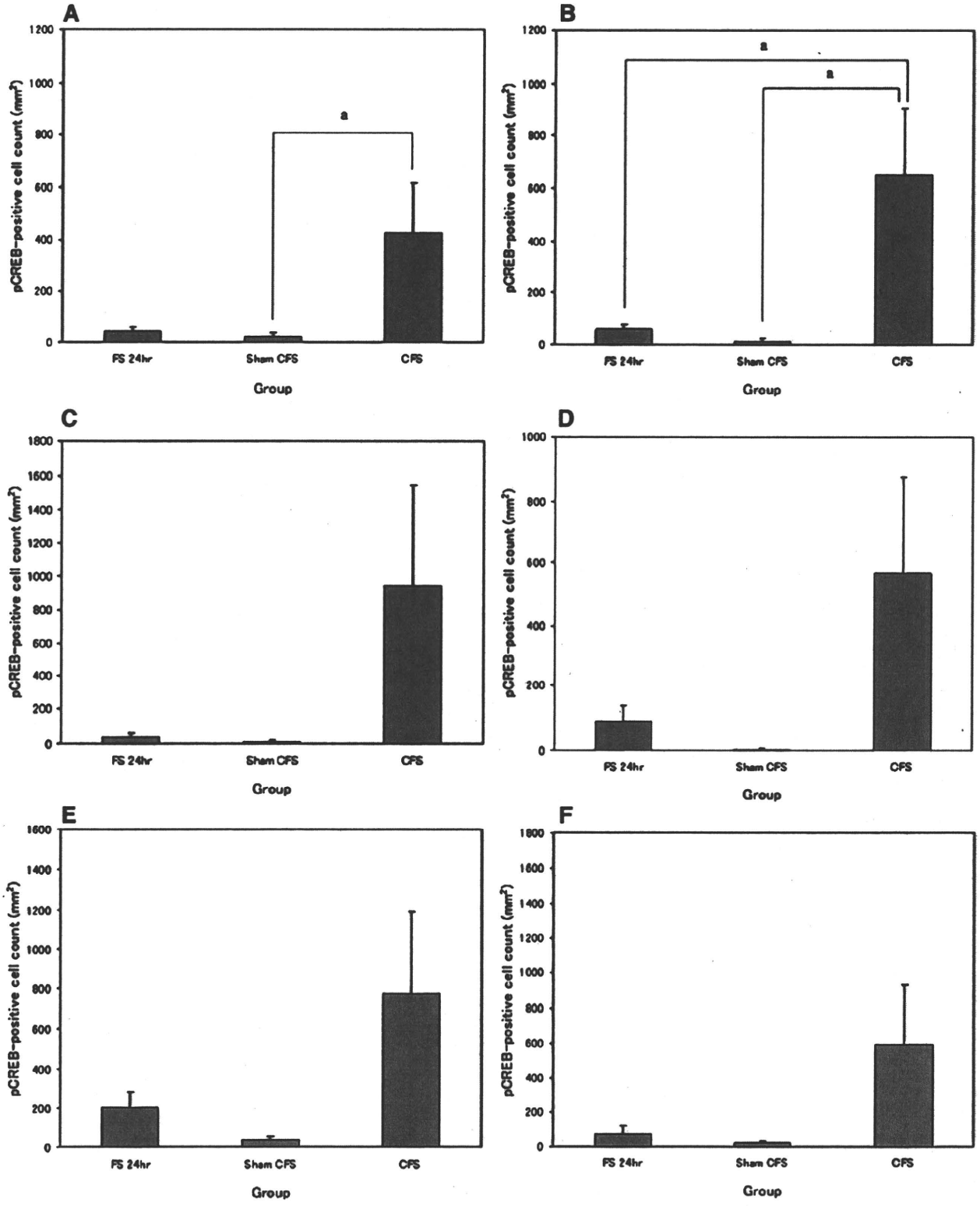

Fig. 3. Effect of sham footshock, footshock, sham conditioned fear stress, and conditioned fear stress on pCREB immunoreactivity in the amygdala of rats. Results are means with S.E.M. and are expressed as pCREB positive cell nuclei per mm^2. (A) lateral nucleus of the amygdala; (B) basal nucleus of the amygdala; (C) accessory basal nucleus of the amygdala; (D) central nucleus of the amygdala; (E) medial nucleus of the amygdala; (F) cortical nucleus of the amygdala. $^a p < 0.05$ The number of rats per group was as follows: FS 24 h group, $n=5$; sham CFS 2 h group, $n=6$; CFS 2 h group, $n=5$. FS, footshock; CFS, conditioned fear stress.

value=50% higher than the background gray value), (2) nuclei diameter of 4–12 μm (to exclude cell debris and artifacts). The background gray value was determined in a part of each unit area containing no nuclei. The measurement of the background gray value and the pCREB positive cell counting were repeated 3 times, and values were averaged. Semiquantitative cell counting was performed by an investigator who was blinded to the treatment.

2.5. Experimental procedures

2.5.1. Experiment 1: CREB phosphorylation in the amygdala under various conditions

Amygdala CREB phosphorylation was assessed under various experimental conditions. The rats were assigned to one of the following 5 groups. (1) Sham footshock 2 h group: The rats were individually placed in the shock chamber without footshocks and 5 min later, they were returned to their homecages (sham footshock). One hour and 45 min later, they were anesthetized in their homecages and then perfused 15 min later: (2) Footshock 2 h group: After the 5-min footshock, the rats were returned to their homecages. One hour and 45 min later, they were anesthetized and perfused similarly: (3) Footshock 24 h group: After the 5-min footshock, the rats were returned to their homecages. Twenty-four hours later, they were anesthetized and perfused similarly: (4) Sham CFS 2 h group: The rats were individually placed in the shock chamber without footshocks and 5 min later, they were returned to their homecages. Twenty-four hours later, they were individually placed in the shock chamber again without footshocks and 5 min later, they were returned to their homecages (sham CFS). One hour and 45 min later, they were anesthetized and perfused similarly: (5) CFS 2 h group: After the 5-min footshock, the rats were returned to their homecages. Twenty-four hours later, they were individually placed in the shock chamber again without footshocks and 5 min later, they were returned to their homecages. One hour and 45 min later, they were anesthetized and perfused similarly. The number of rats per group was as follows: sham footshock 2 h, $n=6$; footshock 2 h, $n=2$; footshock 24 h, $n=5$; sham CFS 2 h, $n=6$; CFS 2 h, $n=5$. Because the number of rats in the footshock 2 h group was relatively small, statistical analysis was performed to compare only the footshock 24 h, sham CFS 2 h and CFS 2 h groups.

2.5.2. Experiment 2: Effect of extinction on CFS-induced freezing behavior and CREB phosphorylation in the amygdala

Changes of CFS-induced freezing behavior and CREB phosphorylation in amygdala were assessed relative to the extinction process of CFS. Rats were assigned to one of the following 6 groups. (1) Sham CFS expression×1 group: One hour and 45 min after above-mentioned sham CFS, the rats were anesthetized and perfused similarly: (2) Sham CFS expression×2 group: Twenty-four hours after sham CFS, the rats were subjected to the second sham CFS. One hour and 45 min later, they were anesthetized and perfused similarly: (3) Sham CFS expression×3 group: Twenty-four hours after the second sham CFS, the rats were subjected to the third sham CFS. One hour and 45 min later, they were anesthetized and perfused similarly: (4) CFS expression×1 group: One hour and 45 min after CFS, the rats were anesthetized and perfused similarly: (5) CFS expression×2 group: Twenty-four hours after CFS, the rats were subjected to the second CFS. One hour and 45 min later, they were anesthetized and perfused similarly: (6) CFS expression×3 group: Twenty-four hours after the second CFS, the rats were subjected to the third CFS. One hour and 45 min later, they were anesthetized and perfused similarly. During the 5-min sham CFS or CFS operation, the behavior of the rats was recorded on videotape. In the behavior experiment, the number of rats per group was 6, and in the histological experiment, the number of rats per group was as follows: sham CFS expression×1–3 groups, $n=6$; CFS expression×1 group, $n=5$; CFS expression×2, 3 groups, $n=4$.

2.6. Data analysis

Multiple group comparisons were performed using one-way and two-way analysis of variance (ANOVA) and the Bonferroni–Dunn's post hoc test. Simple linear regression analysis was used to evaluate the correlations between percent freezing rate and pCREB positive cell count and between times of expression and pCREB positive cell count using the data from sham CFS expression×1–3 and CFS expression×1–3 groups.

3. Results

3.1. Experiment 1: CREB phosphorylation in the amygdala under various conditions

One-way ANOVA indicated a significant effect of CFS footshock compared to the footshock 24 h group and sham CFS group in the lateral nucleus ($F(2, 9)=4.9$, $p=0.026$) and basal nucleus ($F(2, 9)=6.6$, $p=0.011$), but not in the accessory basal nucleus, ($F(2, 9)=6.6$, $p=0.12$), central nucleus ($F(2, 9)=6.6$, $p=0.074$), medial nucleus ($F(2, 9)=6.6$, $p=0.093$) and cortical nucleus ($F(2, 9)=6.6$, $p=0.092$) of the amygdala (Fig. 2, Fig. 3A–3F). In the lateral nucleus, post hoc comparison showed that the number of pCREB positive cells was greater in the CFS 2 h group than in the sham footshock 2 h group ($p=0.014$) (Fig. 2, Fig. 3A). In the basal nucleus, post hoc comparison showed that the number of pCREB positive cells was greater in the CFS 2 h group than in the sham footshock 2 h group ($p=0.011$) and the footshock 24 h group ($p=0.0054$) (Fig. 2, Fig. 3B).

3.2. Experiment 2: Effect of extinction on CFS-induced freezing behavior

Two-way ANOVA (CFS×expression) indicated a significant main effect of CFS ($F(2, 66)=47.1$, $p<0.0001$) and expression ($F(2, 66)=19.0$, $p<0.0001$) in freezing behavior. Two-way ANOVA also indicated a significant interaction between CFS ($F(2, 66)=47.1$, $p<0.0001$) and expression ($F(2, 30)=21.1$, $p<0.0001$). One-way ANOVA across 6 groups indicated a significant effect of treatment ($F(5, 66)=36.3$, $p<0.0001$). Post hoc comparison indicated a significant difference between CFS expression×1 group and CFS expression×2 group ($p<0.0001$) and

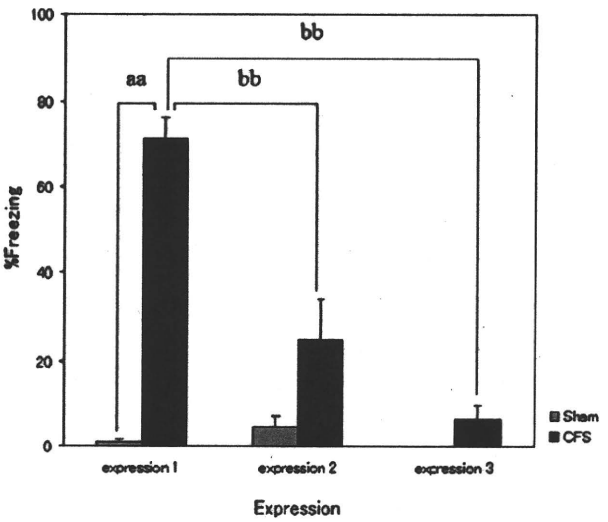

Fig. 4. Effect of repeated exposure to shock box on CFS-induced freezing behavior in rats. Results are the mean percentage with S.E.M. of freezing scored for a 5-min observation period. $^{aa}p<0.01$, $^{bb}p<0.01$, $n=6$. Expression 1: groups subjected to sham CFS or CFS one time. Expression 2: groups subjected to sham CFS or CFS two times. Expression 3: groups subjected to sham CFS or CFS three times. Sham, sham conditioned fear stress; CFS, conditioned fear stress; N.S., not significant.

between CFS expression×1 group and CFS expression×3 group ($p<0.0001$). Post hoc comparison also indicated significant difference between sham CFS expression×1 group and CFS expression×1 group ($p<0.0001$), but the difference between sham CFS expression×2 group and CFS expression×2 group and the difference between sham CFS expression×3 group and CFS expression×3 group were not statistically significant (Fig. 4).

3.3. Experiment 2: Effect of extinction on CFS-induced CREB phosphorylation in the amygdala

In the basal nucleus of the amygdala, two-way ANOVA (CFS× expression) indicated a significant main effect of CFS ($F(2, 25)=10.2$, $p=0.0037$) and a significant interaction between CFS and expression ($F(2, 25)=21.1$, $p<0.0001$), but the main effect of expression was not statistically significant ($F(2, 25)=10.2$, $p=0.10$). One-way ANOVA across 6 groups indicated a significant effect of treatment ($F(5, 25)=4.7$, $p=0.0037$) in the basal nucleus. Post hoc comparison indicated a significant difference between the sham CFS expression×1 group and CFS expression×1 group ($p=0.0003$), but the difference between the sham CFS expression×2 group and CFS expression×2 group and the difference between the sham CFS expression×3 group and CFS expression×3 group were not statistically significant (Fig. 5B). In the lateral and accessory basal nucleus of the amygdala, the main effects of CFS ($F(2, 25)=1.4$, $p=0.25$; $F(2, 25)=3.8$, $p=0.064$, respectively), the main effects of expression ($F(2, 25)=1.09$, $p=0.35$; $F(2, 25)=1.6$, $p=0.21$, respectively) and the interactions between CFS and expression ($F(2, 25)=2.8$, $p=0.080$; $F(2, 25)=1.8$, $p=0.19$, respectively) were not significant (Fig. 5A, C). In the central, medial and cortical nucleus of amygdala, two-way ANOVA (CFS×session) indicated that the main effects of CFS ($F(2, 25)=5.7$, $p=0.025$; $F(2, 25)=7.1$, $p=0.013$; $F(2, 25)=7.1$, $p=0.014$, respectively) were significant, but the main effects of expression ($F(2, 25)=1.8$, $p=0.19$; $F(2, 25)=1.6$, $p=0.22$; $F(2, 25)=1.4$, $p=0.28$, respectively) and the interactions between CFS and expression ($F(2, 25)=2.4$, $p=0.12$; $F(2, 25)=1.7$, $p=0.20$; $F(2, 25)=1.4$, $p=0.28$, respectively) were not significant. Post hoc comparisons of

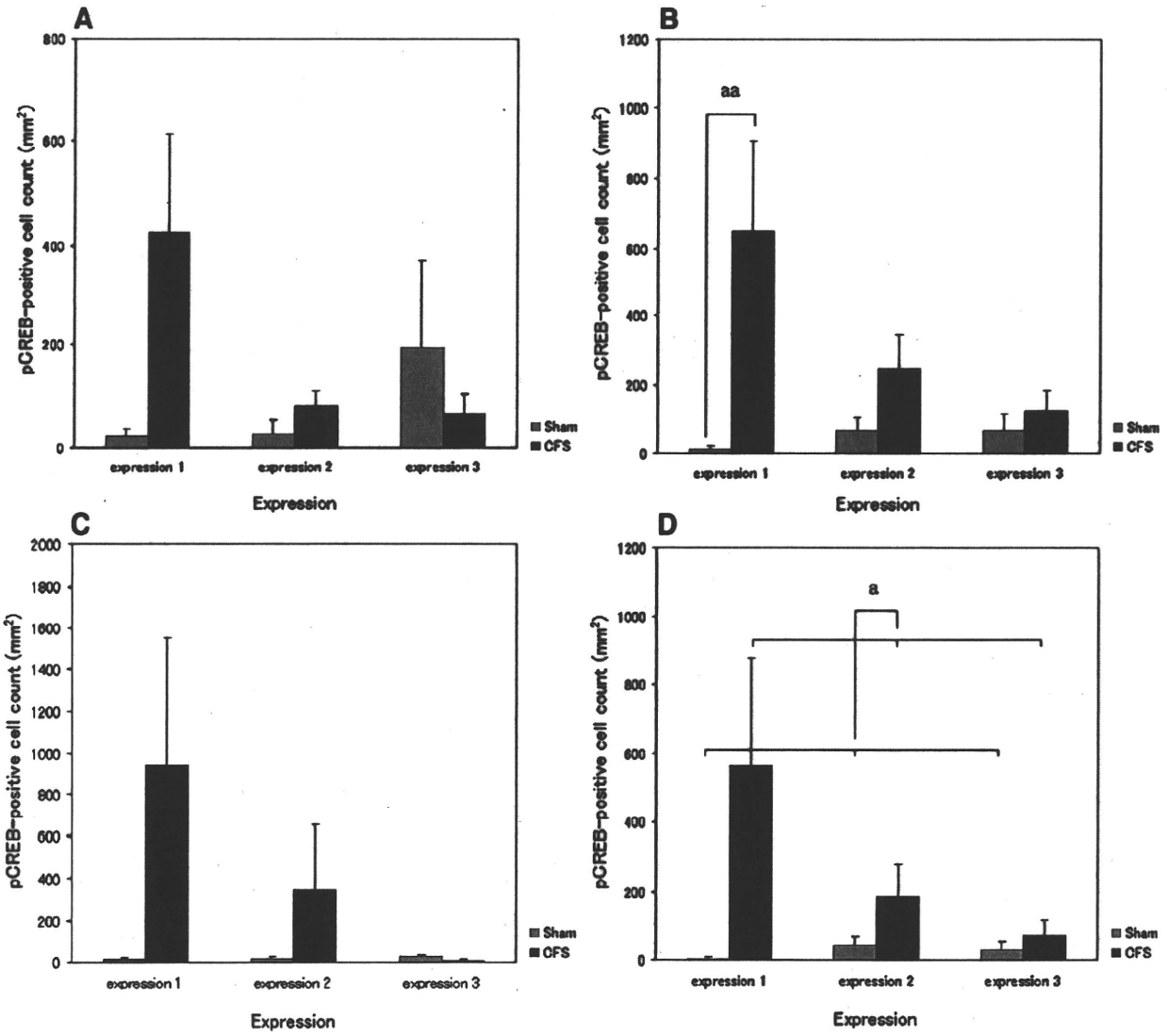

Fig. 5. Effect of repeated exposure to shock box on pCREB immunoreactivity in the amygdala of rats. Results are means with S.E.M. and are expressed as pCREB positive cell nuclei per mm². (A) lateral nucleus of the amygdala; (B) basal nucleus of the amygdala; (C) accessory basal nucleus of the amygdala; (D) central nucleus of the amygdala; (E) medial nucleus of the amygdala; (F) cortical nucleus of the amygdala. $^{a}p<0.05$, $^{aa}p<0.01$ The number of rats per group was as follows: sham CFS expression×1–3 groups, $n=6$; CFS expression×1 group, $n=5$; CFS expression×2, 3 groups, $n=4$. Expression 1: groups subjected to sham CFS or CFS one time. Expression 2: groups subjected to sham CFS or CFS two times. Expression 3: groups subjected to sham CFS or CFS three times. CFS, conditioned fear stress; Sham, sham conditioned fear stress; N.S., not significant.

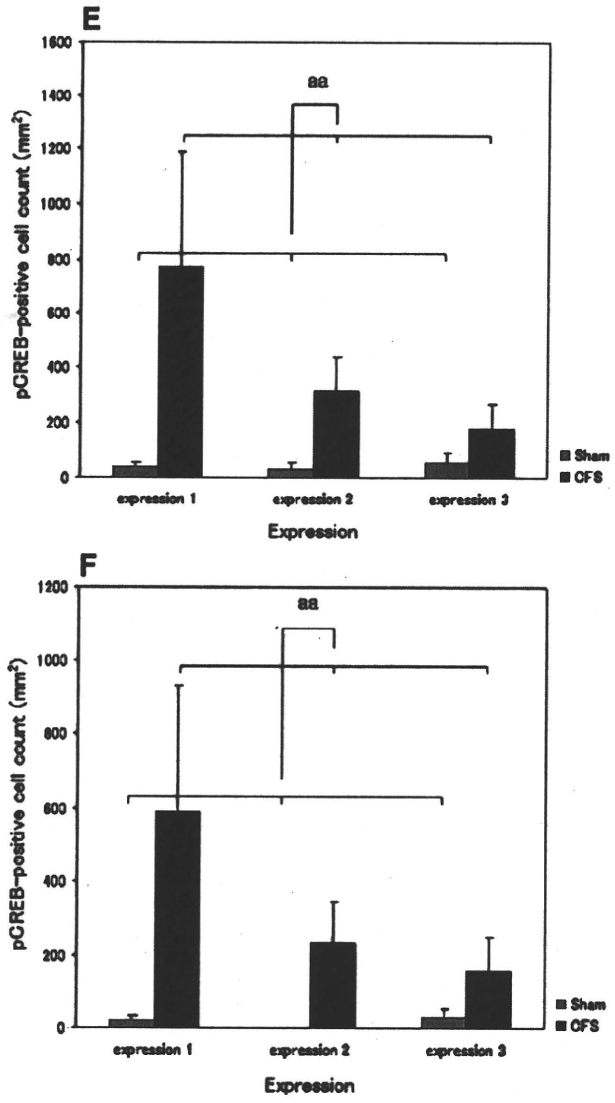

Fig. 5 *(continued).*

two-way ANOVA indicated a significant difference between the sham CFS groups (expression×1 group+expression×2 group+expression×3 group) and CFS groups (expression×1 group+expression×2 group+expression×3 group) in these 3 nuclei (p=0015; p=0.083; p=0.0088, respectively) (Fig. 5D, E, F).

Regression analysis indicated a significant correlation between percent freezing rate and pCREB positive cell count in the lateral (r=0.43, p=0.015), basal (r=0.63, p=0.001), accessory basal (r=0.51, p=0.031), central(r=0.63, p=0.002), medial (r=0.55, p=0.0015) and the cortical (r=0.48, p=0.0064) nucleus of amygdala, but there was no significant correlation between times of expression and pCREB positive cell count in any of the subnuclei of amygdala.

4. Discussion

Two different studies have investigated the time course of stress-induced CREB phosphorylation in brain. Stanciu et al. (2001) indicated that contextual-dependent conditioned fear resulted in two peaks of CREB phosphorylation in the parietal cortex, CA1, dentate, basolateral amygdala, and the central amygdala of mice. The first peak occurred 7 min after stress and disappeared at 90 min after stress. The second peak appeared at 180 min after stress and persisted at 360 min after stress. However, they did not investigate CREB phosphorylation at 120 min after stress. Bilang-Bleuel et al. (2002) also showed that forced swimming resulted in two peaks of CREB phosphorylation in

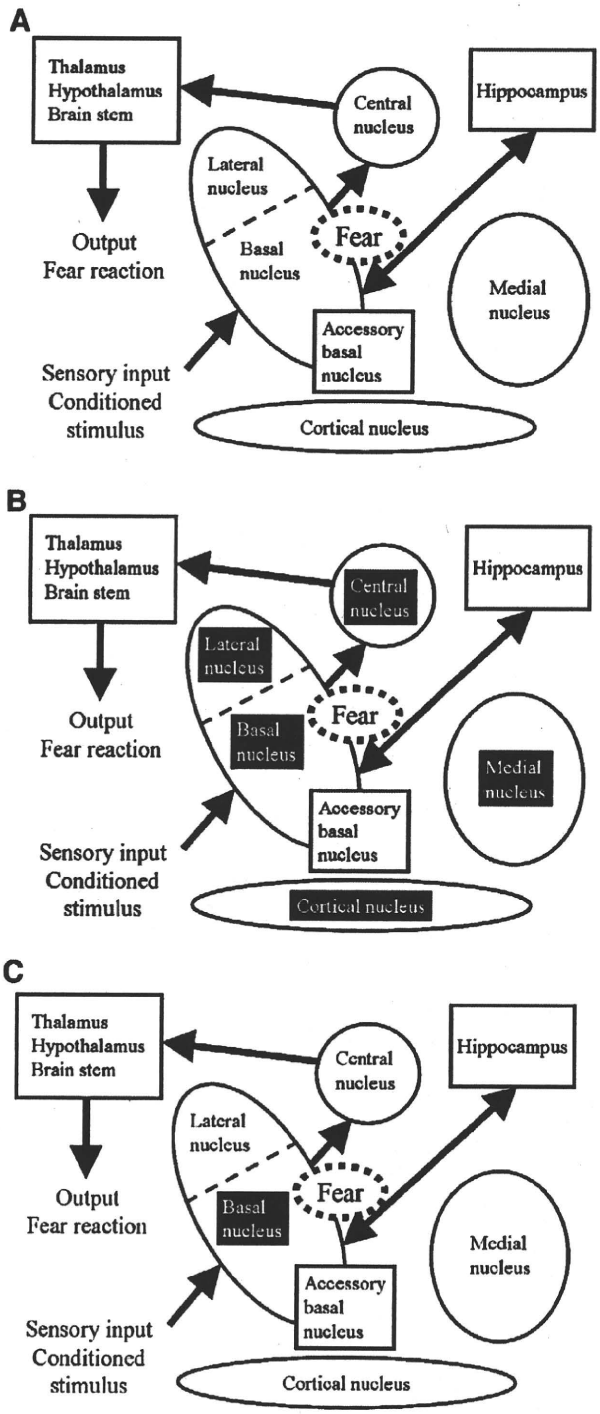

Fig. 6. Scheme representing the changes of CREB phosphorylation in amygdala subnuclei by the expression and extinction of contextual conditioned fear. (A) Neural pathway concerning the expression of contextual conditioned fear. (B) The region where CREB phosphorylation was increased by the expression of conditioned fear (indicated by white letters and black background). (C) The region where CREB phosphorylation was increased in association with the extinction of conditioned fear (indicated by white letters and black background).

the dentate and the neocortex in rats. The first peak occurred at 15 min after stress and disappeared at 60 min after stress. The second peak appeared at 120 min after stress and persisted at 48 h after stress. Based on these studies, the expectation was that stress-induced CREB phosphorylation would result in two peaks, the first within 1 h and the second at more than 2 h. However, the time point of peaks is variable according to the animal species, type of stress, intensity and duration of stress, and the brain location. Data from the present study reflect the second peak of CREB phosphorylation. Further study would be of benefit to investigate the difference in the biological mechanisms between these two peaks.

In Experiment 1, pCREB levels of the lateral and basal nucleus of the amygdala were also increased in the CFS 2 h group. Because amygdala pCREB levels did not increase in the footshock 24 h group, increases in pCREB levels in the amygdala in the CFS 2 h group were not considered to be the result of the footshock 24 h before. Moreover, amygdala pCREB levels in the sham CFS 2 h group did not increase, suggesting that placing rats in the shock chamber without administrating shocks itself did not play a role in this phenomenon. Thus, the increase in pCREB levels in the lateral and basal nucleus in CFS 2 h group likely reflect the neural process of the expression of context-dependent CFS. Indeed, Hall et al. (2001) reported expression of tone-dependent CFS-induced CREB phosphorylation in the lateral and basal nucleus of the amygdala in rats, which is consistent with data from the present study.

In Experiment 2, CFS-induced freezing behavior significantly decreased as the expression of CFS was repeated; this suggests that extinction of CFS occurred. CFS-induced CREB phosphorylation in the basal nucleus also decreased as the expression was repeated. While there was a significant difference between the sham CFS group and the CFS group in expression 1, there was no difference between these groups in expressions 2 and 3, which suggests that CREB phosphorylation in the basal nucleus decreased in parallel with the extinction of CFS-induced freezing behavior. In the central, medial and the cortical nucleus of amygdala, CREB phosphorylation was greater in CFS groups than in the sham CFS groups; however, no time-course effect was observed, as seen in the basal nucleus.

Experiments 1 and 2 demonstrated that CREB phosphorylation was increased by CFS in the lateral, basal, central, medial and the cortical nucleus of amygdala, but CREB phosphorylation was decreased in association with extinction of CFS only in the basal nucleus (Fig. 6B, C). Indeed, Lin et al. (2003) reported that the tone-dependent CFS-induced CREB phosphorylation in the amygdala including the lateral and basal nuclei, decreased as the extinction session was repeated in rats, which is at least partially consistent with our result.

In a previous study, we reported that CFS-induced c-Fos expression in the basal nucleus, but not in the central nucleus of amygdala (Izumi et al., 2006). Although other studies have reported that CFS-induced c-Fos expression in many cortical and subcortical region, the basal nucleus of amygdala was the common location that was associated with CFS (Izumi et al., 2006). Further, pCREB is upstream of c-Fos in the intracellular signal pathway, and pCREB enhances c-Fos production as well as production of other transcription factors. Therefore, functional mapping of pCREB is likely distinct from that of c-Fos. Regardless, the present and previous studies have consistently demonstrated a significant change in the basal nucleus with contextual CFS.

Anatomical and lesion studies have characterized the nuclei of the amygdala that participate in the expression of CFS. Anatomical study indicated that the lateral, basal and central nuclei of the amygdala receive moderate to heavy projections from the sensory-related cortices and the thalamus and that these nuclei are expected to mediate sensory input to the amygdala (Pitkanen, 2000). Further, the central and medial nuclei provide moderate to heavy projections to the subcortical nuclei and the brain stem, and these nuclei are expected to mediate motor, autonomic and hormonal output from the amygdala (Pitkanen, 2000).

Studies regarding tone-dependent CFS indicated that the tone stimulus is received at the auditory organ and is converted to neural information, which subsequently passes through the thalamus and auditory cortex. This neural information is received by the lateral nucleus, which subsequently induces a fear reaction via the central nucleus (LeDoux, 2000). In context-dependent CFS, the contextual stimulus (e.g., exposure to the shock chamber), is received at the sensory organ and is converted to the neural information, which subsequently passes through the thalamus and sensory-related cortices. After being checked against memory in the hippocampus, this information is received by the basal nucleus, which induces a fear reaction via the central nucleus (LeDoux, 2000) (Fig. 6A). In the present study, context-dependent CFS-induced CREB phosphorylation in the basal nucleus and in the lateral nucleus, suggesting that the sensory stimuli except context (e.g., tone or smell) is partly related to fear-conditioning.

Two different neural plasticity-related phenomena occur in brain after the fear memory is retrieved; reconsolidation and extinction. The effects of these two processes on rat behavior are contrary, the former maintains fear related behavior, while the latter reduces it when the fear memory retrieval is repeated (Nader et al., 2000; Debiec et al., 2002). In the present study, the percent freezing rate approximately 70% in the first retrieval, 20% in the second, and 10% in the third. The fact that freezing behavior reduced rapidly in parallel with levels of CREB phosphorylation suggests that the extinction process was dominant over the reconsolidation process in our experimental design. Thus, neural activity of the basal amygdala reflects the extinction process in the case of context-dependent aversive classical conditioning.

We previously reported that CFS-induced c-Fos expression in the basal nucleus (Izumi et al., 2006). The present study demonstrated that CREB phosphorylation in the basal nucleus of the amygdala decreased with the extinction of context-dependent conditioned fear-induced freezing behavior. These data suggest that the basal nucleus of the amygdala plays an essential role in the expression of context-dependent conditioned fear, and this is the first study to demonstrate that CREB phosphorylation in the basal nucleus of the amygdala changes in parallel with the extinction of context-dependent conditioned fear.

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A possible association between missense polymorphism of the breakpoint cluster region gene and lithium prophylaxis in bipolar disorder

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Abstract

Lithium is one of the most commonly used drugs for the treatment of bipolar disorder. To prescribe lithium appropriately to patients, predictors of response to this drug were explored, and several genetic markers are considered to be good candidates. We previously reported a significant association between genetic variations in the breakpoint cluster region (BCR) gene and bipolar disorder. In this study, we examined a possible relationship between response to maintenance treatment of lithium and Asn796Ser single-nucleotide polymorphism in the BCR gene. Genotyping was performed in 161 bipolar patients who had been taking lithium for at least 1 year, and they were classified into responders for lithium monotherapy and non-responders. We found that the allele frequency of Ser796 was significantly higher in non-responders than in responders. Further investigation is warranted to confirm our findings.

Keywords: BCR (breakpoint cluster region); Bipolar disorder; Lithium; SNP (single-nucleotide polymorphism)

1. Introduction

Bipolar disorder (BPD) is one of the most distinct syndromes in psychiatry, which is characterized by recurrent episodes of mania and depression. Three representative mood stabilizers, lithium, valproate and carbamazepine, are used worldwide for its treatment, and American Psychiatric Association guideline listed lithium as a first line agent (American Psychiatric Association, 2002). However, these treatments are associated with variable rates of efficacy and often with intolerable side effects. Therefore, many researchers explored psychopathological and biological markers for good response to lithium treatment (Gelenberg and Pies, 2003; Ikeda and Kato, 2003). To date, several studies investigated possible molecular predictors of lithium efficacy. The functional polymorphism in the upstream regulatory region of the serotonin transporter gene (5-HTTLPR) has been associated with lithium efficacy in two independent studies (Serretti et al., 2001;

Abbreviations: ANOVA, analysis of variance; BCR, breakpoint cluster region; BDNF, brain-derived neurotrophic factor; BPD, bipolar disorder; BP I, bipolar I disorder; BP II, bipolar II disorder; PH domain, pleckstrin homology domain; SNP, single-nucleotide polymorphism.

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Del Zompo et al., 1999), although the polymorphism associated with better lithium response was opposite. Other numerous genetic variants including catechol-*O*-methyltransferase were not associated with lithium response (Serretti et al., 2002). The association between prophylactic lithium response and the polymorphism of the brain-derived neurotrophic factor (BDNF) gene was reported (Rybakowski et al., 2005); however, this association was not replicated in subsequent studies (Masui et al., 2006; Michelon et al., 2006).

We previously reported a significant association between genetic variants in the breakpoint cluster region gene (*BCR*), which is located on chromosome 22q11, and BPD (Hashimoto et al., 2005). The *BCR* is highly expressed in hippocampal pyramidal cell layer and dentate gyrus (Fioretos et al., 1995), and encodes a Rho GTPase-activating protein (GAP), which inactivate the Rho GTPase playing an important role in neuronal development (Diekmann et al., 1991; Negishi and Katoh, 2002). The A2387G single-nucleotide polymorphism (SNP) in the *BCR* gene [National Center for Biotechnology Information (NCBI) SNP ID: rs140504] is the non-conservative SNP giving rise to an amino acid change of asparagine to serine at codon 796 (Asn796Ser; NCBI Protein ID: NP_004318). Ser796 allele showed a significant association with BPD and stronger evidence for an association with bipolar II disorder (BPII) than bipolar I disorder (BPI) (Hashimoto et al., 2005). It has been reported that patients with BPII have greater number of abnormal mood episodes and comorbidity of other psychiatric illnesses than patients with BPI (Ayuso-Gutierrez and Ramos-Brieva, 1982; Berk and Dodd, 2005). These clinical features of BPII have been also considered as markers for poor response to lithium treatment (Ikeda and Kato, 2003). Therefore, Ser796 allele of the *BCR* gene may contribute to poorer response to lithium therapy in BPD.

In this study, we examined the possible association between prophylactic effect of lithium and Asn796Ser SNP of the *BCR* gene in Japanese patients with BPD.

2. Methods

2.1. Subjects

Subjects were 161 patients with BPD (83 patients were BPI, and 78 patients were BPII). Consensus diagnosis was made for each patient by at least two psychiatrists according to the Diagnostic and Statistical Manual of Mental Disorders 4th edition (DSM-IV) criteria. The presence of concomitant diagnoses of mental retardation, drug dependence, or other Axis I disorder, together with somatic or neurological illnesses that impaired psychiatric evaluation, represented exclusion criteria. They were composed of 76 males and 85 females with mean age of 48.2± 12.8 years (mean±S.D.). All the subjects were biologically unrelated Japanese. Patients had been treated with lithium carbonate and its serum concentration was maintained between 0.4 and 1.2 mEq/L at least for one year, in a completely naturalistic setting.

Response to lithium treatment was retrospectively determined for each patient from all available information including clinical interview and medical records, by at least two psychiatrists, and the patients were classified into lithium responders and non-responders. The phenotype definition of lithium prophylaxis is a very difficult issue. Lithium responders were defined as those patients without any affective episodes during the maintenance period of lithium mono-therapy. During the maintenance period, the addition of antidepressants, antipsychotics, or anticonvulsants was regarded as a relapse, and excluded from the responder group. However, coadministration of hypnotics for sleep disturbance was allowed, and was not regarded as a relapse when subsequent affective episode did not appear.

Our definition of response to lithium treatment is full response without any affective episode during lithium treatment. This definition is similar to "excellent lithium responders" used as clinical endophenotypic marker of BPD in some molecular-genetic research (Rybakowski et al., 2005; Mamdani et al., 2007). On the other hand, recurrence index [number of episodes/duration of illness (years)] before and during lithium treatment is a better method to measure the response to lithium including partial response (Gasperini et al., 1993; Serretti et al., 2002). However, more clinical information is necessary to calculate the recurrence index. We investigated the association between the change of recurrence index and clinical variables in parts of total subjects (24 patients) whose recurrence pattern were clearly established during more than 1 year [mean 5.8±5.0 (range 1.3–21.0) years] before lithium treatment. They were composed of 9 BPI and 15 BPII patients, whose age of onset was 35.4± 9.5 years old, duration from onset of illness to lithium treatment was 9.5±7.0 (range 1.3–22.0) years, number of episodes which could be clearly identified before lithium treatment was 16.3± 30.3 (range 3.0–150.0), duration of lithium treatment was 6.0± 4.3 (range 1.0–14.3) years, number of episodes during lithium treatment was 6.8±6.0 (range 0.0–26.0) and recurrence index before and during lithium were 2.7±2.8 (range 0.6–14.2) and 1.8±1.5 (range 0.0–5.3), respectively.

After complete description of the study, written informed consent was obtained from every subject. The study protocol was approved by institutional ethics committees.

Table 1
Clinical characteristics of subjects, sorted by response to lithium treatment

	Response to lithium treatment		
	Responders (N=43)	Non-responders (N=118)	
Subtype			χ^2test
BPI	29 (34.9%)	54 (65.1%)	$p<0.05$
BPII	14 (18.0%)	64 (82.0%)	
Gender			
Male	25 (32.9%)	51 (67.1%)	NS
Female	18 (21.2%)	67 (78.8%)	
			t-test
Age at last observation	54.4±11.8	46.1±12.4	$p<0.01$
Age of onset	41.5±13.6	32.9±10.7	$p<0.01$
Duration of illness	12.9±9.0	13.2±9.9	NS

Continuous values were represented as the mean±SD.
BPI=bipolar I disorder, BPII=bipolar II disorder,
NS=not significant.

Table 2
Allele frequencies and genotype of the Asn796Ser polymorphism of the BCR gene and response to lithium treatment

Response to lithium treatment	Allele frequency		χ^2test	Genotype		χ^2test
	Asn	Ser	p value (OR)	Asn/Asn Asn/Ser	Ser/Ser	p value
Responders (n=43)	49 (57.0%)	37 (43.0%)		35 (81.4%)	8 (18.6%)	
Non-responders (n=118)	101 (42.8%)	135 (57.2%)		77 (65.3%)	41 (34.7%)	
			0.024 (1.77)			0.049
Total patients (n=161)	150 (46.6%)	172 (53.4%)		112 (69.6%)	49 (30.4%)	

OR: Odds ratio.

2.2. Genotyping

Venous blood was drawn from the subjects and genomic DNA was extracted from whole blood according to the standard procedures. The genotype of the Asn796Ser SNP (rs140504) of the *BCR* gene was determined by TaqMan 5′-exonuclease allelic discrimination assay, described previously (Hashimoto et al., 2005). Briefly, probes and primers for detection of the polymorphism were: forward primer 5′-AGCTGGACGCTTTGAAGATCA-3′, reverse primer 5′-TGGTGTGCACCTTCTCTCTCT-3′, probe 1 5′-VIC-CCAGATCAAGAATGACAT-MGB-3′, and probe 2 5′-FAM-CCAGATCAAGAGTGACAT-MGB-3′. PCR cycling conditions were: at 95 °C for 10 min, 50 cycles of 92 °C for 15 s and 60 °C for 1 min.

2.3. Statistical analysis

Difference in clinical characteristics between responders and non-responders to lithium treatment was analyzed using the χ^2 tests for categorical variables and t tests for continuous variables. The presence of Hardy–Weinberg equilibrium was examined by using the χ^2 test for goodness of fit. Subsequently, multiple logistic regression analysis was performed to correct background difference between responders and non-responders for lithium treatment. Possible predictors (genotype of the *BCR* gene, subtype of bipolar disorder, age of onset, age at last observation, and gender) were included in the original model. Backward stepwise regression was performed, and p-value greater than 0.10 was used for variable removal. Pearson coefficient of correlation test was used for comparison between recurrence index and clinical variables. The effect of the Asn796Ser SNP on recurrence index was assessed by analysis of variance (ANOVA). All p-values reported are two-tailed. Statistical significance was defined at $p<0.05$.

3. Results

Among 161 patients with BPD, 43 patients were determined as responders and 118 patients as non-responders for the maintenance treatment of lithium. The clinical characteristics sorted by response to lithium treatment and genotype distribution were presented in Table 1. There were significant differences between responders and non-responders in subtype of bipolar disorder (BPI and BPII), age at last observation, and age of onset.

The genotype distributions for the total patients, responders, and non-responders were in Hardy–Weinberg equilibrium (total patients: $\chi^2=0.94$, $df=1$, $p=0.33$; responders: $\chi^2<0.001$, $df=1$, $p=0.98$; non-responders: $\chi^2=0.81$, $df=1$, $p=0.37$). Allele frequencies and genotype distributions of the Asn769Ser polymorphism of the *BCR* gene among responders and non-responders for lithium treatment are presented in Table 2. The Ser796 allele was in excess in the non-responders rather than responders ($\chi^2=5.09$, $df=1$, $p=0.024$; OR 1.77, 95% CI 1.08–2.92). Then, we examined patients homozygous for the Ser796 allele and the Asn796 allele carriers, separately. Patients homozygous for the Ser796 allele were significantly more common in the non-responders than the Asn796 carriers ($\chi^2=3.88$, $df=1$, $p=0.049$; OR 2.33, 95% CI 0.99–5.49). After backward stepwise regression, the final logistic regression model included subtype of bipolar ($p<0.01$), age of onset ($p<0.01$), and genotype which is separated to the Asn796 carrier and homozygous for the Ser796 ($P=0.04$).

We next investigated the association between lithium response using recurrence index and clinical variables in 24 subjects with BPD. The change of recurrence index before to during lithium treatment was not associated with subtype ($t=0.79$, $df=22$, $p=0.44$), age of onset (correlation coefficient=−0.29, $p=0.17$), duration from onset of illness to lithium treatment (correlation coefficient=0.12, $p=0.57$), duration during treatment (correlation coefficient=0.11, $p=0.60$), or the Asn796Ser SNP ($df=2$, $F=0.03$, $p=0.97$).

We also examined the association between age of onset and recurrence index before lithium treatment, which reflects severity of illness. There was a negative trend between age of onset and recurrence index (correlation coefficient=−0.37, $p=0.074$). Although difference among genotype of Asn796Ser SNP was not statistically significant, the number of Ser796 allele was associated with higher recurrence index before lithium treatment (Asn/Asn=1.63±1.19, Asn/Ser=2.89±0.84, and Ser/Ser=3.23±1.19. $df=2$, $F=0.53$, $p=0.60$). Therefore, the Ser796 allele might also be associated with both early onset and severity of illness, which could result in poorer lithium response.

4. Discussion

We investigated a possible association between the *BCR* gene and the prophylactic effect of lithium treatment in patients with BPD for the first time. As expected, our results suggested that lithium treatment might be less effective in patients homozygous for the Ser796 allele of the *BCR* gene than in patients with the Asn796 allele. In addition, allele frequencies of the Ser796 associated with poorer lithium response were 43.0%

in responders and 57.2% in non-responders. As allele frequency of the Ser796 in healthy subjects in our previous study was 48.1% (Hashimoto et al., 2005), allele frequency of the Ser796 of responders is similar to the general population.

Comparing clinical characteristics of responders and non-responders, there were more BPII patients in non-responder group. Clinical characteristics predicting poorer response to lithium therapy and that of BPII seem to overlap each other, but better lithium response in BPI is not universally accepted. We excluded any Axis I comorbidity in this study. This would leave in more typical bipolar II patients who would be more likely to respond to lithium, however, other clinical factors such as Axis II comorbidity might influence our results. The presence of positive family history of lithium responsive BPD has been reported as indicative of favorable response (Grof et al., 2002). However, it was not assumed that our sample size was enough to investigate this issue because only 8.7% of BPD had positive family history of the same disease in 1st degree relatives (Smoller and Finn, 2003). Therefore, information about family history of lithium response was not collected in this study.

Age at onset was also different between responders and non-responders, and early age of onset was associated with poorer response to lithium treatment in our subjects. This observation is consistent with recent meta-analysis (Kleindienst et al., 2005). As the objective of this study is to examine the association between response to lithium treatment and a SNP in the *BCR* gene, the differences in demographic parameters of responders and non-responders might not be preferable. Therefore, we conducted a multiple logistic regression analysis, and homozygous for the Ser796 allele of the *BCR* gene was still significantly associated with poorer response to lithium treatment.

The evaluation of lithium prophylaxis is considerably difficult because of complex clinical course of BPD, and each researcher has used different methodologies. Although our finding was based on the simple definition, in which lithium responders didn't have any affective recurrences during lithium, one of the limitation of this study is lack of detailed clinical information, e.g. duration from onset of illness to lithium treatment and number of episodes which could be clearly identified before lithium treatment in total subjects. To evaluate lithium efficacy including partial response, calculating recurrence index before and during lithium treatment is used in several researches. This would be a correct measure of lithium prophylaxis, but evaluating mood recurrence accurately before the first contact to mental professionals is difficult. We tried to evaluate lithium response with recurrence index; however, we could examine it in only 24 subjects out of 161 subjects due to the difficulty of collecting this clinical information. We did not found any association between the recurrence index and clinical variables and the SNP in the *BCR* gene, except for the trend between the recurrence index and age of onset. As these results were from subgroup analysis with smaller number, further investigation is needed in a larger sample size.

In this study, the same variant associated with the illness was also associated with poorer outcome. This situation is similar to that of the Val allele of the *BDNF* Val66Met polymorphism (Rybakowski et al., 2005), and it is possible that the *BCR* Ser796 and the *BDNF* Val66 alleles are associated with severer illness presentation. The trend between the recurrence index and age of onset in our subgroup analysis might imply this possibility. In case of the *BDNF* Val66Met SNP, the functional differences arisen from each allele were reported (Eagan et al., 2003). While biological functional of the *BCR* Asn796Ser SNP is still unknown, this SNP may produce functional difference in the brain, like the *BDNF* Val66Met SNP. To speculate this issue, it is noteworthy that this SNP is in the pleckstrin homology (PH) domain of the *BCR*. As PH domain is known for its ability to bind phosphatidylinositol and this binding regulates the activity of PH domain containing protein (Lemmon et al., 2002), signal transduction from inositol cycle to the *BCR* products might be affected by this SNP. As the *BCR* is RhoGAP, this change may influence on the activity of its downstream target, RhoGTPase, which activates many kind of effectors associated with constructing neuronal network, and subsequently influence on neuronal development. Additionally, as inositol cycle is considered as one of therapeutic targets of lithium (Harwood, 2005), this SNP could alter the clinical efficacy of lithium. To understand the mechanism of our findings, it is worth investigating whether the Asn796Ser SNP alters the binding ability of PH domain to inositol.

5. Conclusion

This is the first report demonstrating that long-term lithium treatment may be less effective in BPD patients homozygous for Ser796 allele of the *BCR* gene than in patients with the Asn796 allele. The limitations of this study are retrospective design without placebo control group, small sample size, and lack of clinical information such as presence of rapid cycling and/or psychotic symptoms, and detailed lithium levels. Further investigations are needed to confirm our findings.

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Reduced expression of glyoxalase-1 mRNA in mood disorder patients

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ABSTRACT

Glyoxalase-1 (Glo1) is an antioxidant enzyme which detoxifies α-ketoaldehydes to prevent the accumulation of pro-oxidant compounds, such as methylglyoxal, in all cell types. Glo1 has been suggested to be involved in anxiety disorders, autism, and Alzheimer's disease. Mood disorders have a high rate of comorbidity with anxiety disorders although, to date, little is known of the involvement of Glo1 in the pathophysiology of these conditions. In the present study, we examined the expression levels of Glo1 mRNA in peripheral white blood cells of mood disorder patients to understand the role of Glo1 in mood disorders. Quantitative real-time polymerase chain reaction experiments revealed that reduced expression of Glo1 mRNA was observed in major depressive and bipolar disorder patients in a current depressive state, as compared with healthy control subjects. In contrast, the expression of Glo1 mRNA in major depressive and bipolar patients, in a remissive state, showed no significant alteration when compared with healthy control subjects. These results suggest that the aberrant expression of Glo1 might be involved in the pathophysiology of mood disorders.

A growing body of evidence has implicated a role of chronic or moderate oxidative stress in the pathogenesis of anxiety in humans [2]. Previous clinical investigations have reported an imbalance of antioxidant enzyme activities in patients with social phobia and obsessive-compulsive disorder [17]. Glyoxalase-1 (Glo1) is an antioxidant enzyme that, together with the cofactor glutathione, is involved in the detoxification of α-ketoaldehydes, thereby preventing the accumulation of pro-oxidant compounds such as methylglyoxal [27,28]. The association between altered Glo1 expression levels and anxiety disorders in mice supports the hypothesis that Glo1 is involved in the pathogenesis of these conditions [11,15].

The manifestation of anxiety in a number of psychiatric disorders such as generalized anxiety disorder, depressive disorder, panic disorder, phobia, obsessive-compulsive disorder and post-traumatic stress disorder [6] highlights the importance of gaining a better understanding of common biomarkers for these disorders. The significant association between anxiety and depression in behavioral studies [15] resembles the clinical situation of a high comorbidity between anxiety disorders and major depressive disorder [18]. Although Glo1 has been reported to be associated with anxiety [22], little is known about the involvement of Glo1 in the pathophysiology of mood disorders. To investigate the role of Glo1 in the pathophysiology of mood disorders, we examined the expression levels of Glo1 mRNA in the peripheral white blood cells of major depressive and bipolar disorder patients in a depressive, as well as a remissive, state.

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) [1]. 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 being 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 six on HDRS and did not show any symptoms of a 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 and 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. This protocol was approved by the Institutional Review Board of Yamaguchi University Hospital. Informed written consent was obtained for all subjects.

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Blood sample preparation, total RNA isolation and cDNA synthesis were performed as previously described [19]. In brief, blood was obtained by vein puncture between 10:00 a.m. and 11:00 a.m. and total RNA was isolated using the QIAamp RNA blood mini kit (Qiagen, Chatsworth, CA) according to the manufacturer's manual. One microgram of total RNA was used for cDNA synthesis using random hexamer primers and omniscript reverse transcriptase (Qiagen). The cDNA was stored at −80 °C until use. Quantitative real-time polymerase chain reaction (PCR) was performed in an Applied Biosystems 7300 fast real-time PCR system with SYBR green PCR master mix (Applied Biosystems, Foster City, CA), as previously reported [19]. PCR conditions were 15 min at 95 °C, 45 cycles of 15 s at 95 °C and 30 s at 60 °C. Amplification of the single PCR product was confirmed by monitoring the dissociation curve and electrophoresis on 1% agarose gels stained with ethidium bromide. The expression level of GAPDH mRNA was used for normalization and the expression value was normalized by dividing the mean of the value for control subjects. All measurements were performed in duplicate and two-independent experiments were conducted. The following PCR primers were used: Glo1 forward, 5′-CGAGGATTCGGTCATATTGG-3′; Glo1 reverse, 5′-CCAGGCCTTTC-ATTTTACCA-3′; GAPDH forward, 5′-CAGCCTCAAGATCATCAGCA-3′; GAPDH reverse, 5′-TGTGGTCATGAGTCCTTCCA-3′. A subgroup of subjects in a current depressive state underwent the dexamethasone (Dex)/corticotropin-releasing hormone (CRH) test as previously reported [19]. All data are expressed as means ± standard error of the mean (SEM). Statistical analysis was performed with commercial software (SPSS version 16.0; Chicago, IL). Multivariable analysis was conducted using Glo1 mRNA level as a dependent variable and with age, gender, state (depressive and remissive states) and type of drugs used (antidepressants and mood stabilizers) as independent variables. Gender distribution was analyzed by the χ^2-test. The data of Glo1 mRNA levels were subjected to a factorial analysis of variance (ANOVA) followed by *post hoc* comparison (Dunnett test). The Spearman rank correlation was calculated to assess the correlation between data. Two group comparisons, such as suppressors and non-suppressors of the Dex/CRH test on Glo1 mRNA expression, were performed using the Student's *t*-test. In all cases, *p*-values were two-tailed, and comparisons were considered to be statistically significant for $p < 0.05$.

Table 1 shows the demographic and clinical characteristics of the subjects used in this study. The mean ages were not significantly different among major depressive disorder patients, bipolar disorder patients and healthy control subjects ($F_{(2, 104)} = 1.84$, $p = 0.16$). Regarding the gender distribution, bipolar disorder patients showed a significantly larger ratio of females to males

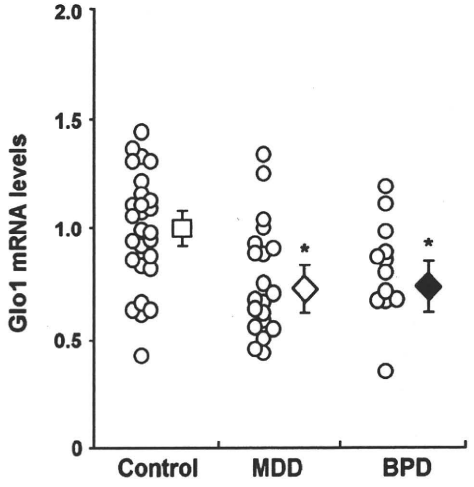

Fig. 1. Expression levels of Glo1 mRNA for mood disorder patients in a current depressive state. Quantitative real-time PCR experiments revealed reduced expression levels of Glo1 mRNA (open circles) for major depressive disorder patients in a current depressive state (MDD, $n = 20$) and bipolar disorder patients (BPD, $n = 13$), as compared to normal control subjects ($n = 28$). Data is represented as means ± S.E.M. (control, open square; MDD, open diamond; BPD, closed diamond). Asterisk represents statistically significant difference at $p < 0.05$.

($\chi^2 = 11.77$, $p = 0.001$). Multivariable analyses demonstrated that the variable "state (depressive and remissive states)" was solely and significantly associated with the expression level of Glo1 mRNA ($p = 0.004$), when analyzed together with the control variables: age, gender, and type of drugs used (antidepressants and mood stabilizers). Quantitative real-time PCR experiments revealed that reduced expression of Glo1 mRNA was observed in major depressive disorder patients ($F_{(2, 58)} = 5.70$, $p < 0.01$) and bipolar disorder patients in a current depressive state ($F_{(2, 58)} = 5.70$, $p < 0.05$), compared with healthy control subjects (Fig. 1). In a remissive state, by contrast, there was no significant difference in the expression levels of Glo1 mRNA in major depressive disorder patients ($F_{(2, 98)} = 0.19$, $p = 0.82$) or bipolar disorder patients ($F_{(2, 98)} = 0.19$, $p = 1.00$), compared with healthy control subjects (Fig. 2). There was a significant correlation between Glo1 mRNA levels and HDRS scores in major depressive disorder patients ($r = -0.358$, $p = 0.005$) (Fig. 3), but not in bipolar disorder patients ($r = -0.198$, $p = 0.187$).

Dysfunction of the hypothalamic–pituitary–adrenal (HPA) system is the most characteristic biological alteration found in the majority of depressed patients. Accumulating evidence suggests

Table 1
Demographic and clinical characteristics of subjects

	Controls	Patients			
		MDD		BPD	
		Depressive	Remissive	Depressive	Remissive
Number of subjects	28	20	40	13	33
Mean age (years)	50.0 ± 1.8	52.3 ± 3.5	57.2 ± 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 ± 1.0	2.8 ± 0.2
Medication					
No medication	28	3	4	1	0
SSRI/SNRI	0	10	38	9	9
TCA/other antidepressants	0	23	28	6	14
Li	0	0	2	4	17
VPA	0	0	0	7	15
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–noradrenaline reuptake inhibitor; TCA, tricyclic antidepressant; Li, lithium; VPA, valproic acid; CBZ, carbamazepine.

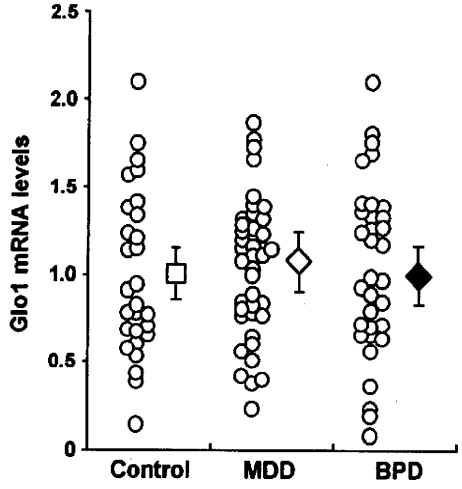

Fig. 2. Expression levels of Glo1 mRNA for mood disorder patients in a remissive state. Quantitative real-time PCR experiments revealed that expression of Glo1 mRNA (open circles) for major depressive disorder patients in a remissive state (MDD, $n = 40$) and bipolar disorder patients (BPD, $n = 33$) were not significantly different to that of normal control subjects ($n = 28$). Data is represented as means ± S.E.M. (control, open square; MDD, open diamond; BPD, closed diamond).

that the combined Dex/CRH test is highly sensitive and is able to detect HPA system abnormalities [10]. ACTH and cortisol responses to this test are exaggerated in depressed patients [7,8]. To examine the association between Glo1 mRNA levels and HPA axis activity, the mRNA levels for Glo1 of mood disorder patients in a current depressive state were compared between suppressors ($n = 11$; 8 major depressive disorder patients and three bipolar disorder patients) and non-suppressors ($n = 15$; 8 major depressive disorder patients and seven bipolar disorder patients) of the Dex/CRH test. There was no significant difference in the expression levels of Glo1 mRNA between suppressors and non-suppressors ($F_{(1,24)} = 3.68$, $p = 0.67$). In addition, there was no significant correlation between Glo1 mRNA levels and the plasma cortisol concentration in healthy control subjects ($r = -0.09$, $p = 0.72$), major depressive disorder patients ($r = 0.42$, $p = 0.27$) or bipolar disorder patients ($r = -0.50$, $p = 0.39$).

Previous reports have suggested the involvement of Glo1 in neuropsychiatric disorders, including anxiety disorders and autism. A significant association of the Glo1 Ala111Glu polymorphism has been observed in a subgroup of patients with panic disorder without agoraphobia [22] and patients with autism [12]. Reduced Glo1 enzyme activity has also been observed in the brains of patients with autism [12]. Moreover, a possible association between Glo1 and mood disorders has been found in a linkage study of families with mood disorders [26]. There is a wealth of data demonstrating the comorbidity of mood disorders with anxiety disorders [3,31,24], including panic disorder [5,13,25]. Genetic data with regard to panic disorder and major depressive disorder have been inconsistent, although there is some evidence for a shared diathesis for anxiety and depression [29]. These data suggest an important role for Glo1 in the pathophysiology of many neuropsychiatric disorders, especially with regard to the anxiety symptoms of these conditions.

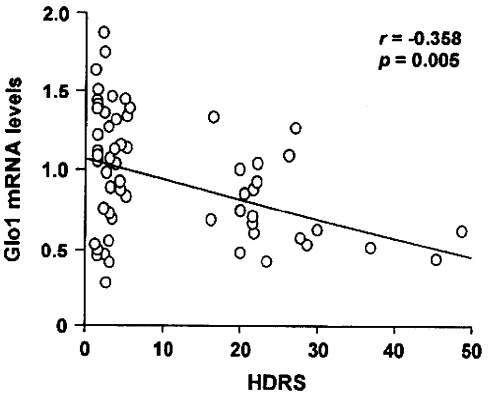

Fig. 3. Significant inverse correlation between HDRS scores and Glo1 mRNA levels was found in the major depressive disorder patients ($n = 60$). HDRS, Hamilton depression rating scale.

Krömer et al. [15] have reported an association between reduced Glo1 expression and high anxiety-like behaviors in mice. Importantly, the reduced expression of Glo1 was observed not only in the amygdala, but also in peripheral red blood cells [15], suggesting that the expression levels of Glo1 in the brain is well correlated with that in peripheral blood cells. These data and our present study raise the possibility that the expression levels of Glo1 in mood disorder patients may be reduced in multiple systems. However, a recent study has shown that local overexpression of Glo1 in various brain regions, e.g. cingulate cortex, resulted in increased anxiety-like behavior [11]. This finding is discordant with that of Krömer et al. [15] and thus, it is still unclear how Glo1 is involved in the pathophysiology of anxiety and depression.

A previous report has shown that the number of Glo1 immunopositive neurons and astroglia increase up to, approximately, 55 years of age and decrease progressively thereafter in humans [16]. Glo1 mRNA levels also showed a biphasic course similar to those observed with protein determination [16], suggesting that the expression of Glo1 is primarily regulated at the transcriptional level. The promoter region of the human Glo1 gene contains several consensus sequences for known transcriptional regulatory elements, including: insulin responsive element, metal responsive element and glucocorticoid responsive element [23]. The existence of the glucocorticoid responsive element in the human Glo1 promoter is particularly interesting, because the glucocorticoid receptor (GR) has been shown to be associated with mood disorders and in the adaptation to stress [4,9,20]. Reduced expression of GRα has been observed in the cerebral cortex, hippocampus and amygdala in mood disorder patients [30,14,21]. In addition, we have previously reported that the expression of GRα mRNA is also reduced in the peripheral white blood cells of mood disorder patients [19]. This raises the possibility that dysfunction of GR plays a causal role in the aberrant Glo1 expression observed in mood disorder patients.

Considering our results from multivariable analysis and the significant correlation between Glo1 mRNA levels and HDRS scores in major depressive disorders, it could be interpreted that the reduced expression of Glo1 mRNA is "state-dependent" at least in major depressive disorders. However, our study has the limitation that all the patients were on medication; therefore, we cannot exclude completely the influence of medication on the expression levels of Glo1 mRNA. To our knowledge, however, there is no evidence showing altered levels of Glo1 expression by treatment with antidepressants or mood stabilizers *in vitro* or *in vivo*. Further study conducted in medication-free subjects is needed to elucidate this issue.

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Characterization of the vulnerability to repeated stress in Fischer 344 rats: possible involvement of microRNA-mediated down-regulation of the glucocorticoid receptor

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Abstract

In the present study, we established and characterized an animal model of vulnerability to repeated stress. We found that control Sprague–Dawley (SD) rats showed a gradual decrease in the HPA axis response following 14 days of repeated restraint stress, whereas Fischer 344 (F344) rats did not show such HPA axis habituation. Similar habituation was observed in the expression of c-fos mRNA, corticotropin-releasing hormone hnRNA, and phospho-CREB and phospho-ERK proteins in the hypothalamic paraventricular nucleus (PVN) of SD rats, but not in the F344 rats. In addition, repeatedly restrained F344 rats exhibited decreased cell proliferation in the dentate gyrus of the hippocampus and increased anxiety-related behaviours, while repeatedly restrained SD rats exhibited a selective enhancement of hippocampal cell proliferation in the ventral area. Moreover, we found a lower expression of glucocorticoid receptor (GR) protein, but not mRNA, in the PVN of F344 rats compared to SD rats. We also identified that microRNA (miR)-18a inhibited translation of GR mRNA in cultured neuronal cells and that increased expression of miR-18a in the PVN was observed in F344 rats compared with SD rats. These strain differences in GR protein levels were not found in the hippocampus and prefrontal cortex, and the expression of miR-18a was much lower in these brain regions than in the PVN. Our results suggest that F344 rats could be a useful animal model for studying vulnerability to repeated stress, and that miR-18a-mediated down-regulation of GR translation may be an important factor to be considered in susceptibility to stress-related disorders.

Introduction

The hypothalamic–pituitary–adrenal (HPA) axis controls the production and release of adrenal glucocorticoids in response to stress and daily circadian rhythm. Dysregulation of the HPA axis is known to be associated with vulnerability to a number of psychiatric diseases including major depression, anxiety disorders and post-traumatic stress disorder (de Kloet *et al.*, 2005; Seckl & Holmes, 2007).

Several lines of evidence have indicated that chronically stressed animals often exhibit suppressed or decreased HPA responses upon re-exposure to the same, or a homotypic, stressor. This decrement, termed habituation, has been observed with various stress paradigms, including restraint (Melia *et al.*, 1994; Dhabhar *et al.*, 1997; Ma *et al.*, 1999; Cole *et al.*, 2000; Viau & Sawchenko, 2002; Girotti *et al.*, 2006), cold (Bhatnagar & Meaney, 1995) and immobilization (Garcia *et al.*, 2000). This plasticity in the regulation of HPA activity as a consequence of repeated stress is thought to protect the organism from the potentially damaging effects of hypercorticosteroidism (Armario *et al.*, 2004). Habituation is thought to be partly regulated by corticosterone-mediated negative feedback, a regulatory mechanism that restores the stress-stimulated HPA axis to basal levels via activation of glucocorticoid type I (mineralocorticoid receptor; MR) and/or type II (glucocorticoid receptor; GR; Dallman *et al.*, 1987; Cole *et al.*, 2000; Jaferi & Bhatnagar, 2006). However, there is very little understanding of the mechanisms responsible for stress habituation.

Different strains of mouse or rat have different neuroendocrine, neurogenic, physical or behavioural phenotypes that are heritable and stable (Dhabhar *et al.*, 1995, 1997; Kempermann *et al.*, 1997; Fernandes *et al.*, 2004; Hovatta *et al.*, 2005). In particular, Fischer 344 (F344) rats have been widely used in the study of HPA axis function (Kosten & Ambrosio, 2002). F344 rats are known to consistently present an exaggerated acute stress-induced corticosterone secretion relative to Sprague–Dawley (SD) and Lewis strains (Dhabhar *et al.*, 1995, 1997). F344 rats have also been reported to exhibit no habituation of HPA axis activity during restraint stress episodes over a period of 10 days (Dhabhar *et al.*, 1997). These observations suggest that F344 rats are a stress-hyperresponsive strain and may have a vulnerability to repeated restraint stress (RRS). However, little is known about the biochemical, physiological and behavioural effects of repeated stress in F344 rats. Therefore, in the first experiment of the present study, we characterized the neuroendocrine and biochemical responses to RRS in F344 and control SD rats. In the second

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experiment, hypothesizing the aberrant expression of GR and/or MR in the HPA dishabituation in F344 rats, we measured the expression of these mRNAs and proteins in several brain regions. In the third experiment, we focused on a class of small noncoding transcripts called microRNAs (miRNAs), which promote translational repression or mRNA degradation, to investigate the molecular mechanism underlying the aberrant GR translation in F344 rats. Finally, we measured behaviour and hippocampal cell proliferation as a consequence of dishabituation to RRS in F344 rats.

Materials and methods

Animals

Male SD and Fischer 344 (F344) rats (Japan SLC Inc., Hamamatsu, Japan) were housed three per cage in clear polycarbonate cages with wood chip bedding at 24 °C in a humidity-controlled room on a 12-h light–dark cycle (light on at 08.00, off at 20.00 h) and maintained for 1 week before experimental use (9 weeks old at stress onset). Food and water were continuously available except during stress sessions and behavioural tests. All experimental procedures were performed according to the Guidelines for Animal Care and Use at Yamaguchi University School of Medicine and in accordance with the Japanese Neuroscience Society. Experimental protocols were approved by the Committee on the Ethics of Animal Experiments at Yamaguchi University School of Medicine.

Stress procedures

Rats were weighed and individually subjected to restraint stress by placing them into wire mesh restrainers secured at the head and tail ends with clips as previously reported (Watanabe *et al.*, 1992). Non-restrained rats were weighed and then returned to their home cage.

General experimental procedures

Each strain of rats was divided into nonrestrained, single- and repeatedly (3-, 7- or 14-session) restrained groups. Single-restraint animals were left in their home cage until the test day. Repeatedly restrained rats were placed into restrainers for 2 h (10.00–12.00 h) for 2, 6 or 13 consecutive days prior to the test day. On the test day, rats from singly- and repeatedly restrained groups were killed by decapitation at 30 min after the initiation of restraint. Non-restrained control rats were rapidly removed from their cages and decapitated. Trunk blood was collected in heparinized tubes and plasma was separated by centrifugation and stored at −80 °C for corticosterone determination. Adrenal glands were removed and their weights were calculated as a percentage of body weight. To determine the expression levels of stress-related molecules, including c-fos mRNA, corticotropin-releasing hormone (CRH) heteronuclear RNA (hnRNA), cyclic AMP response-element binding protein (CREB), phosphorylated CREB (pCREB), extracellular signal-regulated kinase (ERK)1/2 and phophorylated ERK (pERK)1/2, the hypothalamic tissue containing the paraventricular nucleus (PVN) was dissected according to the technique of Palkovits (1973). The tissues were frozen in liquid nitrogen, and then stored at −80 °C until use. For *in situ* hybridization, brains were rapidly removed and frozen with prechilled 2-methylbutane with dry ice and then stored at −80 °C until slice preparation. To evaluate the levels of GR and MR mRNAs and proteins, and miRNA-18a (miR-18a), nonrestrained and 14-session repeatedly restrained animals (24 h after the final stress session) were deeply anaesthetized and the PVN region, hippocampus and prefrontal cortex were dissected, frozen in liquid nitrogen and then stored at −80 °C until use.

Corticosterone assay

Corticosterone concentration was determined using a commercial enzyme immunoassay kit (Assay designs, Ann Arbor, MI, USA). The sensitivity of this assay is 26.99 pg/mL and the intra- and interassay coefficients of variation were 6.6 and 7.8%, respectively.

RNA isolation and cDNA synthesis

Total RNA from dissected tissues or cells was extracted by using the mirVana miRNA isolation kit (Ambion, Austin, TX, USA) and treated with DNase (DNA-free kit, Ambion). One microgram of total RNA was used for cDNA synthesis by QuantiTect Reverse Transcription kit (Qiagen, Chatsworth, CA, USA). The primer mix of this kit contains oligo-dT and random primers to ensure cDNA synthesis from all regions of RNA transcripts. The cDNA was stored at −80 °C until use.

Quantitative real-time polymerase chain reaction (qRT-PCR) and reverse transcription-PCR

qRT-PCR was performed in an Applied Biosystems 7300 Fast Real-Time PCR System with SYBR green PCR master mix (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. PCR conditions were 15 min at 95 °C, 45 cycles of 15 s at 95 °C and 30 s at 60 °C. Amplification of the single PCR product was confirmed by monitoring the dissociation curve and electrophoresis on 1.2% agarose gels stained with ethidium bromide. Amplification curves were visually inspected to set a suitable baseline range and threshold level. The relative quantification method was employed for quantification of target molecules according to the manufacturer's protocol, in which the ratio between the amount of target molecule and a reference molecule within the same sample was calculated. All measurements were performed in triplicate. Levels of GAPDH mRNA and U6 snRNA were used to normalize the relative expression levels of target mRNA or miRNA, respectively. Reverse transcription-PCR was performed using Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. PCR conditions were 1 min at 95 °C, 30 cycles of 30 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C. Amplification of the single PCR product was visualized by electrophoresis on 1.5% agarose gels stained with ethidium bromide. The PCR primers used were as follows (5′ to 3′): c-fos forward, GAGGGAGCTGACAGATACGC; c-fos reverse, GGC TGCCAAAATAAACTCCA; CRH hnRNA forward, GGCAGGAA TGGAGACAGAGA; CRH hnRNA reverse, TAAGCTATTCGCCCG CTCTA; GR forward, GTCCATGGGGCTGTATATGG; GR reverse, TCCAGAAGCCGAAAGTCTGT; MR forward, TCTTTGGAGGAG GTCAGAGC; MR reverse, AAAATGGACTCCACGTTTGTG; pre-miR-18a forward, TGCGTGCTTTTTGTTCTAAGG; pre-miR-18a reverse, TGCCAGAAGGAGCACTTAGG; pre-miR-124a forward, TCTCTCTCCGTGTTCACAGC; pre-miR-124a reverse, ACCGCG TGCCTTAATTGTAT; GR-3′-UTR site A forward, AGGTTGTGCAA ATTAACAGTCC; GR-3′-UTR site A reverse, CCACAGTTTACCCA GCAGGT; GR-3′-UTR site B forward, CCTGTGAATTTCTTCACT GTTGA; GR-3′-UTR site B reverse, TTTGGCCACCTTGAATAGA AA; GAPDH forward, TGCCACTCAGAAGACTGTGG; GAPDH reverse, TTCAGCTCTGGGATGACCTT; U6 snRNA forward, TGCTTCGGCAGCACATATAC; U6 snRNA reverse, AGGGGCCAT GCTAATCTTCT.

Northern blotting

For miRNA Northern blotting, 10 μg of total RNA was separated on a 15% denaturing polyacrylamide gel. The total RNA was transferred onto Hybond N+ membranes (GE Healthcare Bio-Sciences, Piscataway, NJ, USA), UV-crosslinked, baked for 60 min at 80 °C and hybridized using ULTRAhyb-Oligo buffer (Ambion) according to the manufacturer's protocol. Oligonucleotides complementary to mature miRNAs and ^{32}P-end-labelled with T4 kinase (Promega, Madison, WI, USA), were used as probes. Probe sequences were as follows: miR-18a, 5′-CTATCTGCACTAGATGCACCTTA-3′; U6 snRNA, 5′-GAATTTGCGTGTCATCCTTGCGCAGGGGCCATGCTAA-3′. Levels of ribosomal RNA were visualized on gels stained with ethidium bromide. A U6 snRNA probe was applied to normalize the relative miRNA expression levels. Densitometric analysis was performed by FLA2000 (Fujifilm, Tokyo, Japan).

In situ *hybridization*

In situ hybridization for c-fos mRNA was performed as previously described (Watanabe *et al.*, 1994; Kato *et al.*, 1996). In brief, 16-μm-thick sections were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 5 min, acetylated in 0.25% acetic anhydride in 0.1 M triethanolamine–HCl (pH 8.0), rinsed in 2 × SSC and allowed to air-dry. The hybridization mixture containing ^{35}S-labelled c-fos riboprobes was applied to slides and the sections were incubated at 52 °C overnight in a humidified chamber. Following hybridization, the sections were rinsed in 2 × SSC at room temperature, treated with Rnase A for 30 min at room temperature and subsequently washed in 0.2 × SSC for 1 h at 52 °C. After air-drying, the slides were exposed to Kodak X-OMAT film for 1 week at room temperature. Film images of the brain sections were captured by an image analysis system (Neuroscience Inc., Tokyo, Japan).

Protein extraction and Western blotting

Western blotting was performed as previously described with minor modifications (Funato *et al.*, 2006). In brief, 20 or 60 μg of proteins were separated on 7% or 12% Tris–glycine gels and transblotted onto polyvinylidene difluoride membranes (GE Healthcare Bio-Sciences). The membranes were incubated with antibodies directed against: pERK1/2 (1 : 400); ERK1/2 (1 : 1000); pCREB (1 : 500); CREB (1 : 1000; Cell Signalling, Beverly, MA, USA); histone H3 (1 : 1000); phosphorylated histone H3 (p-H3, 1 : 500; Upstate Cell Signalling Solutions, Beverly, MA, USA); GR (M-20, 1 : 1000); MR (C-19, 1 : 500) or β-actin (1 : 2000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). After incubation with an appropriate horseradish peroxidase-conjugated secondary antibody, the blots were developed with an ECL-Plus detection kit (GE Healthcare Bio-Sciences). Densitometric analysis was performed by Inquiry software (Neuroscience Inc.).

Bromodeoxyuridine (BrdU) immunohistochemistry

BrdU administration

BrdU (Sigma, St Louis, MO, USA) was prepared in 0.9% saline to a dilution of 20 mg/mL BrdU and 0.007 M NaOH. On the final stress session, repeatedly restrained and nonrestrained rats were administered BrdU (100 mg/kg) intraperitoneally (i.p.) twice after the termination of restraint stress. A total of 200 mg/kg BrdU was given via two i.p. injections with a 6-h interval (12.00 and 18.00 h).

Perfusion and slice preparation

Twenty-four hours after the first BrdU injection (on the 15th day), rats were deeply anaesthetized with Nembutal and perfused transcardially with saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4). Their brains were removed, postfixed in the same fixative for 24 h and then cryoprotected in 30% sucrose in 0.1 M PB for 3 days at 4 °C. Brains were frozen in 2-methylbutane prechilled with dry ice and then stored at −80 °C until use. Forty-micrometer-thick coronal sections throughout the entire dentate gyrus of the hippocampus were cut on a freezing microtome and collected in six-well plates containing cryoprotectant (25% ethylene glycol and 25% glycerol in 0.05 M PB, pH 7.4) and then stored at −20 °C until use.

BrdU immunohistochemistry

The free-floating sections were incubated in 50% formamide in 2 × SSC at 65 °C for 2 h followed by PBS washes. The sections were then incubated in 2 M HCl for 30 min and in 0.1 M boric acid solution (pH 8.5) for 10 min. After the PBS washes, the sections were pretreated with 0.6% hydrogen peroxide in PBS for 30 min, washed with PBS and incubated for 1 h at room temperature with a blocking solution composed of 0.3% TritonX-100 and 5% normal goat serum in PBS. Sections were then incubated with the primary antibody to BrdU (1 : 400; Chemicon, Temecula, CA, USA) in the blocking solution for 48 h at 4 °C. After rinsing in PBS, the sections were incubated in biotinylated horse antimouse secondary antibody (Vectastain Elite ABC kit, Vector Laboratory, Burlingame, CA, USA) for 2 h at room temperature. Following PBS washes, the sections were incubated in avidin–biotin complex for 1 h at room temperature. Finally, the sections were developed with a solution of 0.03% DAB (Sigma) and 0.03% hydrogen peroxide in Tris-buffered saline and were mounted onto slides, counterstained with cresyl violet, dehydrated, cleared and covered with a coverslip under Permount (Fisher Scientific, Pittsburgh, PA, USA).

Quantification of BrdU-labelled cells in the dentate gyrus

The number of BrdU-labelled cells on every tenth bilateral section throughout the whole dentate gyrus and dorsal (bregma −2.80 to −4.00 mm; Paxinos & Watson, 1998) and ventral hippocampus (bregma −5.20 to −6.80 mm) were counted using a light microscope (Nikon, Tokyo, Japan). The number of BrdU-labelled cells was multiplied by 10 to estimate the total number of BrdU-labelled cells throughout the target regions. To correct the overestimation linked to counting the same nucleus on two adjacent sections, the following formula (Abercrombie, 1946) was applied: $N = n[t/(t + d)]$, where N corresponds to the 'true' number, t is the section thickness, d is the nucleus diameter and n is the estimated number.

Behavioural procedures

Twenty-four hours after the final stress session (on the 15th day), rats were subjected to the following behavioural experiments. All experiments were performed in a blind fashion.

Social interaction test

Each subject was placed in a measuring cage and allowed to stay for 120 min. A male conspecific juvenile was then introduced into the cage and the amount of time spent in social interaction (e.g. grooming, licking, sniffing, crawling over or under) of the testing animal was recorded during a 3-min session.

Novelty-suppressed feeding test

Subjects were weighed (body weight A) and singly housed after the termination of the final restraint stress session on the 14th day, and food pellets were removed from their cages. Water remained available *ad libitum*. Twenty-four hours after food removal, rats were weighed (body weight B) and transferred to a clean holding cage in the testing room. The testing apparatus consisted of a circular arena (60 cm in diameter). A piece of rat chow was placed in the centre of the arena. Each subject was placed in the testing area and the time to the first feeding episode was recorded for 5 min. After the termination of the test, the animal was returned to the home cage with food pellets and the amount of food consumed was measured for 4 h. The percentage loss of body weight was estimated as: [(body weight B)/(body weight A)] × 100.

Generation of DNA constructs

The human GR-3′-UTR (Miesfeld *et al.*, 1986) containing two putative target sites of miR-18a (see Fig. 5A) was amplified from human cDNA (SH-SY5Y cells) using the following primers: GR-3′-UTR forward, 5′-AGGTTGTGCAAATTAACAGTCC-3′; GR-3′-UTR reverse, 5′-TTTGGCCACCTTGAATAGAAA-3′. PCR products were cloned into the pGL3 control vector (Promega), downstream of the luciferase coding sequence. To express the miR-18a gene in SH-SY5Y cells, the genomic sequence containing the pre-miR-18a gene sequence plus 50 base pairs flanking each side were amplified from human DNA (SH-SY5Y cells) using the following primers: miR-18a forward, 5′-TCGGGAAGCCAAGTTGGGGT-3′; miR-18a reverse, 5′-CTATTAAAACACCTATATAC-3′, and then inserted into pcDNA3 (Invitrogen).

Cell culture, transfection and assay

Cell culture experiments were performed as previously described with minor modifications (Uchida *et al.*, 2006). Human neuroblastoma SH-SY5Y cells were maintained in DMEM (Invitrogen) supplemented with 10% fetal bovine serum, penicillin (100 U/mL), streptomycin (100 μg/mL) and insulin (60 ng/mL) at 37 °C in 5% CO_2. For the reporter assay, SH-SY5Y cells were transiently cotransfected in 24-well plates by using the Lipofectamine and PLUS reagent (Invitrogen) with the pGL3 or pGL3-GR-3′-UTR vector (0.2 μg/well) together with the miR-18a expression vector (0.2, 0.4 or 0.8 μg/well) and/or empty vector (pcDNA3). The pCMV-β-galactosidase vector (0.2 μg/well) was also cotransfected as a control for transfection efficiency. Thirty hours after the transfection, luciferase and β-galactosidase activity were measured by using a Luciferase assay system (Promega) and β-galactosidase assay system (Promega), respectively. Luciferase activity was normalized to β-galactosidase activity. All reporter assays were performed in triplicate in three independent experiments. To evaluate the endogenous GR expression levels, SH-SY5Y cells were transiently transfected in six-well plates using the Lipofectamine and PLUS reagent (Invitrogen) with the miR-18a expression vector (1, 2 or 4 μg/well) and/or empty vector (pcDNA3). Forty-eight hours after the transfection, protein and total RNA were isolated from whole-cell extracts, and Western blot analysis, Northern blotting and qRT-PCR were performed as described above.

Statistical analysis

Data are presented as the mean ± SEM. The data on body weight gain were analysed using ANOVA for repeated measures. Grouped data obtained from Figs 2 and 3 were analysed by multifactor repeated-measures ANOVA with strain (SD and F344) as a main factor and session number (NRS, acute, 3rd, 7th and 14th). Grouped data obtained from Figs 4 and 6–8 were analysed by two-way ANOVA with strain (SD and F344) and stress (nonrestraint and repeated restraint). Significant effects or interactions obtained from ANOVA were further analysed by *post hoc* comparisons using Bonferroni's correction. In all cases, *P*-values < 0.05 were considered statistically significant.

Results

Neuroendocrine response to restraint stress in F344 rats

To examine the neuroendocrine effects of acute and repeated restraint stress, we measured changes in daily body weights, adrenal weights and plasma corticosterone levels. Changes in body weight for subjects, across 6 days of prestress and during the 14-day stress period, are shown in Fig. 1A. In the nonrestrained group, there was

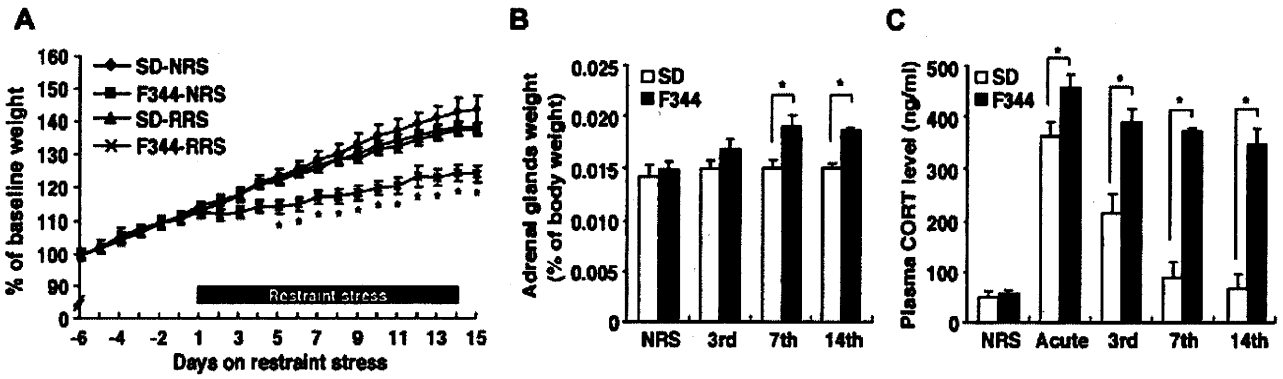

FIG. 1. Effects of restraint stress on body weight gain, adrenal gland weight and plasma corticosterone (CORT) levels in SD and F344 rats. (A) Body weight gain is shown as a percentage of the initial body weight ($n = 6$ for all groups). (B) Weight of adrenal glands is shown as a percentage of body weight at the end of the 3-, 7- and 14-session and nonstressed condition (NRS; $n = 6$ for all groups). (C) Plasma CORT levels in NRS rats and rats exposed to acute, 3-, 7- and 14-session 30 min after the initiation of restraint stress ($n = 6$ for all groups). Data are presented as mean ± SEM. *$P < 0.05$ vs. SD rats in the corresponding stress session.

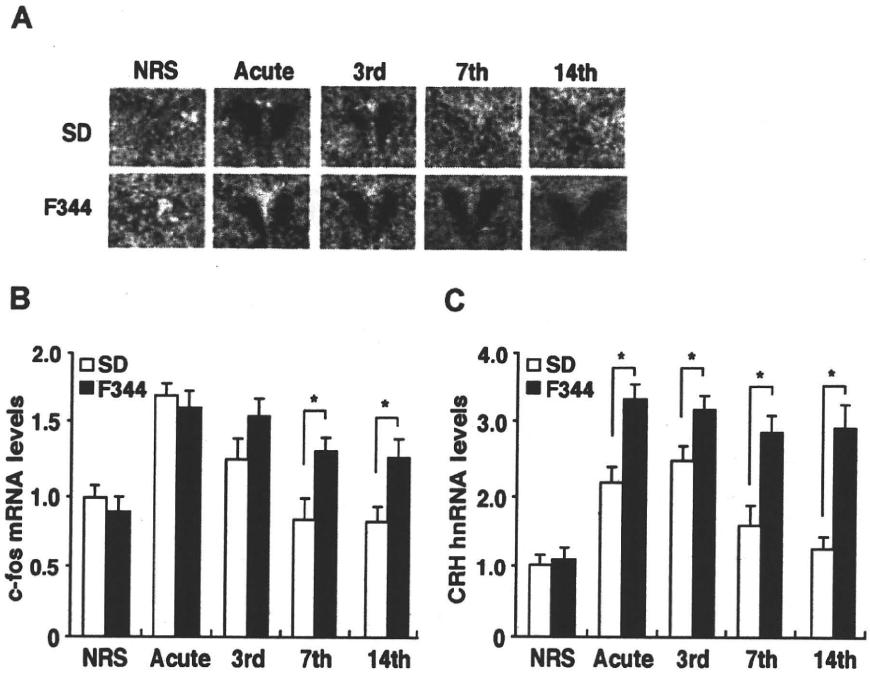

FIG. 2. Changes in c-fos mRNA and CRH hnRNA expression in the PVN of SD and F344 rats following acute and repeated restraint stress exposure. (A) Sample autoradiograms obtained from *in situ* hybridization experiments for c-fos mRNA expression 30 min after the initiation of restraint stress in the PVN of nonrestrained (NRS), acutely and repeatedly 3-, 7- and 14-session restrained SD (open bar) and F344 (closed bar) rats. (B) Quantitative analyses of c-fos mRNA levels in the PVN obtained from qRT-PCR ($n = 6$ for all groups). (C) CRH hnRNA levels 30 min after the initiation of restraint stress in the PVN of NRS, acutely and repeatedly 3-, 7- and 14-session restrained SD and F344 rats ($n = 6$ for all groups) were quantified by qRT-PCR. Data are presented as mean ± SEM. *$P < 0.05$ vs. SD rats in the corresponding stress session.

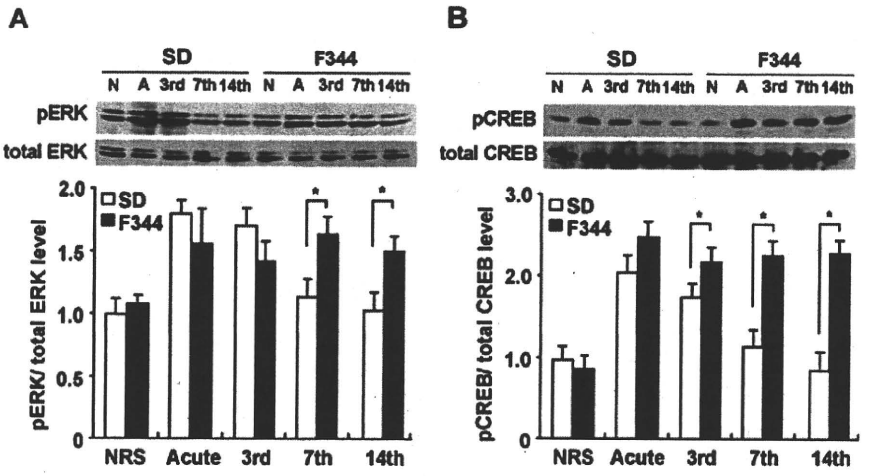

FIG. 3. Effects of acute and repeated restraint stress on the expression of pCREB and pERK in the PVN. (A and B) Western blot analysis of pERK1/2, total ERK1/2, pCREB and total CREB levels 30 min after the initiation of restraint stress in the PVN of acutely and repeatedly 3-, 7- and 14-session restrained SD (open bar) and F344 (closed bar) rats as well as nonrestrained rats (N). The histograms show a quantitative analysis of pERK1/2 and pCREB levels ($n = 8$ for all groups). Data are presented as mean ± SEM. *$P < 0.05$ vs. SD rats in the corresponding stress session.

no significant effect on body weight gain between the SD and F344 rats ($F_{1,200} = 1.06$, $P > 0.05$). In contrast, there was a significant effect of strain upon body weight gain in the repeatedly restrained group ($F_{1,200} = 7.72$, $P < 0.05$) between the SD and F344 rats. Body weight gain of restrained F344 rats was significantly less than that of nonrestrained F344 rats and that of restrained SD rats after the 4th restraint stress presentation. Adrenal weights of rats from the 3-, 7- and 14-session are shown in Fig. 1B. There were significant effects of strain ($F_{1,30} = 30.29$, $P < 0.01$), session number ($F_{3,30} = 9.00$, $P < 0.01$) and the combination of these two factors ($F_{3,30} = 5.06$, $P < 0.01$). F344 rats from the 7th and 14th restraint stress sessions exhibited a significantly increased adrenal weight compared with restrained SD and nonrestrained F344 rats.