mPFC of HMS180 rats was comparable to that of AFR and HMS15 rats during all stages of development examined ($F_{(14,75)} = 1.01$, $p > 0.05$) (Fig. 2*A*). In contrast, the mRNA expression of *Rest4* in HMS180 rats was significantly higher than that of AFR and HMS15 rats at P7 ($F_{(14,75)} = 33.65$, $p < 0.01$; *post hoc*, $p < 0.01$) and P14 (*post hoc*, $p < 0.01$) (Fig. 2*B*). The altered expression of *Rest4* mRNA in HMS180 rats was not observed in P21, P35, and adult rats (Fig. 2*B*). We also examined the expression of mRNA for *Rest2* and *Rest3* isoforms in the mPFC of rats, but these expressions were quite low or nondetectable (data not shown). Western blotting revealed that the level of REST protein in the mPFC of HMS180 rats at P14 was significantly decreased compared with those of AFR and HMS15 rats ($F_{(2,15)} = 4.51$, $p < 0.05$; *post hoc*, $p < 0.05$), and the expression of REST4 protein was significantly increased compared with those in AFR and HMS15 rats ($F_{(2,15)} = 8.26$, $p < 0.01$; *post hoc*, $p < 0.05$) (Fig. 2*C*,*D*). In P14 rats, there were no significant differences in the levels of *Rest* and *Rest4* mRNAs among the three groups in either the hippocampus (*Rest*, $F_{(2,15)} = 0.86$, $p > 0.05$; *Rest4*, $F_{(2,15)} = 0.45$, $p > 0.05$) or the amygdala (*Rest*, $F_{(2,15)} = 0.11$, $p > 0.05$; *Rest4*, $F_{(2,15)} = 0.61$, $p > 0.05$) (Fig. 2*E*,*F*; data not shown). These results suggest an aberrant RE-1-mediated gene regulation induced by the altered REST4 and/or REST expression in the mPFC of the HMS180 rats during early postnatal periods.

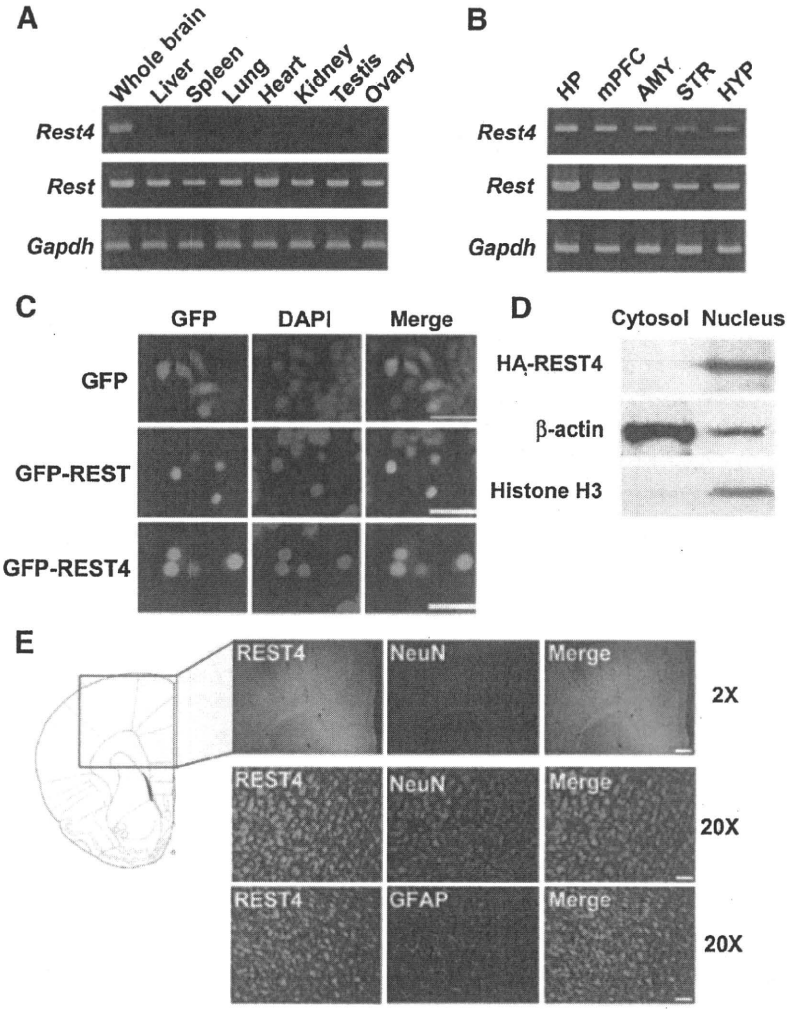

Figure 4. REST4 is specifically expressed in the brain and is localized in the nucleus of mPFC neurons. ***A***, Ethidium bromide stained gels of products of reverse-transcription PCR with cDNA isolated from a variety of mouse tissues showing that *Rest4* is expressed only in the brain, whereas *Rest* is ubiquitously expressed in the liver, lung, heart, spleen, kidney, testis and ovary. ***B***, Within the brain, *Rest4* mRNA was detected in the hippocampus (HP), mPFC, amygdala (AMY), striatum (STR), and hypothalamus (HYP). ***C***, Fluorescence micrographs of Neuro2a cells transfected with either EGFP-REST4, EGFP-REST or EGFP expression vectors. EGFP fluorescence was detected in the nucleus of the EGFP-REST4- and EGFP-REST-transfected cells. Scale bar represents 50 μm. ***D***, Western blots of nuclear and cytosolic fractions of Neuro2a cells transfected with pcDNA3-HA-REST4 then stained with antibodies against HA, β-actin (a marker for cytosolic protein), or histone H3 (a marker for nuclear protein). ***E***, Anti-REST4 immunohistochemical analysis of rat coronal sections showing that REST4 immunoreactivity is colocalized with the neuronal nuclear marker NeuN in the mPFC and not with the astrocyte marker GFAP. Scale bars represent 500 and 50 μm for 2× and 20×, respectively.

Altered mRNA expression of RE-1-containing genes in the maternally separated rats

To test whether the expressions of RE-1-containing genes were affected in the HMS180 rats, we examined the mRNA levels of such genes in the mPFC. As shown in Figure 3*A*, the expressions of mRNAs for the AMPA glutamate receptor 2 subunit (*Glur2*), NMDA receptor 1 subunit (*Nr1*), *Crh*, calcium/calmodulin-dependent protein kinase II α (*CamKII*α), cell adhesion molecule L1 (*L1*), adenylate cyclase 5 (*Adcy5*), *5htr1a*, and voltage-gated potassium channel subunit Kv3.1 (*Kcnc1*) genes in the HMS180 rats at P14 were significantly increased compared with those in AFR and HMS15 rats. In contrast, the expression of mRNAs for the *Bdnf*, dopamine β hydroxylase (*Dbh*), agouti-related protein (*Agrp*), amiloride-sensitive cation channel 1 (*Accn1*), and neurogenic differentiation 1 (*Neurod1*) genes in the mPFC of the HMS180 rats were comparable to those of AFR rats, and the expression of mRNA for the neuron navigator 1 (*Nav1*) gene was significantly decreased in HMS180 rats. We also checked the validity of our real-time PCR data using 18S rRNA as an internal control. Similar to the analysis using *Gapdh* normalization, the expression of mRNAs for *Glur2*, *Nr1*, *Crh*, *CamKII*α, *L1*, *Adcy5*, and *5htr1a* genes in the HMS180 rats were significantly increased compared with those in the AFR and HMS15 rats (supplemental Fig. S1, available at www.jneurosci.org as supplemental material).

Effect of maternal separation on miRNAs expression in the mPFC

REST regulates some of the brain-enriched microRNAs (miRNAs) (Conaco et al., 2006; Otto et al., 2007) that are thought to be involved in such neuronal functions as brain development and plasticity (Vo et al., 2005; Conaco et al., 2006; Kosik, 2006; Rajasethupathy et al., 2009). Therefore, to examine whether maternal separation affects the expression of such miRNAs in the mPFC, we measured the expression levels of 10 brain-enriched miRNAs. Real-time PCR analyses showed significantly increased expressions of pre-*Mir132*, *-124-1*, *-9-1*, *-9-3*, *-212*, and *-29a* in HMS180 rats at P14 compared with those in AFR rats (Fig. 3*B*). In contrast, there were

no significant differences among the three groups in the expression levels of pre-*Mir330*, *-153*, *-135b*, and *-134*. Northern blotting revealed that the expression levels of mature *Mir132*, *-124*, *-9*, and *-29a* in HMS180 rats at P14 were significantly higher than those in the other groups (Fig. 3*C*,*D*), whereas the expression levels of mature *Mir134* were not significantly different among the three groups. It should be noted that *Mir132*, *-124-1*, *-9-1*, *-9-3*, *-212* and *-29a* all have an RE-1 site within 50 kb of their promoter regions (Otto et al., 2007). Thus, our data suggest that the altered expressions of mRNAs and miRNAs of RE-1-containing genes in the mPFC of HMS180 rats might be due to the activation of RE-1-mediated gene transcription. We also examined the expressions of mRNA and miRNAs for some of the RE-1-containing genes in the mPFC of adult rats (P58-P60), and found increased expressions of mRNA for the *Glur2*, *CamKIIα* and *Adcy5* genes and for the precursors for *Mir132*, *-124*, and *-212* (Fig. 3*E*).

Characterizations of the expression and function of REST4

Although REST4 is a splicing variant of REST, its role in transcriptional regulation is not completely understood. Therefore we characterized the expression and function of REST4. As shown in Figure 4*A*, the expression of *Rest4* mRNA in mice was restricted to the brain, whereas that of *Rest* was ubiquitously expressed in a variety of tissues, including liver, spleen, lung, heart, kidney, testis, and ovary. These results concur with previous data from rat tissues (Palm et al., 1998). Within the brain, the expression of *Rest4* mRNA was detected in the hippocampus, mPFC, amygdala, striatum, and hypothalamus (Fig. 4*B*). We next examined the subcellular localization of REST4. When we transiently transfected Neuro2a cells with an EGFP-REST4 expression vector, we detected EGFP fluorescence in the cell nuclei (Fig. 4*C*). Western blotting also demonstrated that REST4 was localized in the nucleus and not in the cytoplasm of the Neuro2a cells (Fig. 4*D*). To investigate the subcellular localization of REST4 *in vivo*, we generated antibodies specific to the C-terminal epitopes of REST4, and performed immunohistochemistry in rat brain sections. REST4-immunofluorescence was colocalized with that for the neuronal nuclear marker NeuN, while colocalization with the astrocyte marker GFAP was very low (Fig. 4*E*). These results indicate that REST4 is localized in neuronal nuclei in the mPFC, and that it may act there as a brain-specific regulator of gene transcription.

To investigate the role of REST4 in the transcriptional regulation of RE-1-containing genes *in vivo*, we overexpressed REST4 in the mPFC of neonatal mice by injecting a plasmid vector containing either the *Egfp* or *Rest4* cDNA using the PEI delivery system, and analyzed the expression of eight mRNAs and six pre-miRNAs, whose expressions were increased in HMS180 rats. To confirm the successful transduction of the PEI delivery system, EGFP or HA-REST4 expression plasmids complexed with PEI were injected into the mPFC of neonatal mice at P6. EGFP-positive cells were observed 3 d after the injection (Fig. 5*A*). In addition, Western blotting revealed the induction of exogenous REST4 (HA-REST4) in the mPFC of mice 3 d after the injection (Fig. 5*B*). We thus concluded that the foreign gene was successfully transduced in the mPFC by PEI. Using this method, we found that in the presence of the overexpression of REST4 in the mPFC of neonatal mice the mRNAs for *Crh*, *5htr1a*, *CamKIIa*, *Glur2*, *Nr1*, *Adcy5* (Fig. 5*C*), and the precursors of *Mir132*, *Mir212*, and *Mir9-3* (Fig. 5*D*) were significantly increased. These results suggest that REST4 enhances the transcription of the some of the RE-1-containing genes *in vivo*. However, the expression of

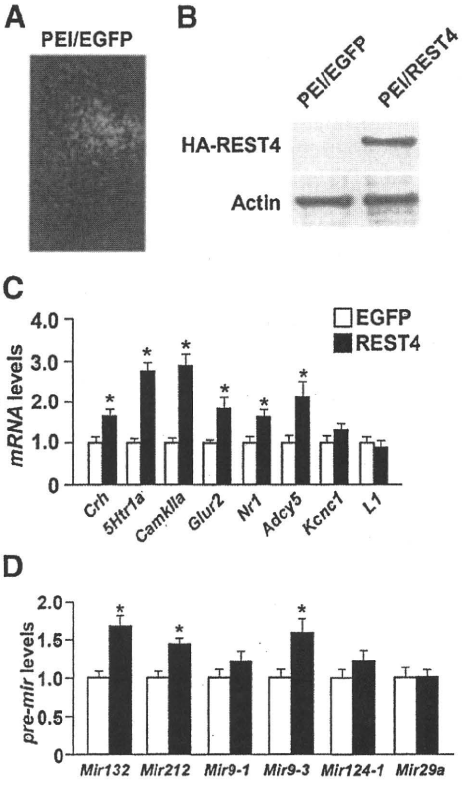

Figure 5. PEI-mediated REST4 overexpression in the mPFC of neonatal mice increases the expression of RE-1-containing genes. ***A***, Fluorescence micrograph of neonatal mouse mPFC after being injected (P6–P7) with EGFP expression plasmids complexed with PEI. Three days after the injection, EGFP fluorescence was detected in the mPFC region. ***B***, Western blotting with anti-HA antibody shows the transduction of HA-REST4 in the mPFC of mice 3 d after the injection of PEI/*Rest4* complexes. ***C***, ***D***, Bar graphs showing Q-PCR of the levels of mRNAs (***C***) and microRNAs (***D***) in the mPFC of mice 3 d after injection of either the PEI/*Egfp* or PEI/*Rest4* complexes ($n = 6$ for each group). $^*p < 0.05$.

Kcnc1 and *L1* mRNAs and pre-*Mir9-1*, *-124-1*, and *-29a* were not affected by the overexpression of REST4 *in vivo*, suggesting that REST4-mediated gene regulation is cell-type specific and/or context-dependent, and that other molecules may be involved in the regulation of the expressions of these genes in HMS180 rats.

PEI-mediated REST4 gene delivery in the mPFC of neonatal mice increases stress vulnerability

We investigated the role of postnatal REST4-mediated gene regulation in the development of stress vulnerability. The experimental design is shown in Figure 6*A*. We overexpressed REST4 in the mPFC of neonatal mice at P6-P7 by injecting a plasmid vector containing either the *Rest4* or control *Egfp* cDNA using the PEI delivery system. To examine the neuroendocrine effect of neonatal REST4 overexpression in the adult mice, we assessed plasma corticosterone levels in response to acute restraint stress (Fig. 6*B*). Two-way ANOVA revealed that there was significant effect of time ($F_{(2,35)} = 23.89$, $p < 0.01$) and postnatal manipulation ($F_{(1,35)} = 8.64$, $p < 0.01$). Plasma corticosterone levels in the REST4 overexpression mice 30 min after the initiation of restraint stress were significantly greater than those of the corresponding EGFP overexpression mice ($p < 0.05$). To examine the effect of neonatal REST4 overexpression on hedonic behavior after exposure to repeated restraint stress, we assessed the sucrose preference test (Fig. 6*C*,*D*). There were significant effects of postnatal manipulation ($F_{(1,47)} = 5.24$, $p < 0.05$) and restraint stress

exposure ($F_{(1,47)} = 5.48, p < 0.05$) on the sucrose preference. The sucrose preference of REST4 overexpression mice following repeated restraint stress was significantly decreased compared with that of the nonrestrained REST4 mice (*post hoc*, $p < 0.05$) and the nonrestrained EGFP overexpression mice (*post hoc*, $p < 0.05$) (Fig. 6*C*). Total fluid consumptions were stable and were not significantly affected by any of the treatments (Fig. 6*D*). We next examined depression-like behavior using the forced swim test (Figs. 6*E*,*F*). There were significant effects of postnatal manipulation ($F_{(1,47)} = 23.31$, $p < 0.01$) and the interaction of postnatal manipulation and restraint stress exposure ($F_{(1,47)} = 6.20$; $p < 0.05$) on the immobility time. REST4 overexpression in the mPFC did not affect the immobility time in nonrestrained mice (Fig. 6*E*), but following repeated restraint stress the REST4 overexpression mice exhibited a significant increase in immobility time compared with the nonrestrained EGFP overexpression control mice (*post hoc*, $p < 0.01$) (Fig. 6*E*). However, there was no significant effect of postnatal manipulation ($F_{(1,47)} = 0.96, p > 0.05$), restraint stress exposure ($F_{(1,47)} = 1.74, p > 0.05$) and their interaction ($F_{(1,47)} = 0.28, p > 0.05$) on the latency to immobility (Fig. 6*F*). To examine whether neonatal REST4 overexpression mice with or without additional repeated restraint stress during adulthood were anxious, we assessed the novelty-suppressed feeding test (Fig. 6*G*,*H*). There were significant effects of postnatal manipulation ($F_{(1,47)} = 35.26$; $p < 0.01$). Nonrestrained and repeatedly restrained REST4 overexpression mice exhibited significantly longer latencies to begin eating than did the EGFP overexpression groups (*post hoc*, $p < 0.05$), with no differences in weight loss induced by food deprivation (Fig. 6*G*,*H*).

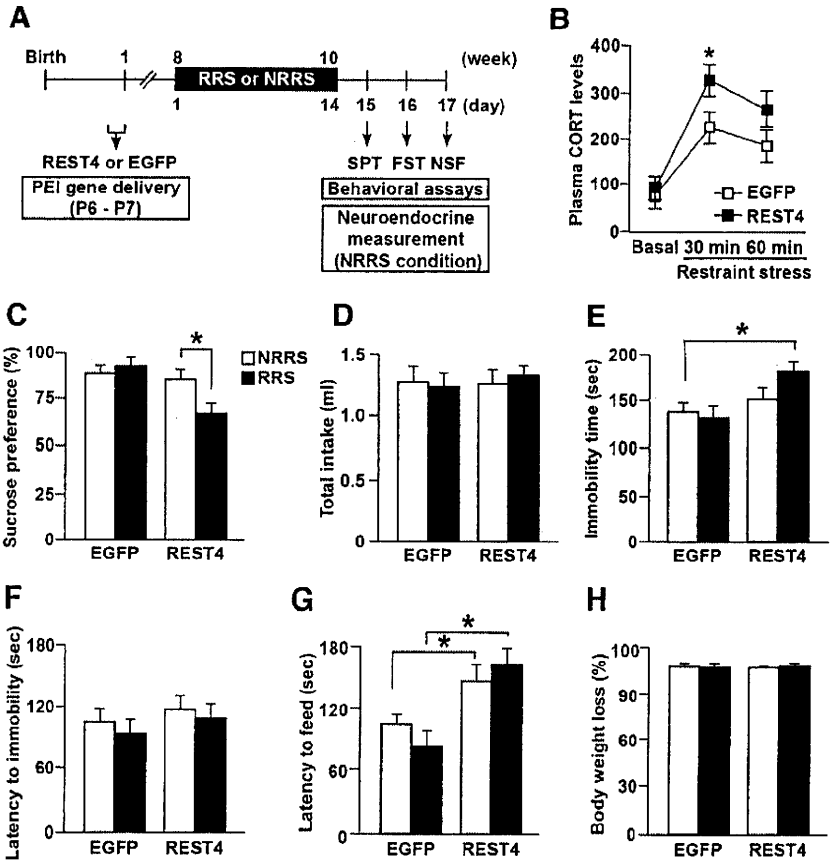

Figure 6. PEI-mediated REST4 overexpression in the mPFC of neonatal mice increases stress vulnerability. ***A***, Schematic of experimental design. PEI/*Rest4* or control PEI/*Egfp* complexes were injected to the mPFC of mice at P6–P7. Adult mice were subjected to repeated restraint stress (RRS) or nonrepeatedly restrained stress (NRRS) for 14 consecutive d, and then assessed for anxiety- and depression-like behaviors in the sucrose preference (SPT), forced swim (FST) and novelty-suppressed feeding (NSF) tests ($n = 14–16$ for each group; see Materials and Methods for details). ***B***, Plasma corticosterone (CORT) levels before and 30 and 60 min after the initiation of restraint stress in mice injected with either PEI/*Egfp* or PEI/*Rest4* complexes ($n = 5–7$ for each group). Plasma CORT levels in response to restraint stress were higher in the mice with REST4 overexpression compared with those with EGFP overexpression (*$p < 0.05$). ***C***, ***D***, Results of the sucrose preference test after RRS or NRRS, showing sucrose preference (***C***) and the total intake of fluids (***D***). REST4 overexpression mice subjected to RRS showed significantly deceased sucrose preference compared with nonrestrained REST4 overexpression mice (*$p < 0.05$). ***E***, ***F***, Results of the forced swim test after RRS or NRRS, showing immobility time (***E***) and the latency to first immobility (***F***). REST4 overexpression mice subjected to RRS showed significantly increased immobility time (***E***) but normal latency to first immobility (***F***) compared with the nonrestrained EGFP overexpression mice (*$p < 0.01$). ***G***, ***H***, Results of the novelty-suppressed feeding test after RRS or NRRS, showing the latency to feed (***G***) and the percentage body weight loss (***H***) in each group. REST4 overexpression mice with and without RRS showed significantly increased latencies to feed compared with corresponding EGFP overexpression mice (*$p < 0.05$).

To exclude the possibility that overexpression of the nuclear gene causes behavioral changes apart from any specific effects of REST4, we also used the NLS-EGFP vectors as controls. We overexpressed either NLS-EGFP, EGFP, or REST4 in the mPFC of neonatal mice (P6-P7) using the PEI delivery system, and analyzed anxiety behavior using the novelty-suppressed feeding test during adulthood (supplemental Fig. S2, available at www.jneurosci.org as supplemental material). We found that REST4 overexpression mice showed an increased latency to feed in the novel environment compared with NLS-EGFP and EGFP overexpression mice, whereas the latency to feed in the home cage and body weight loss were comparable among the groups (supplemental Fig. S2, available at www.jneurosci.org as supplemental material). Thus, our data indicate that the overexpression of REST4 in the mPFC during the neonatal period increases anxiety behavior and the behavioral vulnerability to repeated restraint stress in adulthood.

Viral-mediated REST4 overexpression in the mPFC of adult mice did not affect stress vulnerability

Finally, we examined whether overexpression of REST4 in the mPFC of adult mice could also enhance behavioral vulnerability to repeated restraint stress. The experimental design is shown in Figure 7*A*. We overexpressed REST4 and control EGFP in the mPFC of adult mice by injecting rAAV expressing REST4 or EGFP. Successful transductions of REST4 are shown in Figure 7, *B* and *C*. Two weeks after the virus injection, mice were subjected to 2 weeks of repeated restraint stress then to behavioral assays. In the sucrose preference test (Fig. 7*D*), there was no significant effects of gene delivery ($F_{(1,46)} = 0.42, p > 0.05$), restraint stress exposure ($F_{(1,46)} = 0.17, p > 0.05$), or their interaction ($F_{(1,46)} = 0.06, p > 0.05$) on sucrose preference. In the forced swim test, there were no significant effects of gene delivery ($F_{(1,46)} = 1.84$, $p > 0.05$), restraint stress exposure ($F_{(1,46)} = 0.08, p > 0.05$), or

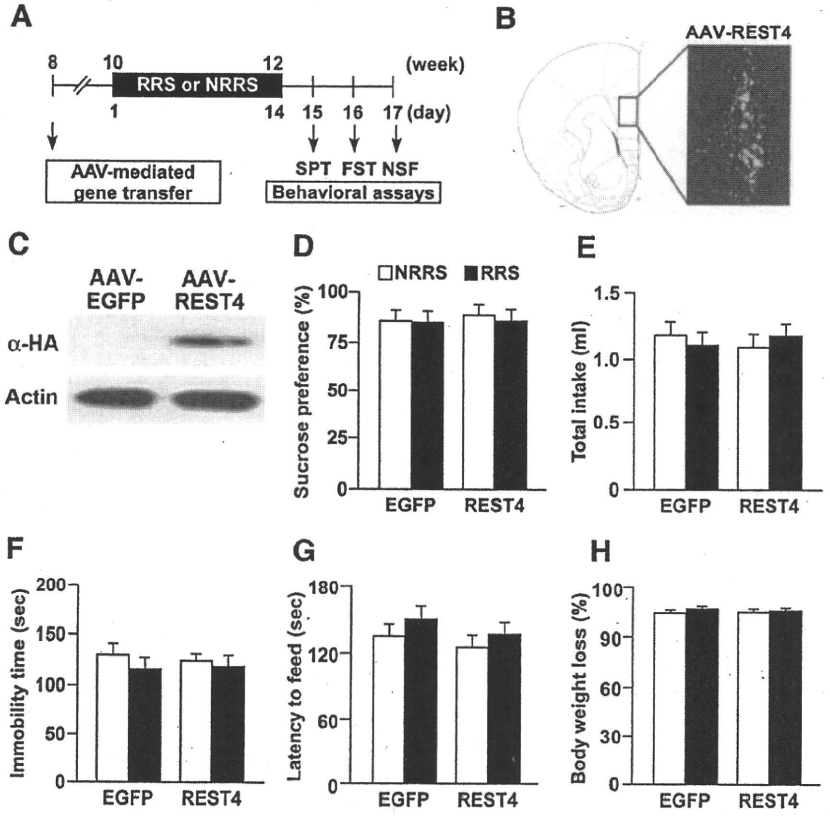

Figure 7. Effects of REST4 overexpression in the mPFC of adult mice on stress vulnerability. ***A***, Schematic of experimental design. rAAV-REST4 or control rAAV-EGFP were injected into the mPFC of adult mice at P56. Two weeks after the injection, mice were subjected to repeated restraint stress (RRS) or nonrepeatedly restrained stress (NRRS) for 14 consecutive days, and then assessed for anxiety- and depression-like behaviors in the sucrose preference (SPT), forced swim (FST), and novelty-suppressed feeding (NSF) tests ($n = 11$–14 for each group). ***B***, Fluorescence micrograph of adult mouse mPFC after injection of AAV-REST4. Four weeks after the injection, HA-REST4 immunofluorescence was detected in the mPFC region. ***C***, Western blotting with anti-HA antibody shows the overexpression of HA-REST4 in the mPFC of mice 4 weeks after the injection of AAV-REST4. ***D***, ***E***, Results of the sucrose preference test after RRS or NRRS, showing sucrose preference (***D***) and the total intake of fluids (***E***). ***F***, Results of the forced swim test after RRS or NRRS, showing immobility time. ***G***, ***H***, Results of the novelty-suppressed feeding test after RRS or NRRS, showing the latency to feed (***G***) and the percentage body weight loss (***H***) in each group.

their interaction ($F_{(1,46)} = 0.99, p > 0.05$) on the immobility time (Fig. 7*E*). Also in the novelty-suppressed feeding test (Fig. 7*F*), there were no significant effects of gene delivery ($F_{(1,46)} = 1.28$, $p > 0.05$), restraint stress exposure ($F_{(1,46)} = 0.67, p > 0.05$), or their interaction ($F_{(1,46)} = 0.12, p > 0.05$) on the latency to begin feeding. Thus, these behavioral data indicate that the overexpression of REST4 in the mPFC of adult mice did not affect anxiety behavior or the behavioral vulnerability to repeated restraint stress, and suggest that the network of REST4-mediated genes in the mPFC during the early postnatal period plays an important role in the development of stress vulnerability.

Discussion

In the present study, we found that maternal separation enhances stress vulnerability to repeated restraint stress exposure in adulthood. At the molecular level, maternal separation increased the expression of REST4 mRNA and protein, and that of several mRNAs and miRNAs of RE-1-containing genes in the mPFC. We also found that REST4 derepresses and upregulates the expression of some RE-1-containing genes *in vivo*. Importantly, transient overexpression of REST4 in the mPFC of neonatal mice produced depression-like behaviors in adults following repeated exposure to restraint stress, suggesting a crucial role of REST4 in the development of stress vulnerability. Thus, our data provide evidence suggesting that an aberrant REST-4-mediated network of genes in the early postnatal mPFC is involved in the increasing risk for the development of stress-related disorders, such as depression, in adulthood.

Early life stress and stress vulnerability

Depression, anxiety, and posttraumatic stress disorders are known to be associated, in part, with dysregulation of the HPA axis (de Kloet et al., 2005; Müller and Holsboer, 2006). In the present study, HMS180 rats showed greater plasma corticosterone levels in response to restraint stress, which is consistent with previous reports (Francis et al., 2002; Lippmann et al., 2007; Murgatroyd et al., 2009). In addition, we found that HMS180 rats exhibited increased anxiety as adults, even in the absence of stress-inducing factors, and increased depression-like behaviors in the forced swim and sucrose preference tests after episodes of repeated restraint. Inhibition of the HPA axis response to stress is thought to protect organisms from the potentially damaging effects of long-term exposure to corticosterone (Armario et al., 2004). It should be noted that daily exposure to corticosterone and hyperreactivity of the HPA axis to repeated stress in rodents can increase depression- and anxiety-related behaviors in a stressful situation (McEwen, 2001; de Kloet et al., 2005; Uchida et al., 2008; David et al., 2009). Together, these findings suggest that the increased depression-like behaviors of stressed HMS180 rats might be associated with the increased HPA axis response to stress.

In human, early life adversity is one of the most prominent environmental factors associated with an increased risk of developing mood and anxiety disorders (Heim and Nemeroff, 2001; Gross and Hen, 2004). Previous reports indicated a direct relationship between maternal care and the development of the HPA axis and/or behaviors in rodents (Liu et al., 2000; Francis et al., 2003; Prakash et al., 2006). Early life stress is thought to act on the maturing neural circuitry to predispose an individual to a vulnerability to mood and anxiety disorders, whereas stressful events occurring in adulthood activate or amplify the expression of such symptoms (Leonardo and Hen, 2008). Supporting this notion, our data suggest that early life stress in combination with stressful events in adulthood prime the susceptibility to depression.

Role of REST4-mediated gene transcription in stress vulnerability

REST is a transcriptional regulator with genome-wide effects important for orchestrating neuronal development (Chong et al., 1995). REST4 is generated by alternative RNA splicing of the *Rest* gene, is expressed specifically in the brain (Palm et al., 1998), and may function as a dominant negative or "anti-silencer" when expressed in neuronal cells (Shimojo et al., 1999). Consistent

with these reports, our data indicate that *Rest4* mRNA was expressed only in the brain, whereas *Rest* mRNA was ubiquitously expressed in various tissues. In addition, we found that REST4 is localized to the nucleus of mPFC neurons, suggesting that it acts as a modulator of gene expression. Furthermore, we demonstrated that REST4 enhances transcription of some of the RE-1-containing genes *in vivo*. However, the results of expression analyses of RE1-containing genes were inconsistent between the HMS180 rats and REST4-overexpression mice. Maternal separation also affects the expression of other transcription factors, including the glucocorticoid receptor, mineralocorticoid receptor, and cAMP responsive element binding protein, in the brain (Ladd et al., 2004; Nair et al., 2007). Thus, the increased expressions of RE-1-containing genes in the HMS180 rats might be regulated not only by REST4, but also by other transcription factors. However at a minimum, the increased expressions of *Glur2*, *Nr1*, *Crh*, *CamKIIa*, *Adcy5*, *5htr1a*, *miR132*, *miR121* and *miR-9-3* genes in HMS180 rats might be regulated by REST4, as those expressions were elevated by REST4 overexpression. It is important to note that CRH, GluR2, NR1, CaMKIIα, Adcy5 and 5-HT1A, whose mRNA levels were upregulated in this study, are suggested to be involved in stress vulnerability and anxiety (Chen et al., 1994; Stenzel-Poore et al., 1994; Liu et al., 1997; Gross et al., 2002; Mead et al., 2006; Krishnan et al., 2008; Halene et al., 2009; Hasegawa et al., 2009). More recently, augmented maternal stimulation of pups, which results in reduced stress responses (Plotsky and Meaney, 1993), was reported to enhance REST expression and subsequent reduction of *Crh* mRNA expression in the hypothalamic paraventricular nucleus (Korosi et al., 2010). Together, these data suggest that the activation of a network of RE-1-mediated genes that is induced by an increased REST4 and decreased REST during early postnatal period may account for the development of stress vulnerability in rodents.

Another finding of this study is the increased expressions of *Glur2*, *CamKIIa*, and *Adcy5* mRNAs and of the precursors for *Mir132*, *-212* and *-124-1* in HMS180 rats both at P14 and adulthood. The mechanism for such a persistent effect of maternal separation on gene expression is unclear, but it may be due to epigenetic regulation. It has been suggested that epigenetic mechanisms underlie brain plasticity that requires stable modulation of gene expression (Tsankova et al., 2007; Flavell and Greenberg, 2008). Recent reports suggested that early life stress affects DNA methylation, one of the most intensely studied epigenetic mechanisms, of the glucocorticoid receptor and arginine vasopressin genes, the consequences of which are associated with altered gene expression (Weaver et al., 2004; Murgatroyd et al., 2009). Interestingly, promoters of the genes that encode GluR2, Adcy5, Mir132, and -212 all contain a CpG island (Myers et al., 1999; Vo et al., 2005; Kuang et al., 2008). In addition, REST can interact with the corepressor CoREST, which in turn, recruits histone deacetylases (Andrés et al., 1999; Grimes et al., 2000; Ballas et al., 2001). CoREST also interacts with methyl-CpG-binding protein 2 (MeCP2) and chromatin remodeling enzymes (Battaglioli et al., 2002; Lunyak et al., 2002; Shi et al., 2003; Roopra et al., 2004). Thus, REST regulates the transcription of its target genes via chromatin modifications by recruiting multiple cofactor complexes to the RE-1 site. We speculate that the persistent gene expression observed in the mPFC of HMS180 rats may be induced by such epigenetic mechanisms. However, it is still unclear how REST4 modulates the REST complexes and their functions in gene expression. Also, regulations of the expression of RE-1-containing genes are complex, cell type- and developmental stage-specific. Further examinations are needed to clarify the effects of early life stress on the transcription regulations of RE-1-mediated gene expression.

Role of mPFC in the development of stress vulnerability

The mPFC plays an important role in modulating the neural circuitry that mediates the HPA axis and emotional responses to stress (Arnsten, 2009; Ulrich-Lai and Herman, 2009). Early postnatal life is a critical period for development of the mPFC (Benes et al., 2000). We found increased expressions of RE-1-containing miRNAs in the mPFC of HMS180 rats, and some of their expressions were regulated by REST4 *in vivo*. This is the first report showing the altered expressions of miRNAs by early life stress and REST4. Importantly, miRNAs are strongly suggested to be involved in neuronal functions, including brain development and plasticity (Vo et al., 2005; Conaco et al., 2006; Kosik, 2006; Rajasethupathy et al., 2009). REST-controlled miRNAs (e.g., Mir132, -124) are already known to regulate neuronal morphogenesis, differentiation, and synaptic plasticity (Vo et al., 2005; Conaco et al., 2006; Rajasethupathy et al., 2009). It is interesting to note that early life stress results in a decrease in dendritic length (Pascual et al., 2007), a decrease in the number of astrocytes (Musholt et al., 2009), abnormally high synaptic density (Ovtscharoff and Braun, 2001), and an attenuated basal neuronal activity (Stevenson et al., 2008) within the mPFC. More recently, Goodfellow et al. (2009) reported an increased 5-HT1A-mediated outward current and an increased expression of *5htr1a* mRNA in the mPFC during postnatal development of maternally separated rats, the latter of which was replicated by our present expression analyses. Based on these data, we propose that an aberrant RE-1-mediated network of genes may affect structural and synaptic plasticity within the mPFC of developing animals, which then lead to the stress vulnerability in adulthood.

Conclusions

Our results support to concept that early adverse life events can modulate the neuroendocrine and behavioral responses to stress. More importantly, our data suggest that the activation of a REST4-mediated gene network in the mPFC at an early stage of postnatal development may enhance and contribute to mood and anxiety disorders in response to chronic stressful life events during adulthood. Similarities between effects in mice and rats support this conclusion.

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Journal of Psychiatric Research 44 (2010) 378–384

Contents lists available at ScienceDirect

Journal of Psychiatric Research

journal homepage: www.elsevier.com/locate/jpsychires

Aberrant REST-mediated transcriptional regulation in major depressive disorder

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ARTICLE INFO

Article history:
Received 16 June 2009
Received in revised form 10 September 2009
Accepted 24 September 2009

Keywords:
Major depression
Bipolar disorder
REST/NRSF
Gene expression

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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0022-3956/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jpsychires.2009.09.009

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).

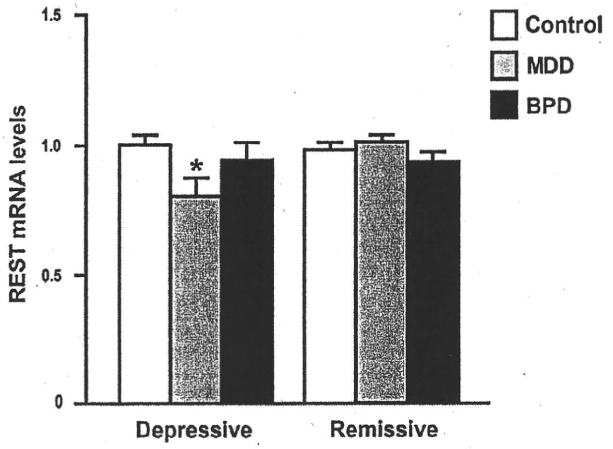

Fig. 1. The mRNA expression of REST in mood disorder patients in a depressive state and a remissive state. Quantitative real-time PCR revealed decreased mRNA expression of REST in a depressive state only in MDD patients ($n = 20$) compared with healthy control subjects ($n = 28$) but not in a remissive 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.

Next, we examined the mRNA levels of the known and putative REST target genes, including CRH, 5-HT1A, Adcy5, CaMKIIa, Epor, IGF1R, Tnfsf10, Tnfsf11, and Tnfsf12–13 in major depressive disorder patients in a current depressive state. qRT-PCR revealed that the mRNA expression of CRH was significantly increased sin major depressive disorder patients compared with healthy control subjects ($F = 5.885$, $df = 2,58$, $p = 0.005$, *post hoc* p = 0.009) (Fig. 2). In addition, Adcy5 and Tnfsf12–13 mRNA levels were significantly increased in major depressive disorder patients compared with healthy control subjects (Adcy5: $F = 3.38$, $df = 2,58$, $p = 0.041$; *post hoc* $p = 0.032$, Tnfsf12–13: $F = 13.173$, $df = 2,58$, $p < 0.001$; *post hoc* $p < 0.001$) (Fig. 2). There were no significant differences in the mRNA levels of 5-HT1A, CaMKIIa, Epor, IGF1R, Tnfsf10, and Tnfsf11 among major depressive, bipolar disorder patients and healthy control subjects (5-HT1A: $p = 0.176$, CaMKIIa: $p = 0.129$, Epor: $p = 0.413$, IGF1R: $p = 0.955$, Tnfsf10: $p = 0.166$, Tnfsf11: $p = 0.131$) (Fig. 2). We examined correlation between the mRNA expression of REST and the mRNA expression of CRH, Adcy5, and Tnfsf12–13, but there was no significant correlation (CRH: $r = 0.324$, $p = 0.164$, Adcy5: $r = 0.34$, $p = 0.143$, Tnfsf12–13: $r = -0.283$, $p = 0.227$). Next, we examined the mRNA expression of CRH, Adcy5, and Tnfsf12–13 in mood disorder patients in a remissive state. There was no significant difference in the mRNA expression of CRH, Adcy5, and Tnfsf12–13 among the three groups (CRH: $F = 0.349$, $df = 2,98$, $p = 0.706$, Adcy5: $F = 0.822$, $df = 2,98$, $p = 0.443$, Tnfsf12–13: $F = 1.592$, $df = 2,58$, $p = 0.209$) (Fig. 3). We also longitudinally examined the mRNA levels of REST, CRH, Adcy5, and Tnfsf12–13 in the same patients with major depressive ($n = 15$) and bipolar disorder ($n = 9$) before and after remission. qRT-PCR revealed that the mRNA expression of REST was significantly decreased only in patients with major depressive disorder in a depressive state compared with those in a remissive state ($t = -2.175$, $df = 28$, $p = 0.038$) (Fig. 4). The mRNA expression of CRH, Adcy5, and Tnfsf12–13 was significantly increased only in patients with major depressive disorder in a current depressive state compared with those in a remissive state (CRH: $t = 2.231$, $df = 28$, $p = 0.034$, Adcy5: $t = 2.42$, $df = 28$, $p = 0.022$, Tnfsf12–13: $t = 3.848$, $df = 28$, $p = 0.001$) (Fig. 4). There was no significant difference in the mRNA expression of REST, CRH, Adcy5 and Tnfsf12–13 in patients with bipolar disorder between a depressive and a remissive state (REST: $t = 0.68$, $df = 16$, $p = 0.506$, CRH: $t = -0.828$, $df = 16$, $p = 0.42$, Adcy5: $t = 0.789$, $df = 16$, $p = 0.442$, Tnfsf12–13: $t = 1.843$, $df = 16$, $p = 0.084$). Importantly, there was no significant difference in the length of treatment before remission between the major depressive disorder (2.93 ± 0.36 months) and the bipolar disorder patients (2.78 ± 0.41 months) ($t = 0.274$, $df = 22$, $p = 0.787$). In addition, there was no difference in the medication (imipramine equivalent) between the major depressive patients in a depressive or a remissive state ($t = 0.120$, $df = 28$, $p =$

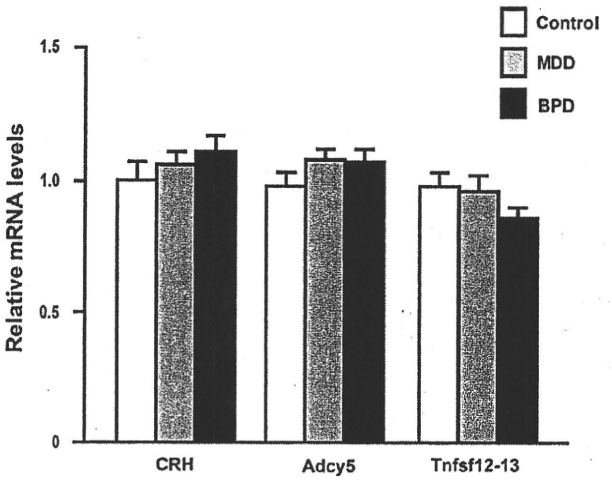

Fig. 3. The mRNA expression of CRH, Adcy5, and Tnfsf12–13 in mood disorder patients in a remissive state. There were no significant differences in the levels of CRH, Adcy5, and Tnfsf12–13 mRNAs among the three groups examined. MDD, major depressive disorder; BPD, bipolar disorder. Values are mean ± standard error.

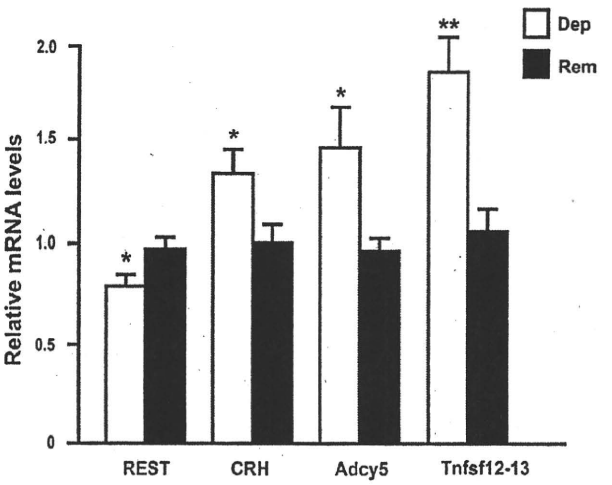

Fig. 4. The mRNA expression of REST, CRH, Adcy5, and Tnfsf12–13 in the same patients with mood disorders before and after remission. Quantitative real-time PCR analysis of REST, CRH, Adcy5, and Tnfsf12–13 mRNA levels in patients with major depressive disorder before and after remission ($n = 15$). Dep, depressive state; Rem, remissive state. Values are mean ± standard error. $^{*}p < 0.05$; $^{**}p < 0.01$ versus remissive state.

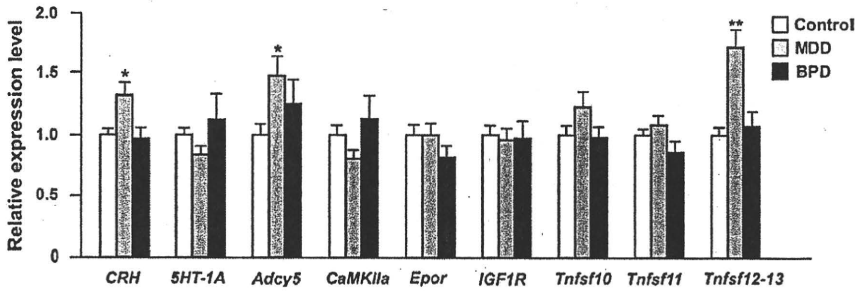

Fig. 2. The mRNA expression of target genes of REST in mood disorder patients in a depressive state. Quantitative real-time PCR revealed increased CRH, Adcy5, and Tnfsf12–13 mRNA expression only in MDD patients ($n = 20$) compared with healthy control subjects ($n = 28$). There was no significant difference in the mRNA levels of 5-HT1A, CaMKIIa, Epor, IGF1R, Tnfsf10 and Tnfsf11 mRNAs among the three groups examined. MDD, major depressive disorder; BPD, bipolar disorder. Values are mean ± standard error. $^{*}p < 0.05$; $^{**}p < 0.01$.

0.906). Furthermore, there was no correlation between the mRNA expression of REST, CRH, Adcy5 and Tnfsf12–13 and the medication (imipramine equivalent) in the major depressive patients (REST: $r = 0.076$, $p = 0.688$, CRH: $r = -0.097$, $p = 0.608$, Adcy5: $r = -0.022$, $p = 0.909$, Tnfsf12–13: $r = 0.087$, $p = 0.647$). In addition, we broadly investigated the effect of each antidepressants and sulpiride on the expression of the altered genes in the patients with major depressive disorder and bipolar disorder. There was no significant difference in the expression level of REST, CRH, Adcy5, and Tnfsf12–13 mRNA among mood disorder patients receiving any type of antidepressants (tricyclics, tetracyclics, selective serotonin reuptake inhibitor, serotonin norepinephrine reuptake inhibitor) and sulpiride (Supplement 1). These data suggest that the observed effects are unlikely to be due to the effects of the medication.

Since it has been reported that the secretion of corticosteroids is increased under conditions of chronic stress, and many patients with mood disorders have disturbed regulation of the hypothalamic–pituitary–adrenal axis (de Kloet et al., 2005), we investigated the correlation between CRH mRNA levels and the serum cortisol concentration. There was no significant correlation between CRH mRNA levels and the serum cortisol concentration for healthy control subjects, major depressive disorder patients or bipolar disorder patients (data not shown). Further, for other altered genes (REST, Adcy5, Tnfsf12–13), there was no significant correlation between the serum cortisol concentration and the mRNA expression levels (data not shown). To examine the association between CRH mRNA levels and hypothalamic–pituitary–adrenal axis activity; the mRNA levels of CRH in mood disorder patients in a current depressive state were compared between suppressors and non-suppressors of the Dex/CRH test. There was no significant difference in the expression levels of CRH mRNA between suppressors and non-suppressors (data not shown). Further, for other altered genes (REST, Adcy5, Tnfsf12–13), there was no significant difference in the mRNA expression levels between suppressors and non-suppressors (data not shown).

To examine the change of gene expression in relationship to depression severity, we investigated the correlation between HDRS score and the mRNA expression level of the altered genes in major depression. There was no significant correlation between HDRS score and the mRNA expression of REST, CRH, Adcy5, and Tnfsf12–13 in major depression (REST: $r = 0.092$, $p = 0.699$, CRH: $r = -0.259$, $p = 0.271$, Adcy5: $r = 0.076$, $p = 0.751$, Tnfsf12–13: $r = 0.084$, $p = 0.725$). We also examined the influence of the number of previous episodes on the mRNA expression of the altered genes in mood disorders. There was no significant correlation between the number of previous episodes and the mRNA expression of REST, CRH, Adcy5, and Tnfsf12–13 both in major depression (REST: $r = -0.291$, $p = 0.213$, CRH: $r = 0.222$, $p = 0.346$, Adcy5: $r = -0.365$, $p = 0.114$, Tnfsf12–13: $r = 0.249$, $p = 0.289$), and in bipolar disorder (REST: $r = 0.112$, $p = 0.716$, CRH: $r = -0.252$, $p = 0.406$, Adcy5: $r = -0.239$, $p = 0.433$, Tnfsf12–13: $r = -0.386$, $p = 0.193$).

4. Discussion

In the present study, we found reduced mRNA expression of REST and increased mRNA expression of CRH, Adcy5, and Tnfsf12–13 in peripheral white blood cells of major depressive disorder patients in a current depressive state. In contrast, in a remissive state, the mRNA expression of REST, CRH, Adcy5 and Tnfsf12–13 in major depressive disorder patients were comparable to those of healthy controls. These results suggest that the reduced mRNA expression of REST and the increased mRNA expression of CRH, Adcy5, and Tnfsf12–13 are state-dependent, and that the altered mRNA levels of these genes are independent of genetic factors, such as single-nucleotide polymorphisms, but are affected by epigenetic factors.

A limitation of our study is that the majority of the patients were on medication, thus we cannot exclude the influence of medication on the mRNA expression of the examined genes. Previous reports have indicated that acute treatment with fluoxetine did not alter CRH mRNA levels in the paraventricular nucleus (PVN) of mice and chronic treatment with imipramine decreased CRH mRNA levels in the PVN of rats (Brady et al., 1991; Stewart et al., 2008). In our study, however, the mRNA levels of CRH were only affected in a depressive state and not in a remissive state. In addition, we found that there was a significant difference in the expression of CRH mRNA, as well as Adcy5 and Tnfsf12–13 mRNAs, in the same patients with major depressive disorder before and after remission, although they received similar medication before and after remission. It is important to note that there was no correlation between the mRNA expression of CRH, Adcy5 and Tnfsf12–13 and medications in the same patients with major depressive disorder before and after remission. There was no significant difference in the expression of mRNA for REST, CRH, Adcy5, and Tnfsf12–13 among mood disorder patients receiving any type of antidepressants and sulpiride. These results suggest that there is no effect of medications on the mRNA expression of CRH, Adcy5 and Tnfsf12–13, and the observed alterations of mRNA expression is likely to be due to the disease state.

Bioinformatic studies have shown that there are more than 1300 putative RE-1 sites in the human genome (Bruce et al., 2004; Johnson et al., 2006). In this study, we examined the mRNA expression of nine selected genes that are reported to have putative RE-1 sites within their regulatory regions (Otto et al., 2007). Among them, the transcriptional activity of CRH has been reported to be repressed by REST (Seth and Majzoub, 2001). In addition, Adcy5 and Tnfsf12–13 were identified as REST target genes by using the serial analysis of chromatin occupancy (SACO) methodology (Otto et al., 2007). Thus, we suggest that the increased mRNA expression of CRH, as well as Adcy5 and Tnfsf12–13, in major depressive disorder patients would be the result of, at least in part, the dysregulation of REST. However, we could not detect any alteration in the mRNA expression of 5-HT1A, CaMKIIa, Epor, IGF1R, Tnfsf10 and Tnfsf11 in major depressive disorder patients, despite the fact that these genes also contain functional and/or putative RE-1 sites within their regulatory regions (Lemonde et al., 2004; Otto et al., 2007). In addition, although the expression of brain-derived neurotrophic factor and L1 cell adhesion molecule is known to be modulated by REST (Kallunki et al., 1997; Timmusk et al., 1999), our previous studies have indicated that the expression levels of these mRNAs in major depressive disorder patients are comparable to those in healthy controls (Otsuki et al., 2008; Wakabayashi et al., 2008). The reason for this contradiction is unclear, but some reports suggest that REST-mediated transcriptional regulation is cell-type specific. Several groups have suggested that REST can act as either a repressor or an activator, depending on the spatial and temporal context of its expression (Bessis et al., 1997; Chen et al., 1998; Palm et al., 1998; Kallunki et al., 1998). Thus, further study is required to clarify the molecular mechanism of REST in the regulation of target gene expression.

Dysfunction of the CRH system is well documented with mood disorders. Central administration of CRH to rodents as well as CRH overexpression in transgenic mice resulted in behavioral changes including anxiety- and depression-related symptoms (Britton et al., 1986; Pepin et al., 1992; Stenzel-Poore et al., 1994; Strohle et al., 1998). In humans, CRH levels was elevated in the cerebrospinal fluid of patients with depression (Nemeroff et al., 1984; Landgraf, 2006), and there was an elevated number of CRH-producing

neurons in the PVN of patients with depression (Raadsheer et al., 1994). Besides central CRH, several studies reported that lymphocytes are important sources of immunoreactive CRH (Ekman et al., 1993; Kravchenco and Furalev, 1994) and CRH has suggested to be involved in the production of cytokines (Singh and Leu, 1990). Furthermore, several reports showed that increased concentration of several cytokines including tumor necrosis factor-α (TNF-α), interleukin-1β, and interleukin-6 in the peripheral blood of major depressive disorder patients (Mikova et al., 2001; Alesci et al., 2005; Thomas et al., 2005). Taken together with our observations, the increased CRH mRNA in white blood cells may be involved in the upregulation of cytokines. The TNF superfamily activates signaling pathways for cell survival, death, and differentiation that orchestrate the development, organization and homeostasis of lymphoid, mammary, neuronal and ectodermal tissues (Ware, 2003). To our knowledge, this is the first report indicating increased mRNA expression of Tnfsf12–13 in mood disorder patients. Adenylate cyclases can regulate the production of cyclic adenosine monophosphate, a key intracellular second messenger, in response to calcium entry. Recent reports have indicated that Adcy5 knockout mice showed anxiolytic and antidepressant phenotypes, suggesting that a selective Adcy5 antagonist could be of therapeutic value against depression and anxiety (Krishnan et al., 2008). Thus, these results suggest that the altered expression of CRH, Adcy5 and Tnfsf12–13 might be associated with the pathophysiology of major depression.

Although it is unknown whether REST expression is also changed in the brain of major depressive disorder patients, it should be noted that there have been an increasing number of molecules that showed altered expression in both the brain and peripheral blood cells of mood disorder patients, including glucocorticoid receptor α mRNA, heat shock protein 40, PCNT2, and LIM protein (Webster et al., 2002; Iwamoto et al., 2004; Knable et al., 2004; Matsubara et al., 2006; Anitha et al., 2008). To confirm the relevance of the altered mRNA expression of REST, as well as CRH, Adcy5 and Tnfsf12–13 in the pathophysiology of mood disorders, it is necessary to examine the expression level of these genes in postmortem brains of depressed suicide victims, and establish an animal model for mood disorders, in which the REST gene and/or its target genes are genetically modified. Recently, gene expression analysis using mRNA isolated from the peripheral leukocytes of neuropsychiatric disorder patients has been widely studied (Iga et al., 2007; Anitha et al., 2008; Miller et al., 2008; Su et al., 2009). These studies will give us clues to assess mood disorders because circulating peripheral leukocytes are influenced by systems that underlie mood disorders, and the quantification of their mRNAs is methodologically precise and easier than that of protein (Iga et al., 2008).

In conclusion, our results suggest that the changes in mRNA expression of REST and its target genes, CRH, Adcy5, and Tnfsf12–13, in peripheral white blood cells might be state-dependent and associated with the pathophysiology of major depression. These genes could be important biological markers for the differentiation between major depressive disorder and bipolar disorder.

Conflict of interest

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

Contributors

K. Otsuki, S. Uchida and Y. Watanabe designated the research. K. Otsuki, Y. Wakabayashi, T. Matsubara, T. Hobara, and H. Funato performed the experiments. The manuscript was written by K. Otsuki, S. Uchida and Y. Watanabe. All authors discussed results and commented on the manuscript.

Role of funding sources

This study was supported in part by a Grant-in-Aid for Scientific Research from Japanese Ministry of Education, Culture, Sports, Science and Technology and a grant for Research on Psychiatric and Neurological Diseases and Mental Health from Japanese Ministry of Health, Labor and Welfare.

Acknowledgements

The authors thank Ms. Ayumi Kobayashi and Ms. Kumiko Hara for their excellent technical assistance.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jpsychires.2009.09.009.

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Journal of Psychiatric Research 44 (2010) 263–270

Contents lists available at ScienceDirect

Journal of Psychiatric Research

journal homepage: www.elsevier.com/locate/jpsychires

Altered gene expression of histone deacetylases in mood disorder patients

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ARTICLE INFO

Article history:
Received 8 May 2009
Received in revised form 18 August 2009
Accepted 27 August 2009

Keywords:
Major depression
Bipolar depression
Histone deacetylase
Leukocyte
Chromatin regulation
Gene expression

ABSTRACT

Chromatin remodeling such as changes in histone acetylation has been suggested to play an important role in the pathophysiology and treatment of mood disorders. In the present study, we investigated whether the expression of histone deacetylase (HDAC) genes are altered in mood disorder patients. We used quantitative real-time PCR to measure the mRNA levels of 11 HDACs (HDAC1-11) in peripheral white blood cells of major depressive disorder (MDD) and bipolar disorder (BPD) patients during depressive and remissive episodes and in the first-degree relatives of BPD patients. In addition, we investigated the effect of antidepressants and mood stabilizers on the mRNA levels of HDACs using mice. In MDD, the expression of HDAC2 and −5 mRNA was increased in a depressive state, but not in a remissive state, compared to controls. In BPD, the expression of HDAC4 mRNA was increased only in a depressive state, and the expression of HDAC6 and −8 was decreased in both depressive and remissive states compared to controls, whereas the first-degree relatives did not show any significant alteration in expression levels. Animal study showed that the expression of HDAC2 and −5 or HDAC4, −6 and −8 mRNAs in the mouse leukocytes were not affected by chronic treatment with antidepressants or mood stabilizers. Our data suggest that aberrant transcriptional regulation caused by the altered expression of HDACs is associated with the pathophysiology of mood disorders.

1. Introduction

There is growing evidence demonstrating that aberrant transcriptional regulation is one of the key components of the pathophysiology of many neuropsychiatric disorders, including mood disorders, schizophrenia and drug addiction (Colvis et al., 2005; Tsankova et al., 2007). The altered expression and/or activity of a variety of transcription factors such as cAMP-responsive element binding protein (CREB) and glucocorticoid receptor (GR) have been reported in patients with mood disorder, as well as in stressed animals (Carlezon et al., 2005; de Kloet et al., 2005; Holsboer, 2000; Laifenfeld et al., 2005; Nestler et al., 2002; Webster et al., 2002). In addition, antidepressant treatment and electroconvulsive therapy alter gene expression patterns in the brain (Coyle and Duman, 2003; Manji et al., 1999). Mood disorder patients and chronically stressed humans have also been reported to show altered gene expression in peripheral blood cells (Anitha et al., 2008; Matsubara et al., 2006; Miller et al., 2008; Otsuki et al., 2008).

Chromatin remodeling is suggested to play important roles in many phenomena in the brain, including circadian rhythm (Etchegaray et al., 2003), memory formation (Guan et al., 2002; Korzus et al., 2004; Levenson and Sweatt, 2005), drug addiction (Kumar et al., 2005) and depression (Berton and Nestler, 2006; Newton and Duman, 2006; Tsankova et al., 2006). It has been widely recognized that changes in chromatin structure play a role in the regulation of gene expression attributable to covalent histone modification via acetylation and deacetylation (Grunstein, 1997; Hsieh and Gage, 2005). Histone deacetylases (HDACs) are one of the major enzymes involved in chromatin remodeling (Grunstein, 1997; Hsieh and Gage, 2005). HDACs can remove acetyl groups from lysine/arginine residues in the amino-terminal tails of core histones, thereby allowing histones in the deacetylated state to pack DNA into more condensed chromatin, which prevents access of transcriptional activators to their target sites, resulting in transcriptional repression (Grunstein, 1997; Hsieh and Gage, 2005).

A recent report has indicated that hippocampal HDAC5 mRNA is downregulated after chronic treatment with the tricyclic antidepressant imipramine, and viral-mediated HDAC5 overexpression in the hippocampus blocks the effect of imipramine on depression-like behaviors in mice (Tsankova et al., 2006). Another report suggested that sodium butyrate, an HDAC inhibitor, has antidepressant-like effects on behavioral despair in mice (Schroeder et al., 2007). Valproate, a therapeutic agent for bipolar disorder, is a direct inhibitor of HDAC (Gottlicher et al., 2001; Phiel et al.,

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doi:10.1016/j.jpsychires.2009.08.015

2001). These observations suggest that HDACs are involved in the pathophysiology of mood disorders and the action of antidepressants and mood stabilizers. However, there is very little understanding of the role of HDACs in mood disorders.

In this study, we aimed to determine whether there are alterations in the expression of multiple HDAC mRNA in peripheral white blood cells of mood disorder patients. Also, to examine whether the altered mRNA expression of HDACs is state- or trait-dependent, mood disorder patients in remission and their first-degree relatives were also assessed. We also examined the mRNA levels of HDACs in mice receiving antidepressants or mood stabilizers to investigate the effects of these drugs on the expression of HDACs.

2. Methods and materials

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 (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 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, renal, liver or thyroid function. Female subjects who were pregnant or took oral contraceptives were also excluded. All healthy controls subjects and the first-degree relatives of bipolar disorders did not have any past history of mental illness. 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 am and 11:00 am and processed for total RNA purification from peripheral blood cells by using the QIAamp RNA Blood Mini Kit (Qiagen, Chatsworth, California) according to the manufacturer's manual. The quality of RNA was determined based on A_{260}/A_{280} ratio, which was 1.7–2.0 for all RNA preparations.

2.3. Quantitative real-time polymerase chain reaction

Quantitative real-time polymerase chain reaction (Q-PCR) was performed in the Applied Biosystems 7300 Fast Real-Time PCR System with SYBR green PCR master mix (Applied Biosystems, Foster City, California) as previously reported (Otsuki et al., 2008; Uchida et al., 2008). Supplementary Material 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

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

2.6. Animals study

2.6.1. Animals

The animal studies were performed according to the Guidelines for Animal Care and Use at Yamaguchi University School of Medicine. Experimental protocols were approved by the Committee on the Ethics of Animal Experiments at Yamaguchi University School of Medicine. Male C57BL/6 and BALB/c mice (Charles River Japan, Kanagawa, Japan), eight weeks old, were housed in groups of five in plastic cages with a 12 h light/dark schedule at 24 °C, 60% humidity, with food and tap water available *ad libitum*.

2.6.2. Drugs

Paroxetine (Wako Pure Chemical Industries, Saitama, Japan) and clomipramine (Sigma, St. Louis, Missouri) were dissolved in tap water at a concentration of 40 or 80 and 80 or 160 mg/L, respectively. These concentrations were estimated to achieve a final dose of 6 or 10 (paroxetine) and 10 or 19 (clomipramine) mg/kg/day based on the average drinking amount and average weight of the mice used in the study. Lithium chloride and valproate (Sigma) were dissolved in tap water at a concentration of 600 mg/L and 0.4% w/v, respectively. These concentrations were estimated to achieve a final dose of 90 (lithium) and 650 (valproate) mg/kg/day. These doses are generally used and achieve the therapeutic serum or plasma levels (Dehpour et al., 2000; Kelley, 2007; Shabbeer et al., 2007). Drug solutions were protected from light in opaque water bottles. Vehicle-treated animals received regular (tap) drinking water. The drug solutions were replaced every 3 days.

2.6.3. Forced swim test

Each mouse was placed in a glass cylinder (height, 23 cm; diameter, 11 cm) containing 15 cm of water at 22–23 °C for 5 min. At the end of each videotaped trial, immobility time (floating) was measured. A mouse was judged immobile when it stopped any movements except those that were necessary to keep its head above water.

2.6.4. RNA extraction, cDNA synthesis and Q-PCR

Mice were killed under anesthesia and their trunk blood was collected in heparinized tubes. Total RNA from the lymphocytes was extracted using the PureLink Total RNA Blood Purification Kit (Invitrogen, Carlsbad, California), and treated with DNase (DNA-free kit, Applied Biosystems), according to the manufacturer's protocols. One hundred nanograms of total RNA were used for cDNA synthesis by the QuantiTect Reverse Transcription kit (Qiagen), according to the manufacturer's protocol. Q-PCR experiments were performed as described above, and the primer sequences used are shown in Supplementary Material 1.

2.7. 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). Multivariable analysis was conducted using the mRNA level of each HDAC as a dependent variable and with age, gender, and type of drugs used (antidepressants and mood stabilizers) as independent variables. Gender distribution was analyzed by the χ^2 test. The mRNA levels of HDACs were subjected to one-way analysis of variance (ANOVA) followed by post hoc analysis (Dunnett test). Two group comparisons in the same patients of mood disorders before and after remission and in the suppressors and non-suppressors of the Dex/CRH test on the mRNA levels of HDACs were performed using the paired *t* test and unpaired *t* test, respectively. Two group comparisons in the first-degree relatives of bipolar disorder patients and controls on the mRNA levels of HDACs were performed using the Mann–Whitney U-test. In all cases, *p* – values were two-tailed, and comparisons were considered to be statistically significant for $p < 0.05$.

3. Results

3.1. mRNA levels of HDACs in mood disorder patients

Table 1 shows the demographic and clinical characteristics of the subjects. The majority of the patients were on medication. The mean ages were not significantly different among major depressive disorder patients, bipolar depressive patients and healthy control subjects [$F(4,127) = 1.164$, $p = 0.330$]. Regarding the gender distribution, bipolar disorder patients showed a significantly larger ratio of females to males ($\chi^2 = 16.20$, $p < 0.001$). In multivariable analyses ($n = 104$), the mRNA levels of all the HDACs examined were not associated with age, gender or type of drugs used (antidepressants or mood stabilizers) (data not shown).

We examined the expression levels of the HDAC members (HDAC1-11) in patients with mood disorder in a current depressive (Fig. 1A) and a remissive state (Fig. 1B). Q-PCR experiments revealed that the expression of HDAC2 and −5 mRNA were significantly increased in major depressive disorder patients in a depressive state compared to healthy control subjects [HDAC2, $F(2,57) = 17.376$, $p < 0.001$; HDAC5, $F(2,57) = 7.549$, $p = 0.001$] (Fig. 1A). The expression of HDAC4 mRNA was significantly increased in bipolar disorder patients in a depressive state [$F(2,57) = 54.175$, $p = 0.002$], whereas HDAC6 and −8 mRNA levels were significantly decreased compared to those of healthy control subjects [HDAC6, $F(2,57) = 6.804$, $p = 0.002$; HDAC8, $F(2,57) = 4.205$, $p = 0.002$] (Fig. 1A). In a remissive state, the expression of HDAC2 and −5 mRNA in major depressive disorder patients was comparable to those of healthy control subjects [HDAC2, $F(2,97) = 0.027$, $p = 0.975$; HDAC5, $F(2,97) = 3.35$, $p = 0.506$], meanwhile, HDAC8 and −9 mRNA levels were decreased compared to those of healthy control subjects [HDAC8, $F(2,97) = 4.51$, $p = 0.025$; HDAC9, $F(2,97) = 11.16$, $p = 0.004$] (Fig. 1B). In the bipolar disorder patients, there was no significant difference in the expression of HDAC4 mRNA compared to healthy control subjects [HDAC4: $F(2,97) = 0.766$, $p = 0.506$] (Fig. 1B). In contrast, the expression of HDAC6 and −8 mRNA was significantly decreased compared to healthy control subjects [HDAC6, $F(2,97) = 3.52$, $p = 0.033$; HDAC8, $F(2,97) = 4.512$, $p = 0.013$] (Fig. 1B), as observed in a depressive state (Fig. 1A). The expression levels of HDAC5, −7, −9 and −10 mRNA in bipolar disorder patients in a remissive state were significantly decreased compared to those of healthy control subjects [HDAC5, $F(2,97) = 3.35$, $p = 0.032$; HDAC7, $F(2,97) = 5.778$, $p = 0.003$; HDAC9, $F(2,97) = 9.106$, $p < 0.001$; HDAC10, $F(2,97) = 5.229$, $p = 0.005$] (Fig. 1B).

We next examined the mRNA levels of HDACs in the same patients with major depressive disorder ($n = 15$) or bipolar disorder ($n = 11$) before and after remission. In major depressive disorder patients, the expression levels of HDAC2 and −5 mRNA were significantly decreased in a remissive state compared with those in a depressive state (HDAC2, $t = 5.277$, $p < 0.001$; HDAC5, $t = 5.085$, $p < 0.001$) (Fig. 2A and B). However, the expression of HDAC8 and −9 mRNA did not differ between the depressive and remissive state of major depressive disorder patients (Fig. 2C and D). In bipolar disorder patients, the expression levels of HDAC4, −5, −7 and −9 mRNA were significantly decreased in the bipolar disorder patients in a remissive state compared with those in a depressive state (HDAC4, $t = 4.419$, $p < 0.001$; HDAC5, $t = 4.398$, $p < 0.001$; HDAC7, $t = 4.356$, $p = 0.01$; HDAC9, $t = 4.366$, $p = 0.012$) (Fig. 2E, F, H and J). In contrast, there was no significant difference in the expression of HDAC6 and −8 mRNA, whose expression was decreased in both depressive and remissive states of bipolar disorder patients (Fig. 1), in the same patients before and after remission (HDAC6, $t = 4.042$, $p = 0.104$; HDAC8, $t = 3.742$, $p = 0.505$) (Fig. 2G and I). These results suggest that the increased expression of HDAC2 and −5 mRNA were state-dependent in major depressive disorder patients. In addition, the increased expression of HDAC4 mRNA and the decreased expression of HDAC6 and −8 mRNA in bipolar disorder patients are suggested to be state- and trait-dependent, respectively. It is possible that the decreased expression of HDAC5, −7 and −9 mRNA in a remissive state may be a biological marker for the therapeutic action of drugs in bipolar disorder patients.

As the expression of HDAC6 and −8 mRNA were decreased in bipolar disorder patients in both remissive and depressive states,

Table 1
Demographic and clinical characteristics of subjects.

	Controls ($n = 28$)	Patients				Relatives
		MDD		BPD		BPD
		Depressed $n = 20$	Remission $n = 39$	Depressed $n = 12$	Remission $n = 33$	n = 13
Age (years)	50.0 ± 1.8	52.3 ± 3.5	54.8 ± 2.2	54.8 ± 3.9	52.7 ± 2.6	42.5 ± 4.9
Gender (Male/Female)	15/13	10/10	15/24	2/10	7/26	5/8
HDRS		25.9 ± 1.9	3.2 ± 0.2	24.5 ± 1.1	2.8 ± 0.2	
Serum cortisol (mg/dl)	8.6 ± 0.8	10.3 ± 1.3	7.3 ± 1.3	11.2 ± 3.0	10.3 ± 0.9	
Medication						
No medication	28	3	4	1	0	13
SSRI	0	5	24	3	6	0
TCA	0	8	7	1	3	0
Li	0	0	0	3	17	0
VPA	0	0	0	7	15	0
CBZ	0	0	0	2	8	0

MDD, major depressive disorder; BPD, bipolar disorder; Relatives, first-degree relatives of BPD patients; HDRS, Hamilton Depression Rating Scale; SSRI, selective serotonin reuptake inhibitor; TCA, tricyclic antidepressant; Li, lithium; VPA, valproic acid; CBZ, carbamazepine.

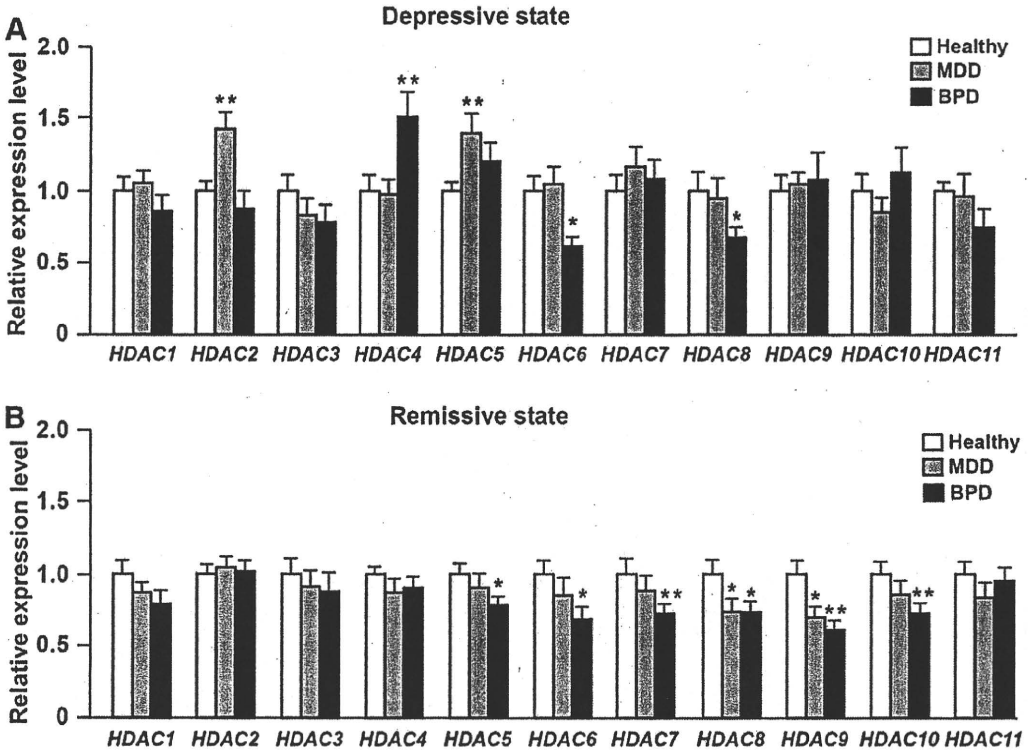

Fig. 1. mRNA levels of HDACs in mood disorder patients in a depressive and remissive state. (A) Quantitative real-time PCR analysis of mRNA levels of HDACs in major depressive disorder ($n = 20$) and bipolar disorder patients ($n = 12$) in a current depressive state and healthy control subjects ($n = 28$). (B) Quantitative real-time PCR analysis of mRNA levels of HDACs in major depressive disorder ($n = 39$) and bipolar disorder patients ($n = 33$) in a remissive state and healthy control subjects ($n = 28$). Values are mean ± standard error. $^{*}p < 0.05$; $^{**}p < 0.01$ versus controls. MDD; major depressive disorder patients, BPD; bipolar disorder patients.

we also examined the expression of these mRNAs in the first-degree relatives of bipolar disorder patients. However, the expression of these mRNAs was similar to those of controls (Mann–Whitney test: HDAC6, $U = 173.0$, $p = 0.814$; HDAC8, $U = 142.0$, $p = 0.272$) (Fig. 3).

3.2. mRNA levels of HDACs and the HPA axis

Dysfunction of the hypothalamic–pituitary–adrenal (HPA) system is the most characteristic biological alteration found in the majority of depressed patients. Accumulating evidence suggests that the combined Dex/CRH test is highly sensitive and is able to detect HPA system abnormalities (Holsboer et al., 1995). ACTH and cortisol responses to this test are exaggerated in depressed patients (Heuser et al., 1994, 1996). To examine the association between the mRNA levels of HDACs and HPA axis activity, the mRNA levels for HDACs of mood disorder patients in a current remissive state were compared between suppressors ($n = 10$; 8 major depressive disorder patients and 2 bipolar disorder patients) and non-suppressors ($n = 10$; 3 major depressive disorder patients and 7 bipolar disorder patients) of the Dex/CRH test. There was no significant difference in the expression levels of any HDACs between suppressors and non-suppressors (Supplementary Material 2. In addition, there was no significant correlation between the mRNA levels of HDACs and the serum cortisol concentration in healthy control subjects, major depressive disorder patients or bipolar disorder patients (data not shown).

3.3. Effect of antidepressants and mood stabilizers on the expression of mRNA for HDACs in humans

There was no significant difference in the expression of mRNA for HDACs among mood disorder patients receiving any type of antidepressants (tricyclics, tetracyclics, SSRI, SNRI) and sulpiride (Supplementary Material 3), as well as mood stabilizers (lithium, valproate, carbamazepine) (Supplementary Material 4). In addition, there was no significant difference in the medication (imipramine equivalent) of the patients with major depression and bipolar disorders between a current depressive and a remissive state (major depressive disorder; $t = 0.779$, $df = 28$, $p = 0.205$; bipolar disorder; $t = 0.530$, $df = 28$, $p = 0.751$). There was also no correlation between the expression level of each HDAC mRNA and the medication (imipramine equivalent) in patients with major depressive disorders and bipolar disorders (data not shown). These data suggest that the observed alterations of HDAC mRNA expression in the patients are unlikely to be due to the effects of the medication.

3.4. Effect of antidepressants and mood stabilizers on the mRNA levels of HDACs in the mouse

To address more directly the question whether antidepressants and mood stabilizers could affect the expression of HDAC2, −4, −5, −6 and −8 mRNAs, whose expression was altered in the leukocytes of mood disorder patients, we examined the mRNA levels of these HDACs in the leukocytes of mice receiving antidepressants (clomipramine and paroxetine) or mood stabilizers (lithium chloride and valproate) for 21 days. First, we evaluated the antidepressant effects of clomipramine, paroxetine, lithium and valproate using the forced swim test, which is highly reliable and has a strong predictive validity for antidepressant compounds (Cryan et al., 2002; Dulawa et al., 2004; Porsolt et al., 1977). C57BL/6 mice receiving both doses of clomipramine (10 or 19 mg/kg/day) or paroxetine (6 or 10 mg/kg/day) did not show a reduction of immobility time in the forced swim test (Supplementary Material 5A). In contrast, BALB/c mice receiving clomipramine (19 mg/kg/day) or paroxetine

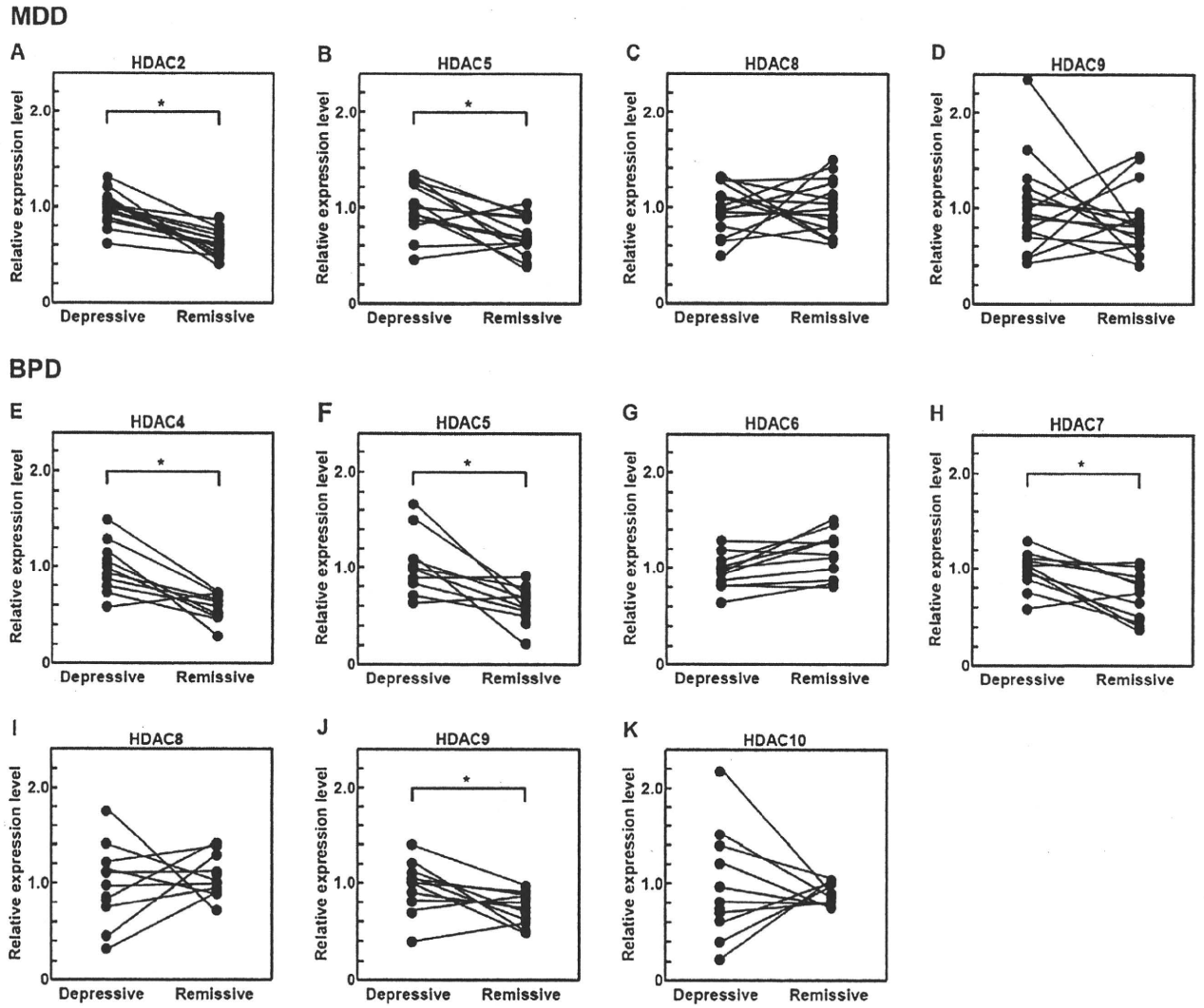

Fig. 2. mRNA levels of HDACs in the same patients with mood disorders before and after remission. (A–D) Quantitative real-time PCR analysis of mRNA levels of HDACs in patients with major depressive disorder before and after remission ($n = 15$). (E–K) Quantitative real-time PCR analysis of mRNA levels of HDACs in patients with bipolar disorder before and after remission ($n = 11$). Values are mean ± standard error. $^{*}p < 0.05$ versus depressive state. MDD; major depressive disorder patients, BPD; bipolar disorder patients.

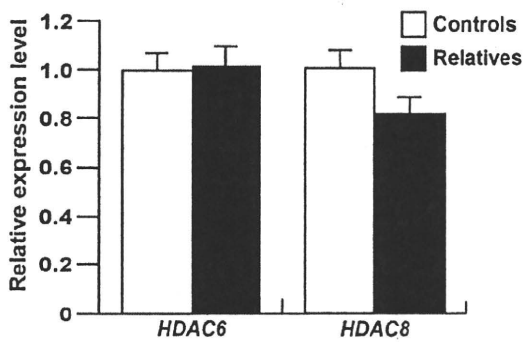

Fig. 3. The expression of HDAC6 and −8 mRNA in the first-degree relatives of bipolar disorder patients. Quantitative real-time PCR analysis revealed no significant change in either HDAC6 or −8 mRNA expression in the first-degree relatives of bipolar disorder patients ($n = 13$), who did not have a past history of mental illness, compared to controls ($n = 28$). Values are mean ± standard error.

(10 mg/kg/day) showed a reduction of immobility time (clomipramine, $p < 0.001$; paroxetine, $p < 0.001$) (Supplementary Material 5B). These results were consistent with a previous report showing that the BALB/c strain exhibited sensitivity to fluoxetine in the forced swim test, but not the C57BL/6 strain (Dulawa et al., 2004). In addition, both strains of mice receiving lithium (90 mg/kg/day) showed a reduced immobility time (C57BL/6, $p < 0.001$; BALB/c, $p < 0.001$), whereas valproate (650 mg/kg/day) did not affect the immobility time in both strains (C57BL/6, $p = 1.0$; BALB/c, $p = 0.67$) (Supplementary Material 5A and B). Therefore, we used the BALB/c strain for the expression analysis of the mRNA for HDACs. The expression of HDAC2 and −5 mRNA in the lymphocytes of mice receiving either clomipramine or paroxetine were comparable to those of control mice [HDAC2, $F(2,27) = 0.335$, $p = 0.718$; HDAC5, $F(2,27) = 0.169$, $p = 0.846$] (Fig. 4A). Also, the expression of HDAC4, −6 and −8 mRNA in the lymphocytes of mice receiving either lithium or valproate were comparable to those of control mice [HDAC4, $F(2,27) = 0.120$, $p = 0.887$; HDAC6, $F(2,27) = 0.203$, $p = 0.817$; HDAC8, $F(2,27) = 0.361$, $p = 0.700$] (Fig. 4B). These results suggest that the medication *per se* does not have a causal role in the altered expression of HDAC2, −4, −5, −6, and −8 mRNA in mood disorder patients.

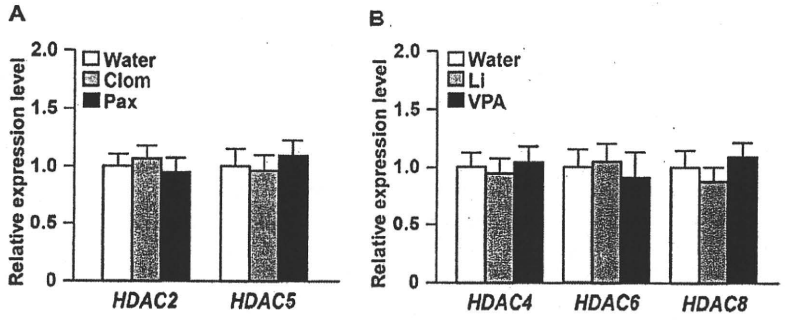

Fig. 4. Effects of antidepressants and mood stabilizers on the mRNA levels of HDACs in murine leukocytes. (A) Quantitative real-time PCR analysis of HDAC2 and −5 mRNA expression levels in mice receiving clomipramine (Clom) or paroxetine (Pax) for 21 days. (B) Quantitative real-time PCR analysis of HDAC4, −6, and −8 mRNA expression levels in mice receiving lithium (Li) or valproate (VPA) for 21 days. Values are mean ± standard error.

4. Discussion

In this study, we found different alteration pattern of HDACs mRNA expression levels in peripheral blood cells of major depressive disorder and bipolar disorder patients. Our data clearly suggest that the increased expression of HDAC2 and −5 mRNA is state-dependent in major depressive disorder patients. In bipolar disorder patients, the increased expression of HDAC4 mRNA is state-dependent, whereas the reduction of HDAC6 and −8 mRNA expression is trait-dependent.

Although we found the altered expression of HDAC8 and −9 mRNA in major depressive disorder patients in a current remissive state, but not in a current depressive state, compared to healthy controls (Fig. 1B), there was no significant difference in the expression level of these mRNA in the same patients with major depressive disorder before and after remission (Fig. 2C and D). Similar discrepancy was also observed regarding the expression of HDAC10 mRNA in bipolar disorder patients (Figs. 1B and 2K). These discrepancies could be due to the small size of subjects, and further study is needed to resolve this issue.

The principal limitation of this study is that the majority of patients were on medication, so that we should consider the effect of therapeutic agents used on the mRNA levels of HDACs. A previous report has indicated that the hippocampal HDAC9 and −5 mRNA expression levels were decreased in non-stressed and socially defeated mice receiving imipramine, respectively, whereas hippocampal HDAC1, −2, −4 and −7 mRNA levels were not altered by imipramine in both non-stressed and stressed mice (Tsankova et al., 2006). In addition, one report has shown that HDAC5 mRNA levels in peripheral leukocytes of major depressive disorder patients without any antidepressants treatment were significantly higher than those of control subjects, and were significantly decreased to baseline by paroxetine treatment for 8 weeks (Iga et al., 2007). These reports suggest that antidepressant treatment could be responsible for the reduction of HDAC5 mRNA levels observed in our major depressive disorder patients in a remissive state compared to those in a depressive state. However, it should be noted that there was no difference in the medication (imipramine equivalent) of our patients with major depressive disorder in between a current depressive and a remissive state. Further, our data with mice indicate that the expression of HDAC2, −4, −5, −6, and −8 mRNA was not affected by treatment with clomipramine, paroxetine, lithium or valproate. Taken together, these results suggest that the observed alterations, at least, in the expression of HDAC2, −4, −5, −6 and −8 mRNAs in the mood disorder patients are unlikely to be due to the effects of the dugs used.

It has been reported that HDAC2 expression and its activity were increased by treatment with Dex, a GR agonist, *in vitro* (Ito et al., 2000), suggesting that activated GR enhances HDAC2 expression. However, our patients used in this study did not show the hypercortisolaemia (Table 1), and the reduced expression of GRα mRNA in mood disorder patients that was reported previously (Matsubara et al., 2006). In addition, there was no significant correlation between GRα and HDAC2 mRNA expressions in the peripheral blood cells of mood disorder patients as well as healthy controls (data not shown). These results suggest that the increased expression of HDAC2 in the major depressive disorder patients was in a glucocorticoid-independent manner. There is little understanding of the transcriptional mechanisms for the expression of the other HDAC genes, thus the molecular mechanisms responsible for the altered mRNA levels of HDACs in the mood disorder patients remain unclear, and further studies are required to resolve this issue.

HDACs are known to be recruited by multiple transcription factors, leading to the deacetylation of histones, resulting in transcriptional repression. For example, HDAC2 was reported to be recruited by the repressor element-1 silencing transcription factor/neuron-restrictive silencing factor (Ballas et al., 2001), which has been reported to have a variety of putative target genes, including brain-derived neurotrophic factor (BDNF), CRH, tryptophan hydroxylase 2, artemin and L1cam (Otto et al., 2007), which are suggested to be associated with mood disorders in humans and depressive states in rodents (Berton and Nestler, 2006; Clark et al., 2008; Law et al., 2003; Otsuki et al., 2008; Wakabayashi et al., 2008; Zhang et al., 2005). Also, chronic, but not acute, treatment with imipramine increased histone acetylation at specific promoters of the gene encoding BDNF, in part by reducing HDAC5 mRNA expression in the hippocampus of socially defeated mice (Tsankova et al., 2006). These observations strongly suggest that the aberrant expression of HDAC2 and HDAC5 might be associated with the changes in large gene networks in patients with major depression.

In bipolar disorders, the increase in the expression of HDAC4 mRNA was state-dependent, and this molecule has been reported to be involved in cell death through the repression of myocyte enhancer factor 2 and CREB-dependent transcription (Bolger and Yao, 2005). Interestingly, increased levels of cell death have been observed in peripheral blood cells of depressive patients (Eilat et al., 1999; Ivanova et al., 2007). Together with our data, it is plausible that the altered expression of HDAC4 mRNA may contribute to cell death of the peripheral blood cells of mood disorder patients, and this might be one of the mechanisms responsible for the immune inadequacy observed in mood disorder patients (Riccardi et al., 2002). In contrast to HDAC4 mRNA, the expression levels of HDAC6 and −8 were decreased in bipolar disorder patients in both a depressive and a remissive state. However, we did not identify similar alterations of the levels of these mRNAs in the first-degree relatives of bipolar disorder patients. Thus, the decreased