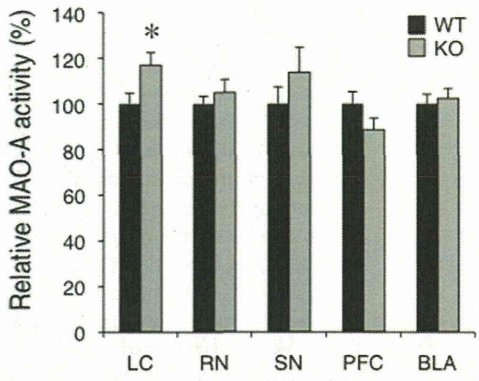

Figure 5. MAO-A enzymatic activities in Rines KO and WT mice. The enzymatic activities were measured in the LC, raphe nuclei (RN), substantia nigra (SN), prefrontal cortex (PFC), and basolateral nucleus of the amygdala (BLA) in WT and Rines KO mouse brains. Increased MAO-A activity was observed in the LC region of Rines KO mice. WT and KO, $n = 7–14$; $*p < 0.05$, Student's t test. WT values are indicated as 100%.

substantia nigra showed comparable monoamine levels between WT and KO mice regardless of foot shock (Table 2). These results suggest that Rines KO mice have altered LC function and monoamine levels in certain brain regions.

MAO-A has an essential role in the metabolism of NE and 5-HT, and its expression is concentrated in the LC (Shih et al., 1999). MAO-A-deficient mice have increased levels of monoamines such as NE and 5-HT in the brain (Cases et al., 1995; Kim et al., 1997) and show behavioral phenotypes that are partially opposite to those of Rines KO mice (see Discussion). To clarify the effects of MAO-A in the Rines KO brain, we measured MAO-A activity in the LC and found that it was significantly higher than that in WT mice (Fig. 5: Student's t test, $t = 2.33$, $p = 0.028$). There were no significant differences of MAO-A activity in other brain regions such as the raphe nuclei, substantia nigra, prefrontal cortex, or amygdala (Fig. 5: RN, Student's t test, $t = 0.75$, $p = 0.47$; SN, Student's t test, $t = 1.03$, $p = 0.32$; PFC, Student's t test, $t = 1.48$, $p = 0.16$; BLA, Student's t test, $t = 0.39$, $p = 0.70$). Moreover, Rines KO mice had increased MAO-A protein levels in the LC, as revealed by quantitative immunostaining (Fig. 6*A–C*: MAO-A intensity, Student's t test, $t = 3.49$, $p = 0.00064$; relative intensity of MAO-A/TH, Student's t test, $t = 2.24$, $p = 0.0264$). In control experiments, neither the immunostaining signal intensity for the catecholamine synthesizing enzyme TH (Fig. 6*A–C*: TH intensity, Student's t test, $t = 0.76$, $p = 0.45$) nor the area or number of TH-positive cells (Fig. 6*D,E*: relative area in LC region, Student's t test, $t = 1.04$, $p = 0.30$; total cell number in LC region, Student's t test, $t = 0.35$, $p = 0.73$) in the LC region differed between WT and KO mice. In addition, there were no intergenotype differences in the mRNA levels of MAO-A or other proteins involved in the synthesis, transport, or metabolism of monoamines (Fig. 6*F*: MAO-A, Student's t test, $t = 0.15$, $p = 0.88$; MAO-B, Student's t test, $t = 0.35$, $p = 0.73$; DBH, Student's t test, $t = 1.09$, $p = 0.30$; NET, Student's t test, $t = 0.31$, $p = 0.76$; VMAT2, Student's t test, $t = 0.20$, $p = 0.84$; COMT, Student's t test, $t = 1.58$, $p = 0.14$; Rines, Welch's t test, $t = 27.28$, $p < 0.0001$). These results suggest that Rines may reduce MAO-A protein levels posttranslationally in the LC, with a cognate reduction in MAO-A activity.

To determine the specificity and regulation of MAO-A by Rines, we investigated whether ubiquitination and subsequent degradation of MAO-A protein was mediated by Rines in cell culture. First, we verified the interaction between Rines and MAO-A. When Flag–Rines was coexpressed with HA-MAO-A in epoxomicin (a proteasome inhibitor)-treated HEK293T cells and immunoprecipitated with an anti-Flag antibody, we detected the coprecipitated HA-MAO-A (Fig. 7*A*). We also investigated whether Rines could promote the ubiquitination of the MAO-A protein. Flag–MAO-A and HA–ubiquitin were coexpressed in NIH3T3 cells in the presence or absence of Myc-Rines, and cell lysates were subjected to immunoprecipitation with an anti-Flag antibody, followed by immunoblotting with an anti-HA antibody to detect ubiquitin-conjugated MAO-A. As a result, Myc-Rines enhanced a broad band with high molecular weight corresponding to polyubiquitinated Flag–MAO-A (Fig. 7*B*), indicating that MAO-A was polyubiquitinated by Rines. Myc-Rines was detected in Flag–MAO-A coprecipitates (Fig. 7*B*), which is consistent with the results presented in Figure 7*A*. Furthermore, we observed that HA-MAO-A protein levels decreased in the presence of Flag–Rines and this reduction was rescued by epoxomicin treatment (Fig. 7*C*). In addition, we verified the ubiquitination of endogenous MAO-A by Rines in the brain LC region (Fig. 7*D*). Tissue lysates of the LC regions from WT or Rines KO mice were immunoprecipitated with an anti-MAO-A antibody and then immunoblotted with an anti-ubiquitin antibody to detect polyubiquitinated MAO-A protein. We determined the densities of the broad band with high molecular weight corresponding to polyubiquitinated endogenous MAO-A. The densities significantly decreased in Rines KO mice (Fig. 7*D*: Student's t test, $t = 3.42$, $p = 0.0090$). Furthermore, a band corresponding to unubiquitinated MAO-A protein in Rines KO mice was more intense than that in WT mice (Fig. 7*D*: Student's t test, $t = 2.70$, $p = 0.027$), a result consistent with the MAO-A immunostaining (Fig. 6*A–C*). These results indicate that Rines promotes ubiquitination and proteasomal degradation of MAO-A both in cell culture and brain LC.

The above results suggest that the altered emotional responses in Rines KO mice might be at least partially due to increased MAO-A activity in the absence of Rines-dependent protein degradation. To address this possibility, we examined the effect of the nonselective and irreversible MAO inhibitor TCP on emotional responses (Fig. 8*A–E*). TCP is used as an antidepressant and anxiolytic agent in the clinical treatment of mood and anxiety disorders (O'Donnell and Shelton, 2010). Rines KO and WT mice were orally administered TCP or vehicle (water) chronically for 11–23 d and then tested behaviorally. In the open field test, TCP increased the number of movements in Rines KO mice and rescued behavioral function to a level comparable to control WT mice (Fig. 8*A*: two-way ANOVA, $F_{\text{genotype }(1,91)} = 8.63$, $p = 0.0042$; $F_{\text{drug }(1,91)} = 4.52$, $p = 0.036$; $F_{\text{genotype} \times \text{drug }(1,91)} = 4.04$, $p = 0.046$; WT-water vs KO-water, Student's t test, $t = 3.44$, $p = 0.0012$; KO-water vs KO-TCP, Student's t test, $t = 2.84$, $p = 0.0070$). A similar tendency, although not significant, was observed in the total time spent in the center of the field in the open field test (Fig. 8*C*: two-way ANOVA, $F_{\text{genotype }(1,91)} = 2.18$, $p = 0.14$; $F_{\text{drug }(1,91)} = 0.14$, $p = 0.71$; $F_{\text{genotype} \times \text{drug }(1,91)} = 0.48$, $p = 0.49$) and TCP increased the total distance only in WT mice but not in KO mice (Fig. 8*B*: WT-water vs WT-TCP, Student's t test, $t = 3.43$, $p = 0.0012$; KO-water vs KO-TCP, Student's t test, $t = 1.60$, $p = 0.12$; two-way ANOVA, $F_{\text{genotype }(1,91)} = 1.22$, $p = 0.27$; $F_{\text{drug }(1,91)} = 11.72$, $p = 0.00093$; $F_{\text{genotype} \times \text{drug }(1,91)} = 0.77$, $p = 0.38$). In addition, a two-way ANOVA demonstrated significant genotype-treatment interaction effects on the immobility time on the second day of the forced swimming test (Fig. 8*D*: two-way ANOVA, $F_{\text{genotype }(1,131)} = 0.79$, $p = 0.37$; $F_{\text{drug }(1,131)} = 5.88$, $p = 0.017$; $F_{\text{genotype} \times \text{drug }(1,131)} = 4.04$, $p = 0.047$; WT-water vs WT-TCP, Student's t test, $t = 3.35$, $p = 0.0013$;

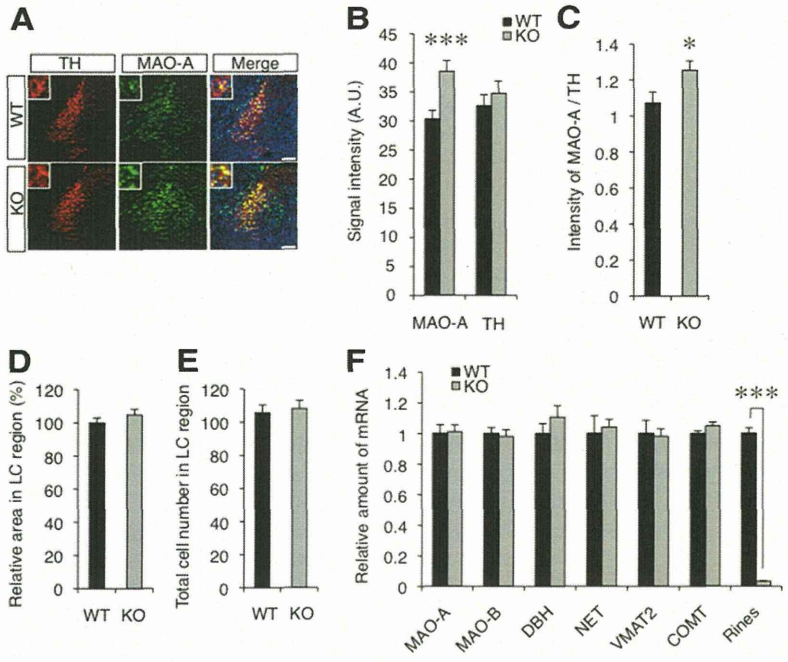

Figure 6. Rines regulated MAO-A protein level. ***A–C***, Rines KO mice showed significantly increased MAO-A protein levels in the LC region. ***A***, Representative immunofluorescence staining of TH and MAO-A proteins in the LC region from WT and Rines KO mice. High-magnification images of the center regions are shown in insets. WT, $n = 5$; KO, $n = 5$. Scale bar, 100 μm. ***B***, Quantitative analyses of signal intensities for MAO-A and TH. $^{***}p < 0.001$, Student's t test. AU, arbitrary unit. ***C***, Intensities of MAO-A relative to TH. $^{**}p < 0.01$, Student's t test. ***D***, ***E***, Comparison of the areas and total cell numbers in the LC regions of WT and Rines KO mice. There were no significant differences in the area (***D***) or number (***E***) of TH-expressing cells in the LC region between the two genotypes. WT mean values = 100% (***D***). WT, $n = 5$; KO, $n = 5$. ***F***, The relative amount of mRNA in the LC region isolated from WT and Rines KO mice measured with quantitative RT-PCR. DBH, dopamine β-hydroxylase; NET, NE transporter; VMAT2, vesicular monoamine transporter protein 2; COMT, catechol-O-methyltransferase. No differences were detected in the mRNA levels of these proteins between the genotypes. WT, $n = 7$; KO, $n = 7$; $^{***}p < 0.001$, Welch's t test. WT values are indicated as 1.

WT-TCP vs KO-TCP, Student's t test, $t = 2.08$, $p = 0.042$) and the latency to enter the dark box in the light–dark box test (Fig. 8*E*: two-way ANOVA, $F_{\text{genotype }(1,131)} = 0.012$, $p = 0.91$; $F_{\text{drug }(1,131)}, < 0.0001$, $p = 0.999$; $F_{\text{genotype} \times \text{drug }(1,131)} = 4.17$, $p = 0.043$). In addition, we examined the effect of a specific MAO-A inhibitor, clorgyline, on emotional memory formation in the passive avoidance test (Fig. 8*F*). Two and a half hours after the intraperitoneal administration of clorgyline or vehicle (saline), mice were conditioned with an electric foot shock (Fig. 3*C*). In a test performed 24 h later, clorgyline-treated KO mice exhibited significantly lower latencies than did saline-treated KO mice, although there were no significant differences between saline-treated and clorgyline-treated WT mice (Fig. 8*F*: WT-saline vs WT-clorgyline, Student's t test, $t = 0.25$, $p = 0.81$; KO-saline vs KO-clorgyline, Student's t test, $t = 2.78$, $p = 0.011$; WT-saline vs KO-saline, Student's t test, $t = 2.08$, $p = 0.049$; WT-clorgyline vs KO-clorgyline, Welch's t test, $t = 3.57$, $p = 0.0028$). The differential response to TCP or clorgyline in these emotional behaviors supports the idea that Rines is involved in MAO-A proteostasis in connection with some emotional responses.

Discussion

Rines controls emotional and social behaviors

Rines KO mice exhibited several types of abnormal responses to environmental changes and social inputs. First, they showed enhanced anxiety-like behaviors in response to exposure to new and unexpected nonpainful sensory stimuli (i.e., open space in the open field test, open arms in the elevated plus maze test, Fig. 3*A*,*B*). Second, altered stress reactivity was indicated by the exposure to aversive stimuli over two successive days (the passive avoidance and forced swimming tests, Fig. 3*C*,*D*). Second-day responses were similar to those on the first day, whereas these two responses were significantly different in WT mice. Third, affiliative social behaviors increased in both familiar and unfamiliar environments (the social interaction and resident-intruder tests, Fig. 4*A*,*B*). Therefore, Rines KO mice, which show altered reactivity to new stimuli, would be interesting model animals to investigate the molecular mechanisms underlying proper emotional and social behaviors.

Rines regulates monoamine levels in the brain

This study shows that Rines KO mice have altered monoamine levels in some brain regions. Among the three major monoamine sources (LC, raphe nuclei, and substantia nigra), significantly less NE and 5-HT were observed in the LC after aversive stimuli (Table 2). Exposure to new, unexpected, or non-noxious stimuli activates the rodent LC (Aston-Jones and Bloom, 1981). Acute emotional responses, including activation of fear memories and stress responses, involve activation of LC neurons (Berridge and Waterhouse, 2003; Alsene and Bakshi, 2011).

Consistent with this idea, studies in humans and primates demonstrate that LC-NE neurotransmission plays a vital role in cognitive and attentional processes (Berridge and Waterhouse, 2003; van Stegeren, 2008; Kindt et al., 2009). Dysregulation of this system contributes to cognitive and emotional dysfunction associated with a variety of psychiatric disorders, including stress- and/or anxiety-related disorders (Berridge and Waterhouse, 2003; van Stegeren, 2008; Kindt et al., 2009; Alsene and Bakshi, 2011).

In addition to LC monoamines, lower 5-HT levels observed in the prefrontal cortex are intriguing in view of the following points. First, the serotonergic system has been reported to play an important role in the prefrontal cortex, a major area regulating emotion and cognition (Barnes and Sharp, 1999; Gross et al., 2002; Weisstaub et al., 2006; Meltzer and Huang, 2008; Puig and Gulledge, 2011). In rodents, intraprefrontal cortex administration of a 5-HT1A or 5-HT1B agonist–antagonist alters the amount of time in the open arms of an elevated plus maze (Solati et al., 2011), and disruption of 5-HT2A receptor signaling in cerebral cortex affects the time spent in the center of an open field (Weisstaub et al., 2006). The anxiety-like behavior in the Rines KO mice could reflect altered 5-HT levels in the prefrontal cortex. Second, 5-HT levels in the Rines KO prefrontal cortex is reciprocally altered upon exposure to foot shock stress compared with WT (Table 2). This indicates the altered stress reactivity of the Rines KO brain, which could be the basis of the abnormalities in the forced swimming and passive avoidance tests. Third, the 5-HT system is involved in controlling aggressive behaviors in mice (Saudou et al., 1994; Korte et al., 1996; Popova et al., 1996; Popova, 2008). The altered social behavior in Rines KO mice could also be related to the 5-HT dysregulation.

We also observed that NE levels increased in the stressed prefrontal cortex and unstressed amygdala of Rines KO mice (Table 2). Considering that the NE fibers from the LC have auto-NE receptors that inhibit NE release (Starke, 2001), the reduction of NE in the LC might lead to impaired NE feedback, thus increasing the apparent levels of NE in the prefrontal cortex and amygdala. Similar observations have been reported in the brain of SIRT1-overexpressing mice. These mice showed higher MAO-A activity and lower 5-HT levels in their brain regions than WT mice. However, in contrast to 5-HT, they showed higher NE levels than those of WT mice (Libert et al., 2011).

Rines is a critical regulator of the MAO-A protein level

We have shown that MAO-A activity and protein abundance increases in the LC region of Rines KO mice (Figs. 5, 6*A–C*, 7*D*). Furthermore, Rines can bind and ubiquitinate MAO-A and can enhance its proteosomal degradation (Fig. 7). The ubiquitination of endogenous MAO-A by Rines was verified by the ubiquitination assay using the LC regions from WT and Rines KO mice. The polyubiquitination of MAO-A in the LC region decreased in Rines KO mice (Fig. 7*D*). In addition, part of the behavioral phenotype of the Rines KO was the opposite of that of the MAO-A loss-of-function mutant mice; that is, MAO-A KO mice exhibit emotional behavior abnormalities including increased time spent in the center of the platform in the open field test (Cases et al., 1995) and enhanced fear conditioning in the passive avoidance test (Kim et al., 1997; Dubrovina et al., 2006). Furthermore, social investigation and interaction are reduced in both MAO-A-KO and MAO-A-hypomorphic mice (Cases et al., 1995; Vishnivetskaya et al., 2007; Scott et al., 2008; Bortolato et al., 2011).

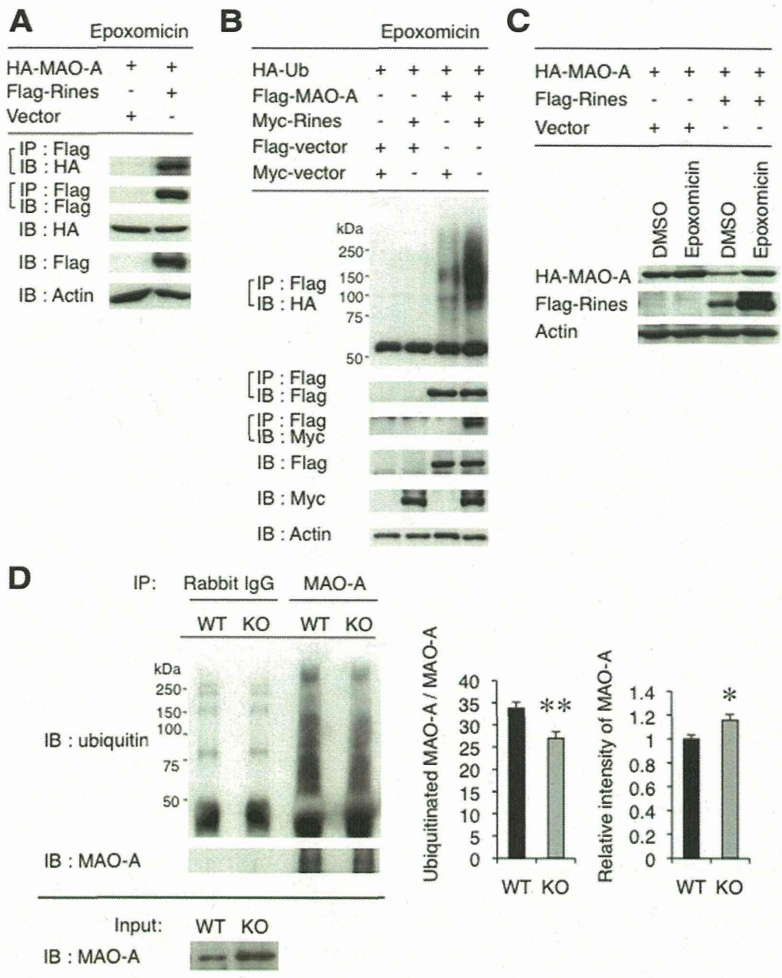

Figure 7. Rines promotes the ubiquitination and degradation of MAO-A in the proteasome. ***A***, Coimmunoprecipitation of the Rines protein with MAO-A protein from HEK293T cells transfected with Rines and MAO-A expression vectors and treated with a proteasome inhibitor, epoxomicin (5 μM). ***B***, NIH3T3 cells transfected with the indicated vectors were treated with epoxomicin, immunoprecipitated with an anti-Flag antibody, and then immunoblotted with an anti-HA antibody to detect polyubiquitinated MAO-A (apparent as smear bands). ***C***, Enhanced MAO-A protein decrement by Rines was blocked by application of epoxomicin to NIH3T3 cells. ***D***, Ubiquitination of endogenous MAO-A in LC decreased in Rines KO mice. Left: Equal protein amounts of tissue lysates of LC region from WT or Rines KO mice were immunoprecipitated with an anti-MAO-A antibody and then immunoblotted with an anti-ubiquitin antibody (IP) to detect polyubiquitinated endogenous MAO-A (apparent as smear bands) or were directly immunoblotted with anti-MAO-A antibody (input). Middle: Intensities of polyubiquitinated MAO-A relative to that of unubiquitinated MAO-A. $^{**}p < 0.01$, Student's *t* test. WT, $n = 5$; KO, $n = 5$. Right: Intensities of unubiquitinated MAO-A in LC regions of WT and Rines KO mice. Endogenous MAO-A protein levels in LC region from Rines KO mice increased relative to those from WT mice. WT, $n = 5$; KO, $n = 5$; $^{*}p < 0.05$, Student's *t* test. WT values are indicated as 1.

These behavioral and monoamine abnormalities in Rines KO mice and our biochemical analysis suggest that Rines regulates MAO-A protein levels in specific brain regions. The basis of the selective MAO-A deregulation in LC (Fig. 5) remains to be elucidated. We surmise that the altered protein degradation rate might be most easily detected in LC with the highest MAO-A expression. Otherwise, other factors cooperating with or targeted by Rines might be differentially distributed in the brain regions. However, the distinct response of MAO inhibitors on some Rines KO behaviors supports the idea that the control of emotional behavior by Rines is partly due to the deregulation of brain MAO-A subset (Fig. 8).

MAO-A expression is known to be transcriptionally regulated (Chen et al., 2005; Libert et al., 2011). However, this study is the first to reveal the regulation of MAO-A protein level via the UPS. It is likely that MAO-A levels are controlled both at the level of gene expression and proteasomal degradation, and the balance between anabolism and catabolism finely tunes the optimal amount. Although the UPS is proposed to be involved in mood disorder pathogenesis based on genome-wide association and gene expression analyses (Ryan et al., 2006; Garriock et al., 2010; Gormanns et al., 2011), little is known of the E3 ubiquitin ligase that plays a role in affective behaviors by alteration of the monoaminergic system. In addition, to our knowledge, no studies have found that the E3 ubiquitin ligase targets monoamine-metabolizing enzymes. Considering the role of the monoamine system in controlling overall brain functions (Bear et al., 2006), our Rines KO mice may become an important animal model for understanding the molecular

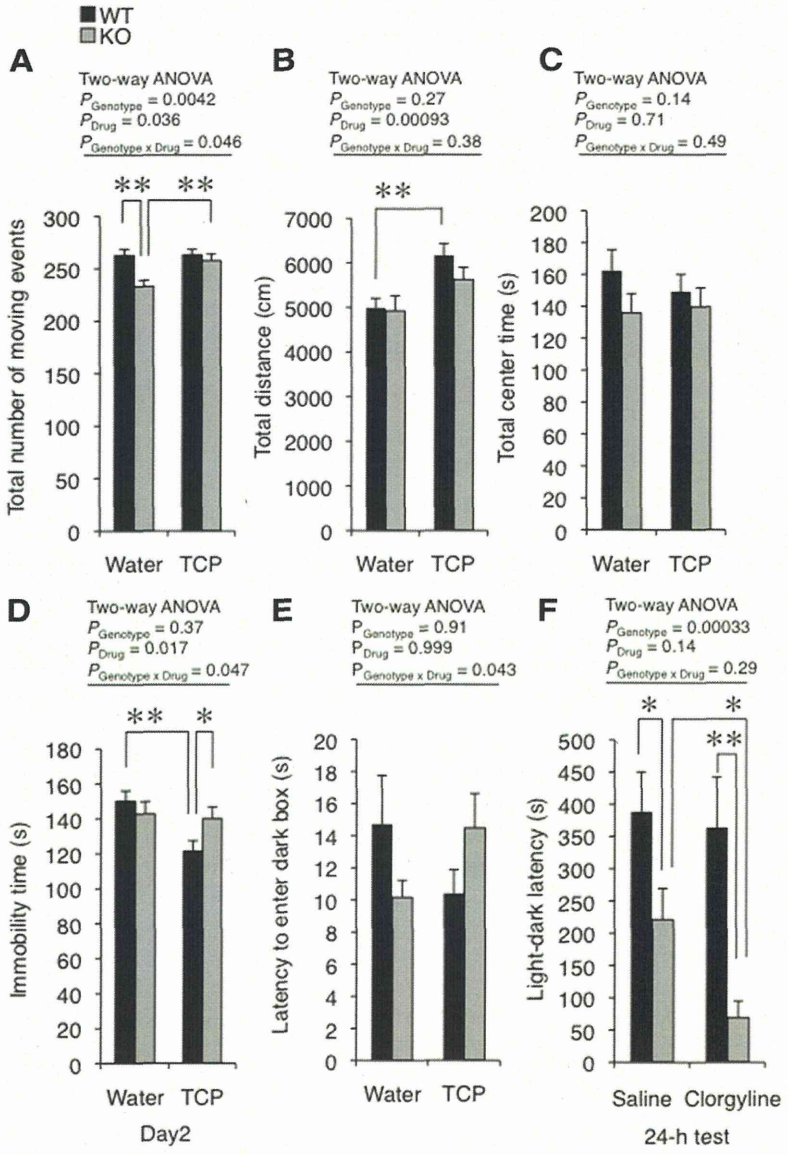

Figure 8. Emotional behaviors in Rines KO and WT mice were differentially affected by MAO inhibitors. ***A–E***, Water or water containing the MAO inhibitor TCP (3 mg/kg) was administered to mice chronically. The total number of movements (***A***), total distance (***B***), and total center time (***C***) in the open field test were measured. ***A***, TCP rescued the reduction in the total number of movements in KO mice. ***D***, ***E***, Rines KO mice exhibited altered responses to TCP in the immobility time on the second day of the forced swimming test (***D***) and the latency to enter dark box in the light–dark box test (***E***). $n = 22–25$ mice per condition in the open field test; $n = 32–36$ mice per condition in the forced swimming test; $n = 32–36$ mice per condition in the light–dark box test; $^{*}p < 0.05$, $^{**}p < 0.01$, Student's t test. ***F***, Saline or the selective MAO-A inhibitor clorgyline (0.1–1 mg/kg) was intraperitoneally administered to mice 2.5 h before electric foot shock on the conditioning day in passive avoidance test. Data are presented as light–dark latencies in the 24 h test. Rines KO mice showed a distinct response to the clorgyline. $n = 12–14$ mice per condition; $^{*}p < 0.05$, $^{**}p < 0.01$, Student's t test or Welch's t test.

mechanisms underlying the regulation of emotional responses and social behaviors.

Clinical implications of Rines-mediated regulation of the monoaminergic pathway

Abnormalities evident in Rines KO mice may have clinical implications. Interestingly, MAO-A-H (the higher transcriptional efficiency variant) is associated with anxiety and mood disorders (Deckert et al., 1999; Schulze et al., 2000; Samochowiec et al., 2004; Yu et al., 2005; Meyer et al., 2006). We speculate that the pathophysiological status of patients is related to the enhanced anxiety and altered stress reactivity in Rines KO mice. Furthermore, the MAO-A level in human brain is inversely correlated with the personality traits of aggression (Alia-Klein et al., 2008). The contrasting sociality between Rines KO mice (highly affiliative behaviors) and MAO-A KO hypomorphic mice (low affiliative behaviors) may reflect the difference in MAO-A activity (Rines KO, high; MAO-A mutants, low). In sum, the Rines KO behavioral phenotypes found in this study may be linked to MAO-A activity.

Consistent with this view, a recent study showed that SIRT1 activates transcription of MAO-A, and transgenic mice overexpressing SIRT1 in the brain show enhanced anxiety-like behavior (Libert et al., 2011). Moreover, SIRT1 variants are associated with risk of anxiety in human population samples (Libert et al., 2011), providing evidence that a protein that regulates MAO-A levels can be a disease-causing factor.

In contrast, studies on human subjects indicate that the prevalence of aggressive and antisocial behavior in adults in the MAO-A-L (the lower transcriptional efficiency variant) is affected by their history of stress during childhood, such as abuse and maltreatment (Caspi et al., 2002; Kim-Cohen et al., 2006). In addition, administration of a 5-HT synthesis inhibitor to MAO-A-KO mice during brain development (postnatal days 0–6) reverses aggressive behavior in adulthood (Cases et al., 1996). Therefore, emotional behavior abnormalities in Rines KO mice may be affected by age and environmental factors. Longitudinal and gene–environment interaction studies with Rines KO mice would contribute to a more comprehensive understanding of the pathophysiology of aggression and antisocial behavior.

Finally, Rines is the first reported E3 ubiquitin ligase the deletion of which causes altered MAO-A levels in the LC, monoamine levels in some brain regions, and emotional behaviors including social behavior. Further clarification of the role of this enzyme in emotional responses along with genetic analyses of human Rines polymorphisms should offer new insights into psychiatry and the treatment of anxiety, stress-related disorders, and impaired social functions.

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SOFT-DIET FEEDING AFTER WEANING AFFECTS BEHAVIOR IN MICE: POTENTIAL INCREASE IN VULNERABILITY TO MENTAL DISORDERS

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Abstract—Mastication is one of the most important oral functions, and the period during which mastication is acquired overlaps with the term of rapid development and maturation of the neural systems. In particular, the acquisition period after weaning is related to the potential onset of mental disorders. However, the roles of mastication during this period for brain development remain largely unknown. Therefore, we used a series of standard behavioral analyses, assessment of hippocampal cell proliferation, and the expression of brain-derived neurotrophic factor (BDNF), TrkB, and Akt1 in the hippocampus and frontal cortex of mice to investigate the effects of post-weaning mastication on brain function. We fed 21-day-old C57BL6/J male mice either a hard or a soft diet for 4 weeks and conducted a series of standard behavioral tests from 7 weeks of age. Further, histological analysis with bromodeoxyuridine was performed to compare hippocampal cell proliferation at 7 and 14 weeks of age. Real-time polymerase chain reaction was performed to compare *BDNF*, *TrkB*, and *Akt1* expression in the hippocampus and frontal cortex of 14-week-old mice. Compared to mice fed a hard diet (HDM), soft-diet mice (SDM) showed behavioral impairments, including decreased home cage activity, increased open field test activity, and deficits in prepulse inhibition. These results were similar to those observed in mouse models of schizophrenia. However, no effects were observed on anxiety-like behaviors or memory/learning tests. Compared to HDM, SDM showed significantly decreased hippocampal cell proliferation and hippocampal *BDNF* and *Akt1* gene expression at 14 weeks of age. A soft diet after weaning may have resulted in histological and molecular changes in the hippocampus and influenced outcomes of behavioral tests related to mental disorders. Our findings suggest that soft-diet feeding after weaning may affect both physical and mental development of mice, and may increase vulnerability to mental disorders.

Key words: soft-diet feeding, prepulse inhibition; behavioral analysis; mental disorder; neurogenesis; brain-derived neurotrophic factor.

INTRODUCTION

Mastication is one of the most important oral functions. Infancy is an extremely important period for acquiring the complex ability to masticate, which consists of the actions of chewing and swallowing (Bosma, 1976; Gisel, 1991; Qureshi et al., 2002). Epidemiological studies of human infants have examined mastication acquisition, which is comprised of chewing, swallowing, and coordinating the timing of breathing and chewing rhythm. Such studies indicated that the acquisition of masticatory ability, which occurs simultaneously with anatomical development of the brain, is made possible by the interaction of mastication experience with central nervous system (CNS) development and maturation (Morris, 1989; Gisel, 1991; Fucile et al., 2005).

Additional studies have been conducted to investigate masticatory motor development in other mammals. These studies indicated that masticatory ability is acquired through various factors, such as craniofacial development, CNS maturation, peripheral sensory nerve input, and motor learning (Iriki et al., 1988; Westneat and Hall, 1992; Huang et al., 1994). In particular, it has been reported that the development of proper masticatory function is inhibited by soft-diet feeding (Liu et al., 1998; Okayasu et al., 2003).

Mastication has been reported to be related to maxillofacial development, particularly the growth and

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Abbreviations: ANOVA, analysis of variance; ASR, acoustic startle response; BDNF, brain-derived neurotrophic factor; Brd-U, bromodeoxyuridine; CCD, charge-coupled device camera; CNS, central nervous system; DG, dentate gyrus; fMRI, functional magnetic resonance imaging; HDM, hard diet mice; OCT, optimal cutting temperature; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PPI, prepulse inhibition; PTSD, post-traumatic stress disorder; RT, reverse transcriptase; RT-PCR, real-time polymerase chain reaction; SDM, soft-diet mice.

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development of the mandible (Beecher and Corruccini, 1981; Kiliaridis et al., 1999; Luca et al., 2003; Maki et al., 2003). Our previous studies in mice fed a soft diet indicated that mastication after weaning influences gene expression in mandibular condylar cartilage (Watahiki et al., 2004) and produces major differences in mandibular morphology (Enomoto et al., 2010). Mastication has recently been reported to influence not only craniofacial development, but also brain function. For example, mastication has been reported to be involved in improving memory and learning in adults (Wilkinson et al., 2002). In addition, studies using functional magnetic resonance imaging (fMRI) suggested that mastication may simultaneously activate the prefrontal cortex and parietal cortex and may contribute to higher cognitive function (Takada and Miyamoto, 2004; Hirano, 2008).

Furthermore, human studies showed that reduced bite force and the number of teeth lost are related to the onset and progression of dementia and Alzheimer's disease (Okimoto et al., 1991; Shigetomi et al., 1998; Okamoto et al., 2010).

In an animal study in which senescence-accelerated model SAMP8 mice were fed a long-term soft diet, significant declines were observed in behavioral tests of memory and learning ability (Yamamoto and Hirayama, 2001). Yamazaki et al. (2008) also reported that tooth-extracted rats showed significantly reduced performance on behavioral tests that evaluate spatial memory (Yamazaki et al., 2008). In addition, it has been reported that rats from which the molar teeth have been removed show accumulation of amyloid-β in the hippocampus, which is related to decreased hippocampal neurogenesis (Ekuni et al., 2011a,b).

The studies mentioned above clearly demonstrated that a long-term soft diet and tooth loss influence brain functions related to memory and learning, and may also be related to the onset of dementia and Alzheimer's disease.

Previous work has focused primarily on the relationship between mastication and brain function in senescence. However, there have been no reports regarding the relationship between mastication and brain function during growth and development, particularly the relationship between masticatory alterations and mental disorders.

Mental disorders, including schizophrenia, are multifactorial diseases that result from the interaction of complex genetic and environmental factors. All periods of CNS formation, including the fetal stage, the perinatal period, and infancy, are crucial periods in the onset of schizophrenia (Weinberger, 1987; Lillrank et al., 1995). Epidemiological studies have revealed many environmental factors that exacerbate the incidence rate of schizophrenia (Lewis et al., 1992; Mortensen et al., 1999; Cannon et al., 2002). Many aspects of the relationship between environmental factors and the prodromal stage lasting from infancy to adolescence remain unclear. Therefore, many studies using animal models have been conducted to examine the relationships between environmental factors and onset of mental disorders (Deminière et al., 1992; Eyles et al., 2003).

As behaviors are assumed to express internal mental activity, behavioral assessment is likely to be an effective means of studying mental disorders in animal models. Prepulse inhibition (PPI) is a behavioral test procedure that is widely used to assess sensorimotor gating (a type of information processing). PPI is typically observed in healthy subjects, while a decrease in PPI reflects deficits in sensorimotor gating that may indicate impaired information processing in schizophrenia and other mental disorders (Braff et al., 1978; Braff, 2001; Ludgewig, 2003; Geyer, 2006). In addition, decreased PPI has often been reported in behavioral analyses of genetically modified animals related to schizophrenia and other mental disorders (Swerdlow et al., 1994; Lipska and Weinberger, 2000; Powell et al., 2009).

Along with the behavioral measurements, anatomical and molecular analyses of the nervous system are also essential to understand mental disorders. Reduced hippocampal volume and cell count are frequently observed in MRI and histological analyses of the brains of patients with mental disorders (schizophrenia, bipolar disorder, depression, and post-traumatic stress disorder [PTSD]; Saddath, 1990; Bremmner, 2008). Hippocampal neurogenesis has also been reported to occur in adulthood in both humans and animals (Altman and Das, 1965; Cameron et al., 1993; Eriksson et al., 1998; Gage, 2000). Strong relationships between hippocampal neurogenesis and vulnerability to mental disorders have been reported in animal models (Weinberger, 1987; Lillrank et al., 1995; Harrison, 2004; Watanabe et al., 2007; Maekawa et al., 2009). In addition, decreased levels of neurotrophin brain-derived neurotrophic factor (BDNF) were reported to be related to decreased hippocampal neurogenesis and may be involved in dementia (Durany et al., 2001; Szeszko et al., 2005; Tan et al., 2005). Other studies have also suggested that long-term soft-diet feeding may be related to decreased hippocampal neurogenesis (Mitome et al., 2004; Tsutsui et al., 2007). Furthermore, mastication ability is related to BDNF levels in adult mice or senescence-accelerated mice fed a soft diet (Yamamoto and Hirayama, 2001; Aoki et al., 2005; Yamamoto et al., 2008; Yamazaki et al., 2008).

We focused on the observation that the period of masticatory acquisition coincides with that of brain development related to the onset of mental disorders. We hypothesized that masticatory alterations after weaning may affect emotional development and potentially increase vulnerability to mental disorders. To verify this hypothesis, we examined the relationships between mastication after weaning and mental disorders in mice fed either a hard or soft diet.

EXPERIMENTAL PROCEDURES

Animals

Male C57BL6/J mice (CLEA Japan Inc., Tokyo, Japan) were used in this study, and the experiment was started

with animals at the age of 21 days. All animal experiments were approved by the Animal Experiment Committee of the RIKEN Brain Science Institute (approval No. H22-ER082), and the mice were maintained at the Experimental Animal Facility of the institute. The mice were housed three to a cage after weaning. The mice were kept under a 12-h light/dark cycle, with lights off from 20:00 to 08:00. Food and water were freely available except during experiments. The hard diet was comprised of ordinary laboratory chow for mice in typical hard pellet form (Nosan Corp., Kanagawa, Japan), while the soft diet consisted of the same nutritional components mixed with water in standardized proportions (Enomoto et al., 2010).

Behavioral tests

For behavioral analysis, at the time of weaning (21 days of age), mice were randomly divided into the following three groups (n = 7 or 10 per group): (1) male mice fed a hard diet (HDM), (2) male mice fed a soft diet (SDM), and (3) male mice changed to a hard diet from a soft diet at the beginning of the behavioral analysis (SHDM; Fig. 1). Mice were housed individually in cages from one week before commencement of behavioral analyses. The behavioral tests were started when the mice were 7 weeks old and completed before they reached the age of 14 weeks.

Two sets of behavioral tests were conducted independently. Each test was performed with a 1-day to 1-week interval. In the first batch (n = 7 per group), the following basic tests were conducted: home cage activity test (1st to 2nd week), open field test, light/dark box test, and elevated plus maze test (3rd week), Y-maze test, and auditory startle response and PPI test (4th week), Morris water maze test (5th week), rotarod test (6th week), and classical fear conditioning (7th week). Replication of some basic tests in batch 1 and two optional tests were conducted in the second batch (n = 10 per group). We replicated the basic behavioral tests and some optional tasks to assess the emotionality of mice, because those in the SDM group showed abnormal open field behavior and PPI in the first batch: home cage activity test (1st to 2nd week), open field test (2nd week), auditory startle response and PPI test (3rd week), social interaction test (4th week), and tail suspension test (5th week). The detailed procedures were described previously (Sakatani et al., 2009; Katayama et al., 2010; Takashima et al., 2011; Hattori et al., 2012).

These tests were performed in sequence to keep the general principle of the arrangement to avoid a more stressful task before a less stressful task and to minimize carryover effects.

Dimensions of the experimental apparatus are represented as (width × length × height). After each trial (except the auditory startle response test and the water maze test), the apparatus was wiped and cleaned with 80% alcohol and a damp towel. In the auditory startle response test, holding chambers were washed with tap water, wiped with a paper towel, and dried after each trial. All experimental protocols were approved by the RIKEN Institutional Animal Care and Use Committee.

Home cage activity (1st to 2nd week)

Spontaneous activity of each mouse in its home cage was measured using a 24-channel activity monitoring system (O'Hara & Co., Tokyo, Japan). Cages were individually set into compartments made of stainless steel in a negative breeding rack (JCL, Tokyo, Japan).

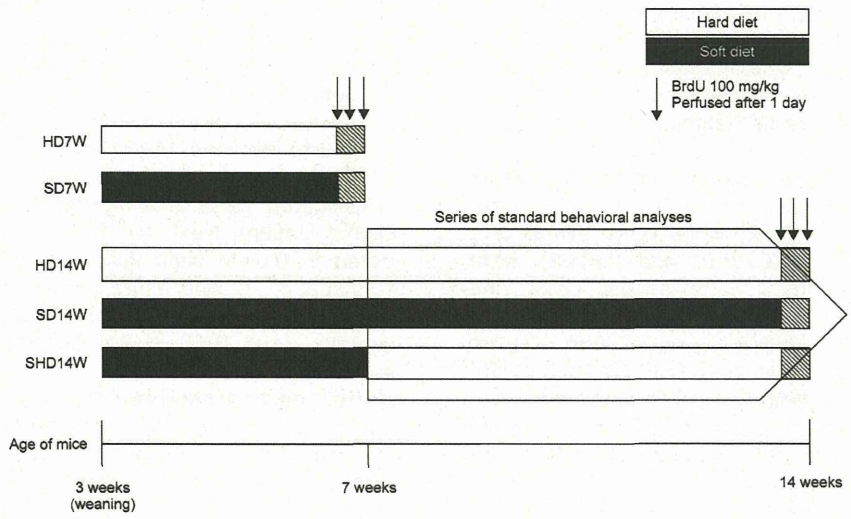

Fig. 1. Time schedule of the experiment. At the time of weaning (21 days of age), male mice were randomly divided into the following three groups (n = 7 or 10 per group): (1) male mice fed a hard diet (HDM); (2) male mice fed a soft diet (SDM); (3) male mice changed to a hard diet from a soft diet at 7 weeks of age (SHDM). Behavioral tests began when mice were 7 weeks old. In addition, at the time of weaning, male mice were randomly divided into the following five groups: (4) male mice fed a hard diet for 4 weeks (HD7W); (5) male mice fed a soft diet for 4 weeks (SD7W); (6) male mice fed a hard diet for 11 weeks (HD14W); (7) male mice changed to a hard diet at 7 weeks of age from a soft diet for 4 weeks (SHD14W); and (8) male mice fed a soft diet for 11 weeks (SD14W).

A piezoelectric sensor was equipped on the ceiling of each compartment to detect the movements of mice. Activity counts represent the number of active time bins (approximately 0.20–0.25 s each) in which spontaneous activities, including locomotor activity, rearing, and other voluntary stereotypic movements, were detected. Home cage activity was measured for 6 consecutive days during which bedding materials were not changed.

Open field test (2nd week)

The open field test apparatus was placed in a small soundproof room (185 × 185 × 225 cm). The apparatus consisted of four white plastic boxes (50 × 50 × 40 cm), two electric fans for ventilation and background noise (35 dB), and a white LED light source (70 lux at the center of the field), which served as the sole source of illumination during the experiment. For each box, a charge-coupled device camera (CCD) was attached to the ceiling to monitor the mice. Mice were individually introduced into the center of the arena and were allowed to move freely for 15 min. Distance traveled (cm) and % duration remaining at the center area of the field (defined as the central 36% of the field) were adopted as the test indices, and they were collected for each 1-min time bin.

Auditory startle response (3rd week)

Each mouse was placed into a small cage (30 or 35 mm in diameter, 12 cm in length) that was set on a sensor block within a soundproof chamber (60 × 50 × 67 cm, Mouse startle; O'Hara & Co.). A dim light was affixed to the ceiling of the soundproof chamber (10 lux at the center of the sensor block), and 65-dB white noise was presented as background noise.

In the acoustic startle response (ASR) test, each mouse was acclimatized to the experimental conditions for 5 min, and the experimental session began immediately after this acclimation period. In the first session, a 120-dB startle stimulus (40 ms) was presented to the mouse 10 times at random intertrial intervals (10–20 s). In the second session, the startle response to stimuli at various intensities was assessed. Five repetitions of 70–120-dB white noise stimuli (70, 75, 80, 85, 90, 95, 100, 110, and 120 dB) were presented for 40 ms in a quasi-random order and random intertrial intervals (10–20 s). In the PPI session, the mouse experienced five types of trial: (1) no stimulus, (2) startle stimulus only (120 dB, 40 ms), (3) 70-dB prepulse (20 ms, lead time 100 ms) followed by a 120-dB pulse, (4) 75-dB prepulse (20 ms, lead time 100 ms) followed by a 120-dB pulse, and (5) 80-dB prepulse (20 ms, lead time 100 ms) followed by a 120-dB pulse. Each trial was repeated 10 times in quasi-random order at random intertrial intervals (10–20 s). In the final session, a 120-dB startle stimulus (40 ms) was again presented to the mouse 10 times at random intertrial intervals (10–20 s). The total duration of an ASR test was about 35–40 min.

Tail suspension test (5th week)

As C57BL/6 mice have a tendency to climb their tail and/or the wire from which they are hung in the tail-suspension test, we used a metal blade (about 15 mm in width and 1 mm in thickness) in place of a wire to hang the mice to prevent their climbing behavior. Mice were individually taped by the tail to a metal blade held horizontally 45 cm above the bench, and the trial was started. Typically, mice would initially struggle to climb up the blade, but soon they showed increased bouts of immobility. A trained observer who was blind to the experimental conditions measured the duration of immobility over a 5-min period. Statistical analysis was carried out using the immobility scores.

5-Bromo-2′-deoxyuridine labeling analysis. Bromodeoxyuridine (Brd-U) analysis was conducted using another set of mice. At the time of weaning (21 days of age), mice were randomly divided into the following five groups: (1) male mice fed a hard diet for 4 weeks (HD7W, $n = 8$), (2) male mice fed a soft diet for 4 weeks (SD7W, $n = 10$), (3) male mice fed a hard diet for 11 weeks (HD14W, $n = 10$), (4) male mice changed to a hard diet at 7 weeks of age from a soft diet for 4 weeks (SHD14W, $n = 9$), and (5) male mice fed a soft diet for 11 weeks (SD14W, $n = 7$; Fig. 1).

Mice in the HD7W, SD7W, HD14W, SD14W, and SHD14W groups received three intraperitoneal injections of 5-bromo-2′-deoxyuridine (BrdU; Sigma, St. Louis, MO) at 100 mg/kg body weight, spaced 2 h apart and were sacrificed one day after BrdU injection.

After deeply anesthetizing the mice with 100 mg/kg sodium pentobarbital (Kyoritsu Pharmaceuticals, Tokyo, Japan), the mice were transcardially perfused with 4% paraformaldehyde containing 0.5% picric acid in 0.1 M phosphate-buffered saline (PBS). The brains were then removed and immersion-fixed for 24 h at 4 °C in the same fixative. After washing in PBS, the brains were successively equilibrated in 5%, 10%, and 20% sucrose in PBS, embedded in Tissue-Tek optimal cutting temperature (OCT) compound (Sakura, Tokyo, Japan), and frozen on dry ice. The frozen brains were coronally sliced into sections 14-μm thick using a cryostat (CM-3000; Leica, Nussloch, Germany) and mounted on MAS-coated glass slides (SUPERFROST; Matsunami, Osaka, Japan). Next, the 14-μm frozen sections were boiled in 0.01 M citric acid, incubated in 2 N HCl for 10 min at 37 °C, and washed in 0.01 M PBS.

BrdU-positive cells were identified throughout the dentate gyrus (DG) in its rostrocaudal extension. Of each set of 12 sections, three consecutive sections (14 μm each) were used for counting, and the total number of positive cells was determined by multiplying the value by 4. Roughly, 20 sets of counts were performed per organism (Maekawa et al., 2005).

Real-time semiquantitative polymerase chain reaction

BDNF, TrkB, and Akt1 expression levels in the hippocampus and frontal cortex were compared by