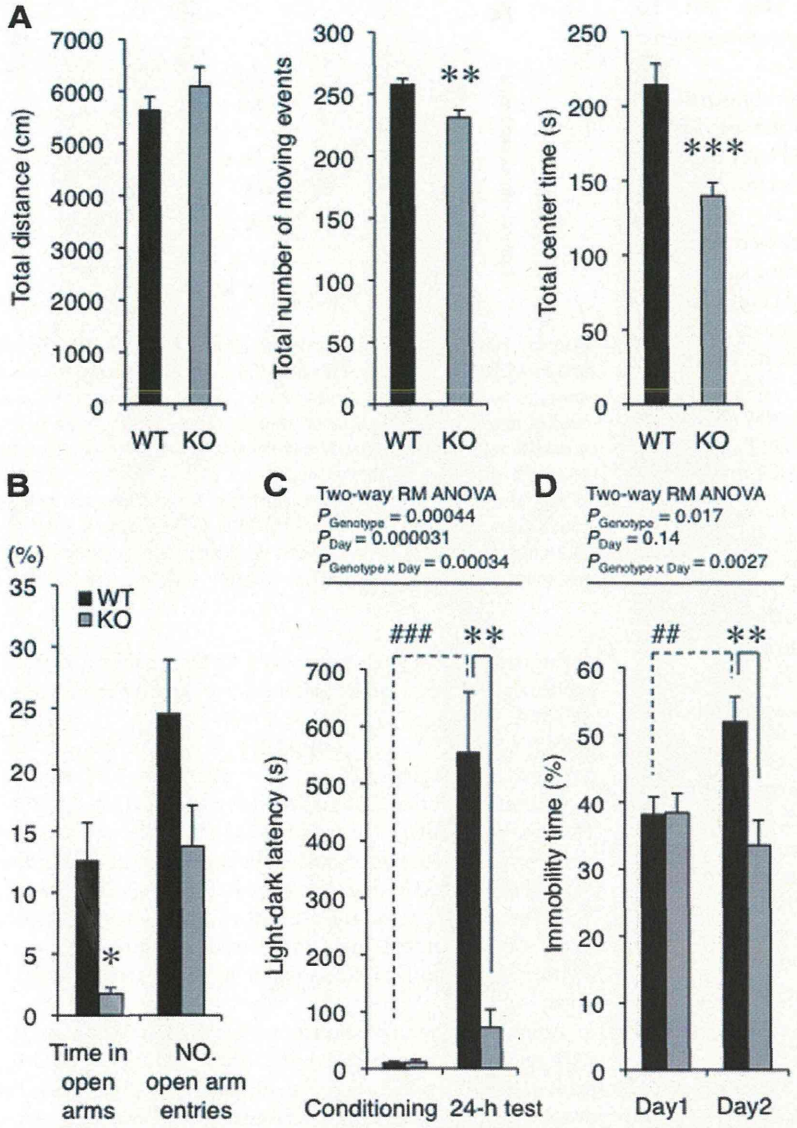

Figure 3. Rines KO mice showed changes in emotional behaviors. ***A***, Open field test. Horizontal locomotor activities of WT and Rines KO mice in the open field arena (50 × 50 cm) were assessed for 15 min. The traveled distance of KO mice was not significantly different from that of WT mice (left). The number of total moving events and the time spent in the center zone (36%) of the open field arena significantly decreased in KO mice (middle, right). In a moving event, a mouse continued moving for 2 s or more. WT, $n = 16$; KO, $n = 12$; $^{**}p < 0.01$, $^{***}p < 0.001$ by Student's t test. ***B***, Elevated plus maze test. Both time spent in open arms and numbers of entries into open arms decreased in Rines KO. WT, $n = 37$; KO, $n = 30$; $^{*}p < 0.05$ by Mann–Whitney U test. ***C***, Passive avoidance test. After habituation to a light– dark box, mice were conditioned with an electric foot shock upon entering the dark compartment (conditioning). Twenty-four hours after conditioning, the mice were reintroduced into the box and the latency to enter the dark compartment (light– dark latency) was measured (24 h test). The light– dark latency of KO mice significantly decreased relative to that of WT mice in the 24 h test. WT, $n = 10$; KO, $n = 10$; $^{**}p < 0.01$, Welch's t test (solid lines); $^{###}p < 0.001$, paired t test (dotted lines). ***D***, Forced swimming test. Mice were confined to an inescapable test chamber filled with water on two successive days and immobility time (i.e., time spent without swimming) was measured for 15 min (Day1) and 5 min (Day2). The immobility time of KO mice was significantly less than that of WT mice on day 2. WT, $n = 22$; KO, $n = 20$; $^{**}p < 0.01$, Mann–Whitney U test; $^{##}p < 0.01$, Wilcoxon signed-rank test (dotted lines).

pcDNA3.1 vector (Invitrogen). Other expression vectors were constructed as described previously (Ogawa et al., 2008).

Immunoblotting. The proteins and gene products were separated by 7.5%–10% SDS-PAGE and transferred to PVDF membranes (Immobilon; Millipore). The membranes were immersed in 5% skim milk overnight at 4°C and incubated with the first antibody. The bound antibodies were detected using horseradish peroxidase-conjugated secondary antibodies (anti-mouse, rabbit IgG) and ECL or ECL Plus reagents (GE Healthcare).

Immunoprecipitation, ubiquitination, and degradation assays. In the immunoprecipitation assay, HEK293T cells transfected with vectors were treated with the proteasome inhibitor epoxomicin (5 μM; Peptide Institute) for 9 h and lysed in an immunoprecipitation buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM EDTA, 10% glycerol, 0.5% Triton X-100, 0.5 mM *N*-ethylmaleimide, 0.5 mM iodoacetamide) and a complete protease inhibitor mixture (Roche). The lysates were subjected to immunoprecipitation as described previously (Ogawa et al., 2008). In the ubiquitination assay with cultured cells, NIH3T3 cells were lysed in an immunoprecipitation buffer with 20 mM *N*-ethylmaleimide, 5 nM ubiquitin aldehyde (Calbiochem), 20 mM sodium fluoride, 2 mM sodium orthovanadate, and 50 μM MG132 (Peptide Institute). The lysates were subjected to immunoprecipitation as described previously (Ogawa et al., 2008; Kawabe et al., 2010). In the degradation assay, NIH3T3 cells were lysed in SDS lysis buffer (50 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 1% SDS, 1 mM dithiothreitol), boiled for 10 min, diluted 5-fold by adding 0.5% NP-40 with a complete protease inhibitor mixture (Roche), and subjected to immunoblotting as described previously (Ogawa et al., 2008). In the ubiquitination assay for LC, circular tissue punches of the LC (2 mm in diameter) were collected from 150-μm-thick frozen coronal brain sections of 8.5-month-old WT or Rines KO male mice. LC tissue punches were lysed in an immunoprecipitation buffer and sonicated for 20 s. The lysates were subjected to immunoprecipitation using anti-MAO-A antibody (MAO-A IP-WB antibody pair), after which time the ubiquitinated MAO-A was detected with an anti-ubiquitin–protein conjugate antibody.

Statistics. Statistical analyses were conducted using the Prism 4 statistical package (GraphPad). Parametric data were analyzed using Student's t test, Welch's t test, or the paired t test; nonparametric data were analyzed using Mann–Whitney U test or Wilcoxon's signed-rank test. The effects of factors were analyzed using two-way ANOVA or two-way repeated-measures ANOVA. Data are presented as mean ± SEM. Differences were defined as statistically significant when $p < 0.05$.

Results

Rines possesses RING finger-dependent E3 ubiquitin ligase activity and shows high expression levels in the postnatal brain (Fig. 1; Ogawa et al., 2008). Here, Rines mRNA in the brain was consistently detected in 1- to 10-month-old mice (Fig. 1*A*) and Rines mRNA and protein were observed in each brain region (Fig. 1*B*,*C*). However, the authentic target molecule and physiological function of Rines remained unknown. To determine the physiological role of Rines, we generated Rines KO mice that lack exon 6, which includes the RING finger domain (Fig. 2*A*–*C*). We found that Rines KO mice had normal survival rates (Fig. 2*D*), body growth (Fig. 2*E*), gross brain mor-

phology (Fig. 2*F*), and external appearance (Fig. 2*G*). To explore the role of Rines in higher cognition, we performed a series of behavioral tests (Table 1).

Rines KO mice exhibited emotional behavior abnormalities (Fig. 3). The total number of moving events and time spent in the center of an open field decreased in KO compared with WT mice (Fig. 3*A*: total number of moving events, Student's *t* test, $t = 3.37$, $p = 0.0023$; total center time, Student's *t* test, $t = 4.43$, $p = 0.00018$). These results suggest that Rines KO mice were reluctant to explore a novel environment. In addition, the time spent in the open arm of the elevated plus maze test decreased (Fig. 3*B*: Mann–Whitney *U* test, $U = 362.50$, $p = 0.015$). However, the total distances in the open field test and home cage activity were unaltered (Fig. 3*A*: total distance, Student's *t* test, $t = 1.05$, $p = 0.31$; Table 1: home cage activity, whole day, two-way repeated-measures ANOVA, $F_{\text{genotype (1,108)}} = 0.095$, $p = 0.76$; $F_{\text{day (6,108)}} = 1.96$, $p = 0.078$; $F_{\text{genotype} \times \text{day (6,108)}} = 1.65$, $p = 0.14$; dark phase, two-way repeated-measures ANOVA, $F_{\text{genotype (1, 126)}} = 0.054$, $p = 0.82$; $F_{\text{day (7,126)}} = 1.48$, $p = 0.18$; $F_{\text{genotype} \times \text{day (7,126)}} = 1.49$, $p = 0.18$; light phase, two-way repeated-measures ANOVA, $F_{\text{genotype (1,108)}} < 0.0001$, $p = 0.999$; $F_{\text{day (6,108)}} = 1.49$, $p = 0.19$; $F_{\text{genotype} \times \text{day (6,108)}} = 0.38$, $p = 0.89$). In addition, the latencies to fall in the rotarod test were also comparable to those of WT mice (Table 1: two-way repeated-measures ANOVA, $F_{\text{genotype (1,54)}} = 0.016$, $p = 0.90$; $F_{\text{day (3,54)}} = 14.62$, $p < 0.0001$; $F_{\text{genotype} \times \text{day (3,54)}} = 1.71$, $p = 0.18$). These results indicate that Rines KO mice show normal locomotor and motor coordination abilities. Together with these results, the abnormalities observed in the open field and the elevated plus maze tests indicate that anxiety-like behavior in a novel environment was enhanced in Rines KO mice. Abnormal responses to stressful events were also observed in the passive avoidance and forced swimming tests (Fig. 3*C*,*D*). In the former, the latency of KO mice to enter the dark area after conditioning was significantly reduced compared with WT mice (Fig. 3*C*: conditioning, WT vs KO, Welch's *t* test, $t = 0.13$, $p = 0.90$; 24 h test, WT vs KO, Welch's *t* test, $t = 4.35$, $p = 0.0014$; WT, conditioning vs 24 h test, paired *t* test, $t = 5.15$, $p = 0.00061$; KO, conditioning vs 24 h test, paired *t* test, $t = 2.06$, $p = 0.070$; two-way repeated-measures ANOVA, $F_{\text{genotype (1,18)}} = 18.42$, $p = 0.00044$; $F_{\text{day (1,18)}} = 30.37$, $p = 0.000031$; $F_{\text{genotype} \times \text{day (1,18)}} = 19.40$, $p = 0.00034$). In addition, forced swimming tests performed on two successive days to observe stress reactivity revealed that the percentage of time spent immobile by WT mice on the second day was significantly higher than that on the first day (Fig. 3*D*: WT, Wilcoxon signed-rank test, $W = 177$, $p = 0.0043$). These results indicate a despaired response to the stress suffered from the long period of forced swimming on the first day (Duncan et al., 1993). However, KO mice did not show such a response (Fig. 3*D*: KO, Wilcoxon signed-rank test, $W = 64$, $p = 0.24$) and their time spent immobile was significantly less than that of WT mice on the second day (Fig. 3*D*: Day1, Mann–Whitney *U* test, U = 208, $p = 0.77$; Day2, Mann–Whitney *U* test, $U = 102$, $p = 0.0031$, two-way repeated-measures ANOVA, $F_{\text{genotype (1,40)}} = 6.23$, $p = 0.017$; $F_{\text{day (1,40)}} = 2.31$, $p = 0.14$; $F_{\text{genotype} \times \text{day (1,40)}} = 10.20$, $p = 0.0027$).

To determine whether nociception was altered in Rines KO mice, we performed the tail flick and hot plate tests. Latencies to remove the tail from the heat and responses to the hot plate were unchanged in Rines KO mice (Table 1: tail flick, Student's *t* test, $t = 1.02$, $p = 0.31$; hot plate, licking of forepaws, Welch's *t* test, $t = 0.60$, $p = 0.55$; flinch of hindpaws, Student's *t* test, $t = 0.33$, $p = 0.74$). In addition, in the Morris water maze test, there were no differences between WT and KO mice in the escape latency during training or the time in the target quadrant during the probe trials (Table 1: escape latency, two-way repeated-measures ANOVA, $F_{\text{genotype (1,54)}} = 0.24$, $p = 0.63$; $F_{\text{day (3,54)}} = 42.8$, $p < 0.0001$; $F_{\text{genotype} \times \text{day (3,54)}} = 0.12$, $p = 0.95$; probe test, time in target, Mann–Whitney *U* test, $U = 49.5$, $p = 0.97$; number of times crossing target, Mann–Whitney *U* test, $U = 50$, $p = 1$). These results indicate that Rines KO mice are not deficient in nociception or various cognitive functions. Accordingly, the abnormal responses of Rines KO mice in the passive avoidance and forced swimming tests suggest that emotional responses, which might be interpreted in terms of reduced attention, aversive memory formation, or stress reactivity, are altered in Rines KO mice.

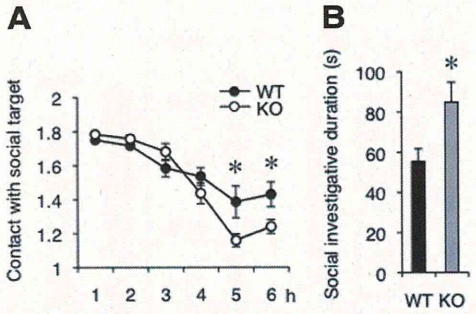

Figure 4. Increased affiliative behaviors in Rines KO mice. ***A***, Contact between Rines KO and unfamiliar C57BL/6J mice in a novel environment. In the computer analysis, each mouse was represented by a particle. Particle numbers 2 and 1 indicate the statuses of separated and contacted, respectively. The mean particle numbers, as contacts with a social target, were calculated for each time bin (1 h). Rines KO mice showed a significant increase in the duration of contact with unfamiliar mice compared with WT mice. WT, $n = 10$; KO, $n = 10$; $^*p < 0.05$, Student's *t* test or Welch's *t* test. ***B***, Resident-intruder test. The bar graph shows the time spent in investigation (e.g., sniffing and following) of WT or KO mice (resident) to intruder mice (BALB/c male mice). Rines KO mice showed a significant increase in the duration of social investigation compared with WT mice. WT, $n = 10$; KO, $n = 10$; $^*p < 0.05$, Student's *t* test.

Altered emotional responses were also observed upon the receipt of social inputs (Fig. 4*A*,*B*). Rines KO mice showed significant increases in the duration of contact with conspecifics in unfamiliar space during a social interaction test (Fig. 4*A*: 5 h, Welch's *t* test, $t = 2.23$, $p = 0.047$; 6 h, Student's *t* test, $t = 2.19$, $p = 0.043$). When they encountered unfamiliar BALB/c intruder mice in a resident-intruder test, the time spent in social investigation (e.g., sniffing or following the intruder) significantly increased in Rines KO mice (Fig. 4*B*: Student's *t* test, $t = 2.48$, $p = 0.024$). Considering that there were no significant alterations in the hidden cookie, auditory startle response, or prepulse inhibition tests (Table 1: latency to find food in hidden cookie test, Student's *t* test, $t = 0.33$, $p = 0.75$; auditory startle response, two-way repeated-measures ANOVA, $F_{\text{genotype (1,144)}} = 0.60$, $p = 0.45$; $F_{\text{dB (8,144)}} = 91.27$, $p < 0.0001$; $F_{\text{genotype} \times \text{dB (8,144)}} = 1.33$, $p = 0.23$, prepulse inhibition, two-way repeated-measures ANOVA, $F_{\text{genotype (1,36)}} = 0.036$, $p = 0.85$; $F_{\text{dB (2,36)}} = 26$, $p < 0.0001$; $F_{\text{genotype} \times \text{dB (2,36)}} = 2.82$, $p = 0.073$), the increased affiliative behaviors may not reflect altered olfactory or auditory functions.

Given the behavioral abnormalities, we investigated whether monoamine levels were altered in the LC, raphe nuclei, and substantia nigra of Rines KO mice because these brain areas are the principal sources of brain NE, 5-HT, and dopamine (Table 2). Because the behavioral abnormalities in Rines KO mice were observed during exposure to stressful stimuli, we also measured monoamine levels in mice in the presence of an aversive stimulus

Table 2. Amounts of monoamines and their metabolites

		LC		Prefrontal cortex		Amygdala	
		Control	Foot shock	Control	Foot shock	Control	Foot shock
5-HT	WT	4667.6 ± 58.4	5223.4 ± 188.8 #	3873.8 ± 471.7	2698.7 ± 214.9 §§	5698.8 ± 161.6	6596.1 ± 120.5
	KO	4531.2 ± 129.3	4553.4 ± 198.7*	2716.9 ± 221.4*	3172.0 ± 165.6	6064.8 ± 227.4	6532.9 ± 208.7
NE	WT	6948.6 ± 276.1	6723.0 ± 155.7 #	5460.0 ± 422.4	4209.5 ± 233.0 §	2932.9 ± 54.9	2453.5 ± 72.9 ##
	KO	6466.7 ± 273.8	5990.4 ± 193.6*	5111.1 ± 207.6	4934.5 ± 90.8*	3239.4 ± 107.8*	2631.1 ± 67.2
DA	WT	499.6 ± 28.2	792.3 ± 28.5	1266.0 ± 141.7	1103.0 ± 183.0	5228.7 ± 597.3	8878.3 ± 1260.1
	KO	518.3 ± 25.3	726.2 ± 36.6	1010.6 ± 80.3	1029.1 ± 46.5	6744.2 ± 837.3	7486.9 ± 1157.6
5-HIAA	WT	5186.5 ± 248.4	6439.5 ± 363.0	2539.4 ± 204.8	2262.9 ±121.4	3014.0 ± 110.2	4045.5 ± 248.7
	KO	5149.1 ± 531.2	5161.3 ± 287.5*	2161.6 ± 234.9	2392.3 ± 103.7	3323.2 ± 188.4	3807.7 ± 188.5
MHPG	WT	977.5 ± 32.3	1811.5 ± 76.3	ND	ND	363.5 ± 20.1	894.4 ± 32.5
	KO	973.9 ± 55.1	1590.6 ±79.5	ND	532.3 ± 78.5	421.3 ± 21.5	880.7 ± 38.1
DOPAC	WT	498.0 ± 23.0	1390.8 ± 67.9	654.6 ± 50.7	1350.6 ± 96.3	1292.4 ± 112.5	2861.4 ± 251.5
	KO	534.1 ± 41.7	1247.7 ± 53.7	569.0 ± 37.6	1503.4 ± 65.3	1510.3 ± 112.5	2549.0 ± 223.8
HVA	WT	691.2 ± 32.7	1238.3 ± 65.2	6948.6 ± 276.1	6723.0 ± 155.7	1792.5 ± 135.2	4077.8 ± 344.7 §
	KO	722.8 ± 63.2	1076.6 ± 40.9	1081.5 ± 143.3	2286.1 ± 87.9	2439.5 ± 262.3	3473.3 ± 380.9

		Raphe nucleus		Substantia nigra	
		Control	Foot shock	Control	Foot shock
5-HT	WT	8599.4 ± 651.0	10420.1 ± 516.1	9365.1 ± 461.1	9068.4 ± 478.3
	KO	10158.6 ± 655.4	10103.7 ± 456.0	9942.0 ± 368.5	9168.7 ± 551.1
NE	WT	8149.3 ± 262.4	6229.8 ± 183.2	3849.3 ± 192.8	4263.6 ± 340.3
	KO	8118.8 ± 327.1	6756.8 ± 222.9	4629.3 ± 349.0	4514.2 ± 309.8
DA	WT	2749.8 ± 1569.9	1635.2 ± 81.5	5895.4 ± 364.9	5458.6 ± 339.2
	KO	1470.9 ± 60.7	1703.4 ± 65.1	5785.5 ± 407.5	5049.2 ± 453.4
5-HIAA	WT	8839.8 ± 836.3	12659.1 ± 1424.6	6149.4 ± 386.8	7155.7 ± 281.0
	KO	11292.1 ± 1235.3	11222.7 ± 714.6	6904.7 ± 501.1	6831.4 ± 418.0
MHPG	WT	800.8 ± 434.6	1187.7 ± 68.0	460.6 ± 33.1	1026.8 ± 63.6
	KO	454.1 ± 23.5	1191.3 ± 91.1	546.1 ± 28.9	1020.6 ± 100.7
DOPAC	WT	772.9 ± 139.3	1400.0 ± 60.5	2239.3 ± 89.9	2495.0 ± 130.4
	KO	786.1 ± 23.7	1395.6 ± 44.3	2277.3 ± 140.4	2354.0 ± 100.0
HVA	WT	1591.2 ± 213.1	2291.5 ± 158.2	3119.6 ± 124.8	3585.8 ± 159.1
	KO	1784.0 ± 89.4	2159.3 ± 63.0	3293.7 ± 217.5	3294.2 ± 153.2

Values are presented as mean ± SEM (pg/mg protein). $n = 7$–9 mice per condition; $^{*}p < 0.05$, Student's t test compared with WT mice. There were significant genotype effects for 5-HT and NE contents in LC and for NE contents in amygdala analyzed by two-way ANOVA ($^{\#}p_{\text{genotype}} < 0.05$, $^{\#\#}p_{\text{genotype}} < 0.01$). There were significant effects of the interaction of genotype by treatment for 5-HT and NE contents in the prefrontal cortex and HVA contents in the amygdala analyzed by two-way ANOVA ($^{\S}p_{\text{genotype} \times \text{treatment}} < 0.05$, $^{\S\S}p_{\text{genotype} \times \text{treatment}} < 0.01$). DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid; MHPG, 3-methoxy-4-hydroxyphenylethyleneglycol; 5-HIAA, 5-hydroxyindoleacetic acid; ND, not detected.

(electric foot shock). We observed significantly less NE and 5-HT levels in the LC of Rines KO mice than in the LC of WT mice after the aversive stimulus (Table 2: NE, Student's t test, $t = 2.89$, $p = 0.013$; 5-HT, Student's t test, $t = 2.42$, $p = 0.031$; NE, two-way ANOVA, $F_{\text{genotype}\ (1,27)} = 6.52$, $p = 0.017$; $F_{\text{treatment}\ (1,27)} = 2.18$, $p = 0.15$; $F_{\text{genotype} \times \text{treatment}\ (1,27)} = 0.28$, $p = 0.60$; 5-HT, two-way ANOVA, $F_{\text{genotype}\ (1,27)} = 6.65$, $p = 0.016$; $F_{\text{treatment}\ (1,27)} = 3.42$, $p = 0.075$; $F_{\text{genotype} \times \text{treatment}\ (1,27)} = 2.91$, $p = 0.099$). NE and 5-HT contents were also altered in the prefrontal cortex and amygdala, which receive projections from the LC and play important roles in emotional processing (Table 2). 5-HT levels in the prefrontal cortex of Rines KO mice were significantly less than those of WT mice under normal conditions (Table 2: Student's t test, $t = 2.39$, $p = 0.031$; two-way ANOVA, $F_{\text{genotype}\ (1,27)} = 1.48$, $p = 0.23$; $F_{\text{treatment}\ (1,27)} = 1.64$, $p = 0.21$; $F_{\text{genotype} \times \text{treatment}\ (1,27)} = 8.41$, $p = 0.0073$). Other than these regions, the raphe nuclei and

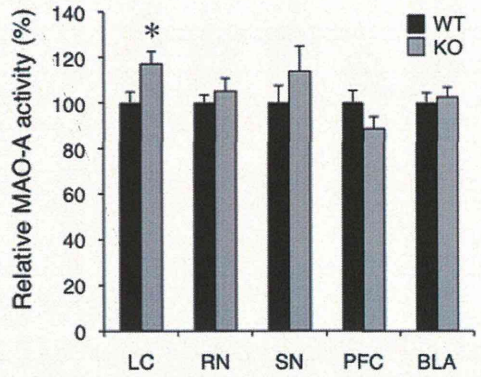

Figure 5. MAO-A enzymatic activities in Rines KO and WT mice. The enzymatic activities were measured in the LC, raphe nuclei (RN), substantia nigra (SN), prefrontal cortex (PFC), and basolateral nucleus of the amygdala (BLA) in WT and Rines KO mouse brains. Increased MAO-A activity was observed in the 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, $t = 3.35$, $p = 0.0013$;

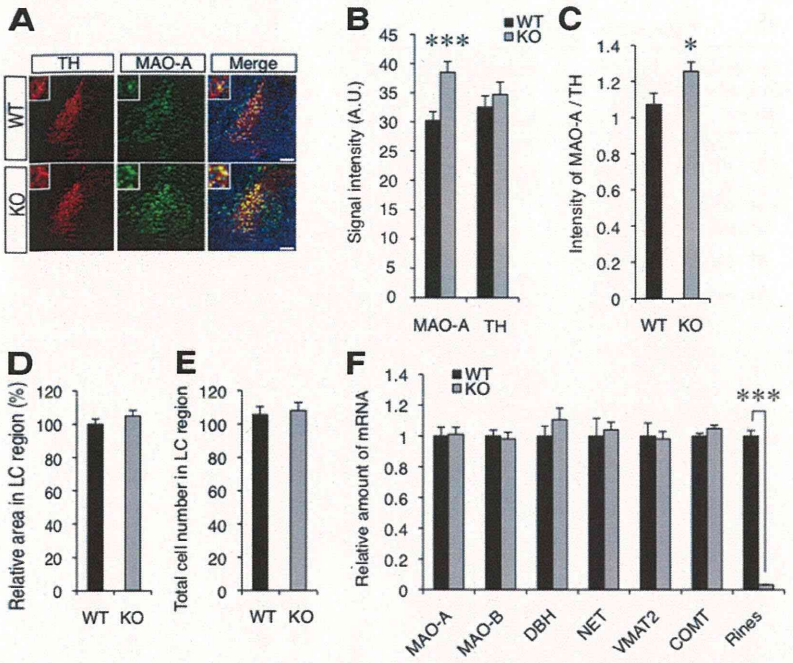

Figure 6. Rines regulated MAO-A protein level. ***A–C***, Rines KO mice showed significantly increased MAO-A protein levels in the LC region. ***A***, Representative immunofluorescence staining of TH and MAO-A proteins in the LC region from WT and Rines KO mice. High-magnification images of the center 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).

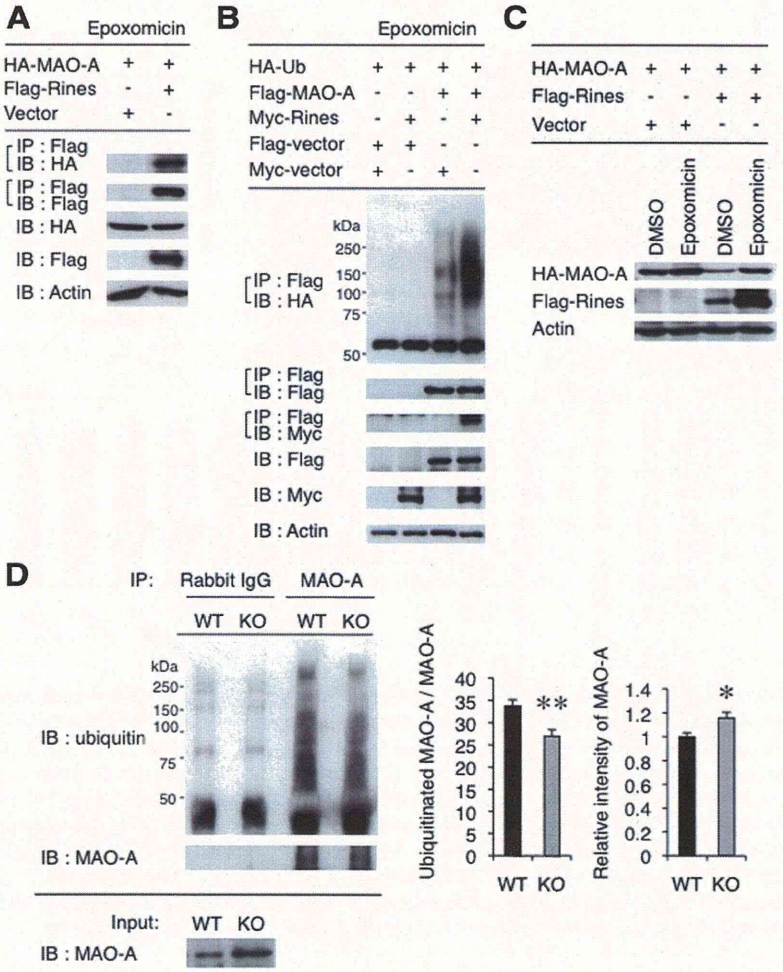

Figure 7. Rines promotes the ubiquitination and degradation of MAO-A in the proteasome. ***A***, Coimmunoprecipitation of the Rines protein with MAO-A protein from HEK293T cells transfected with Rines and MAO-A expression vectors and treated with a proteasome inhibitor, epoxomicin (5 μM). ***B***, NIH3T3 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

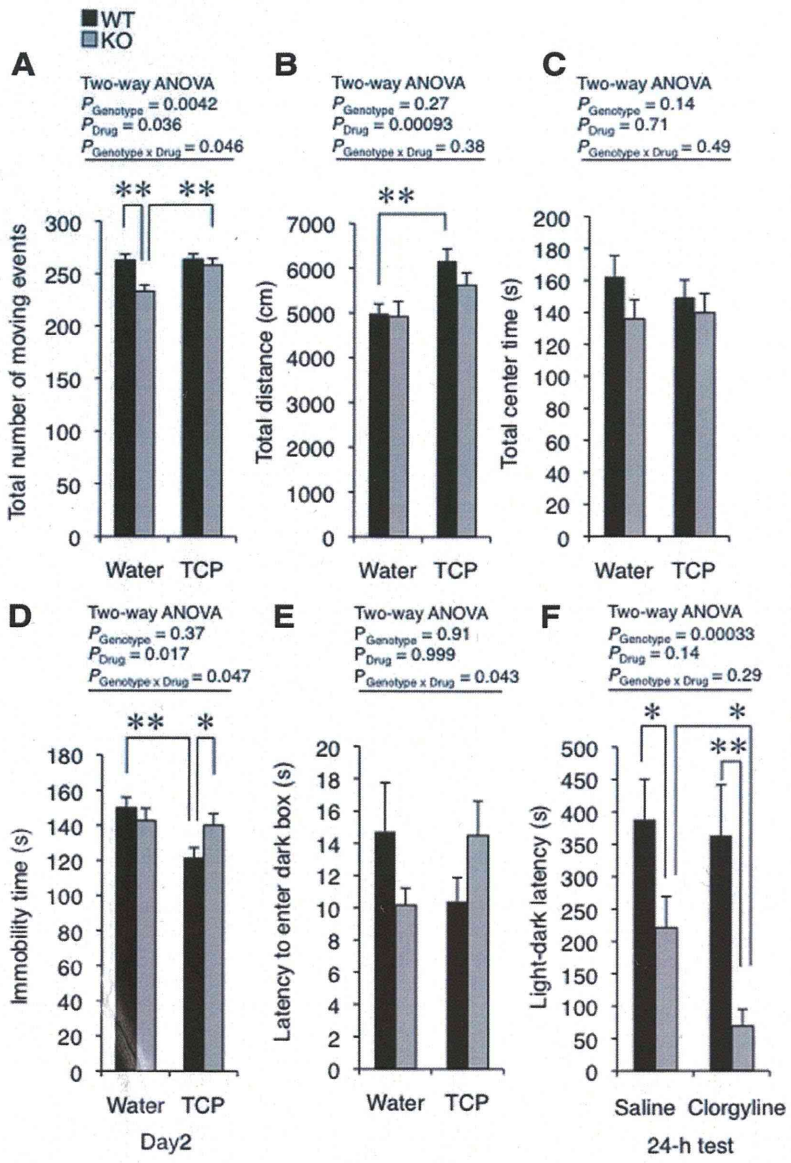

Figure 8. Emotional behaviors in Rines KO and WT mice were differentially affected by MAO inhibitors. ***A–E***, Water or water containing the MAO inhibitor TCP (3 mg/kg) was administered to mice chronically. The total number of movements (***A***), total distance (***B***), and total center time (***C***) 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.

References

Alia-Klein N, Goldstein RZ, Kriplani A, Logan J, Tomasi D, Williams B, Telang F, Shumay E, Biegon A, Craig IW, Henn F, Wang GJ, Volkow ND, Fowler JS (2008) Brain monoamine oxidase A activity predicts trait aggression. J Neurosci 28:5099–5104. CrossRef Medline

Alsene KM, Bakshi VP (2011) Pharmacological stimulation of locus coeruleus reveals a new antipsychotic-responsive pathway for deficient senso-

rimotor gating. Neuropsychopharmacology 36:1656–1667. CrossRef Medline

Aston-Jones G, Bloom FE (1981) Norepinephrine-containing locus coeruleus neurons in behaving rats exhibit pronounced responses to non-noxious environmental stimuli. J Neurosci 1:887–900. Medline

Barnes NM, Sharp T (1999) A review of central 5-HT receptors and their function. Neuropharmacology 38:1083–1152. CrossRef Medline

Bear MF, Conors BW, Paradiso MA (2006) Neuroscience, exploring the brain, Ed 3. Philadelphia: Lippincott Williams & Wilkins.

Berridge CW, Waterhouse BD (2003) The locus coeruleus-noradrenergic system: modulation of behavioral state and state-dependent cognitive processes. Brain Res Brain Res Rev 42:33–84. CrossRef Medline

Bortolato M, Chen K, Shih JC (2008) Monoamine oxidase inactivation: from pathophysiology to therapeutics. Adv Drug Deliv Rev 60:1527–1533. CrossRef Medline

Bortolato M, Chen K, Godar SC, Chen G, Wu W, Rebrin I, Farrell MR, Scott AL, Wellman CL, Shih JC (2011) Social deficits and perseverative behaviors, but not overt aggression, in MAO-A hypomorphic mice. Neuropsychopharmacology 36:2674–2688. CrossRef Medline

Brunner HG, Nelen M, Breakefield XO, Ropers HH, van Oost BA (1993) Abnormal behavior associated with a point mutation in the structural gene for monoamine oxidase A. Science 262:578–580. CrossRef Medline

Carninci P, Kasukawa T, Katayama S, Gough J, Frith MC, Maeda N, Oyama R, Ravasi T, Lenhard B, Wells C, Kodzius R, Shimokawa K, Bajic VB, Brenner SE, Batalov S, Forrest AR, Zavolan M, Davis MJ, Wilming LG, Aidinis V, et al.; FANTOM Consortium; RIKEN Genome Exploration Research Group and Genome Science Group (Genome Network Project Core Group) (2005) The transcriptional landscape of the mammalian genome. Science 309:1559–1563. CrossRef Medline

Cases O, Seif I, Grimsby J, Gaspar P, Chen K, Pournin S, Müller U, Aguet M, Babinet C, Shih JC, et al. (1995) Aggressive behavior and altered amounts of brain serotonin and norepinephrine in mice lacking MAOA. Science 268:1763–1766. CrossRef Medline

Cases O, Vitalis T, Seif I, De Maeyer E, Sotelo C, Gaspar P (1996) Lack of barrels in the somatosensory cortex of monoamine oxidase A-deficient mice: role of a serotonin excess during the critical period. Neuron 16:297–307. CrossRef Medline

Caspi A, McClay J, Moffitt TE, Mill J, Martin J, Craig IW, Taylor A, Poulton R (2002) Role of genotype in the cycle of violence in maltreated children. Science 297:851–854. CrossRef Medline

Chen K, Ou XM, Chen G, Choi SH, Shih JC (2005) R1, a novel repressor of the human monoamine oxidase A. J Biol Chem 280:11552–11559. CrossRef Medline

Deckert J, Catalano M, Syagailo YV, Bosi M, Okladnova O, Di Bella D, Nöthen MM, Maffei P, Franke P, Fritze J, Maier W, Propping P, Beckmann H, Bellodi L, Lesch KP (1999) Excess of high activity monoamine oxidase A gene promoter alleles in female patients with panic disorder. Hum Mol Genet 8:621–624. CrossRef Medline

Dubrovina NI, Popova NK, Gilinskii MA, Tomilenko RA, Seif I (2006) Acquisition and extinction of a conditioned passive avoidance reflex in mice with genetic knockout of monoamine oxidase A. Neurosci Behav Physiol 36:335–339. CrossRef Medline

Duncan GE, Johnson KB, Breese GR (1993) Topographic patterns of brain activity in response to swim stress: assessment by 2-deoxyglucose uptake and expression of Fos-like immunoreactivity. J Neurosci 13:3932–3943. Medline

Garriock HA, Kraft JB, Shyn SI, Peters EJ, Yokoyama JS, Jenkins GD, Reinalda MS, Slager SL, McGrath PJ, Hamilton SP (2010) A genomewide association study of citalopram response in major depressive disorder. Biol Psychiatry 67:133–138. CrossRef Medline

Gong B, Cao Z, Zheng P, Vitolo OV, Liu S, Staniszewski A, Moolman D, Zhang H, Shelanski M, Arancio O (2006) Ubiquitin hydrolase Uch-L1 rescues beta-amyloid-induced decreases in synaptic function and contextual memory. Cell 126:775–788. CrossRef Medline

Gormanns P, Mueller NS, Ditzen C, Wolf S, Holsboer F, Turck CW (2011) Phenome-transcriptome correlation unravels anxiety and depression related pathways. J Psychiatr Res 45:973–979. CrossRef Medline

Gross C, Zhuang X, Stark K, Ramboz S, Oosting R, Kirby L, Santarelli L, Beck S, Hen R (2002) Serotonin1A receptor acts during development to establish normal anxiety-like behaviour in the adult. Nature 416:396–400. CrossRef Medline

Hershko A, Ciechanover A (1998) The ubiquitin system. Annu Rev Biochem 67:425–479. CrossRef Medline

Hunter P (2010) The psycho gene. EMBO Rep 11:667–669. CrossRef Medline

Inoue T, Hatayama M, Tohmonda T, Itohara S, Aruga J, Mikoshiba K (2004) Mouse Zic5 deficiency results in neural tube defects and hypoplasia of cephalic neural crest derivatives. Dev Biol 270:146–162. CrossRef Medline

Irie F, Badie-Mahdavi H, Yamaguchi Y (2012) Autism-like socio-communicative deficits and stereotypies in mice lacking heparan sulfate. Proc Natl Acad Sci U S A 109:5052–5056. CrossRef Medline

Jiang H, Jiang Q, Liu W, Feng J (2006) Parkin suppresses the expression of monoamine oxidases. J Biol Chem 281:8591–8599. CrossRef Medline

Johnston JA, Madura K (2004) Rings, chains and ladders: ubiquitin goes to work in the neuron. Prog Neurobiol 73:227–257. CrossRef Medline

Katayama K, Yamada K, Ornthanalai VG, Inoue T, Ota M, Murphy NP, Aruga J (2010) Slitrk1-deficient mice display elevated anxiety-like behavior and noradrenergic abnormalities. Mol Psychiatry 15:177–184. CrossRef Medline

Kato T, Ishiwata M, Yamada K, Kasahara T, Kakiuchi C, Iwamoto K, Kawamura K, Ishihara H, Oka Y (2008) Behavioral and gene expression analyses of Wfs1 knockout mice as a possible animal model of mood disorder. Neurosci Res 61:143–158. CrossRef Medline

Kawabe H, Neeb A, Dimova K, Young SM Jr, Takeda M, Katsurabayashi S, Mitkovski M, Malakhova OA, Zhang DE, Umikawa M, Kariya K, Goebbels S, Nave KA, Rosenmund C, Jahn O, Rhee J, Brose N (2010) Regulation of Rap2A by the ubiquitin ligase Nedd4-1 controls neurite development. Neuron 65:358–372. CrossRef Medline

Kim JJ, Shih JC, Chen K, Chen L, Bao S, Maren S, Anagnostaras SG, Fanselow MS, De Maeyer E, Seif I, Thompson RF (1997) Selective enhancement of emotional, but not motor, learning in monoamine oxidase A-deficient mice. Proc Natl Acad Sci U S A 94:5929–5933. CrossRef Medline

Kim-Cohen J, Caspi A, Taylor A, Williams B, Newcombe R, Craig IW, Moffitt TE (2006) MAOA, maltreatment, and gene-environment interaction predicting children's mental health: new evidence and a meta-analysis. Mol Psychiatry 11:903–913. CrossRef Medline

Kindt M, Soeter M, Vervliet B (2009) Beyond extinction: erasing human fear responses and preventing the return of fear. Nat Neurosci 12:256–258. CrossRef Medline

Kitanaka N, Kitanaka J, Takemura M (2006) Modification of morphine-induced hyperlocomotion and antinociception in mice by clorgyline, a monoamine oxidase-A inhibitor. Neurochem Res 31:829–837. CrossRef Medline

Koen N, Stein DJ (2011) Pharmacotherapy of anxiety disorders: a critical review. Dialogues Clin Neurosci 13:423–437. Medline

Korte SM, Meijer OC, de Kloet ER, Buwalda B, Keijser J, Sluyter F, van Oortmerssen G, Bohus B (1996) Enhanced 5-HT1A receptor expression in forebrain regions of aggressive house mice. Brain Res 736:338–343. CrossRef Medline

Lee SH, Choi JH, Lee N, Lee HR, Kim JI, Yu NK, Choi SL, Lee SH, Kim H, Kaang BK (2008) Synaptic protein degradation underlies destabilization of retrieved fear memory. Science 319:1253–1256. CrossRef Medline

Libert S, Pointer K, Bell EL, Das A, Cohen DE, Asara JM, Kapur K, Bergmann S, Preisig M, Otowa T, Kendler KS, Chen X, Hettema JM, van den Oord EJ, Rubio JP, Guarente L (2011) SIRT1 activates MAO-A in the brain to mediate anxiety and exploratory drive. Cell 147:1459–1472. CrossRef Medline

Maki Y, Inoue T, Izumi T, Muraki I, Ito K, Kitaichi Y, Li X, Koyama T (2000) Monoamine oxidase inhibitors reduce conditioned fear stress-induced freezing behavior in rats. Eur J Pharmacol 406:411–418. CrossRef Medline

Meltzer HY, Huang M (2008) In vivo actions of atypical antipsychotic drug on serotonergic and dopaminergic systems. Prog Brain Res 172:177–197. CrossRef Medline

Meyer JH, Ginovart N, Boovariwala A, Sagrati S, Hussey D, Garcia A, Young T, Praschak-Rieder N, Wilson AA, Houle S (2006) Elevated monoamine oxidase a levels in the brain: an explanation for the monoamine imbalance of major depression. Arch Gen Psychiatry 63:1209–1216. CrossRef Medline

Millan MJ (2003) The neurobiology and control of anxious states. Prog Neurobiol 70:83–244. CrossRef Medline

Moriyoshi K, Iijima K, Fujii H, Ito H, Cho Y, Nakanishi S (2004) Seven in

absentia homolog 1A mediates ubiquitination and degradation of group 1 metabotropic glutamate receptors. Proc Natl Acad Sci U S A 101:8614–8619. CrossRef Medline

O'Donnell JM, Shelton RC (2010) Drug therapy of depression and anxiety disorders. In: Goodman and Gilman's the pharmacological basis of therapeutics, Ed 12 (Brunton LL, ed), pp 397–415. New York: McGraw-Hill.

Ogawa M, Mizugishi K, Ishiguro A, Koyabu Y, Imai Y, Takahashi R, Mikoshiba K, Aruga J (2008) Rines/RNF180, a novel RING finger gene-encoded product, is a membrane-bound ubiquitin ligase. Genes Cells 13:397–409. CrossRef Medline

Pickart CM (2001) Mechanisms underlying ubiquitination. Annu Rev Biochem 70:503–533. CrossRef Medline

Popova NK (2008) From gene to aggressive behavior: the role of brain serotonin. Neurosci Behav Physiol 38:471–475. CrossRef Medline

Popova NK, Kulikov AV, Avgustinovich DF, Shigantsov SN (1996) The characteristics of the brain serotonin system and anxiety in the C57BL and CBA mouse strains [Article in Russian]. Zh Vyssh Nerv Deiat Im I P Pavlova 46:348–354. Medline

Porsolt RD, Anton G, Blavet N, Jalfre M (1978) Behavioural despair in rats: a new model sensitive to antidepressant treatments. Eur J Pharmacol 47:379–391. CrossRef Medline

Porsolt RD, Bertin A, Blavet N, Deniel M, Jalfre M (1979) Immobility induced by forced swimming in rats: effects of agents which modify central catecholamine and serotonin activity. Eur J Pharmacol 57:201–210. CrossRef Medline

Puig MV, Gulledge AT (2011) Serotonin and prefrontal cortex function: neurons, networks, and circuits. Mol Neurobiol 44:449–464. CrossRef Medline

Ravindran LN, Stein MB (2010) The pharmacologic treatment of anxiety disorders: a review of progress. J Clin Psychiatry 71:839–854. CrossRef Medline

Ryan MM, Lockstone HE, Huffaker SJ, Wayland MT, Webster MJ, Bahn S (2006) Gene expression analysis of bipolar disorder reveals downregulation of the ubiquitin cycle and alterations in synaptic genes. Mol Psychiatry 11:965–978. CrossRef Medline

Sakatani S, Yamada K, Homma C, Munesue S, Yamamoto Y, Yamamoto H, Hirase H (2009) Deletion of RAGE causes hyperactivity and increased sensitivity to auditory stimuli in mice. PLoS One 4:e8309. CrossRef Medline

Sakurai M, Sekiguchi M, Zushida K, Yamada K, Nagamine S, Kabuta T, Wada K (2008) Reduction in memory in passive avoidance learning, exploratory behaviour and synaptic plasticity in mice with a spontaneous deletion in the ubiquitin C-terminal hydrolase L1 gene. Eur J Neurosci 27:691–701. CrossRef Medline

Samochowiec J, Hajduk A, Samochowiec A, Horodnicki J, Stepién G, Grzywacz A, Kucharska-Mazur J (2004) Association studies of MAO-A, COMT, and 5-HTT genes polymorphisms in patients with anxiety disorders of the phobic spectrum. Psychiatry Res 128:21–26. CrossRef Medline

Satoh Y, Endo S, Ikeda T, Yamada K, Ito M, Kuroki M, Hiramoto T, Imamura O, Kobayashi Y, Watanabe Y, Itohara S, Takishima K (2007) Extracellular signal-regulated kinase 2 (ERK2) knockdown mice show deficits in long-term memory; ERK2 has a specific function in learning and memory. J Neurosci 27:10765–10776. CrossRef Medline

Saudou F, Amara DA, Dierich A, LeMeur M, Ramboz S, Segu L, Buhot MC, Hen R (1994) Enhanced aggressive behavior in mice lacking 5-HT1B receptor. Science 265:1875–1878. CrossRef Medline

Schulze TG, Müller DJ, Krauss H, Scherk H, Ohlraun S, Syagailo YV, Windemuth C, Neidt H, Grässle M, Papassotiropoulos A, Heun R, Nöthen MM, Maier W, Lesch KP, Rietschel M (2000) Association between a functional polymorphism in the monoamine oxidase A gene promoter and major depressive disorder. Am J Med Genet 96:801–803. CrossRef Medline

Scott AL, Bortolato M, Chen K, Shih JC (2008) Novel monoamine oxidase A knock out mice with human-like spontaneous mutation. Neuroreport 19:739–743. CrossRef Medline

Shih JC, Chen K, Ridd MJ (1999) Monoamine oxidase: from genes to behavior. Annu Rev Neurosci 22:197–217. CrossRef Medline

Solati J, Salari AA, Bakhtiari A (2011) 5HT(1A) and 5HT(1B) receptors of medial prefrontal cortex modulate anxiogenic-like behaviors in rats. Neurosci Lett 504:325–329. CrossRef Medline

Starke K (2001) Presynaptic autoreceptors in the third decade: focus on alpha2-adrenoceptors. J Neurochem 78:685–693. CrossRef Medline

Swiergiel AH, Leskov IL, Dunn AJ (2008) Effects of chronic and acute stressors and CRF on depression-like behavior in mice. Behav Brain Res 186:32–40. CrossRef Medline

van Stegeren AH (2008) The role of the noradrenergic system in emotional memory. Acta Psychol (Amst) 127:532–541. CrossRef Medline

Vishnivetskaya GB, Skrinskaya JA, Seif I, Popova NK (2007) Effect of MAO A deficiency on different kinds of aggression and social investigation in mice. Aggress Behav 33:1–6. CrossRef Medline

Weber M, Talmon S, Schulze I, Boeddinghaus C, Gross G, Schoemaker H, Wicke KM (2009) Running wheel activity is sensitive to acute treatment with selective inhibitors for either serotonin or norepinephrine reuptake. Psychopharmacology (Berl) 203:753–762. CrossRef Medline

Weisstaub NV, Zhou M, Lira A, Lambe E, González-Maeso J, Hornung JP, Sibille E, Underwood M, Itohara S, Dauer WT, Ansorge MS, Morelli E, Mann JJ, Toth M, Aghajanian G, Sealfon SC, Hen R, Gingrich JA (2006) Cortical 5-HT2A receptor signaling modulates anxiety-like behaviors in mice. Science 313:536–540. CrossRef Medline

Yamada K, Santo-Yamada Y, Wada K (2003) Stress-induced impairment of inhibitory avoidance learning in female neuromedin B receptor-deficient mice. Physiol Behav 78:303–309. CrossRef Medline

Yao I, Takagi H, Ageta H, Kahyo T, Sato S, Hatanaka K, Fukuda Y, Chiba T, Morone N, Yuasa S, Inokuchi K, Ohtsuka T, Macgregor GR, Tanaka K, Setou M (2007) SCRAPPER-dependent ubiquitination of active zone protein RIM1 regulates synaptic vesicle release. Cell 130:943–957. CrossRef Medline

Yu YW, Tsai SJ, Hong CJ, Chen TJ, Chen MC, Yang CW (2005) Association study of a monoamine oxidase a gene promoter polymorphism with major depressive disorder and antidepressant response. Neuropsychopharmacology 30:1719–1723. CrossRef Medline

Neuroscience 263 (2014) 257–268

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

K. NOSE-ISHIBASHI,[a] J. WATAHIKI,[a]* K. YAMADA,[b] M. MAEKAWA,[c] A. WATANABE,[c] G. YAMAMOTO,[e] A. ENOMOTO,[a] Y. MATSUBA,[f] T. NAMPO,[a] T. TAGUCHI,[a] Y. ICHIKAWA,[a] T. C. SAIDO,[f] K. MISHIMA,[e] Y. YAMAGUCHI,[d] T. YOSHIKAWA[c] AND K. MAKI[a]

[a] *Department of Orthodontics, School of Dentistry, Showa University, Tokyo, Japan*

[b] *Research Resource Center, RIKEN Brain Science Institute, Saitama, Japan*

[c] *Laboratory for Molecular Psychiatry, RIKEN Brain Science Institute, Saitama, Japan*

[d] *Neuroinformatics Japan Center, RIKEN Brain Science Institute, Saitama, Japan*

[e] *Department of Oral Pathology and Diagnosis, School of Dentistry, Showa University, Tokyo, Japan*

[f] *Laboratory for Proteolytic Neuroscience, RIKEN Brain Science Institute, Saitama, Japan*

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. **© 2014 IBRO. Published by Elsevier Ltd. All rights reserved.**

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

*Corresponding author. Address: Department of Orthodontics, School of Dentistry, Showa University, 2-1-1, Kitasenzoku, Ohta-ku, Tokyo 145-8515, Japan. Tel: +81-3-3787-1151; fax: +81-3-3784-6641.

E-mail address: junwata2000@ybb.ne.jp (J. Watahiki).

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.

http://dx.doi.org/10.1016/j.neuroscience.2013.12.065
0306-4522/© 2014 IBRO. Published by Elsevier Ltd. All rights reserved.