR504734 (30 mg/kg, intraperitoneal) on conditioned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

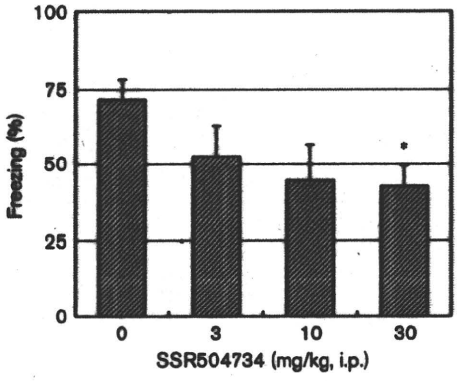
In this study, we investigated the effects of acute administration of SSR504734 on the acquisition and expression of contextual conditioned fear in rats. We found that acute administration of SSR504734 dose-dependently attenuated conditioned freezing in both the

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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Aberrant REST-mediated transcriptional regulation in major depressive disorder

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ABSTRACT

There is growing evidence that aberrant transcriptional regulation is one of the key components of the pathophysiology of mood disorders. The repressor element-1 silencing transcription factor (REST) is a negative regulator of genes that contain the repressor element-1 (RE-1) binding site. REST has many target genes, including corticotropin releasing hormone (CRH), brain-derived neurotrophic factor, serotonin 1A receptor, which are suggested to be involved in the pathophysiology of depression and the action of antidepressants. However, a potential role for REST-mediated transcriptional regulation in mood disorders remains unclear. In this study, we examined the mRNA levels of REST and its known and putative target genes, using quantitative real-time PCR in peripheral blood cells of patients with major depressive and bipolar disorders in both a current depressive and a remissive state. We found reduced mRNA expression of REST and increased mRNA expression of CRH, adenylate cyclase 5, and the tumor necrosis factor superfamily, member 12–13 in patients with major depressive disorder in a current depressive state, but not in a remissive state. Altered expression of these mRNAs was not found in patients with bipolar disorder. Our results suggest that the aberrant REST-mediated transcriptional regulation of, at least, CRH, adenylate cyclase 5, and tumor necrosis factor superfamily, member 12–13, might be state-dependent and associated with the pathophysiology of major depression.

1. Introduction

While the pathophysiology of mood disorder is not completely understood, recent reports have focused on the long-term molecular changes that underlie mood disorders and treatment with antidepressants (Nestler et al., 2002; Duman et al., 2006). Aberrant regulation of gene expression represents a major component of mood disorders and the action of antidepressants (Nestler et al., 2002; Duman et al., 2006). The altered expression of a variety of transcription factors such as the glucocorticoid receptor and the cAMP-responsive element binding protein are associated with mood disorders and stressed animals (Holsboer, 2000; Nestler et al., 2002; Webster et al., 2002; Carlezon et al., 2005; de Kloet et al., 2005; Laifenfeld et al., 2005). In addition, antidepressants and/or mood stabilizers alter gene expression patterns in the brain (Manji et al., 1999; Coyle and Duman, 2003). Mood disorder patients and chronically stressed humans have also been reported to show alterations of gene expression in peripheral blood cells (Matsubara et al., 2006; Anitha et al., 2008; Miller et al., 2008; Otsuki et al., 2008).

Repressor element-1 silencing transcription factor (REST), also termed neuron-restrictive silencing factor, is a modular protein that contains, in addition to a DNA-binding domain with eight consecutive zinc fingers, two independent repression domains located at the N- and C-terminals of the molecule (Chong et al., 1995; Schoenherr and Anderson, 1995; Tapia-Ramirez et al., 1997). This zinc finger protein binds to a conserved consensus sequence called repressor element-1 (RE-1), also called the neuron-restrictive silencing element, allowing the transcriptional repression of RE-1 containing target genes, most of which are expressed in neurons (Chong et al., 1995; Schoenherr and Anderson, 1995; Tapia-Ramirez et al., 1997). In the central nervous system, it is believed that the REST – RE-1 system serves as a molecular switch that helps to distinguish neuronal from non-neuronal cell types, as the repression was thought to occur in non-neuronal cells, which contain REST, but not in neuronal cells, which either lack or contain only relatively low levels of REST (Kraner et al., 1992; Mori et al., 1992; Schoenherr and Anderson, 1995; Chong et al., 1995). Recent evidence suggests that REST and its target genes are involved in the regulation of neuronal terminal differentiation (Chong et al., 1995; Schoenherr et al., 1996), neurogenesis (Ballas et al., 2005; Westbrook et al., 2008) and synaptic plasticity (Schoenherr and Anderson, 1995). Although the dysregulation of REST and its target genes have been implicated in the pathogenesis of Down's

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syndrome (Bahn et al., 2002), Alzheimer's disease (Okazaki et al., 1995), Huntington's disease (Zuccato et al., 2007) and ischemic insults (Calderone et al., 2003), its association with the pathophysiology of mood disorders remains unknown.

A recent report indicated that REST has many target genes including corticotropin releasing hormone (CRH), brain-derived neurotrophic factor, and the serotonin (5-HT) 1A receptor (Otto et al., 2007), which are suggested to be involved in mood disorders and/or the action of antidepressants in humans and rodents (Lemonde et al., 2003; Nestler et al., 2002; Duman et al., 2006). In addition, the repressive activity of REST is modulated by a recruitment of histone deacetylases, chromatin remodeling molecules, which are suggested to be involved in mood disorders and/or the action of antidepressants (Tsankova et al., 2006; Schroeder et al., 2007). Furthermore, depressed individuals often exhibit hypercortisolaemia (de Kloet et al., 2005), and a more recent study demonstrated that REST-mediated transcriptional regulation is involved in the synthesis of cortisol/corticosterone (Somekawa et al., 2009). These observations prompted us to postulate a role for aberrant REST-mediated gene regulation in the pathogenesis of mood disorders.

In this study, to investigate whether the mRNA expression of REST is altered in mood disorder patients, we assessed the mRNA levels of REST and a variety of its target genes, including CRH, 5-HT1A, adenylate cyclase 5 (Adcy5), calcium/calmodulin-dependent kinase II α (CaMKIIa), erythropoietin receptor (Epor), insulin-like growth factor 1 receptor (IGF1R), tumor necrosis factor superfamily, member 10 (Tnfsf10), Tnfsf11, and Tnfsf12–13 using quantitative real-time PCR in peripheral blood cells from major depressive and bipolar disorder patients. Furthermore, to examine whether the altered expression of these mRNAs is state- or trait-dependent, mRNA levels were examined in both current depressive and remissive states.

2. Methods and material

2.1. Subjects

Major depressive and bipolar disorder patients were diagnosed according to the criteria in the Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV; American Psychiatric Association, 1994). These included both outpatients and inpatients of the Division of Neuropsychiatry of the Yamaguchi University Hospital. The extent of the depressive state was assessed by a 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 the major depressive episode in the DSM-IV criteria for more than 2 months. Individuals were excluded from the present study if they had abnormal physical examinations or abnormal results for routine medical laboratory tests such as a complete blood count, renal, liver or thyroid function. Female subjects who were pregnant or took oral contraceptives were also excluded. All healthy control subjects were screened to exclude significant current or past medical or neurological illnesses, significant alcohol or drug abuse and past or current Axis I psychiatric illnesses. Controls and patients were all of Japanese ethnicity and there was no significant population stratification 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 for 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 (Matsubara et al., 2006). In brief, blood was obtained by venipuncture between 10:00 and 11:00 am and processed for total RNA purification from peripheral blood cells using the QIAamp RNA Blood Mini Kit (Qiagen, Chatsworth, CA, USA) according to the manufacturer's manual. The quality of RNA was determined based on the A_{260}/A_{280} ratio, which was 1.7–2.0 for all RNA preparations. One hundred nanograms of total RNA were used for cDNA synthesis using random hexamer primers and Omniscript Reverse Transcriptase (Qiagen). The cDNA was stored at −80 °C until further use.

2.3. Quantitative real- time polymerase chain reaction

Quantitative real-time polymerase chain reaction (qRT-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 (Matsubara et al., 2006; Otsuki et al., 2008). Table 1 lists all primer sequences used. 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.

2.4. Serum cortisol determination

Serum cortisol concentration was measured via radioimmunoassay by the SRL Corporation (Tokyo, Japan).

2.5. Dex/CRH test

A few days after blood samples were taken for RNA isolation and basal cortisol determination, the Dexamethasone (Dex)/CRH test was performed as previously reported (Matsubara et al.,

Table 1
Primer sequences.

	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	Size (bp)
REST	CACCACTGCCAAAGGAAAAT	CCAGGTAGGCTCTCATCTGC	130
CRH	CTGGGGAACCTCAACAAGAG	CAACACGCGGAAAAAGTTG	120
5-HT1A	ACAGGTACTGGGCCATCACG	GCGGGATAGAGATGAGGAAGC	114
Adcy5	GGCAGCTGGAGAAGATCAAG	GCCCACCTTGTCGTAGGTAG	83
CaMKIIa	TACATCCGCATCACGCAGTA	CTGTGGAAGTGGACGATCTG	116
Epor	CTCATCCTCGTGGTCATCCT	CAGGCCAGATCTTCTGCTTC	85
IGF1R	CCATTCTCATGCCTTGGTCT	TGCAAGTTCTGGTTGTCGAG	114
Tnfsf10	TTCACAGTGCTCCTGCAGTC	ATCTGCTTCAGCTCGTTGGT	71
Tnfsf11	CGTCGCCCTGTTCTTCTATT	TGCAGTGAGTGCCATCTTCT	71
Tnfsf12–13	GGAACTGAATCCCCAGACAG	GCCTTTAGGTGCACTTCTGC	88
GAPDH	CAGCCTCAAGATCATCAGCA	TGTGGTCATGAGTCCTTCCA	106

2006). Mood disorder patients were pretreated with an oral dose of 1 mg of Dex (Asahikasei Pharmaceutical Corporation, Tokyo, Japan) at 11:00 pm. On the next day, an intravenous cannulation was carried out at 12:30 pm and 100 μg of human CRH (hCRH, Mitsubishi Pharma Corporation, Tokyo, Japan) was administered intravenously at 1:00 pm, immediately after the first blood collection, followed by four additional blood samples taken through the intravenous catheter 15 min, 30 min, 60 min and 120 min later. Blood samples were immediately centrifuged and stored at −20 °C. The serum level of cortisol and plasma level of ACTH were measured with radioimmunoassay (SRL). We defined as subjects non-suppressors (baseline cortisol > 5 μg/dl), intermediate suppressors (baseline cortisol < 5 μg/dl and peak cortisol > 5 μg/dl), or suppressors (peak cortisol < 5 μg/dl), according to a classification proposed by Kunugi et al. (2004, 2006).

2.6. Statistical analysis

Commercial software (SPSS version 16.0; SPSS Inc., Chicago, Illinois) was used to perform data analysis. All data are expressed as the mean ± standard error of the mean (SEM). Gender distribution was analyzed by the χ^2 test. The mRNA levels were subjected to one-way analysis of variance (ANOVA) followed by *post hoc* analysis (Tukey test). Two group comparisons in the same mood disorder patients before and after remission and in the suppressors and non-suppressors including intermediate suppressors of the Dex/CRH test on the mRNA levels of measured genes were performed using the paired *t* test and unpaired *t* test, respectively. Two group comparisons in the control subjects of male and female on the mRNA level of measured genes were performed using the Mann–Whitney U-test. Pearson 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 for $p < 0.05$.

3. Results

Table 2 shows the demographic and clinical characteristics of the subjects. The majority of the patients were on medications. The mean ages were not significantly different among major depressive disorder patients, bipolar depressive patients and healthy control subjects ($F = 1.263$, $df = 4,129$, $p = 0.288$). Regarding the gender distribution, bipolar disorder patients showed a significantly larger ratio of females to males ($\chi^2 = 19.565$, $p < 0.001$). There was no significant association of gender with the mRNA expression level of all measured genes in control subjects (data not shown).

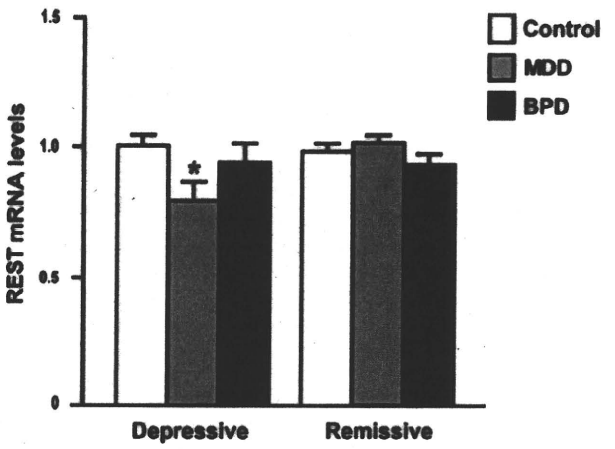

Fig. 1. The mRNA expression of REST in mood disorder patients in a depressive state and a remissive state. Quantitative real-time PCR revealed decreased mRNA expression of REST in a depressive state only in MDD patients ($n = 20$) compared with healthy control subjects ($n = 28$) but not in a remissive state. MDD, major depressive disorder; BPD, bipolar disorder. Values are mean ± standard error. * $p < 0.05$.

We examined the mRNA expression of REST in patients with mood disorder in a current depressive state. qRT-PCR revealed that the mRNA expression of REST was significantly decreased in major depressive disorder patients compared with healthy control subjects ($F = 4.799$, $df = 2,58$, $p = 0.012$; *post hoc* $p = 0.009$) (Fig. 1). In a remissive state, major depressive disorder patients did not show any significant differences in the mRNA levels of REST compared with healthy control subjects ($F = 2.739$, $df = 2,98$, $p = 0.855$) (Fig. 1). We did not find an alteration in the mRNA expression of REST in patients with bipolar disorder in a current depressive ($F = 4.799$, $df = 2,58$, $p = 0.691$) or a remissive state ($F = 2.739$, $df = 2,98$, $p = 0.259$).

Table 2
Demographic and clinical characteristics of subjects.

	Control	Patients			
		MDD		BPD	
		Depressed	Remitted	Depressed	Remitted
	$n = 28$	$n = 20$	$n = 40$	$n = 13$	$n = 33$
Age (years)	50.0 ± 1.8	52.3 ± 3.5	57.1 ± 2.2	55.5 ± 3.5	52.7 ± 2.6
Gender (male/female)	15/13	10/10	15/25	2/11	7/26
HDRS		25.9 ± 1.9	3.3 ± 0.2	24.6 ± 1.1	2.8 ± 0.2
Serum cortisol (μg/dl)	8.6 ± 0.8	10.3 ± 1.3	11.6 ± 1.1	10.9 ± 4.5	10.3 ± 0.9
Dex-CRH test					
Suppressor		8	7	3	2
Intermediate suppressor		5	2	5	5
Non-suppressor		3	1	2	0
Medication					
No medication	28	3	4	1	0
Tricyclics	0	8	8	1	3
Tetracyclics	0	11	15	4	10
SSRI	0	5	24	4	6
SNRI	0	5	13	5	3
Sulpiride	0	4	8	1	1
Mood stabilizers	0	0	0	10	30

MDD, major depressive disorder; BPD, bipolar disorder; HDRS, Hamilton Depression Rating Scale; SSRI, selective rotonin reuptake inhibitor; SNRI, serotonin norepinephrine reuptake inhibitor.