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Levetiracetam-mediated emotional behavior in heterozygous *rolling* Nagoya $Ca_V2.1$ channel mutant mice

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ABSTRACT

$Ca_V2.1$, which is highly expressed in the nervous system, plays an essential role in presynaptic neurotransmitter release. Although recent data suggest that the antiepileptic drug levetiracetam (LEV) inhibits presynaptic $Ca_V2.1$ activity, the precise physiological role of $Ca_V2.1$/LEV-regulated emotional performance has not been elucidated. We examined whether $Ca_V2.1$/LEV mediates emotional behavior using a combined pharmacologic and genetic approach. Heterozygous *rolling* Nagoya (*rol*/+) mice carrying the $Ca_V2.1\alpha_1$ mutation demonstrated normal emotional behavior. Exposure to 75 mg/kg LEV, which had no effect in wild-type controls, reduced anxiety in elevated plus maze and light–dark exploration tests and reduced depression in forced swimming and tail suspension behavioral tests in *rol*/+ mice. Similar behavioral patterns in motor activity were noted in wild-type and *rol*/+ mice injected with 0–150 mg/kg LEV. The phosphorylation of tryptophan hydroxylase at serine-58 and serotonin concentration were increased in the brainstems of *rol*/+ mice injected with 75 mg/kg LEV but not in those of wild-type controls. These results indicate that $Ca_V2.1$/LEV mediates serotonin signaling leading to alterations in emotion. Our results also indicate that a combination of subthreshold pharmacologic and genetic approaches can be used to study functional signaling pathways in neuronal circuits.

1. Introduction

Voltage-gated Ca^{2+} (Ca_V) channels play an important regulatory role in diverse neuronal functions attributed to elevated intracellular Ca^{2+} concentrations (Berridge et al., 2000; Liu et al., 2003). Ca^{2+} influx triggers neurotransmitter production and release in a cooperative process with other components of neurotransmitter-biosynthesizing enzyme activation and vesicle fusion machineries (Catterall, 1998; Mendoza et al., 2003). Given the pivotal role of Ca^{2+} channels in the control of neurotransmitter production and release, defects in the expression, localization, structure, or modulation of presynaptic Ca^{2+} channels may result in aberrant synaptic signaling leading to various patterns of neural network dysfunction. Two major Ca_V2 channel types, $Ca_V2.1$ (P/Q-type) and $Ca_V2.2$ (N-type), have critical roles in presynaptic terminals (Catterall, 1998; Yokoyama et al., 2005).

The α_1 subunit is a pore-forming component, which functions as a voltage sensor and is capable of generating channel activity (Catterall, 1999; Mikami et al., 1989). Mutations in the $Ca_V2.1$ channel α_1 subunit ($Ca_V2.1\alpha_1$) gene have been identified in ataxic mutant mice such as *rolling* mouse Nagoya, *tottering*, and *leaner* (Oda, 1973; Fletcher et al., 1996). *Rolling* mouse Nagoya carries a mutation in the voltage-sensing S4 segment of the third repeat in the $Ca_V2.1\alpha_1$ gene (Mori et al., 2000). Previously, we assessed emotion-related behavior and $Ca_V2.1\alpha_1$ mRNA expression in two- and 22-month-old mice (Takahashi et al., 2009a). Reduced anxiety and depression phenotypes were observed in 22-month-old heterozygous (*rol*/+) mice compared to age-matched wild-type (+/+) mice, suggesting that aged *rol*/+ mice can be used to delineate the interaction between $Ca_V2.1$ function and emotional performance. The mRNA expression of mutant-type $Ca_V2.1\alpha_1$ was increased in the brainstems of 22-month-old *rol*/+ mice. In contrast, no difference in behavior or expression was noted between two-month-old *rol*/+ and +/+ mice. Further, no significant difference was observed between two-month-old *rol*/+ and +/+ mice and between 22-month-old *rol*/+ and +/+ mice in motor-related behavioral tasks, including footprint and traction tests (Takahashi et al., 2009b), suggesting that *rol*/+ mice possess no epileptic or ataxic phenotypes. These findings suggest that *rol*/+ mice show age-related emotional changes but not epileptic or ataxic changes, and that mutant-type $Ca_V2.1\alpha_1$ expression in two-month-old *rol*/+ mice has a sensitive subthreshold dose to emotional performance.

The anti-epileptic drug levetiracetam (LEV) inhibits presynaptic $Ca_V2.1$ function (Lee et al., 2009). However, the precise physiologic role of $Ca_V2.1$/LEV-regulated synaptic function in emotional performance at the system level remains unclear. There may be a subthreshold dose of LEV that triggers an alteration in emotion-related behavior in two-month-old *rol*/+ mice but not in wild-type controls.

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In the present study, we conducted emotion-related behavioral tests and analyzed the levels of the serotonin-biosynthesizing enzyme tryptophan hydroxylase (TPH) and serotonin in the brainstems of two-month-old $Ca_V2.1$ mutant *rol*/+ mice using various concentrations of LEV.

2. Materials and methods

2.1. Animals

All procedures involving animals were approved by the Animal Experiments Committee of RIKEN. All animals were cared for humanely in accordance with institutional guidelines for animal experimentation. The *rolling* Nagoya mouse strain, which was found among descendants of a cross between strains SIII and C57BL/6 (Oda, 1973), was provided by the RIKEN BioResource Center with support from the National BioResource Project of the Ministry of Education, Culture, Sports, Science, and Technology of Japan. Male +/+ and *rol*/+ F1 progeny were derived from a cross between +/+ and *rol*/+ mice and genotyped by PCR using tail DNA (Takahashi et al., 2009a). The mice were given free access to water and food pellets (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan) and were housed under a 12-h/12-h light/dark cycle (lights on from 08:00 to 20:00) at 23 ± 1 °C and 55 ± 5% humidity. All behavioral analyses were conducted between 09:00 and 16:00 by a well-trained experimenter who was blinded to the mouse strains. Anxious behavior was studied using the elevated plus maze (Pellow et al., 1985) and light–dark exploration (Crawley, 1981) behavioral tests. Depressive behavior was studied using the forced swimming (Porsolt et al., 1978) and tail suspension (Steru et al., 1985) tests. The mice were moved into the behavioral testing room at least 1 h before testing. We examined the levels of TPH and TPH phosphorylated at serine-58 (p-TPH) by Western blot analysis and used high-performance liquid chromatography (HPLC) to examine the monoamine concentrations. Separate groups of two-month-old male mice were used for the behavior, expression, and monoamine concentration tests.

2.2. Drug

LEV (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 0.9% NaCl and injected intraperitoneally 30 min before behavioral testing in a final volume of 5 ml/kg. The doses (25–150 mg/kg) used were within the range reported to produce emotional alterations in various rodent models (Gower et al., 2003; Klitgaard and Pitkänen, 2003; Lamberty et al., 2003).

2.3. Open field test

Motor activity was measured by placing individual mice in a clear Plexiglas box (L×W×H: 30×20×15 cm). The box was then positioned in a frame on which infrared beams (Scanet SV-10, Tokyo, Japan) were mounted. The light intensity in the experimental room was 60 lux. Beam interruptions were summed over 5 min. The following mice were used in the open field test: 0 mg/kg LEA-injected +/+ ($n=10$), 0 mg/kg LEA-injected *rol*/+ ($n=10$), 25 mg/kg LEA-injected +/+ ($n=10$), 25 mg/kg LEA-injected *rol*/+ ($n=10$), 75 mg/kg LEA-injected +/+ ($n=10$), 50 mg/kg LEA-injected +/+ ($n=10$), 50 mg/kg LEA-injected *rol*/+ ($n=10$), 75 mg/kg LEA-injected *rol*/+ ($n=10$), 150 mg/kg LEA-injected +/+ ($n=10$), and 150 mg/kg LEA-injected *rol*/+ mice ($n=10$).

2.4. Elevated plus maze test

The apparatus consisted of two open arms (30×5 cm) and two closed arms (30×5×15 cm) that extended from a common central platform (5×5 cm). A small raised lip (0.3 cm) around the perimeter of the open arms prevented the mice from falling. The apparatus was made of Plexiglas with a gray floor and walls, and was elevated 45 cm above the floor. At the beginning of each experiment, a mouse was placed on the center platform. The mice were allowed to explore the apparatus freely for 5 min under 20 lux of illumination. Behavior was recorded with an overhead video camera. Arm entry was defined as four legs entering one of the maze arms. The number of transitions between the arms, the number of entries into open arms, and the time spent in open arms were measured. The following mice were used in the elevated plus maze test: 0 mg/kg LEA-injected +/+ ($n=12$), 0 mg/kg LEA-injected *rol*/+ ($n=12$), 25 mg/kg LEA-injected +/+ ($n=12$), 25 mg/kg LEA-injected *rol*/+ ($n=12$), 50 mg/kg LEA-injected +/+ ($n=12$), 50 mg/kg LEA-injected *rol*/+ ($n=12$), 75 mg/kg LEA injected +/+ ($n=12$), 75 mg/kg LEA-injected *rol*/+ ($n=12$), 150 mg/kg LEA-injected +/+ ($n=12$), and 150 mg/kg LEA-injected *rol*/+ mice ($n=12$).

2.5. Light–dark exploration test

The apparatus consisted of two compartments: a dark compartment (15×10×20 cm) and a light compartment (20×15×20 cm). The dark compartment had a lid on top and was made of black Plexiglas, whereas the light compartment was open at the top and was made of white Plexiglas. A black Plexiglas tunnel (10×7×4.5 cm) separated the dark box from the light box. The light intensity in the experimental room was 100 lux. A mouse was placed in the light compartment and its behavior was recorded on a videotape over a 5-min period. The number of transitions between the compartments and the time spent in the light compartment were measured. A mouse with all four paws in the light compartment was considered to be fully in the light compartment. The following mice were used in the light–dark exploration test: 0 mg/kg LEA-injected +/+ ($n=12$), 0 mg/kg LEA-injected *rol*/+ ($n=12$), 25 mg/kg LEA-injected +/+ ($n=12$), 25 mg/kg LEA-injected *rol*/+ ($n=12$), 50 mg/kg LEA-injected +/+ ($n=12$), 50 mg/kg LEA-injected *rol*/+ ($n=12$), 75 mg/kg LEA injected +/+ ($n=12$), 75 mg/kg LEA-injected *rol*/+ ($n=12$), 150 mg/kg LEA-injected +/+ ($n=12$), and 150 mg/kg LEA-injected *rol*/+ mice ($n=12$).

2.6. Forced swimming test

Each mouse was placed in a 19-cm glass cylinder (11.0 cm in diameter) containing 13 cm of water at 23 ± 1 °C. A mouse was deemed immobile when it floated, its hindlimbs appeared immobile, and only small movements of the forepaws were used to keep the head above water. The light intensity in the experimental room was 150 lux. The behavior of the mice was recorded with a video camera for 7 min. Immobility was recorded at 2 and 7 min. The parameter recorded was the total amount of time (s) spent immobile. The following mice were used in the forced swimming test: 0 mg/kg LEA-injected +/+ ($n=10$), 0 mg/kg LEA-injected *rol*/+ ($n=10$), 25 mg/kg LEA-injected +/+ ($n=10$), 25 mg/kg LEA-injected *rol*/+ ($n=10$), 75 mg/kg LEA-injected +/+ ($n=10$), 75 mg/kg LEA-injected *rol*/+ ($n=10$), 150 mg/kg LEA-injected +/+ ($n=10$), and 150 mg/kg LEA-injected *rol*/+ mice ($n=10$).

2.7. Tail-suspension test

The apparatus consisted of a non-transparent compartment (15.0×16.0×25.0 cm) with a hook (4.0 cm in length). The distance between the hook and floor was 21 cm. Each mouse was hung from the hook using adhesive tape placed 2 cm from the end of its tail, and its behavior was recorded with a video camera for 7 min. The immobility time was evaluated between 2 and 7 min. The light intensity in the experimental room was 150 lux. The following mice were used in the tail suspension test: 0 mg/kg LEA-injected +/+

($n=10$), 0 mg/kg LEA-injected *rol*/+ ($n=10$), 25 mg/kg LEA-injected +/+ ($n=10$), 25 mg/kg LEA-injected *rol*/+ ($n=10$), 75 mg/kg LEA-injected +/+ ($n=10$), 75 mg/kg LEA-injected *rol*/+ ($n=10$), 150 mg/kg LEA-injected +/+ ($n=10$), and 150 mg/kg LEA-injected *rol*/+ mice ($n=10$).

2.8. Measurement of dopamine, noradrenaline, and serotonin

The mice were decapitated and their brainstems were removed quickly and stored at −80 °C for neurochemical analysis. The concentrations of dopamine, noradrenaline, and serotonin were measured using HPLC with an electrochemical detection system (HTEC-500, Eicom, Kyoto, Japan). Tissue samples were prepared for HPLC by homogenization in 0.2 M perchloric acid, and the extracts were used to determine the monoamine concentrations. The mobile phase consisted of 83% phosphate buffer containing 5 mg/L Na_2EDTA, 190 mg/ml sodium octylsulfate, and 17% methanol. The samples were injected into the column (2.1ϕ×150 mm, EICOMPAK SC-50DS; Eicom, Kyoto, Japan) at a flow rate of 0.5 ml/min. The substance was oxidized with a graphite electrode at a potential of +750 mV relative to an Ag/AgCl reference electrode; the electrochemical detector was set at a gain of 0.5 nA at full-scale. The monoamine concentrations were determined using data analysis software (PowerChrom, Bio Research Center, Aichi, Japan) and are expressed as pg/mg tissue. The following mice were used to measure the monoamine concentrations: 0 mg/kg LEA-injected +/+ ($n=8$), 0 mg/kg LEA-injected *rol*/+ ($n=8$), 75 mg/kg LEA-injected +/+ ($n=8$), 75 mg/kg LEA-injected *rol*/+ ($n=8$), 150 mg/kg LEA-injected +/+ ($n=8$), and 150 mg/kg LEA-injected *rol*/+ mice ($n=8$).

2.9. Western blot analysis

The brainstems of the mice were suspended in buffer (PRO-PREP™ Protein Extraction Solution; iNtRON Biotechnology, Gyeonggi, Korea). A 20-μg sample of each protein was subjected to 10% SDS-PAGE, and the bands were transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA, USA). Commercial rabbit monoclonal anti-TPH antibodies (Abcam, Cambridge, MA, USA) and rabbit polyclonal anti-pTPH antibodies (BIOMOL, Plymouth Meeting, PA, USA) were used for immunodetection. The protein concentration was quantified using mouse monoclonal anti-β-actin antibodies (Chemicon International, Temecula, CA, USA). The membranes were stripped in stripping buffer and re-probed for β-actin as a protein loading control. The protein signals were visualized and quantified using NIH Image. The protein bands were boxed, and the integrated intensity of the pixels in each box was calculated against the average background level for a box of the same size. The amount of protein is expressed as the ratio of protein to β-actin. The following mice were used in the Western blot analyses: 0 mg/kg LEA-injected +/+ ($n=10$), 0 mg/kg LEA-injected *rol*/+ ($n=10$), 75 mg/kg LEA-injected +/+ ($n=10$), 75 mg/kg LEA-injected *rol*/+ ($n=10$), 150 mg/kg LEA-injected +/+ ($n=10$), and 150 mg/kg LEA-injected *rol*/+ mice ($n=10$).

2.10. Statistical analysis

The data are presented as the mean ± standard error of the mean (SEM). Statistical analyses were conducted using Excel Statistics 2006 (SSRI, Tokyo, Japan). The data were analyzed using analysis of variance (ANOVA). Bonferroni *post hoc* comparisons between groups were performed when appropriate. The results were considered significant at a probability of error ≤5%. In the Western blot analysis, data were normalized prior to parametric ANOVA and *post hoc* comparisons. The β-actin expression ratio in the 0 mg/kg LEA-injected +/+ mice was used as a standard control against which the ratios for the other strains were normalized. Quantification was based on the average of six independent experiments.

3. Results

3.1. Effects of LEV on motor activity in rol/+ mice

The groups did not differ significantly in terms of their activity counts (genotype effect: $F[1, 90]=0.790$, $P=0.377$; dose effect: $F[4, 90]=0.554$, $P=0.697$; genotype×dose interaction: $F[4, 90]=0.800$, $P=0.528$) (Table 1).

3.2. Effects of LEV on elevated plus maze test performance in rol/+ mice

The groups did not differ significantly in terms of the number of closed-arm entries (genotype: $F[1, 110]=0.837$, $P=0.362$; dose: $F[4, 110]=2.231$, $P=0.070$; genotype×dose: $F[4, 110]=0.565$, $P=0.689$) (Fig. 1A); however, the groups differed significantly in terms of the number of open-arm entries (genotype: $F[1, 110]=5.992$, $P=0.016$; dose: $F[4, 110]=7.107$, $P<0.001$; genotype×dose: $F[4, 110]=2.664$, $P=0.036$) (Fig. 1B). The groups also differed significantly in the amount of time spent on the open arms (genotype: $F[1, 110]=8.172$, $P=0.005$; dose: $F[4, 110]=24.558$, $P<0.001$; genotype×dose: $F[4,110]=2.743$, $P=0.032$) (Fig. 1C). Although the number of open-arm entries and the amount of time spent on the open arms in the 50 mg/kg LEA-injected *rol*/+ mice tended to be greater than that in the 50 mg/kg LEA-injected +/+ mice, the difference was not significant. The number of open-arm entries and the amount of time spent in the open arms in the 75 mg/kg LEA-injected *rol*/+ mice exceeded that in the 75 mg/kg LEA-injected +/+ mice (open-arm entries: $P<0.001$; time spent on the open arms: $P<0.001$, Bonferroni test). The number of open-arm entries and the amount of time spent in the open arms were significantly different between the 0 mg/kg LEA-injected *rol*/+ and 75 mg/kg LEA-injected *rol*/+ mice (open-arm entries: $P<0.001$; time spent on the open arms: $P<0.001$, Bonferroni test), between the 0 mg/kg LEA-injected +/+ and 150 mg/kg LEA-injected +/+ mice (open-arm entries: $P<0.001$; time spent on the open arms: $P<0.001$, Bonferroni test), and between the 0 mg/kg LEA-injected *rol*/+ and 150 mg/kg LEA-injected *rol*/+ mice (open-arm entries: $P<0.001$; time spent on the open arms: $P<0.001$, Bonferroni test).

3.3. Effects of LEV on light–dark exploration test performance in rol/+ mice

Although ANOVA revealed a significant dose effect on the number of transitions, the groups did not differ significantly in terms of the effect of genotype or genotype×dose interaction (genotype: $F[1, 110]=0.005$, $P=0.942$; dose: $F[4, 110]=3.214$, $P=0.016$; genotype×dose: $F[4,110]=0.245$, $P=0.912$) (Fig. 2A). The groups differed significantly in terms of the time spent in the light box (genotype: $F[1, 110]=8.699$, $P=0.004$; dose: $F[4, 110]=13.429$, $P<0.001$; genotype×dose: $F[4, 110]=4.127$, $P=0.004$) (Fig. 2B). Although the number of transitions in the 75 mg/kg LEA-injected *rol*/+ mice was similar to that in the 75 mg/kg LEA-injected +/+ mice, the time spent in the light box in the 75 mg/kg LEA-injected *rol*/+ mice exceeded that in the 75 mg/kg LEA-injected +/+ mice ($P<0.001$, Bonferroni test). Although the number of transitions in the 75 mg/kg LEA-injected *rol*/+ mice was similar to that in the 0 mg/kg LEA-injected *rol*/+ mice, the time spent in the light box in

Table 1
Motor activity.

Genotype	Levetiracetam				
	0 mg/kg	25 mg/kg	50 mg/kg	75 mg/kg	150 mg/kg
+/+	1610 ± 87.74	1681 ± 22.38	1684 ± 29.49	1681 ± 30.27	1662 ± 30.63
rol/+	1714 ± 30.70	1702 ± 26.31	1704 ± 34.39	1684 ± 26.25	1627 ± 34.19

The data are presented as the mean ± standard error of the mean (SEM).

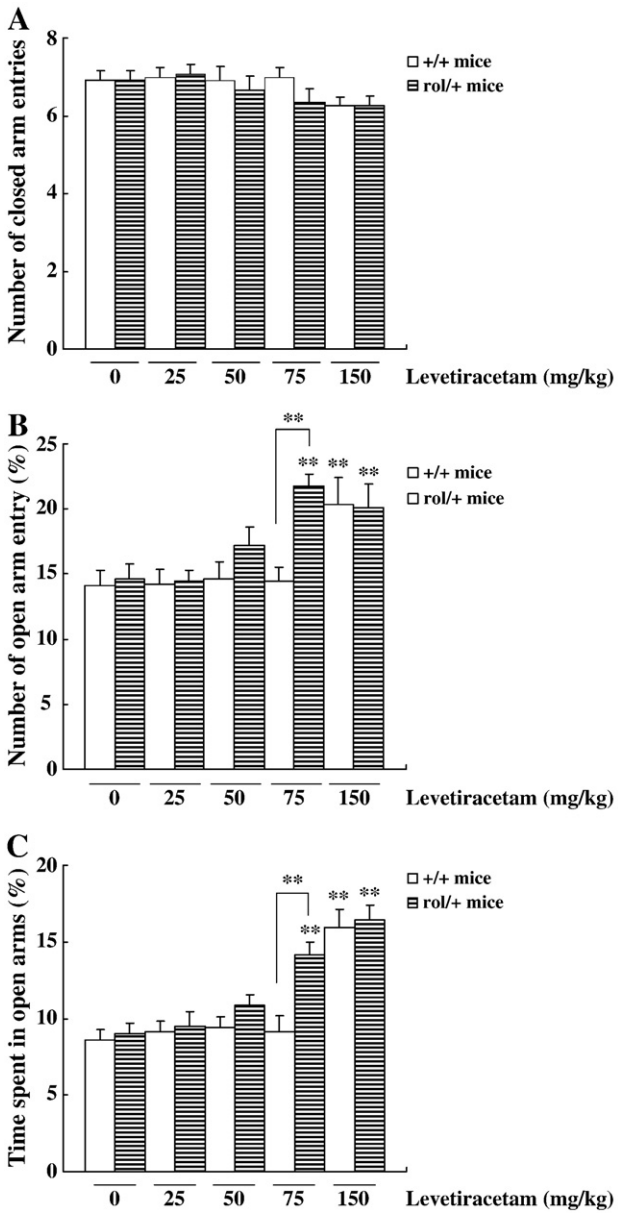

Fig. 1. Elevated plus maze test. The number of closed-arm entries was counted (A). The number of entries on the open arms is expressed as a percentage of the total number of arm entries (B). The time spent on open arms is expressed as a percentage of the total time of arm entries (C). ** $P<0.01$ compared to the appropriate control (Bonferroni test).

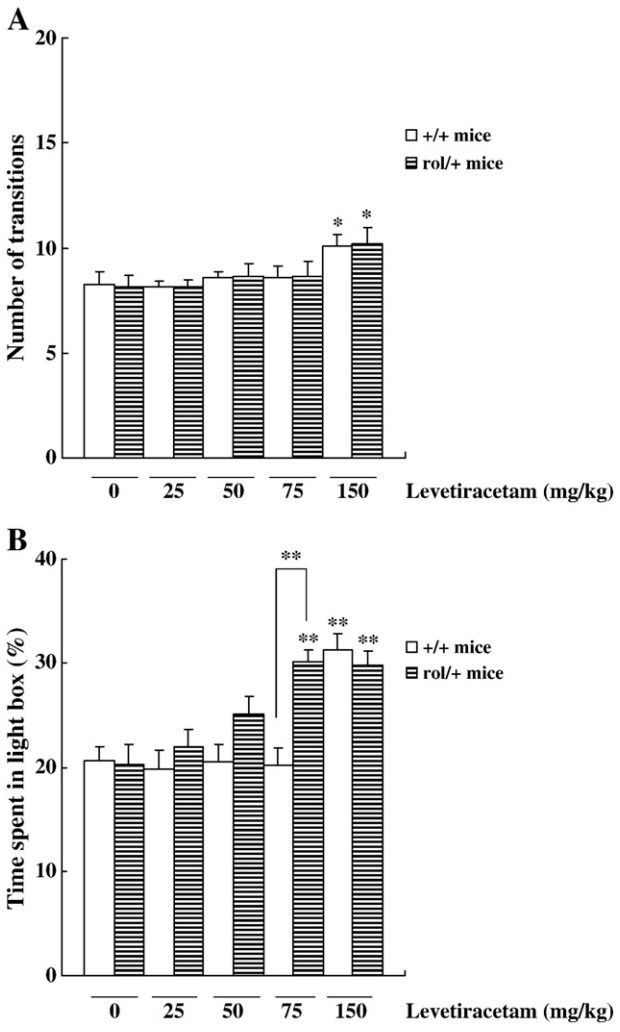

Fig. 2. Light–dark exploration test. The number of transitions between boxes was measured (A). The time spent in the light compartment is expressed as a percentage of the total time in the light and dark compartments (B). * $P<0.05$, ** $P<0.01$ compared to the appropriate control (Bonferroni test).

the 75 mg/kg LEA-injected *rol*/+ mice was greater than that in the 0 mg/kg LEA-injected *rol*/+ mice ($P<0.001$, Bonferroni test). The number of transitions and the amount of time spent on the open arms were significantly different between the 0 mg/kg LEA-injected +/+ and 150 mg/kg LEA-injected +/+ mice (number of transitions: $P<0.05$; time spent in the light box: $P<0.001$, Bonferroni test) and between the 0 mg/kg LEA-injected *rol*/+ and 150 mg/kg LEA-injected *rol*/+ mice (number of transitions: $P<0.05$; time spent in the light box: $P<0.001$, Bonferroni test).

3.4. Effects of LEV on forced swimming test performance in rol/+ mice

There was a significant difference in the time spent immobile among the groups (genotype: $F[1, 72]=4.350$, $P=0.041$; dose: $F[3, 72]=10.962$, $P<0.001$; genotype × dose: $F[3, 72]=3.219$, $P=0.028$) (Fig. 3). The 75 mg/kg LEA-injected *rol*/+ mice spent significantly less time immobile than the dose-matched +/+ mice ($P<0.001$, Bonferroni test). A significant difference was observed between the 0 mg/kg LEA-injected *rol*/+ and the 75 mg/kg LEA-injected *rol*/+ mice ($P<0.001$, Bonferroni test), between the 0 mg/kg LEA-injected +/+ and 150 mg/kg LEA-injected +/+ mice ($P<0.001$, Bonferroni test), and between the 0 mg/kg LEA-injected *rol*/+ and 150 mg/kg LEA-injected *rol*/+ mice($P<0.001$, Bonferroni test).

3.5. Effects of LEV on tail suspension test performance in rol/+ mice

A significant difference in the time spent immobile was found among the groups (genotype: $F[1, 72]=5.913$, $P=0.018$; dose: $F[3, 72]=21.207$, $P<0.001$; genotype × dose: $F[3, 72]=7.891$, $P<0.001$) (Fig. 4). The 75 mg/kg LEA-injected *rol*/+ mice spent significantly less time immobile than the dose-matched +/+ mice ($P<0.001$, Bonferroni test). The time spent immobile was significantly different between the 0 mg/kg LEA-injected *rol*/+ and 75 mg/kg LEA-injected *rol*/+ mice ($P<0.001$, Bonferroni test), between the 0 mg/kg LEA-injected +/+ and 150 mg/kg LEA-injected +/+ mice ($P<0.001$, Bonferroni test), and between the 0 mg/kg LEA-injected *rol*/+ and 150 mg/kg LEA-injected *rol*/+ mice ($P<0.001$, Bonferroni test).

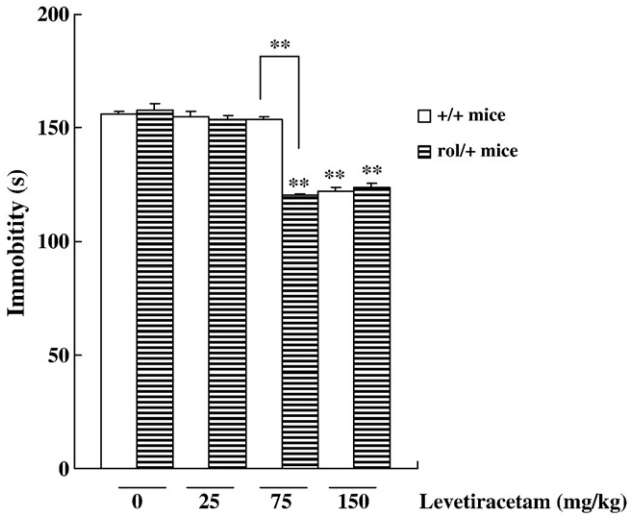

Fig. 3. Forced swimming test. The time spent immobile was evaluated at 2 and 7 min. ** $P<0.01$ compared to the appropriate control (Bonferroni test).

3.6. Effects of LEV on the monoamine levels in rol/+ mice

The groups differed significantly in their serotonin levels (serotonin; genotype: $F[1, 42]=65.888$, $P<0.001$; dose: $F[2, 42]=263.588$, $P<0.001$; genotype × dose: $F[2, 42]=81.993$, $P<0.001$) but not in their dopamine or noradrenaline levels (dopamine; genotype: $F[1, 42]=0.005$, $P=0.943$; dose: $F[2, 42]=0.704$, $P=0.500$; genotype × dose: $F[2, 42]=0.244$, $P=0.784$, noradrenaline; genotype: $F[1, 42]=0.172$, $P=0.680$; dose: $F[2, 42]=0.884$, $P=0.420$; genotype × dose: $F[2, 42]=0.321$, $P=0.727$) (Table 2). The 75 mg/kg LEA-injected *rol*/+ mice showed a significant increase in serotonin concentration compared to the 75 mg/kg LEA-injected +/+ mice ($P<0.001$, Bonferroni test). The serotonin concentrations were significantly different between the 0 mg/kg LEA-injected *rol*/+ and 75 mg/kg LEA-injected *rol*/+ mice ($P<0.001$, Bonferroni test), between the 0 mg/kg LEA-injected +/+ and 150 mg/kg LEA-injected +/+ mice ($P<0.001$, Bonferroni test), and between the 0 mg/kg LEA-injected *rol*/+ and 150 mg/kg LEA-injected *rol*/+ mice ($P<0.001$, Bonferroni test).

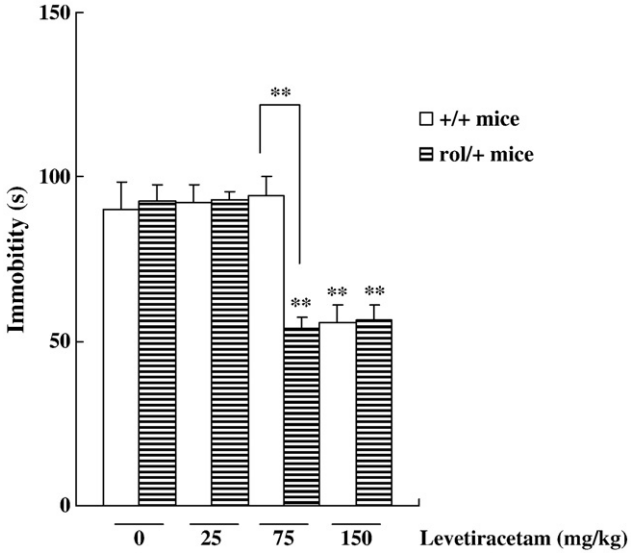

Fig. 4. Tail suspension test. The time spent immobile (s) was evaluated at 2 and 7 min. ** $P<0.01$ compared to the appropriate control (Bonferroni test).

Table 2
Brainstem monoamine concentrations.

Concentration (pg/mg wet tissue)	Genotype	Levetiracetam		
		0 mg/kg	75 mg/kg	150 mg/kg
Dopamine	+/+	31.6 ± 1.55	31.8 ± 1.77	32.0 ± 2.01
	rol/+	31.9 ± 1.98	31.8 ± 2.20	30.9 ± 2.35
Noradrenaline	+/+	775.6 ± 22.22	769.4 ± 25.57	776.1 ± 25.11
	rol/+	772.9 ± 23.32	777.8 ± 27.75	780.8 ± 28.92
Serotonin	+/+	765.6 ± 24.34	759.4 ± 30.82	876.1 ± 30.12**
	rol/+	759.7 ± 24.68	882.7 ± 23.67**	879.7 ± 21.45**

The data are presented as the mean ± standard error of the mean (SEM).
** $P<0.01$ compared to the appropriate control (Bonferroni test).

rol/+ and 150 mg/kg LEA-injected *rol*/+ mice ($P<0.001$, Bonferroni test).

3.7. Effects of LEV on TPH expression in rol/+ mice

The groups differed significantly in their pTPH levels (genotype: $F[1, 54]=85.780$, $P<0.001$; dose: $F[2, 54]=301.268$, $P<0.001$; genotype × dose: $F[2, 54]=70.248$, $P<0.001$) but not in their TPH levels [genotype: $F[1, 54]=0.022$, $P=0883$; dose: $F[2, 54]=0.670$, $P=0.516$; genotype × dose: $F[2,54]=0.022$, $P=1.883$] (Fig. 5). The 75 mg/kg LEA-injected *rol*/+ mice expressed more pTPH than the dose-matched +/+ mice ($P<0.001$, Bonferroni test). Additionally, the pTPH level was significantly different between the 0 mg/kg LEA-injected *rol*/+ and 75 mg/kg LEA-injected *rol*/+ mice ($P<0.001$, Bonferroni test), between the 0 mg/kg LEA-injected +/+ and 150 mg/kg LEA-injected +/+ mice ($P<0.001$, Bonferroni test), and between the 0 mg/kg LEA-injected *rol*/+ and 150 mg/kg LEA-injected *rol*/+ mice ($P<0.001$, Bonferroni test).

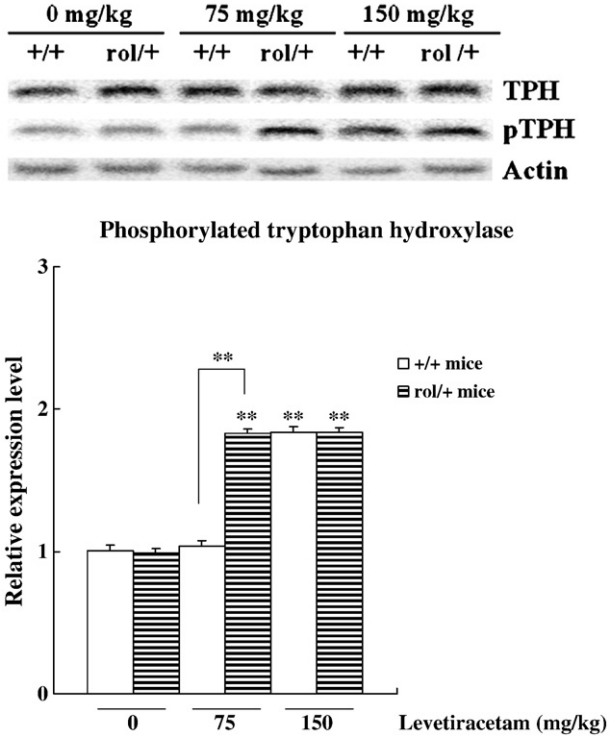

Fig. 5. Western blot analysis. Representative expression patterns of tryptophan hydroxylase (TPH), tryptophan hydroxylase phosphorylated at serine-58 (pTPH), and β-actin (actin) are shown at the top. Quantification of the pTPH expression level is shown at the bottom. The actin expression ratio in 0 mg/kg LEA-injected +/+ mice was used as a standard control against which the ratios for the other strains were normalized. Quantification was based on the average of six independent experiments. ** $P<0.01$ compared to the appropriate control (Bonferroni test).

4. Discussion

In this study, we analyzed the $Ca_V2.1$/LEV-mediated emotional behavior of two-month-old *rol*/+ mice. We also studied the expression of the serotonin-biosynthesizing enzyme TPH and examined the monoamine concentrations in the brainstems of mice injected with LEV. The 0–150 mg/kg LEV-injected +/+ and *rol*/+ mice showed no significant difference in motor activity in open field tests. They made a similar number of closed-arm entries in the elevated plus maze test. These results indicate a lack of dose-dependent changes in motor activity between heterogeneous *rolling* Nagoya $Ca_V2.1$ channel mutant and wild-type mice. Anxious and depressive behavior was decreased in the *rol*/+ mice at a lower dose of LEV compared to +/+ mice. In the elevated plus maze test, the 75 mg/kg LEV-injected *rol*/+ mice spent significantly more time on the open arms compared to dose-matched +/+ mice. Furthermore, less anxiety was apparent during the light–dark exploration test, because the 75 mg/kg LEV-injected *rol*/+ mice spent significantly more time in the light box. Because no behavioral differences were observed between the 0 mg/kg LEV-injected *rol*/+ and +/+ mice, between the 25 mg/kg LEV-injected *rol*/+ and +/+ mice, between the 50 mg/kg LEV-injected *rol*/+ and +/+ mice, and between the 150 mg/kg LEV-injected *rol*/+ and +/+ mice in the elevated plus maze and light–dark exploration tests, our results imply that the subthreshold dose for reduced anxiety in *rol*/+ mice is 75 mg/kg. Our tail suspension and forced swimming test results also indicate that the 75 mg/kg LEV-injected *rol*/+ mice exhibited decreased depressive behavior compared with dose-matched +/+ mice. The neurotransmission of monoamines is thought to control emotional behavior (Rodgers et al., 1994; Zhou and Palmiter, 1995; Gainetdinov et al., 1999; Zhuang et al., 1999). To determine whether the reduced anxious and depressive behaviors observed in the 75 mg/kg LEV-injected *rol*/+ mice were due to a dose effect in monoamine levels, we examined the concentrations of dopamine, norepinephrine, and serotonin in the brainstems of mice of both genotypes injected with LEV. Although no significant differences were observed in their dopamine or noradrenaline levels, the 75 mg/kg LEV-injected *rol*/+ mice showed an increase in brainstem serotonin compared to dose-matched +/+ mice. To determine whether this increase was due to increased expression of the serotonin-biosynthesizing enzyme TPH, Western blotting was used to examine the brainstem expression of TPH and pTPH. Serine-58 phosphorylation plays an important role in the regulation of TPH activity and the subsequent biosynthesis of serotonin (Kuhn et al., 1997). Western blot analysis showed an increase in pTPH, but not in TPH in the 75 mg/kg LEV-injected *rol*/+ mice. Our results suggest that this increase in TPH activity might be at least partly responsible for the observed decrease in anxiety and depressive behavior in the 75 mg/kg LEV-injected *rol*/+ mice. However, the 75 mg/kg LEV-injected +/+ mice showed similar patterns in emotion-related behavioral, protein expression, and monoamine concentration to the 0 and 50 mg/kg LEV-injected +/+ mice. Neither mouse genotype injected with 150 mg/kg LEV showed a difference in any of these tests. Our results suggest an all-or-nothing effect of the drug. LEV specifically binds SV2A, which is abundant in synaptic vesicles (Kaminski et al., 2009), partially inhibits $Ca_V2.2$ (Lukyanetz et al., 2002), and reduces the inhibition of γ-aminobutyric acid (GABA)- and glycine-gated currents (Rigo et al., 2002). Additional studies using LEV and $Ca_V2.1\alpha_1$-specific inhibitors are required to examine the all-or-nothing effect of LEV on $Ca_V2.1$ function.

Previously, we examined the emotional phenotypes of two- and 22-month-old *rol*/+ mice using emotional-behavioral tests (Takahashi et al., 2009a). The 22-month-old *rol*/+ mice demonstrated reduced anxiety in both elevated plus maze and light–dark exploration behavioral tests, and reduced depression in both forced swimming and tail suspension tests compared with age-matched +/+ mice, although there was no difference between two-month-old +/+ and *rol*/+ mice. Expression analyses of the brainstems of the mice showed that mutant-type $Ca_V2.1\alpha_1$ mRNA was expressed at a higher level in the 22-month-old mice compared to the two-month-old *rol*/+ mice (Takahashi et al., 2009a). One factor contributing to these alterations in the emotional behavior of the 22-month-old *rol*/+ mice is an age-related increase in mutant-type $Ca_V2.1\alpha_1$ expression, resulting in abnormalities in presynapses expressing mutant $Ca_V2.1$ channels with reduced anxiety and depression. It was impossible to distinguish the *rol*/+ mice from +/+ mice in the same cage based on observation alone. However, the two-month-old *rol*/+ mice were extremely sensitive to emotional alterations; thus, the level of mutant-type $Ca_V2.1\alpha_1$ expression in the two-month-old *rol*/+ mice represents a subthreshold dose. This possibility was confirmed using a subthreshold dose of LEV, which triggered emotional alterations in two-month-old *rol*/+ mice but not in wild-type controls.

$Ca_V2.1$ channels play a pivotal role in neurotransmitter release (Harvey et al., 1996; Kawata et al., 2001) and in enzyme activation via phosphorylation (Mendoza et al., 2003). No differences were observed in the concentrations of dopamine, norepinephrine, or serotonin in the brainstems of two-month-old *rol*/+ and +/+ mice (Takahashi et al., 2009a). Conversely, although no significant differences were observed in dopamine or noradrenaline, the 22-month-old *rol*/+ mice showed an increase in brainstem serotonin compared to age-matched +/+ mice (Takahashi et al., 2009a). The 22-month-old *rol*/+ mice showed an increase in pTPH but not in TPH, and they exhibited increased serotonin concentrations (Takahashi et al., 2009a). The Ca^{2+} current amplitude showed a 40% reduction in homozygous *rolling* Nagoya mice compared to wild-type controls (Mori et al., 2000). These data indicate that a decrease in depolarization-induced Ca^{2+} influx through $Ca_v2.1$ channels may induce abnormal TPH phosphorylation and serotonin release. An electrophysiological study reported that LEV reduced $Ca_V2.1$ function (Pisani et al., 2004; Lee et al., 2009), suggesting that a subthreshold dose of LEV induces abnormal TPH phosphorylation and serotonin concentrations in two-month-old *rol*/+ mice but not in wild-type controls. In agreement with this, our results showed increased TPH phosphorylation and serotonin concentrations in *rol*/+ mice injected with a subthreshold dose of LEV, but not in +/+ mice. Although emotional behavior may also be mediated by other neurotransmitter systems, our results suggest that increased $Ca_V2.1$-meditaed serotonin signaling is at least partly responsible for the observed decrease in anxiety and depressive behavior in *rol*/+ mice injected with a subthreshold dose of LEV. Interestingly, although the subthreshold dose is 75 mg/kg for reduced anxiety and depression, motor activity between the +/+ and *rol*/+ mice when injected with 0–150 mg/kg LEV was similar. In the light–dark exploration test, the 75 mg/kg LEV-injected *rol*/+ mice spent significantly more time in the light box. In contrast, although intercompartmental transitions are also believed to be linked to anxiety (low transitions = high anxiety; high transitions = low anxiety) (Crawley, 1989), no effect of genotype × dose interaction on the transitions was noted. These results indicate that the subthreshold dose differs in signaling, leading to various phenotypes. Because SV2A interacts with synaptotagmin 1 to enhance the release of various neurotransmitters (Janz et al., 1999; Lynch et al., 2004), additional studies are necessary to examine serotonin release conditions. It would also be interesting to examine the degree of anxiety and depression through behavioral tests, the level of pTPH, and the serotonin concentration using 22-month-old LEV-injected *rol*/+ mice for age-dependent $Ca_V2.1$/LEV-mediated signaling analyses.

Although LEV has been suggested to possess antiepileptogenic properties (Klitgaard and Pitkänen, 2003), it induces anxiolytic- and antidepressant-like effects in rodent models of anxiety and depression (Gower et al., 2003; Husum et al., 2004; Lamberty et al., 2003). The efficacy of the drug has been reported in human clinical studies targeting anxiety and depression (Mazza et al., 2008; Papp, 2006; Simon et al., 2004; Zhang et al., 2005). Our results also showed that LEV induces anxiolytic- and antidepressant-like effects in mice,

indicating that it is effective for a wide spectrum of neuropsychiatric diseases by $Ca_V2.1$-mediated serotonin signaling.

In this study, we showed that $Ca_V2.1$/LEV-mediated signaling may be an important factor in emotional function, at least in part due to an increase in the phosphorylation of TPH and the concentration of serotonin. Our results indicate that a combination of subthreshold doses of different agents and genes is a useful tool for identifying functional signaling pathways in neuronal circuits.

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