Cholinergic Modulation of Locomotion and Striatal Dopamine Release Is Mediated by $\alpha 6\alpha 4^*$ Nicotinic Acetylcholine Receptors

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Dopamine (DA) release in striatum is regulated by firing rates of midbrain DA neurons, striatal cholinergic tone, and nicotinic ACh receptors (nAChRs) on DA presynaptic terminals. DA neurons selectively express $\alpha 6^* $nAChRs, which show high ACh and nicotine sensitivity. To help identify nAChR subtypes that control DA transmission, we studied transgenic mice expressing hypersensitive $\alpha 6^{19^*} $ receptors. $\alpha 6^{19^*} $ mice are hyperactive, travel greater distance, exhibit increased ambulatory behaviors such as walking, turning, and rearing, and show decreased pausing, hanging, drinking, and grooming. These effects were mediated by $\alpha 6\alpha 4^* $ pentamers, as $\alpha 6^{19^*} $ mice lacking $\alpha 4 $ subunits displayed essentially normal behavior. In $\alpha 6^{19^*} $ mice, receptor numbers are normal, but loss of $\alpha 4 $ subunits leads to fewer and less sensitive $\alpha 6^* $ receptors. Gain-of-function nicotine-stimulated DA release from striatal synaptosomes requires $\alpha 4 $ subunits, implicating $\alpha 6\alpha 4\beta^2 $ nAChRs in $\alpha 6^{19^*} $ mouse behaviors. In brain slices, we applied electrochemical measurements to study control of DA release by $\alpha 6^{19^*} $ nAChRs. Burst stimulation of DA fibers elicited increased DA release relative to single action potentials selectively in $\alpha 6^{19^*} $, but not WT or $\alpha 4\beta\kappa/\alpha 6^{19^*} $, mice. Thus, increased nAChR activity, like decreased activity, leads to enhanced extracellular DA release during phasic firing. Bursts may directly enhance DA release from $\alpha 6^{19^*} $ presynaptic terminals, as there was no difference in striatal DA receptor numbers or DA transporter levels or function in vitro. These results implicate $\alpha 6\alpha 4\beta^2 $ nAChRs in cholinergic control of DA transmission, and strongly suggest that these receptors are candidate drug targets for disorders involving the DA system.

Introduction

Dopamine (DA) transmission, both at cell bodies situated in the midbrain and at presynaptic terminals in the striatum and prefrontal cortex, is tightly regulated by cholinergic systems. Patients with neural disorders involving DA signaling may benefit from therapies that augment or reduce cholinergic control over DA release. For example, stimulating DA release from residual DA terminals with nicotinic ACh receptor (nAChR) agonists may be an effective treatment for Parkinson’s disease (PD) (Quik and McIntosh, 2006). Conversely, the use of antagonists or weak partial agonists at midbrain nAChRs may be an effective strategy for smoking cessation drugs such as varenicline (Coe et al., 2005).

Developing and further refining such agonists, however, requires a better understanding of the cell biology and physiology of particular nAChR subtypes.

Midbrain DA neurons and other cells in local and long-range circuits that directly synapse onto DA neurons express distinct nAChR subtypes on their cell surface. GABAergic cells in the substantia nigra pars reticulata and the ventral tegmental area (VTA) express $\alpha 4\beta^2 $ ( $\alpha 4^* $ indicates that other nAChR subunits may be present) nAChRs receptors with at least two sensitivities to ACh and nicotine (Nashmi et al., 2007; Xiao et al., 2009). Midbrain DA neurons express as many as seven distinct nAChR subtypes or stoichiometric variants: ($\alpha 4)_3(\beta 2)_2$, ($\alpha 4)_2(\beta 2)_3$, $\alpha 4\beta 2\alpha 5$, $\alpha 6\beta 2$, $\alpha 6\beta 2\beta 3$, $\alpha 6\alpha 4\beta 2$, and $\alpha 6\alpha 4\beta 2\beta 3$ (Gotti et al., 2005; Salminen et al., 2007). $\beta 2$ subunits are strictly required for nAChR function in midbrain DA neurons (Picciotto et al., 1998; Maskos et al., 2005); $\alpha 4 $ subunits are expressed in nearly all DA neurons, and loss of $\alpha 4 $ subunits eliminates 80% of surface nAChRs in these cells (Azam et al., 2002; Marubio et al., 2003; Nashmi et al., 2007; Xiao et al., 2009). Functional $\alpha 6^* $ nAChRs are highly and selectively expressed in DA neurons (Drenan et al., 2008b), where they assemble with $\beta 2$, and often, $\beta 3$ and/or $\alpha 4 $ subunits (Champtiaux et al., 2002, 2003; Cui et al., 2003; Drenan et al., 2008a).

Previous studies indicate that $\alpha 6\alpha 4\beta 2\beta 3 $ nAChR pentamers are important regulators of DA release. For instance, studies on...
At time (San Diego Instruments). Ambulation events were recorded when locomotor activity was measured with an infrared photobeam activity cage system (2.5 and 5.0 m). Absolute DA concentrations for release were determined using these standards. We hold to convention and indicate pulse trains by 1p, 2p, etc., individual pulses within a train are indicated as p1, p2, etc. For paired-pulse experiments, responses to 1p and 2p (100 Hz) stimulation were recorded. Release due to the second of a pair of pulses (p2) was isolated by subtracting the response to a single pulse (p1, i.e., 1p) from the response to a 2p train.

Dopamine release from striatal synaptosomes. After a mouse was killed by cervical dislocation, its brain was removed and placed immediately on the ice-cold platform and brain regions were dissected. Tissues from each mouse were homogenized in 0.5 mL of ice-cold 0.32 M sucrose buffer with 5 mM HEPES, pH 7.5. A crude synaptosomal pellet was prepared by centrifugation at 12,000 × g for 20 min. The pellets were resuspended in “uptake buffer”: 128 mM NaCl, 2.4 mM KCl, 3.2 mM CaCl2, 1.2 mM KH2PO4, 1.2 mM MgCl2, 2.4 mM CaCl2, 18 mM NaHCO3, and 11 mM glucose. Coordinates for recordings in the caudate/putamen (CPU) were within the following range: (0.0 to +1.0 mm from bregma, −3.0 to −4.0 mm from the surface, and −1.0 to −3.0 mm from the midline).

Carbon fiber (7 μm, unsized; Goodfellow) electrodes were fabricated at Caltech using anodic electodeposition of paint (Schulte and Chow, 1996; Akopian et al., 2008). Voltammetry data were acquired with a Multiclamp 700A amplifier (Molecular Devices). The command micro-electrode potential was −400 mV (against a Ag/AgCl reference electrode), and the potential was scanned from −400 mV to +1000 mV to −400 mV at a rate of 300 mV/ms; ramp frequency was 10 Hz. Dopamine release was elicited by stimulating DA fibers with a bipolar stimulating electrode (FHC), placed 200–300 μm from the surface, and their baseline level of activity was resettled by cardiac perfusion with oxygenated (95% O2/5% CO2) ice-cold glycerol-based artificial CSF (gACSF) containing 252 mM glycerol, 1.6 mM KCl, 1.2 mM NaH2PO4, 1.2 mM MgCl2, 2.4 mM CaCl2, 18 mM NaHCO3, and 11 mM glucose. Following perfusion, brains were removed and retained in gACSF (0–4°C). Coronal slices (300 μm) were cut with a microslicer (DTK-1000; Ted Pella) at a frequency setting of 9 and a speed setting of 3.25. Brain slices were allowed to recover for at least 1 h at 32°C in regular, oxygenated artificial CSF (ACSF) containing 126 mM NaCl, 1.6 mM KCl, 1.2 mM NaH2PO4, 1.2 mM MgCl2, 2.4 mM CaCl2, 18 mM NaHCO3, and 11 mM glucose. Coordinates for recordings in the caudate/putamen (CPU) were within the following range: (0.0 to +1.0 mm from bregma, −3.0 to −4.0 mm from the surface, and −1.0 to −3.0 mm from the midline).

Carbon fiber surface was exposed each recording day. Voltammetry protocols were initially run for 30 min to allow the background currents to settle to an optimal waveform, and were run continually throughout the day when stimulation protocols were not run. Background currents were subtracted from signals acquired during striatal stimulation to isolate transient neurotransmitter release events. DA release recovered fully within 2 min after stimulus trains, and trains were typically delivered at 3 min intervals. Each day, baseline currents were calibrated against DA standards in solution (2.5 and 5.0 μM DA). Absolute DA concentrations for release responses in slices were determined using these standards. We hold to convention and indicate pulse trains by 1p, 2p, etc.; individual pulses within a train are indicated as p1, p2, etc. For paired-pulse experiments, responses to 1p and 2p (100 Hz) stimulation were recorded. Release due to the second of a pair of pulses (p2) was isolated by subtracting the response to a single pulse (p1, i.e., 1p) from the response to a 2p train.

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[uptake buffer containing 0.1% bovine serum albumin (BSA), 1 μM atropine, and 1 μM nomifensine at 0.7 ml/min for 10 min, or buffer for 5 min followed by buffer with 50 mM αCtMII]. Aliquots of synaptosomes were then exposed to nicotine in buffer for 20 s to stimulate release of [3H]dopamine followed by buffer. Fractions (~0.1 ml) were collected for 4 min into 96-well plates every 10 s starting from 1 min before stimulation, using a Gilson FC204 fraction collector with a multichannel micropump (Gilson). Radioactivity was determined by scintillation counting using a 1450 MicroBeta Trilux scintillation counter (PerkinElmer Life Sciences) after addition of 0.15 ml of Optiphase SuperMix scintillation cocktail. Instrument efficiency was 40%.

Data were analyzed using SigmaPlot 5.0 for DOS. Perfusion data were plotted as counts per minute (cpm) versus fraction number. Fractions collected before and after the peak were used to calculate baseline as a single-exponential decay. The calculated baseline was subtracted from the experimental data. Fractions that exceeded baseline by 10% or more were summed to give total released cpm and then normalized to baseline to give units of release [cpm – baseline]/baseline] (Salminen et al., 2007).

Dopamine uptake. Synaptosomes were prepared as described for DA release assays. Synaptosomes were suspended in uptake buffer (described above) at a concentration of 8 ml per mouse for dorsal striatum (ST) and 4 ml per mouse for olfactory tumor (OT). For each well, synaptosomes (25 μl) were incubated in uptake buffer (final volume 100 μl) with either 0.05 μM or 1 μM [3H]dopamine at 22°C for 5 min. Nomifensine (100 μM) was used for blank determination. The reaction was terminated by filtration onto a two layer filter consisting of one sheet of GFA/E glass fiber filter paper (Whatman) and another sheet of 0.5% polyethylenimine using an Inotech Cell Harvester (Inotech). Radioactivity was measured using a Wallac 1420 MicroBeta Trilux scintillation counter (PerkinElmer Life and Analytical Sciences) after addition of 50 μl of Optiphase SuperMix scintillation cocktail (PerkinElmer Life Sciences) to each well of a 96-well counting plate.

Statistical analysis. Home cage locomotor ambulation data were analyzed for significance with a one-way ANOVA with Tukey post hoc analysis (Drenan et al., 2008b). For automated mouse behavior analysis (AMBA) behavioral experiments, data were analyzed for significance with a two-tailed Wilcoxon rank-sum test (nonparametric) (Steele et al., 2007). nAChR ligand binding, DA receptor ligand binding, DAT ligand binding, and DAT uptake data were analyzed for significance with a t test within a brain region (ST or OT) and within a genetic background (WT or αKO). Synaptosomal DA release curves were fit to the Michaelis-Menten equation (Hill coefficient = 1), and Rmax and EC50 values were evaluated for statistical significance using a χ test within a brain region (ST or OT) and within a genetic background (WT or αKO). Voltammetry data were evaluated for significance with a t test within a genotype, where 1p and 4p data were compared.

Materials. All radioactive compounds were obtained from PerkinElmer. αCtMII was synthesized as previously described (Cartier et al., 1996). Ultra centrifugation grade sucrose was obtained from Fisher Chemicals. Sigma-Aldrich was the source for the following compounds: ascorbic acid, ars...
(lights off #1: α4KO, 6057 ± 1392 ambulations/15 min; α4KO/α6L9S, 7821 ± 2225 ambulations/15 min; p > 0.05) (lights on: α4KO, 2174 ± 354 ambulations/15 min; α4KO/α6L9S, 2056 ± 504 ambulations/15 min; p > 0.05) (lights off #2: α4KO, 6328 ± 2014; α4KO/α6L9S, 7021 ± 2187; p > 0.05 (Fig. 1B, C), revealing that α4 subunits are required for the pronounced phenotype seen in α6L9S mice.

α6L9S mice are hypersensitive to nicotine, demonstrating locomotor activation (rather than locomotor suppression seen in WT mice) in response to low doses of nicotine and other nAChR ligands (Drenan et al., 2008b). In response to 0.2 mg/kg nicotine, α4KO/α6L9S mice showed only partial locomotor activation over a 30 min time course when compared to the response seen in α6L9S mice (peak locomotor response: α6L9S, 93 ± 14 ambulations/min; α4KO/α6L9S, 53 ± 4 ambulations/min) (Fig. 1D). To further study this effect, we constructed nicotine dose–response relations for WT, α6L9S, α4KO, and α4KO/α6L9S mice. As seen previously, α6L9S mice demonstrated locomotor activation across a range of nicotine concentrations that do not significantly affect WT mice (Fig. 1F). WT mice responded only to the highest tested dose (1.5 mg/kg) with suppression of locomotor activity, and locomotor activity of α4KO animals was not altered in response to the concentrations tested (Fig. 1E), consistent with previous results (Tapper et al., 2007). The potency and efficacy of nicotine were consistently reduced in α4KO/α6L9S mice compared to α6L9S mice (Fig. 1E), suggesting the emergence of alterations in the sensitivity and/or number of α6* receptors when α4 subunits are removed.

To better understand the altered behavior in α6L9S mice and the effect of α4 subunit removal, we extended the behavioral analysis of these mice using AMBA with a video-based behavioral recognition system. This system has previously been validated on several mutant mouse strains that exhibit altered behavior, including mouse models of Huntington’s disease and prion diseases (Steele et al., 2007, 2008). Mice are singly housed in their standard cage while the AMBA software analyzes a video feed of the mouse’s activity (Fig. 2A). To verify that AMBA analyses correspond with the break-beam break (Fig. 1), we analyzed distance traveled with AMBA. Consistent with the break-beam data (Fig. 1) and our previous work (Drenan et al., 2008b), there was a dramatic and statistically significant increase in the mean distance traveled in α6L9S mice compared to WT littermates (WT, 281 ± 28 m/24 h; α6L9S, 2469 ± 535 m/24 h; p < 0.05) (Fig. 2B). In contrast, we observed no statistically significant increase in distance traveled when α4KO mice were compared to α4KO/α6L9S mice, although there was a trend toward increased distance with the presence of the α6L9S BAC transgene (α4KO, 344 ± 62 m/24 h; α4KO/α6L9S, 1203 ± 568 m/24 h; p > 0.05) (Fig. 2B). It is not clear why the α6L9S behavioral hyperactivity phenotype is highly variable and only partially penetrant (Fig. 2B), but genetic mosaicism or epigenetic changes are possible explanations.

We used the power of AMBA to discriminate among several specific behaviors in the genotypes under investigation. Specific behaviors were extracted by the HomeCageScan 3.0 software and expressed as a percentage of total time recorded (percentage of total frames). Compared to WT littermates, α6L9S mice exhibited robust differences in median values for a variety of behaviors. In particular, α6L9S mice showed the greatest difference from WT control mice in behaviors of activity or ambulatory movement, including “jump,” “walk,” “stretch,” “turn,” and “rear” motion.
(Fig. 2C). We also noted a large increase in the percentage of time α6* mice exhibited “high-velocity,” erratic behaviors that are poorly resolved at the video camera’s frame rate and by the AMBA algorithms (Steele et al., 2007). As expected, the α6* mice traveled greater distance than WT controls. Distance traveled over 24 h was calculated and plotted for each WT, α6* KO, and α4KO/α6* mouse. C, α4 subunits are required for hypersensitive α6* nAChRs to alter mouse behavior. In each of the 4 mouse genotypes indicated, AMBA was used to calculate the percentage of total time that each mouse spent exhibiting each of 17 specific behaviors. Median values for each behavior were compared between genotypes (α6* vs WT, α4KO/α6* vs α4KO), and the fold change in median for each behavior of either α6* or α4KO/α6* over its respective control group (WT or α4KO) is expressed via a heat map with yellow indicating an increase in median value and blue indicating a decrease in median value. D, E, Behavioral differences between α6* and WT control mice largely require α4 nAChR subunits. For either α6* versus WT (D) or α4KO/α6* versus α4KO (E), the percentage of total time during 24 h that mice spent exhibiting each indicated behavior is plotted. Two y-axes are used because several behaviors occupy a large fraction of total time and all behaviors could not be displayed effectively on a single y-axis. Data are expressed as mean ± SEM. The number of mice in each group was as follows: WT, n = 8; α6* KO, n = 21; α4KO, n = 13; α4KO/α6*, n = 14. All statistically significant comparisons are indicated. *p < 0.05, **p < 0.01, ***p < 0.001.
primary effect of the L9' S mutation is to augment the function of α6* receptors, it is possible that the behavioral differences seen between WT and α6* L9' S or between α4KO and α4KO/α6 L9' S are due to differences in receptor expression. To address this, we quantified the number of α4β2* and α6β2* binding sites using [125I]epli- batidine in striatum and olfactory tuber-
cles of these mouse lines. We separately
dissected the ST and the OT, thus roughly
separating the nigrostriatal and mesolimbic
DA pathways (Ikemoto, 2007). We
used cCtxMII to separate α6β2* (MII-
resistant) from α6β2* (MII-sensitive),
the two predominant subtypes in this prepa-
rati
ces in either of these brain
2* receptors in the (non-
4KO/S mice.

Thus, the apparent increase in

seen in behavioral assays comparing α4KO versus α4KO/α6 L9' S mice, but not WT versus α6* L9' S mice.

Functional aspects of presynaptic α4* and α6* nAChRs are effectively studied using agonist-evoked DA release from striatal synaptosomes (Grady et al., 2002). To study native nAChRs on DA terminals, we prepared synaptosomes from WT, α6* L9' S, α4KO, and α4KO/α6 L9' S mice and measured nicotine-stimulated DA release. To separate responses depend-
ent on α6-containing from α4(non-α6)-containing nAChRs, we blocked α6* nAChRs with cCtxMII as described previously (Salminen et al., 2004, 2007). In measurements on OT tissue (Fig. 4A), we noted a significant increase in Rmax and a substantial reduction in Ecp at α6* OT nAChRs (Fig. 4B) comparing WT and α6 L9' S mice (WT: Rmax = 4.6 ± 0.3 units, Ecp = 0.075 ± 0.025 μM; α6 L9' S: Rmax = 1.47 ± 0.7 units, Ecp = 0.015 ± 0.004 μM; Rmax: p < 0.001; Ecp: p < 0.05) (Fig. 4G,H), consistent with previous results (Drenan et al., 2008b). We noted a corresponding decrease in Rmax at (non-α6) α6β2* nAChRs (Fig. 4C) in α6 L9' S OT (WT: Rmax = 14.5 ± 0.5 units; α6 L9' S: Rmax = 7.4 ± 0.4 units; p < 0.001) (Fig. 4G), but also saw a minor, yet significant decrease in Ecp at these receptors as well (WT: Ecp = 0.53 ± 0.09 μM; α6 L9' S: Ecp = 0.24 ± 0.06 μM; p < 0.05) (Fig. 4H).

Thus, the apparent increase in α4β2* receptor (MII-resistant) numbers (Fig. 3F) in α6 L9' S OT had no observed functional consequence. Results for this genotype comparison were very similar in ST (Fig. 4D) at α6β2* (Fig. 4E) (WT: Rmax = 3.2 ± 0.3 units; α6 L9' S: Rmax = 6.6 ± 0.5 units; p < 0.001) (WT: Ecp = 0.031 ± 0.017 μM; α6 L9' S: Ecp = 0.016 ± 0.006 μM; p > 0.05) and (non-α6) α4β2*
Figure 4. Dopamine release from striatal presynaptic terminals is restored to WT levels in α4KO/α6L9S mice. A–E, Hypersensitive α6+ nAChR-mediated DA release in α6L9S OT (A–C) and ST (D–F) is reversed by α4 nAChR subunit deletion. OT or ST from WT, α6L9S, α4KO, and α4KO/α6L9S mice was dissected and synaptosomes were prepared. DA release was stimulated with a range of nicotine concentrations (1 μM, 3 μM, 10 μM, 30 μM, 100 μM, 300 μM, 1 μM, 10 μM, 100 μM, 300 μM), and a concentration–response relation for each mouse line is shown for total release (A, D). To determine the relative contribution of α6+ and non-α6+ receptors, synaptosome samples were incubated with α6α4MII (50 nM). α6α4MII-sensitive (α6+ dependent) release is shown in B and E, and α6α4-independent release is shown in C and F. G, Quantification of DA release measured in A–F. 4KO, and α4KO/α6L9S mice in ST and OT. R_{max} (G) and EC_{50} (H) for α6α4MII-sensitive (α6α4)2* and α6α4MII-resistant (α4α4)2* DA release in ST and OT is shown. Data are expressed as mean ± SEM. The number of mice in each group was as follows: WT, n = 10; α6L9S, n = 7; α4KO, n = 9; α4KO/α6L9S; n = 10. All statistically significant comparisons are indicated.*p < 0.05, **p < 0.01, ***p < 0.001. 

To study the effect of α4 subunit deletion on α6+ nAChR function, we compared DA release results between α4KO and α4KO/α6L9S mice. Previous results with α4KO mice showed decreased R_{max} and increased EC_{50} compared to WT (Salminen et al., 2007), results that were replicated in this study. For α6+ nAChRs in OT tissue (Fig. 4B), we noted only a slight increase in R_{max} for α4KO/α6L9S mice when compared to WT. For α4KO mice (α4KO: R_{max} = 8.33 ± 0.13 units; α4KO/α6L9S: R_{max} = 2.42 ± 0.24 units; p < 0.001) (Fig. 4G). There was a significant reduction in EC_{50} for α4KO/α6L9S compared to α4KO (α4KO: EC_{50} = 0.97 ± 0.15 μM; α4KO/α6L9S: EC_{50} = 0.63 ± 0.15 μM; p < 0.001) (Fig. 4H), but both values were at least one order of magnitude greater than for either WT or α6L9S mice. Results in ST (Fig. 4E) were similar for the α4KO background (α4KO: R_{max} = 3.63 ± 0.15 μM and reduction currents at −200 mV, respectively (Fig. 5B, inset). Responses to pure DA were linear at our CFEs in the range of detection expected from striatal slices (0–5 μA) (Fig. 5B). Electrically evoked DA release from dorsal striatum in slices revealed a voltammogram very similar to that of pure DA (Fig. 5C, inset). 

Burst firing selectively increases DA release in α6L9S dorsal striatum

The results obtained with synaptosomes suggest that the hyperactivity observed in α6L9S mice, which is eliminated or substantially reduced upon removal of α4 subunits, is due to differences in ACh-modulated DA release in striatum. Midbrain DA neurons exhibit both tonic and phasic firing profiles, and the transition to phasic firing is governed in part by pontine cholinergic inputs to DA neuron cell bodies (Lança et al., 2000). DA neuron firing properties, in turn, strongly influence dopamine release. Therefore, we reasoned that α6L9S mice may demonstrate alterations in DA release as a result of α6+ nAChR hypersensitivity in DA neurons.

To study striatal DA release in a preparation with intact DA fibers and functional ACh-modulation of DA release, we measured DA overflow with fast-scan cyclic voltammetry at carbon fiber electrodes (CFEs) in coronal striatal slices (Fig. 5A). We first calibrated the CFEs with solutions of DA dissolved in ACSF. Catecholamines such as DA and 5-HT produce characteristic oxidation and reduction voltage peaks (Zhou et al., 2005). DA detection was confirmed at our CFEs based on oxidation currents at +600 mV and reduction currents at −200 mV, respectively (Fig. 5B, inset).
α6L9S and α4KO/α6L9S mice. Because α6L9S mice display a locomotor phenotype (Figs. 1, 2), we focused our experiments on dorsal striatum, the region most strongly implicated in motor function and the region dissected for the ST samples in the synaptosome experiments. We measured absolute DA release following single-pulse (1p) stimulation and four pulse (4p, 100 Hz) burst stimulation, which resembles the greatest firing rate for DA neurons in mammals (Hyland et al., 2002; Bayer and Glimcher, 2005). In WT slices, we noted a marked short-term synaptic depression for 4p stimulation compared to 1p [Fig. 5C (left)], which is consistent with other reports using acutely cut slices (Schmitz et al., 2002; Rice and Cragg, 2004; Zhang and Sulzer, 2004; Exley et al., 2008). α6L9S slices displayed a substantial reduction in peak DA release compared to WT 1p stimulation (WT 1p: 0.92 ± 0.06 μM; α6L9S 1p: 0.34 ± 0.05 μM; p < 0.001) (Fig. 5C, middle panel). Unlike WT, however, α6L9S slices did not show synaptic depression, as peak DA responses following 4p stimulation were significantly greater than for 1p stimulation (WT: 1p = 0.92 ± 0.06 μM; 4p = 1.04 ± 0.11 μM; p > 0.05) (α6L9S; 1p = 0.34 ± 0.05 μM; 4p = 0.94 ± 0.19 μM; p < 0.01) (Fig. 5C, right). Interestingly, we noted only a slight reduction in peak 1p DA release in α4KO/α6L9S mice compared to WT (WT 1p: 0.92 ± 0.06 μM; α4KO/α6L9S 1p: 0.70 ± 0.08 μM; p < 0.05) [Fig. 5C (right)]. Removal of α4 subunits from α6L9S mice recapitulated the synaptic depression phenomenon seen in WT slices, as 4p stimulation was slightly but not significantly elevated compared to 1p stimulation in α4KO/α6L9S slices (α4KO/α6L9S; 1p = 0.70 ± 0.08 μM; 4p = 0.97 ± 0.11 μM; p > 0.05) [Fig. 5C (right)]. Although neither 1p nor 4p peak DA responses in α6L9S slices exceeded those in WT slices, we noted substantial alterations to the DA response waveform (Fig. 5C, middle panel) in experiments with α6L9S tissue. To better understand the effect on DA waveforms caused by the α6L9S mutation, we analyzed the responses in Figure 5C for 10–90% rise time and decay time constant (τ). In WT slices, there was no significant difference between 1p and 4p for rise time or τ (rise time: WT 1p = 317 ± 14 ms; WT 4p = 375 ± 33 ms; p > 0.05) (τ: WT 1p = 650 ± 82 ms; WT 4p = 842 ± 268 ms; p > 0.05) (Fig. 5E, F). In contrast, rise time following 1p stimulation in α6L9S slices was substantially elevated compared to 1p (or 4p) responses in WT slices (α6L9S; 1p = 617 ± 70 ms; WT: 1p = 317 ± 17 ms; p < 0.01) (α6L9S; 1p = 617 ± 70 ms; WT: 4p = 375 ± 33 ms; p < 0.01) (Fig. 5E). Further, there was an additional increase in rise time for 4p responses compared to 1p in α6L9S slices (α6L9S; 1p = 617 ± 70 ms; 4p = 804 ± 28 ms; p < 0.05) (Fig. 5E). τ values were also significantly increased for 4p versus 1p stimulation in α6L9S slices (α6L9S; 1p = 824 ± 164 ms; 4p = 1990 ± 221 ms; p < 0.01) (Fig. 5F), suggesting that extracellular DA persists in α6L9S dorsal striatum in response to a burst of action potentials. α4 subunits are apparently required for the effect seen in α6L9S mice, as there was no significant effect on rise time or decay time constant in α4KO/α6L9S mice when we compared 1p and 4p stimulation (rise time: α4KO/α6L9S 1p = 305 ± 36 ms; α4KO/α6L9S 4p = 362 ± 59 ms; p > 0.05) (τ: α4KO/α6L9S 1p = 504 ± 131 ms; α4KO/α6L9S 4p = 1019 ± 271 ms; p > 0.05) (Fig. 5E, F). We did notice significant differences in the maximum decay slope of the 1p and 4p responses among the three genotypes studied. WT DA responses exhibited the fastest DA uptake (i.e.,
significant comparisons are indicated. **

paired-pulse depression in genotypes are shown in (see Discussion). The prolonged decay kinetics seen in 1p and 4p Normal DA transporter and DA receptor number and function in

Altered DA release probability in Drenan et al.

0.00007

D in

followed by washout and quantification by scintillation counting. Raw binding values are indicated as fmol/mg protein for mazin

3.0 and 4.0 

S mice were alternately stimulated with 1 pulse (1p) or 2 pulses (2p). 1p waveforms are measured directly as p1 data, while the p2 waveform was isolated by subtracting average 1p waveforms from average 2p waveforms over several trials. Average p1 and p2 waveforms for the indicated genotypes are shown in A. Calibration: 300 nm DA, 500 ms. B, Increased paired-pulse ratio in 6L9 S versus WT controls and 4KO/6L9 S mice. For each dataset in A, paired-pulse ratio was calculated as p2/p1. Data are expressed as mean ± SEM. The number of recording sites for each group was as follows: WT, n = 9; 6L9 S, n = 11; 4KO/6L9 S, n = 9. All statistically significant comparisons are indicated. **p < 0.01.

Figure 6. Altered DA release probability in 6L9 S dorsal striatum. A, Reduced initial release probability and elimination of paired-pulse depression in 6L9 S striatum. Coronal slices from WT, 6L9 S, and 4KO/6L9 S mice were alternately stimulated with 1 pulse (1p) or 2 pulses (2p). 1p waveforms are measured directly as p1 data, while the p2 waveform was isolated by subtracting average 1p waveforms from average 2p waveforms over several trials. Average p1 and p2 waveforms for the indicated genotypes are shown in A. Calibration: 300 nm DA, 500 ms. B, Increased paired-pulse ratio in 6L9 S versus WT controls and 4KO/6L9 S mice. For each dataset in A, paired-pulse ratio was calculated as p2/p1. Data are expressed as mean ± SEM. The number of recording sites for each group was as follows: WT, n = 9; 6L9 S, n = 11; 4KO/6L9 S, n = 9. All statistically significant comparisons are indicated. **p < 0.01.

Figure 7. Normal DA transporter and DA receptor number and function in 6L9 S and 4KO/6L9 S mice. A–C, Normal DA-related pharmacological parameters in 6L9 S and 4KO/6L9 S mice as determined by DAT and DA receptor ligand binding. Brain regions (OT and ST) from WT, 6L9 S, 4KO, and 4KO/6L9 S mice were dissected and labeled with the indicated ligand followed by washout and quantification by scintillation counting. Raw binding values are indicated as fmol/mg protein for mazindol (DAT ligand), SCH23390 (D1 receptor ligand), and raclopride (D2 receptor ligand). D, E, Normal DA transporter function in 6L9 S and 4KO/6L9 S mice. Synaptosomes were prepared as in Figure 2, but [3H]dopamine uptake was assayed at 30 min (D) or 1 min (E) (DA), for all genotypes. F, Ratio of DAT activity at 1 μM (“high”) versus 50 nM (“low”) (DA). Data are ratios of the values in D and E. Data are expressed as mean ± SEM. The number of mice in each group was as follows: n = 9; 6L9 S, n = 11; 4KO, n = 8; 4KO/6L9 S, n = 8. All statistically significant comparisons are indicated. *p < 0.05.

greatest negative-going maximum decay slope) (1p, −0.00100 ± 0.00007 μl/ms; 4p, −0.00120 ± 0.00017 μl/ms), 6L9 S responses were the slowest (1p, −0.00006 ± 0.00003 μl/ms; 4p, −0.00028 ± 0.00002 μl/ms), and 4KO/6L9 S responses exhibited an intermediate value (1p, −0.00043 ± 0.00008 μl/ms; 4p, −0.00053 ± 0.00011 μl/ms). Maximum decay slope indicates the speed of DA uptake at a saturating concentration of DA, thus these differences may reflect changes in DA transporter V_{max} (see Discussion). The prolonged decay kinetics seen in 1p and 4p DA release responses in 6L9 S slices (Fig. 5F) suggested that the integrated DA response in these mice may differ more substantially than peak measurements would suggest. Area-under-the-curve measurements (Zhang et al., 2009) for 1p and 4p responses in the three genotypes under study were normalized to WT 1p responses. Unlike WT and 4KO/6L9 S slices, 6L9 S slices showed a significantly elevated 4p DA response compared to 1p stimulation (WT: 1p = 1.0 ± 0.06; 4p = 1.0 ± 0.14; p > 0.05) (6L9 S: 1p = 0.5 ± 0.1; 4p = 1.6 ± 0.3; p < 0.001) (4KO/6L9 S: 1p = 0.8 ± 0.1; 4p = 1.1 ± 0.2; p > 0.05) (Fig. 5G).

To further isolate and study the effect of the 6L9 S mutation and DA neuron firing frequency on evoked DA release, we conducted paired-pulse experiments. In dorsal striatum, DA release displays marked short-term synaptic depression when pulses are paired at frequencies from 1 to 100 Hz (Cragg, 2003; Rice and Cragg, 2004; Zhang and Sulzer, 2004). In nucleus accumbens, however, initial release probability is lower but paired-pulse depression is not as severe (Cragg, 2003; Zhang et al., 2009). In WT dorsal striatum, we also measured significant synaptic depression comparing a first pulse (p1; 0.89 ± 0.21 μM DA) with a second (p2; 0.09 ± 0.05 μM DA) (Fig. 6A, left). In contrast, experiments in 6L9 S slices revealed no synaptic depression, as peak [DA], was approximately equivalent for p1 (0.20 ± 0.03 μM DA) and p2 (0.19 ± 0.05 μM DA) (Fig. 6A, middle). This lack of synaptic depression was reflected in a significantly increased paired-pulse ratio for 6L9 S mice versus WT (WT: 0.13 ± 0.06; 6L9 S: 0.67 ± 0.15; p < 0.01) (Fig. 6B). Again, the gain in synaptic facilitation afforded by the 6L9 S mutation was completely reversed by loss of α4 subunits: 4KO/6L9 S p1 peak DA (0.85 ± 0.13 μM DA) returned to WT levels, p2 responses (0.14 ± 0.10 μM DA) were similar to those seen in WT, and the paired-pulse ratio for 4KO/6L9 S release (0.12 ± 0.09) was comparable to WT (Fig. 6A, B). Thus, together, these electrochemical experiments suggest that patterns of tonic and phasic activity in DA neuron firing in 6L9 S mice results in substantially altered patterns of DA release, from reduced DA release during tonic firing to augmented synaptic DA during phasic firing.

DA release and uptake, as well as a variety of mouse behaviors, can be influenced by alterations in components of the DA system such as D1-class and D2-class dopamine receptors and the dopamine transporter (DAT). In particular, DA synaptic lifetime in ST is strongly influenced by the rate of uptake by DAT (Giros et al., 1996; Zhuang et al., 2001; Rice and Cragg, 2008). Further, mice lacking D2 receptors have a decreased peak and duration of DA release in response to single pulses (Schmitz et al., 2002). To determine whether any substantial alterations in these compo-
nents of the DA system might account for our behavioral or physiological results, we measured DAT and D₁ and D₂ DA receptors by ligand binding in tissue homogenates. In ST, there were no differences in DAT levels as determined by [3H]-mazindol binding (Fig. 7A), whereas we did note an increase in DAT levels in α6⁹⁹S OT versus WT OT (WT = 1083 ± 102 fmol/mg protein; α6⁹⁹S = 1503 ± 191 fmol/mg protein; p < 0.05) (Fig. 7A). For D₁-class receptors, there was no statistically significant difference in [3H]SCH23390 ligand binding for any genotype comparison in either OT or ST (Fig. 7B). Similarly, there was also no significant difference in D₂-class receptors (measured with [3H]raclopride) in OT or ST for any genotype comparison (Fig. 7C). We took note of the overall reduction in DAT and D₂ receptor levels in the ventral relative to dorsal striatum, which is consistent with a recent report (Lammel et al., 2008). To directly measure DAT function in vitro, we performed DAT uptake assays using synaptosomes similar to those used for DA release. Using established methods for determining DAT function (Grady et al., 2002), we estimated DAT K_M to be 80 nM DA (data not shown), which was consistent with previous studies (Girod et al., 1996; Parish et al., 2005). At a [DA]_o close to the K_M (50 nM), and at a maximal [DA]_o, (1 μM), there was little effect on DA uptake for any genotype comparison (Fig. 7D, E). Interestingly, these two concentrations of DA roughly correspond to the EC₅₀ for activation of D₂ and D₁ receptors, respectively. There was a significant difference between α4KO and α4KO/α6⁹⁹S only in OT but not ST, and only at 1.0 μM [DA]_o (α4KO = 6.4 ± 0.8 pmol of DA/μg protein/min; α4KO/α6⁹⁹S = 4.3 ± 0.4 pmol of DA/μg protein/min; p < 0.05) (Fig. 7E). There was no difference in either brain region for any genotype comparison for the 1.0 μM [DA]_o (~20 × K_M)/0.05 μM [DA]_o (~K_M) ratio of uptake rates (Fig. 7F).

Discussion

Overall, these data are consistent with the idea that cholinergic control over DA release exerts greater influence in α6⁹⁹S mice due to α6⁺ nAChR hypersensitivity (Fig. 8). Similar to other in vitro electrochemistry studies, we show that in WT dorsal striatum, single stimulus pulses or brief trains in DA fibers elicit similar levels of DA release due to short-term synaptic depression (Fig. 8A). Several reports show that decreased presynaptic nAChR activity, via desensitization, pharmacological blockade, or decreased ACh release from striatal cholinergic interneurons, can alter short-term depression and enhance the difference in efficacy between single pulses and bursts (Zhou et al., 2001; Rice and Cragg, 2004; Zhang and Sulzer, 2004; Threlfall et al., 2010). In this study, increased nAChR function can also enhance this difference. In mice with hypersensitive α6⁹⁹S⁺ nAChRs, single APs combine with nAChR activity to result in reduced DA release (Fig. 8B). In these mice, however, released DA is present for longer periods (Fig. 5E,F), paired-pulse depression (PPD) is reduced (Fig. 6B), and bursts of APs lead to more overall DA release (Fig. 5G).

In some respects, our electrochemistry results in dorsal striatum, where DA release following single-pulse stimulation in α6⁹⁹S slices is reduced and PPD in relieved relative to WT and α4KO/α6⁹⁹S tissue, resembles experiments where nAChR activity is blocked before DA fiber stimulation. Indeed, reducing striatal nAChR activity with desensitizing applications of nicotine or antagonists such as dihydro-β-erythroidine (DHβE) or mecamylamine results in less DA release in response to a single stimulus (Zhou et al., 2001; Rice and Cragg, 2004; Zhang and Sulzer, 2004). These studies also demonstrate that PPD is substantially reduced when nAChR activity is eliminated by these applications. We see a similar result in α6⁹⁹S slices relative to WT: reduced peak DA release following single stimulations (Figs. 5C, 6A). We also noted that the DA release profile in α6⁹⁹S dorsal striatum is similar in some respects to the profile in DA D₂ receptor KO mice. In these mice, as in ours, DA release following single-pulse stimulation is reduced relative to WT mice (Schmitz et al., 2002). Interestingly, our results in α6⁹⁹S dorsal striatum more closely resemble the normal pattern of DA release in ventral striatum of mice and primates. Electrochemical recordings in nucleus accumbens shell of mice (Zhang et al., 2009) or primates (Cragg, 2003) reveals that ventral regions of the striatum are characterized by reduced peak DA release following single stimulation, and paired-pulse results showing little PPD or even paired-pulse facilitation. This is thought to occur via differences in Ca²⁺ availability in presynaptic terminals of these two brain areas. Thus, in some respects our DA release results in α6⁹⁹S mice are quite similar to various studies, including reports using pharmacological agents or other genetic mutations to manipulate the DA system, as well as reports in different species.

In other respects, however, our electrochemical results differ from previous studies. For example, whereas many studies involving elimination of nAChR activity demonstrate that brief trains (e.g., 4p) result in augmented peak DA release compared to identical stimulations under control conditions (Rice and Cragg, 2004; Exley et al., 2008; Zhang et al., 2009), we did not see an
increase in peak DA relative to WT slices following 4p stimulus protocols (Fig. 5D). Most importantly, though, DA waveforms in α6L9S dorsal striatum display novel kinetics. We report that DA responses in α6L9S dorsal striatum are slower to reach peak concentration (Fig. 5E), and also decay more slowly following cessation of the stimulus (Fig. 5F). These effects on DA kinetics are not seen in studies where nAChR activity is altered with pharmacological agents, and are typically only apparent when the DA transporter is genetically eliminated/reduced, or pharmacologically blocked (Giros et al., 1996; Zhuang et al., 2001; Cragg, 2003; Senior et al., 2008; Zhang et al., 2009).

What caused the altered DA release pattern seen in α6L9S dorsal striatum? ACh release from cholinergic interneurons, perhaps augmented by electrical stimulation of the tissue, may play a role in α6L9S altered DA release, likely via differences in receptor sensitivity to ligand (Fig. 4H). Although intrinsic differences in ACh release probability (and consequent α6 nAChR activation) by cholinergic interneurons in intact α6L9S versus WT mice are possible, WT and α6L9S cholinergic interneurons have equivalent firing rates and largely similar membrane properties (Drenan et al., 2008b). We cannot rule out that compensatory or adaptive mechanisms, operating as the animals mature, might cause the reduced 1p release and/or prolonged release kinetics. For instance, α6L9S presynaptic terminals may have undergone a change in their channel repertoire that renders them hypersensitive to repetitive stimulation. Changes in the ratio of α4*(non-α6)/α6 function, which are present in α6L9S mice, may be such an adaptation. Alternatively, the reduction in peak DA release following single and/or burst stimulation may be an adaptive response to the prolonged release kinetics, similar to DAT KO mice (Giros et al., 1996).

In a previous study, we showed that hypersensitive α6L9S channels are tonically active in DA neuron somata (Drenan et al., 2008b), suggesting that tonic cholinergic input to midbrain DA areas may depolarize DA neurons and/or increase their firing rate in vivo. Furthermore, ligand-gated cation currents through α6L9S channels in these cells are prolonged compared to their WT counterparts (Drenan et al., 2008b), which reflects the significant increase in single channel burst duration in nAChRs with L9S mutations (Labarca et al., 1995). In striatum, presynaptic and/or axonal α6L9S channels may similarly depolarize DA fibers/terminals due to these changes at the single channel level. Axonal depolarization can significantly alter the action potential waveform (Shu et al., 2006; Kole et al., 2007), and tonic depolarization of the presynaptic terminal would be expected to reduce Vmax for the DA transporter (Huang et al., 1999), resulting in slower DA reuptake following release. Indeed, in PC12 cells expressing DAT and nAChRs, nAChR activation resulted in membrane depolarization and a reduction in DAT uptake velocity (Huang et al., 1999). Although our DAT binding and functional experiments show that there are no intrinsic differences in DAT expression levels or enzymatic function in vitro in the four genotypes examined (Fig. 7), they do not address the possibility that α6L9S channels depolarize DA fibers and reduce DAT function in slices and/or in vivo. Alternatively, α6L9S DA fibers may have normal resting membrane potentials but undergo prolonged depolarization (due to activation of α6L9S channels by ACh) during electrical stimulation of the slice, resulting in extended periods of slowed DAT uptake velocity during the time scale of our measurements.

Future studies are needed to fully determine the mechanism that gives rise to the alterations in DA release seen in α6L9S dorsal striatum. It is important to note that, unlike in vitro slice experiments, studies of DA release in vivo are characterized by much smaller DA signals (Michael and Wightman, 1999) and do not show substantial synaptic depression (Chergui et al., 1994). Thus, it will be important to study DA neuron firing and DA release in awake, behaving α6L9S mice in future studies. Nevertheless, these alterations along with changes in excitability of DA neuron cell bodies in midbrain are sufficient to cause the complex behavioral phenotypes that were observed in α6L9S mice. We suggest that α6L9S mouse behavioral hyperactivity results from both (1) a more efficacious action of ACh on DA neuron dendrites and somata harboring hypersensitive α6α4β2* channels, resulting in more phasic firing, and (2) DA fibers in striatum that have more effective frequency filtering (Exley and Cragg, 2008; Exley et al., 2008), where activation of α6L9S channels by ACh permits DA fibers to diminish their output in response to tonic firing yet augment DA release in response to bouts of phasic firing. In contrast to other recent studies that amplify and isolate phasic DA neuron firing to alter DA-dependent behaviors with gene knock-outs (Zwiebel et al., 2009) and optogenetics (Tsai et al., 2009), we show that the cholinergic system can be manipulated to alter DA release and modify a variety of behaviors in mammals.

More specifically, this study demonstrates the importance of α6α4β2* nAChRs in governing cholinergic control over DA release, and reinforces the idea that compounds capable of selectively targeting α6α4β2* nAChRs could be useful in manipulating the DA system in disorders such as nicotine dependence, PD, schizophrenia, or attention deficit hyperactivity disorder. Such drug therapies that regulate dopaminergic output via modification of somatic and/or presynaptic α6α4β2* receptors may give better outcomes than DA agonists or DA replacement therapies, as the natural spatiotemporal activity patterns of DA neurons would be better preserved. For example, stimulating DA release with α6α4β2* selective compounds in PD may alleviate dyskinesias associated with L-Dopa therapy if coadministration of the nAChR compound allowed the clinician to reduce the necessary dose of L-Dopa (Quik and McIntosh, 2006; Quik et al., 2008; Bordia et al., 2010).

References


