Neuropharmacology 76 (2014) 146–155

Contents lists available at ScienceDirect

Neuropharmacology

journal homepage: www.elsevier.com/locate/neuropharm

Decreased vesicular monoamine transporter 2 (VMAT2) and dopamine transporter (DAT) function in knockout mice affects aging of dopaminergic systems

F.S. Hall [a,*], K. Itokawa [b], A. Schmitt [c], R. Moessner [c], I. Sora [d], K.P. Lesch [c], G.R. Uhl [a]

[a] Molecular Neurobiology Branch, Intramural Research Program, NIDA, NIH/DHHS, Baltimore, MD 21224, USA
[b] Department of Neurology, Saitama Medical School, Saitama, Japan
[c] Department of Psychiatry, University of Würzburg, Germany
[d] Tohoku University Graduate School of Medicine, Department of Neuroscience, Division of Psychobiology, Sendai, Japan

ARTICLE INFO

Article history:
Received 1 May 2013
Received in revised form
22 July 2013
Accepted 30 July 2013

Keywords:
Transgenic mice
Gene knockout
Dopamine
Aging
Amphetamine
Cocaine

ABSTRACT

Dopamine (DA) is accumulated and compartmentalized by the dopamine transporter (DAT; SLC3A6) and the vesicular monoamine transporter 2 (VMAT2; SLC18A2). These transporters work at the plasma and vesicular membranes of dopaminergic neurons, respectively, and thus regulate levels of DA in neuronal compartments that include the extravesicular cytoplasmic compartment. DA in this compartment has been hypothesized to contribute to oxidative damage that can reduce the function of dopaminergic neurons in aging brains and may contribute to reductions in dopaminergic neurochemical markers, locomotor behavior and responses to dopaminergic drugs that are found in aged animals. The studies reported here examined aged mice with heterozygous deletions of VMAT2 or of DAT, which each reduce transporter expression to about 50% of levels found in wild-type (WT) mice. Aged mice displayed reduced locomotor responses under a variety of circumstances, including in response to locomotor stimulants, as well as changes in monoamine levels and metabolites in a regionally dependent manner. Several effects of aging were more pronounced in heterozygous VMAT2 knockout (KO) mice, including aging induced reductions in locomotion and reduced locomotor responses to cocaine. By contrast, some effects of aging were reduced or not observed in heterozygous DAT KO mice. These findings support the idea that altered DAT and VMAT2 expression affect age-related changes in dopaminergic function. These effects are most likely mediated by alterations in DA compartmentalization, and might be hypothesized to be exacerbated by other factors that affect the metabolism of cytosolic DA.

This article is part of the Special Issue entitled 'The Synaptic Basis of Neurodegenerative Disorders'.

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1. Introduction

The neurotransmitter dopamine (DA) is located in neurotransmitter vesicles, extracellular spaces, and extravesicular cytoplasmic compartments. In the extravesicular cytoplasmic compartments of dopaminergic neurons, DA can produce free radicals and/or form adducts with important cellular proteins that can contribute to cellular stress and damage (Uhl, 1998). Extravesicular cytoplasmic DA concentrations are regulated by the serial actions of two transporters: the plasma membrane dopamine transporter (DAT), which mediates neuronal uptake of DA from extracellular spaces into this compartment (Giros et al., 1992; Kilty et al., 1991; Nirenberg et al., 1996b; Shimada et al., 1991; Usdin et al., 1991), and the vesicular monoamine transporter 2 (VMAT2), which translocates DA from this extravesicular cytoplasmic compartment into synaptic vesicles (Erickson et al., 1992; Gonzalez et al., 1994; Liu et al., 1994, 1996, 1992; Merickel et al., 1995; Nirenberg et al., 1996a; Peter et al., 1996, 1995; Roghani et al., 1996; Surratt et al., 1993; Takahashi and Uhl, 1997). Amphetamine-like compounds block VMAT2-mediated transport of DA into synaptic vesicles, elevating extravesicular cytoplasmic DA levels and releasing DA via mechanisms that include DAT-mediated reverse transport. Elevated intracellular levels of DA may contribute to the toxic effects of amphetamine derivatives on DA neurons (Larsen et al., 2002; Lotharius and O'Malley, 2001; Seiden and Ricaurte, 1987).

We and others have cloned the genes that encode these transporters (Giros et al., 1992; Kilty et al., 1991; Shimada et al., 1991;

* Corresponding author. Molecular Neurobiology Branch, NIDA-IRP, 333 Cassell Drive, Baltimore, MD 21224, USA. Tel.: +1 443 740 2796; fax: +1 443 740 2122.
E-mail address: shall@intra.nida.nih.gov (F.S. Hall).

0028-3908/$ – see front matter Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.neuropharm.2013.07.031

Usdin et al., 1991) and produced KO mice with reduced levels of DAT and VMAT2 expression (Donovan et al., 1999; Fon et al., 1997; Giros et al., 1996; Mooslehner et al., 2001; Sora et al., 1998; Takahashi et al., 1997; Wang et al., 1997). Homozygous VMAT2 KO mice die in the first few postnatal days (Takahashi et al., 1997), but heterozygous +/− mice are viable, express about one-half WT levels of VMAT2 mRNA and protein, display relatively modest changes in brain monoamine levels and in a number of motor and behavioral tests (Takahashi et al., 1997). Homozygous DAT KO mice are small and display striking hyperlocomotion (Giros et al., 1996; Sora et al., 1998). However, heterozygous DAT KO mice, expressing one-half of WT levels of DAT mRNA and protein, display weight and locomotion that are nearly identical to those found in WT mice.

Heterozygous mice with one-half of WT levels of VMAT2 and DAT mRNA and protein expression also display differential sensitivities to the dopaminergic toxin MPTP. Dopaminergic toxicity induced by MPTP is profoundly enhanced in VMAT2 +/− mice (Takahashi et al., 1997) and reduced in DAT +/− mice (Fumagalli et al., 1998; Gainetdinov et al., 1997; Takahashi et al., 1997). When DAT is over-expressed by about one-third in transgenic mice, MPTP toxicity is enhanced (Donovan et al., 1999). These data suggest that DAT and VMAT2 expression influences DA compartmentalization of MPP+, the active metabolite of MPTP, which greatly affects the toxicity of this compound (Javitch et al., 1985, 1984). These results also validate these KO strains as reasonable *in vivo* model systems for dopaminergic neuronal toxicity that might depend on mechanisms that regulate DA compartmentalization.

Dopaminergic neurons are especially vulnerable to the effects of aging, as manifested in biochemical, behavioral and pharmacological assessments of many mammalian species (see Morgan and Finch, 1988, for review). DA compartmentalization has been suggested to be an important mediator of the potential toxicity of DA involved in these functional and neurochemical impairments (Hastings et al., 1996a; Lotharius and Brundin, 2002; Rabinovic et al., 2000; Uhl, 1998). Indeed, a human α-synuclein mutant cell line with reduced VMAT2 expression has been shown to have higher cytoplasmic DA and superoxide levels (Lotharius et al., 2002). Mice with reduced levels of expression of DAT and VMAT2 are thus especially good candidates to display altered availability of DA in the extravesicular cytoplasmic neuronal compartments in which oxidation and/or adduct formation could contribute to aging effects on dopaminergic systems. The alterations in transporter expression found in heterozygous KO mice (Sora et al., 1998; Takahashi et al., 1997) lie near the reported ranges of human individual variation in levels of expression of these proteins (Wilson et al., 1996a, 1996b). These mice thus provide models for the effects of common human allelic variations on aging of dopaminergic brain systems. We now report assessments of DA-related behavioral and neurochemical function in aged DAT +/− and VMAT2 +/− mice in comparison to WT littermates. These studies provide data that is consistent with the hypothesis that age-related declines in DA function, and associated behavioral responses, are affected by the levels of expression of genes that influence the cellular compartmentalization of DA.

2. Materials and methods

2.1. Animals

DAT +/− and VMAT2 +/− KO mice and WT littermates were bred from heterozygote–heterozygote crosses of KO mice from mixed C57BL6J-129Sv genetic backgrounds (Sora et al., 1998; Takahashi et al., 1997). Genotypes were confirmed by PCR as previously described in those publications. Mice were group-housed in standard ventilated housing chambers at 24 °C in 50% relative humidity on a 12/12 h light/dark cycle with lights on at 7:00 A.M. and off at 7:00 P.M., with *ad libitum* access to standard mouse chow and water. All experiments were conducted in accordance with American Association for Laboratory Animal Care and all applicable NIH guidelines. Separate groups of mice were examined from 3 to 6 months of age (young) and from 18 to 24 months of age (old) for each experiment. Each study compared heterozygote KO mice to WT littermates.

2.2. Behavioral testing procedures

Locomotor activity was assessed as total distance traveled when mice were placed individually in 46 × 25 × 19 cm clear plastic cages in Optovarimax activity monitors (Columbus Instruments, Columbus, OH), under dark, sound-attenuated conditions. Young (3–5 months) and aged (18–24 months) DAT KO and VMAT KO mice, and WT littermates, were tested for locomotor activity twice. In the first session, mice were habituated to the apparatus for a period of time, 1 h for VMAT2 KO mice ($N = 54–87$/group) and 3 h for DAT KO mice ($N = 30–68$/group), injected with saline and locomotor activity was measured for two more hours. At least 48 h after this initial test session mice were tested again, but divided into separate drug testing groups. In the second session mice were placed in the apparatus to habituate for the same period of time as the first session (1 h for VMAT2 KO mice and 3 h for DAT KO mice), and then injected with amphetamine or cocaine. For drug testing a between-subjects design was used so that each mouse was tested under only one drug (data was combined for the analysis of locomotion after the initial saline injections). Two doses of amphetamine were tested in VMAT2 KO mice (0.5 mg/kg IP; $N = 13–27$/group; or 1 mg/kg IP; $N = 40–71$/group) and one dose of cocaine (20 mg/kg SC; $N = 19–21$/group). DAT KO mice were injected with amphetamine (1 mg/kg IP; $N = 14–18$/group) or cocaine (10 mg/kg SC; $N = 16–52$/group). After injections locomotor activity was monitored for 2 more hours.

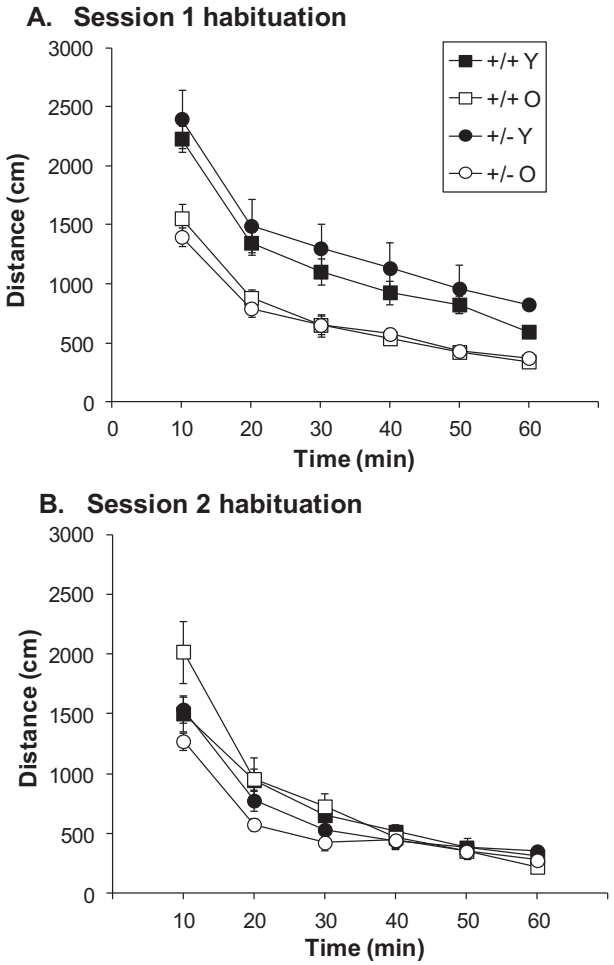

Fig. 1. Effects of aging on the time-course (10 min time bins) of locomotor activity in VMAT2 +/+ and VMAT +/− mice ($N = 54–87$/group) during (A) initial locomotor habituation (novel, session 1) prior to saline injection, and (B) subsequent locomotor habituation (familiar, session 2) prior to drug injection. Data are expressed as mean ± the standard error of the mean (SEM).

2.3. Neurochemical procedures

Tissue levels of monoamines were assessed in old (18–24 months) and young (3–5 months) VMAT2 KO mice ($N = 5–6$/genotype) and DAT KO mice ($N = 9–10$/genotype). Experimentally naïve mice were killed by cervical dislocation, brains were rapidly removed, tissue samples were dissected, frozen in isopentane on dry ice and stored at −70 °C for later analysis. Brains were dissected on an ice-cold plate and tissue samples from the ventral striatum (VS), dorsal striatum (DS) and ventral midbrain (MB), including the substantia nigra and ventral tegmental area, were rapidly dissected and frozen. Prior to analysis by HPLC, tissue samples were homogenized in trichloroacetic acid. Levels of DA, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) were analyzed by HPLC using electrochemical detection at +0.3 V using 10 × 4.6 mm Spherisorb 3 mm ODS-2 reversed-phase chromatography columns (Thomson Instruments, Springfield, VA) with mobile phase consisting of 0.1 M monochloroacetic acid, 8% acetonitrile, 0.5 g/L octanesulfonic acid, 0.3% triethylamine and 10 mM EDTA. Monoamines and metabolites were quantitated relative to standard injections of known amounts and tissue levels calculated as ng per mg wet tissue weight.

2.4. Statistical analyses

Statistical comparisons were made using Statview (SAS Institute). Behavioral and neurochemical data were analyzed by analyses of variance (ANOVA) with the between subjects factors of GENOTYPE (+/+ vs. +/−) and AGE (old vs. young), and the within subjects factor of TIME (10 min time bins) where appropriate. In some cases, where significant effects were demonstrated in overall ANOVA, separate ANOVA were performed on individual groups as *post hoc* comparisons to determine which groups contributed to the significance of overall ANOVA results or by Scheffe's *post hoc* comparisons. Data were analyzed as time-course rather than as summed activity because, in most cases, the effects of GENOTYPE or AGE interacted significantly with TIME. Habituation data obtained prior to saline or drug injections was combined for all subjects of each genotype, and the effects of drug administration analyzed separately.

3. Results

3.1. VMAT2 KO mice

Locomotor data from old and young, VMAT +/+ and VMAT +/−, mice for the two habituation sessions are presented in Fig. 1 to facilitate comparisons. Data for locomotion after administration of saline or psychostimulants are represented in Fig. 2.

Old VMAT +/+ and VMAT2 +/− mice were less active in a novel environment than young VMAT2 mice (Fig. 1A; $F[1,295] = 21.5$, $p < 0.0001$). The effects of age were greater at the beginning of test sessions, resulting in a significant AGE × TIME interaction ($F[5,1475] = 7.7$, $p < 0.0001$). Initial differences in the locomotor behavior of old versus young mice in a novel environment were

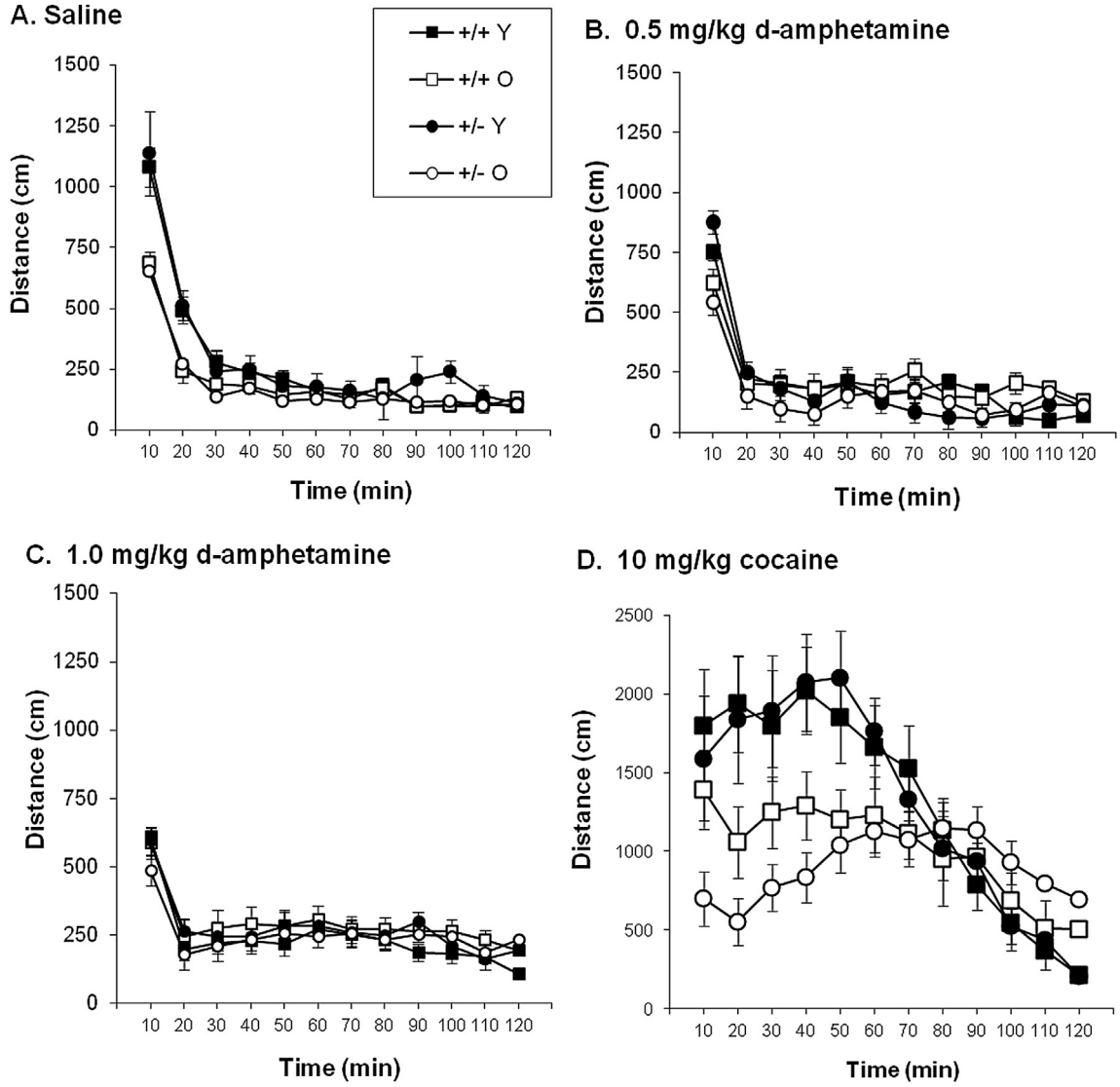

Fig. 2. Effects of aging in VMAT2 +/+ and VMAT +/− mice on the time-course of locomotor responses after injection with (A) saline ($N = 54–87$/group), (B) 0.5 mg/kg D-amphetamine IP ($N = 13–27$/group), (C) 1.0 mg/kg D-amphetamine IP ($N = 40–71$/group), and (D) 10 mg/kg cocaine SC ($N = 19–21$/group). Data are expressed as mean ± SEM.

attenuated by habituation between sessions. Thus, when the mice were tested for a second time, neither the effect of AGE (F[1,279] = 0.8, ns) nor the AGE × TIME interaction (F[5,1395] = 1.0, ns) was significant overall. However, during locomotor habituation in a familiar environment prior to drug testing (e.g. session 2), old VMAT2 +/− mice exhibited less locomotor activity at the beginning of the test sessions than mice in the other groups. There was thus a significant AGE × GENOTYPE × TIME interaction (Fig. 1B; F[5,1395] = 3.9, $p < 0.002$). When old mice were compared in a separate ANOVA, the old VMAT2 +/+ mice had greater activity than old VMAT2 +/− mice (F[5,805] = 5.4, $p < 0.0001$). When young mice were compared in a separate ANOVA, there was no difference between young VMAT2 +/+ and young VMAT2 +/− mice (F[5,590] = 0.45, ns).

Saline injection produced small increases in locomotion, above the level observed at the end of the habituation session (Fig. 1A), that returned to baseline values within 10 min. Activity after saline injections was greater in young than in old animals of both genotypes (Fig. 2A; AGE × TIME, F[11,3245] = 8.1, $p < 0.0001$), consistent with those values at the end of the habituation session. Individual ANOVA for each genotype revealed significant effects of AGE in VMAT2 +/+ mice (F[11,1738] = 5.8, $p < 0.0001$) and in VMAT2 +/− mice (F[11,1507] = 3.2, $p < 0.0003$).

Injection of 0.5 mg/kg D-amphetamine produced modest locomotor stimulation (Fig. 2B), over the values at the end of the habituation period (session 2, Fig. 1B), that were slightly greater than those observed after saline administration. Note that all groups had habituated to the same level of activity by the end of this period. After the amphetamine injection, the most pronounced increase was noted during the first time-bin. Overall, this effect was greater in young than in old mice, resulting in a significant AGE × TIME interaction (F[11,924] = 3.5, $p < 0.0001$). *Post hoc* tests revealed significant results only for young vs. old VMAT2 +/− mice (AGE × TIME: F[11,363] = 2.5, $p < 0.005$); there was no significant effect of age in VMAT2 +/+ mice (AGE × TIME: F[11,561] = 1.7, ns). Aging thus produced greater effects on the locomotor response to an injection of a low dose of amphetamine in the time bin immediately following the injection, a finding similar to that observed after saline injections. By contrast, there were no significant differences in the locomotor response in VMAT2 +/− mice during the first (or subsequent) time bins after injection of 1.0 mg/kg D-amphetamine that resulted from either GENOTYPE or AGING (Fig. 2C). Injections of 10 mg/kg cocaine produced substantial increases in locomotion (Fig. 2D) over those observed at the end of the habituation period (session 2, Fig. 1B). Cocaine-induced locomotion was lower in aged than in young mice during the first half of the testing period (AGE × TIME: F[11,814] = 11.9, $p < 0.0001$). Aged VMAT2 +/− mice displayed less locomotion after cocaine injections than mice in any of the other three groups. Thus, the age-related differences in cocaine-induced locomotion were greater in VMAT2 +/− mice than in VMAT2 +/+ mice. *Post hoc* ANOVA revealed significant AGE × TIME effects for both VMAT2 +/+ (F[11,418] = 3.3, $p < 0.0003$) and VMAT2 +/− (F[11,396] = 9.4, $p < 0.0001$) mice, but the effects were obviously greater in VMAT2 +/− mice. There were no GENOTYPE × TIME effects in young mice (F[11,396] = 0.3, ns). By contrast, old VMAT2 +/− mice displayed significantly less activity, especially in the time intervals assessed just after treatments, when compared to old VMAT2 +/+ mice (F[11,418] = 4.6, $p < 0.0001$).

3.2. DAT KO mice

Locomotor data from old and young, DAT +/+ and DAT +/−, mice for the two habituation sessions are presented in Fig. 3 to facilitate comparisons. Data for locomotion after administration of saline or psychostimulants is represented in Fig. 4.

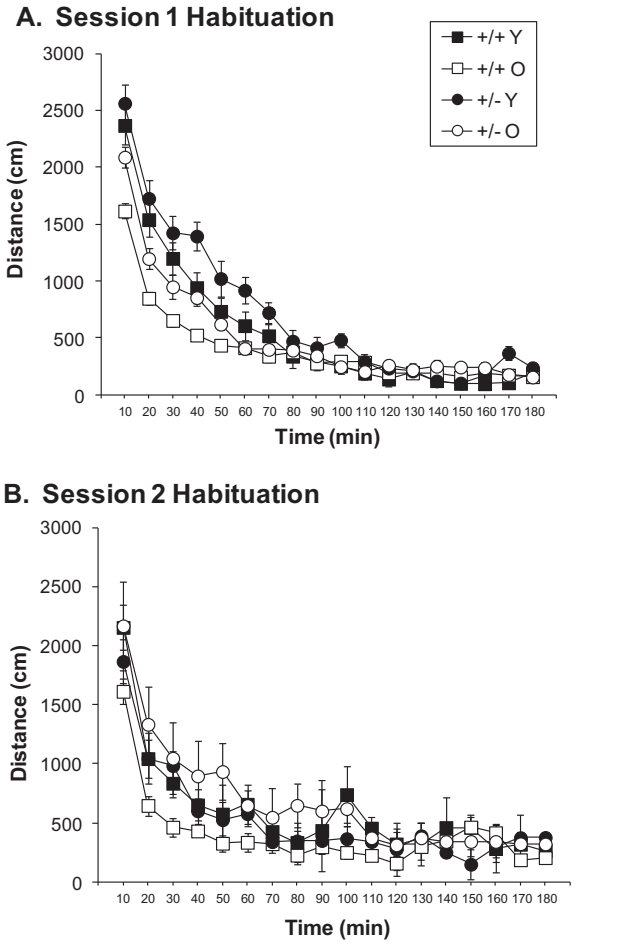

Fig. 3. Effects of aging in DAT +/+ and DAT +/− mice (N = 30–68/group) on the time-course of locomotor activity during (A) initial locomotor habituation (novel, session 1) prior to saline injection, and (B) subsequent locomotor habituation (familiar, session 2) prior to drug injection. Data are expressed as mean ± SEM.

Old DAT mice of both genotypes displayed less activity in a novel environment than younger mice (Fig. 3A; AGING, F[1,191] = 12.6, $p < 0.001$). As locomotor activity habituated over the test period, differences in activity between old and young mice were attenuated (AGE × TIME: F[17,3247] = 15.0, $p < 0.0001$). DAT +/− mice displayed slightly more activity than DAT +/+ mice at the beginning of the test session, but not subsequently (GENOTYPE × TIME: F[17,3247] = 3.2, $p < 0.0001$). There were no significant differences between the activity of young DAT +/+ and DAT +/− mice (F[17,1173] = 0.6, ns), but old DAT +/− mice were significantly more active than old DAT +/+ mice (F[17,2227] = 2.6, $p < 0.0004$). Indeed, the activity levels displayed by old DAT +/− mice were not different from those observed for young DAT +/+ mice.

As observed in VMAT2 +/+ and +/− mice, habituation to an initially novel test environment eliminated many of the differences observed between the young and old DAT +/− groups (e.g. habituation session 2; Fig. 3B). Thus, during the second habituation period, prior to drug injection in DAT +/+ and DAT +/− mice, there were no significant main effects of AGE (F[1,200] = 0.1, ns) or GENOTYPE (F[1,200] = 0.5, ns), nor a significant AGE × GENOTYPE interaction (F[1,200] = 1.4, ns). There were no significant interactions between any of these factors and TIME either. There was a slight trend for old DAT +/− mice to be more active than old DAT +/+ mice, but this difference was not statistically significant.

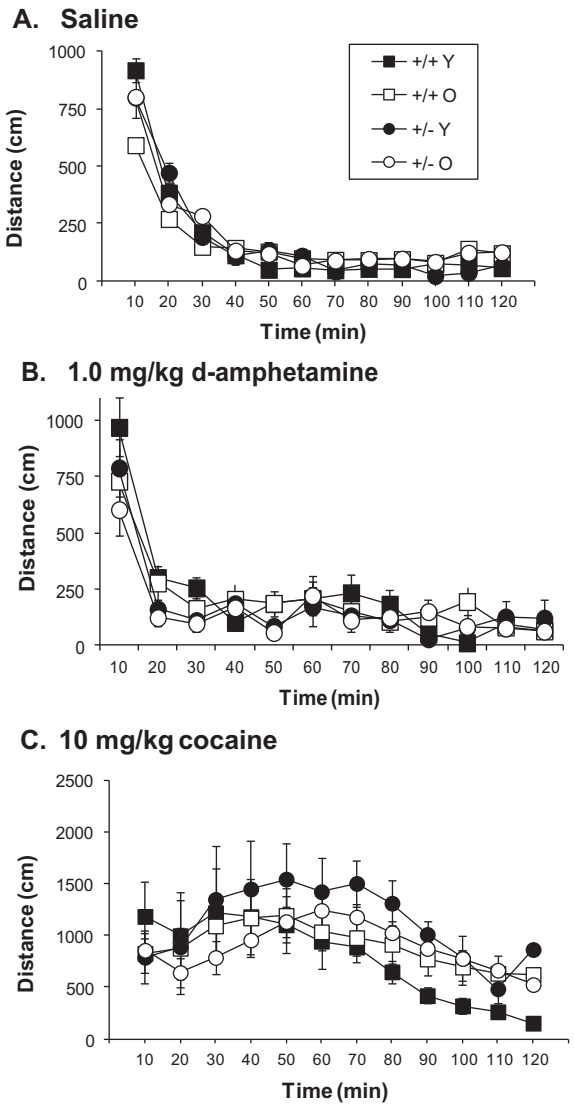

Fig. 4. Effects of aging in DAT +/+ and DAT +/− mice on the time-course of locomotor activity after injection with (A) saline (N = 30–68/group), (B) 1.0 mg/kg D-amphetamine IP (N = 14–18/group) or (C) 10 mg/kg cocaine SC (N = 16–52/group) mice. Data are expressed as mean ± SEM.

Saline injections in DAT +/+ and DAT +/− mice produced transient increases in locomotion (Fig. 4A), above those observed at the end of habituation session 2 (Fig. 3A), that returned to baseline values within 20 min. Old mice displayed reduced responses to saline injection during this initial period (AGE × TIME: $F[11,2101] = 4.0$, $p < 0.0001$). This difference between old and young mice was also affected by genotype. This effect was attenuated in old DAT +/− mice compared to old DAT +/+ mice, yielding a significant AGE × GENOTYPE × TIME interaction ($F[11,2101] = 2.5$, $p < 0.004$). Thus, while old DAT +/+ mice were less active than young DAT +/+ mice at the beginning of the test session, this was not the case in DAT +/− mice. *Post hoc* ANOVA demonstrated a significant AGE × TIME effect in DAT +/+ mice ($F[11,1133] = 7.4$, $p < 0.0001$), but not in DAT +/− mice ($F[11,968] = 1.1$, ns).

Treatment with 1.0 mg/kg D-amphetamine produced modest elevations in locomotor activity (Fig. 4B), above that observed at the end of the second habituation session (Fig. 3B). These increases were slightly greater than those observed after saline injections. These responses to amphetamine injection were smaller in old mice than in young mice (AGE × TIME: $F[11,638] = 2.1$, $p < 0.03$). The effects were again largely limited to the first time bins and thus likely to involve contributions from injection stress. There were no significant effects of GENOTYPE ($F[1,58] = 0.4$, ns) or AGE × GENOTYPE ($F[1,58] = 0.1$, ns) on locomotor responses to amphetamine, nor any interaction of these factors with TIME in DAT mice.

Cocaine produced marked elevations in locomotor activity in all groups of DAT mice (Fig. 4C). There were significant effects of both AGE and GENOTYPE that were dependent on TIME. Locomotor responses to cocaine were thus greater in young mice at the beginning of the test sessions, but approaching basal levels by the end of the test sessions (AGE × TIME: $F[11,1397] = 2.8$, $p < 0.002$), although this was primarily because of reduced levels of locomotion in young DAT +/+ mice compared to the other groups. In young DAT +/+ mice, peak cocaine locomotor responses diminished more quickly with time than those in old DAT +/+ mice, or indeed more quickly than all other groups. Locomotor activity in young DAT +/− mice was elevated compared to young DAT +/+ mice, but locomotor activity in old DAT +/+ and old DAT +/− mice was not substantially different. Overall, locomotion in DAT +/− mice was elevated in the second half of the test session in comparison to DAT +/+ mice, but this effect was entirely produced by low levels of activity in young DAT +/+ mice. This resulted in a significant GENOTYPE × TIME interaction ($F[11,1397] = 2.0$, $p < 0.03$). The AGE × GENOTYPE × TIME interaction was not significant.

3.3. Tissue monoamine levels in heterozygous VMAT2 KO mice

Possible neurochemical correlates of these age-related locomotor effects in VMAT2 +/− mice were examined by assessing levels of DA, 5-HT and their metabolites in ventral striatal, dorsal striatal and midbrain samples dissected from young and old

Table 1
Tissue levels of DA, 5-HT, DOPAC, HVA and 5-HIAA (ng/g wet tissue weight) in the ventral striatum, dorsal striatum and ventral midbrain of young and old, VMAT +/+ and VMAT +/− mice (N = 5–6/group) as determined by HPLC-EC. *Old vs. young, within genotype Scheffe's *post hoc* comparison, $p < 0.05$.

	Genotype	Age	DA	DOPAC	HVA	5-HT	5-HIAA
Ventral striatum	VMAT +/+	Young	4.71 ± 0.9	1.46 ± 0.1	0.740 ± 0.08	0.583 ± 0.11	1.10 ± 0.2
		Old	3.37 ± 0.5	1.72 ± 0.2	0.736 ± 0.06	0.844 ± 0.10	1.21 ± 0.1
	VMAT +/−	Young	5.18 ± 1.1*	1.74 ± 0.4	0.942 ± 0.23	0.937 ± 0.19*	1.56 ± 0.3*
		Old	2.25 ± 0.3	1.12 ± 0.1	0.534 ± 0.05	0.621 ± 0.06	0.91 ± 0.2
Dorsal striatum	VMAT +/+	Young	9.25 ± 1.7	1.72 ± 0.2	0.905 ± 0.1	0.725 ± 0.09	1.38 ± 0.03
		Old	10.28 ± 0.9	1.47 ± 0.1	0.910 ± 0.1	0.706 ± 0.08	1.56 ± 0.2
	VMAT +/−	Young	7.82 ± 1.3	1.71 ± 0.2	1.188 ± 0.1	0.770 ± 0.03	1.17 ± 0.1
		Old	7.58 ± 0.5	1.16 ± 0.1	1.018 ± 0.1	1.049 ± 0.06	1.37 ± 0.1
Ventral midbrain	VMAT +/+	Young	0.280 ± 0.10	0.184 ± 0.04	0.234 ± 0.05	1.26 ± 0.1	1.66 ± 0.3
		Old	0.416 ± 0.05	0.302 ± 0.04	0.314 ± 0.01	1.50 ± 0.1	2.07 ± 0.1
	VMAT +/−	Young	0.398 ± 0.05	0.272 ± 0.04	0.330 ± 0.04	1.41 ± 0.1	1.86 ± 0.2
		Old	0.310 ± 0.05	0.228 ± 0.03	0.334 ± 0.03	1.48 ± 0.1	1.77 ± 0.2

VMAT2 +/− and +/+ mice (Table 1). Changes in neurochemical markers in the ventral striatum provide the closest parallels with the behavioral data. VMAT2 +/+ mice displayed trends toward age-related reductions in ventral striatal DA concentrations that were exacerbated in VMAT2 +/− mice. ANOVA revealed a significant overall effect of AGING on ventral striatal DA levels ($F[1,17] = 7.6$, $p < 0.05$). This aging effect was significant in VMAT2 +/− mice according to *post hoc* comparisons ($p < 0.05$, Scheffe's test), but not in VMAT2 +/+ mice. Similar effects were apparent between old and young mice for ventral striatal levels of DOPAC and HVA, although the effect of AGING did not reach statistical significance in the ANOVA. This age-related pattern was paralleled by similar relationships in ventral striatal 5-HT and 5-HIAA levels, for which there were significant interactions between GENOTYPE and AGING (5-HIAA: $F[1,17] = 5.6$, $p < 0.05$; and 5-HT: $F[1,17] = 5.6$, $p < 0.05$), reflecting reduced levels in aged VMAT2 +/− mice but not VMAT2 +/+ mice.

This age-related pattern was not seen in dorsal striatal or midbrain levels of DA, 5-HT, or their metabolites.

3.4. Tissue monoamine levels in heterozygous DAT KO mice

Possible mechanisms for the age-related locomotor effects in DAT +/− mice were examined by assessing levels of DA, 5-HT and their metabolites in brain tissue samples (ventral striatum, dorsal striatum and midbrain) dissected from young and old DAT +/− and +/+ mice (Table 2). Ventral striatal DA levels were reduced in old DAT mice compared to young DAT mice, as confirmed a significant effect of AGE in the ANOVA ($F[1,36] = 5.4$, $p < 0.03$). The effect of GENOTYPE was not significant, and the effect of AGE was not significant when either DAT +/+ or DAT +/− mice were considered alone in *post hoc* tests. By contrast, levels of DOPAC were elevated in old DAT mice ($F[1,36] = 6.0$, $p < 0.02$). *Post hoc* comparisons showed that this increase in DOPAC levels was significantly different in DAT +/− mice ($p < 0.05$, Scheffe's test), but not DAT +/+ mice. There were no significant differences in HVA levels, or levels of 5-HT. There was a significant effect of AGE on ventral striatal levels of 5-HIAA ($F[1,36] = 5.7$, $p < 0.03$). Aging reduced levels of 5-HIAA in DAT +/+ mice ($p < 0.05$, Scheffe's test), but not DAT +/− mice.

In the dorsal striatum, aged mice actually had higher levels of DA ($F[1,35] = 5.6$, $p < 0.03$). This increase was significant only for old DAT +/− mice, which had higher levels of DA than young DAT +/− mice ($p < 0.05$, Scheffe's test). In the dorsal striatum, there were no significant differences in the levels of either DOPAC or HVA. DAT +/− mice had slightly lower levels of 5-HT than DAT +/+ mice ($F[1,35] = 4.9$, $p < 0.04$). There was also a trend for older mice to have higher 5-HT levels, however this effect was only of marginal statistical significance ($p < 0.06$). Levels of 5-HIAA were lower in aged mice ($F[1,35] = 11.1$, $p < 0.01$) and in DAT +/− mice ($F[1,35] = 5.6$, $p < 0.03$), but there was no significant interaction between these factors. *Post hoc* analysis showed that the effect of age was significant only in DAT +/+ mice, while the effect of genotype was significant only in the young mice ($p < 0.05$, Scheffe's test).

In the midbrain, there were no significant effects of AGE or GENOTYPE on levels of DA, 5-HT or HVA. Midbrain levels of DOPAC were reduced in aged DAT mice ($F[1,35] = 13.1$, $p < 0.001$) of both genotypes when compared to those in young mice ($p < 0.05$, Scheffe's test). There was no effect of genotype. Midbrain levels of 5-HIAA were increased in aged DAT mice ($F[1,35] = 13.1$, $p < 0.001$) of both genotypes compared to young mice ($p < 0.05$, Scheffe's test). There was again no effect of genotype.

4. Discussion

Dopamine neurons display vulnerability in aging that is manifest in biochemical, behavioral and pharmacological assessments in many mammalian species (see Morgan and Finch, 1988, for review). The current results support the idea that alterations in the expression of genes that encode proteins that influence subcellular compartmentalization of DA influence the course of aging effects in dopaminergic systems. Genetic variations in VMAT2 and DAT are thus candidates to play roles in both normal and pathological human age-related processes that include the selective vulnerability of these neurons in Parkinson's disease. The evidence for enhanced effects of aging in VMAT2 +/− mice in the current data is complemented by signs of reduced aging effects in DAT +/− mice. Effects of aging on habituated locomotor activity and injection-stimulated locomotor activity were enhanced in VMAT2 +/− mice but diminished in DAT +/− mice. Effects of aging on cocaine-induced locomotion were enhanced in VMAT2 +/− mice but diminished in DAT +/− mice. In addition, the effects of aging on dopamine levels in the ventral striatum were enhanced in VMAT2 +/− mice, but not in DAT +/− mice.

4.1. Behavior

Aging reduces spontaneous locomotion (Dean et al., 1981; Emerich et al., 1993; Hebert and Gerhardt, 1998; Marshall and Altar, 1986; Marshall and Berrios, 1979; Spangler et al., 1994; Watanabe, 1987b; Yurek et al., 1998) and other measures of motor function that include reaction time (Burwell and Gallagher, 1993; Burwell et al., 1995), coordination (Dean et al., 1981; Emerich et al., 1993; Friedemann and Gerhardt, 1992; Kametani et al., 1995;

Table 2
Tissue levels of DA, 5-HT, DOPAC, HVA and 5-HIAA (ng/g wet tissue weight) in the ventral striatum, dorsal striatum and ventral midbrain of young and old, DAT +/+ and DAT +/− mice ($N = 9$–10/group) as determined by HPLC-EC. *Old vs. young, within genotype Scheffe's *post hoc* comparison, $p < 0.05$. ‡DAT +/+ vs. DAT +/− within genotype Scheffe's *post hoc* comparison, $p < 0.05$.

	Genotype	Age	DA	DOPAC	HVA	5-HT	5-HIAA
Ventral striatum	DAT +/+	Young	7.89 ± 0.39	0.72 ± 0.05	0.62 ± 0.02	0.67 ± 0.06	0.77 ± 0.05*
		Old	6.79 ± 0.40	0.82 ± 0.06	0.58 ± 0.03	0.67 ± 0.04	0.63 ± 0.02
	DAT +/−	Young	7.32 ± 0.25	0.62 ± 0.03*	0.63 ± 0.02	0.59 ± 0.03	0.67 ± 0.03
		Old	6.81 ± 0.25	0.74 ± 0.05	0.61 ± 0.02	0.61 ± 0.03	0.65 ± 0.02
Dorsal striatum	DAT +/+	Young	17.1 ± 1.2	1.10 ± 0.04	1.23 ± 0.05	0.84 ± 0.08	1.00 ± 0.03*
		Old	18.7 ± 0.7	1.03 ± 0.03	1.34 ± 0.06	0.92 ± 0.04	0.86 ± 0.02
	DAT +/−	Young	14.9 ± 0.8*	0.97 ± 0.04	1.33 ± 0.06	0.67 ± 0.05	0.89 ± 0.03‡
		Old	17.5 ± 0.8	1.05 ± 0.07	1.29 ± 0.07	0.83 ± 0.04	0.82 ± 0.03
Ventral midbrain	DAT +/+	Young	0.30 ± 0.01	0.39 ± 0.01*	0.17 ± 0.01	0.46 ± 0.01	1.37 ± 0.08*
		Old	0.31 ± 0.02	0.33 ± 0.01	0.16 ± 0.01	0.41 ± 0.02	1.93 ± 0.11
	DAT +/−	Young	0.28 ± 0.02	0.37 ± 0.02*	0.15 ± 0.02	0.43 ± 0.02	1.29 ± 0.04*
		Old	0.35 ± 0.03	0.29 ± 0.02	0.19 ± 0.02	0.41 ± 0.02	1.90 ± 0.13

Spangler et al., 1994; Yurek et al., 1998), swim performance (Marshall and Altar, 1986; Marshall and Berrios, 1979), screen hang time and rotorod performance (Dean et al., 1981; Wallace et al., 1980). Simpler motor reflexive tasks are often less affected than more complex tasks in ways that are not readily explained solely by generalized morbidity (Spangler et al., 1994). Losses of dopaminergic functions are likely to contribute to at least some of the deficits in these tests, as suggested by pharmacological studies. There is reduced amphetamine-induced rotational behavior and locomotion in aged animals (Crawford and Levine, 1997; Joseph et al., 1978; Yurek et al., 1998), reduced locomotor stimulant responses to the D2 agonist quinpirole and reduced locomotion resulting from treatment with the selective DAT blocker nomifensine (Crawford and Levine, 1997; Hebert and Gerhardt, 1998). The D1 agonist SKF 38393 reduces locomotion in young rats while it increases locomotion in aged rats (Crawford and Levine, 1997). Age-related impairments in swimming tests can be ameliorated by dopamine agonist treatment (Marshall and Berrios, 1979).

For several of the locomotor phenotypes studied here, aging effects were identified in WT mice consistent with this literature, including reduced activity in a novel environment. This was observed in aged DAT +/+ and aged VMAT2 +/+ mice, compared to young mice of the same genotypes. This reduction was attenuated by habituation to the environment, but still apparent at the beginning of the test sessions and immediately after injections. This effect of aging was eliminated by habituation in VMAT2 +/+ mice, but not in VMAT2 +/− mice. DAT +/− mice demonstrated the opposite trend. Another study also observed a reversal of this aging trend on spontaneous locomotion in heterozygous DAT KO mice (Dluzen et al., 2010). Similarly, the transient locomotor-increasing effects of intraperitoneal injection with saline were attenuated in older mice, an effect that was enhanced in VMAT2 +/− mice but reduced in DAT +/− mice. Locomotor responses to cocaine were similarly affected by aging. VMAT2 +/− knockout again exacerbated these effects, while aging effects were not observed in DAT +/− mice. Another VMAT2 transgenic strain has been created in which a mutation in the promoter produces a 90% reduction in VMAT2 expression throughout life, termed VMAT2 deficient (VD) mice (Mooslehner et al., 2001). Examination of the effect of motor function on aging in VD mice identified effects of aging on motor coordination, but not basal locomotion (Colebrooke et al., 2006). However, basal locomotion was substantially reduced even in young VD mice, which may account for the failure to observe aging effects. Similar effects in young mice on motor coordination made it difficult to determine whether or not reductions in VMAT2 expression accelerated the effects of aging on dopaminergic function. In any case this magnitude of reduction in VMAT2 expression is not observed in humans, whereas the magnitude of reduction observed in VMAT2 +/− mice in the present experiments is within the range of variation observed in humans.

In the current experiments, necessarily performed over several years, we carefully compared VMAT2 and DAT heterozygous KO mice to WT littermates. These comparisons are the focus of the current dataset. The two WT strains were bred at different times along with VMAT2 or DAT heterozygote littermates, so data from these strains should thus be compared to each other with caution, since the genetic backgrounds of these two WT strains are likely to differ from each other. Although both strains were on mixed C57BL/6J-129Sv backgrounds, the specific complements of C57 and 129 alleles present in each strain are likely to be different.

4.2. Effects of aging on neurochemical measures

A substantial literature, as well as the present results, indicate that aging affects dopaminergic function. Presynaptic DA system decline with age is manifested by decreased striatal DA and DA metabolite concentrations (Arranz et al., 1996; Burwell et al., 1995; Carlsson et al., 1980; Emerich et al., 1993; Freeman and Gibson, 1987; Friedemann and Gerhardt, 1992; Gozlan et al., 1990; Joseph et al., 1978; Kish et al., 1992; Marshall and Altar, 1986; McIntosh and Westfall, 1987; Osterburg et al., 1981; Watanabe, 1987b). Reduced tissue concentrations of DA could result from reduced synthesis (Kish et al., 1995; Watanabe, 1987a,b), altered DA uptake and transport functions (Felten et al., 1992; Friedemann and Gerhardt, 1992; Gordon et al., 1995; Jonec and Finch, 1975; Shimizu and Prasad, 1991; Stamford, 1989), altered concentrations of DA cells and/or synapses (Felten et al., 1992; Sabel and Stein, 1981), and/or altered DA turnover (Emerich et al., 1993). Furthermore, effects of age differ across brain regions (Felten et al., 1992; Friedemann and Gerhardt, 1992; Osterburg et al., 1981; Yurek et al., 1998). In the present experiments, we observed modest DA reductions in the ventral striatum of aged VMAT2 +/+ mice that were exacerbated by loss of one functional VMAT2 allele. More substantial reductions in DA levels have been observed in VD mice (Colebrooke et al., 2006), but as for behavioral changes in these mice, profound effects are observed even in young mice so that it is not possible to determine whether the effects of aging may be accelerated. The observation of age-related reductions in ventral striatal DA levels, but not dorsal striatal DA levels, agree with previous observations that indicate a greater sensitivity to these age-related effects in the ventral striatum (e.g. Felten et al., 1992; Yurek et al., 1998). Other age-related effects are also described in ventral striatum (Crawford and Levine, 1997; Friedemann and Gerhardt, 1992). In the present study, decreases in ventral striatal levels of 5-HT and 5-HIAA were also observed in VMAT +/− mice. Although it would be parsimonious to hypothesize that loss of DA and 5-HT might be directly related, proof of such a relationship will have to wait for more direct evidence. By contrast to what was observed in VMAT +/− mice, no reductions in ventral striatal dopaminergic or serotonergic markers were observed in DAT +/− mice. Indeed, a recent study in which aged WT mice showed reductions in striatal DA levels, heterozygous DAT KO was found to be protective against this age-related decline in DA levels (Dluzen et al., 2010). In the present study reduced dorsal striatal DA levels were found in young DAT +/− mice compared to old DAT +/− mice which did not differ from either old or young DAT +/+ mice, although it might be better to express this as a reduction of dorsal striatal DA levels in young DAT +/− mice compared to young DAT +/+ mice that is lost with aging. Reduced tissue DA levels have previously been observed in DAT −/− mice, but not DAT +/− mice (Sora et al., 2001), although that study did not differentiate striatal subregions.

The current data support findings of age-related decline in dopaminergic markers that include substantia nigra cell counts (McGeer et al., 1988; Muthane et al., 1998; Snow et al., 1993; Tooyama et al., 1994) and positron emission tomographic (PET) imaging of DAT (Allard and Marcusson, 1989; De Keyser et al., 1990; Ishibashi et al., 2009; van Dyck et al., 1995; Volkow et al., 1994, 1998; Zelnik et al., 1986), and VMAT2 (Bohnen et al., 2006; Naudon et al., 1994; Vander Borght et al., 1995). However, another recent study found that DAT, but not VMAT2, PET binding decreased with age (Troiano et al., 2010), and consequently that the DAT/VMAT2 ratio decreased with age, which was interpreted as a compensatory mechanism that preserved dopaminergic function and motor capability. It would appear that in humans changes in dopamine terminal regions precede changes in cell body regions. A similar pattern is observed in rodents, where dopaminergic cell loss with aging is modest (Cantuti-Castelvetri et al., 2003; McNeill and Koek, 1990) compared to the changes observed in terminal regions noted previously. These observations accord with our failure to

identify significant reductions in midbrain dopamine content or dopamine cell counts (data not shown), as has been the case in most, though not all, previous reports (Burwell et al., 1995; McIntosh and Westfall, 1987; Osterburg et al., 1981; Severson et al., 1981; Yurek et al., 1998). It is interesting to note that deletion of the gene for α-synuclein also reduces a number of indices of dopaminergic function in terminal areas without an appreciable effect on the number of midbrain dopamine neurons (Al-Wandi et al., 2010).

4.3. DA compartmentalization in normal and pathological aging

The current results support the idea that compartmentalization of DA plays a significant role in the vulnerability of DA systems to aging. The magnitude of many of the effects observed in the present studies was small. However, there was definite evidence for reduced effects of aging on dopaminergic function in DAT +/− mice and enhanced effects of aging in VMAT2 +/− mice. There were a number of limitations of the current studies, so in the future it will be important to determine if greater effects of dopaminergic aging can be observed in older mice, in response to a wider range of psychostimulant doses, other behavioral measures and other neurochemical measures. Nonetheless, the present findings are consistent with a study of nitrative damage of midbrain dopamine neurons, using cellular levels of 3-nitrotyrosine as a marker, in rhesus monkeys (Kanaan et al., 2008). In this study, nitrative damage was associated with increased DAT expression and increased DAT/VMAT ratios. The small magnitude of the effects observed in the present studies should not be surprising. Altered dopaminergic compartmentalization is likely to interact with variations in other processes, including altered expression of α-synuclein (Eriksen et al., 2005) or reduced antioxidant functions (Pong, 2003), to produce more profound consequences. The present data thus support the hypothesis that altered DA compartmentalization affects dopaminergic aging but does not exclude roles for compensatory mechanisms likely to emerge in mice with lifelong reductions in DAT and VMAT2 expression.

Changes in transporter expression in these mice provide models for human individual differences in DA compartmentalization. Human DAT and VMAT2 allelic variants have been shown to affect DAT and VMAT2 expression (Drgon et al., 2006; Lin et al., 2005), and common human DAT variants have been shown to affect *in vivo* DAT binding as well (Drgon et al., 2006; van Dyck et al., 2005). By contrast, only rare missense allelic variants alter DAT or VMAT2 amino acid sequences (Grunhage et al., 2000; Heinz et al., 2000; Kim et al., 2000; Persico and Catalano, 1998; Persico and Macciardi, 1997; Tan et al., 2000; Vandenbergh et al., 2000). PET and *post mortem* radioligand binding studies identify 30–100% ranges for age-matched human individual differences in levels of VMAT2 and DAT B_{max} values, but no large variations in K_D values (Erixon-Lindroth et al., 2005; Volkow et al., 1994; Wilson et al., 1996a). A role for DA compartmentalization in mechanisms of "normal" aging also implicates similar mechanisms in pathological aging, such as Parkinson's disease (Cubells et al., 1994; Hastings et al., 1996a,b). *In vitro* and *in vivo* studies of cells with different expression of DAT and VMAT2 correlate with the relative vulnerabilities of these cell types in Parkinsonian-like neurodegeneration (Uhl, 1998; Uhl et al., 1994). Indeed, low VMAT2 gene expression and protein level have been found in the brains of Parkinson's disease patients (Harrington et al., 1996; Miller et al., 1999) and VMAT2 has been observed in Lewy bodies in the substantia nigra (Yamamoto et al., 2006).

Region-specific differences in sensitivity to aging in VMAT2 +/− mice are consistent with evidence that age-related and neurodegenerative disorders often manifest damage to specific neuronal populations but less severe, more broadly distributed abnormalities in other cell groups. Dopaminergic damage in the aged substantia nigra is a prominent alteration in Parkinsonism. It is also present on a background of varying multi-focal pathological changes in other brain regions (Jellinger, 1987). The consistent nature of DA cell loss and consequent motor symptoms of aging suggest that any explanation for brain changes with aging must explain these patterns of cellular loss. Such an overall pattern is consistent with pathology influenced in part by DA compartmentalization influenced by DAT and VMAT2.

5. Conclusions

The present results provide a compelling rationale for seeking genetic mechanisms that modulate transporter-based compartmentalization in dopaminergic neurons. Such mechanisms might interact with the cellular metabolic stresses caused by reactive DA metabolites localized in cytoplasmic/extravesicular neuronal compartments (Mattammal et al., 1995; Uhl, 1998) and play important roles in both normal and pathological aging. Conceivably, drugs that enhance function of VMAT2 or reduce function of DAT would be of potential therapeutic use in slowing the progression of losses of dopaminergic function in these conditions. Such agents could act directly at these transporters, or could modulate transporter function in some other way such as modulation of phosphorylation (Huff et al., 1997; Kitayama et al., 1994) or gene expression. Individual differences in VMAT2 and DAT expression, whether genetically or environmentally driven, could serve as markers for vulnerability to aging in humans, and provide important focal points for prophylactic strategies. Strategies directed at altering dopaminergic compartmentalization in the elderly might slow the progression of both normal aging and even delay the onset of Parkinsonism in otherwise-vulnerable individuals.

Acknowledgments

The authors are grateful for the expert technical assistance of T. Heinemann and R. Burger, and the financial support of the Intramural Research Program of the National Institute on Drug Abuse (NIH/DHHS), the Deutsche Forschungsgemeinschaft (SFB 581, KFO 125/1-2) and the European Commission (NEWMOOD LSHM-CT-2003-503474). The authors declare that there exist no conflicts of interest with regard to the publication of this work.

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