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Exclusive expression of VMAT2 in noradrenergic neurons increases viability of homozygous VMAT2 knockout mice

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ABSTRACT

The vesicular monoamine transporter 2 (VMAT2) translocates monoamine neurotransmitters from the neuronal cytoplasm into synaptic vesicles. Since VMAT2−/− mice die within a few days of birth, it is difficult to analyze the detailed VMAT2 functions using these mice. In this study, we generated human VMAT2 transgenic mice that expressed VMAT2 in noradrenergic neurons with the aim to rescue the lethality of VMAT2 deletion. The expression of human VMAT2 in noradrenergic neurons extended the life of VMAT2−/− mice for up to three weeks, and these mice showed severe growth deficiency compared with VMAT2+/+ mice. These results may indicate that VMAT2 expressed in noradrenergic neurons has crucial roles in survival during the first several weeks after birth, and VMAT2 functions in other monoaminergic systems could be required for further extended survival. Although VMAT2 rescue in noradrenergic neurons did not eliminate the increased morbidity and lethality associated with VMAT2 deletion, the extension of the lifespan in VMAT2 transgenic mice will enable behavioral, pharmacological and pathophysiological studies of VMAT2 function.

1. Introduction

Vesicular monoamine transporters (VMAT) are transmembrane proteins responsible for transporting monoamines from cytoplasm into secretary vesicles in monoamine presynaptic neuronal endings [1]. The vesicular monoamine transporter 1 (VMAT1) is localized in peripheral neurons, whereas the vesicular monoamine transporter 2 (VMAT2) is localized primarily in the central nervous system [2,3]. VMAT2 transports monoamines, including dopamine, serotonin, noradrenaline, and histamine, into synaptic vesicles, and consequently regulates release and metabolism of the monoamine neurotransmitters. VMAT2 is also known to be the main target molecule of a number of psychostimulant drugs, including amphetamine and methamphetamine [3–6], and is therefore potentially associated with many of the severe problems, such as addiction and psychoses, resulting from repetitive use of these drugs. For this reason VMAT2 has been investigated for its possible roles in the pathophysiology of drug abuse and psychosis.

Unfortunately, since homozygous VMAT2 knockout (KO) (VMAT2−/−) mice die within a few days after birth [4,7], physiological and behavioral analyses of many VMAT2 functions are not possible with VMAT2−/− mice. In heterozygous VMAT2 KO (VMAT2+/−) mice, physiological and behavioral effects of VMAT2 deficiency have been evaluated [4,8–10]. However, conclusions drawn solely from studies of heterozygous KO mice are limited. Although there is little evidence to explain the precise mechanisms underlying neonatal mortality in VMAT2−/− mice, VMAT2+/− mice do have longer QT intervals in electrocardiograms [10], which suggests the potential involvement of sympathetic noradrenergic dysfunction in VMAT2−/− related neonatal death. VMAT2 is expressed in noradrenergic sympathetic neurons [11], and in fact, the VMAT blocker reserpine was widely used at one time in the treatment of high blood pressure.

In this report, we generated VMAT2−/− mice with an additional genetic modification that specifically expressed a human VMAT2 transgene in noradrenergic neurons. This would potentially prevent neonatal death in VMAT2 KO mice that might be caused by noradrenergic dysfunctions, including those related to dysfunction sympathetic inputs to the cardiovascular system. This would then allow analysis of homozygous VMAT2 deletions in other monoaminergic neurons. Thus, taking advantage of the increased survival

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produced by this rescue approach, behavioral and pharmacological tests were conducted in VMAT2−/− mice in which the VMAT2 transgene was expressed in noradrenergic neurons in order to evaluate the effect of VMAT2 deletion in other monoaminergic neurons.

2. Materials and methods

2.1. Production of VMAT2−/− mice with transgenic VMAT2 recovery in noradrenergic neurons

A human VMAT2 cDNA cassette [12] was substituted for an interleukin 2Rα DNA fragment in a complementary DNA clone construct (DBH-IL-2Rα) containing the dopamine beta hydroxylase promoter upstream to the interleukin 2Rα fragment [13], at the *EcoRI* restriction enzyme site. The construct was microinjected into fertilized C57BL/6 mouse ova, which were implanted into pseudopregnant female C57BL/6 mice. Integration of the transgene (indicated as '$^{NE+}$') was confirmed by Southern blot analysis. For Southern blot analysis, 6.5 μg of genomic DNA extracted from tail was digested with *KpnI* and loaded on 1% agarose gels. DNA samples were subjected to electrophoresis and transferred to nylon membranes. The membranes were hybridized to ^{32}P-labeled probes. Probes were obtained by PCR from transgene specific region of transgenic vector construct following digestion with *ApaI*, and labeled with Ready-To-Go DNA Labelling Beads (−dCTP) (Amersham-Pharmacia Biotech Inc., Little Chalfont, UK) using [^{32}P]-dCTP. VMAT2+/+$^{NE+}$ mice and VMAT2+/− mice that were backcrossed to C57BL/6 genetic background were mated to produce VMAT2+/−$^{NE+}$ mice. VMAT2+/−$^{NE+}$ mice were mated to produce VMAT2−/− /transgenic (VMAT2−/−$^{NE+}$) mice. All animal care procedures were approved by the Animal Care and Use Committee, Tohoku University Graduate School of Medicine, and complied with the procedures in the Guide for the Care and Use of Laboratory Animals of Tohoku University.

2.2. Enhancement of dopaminergic neurotransmission

To enhance dopaminergic neurotransmission, and promote survival, VMAT2−/−$^{NE+}$ mice were injected with 1 mg/kg or 10 mg/kg methamphetamine (Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) subcutaneously, 3 times per week, beginning 3 days after birth. In an additional group of mice, 2 mg/kg apomorphine (Sigma–Aldrich Corporation, St. Louis, MO, USA) was administered to the VMAT2−/−$^{NE+}$ mice. Apomorphine was injected subcutaneously 3 times per week, beginning 1 week after birth. Each drug was diluted with saline in order to adjust the volume of each injection to 0.1 ml per 10 g bodyweight.

2.3. Crossbreeding

To examine the effect of these genetic backgrounds on VMAT2−/− viability, VMAT2+/−$^{NE+}$ mice with C57BL6 genetic background were crossbred with C3H (Charles River Laboratories Japan, Inc., Yokohama, Japan), DBA/1 J (Charles River Laboratories Japan, Inc., Yokohama, Japan) and BALB/c strains (CLEA Japan, Inc., Tokyo, Japan). VMAT2−/−$^{NE+}$ mice that had mixed genetic background of each strain were obtained by crossbreeding with F1 offsprings.

2.4. Immunohistochemistry

Brain tissues from 2 week-old VMAT2+/+ and VMAT2−/−$^{NE+}$ mice with C57BL/6 genetic background were fixed in 2% paraformaldehyde for 3 days, placed in 15% glucose solution for another 3 days before being frozen for tissue slicing. Sixteen micrometers thick frozen coronal sections at the levels of the striatum, the dorsal raphé nucleus, and the locus coeruleus were prepared using a cryostat. In the locus coeruleus region, VMAT2 was detected with rabbit anti-VMAT2 antibody AB1767 (Chemicon, Temecula, CA, 1:500 dilution) and FITC labeled donkey anti-rabbit antibody 711-095-152 (Jackson, West Grove, PA, 1:500 dilution). Additionally, double-staining of dopamine transporter (DAT) and VMAT2 was conducted in striatal sections, and double-staining of tryptophan hydroxylase (TPH) and VMAT2 was processed in the raphé nuclei sections. Dopaminergic neurons were detected with rat anti-DAT monoclonal antibody MAB369 (Chemicon, Temecula, CA, 1:1000 dilution) and Cy5 labeled anti-rat antibody 712-175-153 (Jackson, West Grove, PA, 1:300 dilution). Serotonergic neurons were detected with sheep anti-TPH polyclonal antibody AB1541 (Chemicon, Temecula, CA, 1:300 dilution) and TRITC labeled anti-sheep antibody 713-025-147 (Jackson, West Grove, PA, 1:300 dilution). Both primary and secondary antibodies were incubated for 2 days at room temperature. Signal intensities were recorded and processed using a fluorescence microscope (DMRXA) with FW4000 software (Leica, Wetzlar, Germany).

2.5. Behavioral tests

The behavioral patterns of 2 week-old VMAT2−/−$^{NE+}$ mice were compared to the VMAT2+/+ mice using several paradigms. Mice using behavioral tests had C3H mixed genetic background.

2.6. Evaluation of gait from footprint patterns

As an evaluation of gross motor function gait was examined using a plastic trench [50 cm (length) × 3 cm (width) × 10 cm (depth)]. One end of the trench was closed, while the other end had an opening [3 cm × 3 cm] allowing escape from the brightly light chamber into a light-shielded box [10 cm (length) × 15 cm (width) × 15 cm (depth)]. The bottom of the plastic trench was covered with a white paper sheet [40 cm (length) × 3 cm (width)] [14,15]. Soles of the front and rear feet of each mouse were painted with red dye [20% dietary red dye (monascus based) + 1% gum arabic] and blue dye [20% dietary blue dye (gardenia based) + 1% gum arabic], respectively, and the mice were placed at the closed end of the trench. Gait tracks of the mouse were recorded on paper covering the bottom of the trench as the mouse walked to the light-shielded box at the other end of the apparatus. Before the experiment mice were trained to walk from the closed end to the dark box 3 times, without painting their feet. The footprint patterns of each mouse were measured three times, and strides of 10 steps per measurement were analyzed. Since starting and ending footprints on the recording paper were irregular and faint, only tracks that were clearly spotted for 10 steps in the center of the track were used for the analyses. An average and standard deviation of stride was calculated for each mouse.

2.7. Open-field test

Open-field behavior was examined in a square box [33 cm (length) × 44 cm (width) × 20 cm (depth)], in which the floor was covered with a white paper sheet. VMAT2+/+ ($n = 5$) and VMAT2−/−$^{NE+}$ ($n = 6$) mice were placed at the center of the box, and movements were recorded digitally (NV–GS100; Matsushita Electric Industrial Co., Ltd., Osaka, Japan) for 30 min.

2.8. Statistical analysis

The probability "p" of the observed life length was calculated by the log rank analysis for Kaplan–Meier's survival curve. Body

weight and locomotor activity were analyzed by *Student's t-test*. A *p*-value of less than 0.05 was considered statistically significant. All data were presented as the mean SEM.

3. Results

3.1. Production of VMAT2−/− mice with human VMAT2 expression in noradrenergic neurons

Based on Southern blotting-based genotyping a mouse was identified that had integrated the human VMAT2 transgene into its genome (Fig. 1A). The VMAT2+/+$^{NE+}$ mouse was mated to a VMAT2+/− mouse to produce double transgenic animals. VMAT2−/−$^{NE+}$ mice exhibited severe growth deficiency resulting in about a 50% decrease in body weight (1 week-old:2.2 ± 0.11 g, 2 week-old:2.5 ± 0.2 g) compared to VMAT2+/+ mice (1 week-old:4.3 ± 0.29 g, 2 week-old:6.6 ± 0.34 g) (Fig. 1B and C).

3.2. Lifespan elongation of VMAT2−/− $^{NE+}$ mice

In contrast with neonatal death within 2–3 days after birth observed in VMAT2−/− mice, VMAT2−/−$^{NE+}$ mice survived on average until 11.4 ± 1.4 days postnatal (Fig. 1D). Therefore, the neonatal lethality of the VMAT2−/− mice was ameliorated, but not eliminated, in VMAT2−/−$^{NE+}$ mice, at least that associated with the immediate neonatal period. This lifespan elongation therefore associates VMAT2 functions in noradrenergic neurons with this early mortality, although VMAT2 function elsewhere must also be contributing to this mortality.

A previous report [16] found that amphetamine administration to neonatal VMAT2−/− mice extended the average lifespan to about 14 days. The lifespan of VMAT2−/−$^{NE+}$ mice was nearly as long as after amphetamine treatment, about 11 days. Therefore, in an attempt to further reduce the mortality associated with VMAT KO and to prolong the lives of VMAT2−/−$^{NE+}$ mice beyond this period, methamphetamine was administered to these mice (1 mg/kg or 10 mg/kg, 3 times per week). However, methamphetamine had no effect on average lifespan in the VMAT2−/−$^{NE+}$ mice (13.3 ± 1.4 days, $p = 0.449$ and 10.3 ± 1.1 days, $p = 0.132$, for 1 mg/kg and 10 mg/kg methamphetamine, respectively). Apomorphine was administered in order to stimulate dopaminergic systems directly, but apomorphine (2 mg/kg) also did not extend the lifespan of the VMAT2−/−$^{NE+}$ mice (11.8 ± 0.8 days, $p = 0.326$).

When VMAT2−/−$^{NE+}$ mice were crossbred to the C3H mouse strain, the average lifespan was extended significantly by 5.4 days to 16.8 ± 1.35 days, compared to the original VMAT2−/−$^{NE+}$ strain on the C57BL/6 genetic background (11.4 ± 1.4 days, $p = 0.005$, Fig. 1E). Crossbreeding of the VMAT2−/−$^{NE+}$ mice with BALB/c or DBA/1 J mice had no significant effect on lifespan of the transgenic mice (12.7 ± 0.9 days: $p = 0.969$, and 13.8 ± 2.0 days: $p = 0.328$, respectively).

3.3. VMAT2 expression in noradrenergic neurons of VMAT2−/− $^{NE+}$ mice

Co-localization of VMAT2 and DAT protein expression was examined by immunohistochemistry for DAT and VMAT2 in the striatum (Fig. 2A). In VMAT2+/+ mice (left), DAT labeling (red)

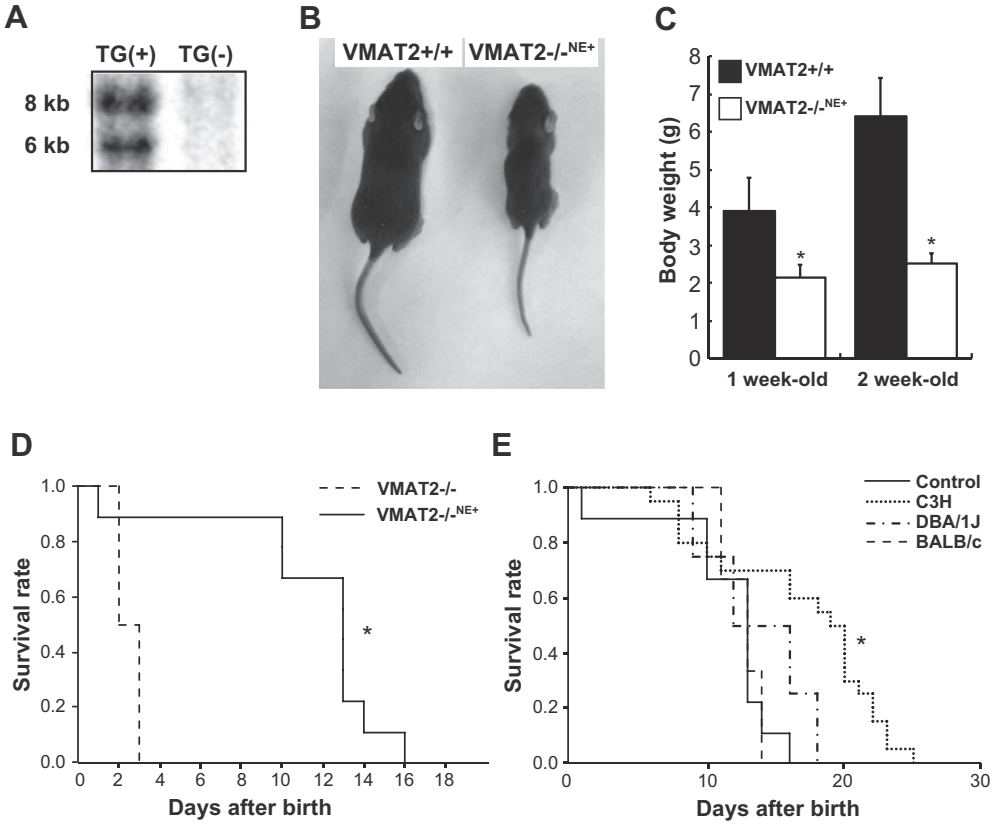

Fig. 1. Generation of VMAT2 transgenic mice. (A) Specific 8 kb and 6 kb bands representing the transgene are displayed in the transgenic mouse. (B) The VMAT2−/−$^{NE+}$ mouse is smaller compared to the VMAT2+/+ mouse at 2 weeks postnatal (C3H crossed VMAT2−/−$^{NE+}$ mouse). (C) Body weight of VMAT2−/−$^{NE+}$ (n = 11–2) versus VMAT2+/+ (n = 11–9) mice (∗ difference between genotypes, $p < 0.05$). (D) and (E) Comparison of Kaplan–Meier's survival curves: *Y*-axis is survival rate, *X*-axis is days after birth. (D) VMAT2−/− (n = 2) mice versus VMAT2−/−$^{NE+}$ (n = 9) mice (∗ difference from VMAT2−/− mice by log-rank test, $p = 0.018$). (E) Control mice (original VMAT2−/−$^{NE+}$) (n = 9) versus crossbred mice (DBA/1J; n = 4, BALB/c; n = 3, C3H; n = 20). (∗ difference from control, $p = 0.005$).

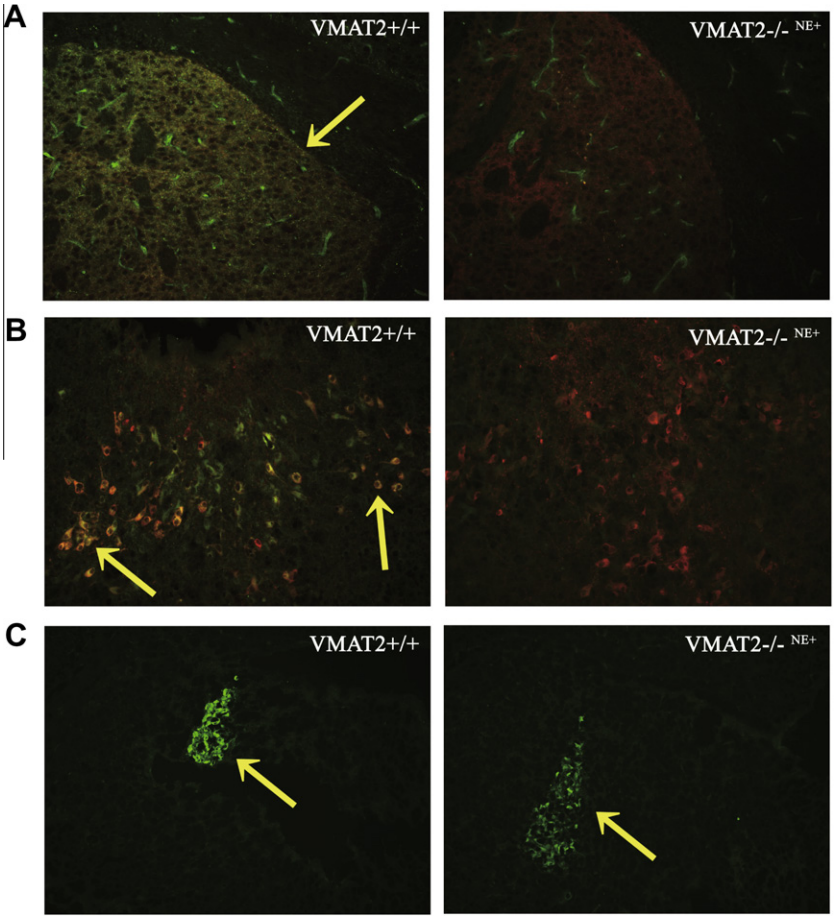

Fig. 2. Immunohistochemistry of transgenic mice. Left: VMAT2+/+ mice Right: VMAT2−/−$^{NE+}$ mice. (A) Striatum area double staining (×100). Yellow arrow indicates expression of both DAT and VMAT2. (B) Raphé nucleus area double staining (×200). Yellow arrows indicate expression of both TPH and VMAT2. (C) Locus coeruleus area (×100). Yellow arrows indicate expression of VMAT2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and VMAT2 labeling (green) overlapped, and appear as yellow labeling in the figure (yellow arrow). However, in VMAT2−/−$^{NE+}$ mice (right), which lacked any VMAT2 labeling (green), only DAT labeling (red) was observed in the striatum. This indicates that VMAT2+/+ mice express VMAT2 in striatal dopaminergic afferents, but VMAT2−/−$^{NE+}$ mice do not express VMAT2 in these neurons. VMAT2 protein expression was also evaluated in serotonergic neurons in the raphé nucleus labeled by anti-tryptophan hydroxylase polyclonal antibody (Fig. 2B). In VMAT2+/+ mice (left), TPH labeling (red) and VMAT2 labeling (green) overlapped, and appear as yellow labeling in the figure (yellow arrows). By contrast, in VMAT2−/−$^{NE+}$ mice (right), which lacked VMAT2 labeling (green), only TPH staining (red) was observable in the raphé nucleus. Thus, whereas VMAT2 co-localization with TPH was observed in serotonergic neurons of VMAT2+/+ mice, it was not detected in VMAT2−/−$^{NE+}$ mice. As expected, a different pattern was observed in the locus coeruleus (Fig. 2C). In noradrenaline-containing neurons of the locus coeruleus of both VMAT2+/+ (left) and VMAT2−/−$^{NE+}$ mice (right), VMAT2 labeling (green) was observed (yellow arrows). Therefore, in confirmation of what was expected to result from the DBH promoter controlling VMAT2 expression of the transgene, VMAT2−/−$^{NE+}$ mice express VMAT2 in noradrenergic neurons, but not in dopaminergic or serotonergic neurons.

3.4. Footprint pattern of VMAT2−/−$^{NE+}$ mice

VMAT2−/−$^{NE+}$ mice with C3H mixed genetic background survived more than 2 weeks, hence, we carried out a behavioral examinations in these mice. Both VMAT2+/+ and VMAT2−/−$^{NE+}$ mice showed frequent overlapping of the front feet (dark red) and the rear feet (dark blue), demonstrating regular gait patterns (Fig. 3A and B). The average stride length (recorded between sequential placements of the same foot, ± SEM) differed vastly between VMAT2+/+ (4.59 ± 0.08 cm) and VMAT2−/−$^{NE+}$ mice (2.45 ± 0.05 cm) (Fig. 3C). The difference in stride length seems to reflect the difference in their body sizes (VMAT2+/+ (8.8 ± 0.42 g bodyweight) vs. VMAT2−/−$^{NE+}$ mice (4.6 ± 0.31 g bodyweight), Fig. 1B and C). These results indicate that VMAT2−/−$^{NE+}$ mice normally develop walking ability, without showing any gross neurological symptoms such as ataxia.

3.5. Locomotor activity of VMAT2−/−$^{NE+}$ mice

Figs. 4A and B show 30 min-open field movement patterns for the two genotypes: VMAT2+/+ and VMAT2−/−$^{NE+}$. The movement of VMAT2+/+ mice took place primarily on the edge of the apparatus, with extensive exploration of the corners, although the mice occasionally made forays into the central area of the apparatus. Longitudinal movements were interrupted by stops, changes in direction, and rearing and turning of the head (Fig. 4A). In contrast to the pattern observed in VMAT2+/+ mice, VMAT2−/−$^{NE+}$ mice scarcely moved during the initial 30 min observation period, and the mice showed little evidence of the typical exploratory pattern in their behavior (Fig. 4B). The total distance moved during the test for VMAT2−/−$^{NE+}$ mice (1.75 ± 1.06 m) was less than 5% of the total distance observed in VMAT2+/+ mice (45.1 ± 12.3 m) (Fig. 4C).

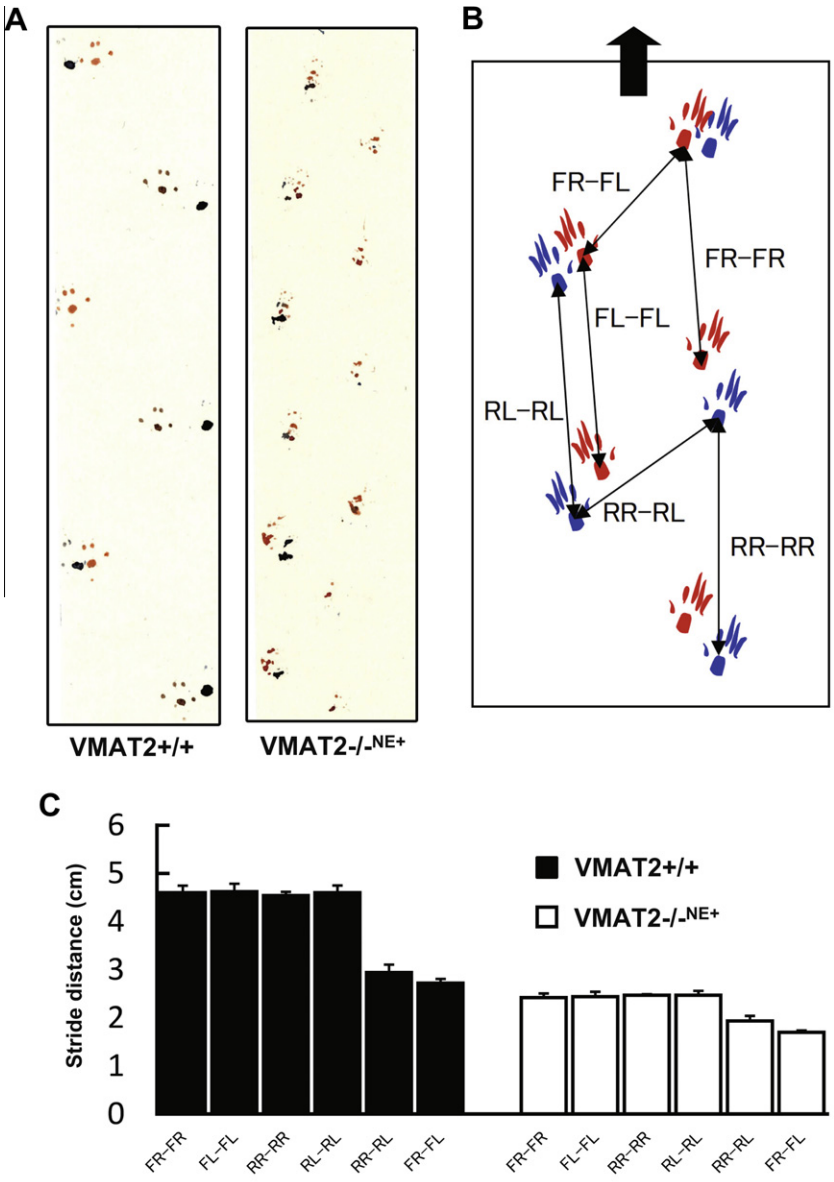

Fig. 3. Footprint analysis of transgenic mice. (A) Footprint records of a VMAT2+/+ mouse (left) and a VMAT2−/−$^{NE+}$ mouse (right). Front foot prints are dark red, and rear foot prints are dark blue. (B) Sample analysis of footprint patterns: front foot prints are red, and rear foot prints are blue; "FR"–right forefoot, "FL"–left forefoot, "RR"–right rear foot, "RL"–left rear foot. (C) Average stride distance, VMAT2+/+ versus VMAT2−/−$^{NE+}$ mice. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

VMAT2 is expressed not only in the central nervous system, but also in postganglionic sympathetic neurons [2,11]. Reduced VMAT2 expression in either the central nervous system or the peripheral sympathetic nervous system in heterozygous mice could contribute to the prolonged QT intervals observed in the mice [10], and these VMAT2 deficits in noradrenergic neurons may contribute to the early death observed in VMAT2−/− mice [4]. In order to rescue the function of sympathetic neurons in VMAT2−/− mice, we generated VMAT2−/− mice that expressed a VMAT2 transgene selectively in noradrenergic neurons. The recovery of VMAT2 expression in noradrenergic neurons extended the lifespan of VMAT2−/− mice up to 2–3 weeks, although the body weight of these mice was about half of that observed in VMAT2+/+ mice. These results may indicate that VMAT2 expression in noradrenergic systems could be essential for the first two weeks of life, and VMAT2 functions in other monoaminergic systems become important for survival after 2–3 weeks of age.

Neither direct nor indirect enhancements of dopaminergic neurotransmission by apomorphine or methamphetamine administration affected average lifespan in VMAT2−/−$^{NE+}$ mice. Therefore, previous results using amphetamine to increase viability up to about 14 days in VMAT2−/− mice [16] is likely to have resulted from enhancement of noradrenergic neurotransmission. In addition, lethality of VMAT2−/−$^{NE+}$ mice after 2–3 weeks could not be counteracted merely by the enhancement of dopaminergic neurotransmission.

VMAT2−/−$^{NE+}$ mice crossbred with the C3H mouse strain, lived 4.5 days longer than the original C57/BL6 strains, while crossbreeding to other strains showed no effect on average lifespan. Since C3H mice have higher levels of the dopamine D1 receptor and tyrosine hydroxylase expression compared with C57BL6/J mice [17], differences in dopaminergic neurotransmission in

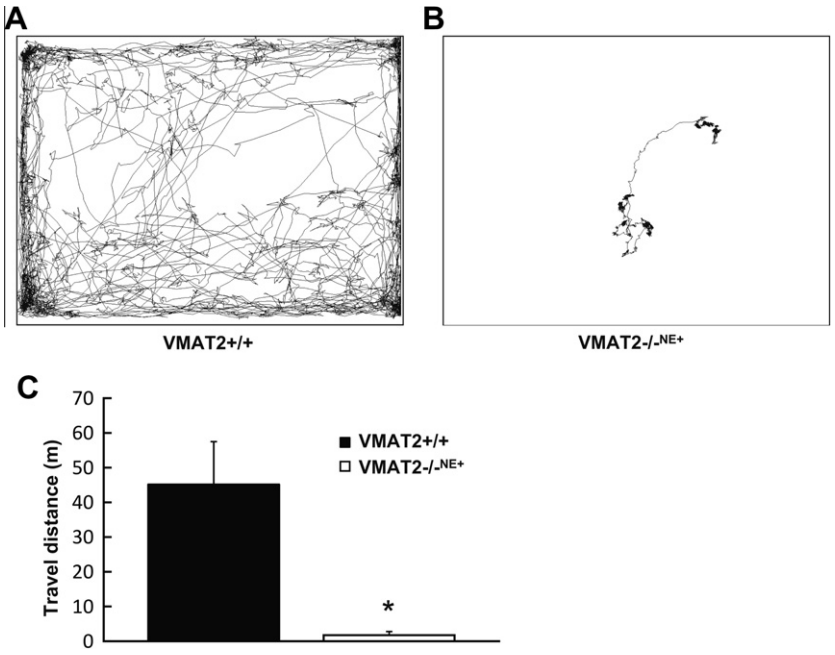

Fig. 4. Patterns of locomotor activity in open-field testing in (A) VMAT2+/+ and (B) VMAT2−/−$^{NE+}$ mice. The average distance traveled is represented in (C) for each genotype.

VMAT2−/−$^{NE+}$ mice with C3H mixed genetic background might underlie the elongation of lifespan observed in the mice.

VMAT2−/− mice show severe growth deficiency in embryonic and neonatal periods of development, and body weight of VMAT2−/− mice is significantly less than VMAT2+/+ mice [7]. Body size of VMAT2−/−$^{NE+}$ mice was also smaller than that of VMAT2+/+ mice at birth, and their body weight was about half of that of VMAT2+/+ mice at 2 weeks of age. Since VMAT2 is expressed in endocrine cells in the digestive system, such as stomach, intestine and pancreas as well as the central nervous system [18–20], deletion of VMAT2 might very well affect digestive functions, and cause lower body weight in VMAT2−/−$^{NE+}$ mice. However, forced feeding of predigested food or glucose had no significant effects on growth or average lifespan in VMAT2−/−$^{NE+}$ mice (data not shown). These data suggest that VMAT2 expression in monoaminergic systems has important roles in growth, and the involvement of VMAT2 function in growth may be independent of feeding function, at least in this study. One possibility may be affects upon pituitary development as DAT KO mice exhibit a pituitary hypoplasia that accounts for reduced size of DAT KO mice [21], however, 2 mg/kg growth hormone treatment had no significant effects on growth or average lifespan of VMAT2−/−$^{NE+}$ mice (data not shown).

The extension of the lifespan in VMAT2−/−$^{NE+}$ mice enabled behavioral evaluation of the effects of VMAT2 deficiency in other monoaminergic neurons (non-noradrenergic neurons) on behavior. Although VMAT2−/−$^{NE+}$ mice showed normal foot print patterns, open field testing revealed that VMAT2−/−$^{NE+}$ pronounced akinesia, with little overall movement and an almost complete elimination of the normal exploratory pattern exhibited in a novel environment, as was observed in VMAT+/+ mice. Decreases in exploratory activity in VMAT2−/−$^{NE+}$ mice may reflect decreased responses to the activating effects of novel stimuli and behavioral activation more generally, given that motor function itself was not profoundly altered. Evidence indicates that dopaminergic neurotransmission is involved in the behavioral activation associated with normal exploratory behavior [22,23], suggesting that the decreased exploration of VMAT2−/−$^{NE+}$ mice may be of the result of reduced VMAT2 expression in dopaminergic neurons, and consequently large reductions in dopamine storage and release [7]. Profound alterations in several aspects of dopaminergic dynamics have been previously reported in VMAT2−/− mice [16] and VMAT transgenics with profound (95%) reductions in VMAT2 expression [24].

In conclusion, the lifespan of VMAT2−/− mice was elongated up to 2–3 weeks postnatal by inducing human VMAT2 transgene expression specifically in noradrenergic neurons. The data suggest that VMAT2 expression in noradrenergic neurons has crucial functions for early neonatal survival, but survival beyond this point must involve impairments of other monoaminergic systems. Nonetheless, the survival of VMAT2−/−$^{NE+}$ mice for even this period of time may provide a valuable animal model to study VMAT2 functions with pharmacological, behavioral and pathophysiological approaches that were not previously possible in VMAT2−/− mice.

Acknowledgments

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