Mice Lacking the α4 Nicotinic Receptor Subunit Fail to Modulate Dopaminergic Neuronal Arbors and Possess Impaired Dopamine Transporter Function

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ABSTRACT

Neuronal nicotinic acetylcholine receptors (nAChRs) at presynaptic sites can modulate dopaminergic synaptic transmission by regulating dopamine (DA) release and uptake. Dopaminergic transmission in nigrostriatal and mesolimbic pathways is vital for the coordination of movement and is associated with learning and behavioral reinforcement. We reported recently that the D2 DA receptor plays a central role in regulating the arbor size of substantia nigra dopaminergic neurons. Given the known effects of nAChRs on dopaminergic neurotransmission, we assessed the ability of the α_4 nAChR subunit to regulate arbor size of dopaminergic neurons by comparing responses of wild-type and α_4 nAChR subunit knockout [$\alpha_4(-/-)$] mice to long-term exposure to cocaine, amphetamine, nicotine, and haloperidol, and after substantia nigra neurotoxic lesioning. We found that dopaminergic neurons in adult drug-naive $\alpha_4(-/-)$ mice had significantly larger terminal arbors, and despite normal short-term behavioral responses to drugs acting on pre- and postsynaptic D2 DA receptors, they were unable to modulate their terminal arbor in response to pharmacological manipulation or after lesioning. In addition, although synaptosome DA uptake studies showed that the interaction of the D2 DA receptor and the dopamine transporter (DAT) was preserved in $\alpha_4(-/-)$ mice, DAT function was found to be impaired. These findings suggest that the α_4 subunit of the nAChR is an independent regulator of terminal arbor size of nigrostriatal dopaminergic neurons and that reduced functionality of presynaptic DAT may contribute to this effect by impairing DA uptake.

The neuronal nicotinic acetylcholine receptor (nAChR) is a ligand-gated ion channel composed of different nicotinic receptor subunits, identified as α_2 to α_{10} and β_2 to β_4. In the brain, α_4 and β_2 transcripts are the most predominant (Zoli et al., 1998; Ross et al., 2000; Drago et al., 2003). The substantia nigra pars compacta (SNpc) and ventral tegmental area (VTA) contain moderate to high levels of α_4, α_5, α_6, β_2, and β_3 nAChR-subunit mRNA, with colocalization of the α_4 subunit and tyrosine hydroxylase (TH)

present on the dopaminergic cell bodies of mesencephalic neurons (Le Novere et al., 1996). The β_3, α_5, α_6, α_3, α_7, and β_4 subunits were detected in decreasing frequency. In addition, an independent in situ hybridization study (Le Novere et al., 1996) and an immunohistochemical study (Hill et al., 1993) confirmed β_2 expression in nearly all dopaminergic neurons. Recent studies (Zoli et al., 2002; Champtiaux et al., 2003; Salminen et al., 2004) have used a number of techniques including immunoprecipitation, ligand binding, and assays of dopamine release in striatal synaptosomes undertaken in mice with null mutations for a number of individual nAChR subunits to define the subunit combination present in dopaminergic terminals. The $\alpha_4\beta_2$ and the $\alpha_4\alpha_5\beta_2$ heteromeric configuration are α-conotoxin MII-resistant in DA release assays, whereas

ABBREVIATIONS: nAChR, nicotinic acetylcholine receptor; DA, dopamine; WT, wild type; DAT, dopamine transporter; $\alpha_4(-/-)$, α_4 nAChR subunit knockout; SNpc, substantia nigra pars compacta; VTA, ventral tegmental area; TH, tyrosine hydroxylase; D2(−/−), dopamine D2 receptor knockout; 6-OHDA, 6-hydroxydopamine; ir, immunoreactivity; CE, coefficient of error; CPu, caudate putamen; GBR 12935, 1-(2-[diphenylmethoxy]ethyl)-4-[3-phenylpropyl]piperazine dihyrochloride; PKC, protein kinase C; ANOVA, analysis of variance; KRH, Krebs-Ringer-HEPES; RU 24213, 3-(2-((N-phenethyl)(N-propyl)amino)ethyl)phenol hydrochloride; YM 09151-2, 5-chloro-2-methoxy-4-(methylamino)-N-(2-methyl-1-(phenyl-methyl)-3-pyrrolidinyl)benzamide; RTI-55, 2-carbomethoxy-3-(4-iodophenyl)tropane.

the α_6-containing heteromeric receptors [$\alpha_4\alpha_6\beta_2(\beta_3)$ and $\alpha_6\beta_2\beta_3$] are α-conotoxin MII-sensitive. Furthermore, measurements of acetylcholine-elicited currents in a number of knockout mice (Champtiaux et al., 2003) have shown that compared with α_6 subunit containing receptors, α_4 subunit-bearing receptors represent the majority of functional heteromeric nAChRs on the dopaminergic somatodendritic compartment.

Nicotine stimulates DA release in the ventral and dorsal striatum (Wonnacott et al., 1989). One mechanism is through the activation of somatodendritic nicotinic receptors on nigral and VTA dopaminergic neurons. Delivery of the nAChR antagonist mecamylamine into the VTA attenuates evoked DA release in the accumbens nucleus (Nisell et al., 1994). However, this study does not exclude an independent, biologically significant effect of nAChR agonists at the synaptic terminal on DA release. Indeed, direct modulation of DA release through presynaptic nAChRs has been shown in vitro using synaptosomes (el-Bizri and Clarke, 1994) and brain slice preparations (Sacaan et al., 1995). Direct application of nicotine to terminals in vivo (Marshall et al., 1997) results in increases in extracellular DA. Furthermore, interactions between nAChR-mediated processes and the DAT may also be important in regulating extracellular DA concentrations (Hart and Ksir, 1996; Gerasimov et al., 2000; Middleton et al., 2004). Nicotinic receptor signaling regulates DAT gene transcription in the midbrain (Li et al., 2004). In addition, there is a relationship between protein kinase C (PKC) activity and DAT expression on the membrane (Melikian and Buckley, 1999), and PKC activity is known to be modulated by nAChRs.

We reported recently several lines of evidence to indicate that the D2 DA receptor plays a central role in regulating SNpc arbor size. First, D2 knockout [D2($-/-$)] mice have abnormally large SNpc axonal arbors and fail to undergo compensatory sprouting after a partial lesion of the SNpc (Parish et al., 2001). Second, D2 antagonists and agonists were shown to regulate arbor size (Parish et al., 2001, 2002b). It was suggested that the D2 DA autoreceptor located on the presynaptic DA terminal regulated arbor size by sending a negative feedback signal on synaptic DA levels to the cell body. Because the nicotinic α_4 nAChR subunit-containing receptor is also located at the presynaptic terminal site and nAChRs regulate the release and uptake of DA, we sought to determine whether this subunit is also involved in regulating DA axonal arbors. Because tree size is a quantifiable, unambiguous anatomical assay of the long-term effects of changes in synaptic DA levels, nAChRs are known to modulate synaptic DA, and the α_4 nAChR is expressed at high levels in the mammalian brain, we examined terminal arbor size of dopaminergic nigrostriatal neurons in drug-naive $\alpha_4(-/-)$ mice and after pharmacological manipulation known to induce sprouting or pruning in WT mice (Parish et al., 2002b). We also examined the role of the α_4 nAChR subunit in regeneration by assessing dopaminergic terminal arbor sizes in $\alpha_4(-/-)$ mice after 6-hydroxydopamine (6-OHDA) lesions of the SNpc. To further examine the role that the α_4 subunit plays in dopaminergic neurotransmission, DAT functionality was also assessed in $\alpha_4(-/-)$ mice, because the DAT is expressed on the presynaptic membrane and is a molecule known to regulate DA levels.

Materials and Methods

A total of 279 mice [140 WT and 139 $\alpha_4(-/-)$ mice] aged 12 weeks and weighing 24 to 28 g were used in the study. The generation of $\alpha_4(-/-)$ mice and their genetic background was as described previously (Ross et al., 2000). Southern analysis was used to genotype mice. All studies have been carried out in accordance with the Declaration of Helsinki and conformed to the Australian National Health and Medical Research Council published code of practice for the use of animals in research.

Drug Administration. The indirect dopamine receptor agonist cocaine (25 mg/kg; Southern Healthcare Pharmacy, Clayton, Australia) or amphetamine (8 mg/kg; Sigma Chemical, St. Louis, MO), or saline (0.25 ml) were administered daily for 8 weeks by subcutaneous injection in WT and $\alpha_4(-/-)$ mice. Mice also received the dopamine receptor antagonist haloperidol (2.5 mg/kg, Serenace; Searle Laboratories, Fairfield, NSW, Australia) or nicotine (50 μg/ml; animals receiving on average 125 μg/day) in drinking water daily for 8 weeks. The concentration of nicotine in the drinking water was increased in increments over a 2-week period to a final concentration of 50 μg/ml. Terminal arbor size was determined for all untreated and drug-administered WT and $\alpha_4(-/-)$ mice. Drug doses and animal numbers for the stereological studies are indicated in Table 1.

Immunohistochemistry for DAT and TH. Dopaminergic terminals in the striatum were identified as DAT-immunoreactive (DAT-ir) structures having the morphology of a synaptic terminal under the light microscope, and dopaminergic cells within the SNpc were identified by the presence of TH immunoreactivity (TH-ir). Alternate sections from the SNpc were either counterstained with 1% Neutral red or left free-floating for TH-ir labeling of dopaminergic SNpc nigrostriatal neurons. Immunohistochemistry was performed using methods described previously (Parish et al., 2001). Double-staining experiments indicated that cells which are TH-ir are also DAT-ir (data not shown).

Fractionator Design for Estimating the Total Numbers of SNpc Neurons and DAT-ir Varicosities in Caudate Putamen. The total number of SNpc cells and the proportion of TH-ir neurons were estimated using a fractionator sampling design (Gundersen et al., 1988; Finkelstein et al., 2000). The boundaries of SNpc were delineated on neutral red-stained sections. Total and TH-ir SNpc counts were made in alternate sections as described previously (Parish et al., 2001).

The rostral 2.5 mm of the striatum was examined, and DAT-ir terminal density within the dorsal portion of the nuclei was determined. The dorsal striatum was defined as the most dorsal 400 μm of the striatum. DAT-positive terminals were identified anatomically as predominantly round swellings in association with axonal processes. Total terminal numbers (DAT number) were estimated, and terminal density was calculated as terminals per cubic micrometer, as described previously (Parish et al., 2001). The coefficient of error (CE) and CV were calculated as estimates of precision, and values of less than 0.1 were accepted (Braendgaard et al., 1990; Parish et al., 2001).

Index of Terminal Tree Size. We derived a representation of the average size of the terminal arbor of SNpc neurons for WT and $\alpha_4(-/-)$ mice using a previously described and extensively used method (Finkelstein et al., 2000; Parish et al., 2001, 2002a,b; Stanic et al., 2003a,b; Tripanichkul et al., 2003). The only way to measure the actual size of the arbor is to anterogradely fill and reconstruct individual axons (Finkelstein et al., 2000), a method which is time-consuming and labor-intensive. Because of these constraints, only a small sample of the neuronal population could be analyzed. Hence, arbor size was obtained by dividing the density of DAT-ir terminals in dorsal caudate putamen (CPu) by the number of neurons counted in SNpc. This calculation provides a representation of arbor size rather than a true measure; however, it is suitable for making comparisons between genotypes and treatment groups in which stri-

TABLE 1
Stereological estimates of SNpc terminal arbors in WT and $\alpha_4(-/-)$ mice after pharmacological manipulation
Data are sometimes presented as mean ± S.D.

Treatment and Genotype	SNpc Counts (CE, CV)	Change[a] %	Change[b] %	TH-ir Neurons[b]	SNpc[c] %	Density of DAT-ir Terminals (CE, CV)	Change[a] %	Change[b] %	TT	Change[a] %	Change[b] %
WT ($n = 14$)	6001 ± 251 (0.077, 0.042)	0.0	0.0	5412 ± 255 (0.068, 0.047)	90.4	$12.3 \pm 0.8 \times 10^{-3}$ (0.045, 0.065)	0.0	0.0	$20.5 \pm 1.3 \times 10^{-7}$	0.0	0.0
$\alpha_4(-/-)$ ($n = 14$)	5684 ± 377 (0.073, 0.066)	5.3	0.0	5024 ± 450 (0.082, 0.057)	88.4	$14.4 \pm 1.5 \times 10^{-3}$ (0.052, 0.095)	17.3*	0.0	$25.4 \pm 1.3 \times 10^{-7}$	23.8*	−0.06
WT Saline ($n = 12$)	5984 ± 402 (0.055, 0.079)	−1.8	−1.7			$12.1 \pm 1.3 \times 10^{-3}$ (0.041, 0.092)	−1.6	−1.6	$20.2 \pm 1.7 \times 10^{-7}$	0.0	0.0
$\alpha_4(-/-)$ Saline ($n = 12$)	5799 ± 342 (0.068, 0.079)	3.4	1.2			$14.7 \pm 1.7 \times 10^{-3}$ (0.042, 0.098)	19.2*	2.1	$24.9 \pm 2.9 \times 10^{-7}$	21.4*	−1.6
WT Haloperidol ($n = 11$)	5940 ± 519 (0.083, 0.087)	−1.0	−1.0			$16.5 \pm 1.2 \times 10^{-3}$ (0.052, 0.07)	34.2*	34.2†	$27.8 \pm 2.0 \times 10^{-7}$	35.6*	35.6†
$\alpha_4(-/-)$ Haloperidol ($n = 11$)	5974 ± 402 (0.073, 0.067)	0.4	3.1			$14.3 \pm 1.6 \times 10^{-3}$ (0.046, 0.067)	15.8*	−0.7	$23.9 \pm 2.2 \times 10^{-7}$	16.6*	−1.4
WT Cocaine ($n = 12$)	5987 ± 170 (0.059, 0.028)	0.2	−0.2			$9.9 \pm 0.5 \times 10^{-3}$ (0.049, 0.046)	−19.4*	−19.4†	$16.2 \pm 2.9 \times 10^{-7}$	−20.8*	−20.8†
$\alpha_4(-/-)$ Cocaine ($n = 12$)	5649 ± 379 (0.049, 0.083)	5.9	−1.3			$14.4 \pm 1.1 \times 10^{-3}$ (0.050, 0.076)	17.3*	0.0	$25.5 \pm 1.9 \times 10^{-7}$	24.6*	0.5
WT Amphetamine ($n = 10$)	6041 ± 524 (0.044, 0.087)	0.7	0.0			$11.2 \pm 1.4 \times 10^{-3}$ (0.088, 0.104)	−9.3*	−9.3†	$18.5 \pm 2.3 \times 10^{-7}$	−9.9*	−9.9†
$\alpha_4(-/-)$ Amphetamine ($n = 10$)	5975 ± 459 (0.062, 0.077)	0.4	3.7			$14.5 \pm 1.5 \times 10^{-3}$ (0.088, 0.104)	18.1*	0.7	$24.3 \pm 2.5 \times 10^{-7}$	18.6*	−4.2
WT Nicotine ($n = 12$)	5775 ± 434 (0.064, 0.077)	−3.7	−3.7			$11.7 \pm 1.1 \times 10^{-3}$ (0.060, 0.095)	−4.9	−4.9	$20.7 \pm 1.6 \times 10^{-7}$	1.1	1.1
$\alpha_4(-/-)$ Nicotine ($n = 10$)	5913 ± 198 (0.064, 0.033)	−1.5	4.0			$14.2 \pm 1.1 \times 10^{-3}$ (0.049, 0.079)	15.6*	−1.4	$24.1 \pm 1.9 \times 10^{-7}$	17.5*	−5.1

n, number of hemispheres; TT, terminal tree size (see *Results*; this number is representative of the size of the terminal arbor and is useful for comparison rather than an accurate reflection of absolute size; TT, DAT-ir terminal density/number of SNpc neurons). *, significantly different from untreated WT; †, significantly different from untreated group of the same genotype.
[a] Percentage of change from untreated WT.
[b] Percentage of change from untreated animals of same genotype.
[c] Percentage of TH-ir neurons in the SNpc.

atal volume is unchanged, as is the case in WT and $\alpha_4(-/-)$ mice (data not shown).

Throughout the neuroanatomical studies of terminal arbor sizes (including SN and DAT-positive terminal counts), statistical significance ($p < 0.05$) was determined by ANOVA with Tukey post hoc tests.

Behavioral Analysis. Single immediate doses of the D2-like agonist RU 24213 (0.1 and 0.25 mg/kg) and the D2-antagonist YM 09151-2 (0.05 and 0.5 mg/kg) were administered to WT and $\alpha_4(-/-)$ mice to determine the functionality of the D2 DA receptor at the level of behavior. RU 24213 at these doses is believed to inhibit DA synthesis and release and to reduce dopaminergic tone via presynaptic D2 autoreceptors and was therefore used to determine autoreceptor-mediated function (McNamara et al., 2002). YM 09151-2 was used to assess postsynaptic receptor-mediated function (McNamara et al., 2003).

Behavioral topographies were assessed in rectangular glass chambers ($36 \times 20 \times 20$ cm). On experimental days, mice were injected with either saline, RU 24213, or YM 09151-2; the effect of drugs on behavior was then assessed for 1 h in both WT and $\alpha_4(-/-)$ mice in an unhabituated state. Behavioral assessments were performed by an observer blinded to genotype and treatment, using a rapid time-sampling behavioral checklist technique (Ross et al., 2000; McNamara et al., 2003), whereby each of 10 randomly selected mice was observed individually for 5-s periods, every 60 s, over 10 consecutive min. In each 5-s period, the presence or absence of each of the following individual behaviors (occurring alone or in any combination) were noted: sniffing; locomotion (coordinated movement of all four limbs producing a change in location); total rearing (rearing of any form); rearing from a sitting position (front paws reaching upwards with hind limbs on floor in sitting position); rearing free (front paws reaching upwards away from any cage wall while standing on hind limbs); rearing to wall (front paws reaching upwards onto or toward a cage wall while standing on hind limbs); sifting (sifting movements of the front paws through cage bedding material); grooming; chewing; and stillness (motionless, with no behavior evident). This cycle of observations was repeated every 10 min continuously over 1 h. Behavioral studies were undertaken between 2 and 6 PM.

6-OHDA Lesions. A partial lesion of SNpc was produced in 17 WT and 16 $\alpha_4(-/-)$ mice by injecting the neurotoxin 6-OHDA into the right SNpc using methods that have been described previously (Parish et al., 2001, 2002a).

Ligand Autoradiography. D1 dopamine receptor and D2 dopamine receptor ligand autoradiography was undertaken as described previously (Padungchaichot et al., 2000). Dopamine transporter autoradiography was completed as described previously (McGregor et al., 2003), with CPu sections from WT and $\alpha_4(-/-)$ mice exposed to 50 pM [^{125}I]RTI-55 and 100 nM fluoxetine to occlude binding to the serotonin and norepinephrine transporters. Nonspecific binding was defined as that remaining in the presence of 10 μM GBR 12935. Images were captured by Micro Computing Imaging Device and quantified using SCION imaging by comparison of the optical densities of images to standard ^{14}C microscales (American Radiolabeled Chemicals, St. Louis, MO).

Measurement of [^{3}H]DA Transport in Synaptosomes. Mice were decapitated, brains were removed and cut in a coronal plane, and the striatum was isolated by dissecting away the nonstriatal brain tissue. For each individual assay, tissue from $n = 3$ mice per genotype was pooled. The dorsal striatum was placed immediately in KRH buffer at 4°C. The KRH buffer consisted of 125 mM NaCl, 1.5 mM K_2HPO_4, 1.5 mM $MgSO_4$, 1.25 mM $CaCl_2$, 10 mM D-glucose, 25 mM HEPES, 0.1 mM ascorbic acid, 1 mM pargyline, and 0.1 mM EDTA, pH 7.4. The buffer was oxygenated with 95% O_2 and 5% O_2 for 10 min before use. The striatal tissue was homogenized in 25 ml of cold sucrose (0.32 M + 5 mM $NaHCO_3$, pH 7.4). The homogenates were centrifuged at 2000g for 10 min at 4°C, and the pellets discarded. The supernatant was centrifuged at 16,000g for 15 min at 4°C to isolate the synaptosomes. The resulting pellets were weighed

and resuspended in KRH buffer so as to obtain a concentration of 100 w/v. Fifty microliters of the synaptosome suspension and 105 μl of KRH buffer were added to each well of a 96-well plate (Falcon, Boston, MA) and incubated at 37°C for 3 min. Twenty-microliter aliquots of either 0.1 mM quinpirole (Tocris Cookson Inc., Ellisville, MO), 0.1 mM mazindol (Sigma-Aldrich Pty Ltd., Castle Hill, Sydney, Australia), or KRH buffer was added and incubated for 2 min to allow compounds to bind. The transport assay was initiated by the addition of 25 μl of KRH buffer (37°C) containing 10 nM [^{3}H]DA (59.7 Ci/mmol; dihydroxyphenylethylamine 3,4-[ring-2,5,6-^{3}H]-; Perkin-Elmer Life and Analytical Sciences, Boston, MA). Blanks consisted of samples of which the suspended pellets containing synaptosomes was substituted for 50 μl of KRH buffer. The volume of each assay was 200 μl. The assay continued for the length of time assigned (2, 5, 7, 10, 15, 20, or 25 min). After the designated time period, solutions were immediately filtered using a 96-well harvester (Tomtec Harvester 96 Mach III M; Skudtek Scientific Pty Ltd, Cranbourne, VIC, Australia) fitted with a Unifilter 96-well microplate with bonded glass-microfiber filters (GF/B; PerkinElmer Life and Analytical Sciences), and extensively washed with ice-cold KRH buffer. The 96-well filter plate was dried overnight at room temperature, and 50 μl of Microscint PS scintillation fluid (PerkinElmer) was added; the plate was sealed with TopSeal-A adhesive sealing film (PerkinElmer). The radioactivity of each well was determined by a liquid scintillation counter. Assays were performed in triplicate and were repeated on three separate occasions per genotype. Results were calculated as [^{3}H]DA uptake in femtomoles per milligram of protein and were normalized as a percentage compared with WT control.

To quantify [^{3}H]DA uptake kinetics, the rate of [^{3}H]DA uptake was measured over a range of substrate concentrations between 0.3 and 100 nM. Synaptosomes were added, and the reaction was allowed to proceed for 5 min at 37°C before being terminated, as described previously. Each concentration was completed in triplicate per assay, and the assay was repeated on four separate occasions. Results were calculated as [^{3}H]DA uptake in femtomoles per milligram of protein per minute, and V_{max} and K_m values were derived from nonlinear regression using GraphPad Prism 3.02 (GraphPad Software Inc., San Diego, CA).

In the synaptosome DA uptake study, statistical significance ($p \leq 0.05$) was determined with a one-way ANOVA with a Tukey post hoc test.

Results

Morphology of the Nigrostriatal Pathway in $\alpha_4(-/-)$ Mice. Previous studies had identified major changes in the nigrostriatal pathway in mutant mice with targeted deletions of genes important in the regulation of sprouting (Parish et al., 2001, 2002a). Differences were seen in both drug-naive mice and in their responses to drugs and lesioning of the dopaminergic pathway. Given the importance of synaptic DA levels in this paradigm and the putative role of nAChRs as regulators of dopaminergic transmission, we began by first describing the neuroanatomy of the dopaminergic arbor in $\alpha_4(-/-)$ mice and then sought to examine the effects of long-term exposure to a number of drugs known to modulate the dopaminergic arbor.

Stereology of the Substantia Nigra Pars Compacta. The number of SNpc neurons was the same in WT and $\alpha_4(-/-)$ mice and was not altered by any of the drug treatments (Fig. 1 and Table 1). In untreated animals, TH-ir SNpc neurons were counted in sections alternate to those in which Neutral Red SNpc cells were counted. As expected, most neurons in the SNpc were TH-ir in WT and $\alpha_4(-/-)$ mice (91 and 88% respectively; Table 1 and Fig. 1). The CE and CV for

SNpc counts for all treatments in WT and $\alpha_4(-/-)$ animals were less than 0.1, indicating an accurate sampling protocol.

Stereology of DAT-ir-Labeled Varicosities. Striatal tissue was processed for DAT immunohistochemistry, and counts of varicosities were estimated stereologically as de-
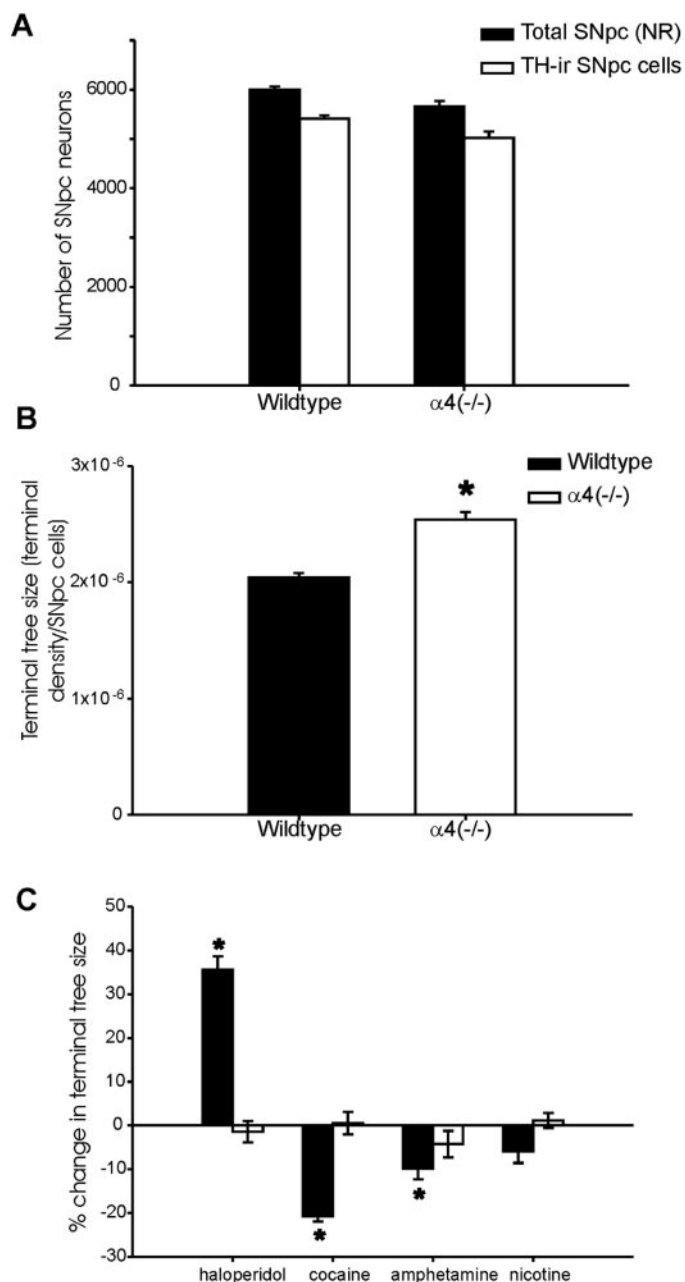

Fig. 1. A, number of neurons counted in the SNpc (mean $\pm$ S.E.) of WT and $\alpha_4(-/-)$ mice. Counts of SNpc neurons stained with neutral red are shown in black, and TH-ir SNpc counts are shown as $\square$. No significant difference was seen in the total number of SNpc neurons (or TH-ir SNpc neurons) between WT and $\alpha_4(-/-)$ mice. B, compared with WT animals, $\alpha_4(-/-)$ mice had significantly (17%) greater terminal arbor sizes ($\star$, $p < 0.05$). C, percentage change in terminal arbor sizes after long-term treatment with haloperidol, cocaine, amphetamine, and nicotine (WT, $\blacksquare$; $\alpha_4(-/-)$ mice, $\square$). Haloperidol caused significantly increased terminal arbor size (34%) in WT, whereas cocaine and amphetamine resulted in significantly reduced arbor size (19 and 9%). Nicotinic treatment showed a (nonsignificant) trend toward reduced terminal arbor size in WT mice (-4.9%). No treatments had any effect on terminal arbor size in $\alpha_4(-/-)$ mice. In all cases, drug-treated percentage changes are calculated relative to saline-treated mice of the same genotype ($\star$, $p < 0.05$).

scribed under *Materials and Methods*. Density was determined as the number of varicosities estimated within the chosen counting area. DAT-ir varicosities and terminals were uniformly distributed within the dorsal striatum. The density of varicosities in the CPu of $\alpha_4(-/-)$ mice was significantly greater (17%, $p < 0.05$) than in WT (14.4×10^{-3} and 12.3×10^{-3}, respectively; Table 1 and Fig. 1B). As described previously (Parish et al., 2001, 2002a,b), haloperidol induced axonal sprouting in dopaminergic neurons, resulting in terminal arbors 34% ($p < 0.05$) larger than normal. In contrast, haloperidol had no effect on terminal arbor size in $\alpha_4(-/-)$ mice (Table 1 and Fig. 1C). Cocaine and amphetamine administration caused significant ($p < 0.05$) decreases in DAT density and resultant terminal tree size in WT mice (20.8 and 9.9%, respectively) but had no significant effect on $\alpha_4(-/-)$ mice (Table 1 and Fig. 1C). The nicotinic administration regime was considered to result in brain nicotine concentrations comparable with concentrations achieved in long-term smokers, because in our hands, it resulted in significant up-regulation ($p < 0.001$) of [³H]nicotine binding in WT mice, with the signal intensity increasing by 30% in the striatum (mean ± S.E.M., WT nicotine 291 ± 13 fmol/mm² compared with mean 222 ± 1.3 fmol/mm² in WT water control mice). In terms of the number of DAT-ir terminals in the dorsal striatum, long-term nicotine administration had no significant

effect on either WT or $\alpha_4(-/-)$ mice; however, WT mice showed a trend toward reduced terminal arbor size (−4.9%). Saline treatment had no significant effect on DAT-ir density counts in WT or $\alpha_4(-/-)$ mice (data not shown).

Effects of 6-OHDA Lesioning of SNpc in $\alpha_4(-/-)$ Mice. Because $\alpha_4(-/-)$ mice were shown to have quite abnormal responses to a DA receptor antagonist and to two indirect-acting DA receptor agonists, we set out to examine the sprouting response of dopaminergic neurons using a non-drug paradigm known to evoke a robust sprouting response in normal mice. The neurotoxin 6-OHDA was injected into the right SNpc of WT and $\alpha_4(-/-)$ mice to produce a partial lesion. Animals were allowed to recover over 2 months, and the number of SNpc neurons and DAT-ir varicosities in the dorsal CPu was estimated, and an index of the terminal tree size was determined. Stereological estimates of SNpc neuron numbers confirmed that lesions ranging in size from 2 to 83% were created. In some WT mice, the number of neurons in the contralateral SNpc was reduced, presumably because of diffusion of 6-OHDA. In these cases, the contralateral hemisphere was included to increase the sample size of lesions less than 30%. There was no difference in the distribution of 6-OHDA induced lesion sizes seen in WT and $\alpha_4(-/-)$ mice (data not shown).

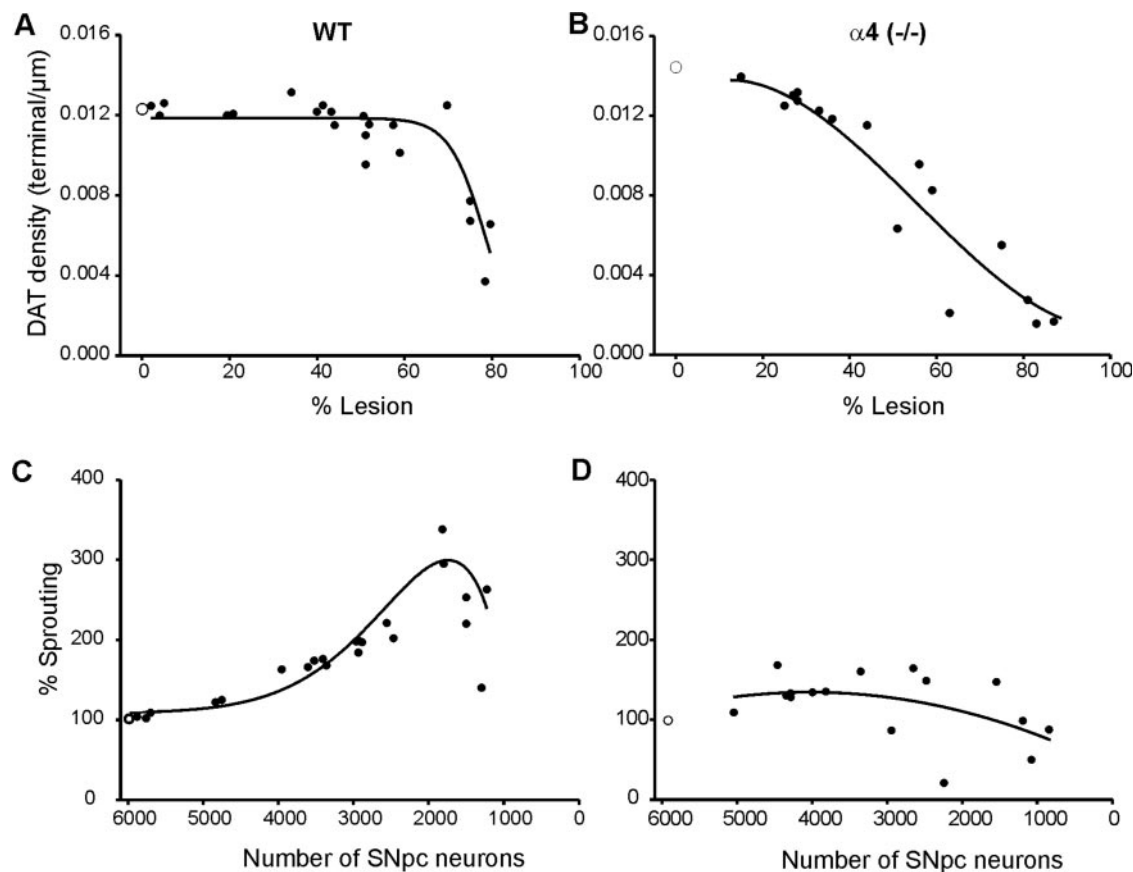As shown previously in rats (Finkelstein et al., 2000) and

Fig. 2. Plots of the density of DAT-ir terminals (A and B) in the dorsal striatum against lesion size and the degree of regenerative sprouting (C and D). A, density of DAT-ir terminals in WT mice is maintained until approximately 75% SNpc neurons are lost, at which point density rapidly falls, presumably because remaining neurons can no longer compensate through sprouting. B, density of DAT-ir terminal in $\alpha_4(-/-)$ mice with respect to varying lesion sizes. Note the persistent decrease in density with increasing lesion size and the absence of sprouting in the remaining neurons in Fig. 2D. C, degree of sprouting in WT mice, with 100% representing normal tree size, and values greater than 100% indicate a degree of sprouting. Note the significant degree of sprouting in WT animals when as few as 1500 (25%) neurons remain. The curves of best fit were computer-generated by the SigmaPlot software (SPSS Inc., Chicago, IL). Each data point on the graph represents one brain hemisphere.

mice (Parish et al., 2001, 2002a), WT mice maintained terminal density within a normal range until more than 75% of SNpc neurons were lost (Fig. 2A). In contrast, in $\alpha_4(-/-)$ mice, terminal density was reduced almost linearly with a slope of 1 [e.g., after a 50% lesion, terminal density fell by approximately 45% of nonlesioned values in $\alpha_4(-/-)$ mice (Fig. 2B)] and showed no evidence of compensatory sprouting (Fig. 2, C and D).

Dopamine Receptor Binding. The $\alpha_4(-/-)$ mice were therefore displaying major abnormalities in the regulation of their dopaminergic arbor. As previous studies have implicated the D2 DA receptor as a significant player in the regulation of plasticity in the dopaminergic arbor, we sought to examine the distribution and density of DA receptors in $\alpha_4(-/-)$ mice using ligand autoradiography. D1- and D2-class receptor binding in the striatum, nucleus accumbens, olfactory tubercle, and substantia nigra was the same in drug-naive WT and $\alpha_4(-/-)$ mice (Table 2).

Determination of D2 DA Receptor Function in $\alpha_4(-/-)$ Mice at the Level of Behavioral Topography. Because D2 DA receptor binding was unchanged in $\alpha_4(-/-)$ mice, we went on to assess the behavioral effects of a DA D2 receptor agonist and antagonist. Administration of the D2-like agonist RU 24213, to determine D2 autoreceptor function, resulted in reduced behavior in both genotypes. Overall drug effects compared with vehicle treatment for the same genotype indicated dose-related reductions in sniffing ($p < 0.01$), locomotion ($p < 0.01$), total grooming ($p < 0.001$), and total rearing ($p < 0.01$), with increased stillness ($p < 0.001$) (Fig. 3). The $\alpha_4(-/-)$ mice showed reduced total rearing relative to WT after vehicle administration ($p < 0.05$); no other behaviors recorded differed significantly between the genotypes.

Likewise, the D2-like antagonist YM 09151-2, given to determine postsynaptic D2 function, reduced behavior to a similar extent in both genotypes. Overall drug effects compared with vehicle treatment indicated reductions for sniffing ($p < 0.05$), locomotion ($p < 0.0001$), total grooming ($p < 0.01$), and total rearing ($p < 0.001$), with increased stillness ($p < 0.01$) (Fig. 3). A significant effect of genotype ($p < 0.05$) was evident for total grooming after a larger (0.5 mg/kg) dose of YM 09151-2, together with a marginal effect of genotype for total rearing after vehicle administration. No other behaviors differed significantly between the genotypes.

DA Uptake in Synaptosomes. Previous studies had shown that DA D2-mediated processes were central to regulation of the dopaminergic arbor (Parish et al., 2001, 2002b), but in the light of the normal D2 receptor binding and functionality in $\alpha_4(-/-)$ mice, we turned our attention to the DAT. Like the D2 DA receptor, the DAT was considered a potential regulatory molecule because it is present on the presynaptic membrane and has a key role in the regulation of synaptic DA levels. Transport of [3H]DA was examined using synaptosomes from the dorsal striatum of WT and $\alpha_4(-/-)$ mice (Fig. 4). To facilitate comparison between experiments, the uptake at each time point was calculated as a percentage of untreated WT values. Synaptosomes from $\alpha_4(-/-)$ mice showed a significant impairment of [3H]DA uptake 7 min after the assay initiation, with an approximate reduction of 35% compared with the WT [percentage values: mean $\pm$ S.E.M., $\alpha_4(-/-)$ 65.9 $\pm$ 6.06%]. Treatment of the synaptosomes with quinpirole, a D2 receptor agonist, caused a significant increase in DA uptake in both WT and $\alpha_4(-/-)$ animals, indicating that the DAT/D2 receptor cooperative interaction is preserved in the $\alpha_4(-/-)$ mice. In fact, the effect of quinpirole was to increase DA uptake by proportionally the same amount (approximately 30%) in both genotypes. This is the third piece of evidence that D2 DA receptor signaling is indeed normal in $\alpha_4(-/-)$ mice. Treatment of synaptosomes with mazindol, a DAT inhibitor, greatly reduced DA uptake in both WT and $\alpha_4(-/-)$ animals (14.5 $\pm$ 2.6 and 18.03 $\pm$ 1.5%, respectively), indicating that the transport of DA into the synaptosomes occurs primarily through the action of DAT.

To further define the characteristics of DA uptake in $\alpha_4(-/-)$ mice, a time course was conducted, and [3H]DA transport was measured in synaptosomes from 2 to 25 min after assay initiation (Fig. 5A). The $\alpha_4(-/-)$ mice had significantly reduced DA uptake from the 7-min time point, as determined by repeated-measures ANOVA and Tukey post hoc tests. Uptake reached a plateau maximum at 15 min in the $\alpha_4(-/-)$ mice compared with 20 min in WT mice, indicating that synaptosomes isolated from the $\alpha_4(-/-)$ mice are unable to internalize DA to the same degree as WT-derived synaptosomes.

To determine differences in DA uptake kinetics between WT and $\alpha_4(-/-)$ mice, a range of DA concentrations was incubated with synaptosomes derived from each genotype at the 5-min time point (Fig. 5B). V_{max} and K_m values were derived from nonlinear regression (Table 3). These results show that whereas the maximal rate of uptake (V_{max}) was not significantly different between genotypes, the K_m values show that affinity of DAT for the [3H]DA substrate was significantly reduced in $\alpha_4(-/-)$ mice compared with WT.

[125I]RTI-55 Binding to DAT on Brain Slices. DAT autoradiography was undertaken to further investigate the reduced DAT functionality identified in our synaptosome uptake studies. Slide-mounted sections of CPu from WT and $\alpha_4(-/-)$ mice were assessed for DAT binding using autoradiography with [125I]RTI-55 (Fig. 6). The conditions used

TABLE 2

D1 and D2-class receptor binding in WT and $\alpha_4(-/-)$ mice

Number in parentheses represents the number of animals investigated Student t tests were used for analysis of all parametric data and Mann-Whitney test for nonparametric data.

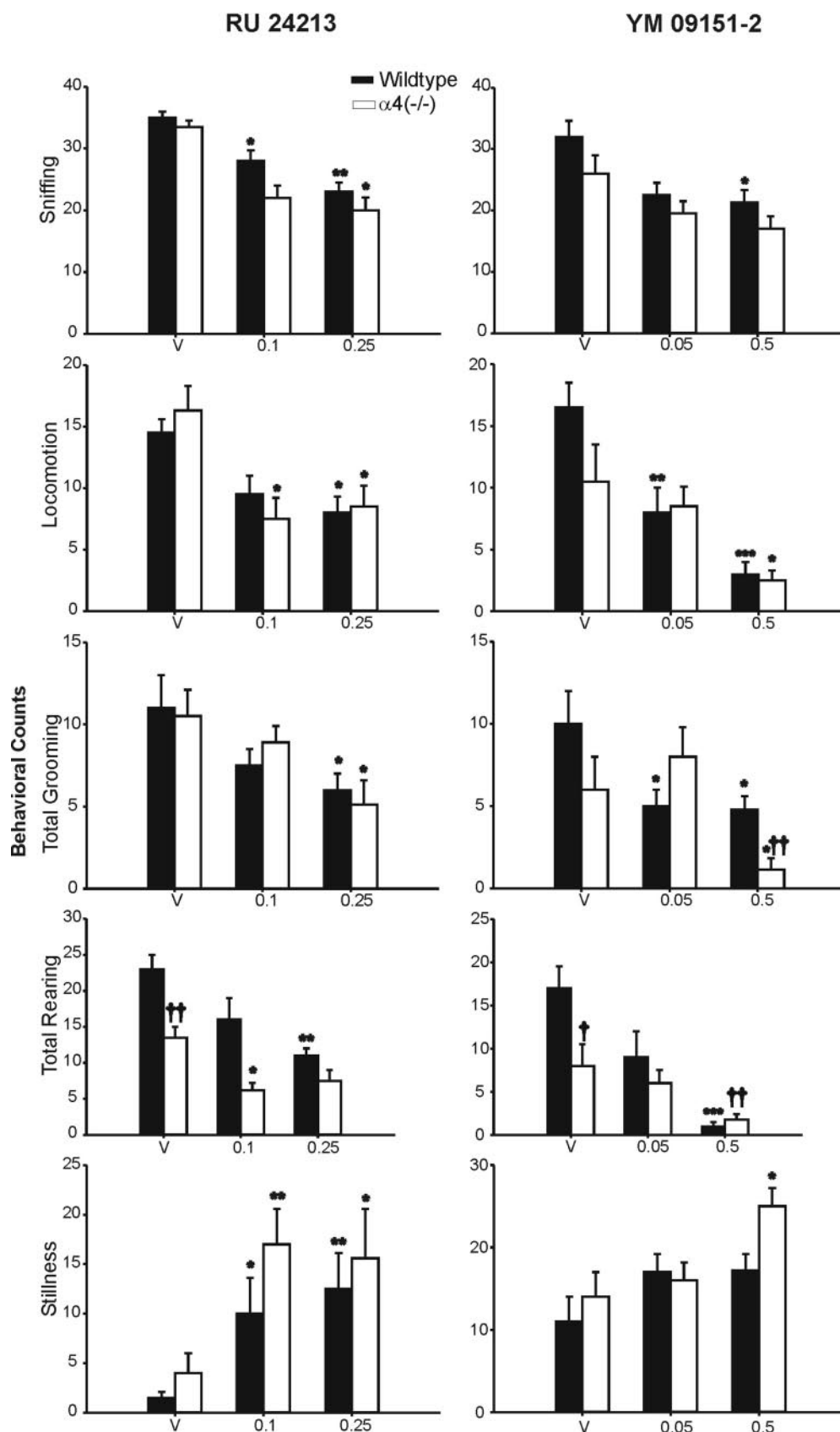|	D1-Class Receptor Binding		D2-Class Receptor Binding	
	WT ($n = 7$)	$\alpha_4(-/-)$ ($n = 11$)	WT ($n = 7$)	$\alpha_4(-/-)$ ($n = 11$)
		fmol/mg		
Striatum	5185 $\pm$ 220	4804 $\pm$ 425	390 $\pm$ 51	448 $\pm$ 45
Nucleus accumbens	4229 $\pm$ 171	3830 $\pm$ 351	269 $\pm$ 5	293 $\pm$ 23
Olfactory tubercle	4585 $\pm$ 240	4213 $\pm$ 475	271 $\pm$ 2	265 $\pm$ 19
Substantia nigra	1559 $\pm$ 136	1486 $\pm$ 115	67 $\pm$ 6	62 $\pm$ 10

Fig. 3. Left , sniffing, locomotion, total grooming, total rearing, and stillness were assessed after administration of vehicle (V) and RU 24213 (0.1 and 0.25 mg/kg) in WT (■) and $\alpha_4(-/-)$ mice (□). Right, behavioral assessments in WT and $\alpha_4(-/-)$ mice after administration of YM 09151-2 (0.05 and 0.5 mg/kg). Data are means ± S.E. of behavioral counts for each treatment group ($n = 8$) over the 1-h unhabituated state. ★, $p < 0.05$; ★★, $p < 0.01$; ★★★, $p < 0.001$ versus vehicle of the same genotype. †, $p < 0.05$; ††, $p < 0.01$ versus mutant.

effectively occluded binding to NET or SERT (note the lack of cortical binding). Densitometric analysis of striatal binding was divided into the four quadrants of the CPu. The apparent density of [^{125}I]RTI-55 binding to slices was reduced in the CPu in $\alpha_4(-/-)$ mice compared with WT, being statistically significant in the dorsolateral, ventromedial, and ventrolateral regions (Fig. 6). This change may reflect a reduction in the affinity or in the number of striatal DAT molecules.

Discussion

We assessed the ability of the α_4 nAChR subunit to regulate arbor size of dopaminergic neurons by comparing responses of WT and $\alpha_4(-/-)$ mice to long-term exposure to cocaine, amphetamine, nicotine, and haloperidol and after lesioning. We found that $\alpha_4(-/-)$ mice were unable to modulate their terminal arbor in response to drugs or after lesioning. There are a number of plausible explanations for the failure of cocaine, amphetamine, and haloperidol to modulate DA arbor size in $\alpha_4(-/-)$ mice. We have evidence to suggest that within the dopaminergic system there exist particular DA thresholds, such that whereas DA levels remain within a specific range, terminal arbor sizes remain unchanged. However, when DA concentrations decrease below a particular threshold, neurons initiate sprouting and produce more terminals, thereby resulting in normalization of DA delivery. On the other hand, when DA concentrations exceed a given level, neurons are triggered to retract and prune their fibers to reduce terminal contacts and DA delivery (Parish et al., 2001, 2002b). We propose that this process of nigrostriatal arbor remodeling requires intact and functional D2 DA receptors and DAT, either of which could respond to synaptic DA, yet-uncharacterized downstream intracellular signaling systems, and the participation of non-neuronal cells such as astroglia, microglia, and cytokines (Parish et al., 2002a).

The synaptosome studies indicate that activation of the D2 DA receptor results in a 30% increase in DA transport in both genotypes, indicating that D2 receptor signaling is normal in $\alpha_4(-/-)$ mice. Although limited to short-term pharmacological responsiveness, our behavioral studies indicate normal functioning of pre- and postsynaptic D2 DA receptors. In support of the behavioral data, there were no significant differences in D2 class receptor binding in the striatum,

nucleus accumbens, olfactory tubercle, and substantia nigra between the drug-naive WT and $\alpha_4(-/-)$ mice. The various data, taken together, suggest that not only is the D2 DA receptor functional in $\alpha_4(-/-)$ mice, it also implies that disturbances with $\alpha_4\beta_2$ nAChR pathway can override D2 DA receptor regulation of sprouting.

An alternative explanation for our results is that DAT functionality may be altered in $\alpha_4(-/-)$ mice. Altered DAT pharmacology has been demonstrated in new dopaminergic terminals (Stanic et al., 2003b). DA uptake by synaptosomes isolated from the reinnervated striatum was shown to be markedly impaired. In the current study, the reduction of DA uptake in $\alpha_4(-/-)$ mice is likely to be an underestimate of the degree of functional impairment of DAT activity, because this figure was not corrected for the fact that $\alpha_4(-/-)$ mice had an increase of 17% in terminal density in the striatum. Implicit in the argument that the DAT may have altered function in $\alpha_4(-/-)$ mice is that presynaptic DAT molecules and nAChRs are functionally coupled. Nicotine has been reported to increase DA clearance in the nucleus accumbens in anesthetized rats (Hart and Ksir, 1996). A recent study has investigated the effects of nicotine on DA concentration in the ventral striatum of rats when injected with cocaine or the stimulant methylphenidate. The effect of nicotine coadministration was additive at low cocaine or methylphenidate doses but became synergistic at higher concentrations, implying a cooperative interaction between DAT and nAChR-mediated processes in dopaminergic neurons in vivo (Gerasimov et al., 2000). Nicotinic receptor-mediated modulation of DAT function was assessed directly using in vivo voltammetry (Middleton et al., 2004). Subcutaneous nicotine was shown to enhance DA clearance in the striatum and medial prefrontal cortex. Furthermore, the effect of nicotine on DA clearance could be inhibited by subcutaneous mecamylamine.

Autoradiography with [^{125}I]RTI-55, under conditions selectively labeling DAT sites, indicated a reduction in apparent binding density in the striatum of $\alpha_4(-/-)$ mice compared with WT. Functional analysis of [^{3}H]DA uptake kinetics revealed a reduction in the affinity of DAT for the substrate. In addition, the time course of DA transport in synaptosomes from $\alpha_4(-/-)$ mice reaches a plateau at a reduced capacity.
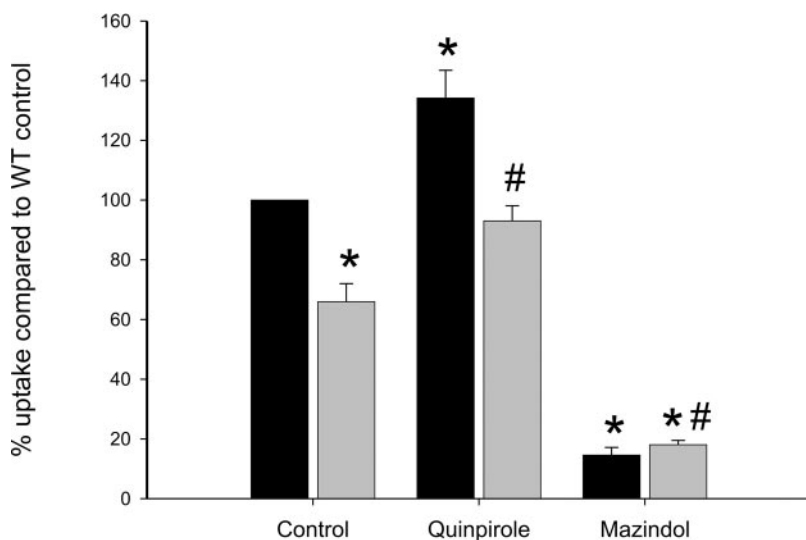

Fig. 4. A histogram of the uptake of [^{3}H]DA into striatal synaptosomes prepared from WT (■) and $\alpha_4(-/-)$ (▨) mice after 7-min incubation. Synaptosomes were treated with 10 μM quinpirole or mazindol before the addition of [^{3}H]DA. The uptake of DA was significantly reduced in $\alpha_4(-/-)$ mice; however, the activation of the D2 dopamine receptor by quinpirole significantly increased DA uptake in both genotypes. Treatment of synaptosomes with the DAT blocker mazindol reduced DA uptake. *, significantly different from WT control; #, significantly different from $\alpha_4(-/-)$ mouse control, $p < 0.05$.

As a group, therefore, these data provide strong evidence for a functional impairment of DAT in $\alpha_4(-/-)$ mice. In vivo microdialysis on an independently generated line of $\alpha_4(-/-)$ mice (Marubio et al., 2003) has shown that baseline levels of extracellular DA are increased and that locomotor responses of $\alpha_4(-/-)$ mice to modest doses of cocaine are increased. Both of these observations would be consistent with our own observations of reduced baseline DAT functionality in our line of $\alpha_4(-/-)$ mice. Despite a large body of literature on the role of nicotinic receptors in general and the α_4 nAChR subunit in particular on the regulation of DA release (Salminen et al., 2004), the in vivo microdialysis study of Marubio and colleagues (2003) and our own observations of heightened anxiety-like behavior, locomotor activity (Ross et al., 2000), and impaired DAT functionality suggest that defective DA reuptake is a major downstream functional consequence of α_4

nAChR subunit gene ablation. Indeed, the present biochemical data would indicate a reduced capacity of DAT in $\alpha_4(-/-)$ mice, noted both in terms of reduced transport and altered profile.

We found previously in rats and mice (and confirmed again in this study) that terminal density was maintained within the normal range until the number of SNpc neurons were
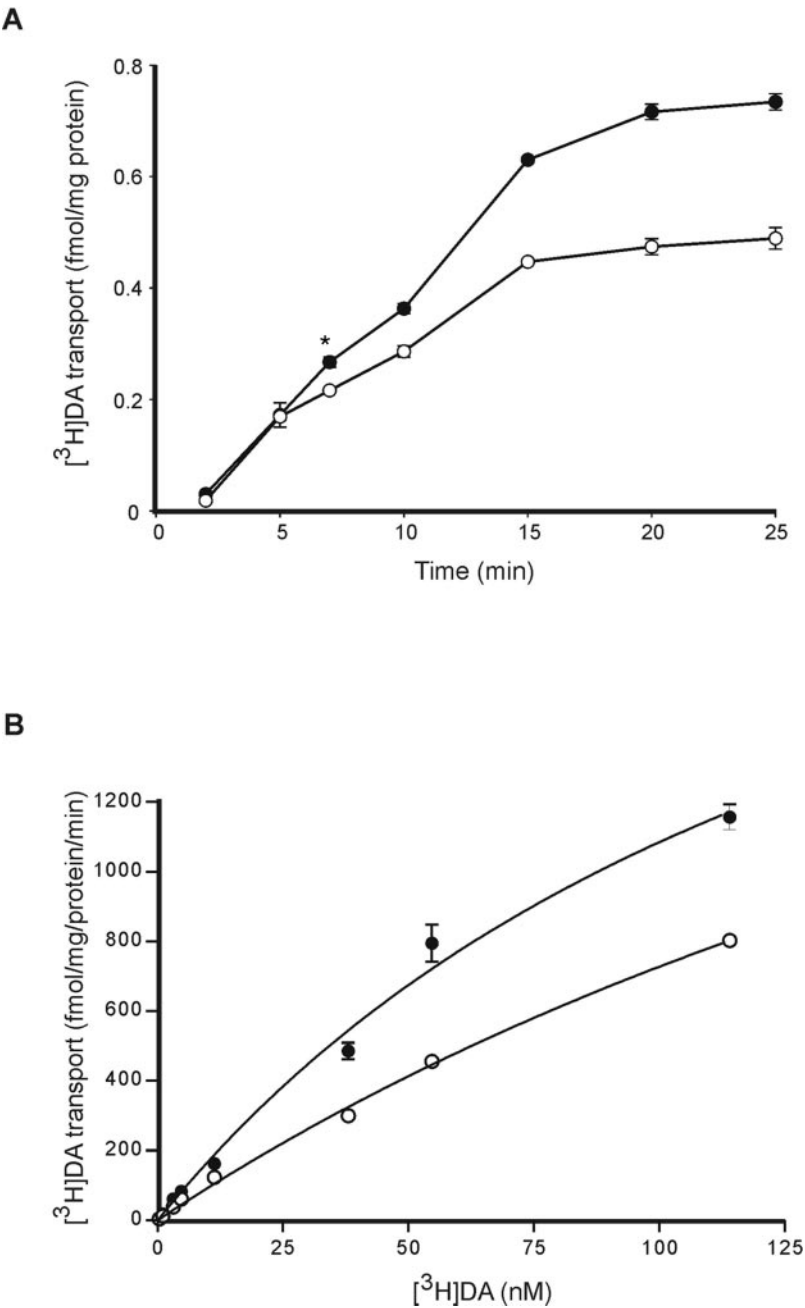
TABLE 3
[^{3}H]DA uptake in WT and $\alpha_4(-/-)$ mice
V_{max} is defined as the maximal rate of [^{3}H]DA uptake under the present experimental conditions.**, $p \leq 0.01$, significantly different from WT values, unpaired t test.

	K_m	V_{max}
	nM	*fmol/mg protein/min*
WT	174.3 ± 15.6	3083.7 ± 188.5
$\alpha_4(-/-)$	292.8 ± 19.3**	3058.0 ± 114.4

Fig. 5. Uptake of [^{3}H]DA into striatal synaptosomes derived from WT and $\alpha_4(-/-)$ mice. Synaptosomes were prepared from WT (■) or $\alpha_4(-/-)$ (○) mice. A, the uptake of [^{3}H]DA (10 nM) into synaptosomes was examined over time. Note that DA uptake was significantly different from the 7-min time point (∗, $p < 0.05$). B, the uptake of a range of [^{3}H]DA concentrations after 5 min. The K_m and V_{max} values are shown in Table 3. Synaptosomes derived from $\alpha_4(-/-)$ mice showed impaired uptake.
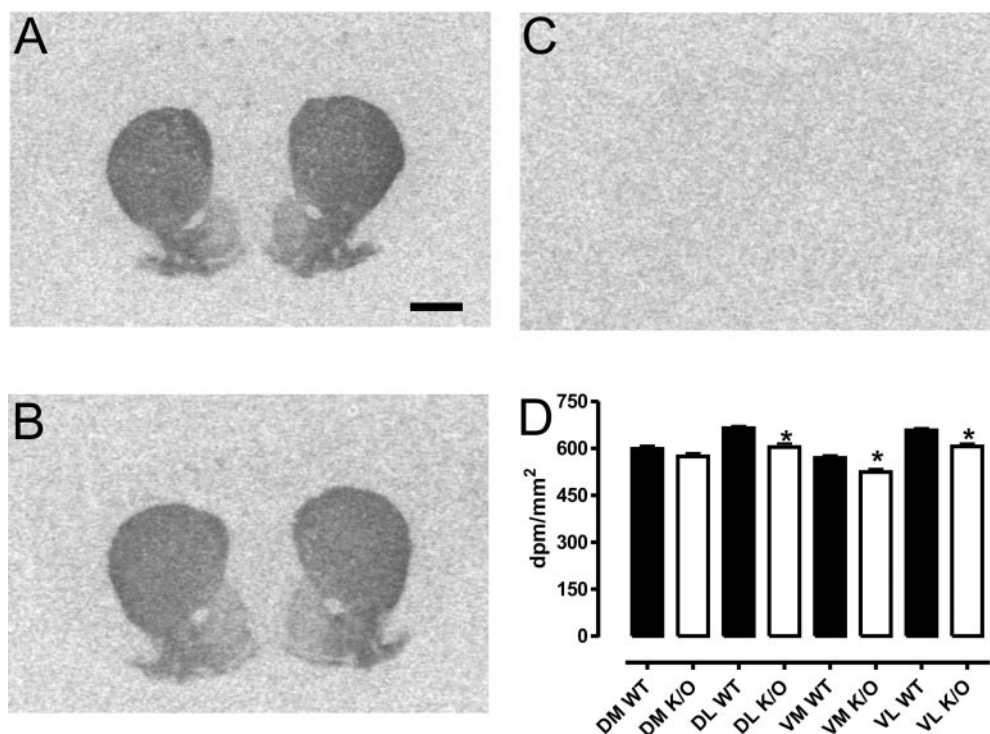
Fig. 6. Autoradiographic analysis of [^{125}I]RTI-55 binding to the CPu of WT (A) and $\alpha_4(-/-)$ (B) mice. Binding was abolished in the presence of GBR 12935 (10 μM) (C). Densitometric analysis of CPu quadrants (D) indicated reduced apparent binding density in dorsomedial (DM), dorsolateral (DL), ventromedial (VM), and ventrolateral (VL) quadrants of $\alpha_4(-/-)$ mice (open symbols) compared with WT (closed symbols) ($n = 5$–6 mice per genotype). $\star$, $p < 0.05$ one-way ANOVA.

reduced by approximately 75% (Parish et al., 2001). The size of the terminal arbors seems to have increased progressively in WT mice until lesions reached approximately 75%; beyond this point, remaining neurons could compensate no further, hence the decreased density. The findings were quite different in $\alpha_4(-/-)$ mice. After lesions in $\alpha_4(-/-)$ mice, terminal density fell progressively in proportion with the size of the lesion, suggesting that the size of the terminal arbor did not increase to compensate for the effect of the lesion. The molecular mechanism of compensatory sprouting after partial lesioning may be similar to that underlying modulation of arbor size with pharmacological manipulation. The effect of impaired DAT functioning in sprouting after lesioning could be tested in heterozygous DAT knockout mice (Jones et al., 1998) and in DAT knockdown mice (Zhuang et al., 2001), although in both of these models, potential compensatory changes in D2 autoreceptor functionality (i.e., desensitization) may complicate the interpretation of results.

There are a number of potential mechanisms underpinning the putative interaction between nAChR-mediated processes and DAT. First, nicotinic receptor stimulation increases DAT mRNA in the midbrain (Li et al., 2004); therefore, nicotinic receptor knockout mice may have reduced DAT levels. This mechanism may be especially relevant, because the α_4 nAChR subunit containing heteromeric nAChRs represents the majority of functional nicotinic receptors on the dopaminergic somatodendritic compartment (Champtiaux et al., 2003). Second, there is a potential interaction between PKC activity and DAT expression on the membrane. Up-regulated PKC activity results in DAT redistribution from the plasma membrane, in which it is active, to the endosomal compartment, in which it is inactive (Melikian and Buckley, 1999). Because nAChRs can regulate PKC activity, DAT membrane trafficking is subject to control by nAChRs. The net effect of targeted deletion of the α_4 subunit on PKC activity in our system is difficult to predict because signaling through

nAChRs can either increase or decrease PKC activity, depending on the preparation [e.g., increase in synaptosomes (Soliakov and Wonnacott, 2001) and in rat prefrontal cortex slices (Drew and Werling, 2003) and decrease in vivo (Sun et al., 2004)] and the sensitization state of the nAChRs (Sun et al., 2004). The situation is further complicated by the fact that the α_4 nAChR subunit itself is phosphorylated by PKC (Pacheco et al., 2003), and the phosphorylation status influences the sensitization state of the molecule (Fenster et al., 1999).

Finally, because α_4 and α_6 heteromeric receptors are both represented on dopaminergic terminals (Zoli et al., 2002; Champtiaux et al., 2003; Salminen et al., 2004) it is possible for α_6-mediated compensatory changes known to occur in $\alpha_4(-/-)$ mice (i.e., a decrease of 75% in α_6 expression) (Champtiaux et al., 2003) to contribute to the phenotype of $\alpha_4(-/-)$ mice.

In summary, this study validates the pivotal role of the α_4 subunit of the nAChR in regulating the DA arbor size. We show that mice with targeted deletion of the α_4 subunit of the nAChR have defective DAT function. The functional impact of this is likely to be substantial in brain regions densely innervated by DA terminals such as the striatum because active transporter-mediated DA reuptake remains the primary mechanism of DA inactivation (Jones et al., 1998; Benoit-Marand et al., 2000).

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