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*).

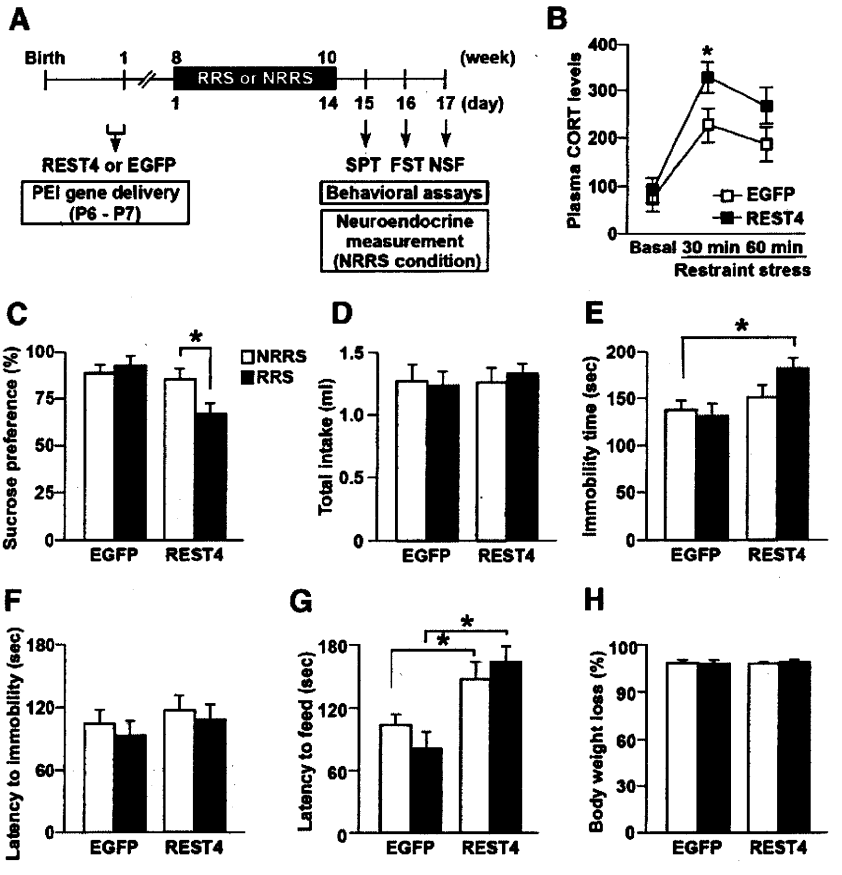

Figure 6. PEI-mediated REST4 overexpression in the mPFC of neonatal mice increases stress vulnerability. *A*, Schematic of experimental design. PEI/*Rest4* or control PEI/*Egfp* complexes were injected to the mPFC of mice at P6–P7. Adult mice were subjected to repeated restraint stress (RRS) or nonrepeatedly 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

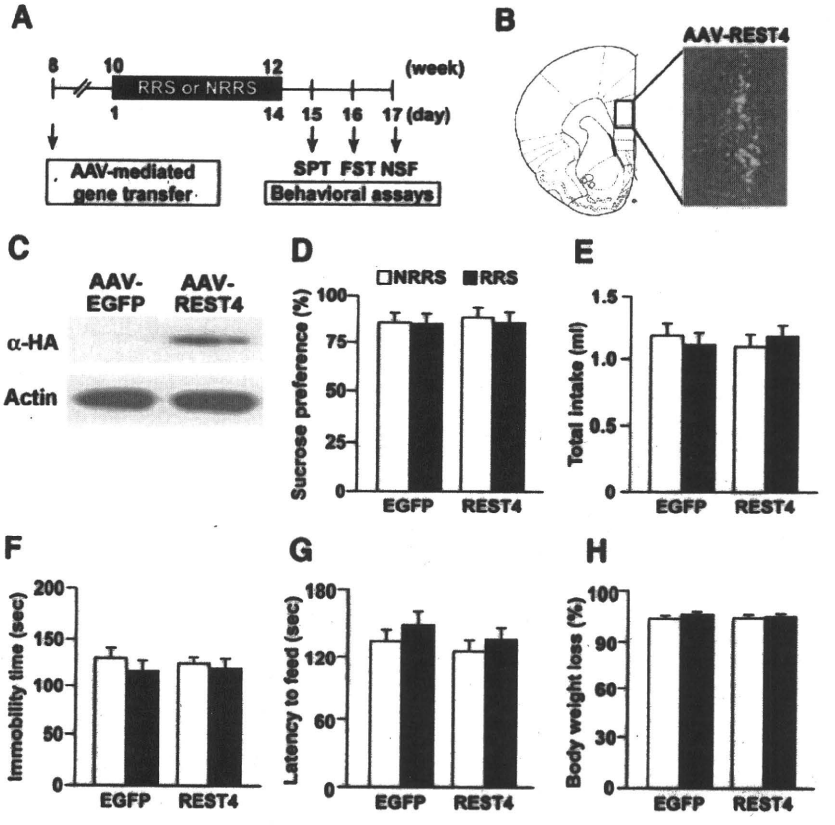

Figure 7. Effects of REST4 overexpression in the mPFC of adult mice on stress vulnerability. *A*, Schematic of experimental design. rAAV-REST4 or control rAAV-EGFP were injected into the mPFC of adult mice at P56. Two weeks after the injection, mice were subjected to repeated restraint stress (RRS) or 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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Different actions for acute and chronic administration of mirtazapine on serotonergic transmission associated with raphe nuclei and their innervation cortical regions

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ABSTRACT

The atypical antidepressant, mirtazapine enhances noradrenergic transmission, but its effects on serotonergic transmission remain to be clarified. The present study determined the effects of acute and chronic administration of mirtazapine on serotonergic transmissions in raphe nuclei and their innervation regions, frontal and entorhinal cortex, using multiple-probes microdialysis with real-time PCR and western blotting. Acute administration of mirtazapine did not affect extracellular serotonin level in raphe nuclei or cortex; however, chronic administration increased extracellular serotonin level in raphe nuclei without affecting that in cortex. Blockade of 5-HT1A receptor, but not that of the 5-HT2A/2C receptor, enhanced the effects of acute administration of mirtazapine on extracellular serotonin level in raphe nuclei. Chronic mirtazapine administration reduced the inhibitory function associated with somatodendritic 5-HT1A receptor in raphe nuclei, but enhanced postsynaptic 5-HT1A receptor in serotonergic innervated cortical regions. Chronic administration reduced the expression of mRNA and protein of serotonin transporter and 5-HT1A receptor in raphe nuclei, but not in the cortices. These results suggested that acute administration of mirtazapine probably activated serotonergic transmission, but its stimulatory action was abolished by activated inhibitory 5-HT1A receptor. Chronic administration of mirtazapine resulted in increased extracellular serotonin level via reduction of serotonin transporter with reduction of somatodendritic 5-HT1A autoreceptor function in raphe nuclei. These pharmacological actions of mirtazapine include its serotonergic profiles as noradrenergic and specific serotonergic antidepressant (NaSSA).

1. Introduction

The atypical antidepressant, mirtazapine (1,2,3,4,10,14b-hexahydro-2-methylpyrazino [2,1-a] pyrido[2,3-c]benzazepin) is a tetracyclic compound with antidepressant activity despite no monoamine transporter affinities (Croom et al., 2009; de Boer, 1995, 1996). A recent meta-analysis study of multiple treatments has demonstrated that in terms of clinical response, mirtazapine is more efficacious than duloxetine, fluoxetine, fluvoxamine, paroxetine and reboxetine (Cipriani et al., 2009). Mirtazapine has attracted attention since its binding profile indicates an antagonistic action to α2 adrenoceptor, serotonin 5-HT2A, 5-HT2C and 5-HT3 receptors without affecting monoamine transporters (Millan et al., 2000; Tatsumi et al., 1997; Van der Mey et al., 2006). Therefore, mirtazapine has a unique binding profile different from that of the typical tricyclic antidepressants and selective serotonin reuptake inhibitor (SSRI), and could be described as a noradrenergic and specific serotonergic antidepressant, abbreviated as NaSSA (Croom et al., 2009; de Boer, 1995, 1996). Furthermore, the inhibitory effects of mirtazapine on 5-HT2A/2C and 5-HT3 receptors reduce the likelihood of some serotonergic adverse effects associated with hyperactivated serotonin receptors induced by serotonin transporter inhibitors (e.g., restlessness, nausea and sexual dysfunction), and this appears to be supported by tolerability data from clinical trials (Croom et al., 2009).

With regard to the antidepressant mechanisms of mirtazapine, previous studies indicated that mirtazapine increases the extracellular norepinephrine level in raphe nuclei through inhibition of

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α2 adrenoceptor on the pre-synaptic terminal from locus coeruleus noradrenergic projection and indirectly enhances serotonergic transmission via activation of α1 adrenoceptor on the postsynaptic membrane of raphe nuclei (NaSSA hypothesis) (**Croom et al., 2009; de Boer, 1995, 1996**). Systemic administration of mirtazapine increased extracellular levels of norepinephrine and dopamine in various brain regions, e.g., frontal cortex (FC), dorsal and ventral hippocampus (**de Boer, 1996; de Boer et al., 1996; Devoto et al., 2004; Millan et al., 2000**). Contrary to norepinephrine, the effects of mirtazapine on the extracellular serotonin level remain controversial, since a report demonstrated that systemic administration of mirtazapine increased extracellular serotonin level in ventral hippocampus (**de Boer et al., 1996**), whereas several other studies could not detect the stimulatory effects on extracellular serotonin level in FC, striatum, dorsal and ventral hippocampus (**Bengtsson et al., 2000; Millan et al., 2000; Nakayama et al., 2004**).

Both the median (MRN) and dorsal (DRN) raphe nuclei receive noradrenergic projections from the locus coeruleus (**Adell et al., 2002**), as well as serotonergic projections from each other raphe nuclei (**Adell et al., 2002; Imai et al., 1986**). The serotonergic neurons in DRN project to FC, ventral hippocampus and striatum, whereas the serotonergic neurons in MRN project to the entorhinal cortex (EC), dorsal hippocampus and nucleus accumbens (**Adell et al., 2002; Imai et al., 1986**). Based on the serotonergic networks, the present study was designed to determine the effects of mirtazapine on serotonergic transmission associated with raphe nuclei. For this purpose, the acute and chronic effects of mirtazapine on extracellular serotonin levels in raphe nuclei and their innervated cortical regions were analyzed in rats using multiple-probes microdialysis. Furthermore, the present study determined the expression of protein and mRNA of serotonin transporter (*Slc6a4*) and 5-HT1A receptor (*Htr1a*) in the same regions.

2. Materials and methods

2.1. Experimental animals

All experiments described in this report were approved by the Ethics Review Committee for Animal Experimentation of Mie University. Male Sprague–Dawley rats (SLC, Shizuoka, Japan), were housed in air-conditioned rooms (temperature, 22 ± 2 °C) set at 12-h light–dark cycle.

2.2. Chemical agents

The following drugs were used in this study: mirtazapine (Sigma, St. Louis, MO), 5-HT1A agonist, 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT, Sigma), 5-HT1A receptor antagonist, WAY100635 (Sigma) and 5-HT2A/2C antagonist, ketanserin (Sigma). The nomenclature used for the above agents and their molecular targets conform to The British Journal of Pharmacology's 'Guide to Receptors and Channels' (**Alexander et al., 2009**). WAY100635 and 8-OH-DPAT were diluted directly in perfusion solution. Mirtazapine and ketanserin were each dissolved in modified Ringer's solution containing less than 0.1% (vol/vol) acetate. The pH of the final solution was adjusted to 7.0 with phosphate buffer.

2.3. Implantation of osmotic pump

To study the effects of chronic administration of mirtazapine on serotonergic transmission system, rats (5 weeks of age) were treated for 21 days with mirtazapine (10 mg/kg/day), using osmotic pumps (2ML4: Alzet, Cupertino, CA) implanted subcutaneously on the dorsal region of the rat under 1.8% isoflurane anesthesia. Mirtazapine was dissolved in 0.1 N HCl, and the pH of the final solution was adjusted to 7.0 with phosphate buffer under the 37 °C temperature.

2.4. Implantation of electrode and microdialysis probe in rat brain

To study the serotonergic networks associated with raphe nuclei, the teflon-coated stainless steel bipolar stimulant electrode (80 μm diameter, Unique Medical Co., Tokyo, Japan) was implanted in DRN ($A = -8.2$ mm, $L = 0.2$ mm, $V = -6.8$ mm, relative to bregma) or MRN ($A = -7.2$ mm, $L = 0.0$ mm, $V = -8.7$ mm, relative to bregma) at an angle of 10° according to the atlas of Paxinos and Watson (**Paxinos and Watson, 1998**). The electrode was connected to a stimulus generator (SEN-3301; Nihon Kohden, Tokyo), and stimuli were delivered through a DC constant current generator isolation unit (SS-201J; Nihon Kohden). Stimuli were delivered in phasic mode (pulses of 250 μs and 700 μA), presented in 0.25 s bursts of six pulses, and the burst frequency was set to twice the spontaneous firing frequency (**Yamamura et al., 2009a**).

Rats were anaesthetized with 1.8% isoflurane and placed in a stereotaxic frame. Rats were implanted with a guide cannula in DRN ($A = -8.2$ mm, $L = 0.2$ mm, $V = -6.8$ mm, relative to bregma) at an angle of 10°, MRN ($A = -7.2$ mm, $L = 0.0$ mm, $V = -8.7$ mm, relative to bregma) at an angle of 10°, FC ($A = +3.2$ mm, $L = 0.8$ mm, $V = -5.2$ mm, relative to bregma) or EC ($A = -7.8$ mm, $L = 5.8$ mm, $V = -6.2$ mm, relative to bregma) according to the atlas of Paxinos and Watson (**Paxinos and Watson, 1998**). Rats, under 1.8% isoflurane anesthesia, were implanted concentric I-type dialysis probe in DRN (0.22 mm diameter; 1 mm exposed membrane; Eicom, Kyoto, Japan), MRN (0.22 mm diameter; 1 mm exposed membrane; Eicom), FC (0.22 mm diameter; 3 mm exposed membrane; Eicom, Kyoto, Japan) or EC (0.22 mm diameter; 2 mm exposed membrane; Eicom) at the 3 days after the insertion of guide cannula.

Especially, to study the chronic effects of mirtazapine on the extracellular serotonin level, at 17 days after implantation of osmotic pump, each rat was implanted the guide cannula in FC, EC and raphe nuclei (DRN or MRN). At 3 days after guide cannula implantation (20 days after implantation of osmotic pump), the I-type microdialysis probes were implanted. At 21 days after implantation of osmotic pump, the perfusion experiments were started.

Perfusion experiments commenced 18 h after recovery from anesthesia. The perfusion rate was set at 2 μL/min, using modified Ringer's solution (MRS) composed of (in mM) 145.0 Na^+, 2.7 K^+, 1.2 Ca^{2+}, 1.0 Mg^{2+}, and 154.4 Cl^-, buffered to pH 7.4 with 2 mM phosphate buffer (**Yamamura et al., 2009b; Zhu et al., 2008**). The perfusion commenced using MRS alone. Extracellular serotonin level was measured at 6 h after starting the perfusion. After collection, each dialyzate sample (40 μL/20 min) was immediately injected into liquid chromatography. When the coefficient of variation of the level of each neurotransmitter reached less than 5% over 60 min (stabilization), control data were obtained over another 60 min period.

At the end of each experiment, the rat was injected with an overdose of pentobarbital and the brain was fixed with 4% paraformaldehyde. Horizontal sections (200 μm thick) were prepared, and the site of the dialysis probe was localized according to the atlas of Paxinos and Watson (**Paxinos and Watson, 1998**). The detailed study designs were described in **"Results"** section.

2.5. Liquid chromatography

The HPLC system used for determination of extracellular serotonin levels was equipped with an electrochemical detector (ECD-300; Eicom) with pump (EP-300; Eicom) and a graphite carbon electrode set at +450 mV (vs. an Ag/AgCl reference electrode). The analytical column (Inertsil ODS-4, 30 × 2.1 mm internal diameter, particle size 2 μm, GL Science, Tokyo, Japan) was maintained at 35 °C and the flow rate of the mobile phase was set at 100 μL/min. The mobile phase was composed of 0.1 M phosphate buffer containing 20% (vol/vol) methanol, 600 mg/L octansulfonic sodium, and 50 mg/L EDTA–2Na; the final pH was 5.9. The quantification limits for serotonin were 0.25 fmol/20 μL (12.5 pM).

2.6. Real-time PCR and western blotting

Twenty-one days after implantation of osmotic pump (during chronic administration of 10 mg/kg/day mirtazapine for 21 days), rats were sacrificed by decapitation, and the brain tissues of raphe nucleus, FC and EC were dissected bilaterally on an ice-cold stage according to the method of Glowinski and Iversen (**Glowinski and Iversen, 1966**). The raphe nucleus composed of both bilateral DRN and MRN. FC composed of medial prefrontal cortex and other frontal cortex regions without olfactory bulb ($A = 5.2–2.7$ mm relative to bregma) according to the atlas of Paxinos and Watson (**Paxinos and Watson, 1998**). Tissues were weighed before being frozen at 80 °C.

RNA was extracted from each brain tissue according to the RNeasy (Qiagen, Valencia, CA), including DNase treatment using RNase-Free DNase set (Qiagen). Each of the total RNA was reverse transcribed with random hexamer using Moloney murine leukemia virus reverse transcriptase (Applied Biosystems, Austin, TX), following the protocol provided by the company. TaqMan primer-probe sets for *Slc6a4* (Rn00564737_m1, Applied Biosystem) and *Htr1a* (Rn00561409_s1*, Applied Biosystem) for a common RNA mass normalizer, and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*, 4352338E, Applied Biosystem) for endogenous control were purchased from Applied Biosystems. Real-time quantitative PCR was performed in StepOnePlus System (Applied Biosystems). Changes in mRNA expression level were calculated after normalization to *Gapdh*. The calibrator sample was cDNA from an arbitrarily selected control rat. The ΔΔCT method provides a relative quantification ratio according to the calibrator that allows statistical comparisons of gene expression among samples (**Pfaffl, 2006**). Values of fold changes in the control sample versus the chronic administrated samples represent averages of triplicate measurements. Changes in gene expression were reported as fold changes relative to the control.

Each brain tissue (raphe nuclei, FC and EC) was homogenized in lysis buffer containing 0.32 M sucrose, 0.5 mM $MgSO_4$ and 5 mM HEPES buffer (pH 8.0) plus protease inhibitors (Protease Inhibitor Cocktail, Nacalai Tesque, Kyoto, Japan),

phosphatase inhibitors (Phosphatase Inhibitor Cocktail, Nakalai Tesque) and 0.2 mM dithiothreitol. The homogenate was centrifuged at 800 × g for 10 min at 4 °C. The supernatant was decanted into a new centrifuge tube and centrifuged at 22,000 × g for 15 min at 4 °C. The pellet was re-suspended in 150 μL of ice-cold dH_2O and an aliquot (75 μL) was centrifuged at 22,000 × g for 15 min at 4 °C. The supernatant was removed and the pellet was re-suspended in ice-cold dH_2O to form the membrane fraction (**Bhide et al., 2009**). Protein value of each brain tissue was determined by the Bradford method. Protein samples were suspended in homogenate buffer containing 2% Triton X-100 (Sigma). When indicated, an identical volume of supernatant (20 μg protein, extracted in the same buffer) was added instead. The total lysates were separated on 10% SDS-PAGE gels, and were blotted to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). SDS-PAGE was performed according to mainly standard procedures using Protean III (BioRad, Hercules, CA). After blocking with 5% nonfat milk containing Tris/HCl (pH 7.5, 100 mM NaCl) and 1% Tween 20, the membranes were treated with anti-5-HT1A receptor (Alomone, Jerusalem, Israel), anti-serotonin transporter (Millipore) and internal control, anti-GAPDH (Abcam, Cambridge, UK) diluted in Can Get Signal solution 1 (TOYOBO: Osaka, Japan) at room temperature for 1 h. Then, the membranes were treated with Cy3-conjugated affinity-purified anti-rabbit IgG (Jackson, West Grove, PA) diluted in Can Get Signal solution 2 (TOYOBO) at room temperature for visualization using Typhoon 9400 (GE Healthcare, Buckinghamshire, UK).

2.7. Statistical analysis

Data were expressed as mean ± SD. The effects of electro-stimulation of DRN and MRN on the extracellular serotonin level in raphe nuclei and cortices were analyzed using repeated measurement of one-way analysis of variance (ANOVA) with Dunnett's multiple comparison. The dose-dependent effects of acute and chronic administrations of mirtazapine on extracellular serotonin levels were analyzed by one-way ANOVA with Tukey's multiple comparison. The effects of perfusion with selective serotonin receptor agents on the extracellular serotonin levels were analyzed by multivariate analysis of variance (MANOVA) with Tukey's multiple comparison. The effects of chronic administration of 10 mg/kg/day for 21 days mirtazapine on the expression of mRNA and protein of serotonin transporter (*Slc6a4*) and 5-HT1A receptor (*Htr1a*) were analyzed by the Student's *t*-test. A *P* value <0.05 was considered statistically significant.

3. Results

The basal extracellular serotonin levels in the DRN and MRN were 83.6 ± 6.9 fmol/sample (40 μL) (2.1 nM; $n = 84$) and 64.5 ± 5.8 fmol/sample (40 μL) (1.6 nM; $n = 84$), respectively. The basal extracellular serotonin levels in the FC and EC were 4.6 ± 0.5 fmol/sample (40 μL) (0.12 nM; $n = 84$) and 3.4 ± 0.4 fmol/sample (40 μL) (0.09 nM; $n = 84$), respectively. The basal extracellular serotonin levels in DRN, MRN, FC and EC were tetrodotoxin-sensitive, Ca^{2+}-dependent, and K^+-sensitive (data not shown).

3.1. Effects of electrical stimulation on the extracellular serotonin levels in raphe nuclei and cortex

To study the serotonergic networks associated with DRN, the bipolar stimulating electrode was implanted in DRN and three concentric I-type dialysis probes were implanted in MRN, FC and EC. To study the serotonergic networks associated with MRN, the bipolar stimulating electrode was implanted in MRN and three concentric I-type dialysis probes were implanted in DRN, FC and EC. After confirming the stabilization of extracellular serotonin level, DRN or MRN was electro-stimulated for 20 min (**Yamamura et al., 2009a**).

Neither electro-stimulations of DRN nor MRN elicited convulsion. Electrical stimulation of DRN increased extracellular serotonin levels in the MRN [$F(6, 30) = 37.1$, $P < 0.01$] and FC [$F(6, 30) = 79.6$, $P < 0.01$] without affecting that in EC (**Fig. 1**). In contrast to DRN stimulation, stimulation of the MRN increased extracellular serotonin level in the EC [$F(6, 30) = 45.8$, $P < 0.01$] without affecting those in the DRN or FC (**Fig. 1**). The results of microdialysis suggest that the presence of serotonergic projections from the MRN to the EC and from the DRN to both the FC and MRN was detectable, but the serotonergic projection from MRN to DRN could not be detected.

3.2. Dose-dependent effects of acute and chronic administrations of mirtazapine on extracellular serotonin levels in raphe nuclei and cortex

To study the acute effects of mirtazapine on the extracellular serotonin level, three concentric I-type dialysis probes were implanted in FC, EC and raphe nuclei (DRN or MRN). After the confirming the stabilization, mirtazapine was injected intraperitoneally (5 and 10 mg/kg, ip). Acute administration of mirtazapine (0, 5 and 10 mg/kg, ip) (**Besson et al., 2000**; **de Boer, 1996**; **de Boer et al., 1996**; **Haddjeri et al., 1996**; **West et al., 2009**) did not affect the extracellular serotonin levels in DRN (**Fig. 2A**), MRN (**Fig. 2B**), FC (**Fig. 2C**) nor EC (**Fig. 2D**).

Contrary to acute administration, chronic administration of mirtazapine (0, 5 and 10 mg/kg/day for 21 days) significantly changed the extracellular serotonin levels in DRN [$F(2, 15) = 7.2$, $P < 0.01$] (**Fig. 2A**) and MRN [$F(2, 15) = 6.5$, $P < 0.01$] (**Fig. 2B**), but not in FC (**Fig. 2C**) or EC (**Fig. 2D**). Chronic administration of mirtazapine (10 mg/kg/day for 21 days) increased extracellular serotonin levels in DRN ($P < 0.05$) and MRN ($P < 0.05$) compared with control, whereas the chronic administration of mirtazapine (5 mg/kg/day for 21 days) did not increase extracellular serotonin levels in raphe nuclei (**Fig. 2A** and B).

3.3. Interaction between mirtazapine and 5-HT2A/2C receptor on extracellular serotonin level in raphe nuclei and cortex

To study the acute effects of mirtazapine on the extracellular serotonin level, three concentric I-type dialysis probes were implanted in FC, EC and raphe nuclei (DRN or MRN). After the confirming the stabilization, the perfusate was switched from MRS to MRS containing 50 μM ketanserin. After the confirming the stabilization, mirtazapine was injected intraperitoneally (5 and 10 mg/kg, ip).

Perfusion with 50 μM ketanserin (5-HT2A/2C receptors antagonist) in DRN and MRN did not alter extracellular serotonin levels in DRN, MRN, FC or EC (data not shown). During blockade of 5-HT2A/2C receptor in DRN by perfusion with 50 μM ketanserin, mirtazapine did not affect the extracellular serotonin levels in DRN, FC or EC (data not shown). During blockade of 5-HT2A/2C receptor in MRN by perfusion with 50 μM ketanserin, mirtazapine did not affect the extracellular serotonin levels in MRN, FC or EC (data not shown).

3.4. Interaction between mirtazapine and 5-HT1A receptor on extracellular serotonin levels in raphe nuclei and cortex

To study the acute effects of mirtazapine on the extracellular serotonin level, three concentric I-type dialysis probes were implanted in FC, EC and raphe nuclei (DRN or MRN). After the confirming the stabilization, the perfusate was switched from MRS to MRS containing 10 μM WAY100635. After the confirming the stabilization, mirtazapine was injected intraperitoneally (5 and 10 mg/kg, ip).

Perfusion of DRN and MRN with 10 μM WAY100635 (5-HT1A receptor antagonist) did not change extracellular serotonin levels in DRN, MRN, FC and EC (data not shown). During blockade of 5-HT1A receptor in DRN by perfusion with 10 μM WAY100635, mirtazapine increased extracellular serotonin levels in DRN [$F_{dose}(2, 15) = 26.8$, $P < 0.01$; $F_{time}(6, 90) = 230.2$, $P < 0.01$; $F_{dose \times time}(18, 135) = 48.3$, $P < 0.01$] (**Fig. 3A**) and FC [$F_{dose}(2, 15) = 10.4$, $P < 0.01$; $F_{time}(6, 90) = 53.9$, $P < 0.01$; $F_{dose \times time}(18, 135) = 32.1$, $P < 0.01$] (**Fig. 3C**) but not in EC (**Fig. 3E**). In contrast to DRN, during blockade of 5-HT1A receptor in MRN by perfusion with 10 μM WAY100635, mirtazapine increased extracellular serotonin levels in MRN [$F_{dose}(2, 15) = 8.5$, $P < 0.01$; $F_{time}(6, 90) = 100.9$, $P < 0.01$; $F_{dose \times time}(18, 135) = 35.8$,

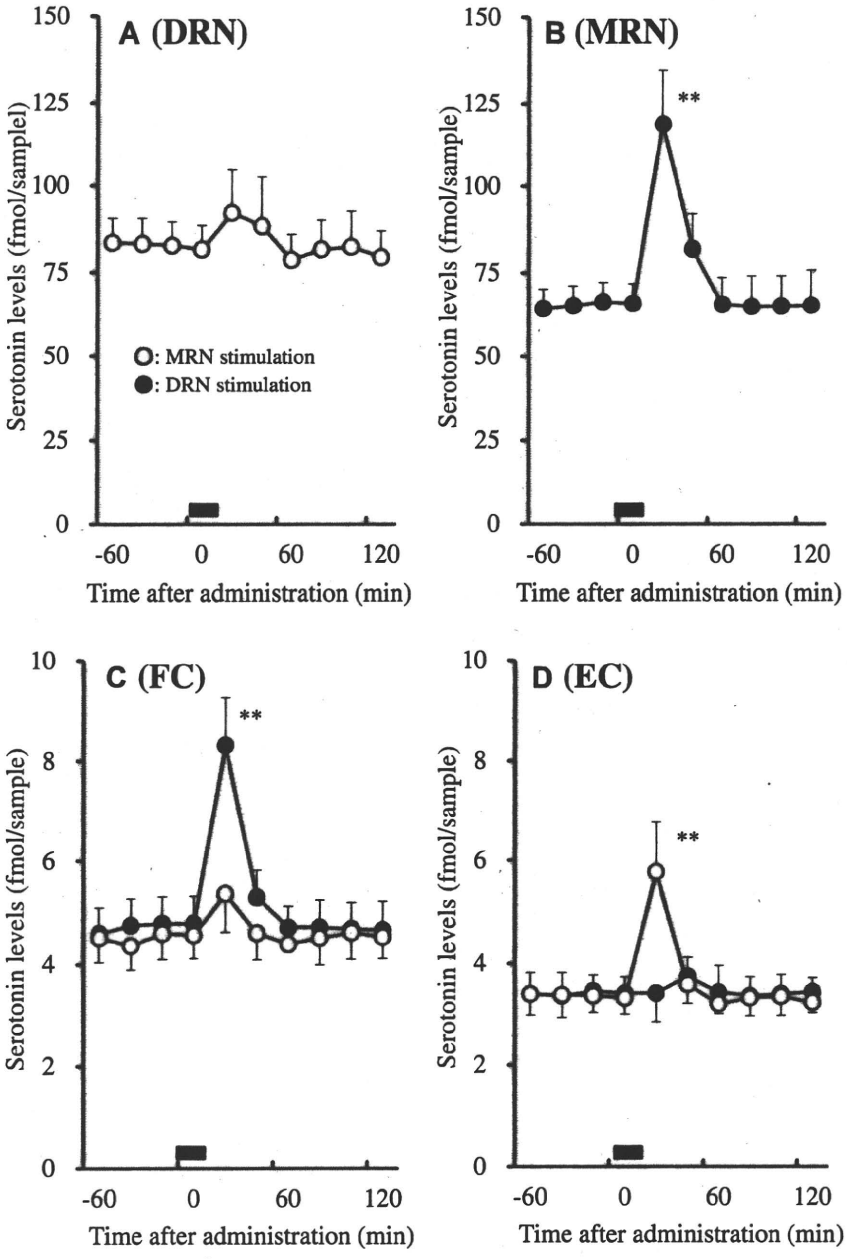

Fig. 1. Effects of electro-stimulation in raphe nuclei on the extracellular serotonin levels in raphe nuclei and cortex. Effects of electrical stimulation in DRN (●) or MRN (○) on extracellular serotonin level in DRN, MRN, FC and EC are indicated in Fig. 1A, B, C and D, respectively. Electrical stimulation in DRN or MRN was applied for 20 min (closed bars). Ordinates and abscissas indicate the mean ± SD ($n = 6$) of extracellular serotonin levels (fmol/sample) and time after electrical stimulation (min). The effects of electro-stimulation on extracellular serotonin level were compared using repeated measurements one-way ANOVA with Dunnett's multiple comparison (*$P < 0.05$; **$P < 0.01$ vs. Pre-stimulation).

$P < 0.01$] (Fig. 3B) and EC [$F_{dose}(2, 15) = 3.7$, $P < 0.05$; $F_{time}(6, 90) = 99.9$, $P < 0.01$; $F_{dose \times time}(18, 135) = 33.9$, $P < 0.01$] (Fig. 3F) but not in FC (Fig. 3D).

3.5. Interaction between chronic administration of mirtazapine and perfusion with 5-HT1A receptor agonist on extracellular serotonin level in raphe nuclei and cortex

After the confirming the stabilization, the perfusate was switched from MRS to MRS containing 100 μM 8-OH-DPAT (5-HT1A receptor agonist) (Suzuki et al., 1995). The interaction between chronic administration of 10 mg/kg/day mirtazapine for 21 days and perfusion with 100 μM 8-OH-DPAT in DRN on the extracellular serotonin levels in DRN [$F_{MTZ}(1, 10) = 82.7$, $P < 0.01$; $F_{time}(6, 60) = 1187.1$, $P < 0.01$; $F_{MTZ \times time}(6, 60) = 318.7$, $P < 0.01$] (Fig. 4A) and FC [$F_{MTZ}(1, 10) = 5.8$, $P < 0.05$; $F_{time}(6, 60) = 133.2$, $P < 0.01$; $F_{MTZ \times time}(6, 60) = 55.9$, $P < 0.01$] (Fig. 4C) were detected. In the control rat brain, perfusion of DRN with 8-OH-DPAT decreased extracellular serotonin level in DRN (Fig. 4A) and FC (Fig. 4C) but not in EC (Fig. 4E). Chronic mirtazapine administration reduced the inhibitory effects of perfusion with 100 μM 8-OH-DPAT in DRN on the extracellular serotonin levels in DRN ($P < 0.01$) (Fig. 4A) and FC ($P < 0.01$) (Fig. 4C).

The interaction between chronic administration of 10 mg/kg/day mirtazapine for 21 days and perfusion with 100 μM 8-OH-DPAT in

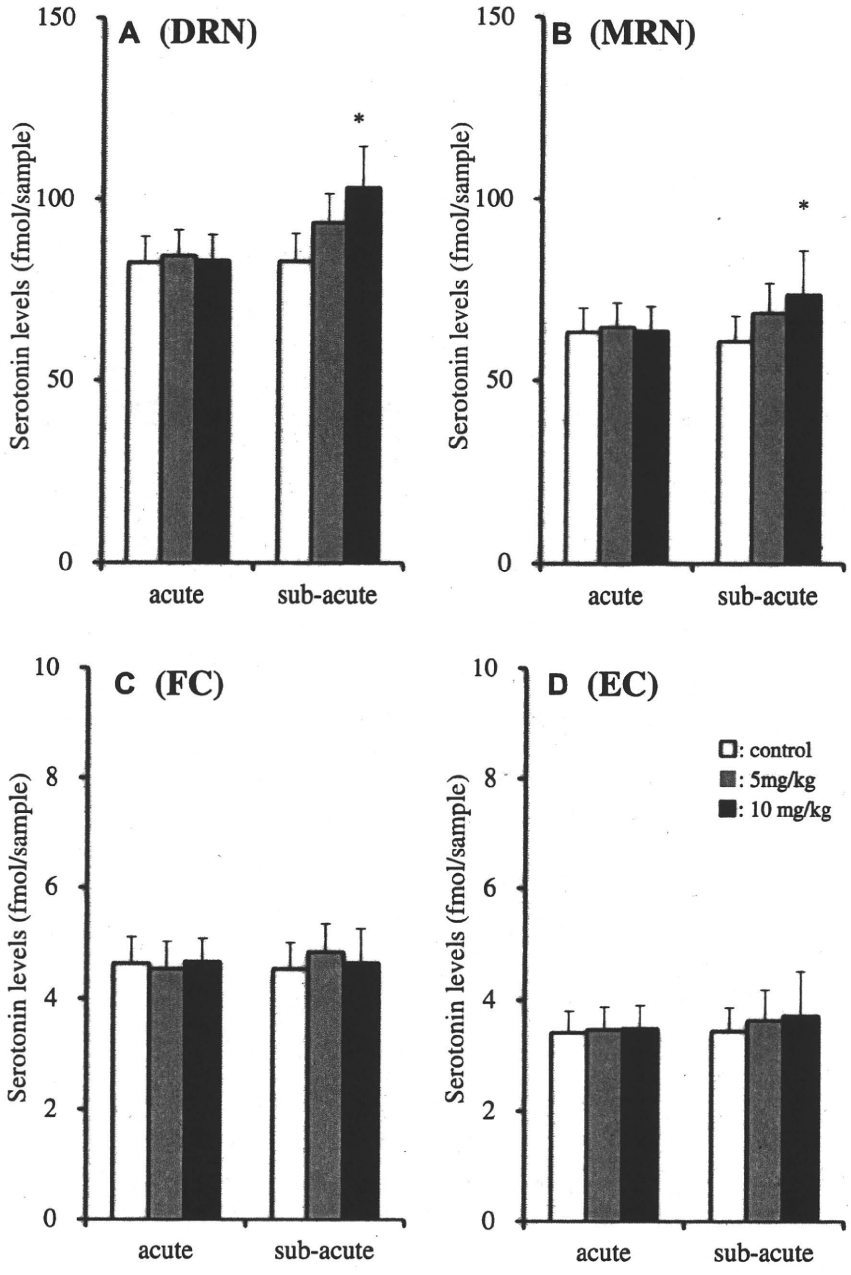

Fig. 2. Dose-dependent effects of acute and chronic administration of mirtazapine on the extracellular serotonin levels in raphe nuclei and cortex. Effects of acute and chronic administrations of mirtazapine (opened bars: control; 0 mg/kg or 0 mg/kg/day for 21 days, gray bars: 5 mg/kg or 5 mg/kg/day for 21 days, and closed bars: 10 mg/kg or 10 mg/kg/day for 21 days) on extracellular serotonin level in DRN, MRN, FC and EC are indicated in Fig. 2A, B, C and D, respectively. Ordinates indicate the mean ± SD ($n = 6$) of extracellular serotonin levels (fmol/sample). The effects of acute and chronic administration of mirtazapine on extracellular serotonin level were compared using one-way ANOVA with Tukey's multiple comparison (*$P < 0.05$ vs. control).

MRN on the extracellular serotonin levels in MRN [$F_{MTZ}(1, 10) = 29.9$, $P < 0.01$; $F_{time}(6, 60) = 943.2$, $P < 0.01$; $F_{MTZ \times time}(6, 60) = 100.2$, $P < 0.01$] (Fig. 4B) and EC [$F_{MTZ}(1, 10) = 5.0$, $P < 0.05$; $F_{time}(6, 60) = 245.9$, $P < 0.01$; $F_{MTZ \times time}(6, 60) = 23.3$, $P < 0.01$] (Fig. 4F) were detected. In the control rat brain, perfusion of MRN with 100 μM 8-OH-DPAT decreased the extracellular serotonin level in MRN (Fig. 4B) and EC (Fig. 4F) without affecting that in FC (Fig. 4D). Chronic mirtazapine administration reduced the inhibitory effects of perfusion with 100 μM 8-OH-DPAT in MRN on the extracellular serotonin levels in MRN ($P < 0.01$) (Fig. 4B) and EC ($P < 0.05$) (Fig. 4F).

The interaction between chronic administration of 10 mg/kg/day mirtazapine for 21 days and perfusion with 100 μM 8-OH-DPAT in FC on the extracellular serotonin levels in FC [$F_{MTZ}(1, 10) = 7.1$, $P < 0.05$; $F_{time}(6, 60) = 1250.5$, $P < 0.01$; $F_{MTZ \times time}(6, 60) = 49.0$, $P < 0.01$] (Fig. 5A) was detected. In the control rat brain, perfusion of FC with 8-OH-DPAT reduced extracellular serotonin levels in FC ($P < 0.01$) (Fig. 5A). Chronic mirtazapine administration enhanced the inhibitory effects of perfusion with 100 μM 8-OH-DPAT in FC on the extracellular serotonin levels in FC ($P < 0.01$) (Fig. 5A).

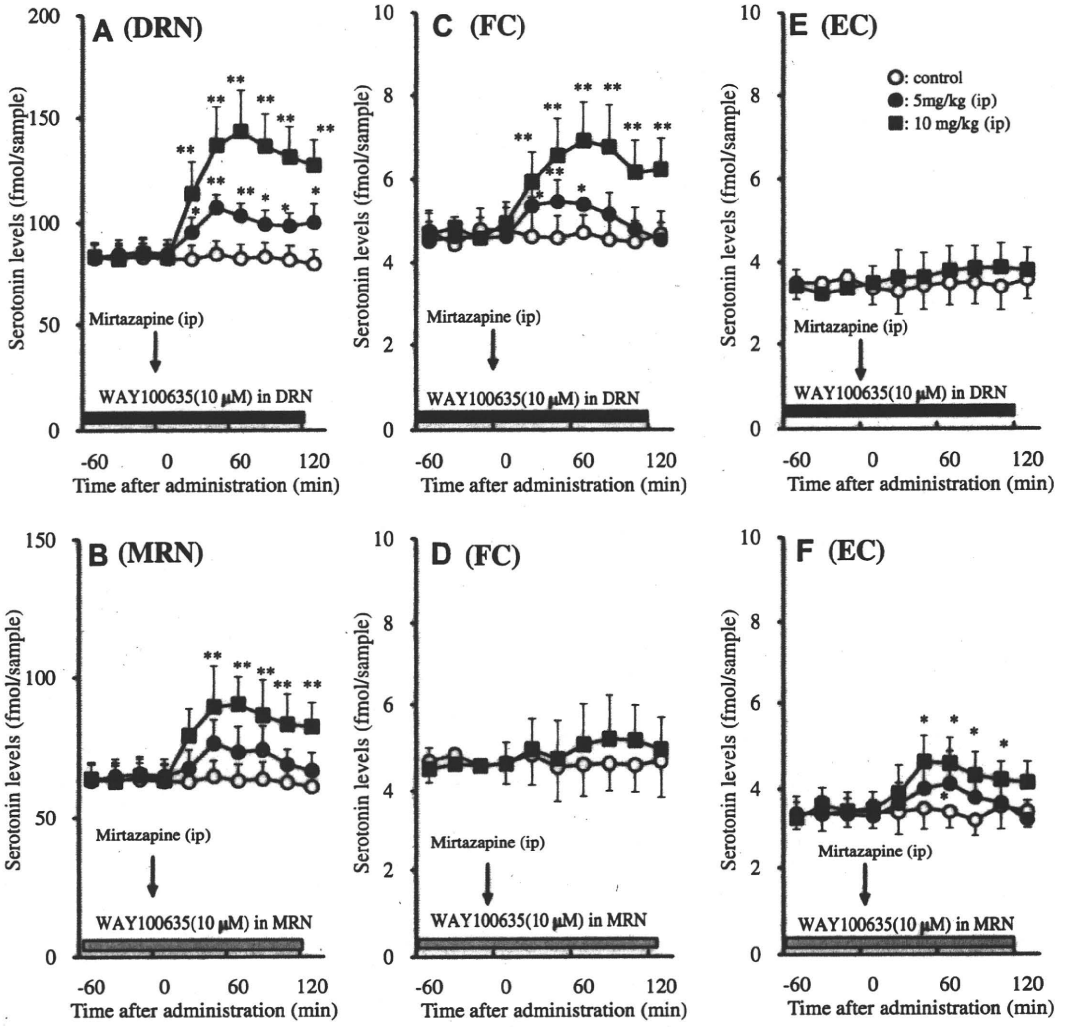

Fig. 3. Dose-dependent effects of acute administration of mirtazapine on extracellular serotonin level in raphe nuclei and cortex, under the blockade of 5-HT1A receptor in raphe nuclei. Effects of acute administration of mirtazapine (○: 0 mg/kg, (●: 5 mg/kg and ■: 10 mg/kg, i.p.) on extracellular serotonin level in DRN, FC and EC, under the condition of 5-HT1A receptor blockade by perfusion with 10 μM WAY100635 in DRN (closed bars) are indicated in Fig. 3A, C and E, respectively. Effects of acute administration of mirtazapine on extracellular serotonin level in MRN, FC and EC, under the condition of 5-HT1A receptor blockade by perfusion with WAY100635 in MRN (gray bars) are indicated in Fig. 3B, D and F, respectively. Ordinates and abscissas indicate the mean ± SD ($n = 6$) of extracellular serotonin levels (fmol/sample) and time after mirtazapine administration (ip). The dose-dependent effects of acute administration of mirtazapine on extracellular serotonin level under the condition of 5-HT1A receptor blockade, were compared using MANOVA with Tukey's multiple comparison (*$P < 0.05$; **$P < 0.01$ vs. control). The data regarding effects of 5 mg/kg mirtazapine in FC (Fig. 3D) and in EC (Fig. 3E) were excluded to avoid over complicating the figure.

The interaction between chronic administration of 10 mg/kg/day mirtazapine for 21 days and perfusion with 100 μM 8-OH-DPAT in EC on the extracellular serotonin levels in EC [$F_{MTZ}(1, 10) = 6.2$, $P < 0.05$; $F_{time}(6, 60) = 441.7$, $P < 0.01$; $F_{MTZ \times time}(6, 60) = 4.4$, $P < 0.01$] (Fig. 5B) was detected. In the control rat brain, perfusion of FC with 100 μM 8-OH-DPAT reduced extracellular serotonin levels in EC ($P < 0.01$) (Fig. 5B). Chronic mirtazapine administration enhanced the inhibitory effects of perfusion with 100 μM 8-OH-DPAT in EC on the extracellular serotonin levels in EC ($P < 0.05$) (Fig. 5B).

3.6. *Effects of chronic administration of mirtazapine on mRNA and protein expression of serotonin transporter and 5-HT1A receptor in raphe nuclei and cortex*

The mRNA expression of *Slc6a4* (serotonin transporter) in raphe nucleus was decreased by chronic administration of mirtazapine (10 mg/kg/day for 21 days) ($P < 0.01$), whereas that in FC and EC was not affected (Fig. 6A). Similar to *Slc6a4*, the expression of *Htr1a* (5-HT1A receptor) in raphe nucleus was decreased by chronic administration of mirtazapine ($P < 0.01$), whereas that in FC and EC was not affected (Fig. 6B).

The protein expression of serotonin transporter in raphe nucleus was decreased by chronic administration of mirtazapine (10 mg/kg/day for 21 days) ($P < 0.05$), whereas that in FC and EC was not affected (Fig. 7A and C). Similar to serotonin transporter, the protein expression of 5-HT1A receptor in raphe nucleus was decreased by chronic administration of mirtazapine ($P < 0.05$), whereas that in FC and EC was not affected (Fig. 7B and C).

4. Discussion

Taken together with the binding profiles of mirtazapine, the potential antidepressant mechanism of mirtazapine is as follows: blockade of α2 adrenoceptor enhances noradrenergic transmission

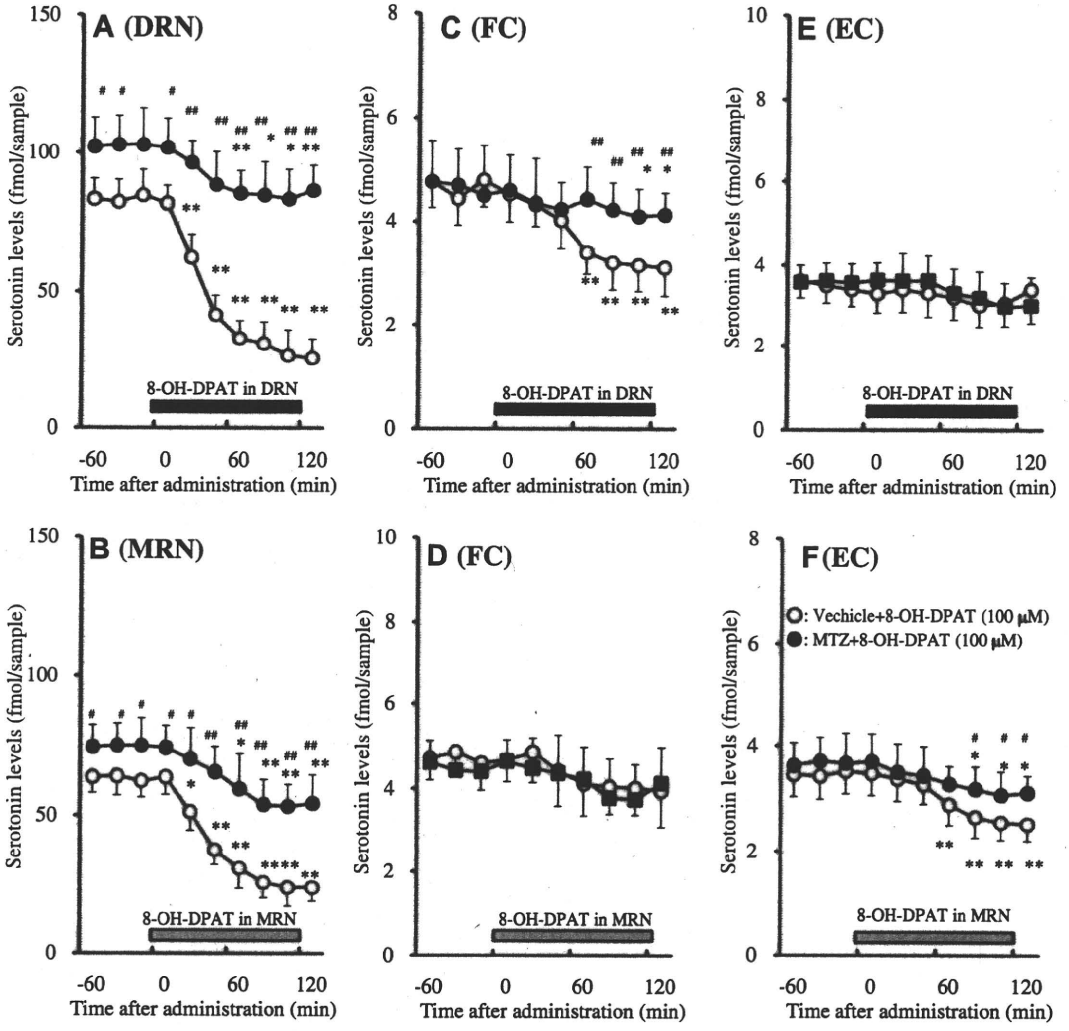

Fig. 4. Effects of perfusion with 5-HT1A receptor agonist in raphe nuclei on extracellular serotonin level in raphe nuclei and cortex, during chronic administration of mirtazapine. During chronic administration with (●: 10 mg/kg/day) or without (○: control) mirtazapine, the effects of perfusion with 100 μM 8-OH-DPAT in DRN (closed bars) on extracellular serotonin level in DRN, FC and EC are indicated in Fig. 4A, C and E, respectively. During chronic administration with or without mirtazapine, the effects of perfusion with 100 μM 8-OH-DPAT in MRN (gray bars) on extracellular serotonin level in MRN, FC and EC are indicated in Fig. 4B, D and F, respectively. After the confirming the stabilization, the perfusate was switched from MRS (pre-treatment) to MRS containing 100 μM 8-OH-DPAT. Ordinates and abscissas indicate the mean ± SD ($n = 6$) of extracellular serotonin levels (fmol/sample) and time after perfusion with 8-OH-DPAT (min). The effects of perfusion with 100 μM 8-OH-DPAT on extracellular serotonin level during chronic administration of mirtazapine, were compared using MANOVA with Tukey's multiple comparison (*$P < 0.05$; **$P < 0.01$ vs. pre-treatment, #$P < 0.05$; ##$P < 0.01$ vs. Vehicle).

and secondarily activates serotonergic transmission via activation of α1 adrenoceptor-mediated noradrenergic facilitation (Croom et al., 2009; de Boer, 1996). Therefore, the antidepressant activity of mirtazapine is considered to be due to enhancement of the noradrenergic and serotonergic effects (NaSSA hypothesis) (Croom et al., 2009; de Boer, 1995, 1996). Indeed, earlier studies demonstrated that systemic administration of mirtazapine enhanced the activities of noradrenergic neurons in locus coeruleus and serotonergic neurons in DRN (Haddjeri et al., 1996), with an increase in hippocampal extracellular serotonin level under the serotonin transporter blockade (de Boer et al., 1996). Contrary to these earlier studies, the later electrophysiological studies also demonstrated that mirtazapine activated noradrenergic neurons in the locus coeruleus but did not affect serotonergic neuronal activity in DRN (Millan et al., 2000). Furthermore, microdialysis studies have demonstrated that mirtazapine increased extracellular norepinephrine level (Adell et al., 2002; Imai et al., 1986; Lechin et al., 1989, 2002); however, under the conditions of serotonin transporter functional, neither acute nor chronic administrations of mirtazapine altered the extracellular serotonin level in regions innervated by the raphe nuclei, dorsal hippocampus, nucleus accumbens, striatum, frontal and occipital cortex (Bengtsson et al., 2000; Devoto et al., 2004; Millan et al., 2000; Nakayama et al., 2004). These critical different results between earlier and later studies suggest that blockade of serotonin transporter might enable an increase induced by α2 adrenoceptor blockade to be seen by blocking the transporter-induce compensation.

In spite of these efforts, the effect of chronic administration of mirtazapine on extracellular serotonin levels in raphe nuclei has not yet been determined. Therefore, the present study focused on the following topics: (1) Effects of both acute and chronic administrations of mirtazapine on the extracellular serotonin level in DRN, MRN and their serotonergic innervation cortical regions, FC and EC, using microdialysis. (2) Effects of chronic administration of mirtazapine on 5-HT1A receptor associated serotonergic transmission in raphe nuclei and cortex. (3) Effects of chronic administration of

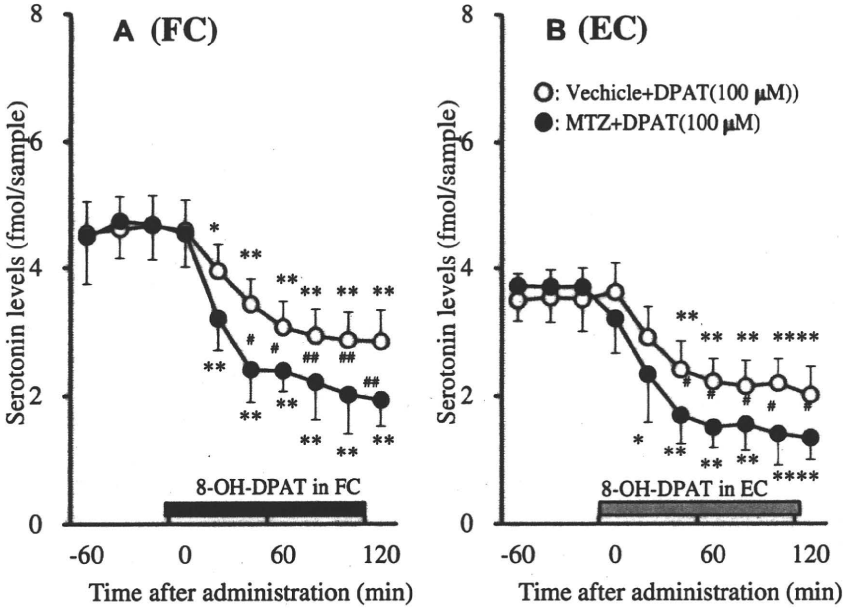

Fig. 5. Effects of perfusion with 5-HT1A receptor agonist in cortex on extracellular serotonin level in raphe nuclei and cortex, during chronic administration of mirtazapine. During chronic administration with (●: 10 mg/kg/day for 21 days) or without (○: control) mirtazapine, the effects of perfusion with 100 μM 8-OH-DPAT in FC (closed bars) and EC (gray bars) on extracellular serotonin level in FC and EC are indicated in Fig. 5A and B, respectively. After the confirming the stabilization, the perfusate was switched from MRS (pre-treatment) to MRS containing 100 μM 8-OH-DPAT. Ordinates and abscissas indicate the mean ± SD ($n = 6$) of extracellular serotonin levels (fmol/sample) and time after perfusion with 8-OH-DPAT (min). The effects of perfusion with 8-OH-DPAT on extracellular serotonin level during chronic administration of mirtazapine, were compared using MANOVA with Tukey's multiple comparison (*$P < 0.05$; **$P < 0.01$ vs. pre-treatment, #$P < 0.05$; ##$P < 0.01$ vs. Vehicle).

mirtazapine on expression of mRNA and protein of *Slc6a4* (serotonin transporter) and *Htr1a* (5-HT1A receptor) in raphe nuclei and cortices.

4.1. Effects of acute administration of mirtazapine on the extracellular serotonin levels in raphe nuclei and cortex

The raphe nuclei house the origin of the mesolimbic serotonergic pathway (Adell et al., 2002; Imai et al., 1986). Previous anatomical studies demonstrated that the serotonergic neurons in DRN and MRN project FC and EC, respectively (Adell et al., 2002; Imai et al., 1986). In the present study, electrical stimulation of DRN and MRN increased extracellular serotonin levels in FC and EC, respectively. Furthermore, the present study showed that electrostimulation of DRN increased extracellular serotonin level in MRN, while that of MRN did not increase extracellular serotonin level in DRN. Both raphe nuclei receive serotonergic projections from each other raphe nuclei (Adell et al., 2002; Imai et al., 1986). The rat MRN contains about 1100 serotonergic neurons, representing less than one tenth of those observed in DRN (Jacobs and Azmitia, 1992). Taken together with the histological evidence, the present study detected the serotonin release from DRN to MRN, but could not detect that from MRN to DRN, suggesting that the level of serotonin release from MRN to DRN is probably lower than the quantification limits for serotonin (12.5 pM).

In the present study, the acute administration of mirtazapine did not affect the extracellular serotonin level in DRN, MRN, FC or EC, similar to the other raphe nuclei innervated regions (Bengtsson et al., 2000; Devoto et al., 2004; Millan et al., 2000; Nakayama et al., 2004). The enhanced serotonin release from raphe nuclei terminals reaches the pre-synaptic 5-HT2A and somatodendritic 5-HT1A receptors in DRN and MRN (Lechin et al., 2006). Activation of 5-HT1A and 5-HT2A receptors generally leads to respective inhibition and activation of serotonergic transmission by multiple mechanisms (Meltzer et al., 2003). To clarify whether does 5-HT2A/2C antagonistic effects of mirtazapine abolish its stimulatory effects on serotonin release or not (Ohoyama et al., in press), the present study determined the effects of mirtazapine on serotonin release under the pre-inhibition of 5-HT2A/2C receptor by ketanserin. Perfusion with ketanserin did not affect serotonin release, and mirtazapine also did not affect extracellular serotonin level under the blockade of 5-HT2A/2C receptors. Thus, this result suggests that the inhibitory effects of mirtazapine on 5-HT2A/2C receptor, at least, probably does not play important roles in the lack of mirtazapine on serotonin release in raphe nucleus. In contrast, under 5-HT1A receptor inhibition in raphe nuclei, mirtazapine dose-dependently increased serotonin release. Therefore, mirtazapine probably activates the serotonin release associated with raphe nuclei; however, the enhanced serotonergic transmission by mirtazapine is prevented via activation of inhibitory somatodendritic 5-HT1A autoreceptor.

4.2. Effects of chronic administration of mirtazapine on the serotonergic transmission

The present results regarding the acute effects of mirtazapine on the extracellular serotonin level suggest that short-term treatment of mirtazapine possibly activates serotonergic transmission associated with raphe nuclei or their serotonergic innervation regions, but its stimulation of serotonergic transmission is abolished via activation of 5-HT1A receptor. Contrary to acute administration, chronic (21 days) administration of mirtazapine resulted in the several changes in serotonergic transmission. The present study demonstrated that chronic administration of mirtazapine weakly but significantly increased the extracellular serotonin level in both DRN and MRN, but did not affect in their innervation cortical regions.

5-HT1A receptors are expressed both pre-synaptically as autoreceptors by serotonin containing neurons in raphe nuclei, and postsynaptically in serotonergic innervation cortical and limbic

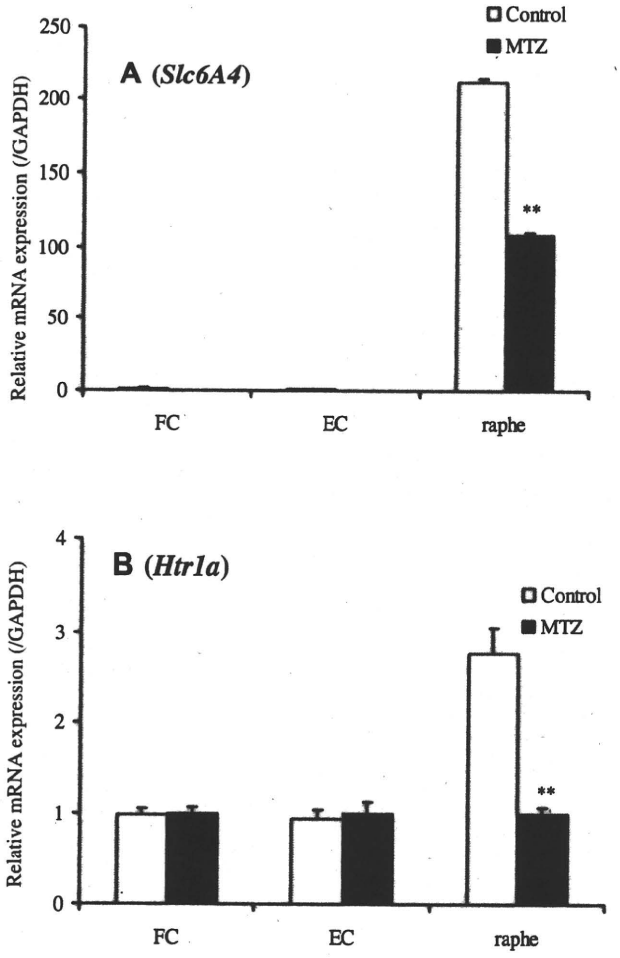

Fig. 6. Effects of *chronic* administration of mirtazapine on mRNA expression of Slc6a4 and Htr1a in raphe nucleus and cortices. Effects of chronic administrations of mirtazapine (□: control, 0 mg/kg/day for 21 days, and ■: MTZ, 10 mg/kg/day for 21 days) on the mRNA expression of *Slc6a4* and *Htr1a* in raphe nucleus, FC and EC are indicated in Fig 6A and B, respectively. Ordinates indicate the mean ± SD ($n = 8$) of control values of the mRNA expression of *Slc6a4* and *Htr1a* adjusted with *Gapdh*. The effects of chronic administration of mirtazapine on expression of *Slc6a4* and *Htr1a* were compared using Student's *t*-test (**$P < 0.01$).

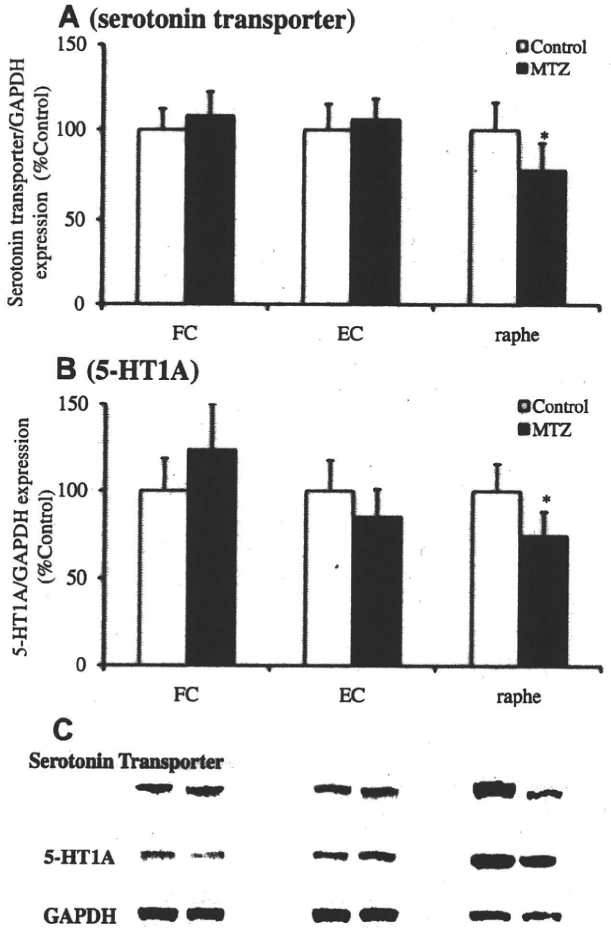

Fig. 7. Effects of chronic administration of mirtazapine on protein expression of serotonin transporter and 5-HT1A receptor in raphe nucleus and cortices. Effects of chronic administrations of mirtazapine (□: control, 0 mg/kg/day for 21 days, and ■: MTZ, 10 mg/kg/day for 21 days) on the protein expression of serotonin transporter and 5-HT1A receptor in raphe nucleus, FC and EC are indicated in Fig 7A and B, respectively. Ordinates indicate the mean ± SD ($n = 8$) of control values of the protein expression of serotonin transporter and 5-HT1A receptor adjusted with GAPDH. The effects of chronic administration of mirtazapine on protein expression of serotonin transporter and 5-HT1A receptor were compared using Student's *t*-test (**$P < 0.01$). Typical florescence photograph of Western blotting of serotonin transporter, 5-HT1A receptor and GAPDH are indicated in Fig. 7C.

regions (**Barnes and Sharp, 1999**; **Pazos and Palacios, 1985**). The activation of cortical postsynaptic 5-HT1A receptor locally reduces the serotonin release in this region (**Casanovas et al., 1999**). In the present study, in cortices, chronic administration of mirtazapine enhanced 8-OH-DPAT-induced reduction of the extracellular serotonin without affecting the expression of *Htr1a* or 5-HT1A receptor protein. Therefore, chronic administration of mirtazapine produced sensitization of postsynaptic 5-HT1A receptor in FC and EC. Indeed, the previous studies have demonstrated that chronic administration of mirtazapine results in the sensitization of postsynaptic 5-HT1A receptor-mediated hypothermia and electrophysiological responses (**Besson et al., 2000**; **McGrath et al., 1998**).

Contrary to postsynaptic 5-HT1A receptor in cortices, in the present study, chronic administration of mirtazapine inhibited the 8-OH-DPAT-induced reduction of the extracellular serotonin in raphe nuclei and their innervation cortical regions. These results suggest that chronic mirtazapine administration reduces the inhibition of serotonergic transmission associated with somatodendritic 5-HT1A receptors in raphe nuclei. The present study cannot confirm whether does chronic administration of mirtazapine produce desensitization of somatodendritic 5-HT1A autoreceptor in raphe nuclei or not, since chronic administration of mirtazapine reduced the expression of *Htr1a* mRNA and 5-HT1A receptor protein in raphe nuclei. Alternatively, chronic administration of mirtazapine reduced the inhibitory regulation associated with somatodendritic 5-HT1A autoreceptor in raphe nuclei.

Chronic administration of SSRI produces the desensitization of both somatodendritic 5-HT1A autoreceptor and postsynaptic 5-HT1A receptor (**Li et al., 1997, 1996**; **Raap et al., 1999**). Unlike the SSRIs, chronic administration of tricyclic antidepressants does not result in the desensitization of somatodendritic 5-HT1A autoreceptor, whereas electrophysiological studies indicate that chronic treatment with tricyclic antidepressants results in the sensitization of hippocampal postsynaptic 5-HT1A receptor (**Chaput et al., 1991**; **Kreiss and Lucki, 1995**). Therefore, the functional profile of 5-HT1A receptor with chronic administration of mirtazapine is intermediate between those of SSRI and tricyclic antidepressants.

Although reduced somatodendritic 5-HT1A autoreceptor function induced by chronic administration of mirtazapine could potentially contribute to the elevation of extracellular serotonin level in raphe nuclei, the present study demonstrated an other mechanism. Both acute and chronic administration of SSRI increased the extracellular serotonin level in raphe nucleus and hippocampus (Popa et al., 2010). Furthermore, chronic administration of SSRI reduced the mRNA expression of *Slc6a4* in hippocampus, parietal cortex, amygdale and other regions (Benmansour et al., 2002). Similar to SSRI, chronic administration of mirtazapine in the present study decreased both expressions of *Slc6a4* and serotonin transporter protein in raphe nuclei without affecting those in cortices. Therefore, the reduction of expression of *Slc6a4* and serotonin transporter protein by chronic administration of mirtazapine probably contributes to the weak but significant increase in the extracellular serotonin levels in DRN and MRN.

5. Conclusion

The present study demonstrated the mechanism of action of mirtazapine on serotonergic transmission associated with raphe nuclei. Acute administration of mirtazapine did not alter the extracellular serotonin level in raphe nuclei or their innervation cortical regions (FC and EC). However, mirtazapine probably enhanced the serotonergic transmission in raphe nuclei, based on the following results. First, under 5-HT1A receptor blockade, acute administration of mirtazapine increased the extracellular serotonin level in both raphe nuclei. Second, chronic administration of mirtazapine increased the extracellular serotonin level, and reduced the serotonin transporter expression in both raphe nuclei, similar to chronic administration of SSRI. Third, chronic administration of mirtazapine reduced inhibitory regulation associated with somatodendritic 5-HT1A autoreceptor with reduction of 5-HT1A receptor expression in raphe nucleus. Thus, with regard to the serotonergic transmission, the effect of short-term administration of mirtazapine is different from that of SSRI; whereas the effect of long-term administration of mirtazapine resembles that of SSRI. Contrary to raphe nuclei, neither acute nor chronic administrations of mirtazapine modulated extracellular serotonin level in the serotonergic innervation region, FC and EC. The expression of serotonin transporter and 5-HT1A receptor were also not affected by chronic administration of mirtazapine. Furthermore, chronic administration of mirtazapine led to the sensitization of postsynaptic 5-HT1A receptor in FC and EC. Thus, the mechanisms of action of mirtazapine involves, at least in part, a reduction of somatodendritic 5-HT1A autoreceptor function and reduction of serotonin transporter induced by chronic administration of mirtazapine.

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Comprehensive analysis of the genes responsible for neuroacanthocytosis in mood disorder and schizophrenia

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ABSTRACT

Neuroacanthocytosis syndromes are mainly comprised of two diseases: chorea-acanthocytosis (ChAc) and McLeod syndrome (MLS). There is a high incidence of psychiatric disorders such as mood disorder and schizophrenia among neuroacanthocytosis patients. We hypothesized that neuroacanthocytosis-related-genes might be associated with susceptibility to these psychiatric disorders. We performed a comprehensive mutation screen of *VPS13A* and *XK*, the gene responsible for ChAc and MLS, respectively, in 85 mood disorder subjects and *XK* in 86 schizophrenia subjects and compared the variants to 100 or more control alleles. We also performed copy number variation (CNV) analysis in 72 mood disorder subjects and 86 schizophrenia subjects. We identified three non-synonymous, two synonymous and six intron variants in mood disorder subjects and a novel GAT triplet repeat polymorphism in *VPS13A*. By CNV analysis, we identified a heterozygous exon 60–61 deletion in *VPS13A* in one mood disorder subject. We identified one non-synonymous and one intron variant in mood disorder and schizophrenia subjects, respectively, in *XK*. The presence of a pathogenic mutation or a potentially functional variant in mood disorder or schizophrenia subjects suggests that neuroacanthocytosis-related-genes might be involved in the pathogenesis of these psychiatric disorders.

1. Introduction

The term neuroacanthocytosis is inclusively used for rare diseases exhibiting neurological and neuropsychiatric abnormalities that occur together with red cell acanthocytosis (Danek et al., 2005). Neuroacanthocytosis has been classified into two groups depending on the presence or absence of movement disorders such as chorea. One group comprises the core neuroacanthocytosis syndrome in which neurodegeneration occurs primarily in the striatum, which causes movement disorders. The core neuroacanthocytosis syndrome consists of two main diseases: chorea-acanthocytosis (ChAc; MIM 200150) and the McLeod syndrome (MLS; MIM 314850).

ChAc is a rare, hereditary, neurodegenerative disease characterized by adult-onset, progressive, involuntary, choreic movements and erythrocyte acanthocytosis. The main neuropathological feature of ChAc is degeneration of the striatum (Danek et al., 2005). Other clinical symptoms, including psychiatric features, epilepsy, peripheral neuropathy, myopathy and oral self-mutilation are often found. The inherited form of ChAc is considered to be autosomal recessive. Previously we identified vacuolar protein sorting 13 homolog A (*VPS13A*), the gene responsible for ChAc, in which there was an exon 60–61 deletion mutation in the patients (Ueno et al., 2001). The *VPS13A* gene is organized into 73 exons spanning approximately 244 kb on chromosome 9q21.

MLS is an X-linked recessive hereditary disease. The X-linked Kx blood group gene (*XK*), which is responsible for MLS, is located on chromosome Xp21 and consists of three exons (Ho et al., 1994). The syndrome is characterized by the absence of the Kx erythrocyte antigen, weak expression of the Kell blood group system antigens and acanthocytosis. The primary neuropathological feature of MLS is degeneration of the striatum. Neuromuscular manifestations include chorea, areflexia, neurogenic muscle atrophy, myopathy and cardiomyopathy (Swash et al., 1983; Kawakami et al., 1999). In addition, generalized seizures and various neuropsychiatric abnormalities such as paranoia, hallucinations, depression, anxiety, personality changes or intellectual decline have also been described (Hardie et al., 1991; Witt et al., 1992; Danek et al., 1994; Swash et al., 1983; Malandrini et al., 1994; Jung and Haker, 2004).

We previously investigated a ChAc pedigree, in which the proband was homozygous for a 3889C > T nonsense mutation in the *VPS13A* gene and presented with a typical ChAc phenotype (Ichiba

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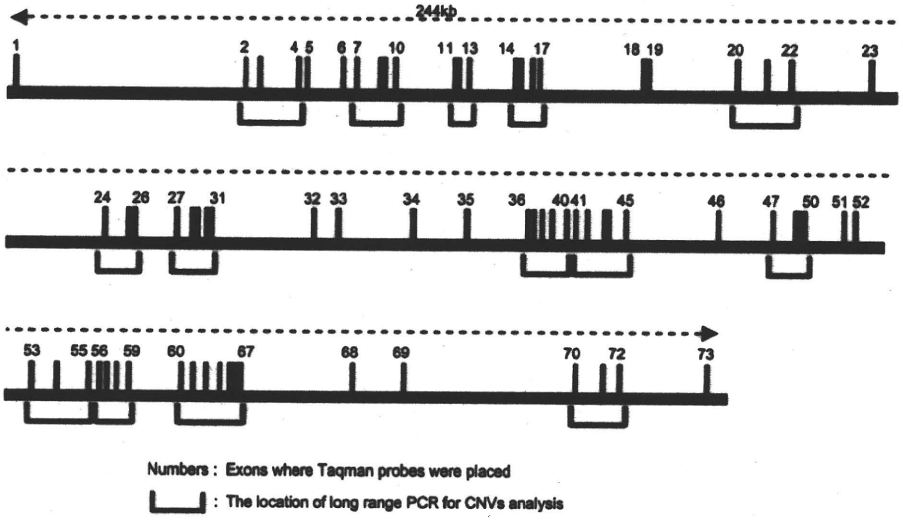

Fig. 1. Genomic structure of the *VPS13A* gene showing the locations of the quantitative real-time PCR and long range PCR assays.

et al., 2007). Four out of five heterozygous mutation carriers in the pedigree showed signs or symptoms potentially attributable to a heterozygous *VPS13A* mutation. Two of them had been given a diagnosis of depression. Thus, we hypothesized that *VPS13A* might be associated with susceptibility to mood disorder. In addition, MLS is associated with a high rate of psychosis such as mood disorder and schizophrenia. Thus, we hypothesized that *XK* might be associated with susceptibility to mood disorder and schizophrenia.

Because an increased number of patients with Parkinson's disease was noted among the probands and obligate carriers of Gaucher disease, the glucocerebrosidase gene (*GBA*), which is responsible for Gaucher disease, has been examined in other patients. Rare, heterozygous, pathogenic variants of *GBA* were identified at high rates in idiopathic Parkinson's disease patients (Aharon-Peretz et al., 2004; Mitsui et al., 2009). This demonstrates the common disease-multiple rare variants hypothesis. Similarly, there is a strong possibility of a genetic link between psychiatric disorders and *VPS13A*, because many patients and carriers with rare variants of neuroacanthocytosis-related-genes develop depression and schizophrenia. The large size of the *VPS13A* gene has thus far prohibited exhaustive sequence analysis in psychiatric patients. In this study, we resequenced the *VPS13A* and *XK* genes in patients with mood disorder, and the *XK* gene in patients with schizophrenia, in an attempt to identify rare, pathogenic variants. We also performed quantitative real-time PCR (Q-PCR) and long-range PCR to quantify *VPS13A* and *XK* copy numbers in 72 patients with mood disorder, and *XK* in 86 patients with schizophrenia.

2. Materials and methods

2.1. Human subjects

Approval for this study was obtained from the Institutional Review Board of Ehime University and Kagoshima University. Subjects with mood disorder and schizophrenia were contacted through their consultant psychiatrists, and written informed consent for the study was obtained before a sample of blood was donated for DNA extraction. Diagnoses were reached by consensus between two trained psychiatrists and were based on The International Statistical Classification of Diseases and Related Health Problems 10th Revision (ICD-10) criteria. No physical symptoms were recognized in all the patients with mood disorder or schizophrenia. Screening for mutations in *VPS13A* and *XK* was conducted in 85 Japanese mood disorder subjects: 18 with bipolar disorder (nine males, nine females) and 67 with depressive disorder (43 males, 24 females). Screening for GAT triplet repeat in *VPS13A* was conducted in 150 Japanese mood disorder subjects. Fifty or more Japanese controls had no known history of psychiatric illness. The screening for copy number variants (CNVs) in *VPS13A* and *XK* was conducted in 72 Japanese mood disorder subjects: 13 with bipolar disorder (six males, seven females) and 59 with depressive disorder (38 males, 21 females). Screening for mutations and CNVs in *XK* was conducted in 86 Japanese schizophrenia subjects (33 males and 53 females).

2.2. DNA resequencing

Genomic DNA was extracted from leukocytes by standard methods. All 73 exons and flanking regions of *VPS13A* (NC_000009.11) and *XK* (NC_000023.10) were amplified from the genomic DNA. Ex Taq was used in amplification of exon1 and exon69B of the genomic DNA. Recombinant Taq was used in amplification of the other exons. The PCR conditions and primer sequences are listed in Supplementary Tables 1 and 2. The annealing temperatures and times were determined according to the primer sequence and sequence abundance. Amplified products were purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany), labeled using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster, CA, USA) and were directly sequenced on an ABI PRISM 3100 Avant Genetic Analyzer (Applied Biosystems). The presence or absence of any variants identified was also analyzed in 100 or more control alleles.

2.3. CNV analysis

To detect CNVs in *VPS13A*, we developed a combinatorial method comprising Q-PCR using Taqman probes and long-range PCR over one or several exon(s). We detected CNVs in *XK* using Q-PCR.

2.4. Q-PCR

As shown in Fig. 1, we designed Taqman probes and primers for 44 out of the 73 exons of *VPS13A*, and for all three exons of *XK*. The sequences of the probes and primers are shown in Supplementary Tables 3 and 4. Q-PCR was performed on an ABI 7300 Real Time

Table 1
Variants of *VPS13A* found in mood disorder.

	NT change	AA change	Exon/intron	Conservation[a]	Cases	Controls
Nonsynonymous variants	c.588T>C[b]	H196R	ex8	C. D. M. R	2/170	0/232
	c.623G>A[b]	R208Q	ex9	C. D. M. R	1/170	0/226
	c.1438A>G	T480A	ex16	C. D. M. R	1/170	1/128
	c.3617G>A	R1206K	ex33	C. D. M. R	1/170	1/124
	c.4026A>C	E1342D	ex35	C. D. R	8/170	4/130
	c.4469G>A	R1490K	ex38	C. D. M. R	22/170	20/130
	c.4927G>A	D1643N	ex40	C. D. R	6/170	7/118
	c.5594C>T	T1865I	ex44	C. D. M. R	2/170	1/118
	c.6905G>A	S2302N	ex50	C. D. M. R	1/170	1/130
	c.7012C>G[b]	L2338V	ex50	C. D. M. R	1/170	0/122
Synonymous variants	c.963G>A[b]	V321V	ex12	C. D. M. R	1/170	0/112
	c.1020A>G	E340E	ex13	C. D. M. R	26/170	18/136
	c.3357A>C[b]	G1119G	ex32	C. D. M. R	1/170	0/124
	c.3498T>C	Y1166Y	ex32	C. D. M. R	9/170	5/132
	c.4041C>T	A1347A	ex35	C. D. M. R	2/170	1/124
Intron variants	c.755-4T>C		in10		1/170	3/116
	c.2964+5G>A[b]		in28		4/170	0/124
	c.3508-44G>T		in32		34/170	15/122
	c.3813-41G>A[b]		in33		1/170	0/122
	c.5575-30A>G[b]		in43		1/170	0/150
	c.6378+15A>T		in47		4/170	6/122
	c.8667+27C>T		in63		2/170	5/126
	c.8668-34C>T[b]		in63		1/170	0/132
	c.8743+74C>A[b]		in66		1/170	0/108
	c.9190-16C>A		in69		2/170	2/102
	c.9474+77G>A[b]		in72		5/170	0/130
	c.9475-43G>T		in72		16/170	13/122
GAT-trinucleotide repeats	c.9498GAT[9]	ex69		2/300	0/260	
	c.9498GAT[10]	ex69		295/300	258/260	
	c.9498GAT[11]	ex69		3/300	2/260	

The numbers of cases and controls represent the number of chromosome.
Nucleotides and amino acids of nonsynonymous variants and synonymous variants are numbered according to the *VPS13A* transcriptA cDNA sequence (Genbank accession no. NM_033305.2).
Nucleotides and amino acids of GAT-trinucleotide repeats are numbered according to the *VPS13A* transcriptB cDNA sequence (Genbank accession no. NM_015186.3).
[a] C, chimpanzee; D, dog; M, mouse; R, rat.
[b] Variants that were absent in controls.

PCR Instrument (Applied Biosystems). Each sample was analyzed in triplicate in 15 μL reaction mixture containing 15 ng of genomic DNA, 2× Taqman Universal PCR Master Mix, 20× working stock of the gene expression assay that includes the probe and primers for each exon of the neuroacanthocytosis-related-genes or the reference gene, *RNaseP*. Amplification was performed for 2 min at 50 °C, 2 min at 95 °C, then 40 cycles of 15 s at 95 °C and 60 s at 60 °C. Data analysis was performed using the ΔΔCt method according to the formula of Livak and Schmittgen (2001) and the manufacturer's instructions. A positive result was confirmed at least twice, and the average relative quantification was calculated.

2.5. Long-range PCR

To detect CNVs in the 29 exons of *VPS13A* for which there were no Taqman probes, we performed long-range PCR covering the exons where the Taqman probes were located, as shown in Fig. 1. Ex Taq was used for this experiment. The primer sequences and PCR conditions are listed in Supplementary Table 5. Co-amplified products from the controls and patients were separated by electrophoresis for 30 min at 100 V on 1% agarose gels. After ethidium bromide staining, they were visualized under UV light. We considered that this method would be able to detect deletion of intervening exons.

3. Results

3.1. VPS13A

Resequencing of *VPS13A* in subjects with mood disorder revealed 10 novel non-synonymous variants, 5 novel synonymous variants and 12 novel intron variants (Table 1). All 10 non-synonymous variants were missense mutations and were present in the heterozygous state. No nonsense mutations, or insertions and deletions leading to frameshift mutations, were found. Three out of 10 non-synonymous variants were absent in at least 100 control alleles. Two out of 5 synonymous variants and six out of 12 intron variants were also absent among control alleles (Fig. 2). In the original *VPS13A* sequence, there are 10 repeats of a GAT-trinucleotide near the 3′-end of the open reading frame of the transcript B variant (Danek et al., 2005). We identified that the GAT triplet repeat is polymorphic, with the number of repeats ranging from 9 to 11. No significant difference in distribution of the repeat length between mood disorder and control were detected. The patients with mood disorder possessing the 9- or 11-GAT triplet repeats were all heterozygous. No pathologically expanded allele was found. As shown in Fig. 3, Q-PCR for *VPS13A* in one mood disorder subject revealed a heterozygous exon 60–61 deletion, which is a known ChAc pathogenic mutation (Ueno et al., 2001). The other CNVs were not found in mood disorder subjects.

3.2. XK

Resequencing of *XK* in subjects with mood disorder revealed one novel non-synonymous variant, Y321C (Table 2 and Fig. 4). It was present in a female patient in the heterozygous state and was absent from 129 normal alleles. Resequencing of *XK* in subjects with schizophrenia revealed one novel intron variant, c.509-13C>G, which was observed in a pyrimidine-rich region at the intron2–exon3 junction (Table 3 and Fig. 4). It was present in a female patient in the heterozygous state and was absent from 109

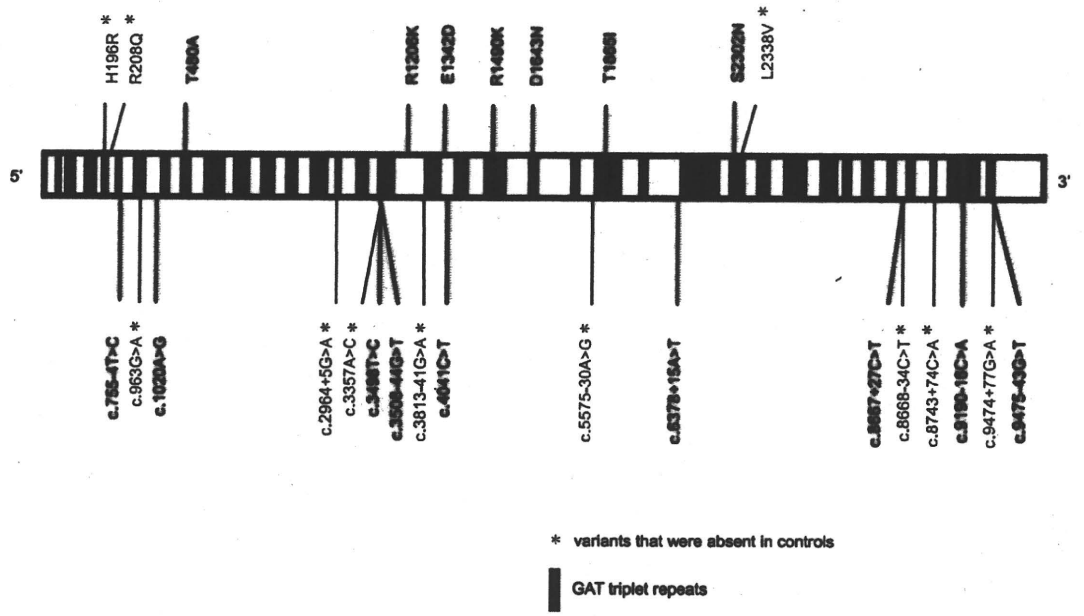

Fig. 2. Schematic diagram of the *VPS13A* cDNA showing the locations of the identified variants. Exons are shown as alternating white and black boxes. Above the cDNA are shown ten non-synonymous variants that have not been previously reported. Three non-synonymous variants were absent in controls (indicated in red and with an asterisk). Below the cDNA are shown 5 synonymous variants and 12 intron variants that have not been previously reported. Two synonymous variants and six intron variants were absent in controls (indicated in red and with an asterisk). The GAT triplet repeat was polymorphic, with the number of repeats ranging from 9 to 11 (indicated in green). (For interpretation of the references to color in this figure caption, the reader is referred to the web version of the article.)

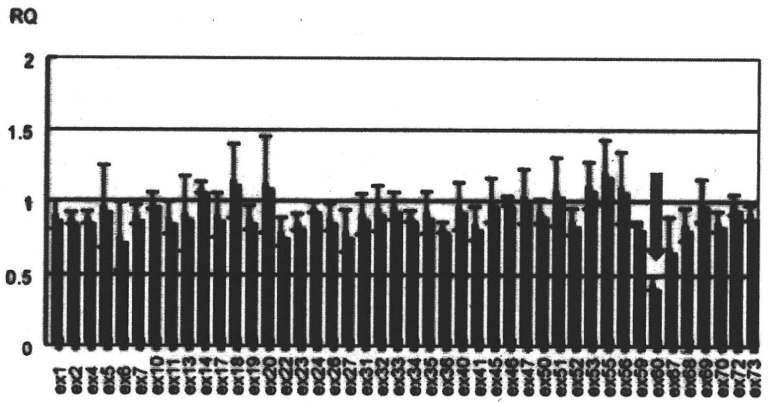

Fig. 3. Results of quantitative real-time PCR for *VPS13A*. The green arrow shows a heterozygous exon 60–61 deletion, a ChAc pathogenic mutation, which was identified in one mood disorder subject. RQ is relative quantity in case normal copy number is one. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of the article.)

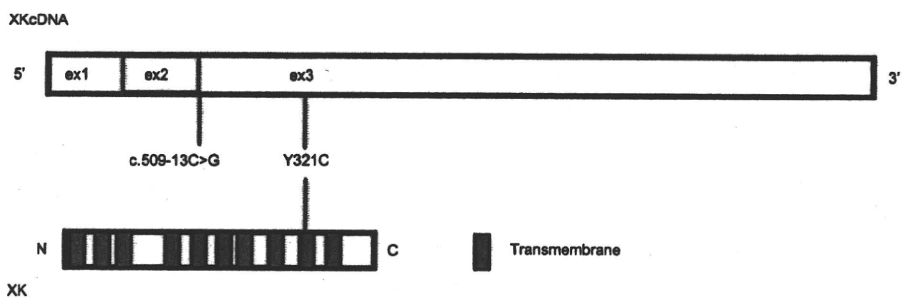

Fig. 4. Schematic diagram of the *XK* cDNA (upper box) and the corresponding XK protein (lower box), showing the locations of the identified variants. Y321C was found in one mood disorder subject. c.509-13C>G was found in one schizophrenia subject. They were present in the heterozygous state and absent in controls. Y321C was identified within a transmembrane domain (green boxes). (For interpretation of the references to color in this figure caption, the reader is referred to the web version of the article.)