0026-895X/10/7805-971–980$20.00
MOLECULAR PHARMACOLOGY
Vol. 78, No. 5
67561/3637778
Mol Pharmacol 78:971–980, 2010
Printed in U.S.A.

$\alpha6\beta2^*$ and $\alpha4\beta2^*$ Nicotinic Receptors Both Regulate Dopamine Signaling with Increased Nigrostriatal Damage: Relevance to Parkinson's Disease

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Received July 19, 2010; accepted August 23, 2010

ABSTRACT

Nicotinic receptors (nAChRs) are important modulators of dopaminergic transmission in striatum, a region critical to Parkinson's disease. The nAChRs mainly involved are the $\alpha6\beta2^*$ and $\alpha4\beta2^*$ subtypes. Lesion studies show that the $\alpha6\beta2^*$ receptor is decreased to a much greater extent with nigrostriatal damage than the $\alpha4\beta2^*$ subtype raising the question whether this latter nAChR population is more important with increased nigrostriatal damage. To address this, we investigated the effect of varying nigrostriatal damage on $\alpha6\beta2^*$ and $\alpha4\beta2^*$ receptor-modulated dopamine signaling using cyclic voltammetry. This approach offers the advantage that changes in dopamine release can be observed under different neuronal firing conditions. Total single-pulse-evoked dopamine release decreased in direct proportion to declines in the dopamine transporter and dopamine uptake. We next used α-conotoxinMII and mecamylamine to understand the role of the $\alpha4\beta2^*$ and $\alpha6\beta2^*$ subtypes in release. Single-pulse–stimulated $\alpha6\beta2^*$ and $\alpha4\beta2^*$ receptor dopamine release decreased to a similar extent with increasing nigrostriatal damage, indicating that both subtypes contribute to the control of dopaminergic transmission with lesioning. Total burst-stimulated dopamine release also decreased proportionately with nigrostriatal damage. However, the role of the $\alpha4\beta2^*$ and $\alpha6\beta2^*$ nAChRs varied with different degrees of lesioning, suggesting that the two subtypes play a unique function with burst firing, with a somewhat more prominent and possibly more selective role for the $\alpha6\beta2^*$ subtype. These data have important therapeutic implications because they suggest that drugs directed to both $\alpha4\beta2^*$ and $\alpha6\beta2^*$ nAChRs may be useful in the treatment of neurological disorders such as Parkinson's disease.

Introduction

The striatal dopaminergic and cholinergic systems play an overlapping role in regulating central nervous system functions linked to motor activity relevant to diseases such as to Parkinson's disease (Zhou et al., 2002; Exley and Cragg, 2008; Quik et al., 2009). The extensive colocalization of dopamine and acetylcholine in the nigrostriatal pathway most likely underlies the functional interdependence of these two systems. For example, acetylcholine regulates neuronal firing in dopamine cell bodies in the substantia nigra. It also modulates dopamine transmission in the striatum, where tonically active cholinergic interneurons provide a pulsed source of acetylcholine that interacts at nicotinic acetylcholine receptors (nAChR) on dopaminergic terminals (Zhou et al., 2001, 2002; Exley and Cragg, 2008; Livingstone and Wonnacott, 2009). A concerted action at these sites is probably responsible for the overall effect of nAChR activation on dopaminergic signaling and behaviors linked to dopaminergic transmission.

One major function of the nigrostriatal dopaminergic system is the control of motor activity, as is readily evident from the neurological deficits observed in Parkinson's disease. This debilitating movement disorder is characterized by rigidity, tremor, and bradykinesia, due to a marked degeneration of the nigrostriatal dopaminergic pathway (Davie, 2008). Accumulating evidence indicates that dopaminergic signaling may be affected by the nicotinic cholinergic system. Long-term nicotine administration is neuroprotective against nigrostriatal damage in Parkinsonian animal models (Quik et al., 2007b; Picciotto

This work was supported by the National Institutes of Health National Institute of Neurological Disorders and Stroke [Grants NS42091, NS59910]; the National Institutes of Health National Institute of Mental Health [Grant MH53631]; the National Institutes of Health National Institute of General Medical Sciences [Grant GM48677]; and the California Tobacco-Related Disease Research Program [Grant 17RT-0119].

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.

doi:10.1124/mol.110.067561.

ABBREVIATIONS: nAChR, nicotinic acetylcholine receptor; RTI-121, 3β-(4-iodophenyl)tropane-2β-carboxylic acid; 6-OHDA, 6-hydroxydopamine; α-CtxMII, α-conotoxinMII; $*$, possible presence of other nicotinic subunits in the receptor complex.

and Zoli, 2008) and improves L-DOPA-induced dyskinesias, a debilitating side effect of dopamine replacement therapy (Quik et al., 2007a, 2009; Bordia et al., 2008).

Nicotine most likely modulates nigrostriatal dopaminergic transmission through an action at nAChRs, the two major subtypes in the nigrostriatal pathway being the $\alpha4\beta2^*$ and $\alpha6\beta2^*$ nAChRs (Grady et al., 2007; Gotti et al., 2009; Livingstone and Wonnacott, 2009; Quik et al., 2009). The $\alpha6\beta2^*$ nAChRs seem to be exclusively expressed on dopaminergic neurons, whereas $\alpha4\beta2^*$ receptors are more widely distributed on presynaptic dopaminergic terminals and on postsynaptic glutamatergic, GABAergic, and serotonergic striatal neurons (Grady et al., 2007; Gotti et al., 2009; Livingstone and Wonnacott, 2009).

Dopaminergic neurons regulate function via tonic firing that involves single-pulse or low-frequency stimulation and also by phasic or burst firing that generally produces a greater dopamine response (Rice and Cragg, 2004; Zhang and Sulzer, 2004; Exley et al., 2008; Meyer et al., 2008; Perez et al., 2008a; Zhang et al., 2009a). Low-frequency firing is thought to play a pacemaker role to maintain dopaminergic tone, whereas phasic signaling may be involved in the initiation or execution of movement and other behaviors (Heien and Wightman, 2006; Sandberg and Phillips, 2009). Fast-scan cyclic voltametric studies have proved very useful in elucidating the contribution of nAChRs to tonic and phasic dopaminergic signaling. The $\alpha6\beta2^*$ receptor plays a prominent role in tonic dopamine release, controlling ~75% of nAChR-mediated release in striatum, whereas $\alpha4\beta2^*$ nAChRs have a greater role in the facilitation of striatal burst-stimulated dopamine release (Exley et al., 2008; Meyer et al., 2008; Perez et al., 2008a, 2009).

The goal of the present study was to understand the role of $\alpha4\beta2^*$ and $\alpha6\beta2^*$ nAChRs in regulating single-pulse and burst stimulated striatal dopamine signaling with progressive nigrostriatal damage. Fast-scan cyclic voltametric data show that the $\alpha6\beta2^*$ and $\alpha4\beta2^*$ subtypes are both important in the control of dopaminergic transmission throughout the neurodegenerative process, suggesting that drugs targeting either subtype may be of relevance for the treatment of neurodegenerative disorders such as Parkinson's disease.

Materials and Methods

Animal Model. Adult male Sprague-Dawley rats (250–270 g) from Charles River Laboratories, Inc. (Wilmington, DE) were housed two per cage under a 12-h light/dark cycle in a temperature-controlled room with free access to food and water. Starting 2 days after arrival, rats were unilaterally lesioned with 6-hydroxydopamine (6-OHDA) HCl (Sigma-Aldrich, St, Louis, MO) as described previously (Bordia et al., 2008). In brief, rats were initially exposed to 5% isoflurane anesthesia and maintained at 2% for the duration of the surgery. They were placed in a Kopf stereotaxic instrument (David Kopf Instruments, Tujunga, CA) and the location of bregma was determined. Burr holes were drilled through the skull at the following coordinates relative to bregma and the dural surface: 1) anteroposterior, bregma −4.4; lateral, midline 1.2; dorsoventral, dura −7.8; tooth bar at −2.4. 2) anteroposterior, bregma −4.0; lateral, midline 0.75; dorsoventral, dura −8.0; tooth bar at +3.4. 6-OHDA was dissolved in 0.02% ascorbic acid/saline and stereotaxically injected at each of these sites to achieve 4 to 12 μg total into the right-ascending, dopamine-fiber bundle. Infusion of 6-OHDA into the target area was over a 2-min period, with the cannula maintained at the site of injection for an additional 2 min before removal. After surgery, rats were administered buprenorphine (0.03 mg/kg s.c.) for postoperative pain. All procedures conformed to the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 1996) and were approved by the Institutional Animal Care and Use Committee.

Limb-Use Asymmetry Test. We used the forelimb asymmetry test as an index of motor function after nigrostriatal denervation. Exploratory behavior was analyzed 2 and 3 weeks after the 6-OHDA lesion as described previously in our laboratory (Bordia et al., 2008) and that of others (Schallert et al., 2000). Rats were placed in a transparent cage and evaluated for contralateral forelimb use for 5 min by a rater blinded to the treatment of the rat. Values are expressed as a percentage of total limb use.

Tissue Preparation. Rats were killed 4 to 5 weeks after the 6-OHDA lesion. The brain was quickly removed and chilled in ice-cold, preoxygenated (95% O_2/5% CO_2) physiological buffer containing 125 mM NaCl, 2.5 mM KCl, 1.2 mM NaH_2PO_4, 2.4 mM $CaCl_2$, 1.2 mM $MgCl_2$, 20 mM HEPES, 11 mM glucose, and 25 mM $NaHCO_3$, pH 7.4, as described previously (Perez et al., 2008a). Coronal corticostriatal slices (400 μm thick) were cut using a Vibratome (VT1000S; Leica Microsystems, Inc., Deerfield, IL) and incubated at room temperature in oxygenated buffer. The remaining portion of the brain, which contained the mid to posterior striatum, was quick-frozen in isopentane on dry ice immediately after the sections were removed, and stored at −80°C. Sections (8 μm) were prepared using a cryostat (Leica Microsystems, Inc., Deerfield, IL) at −20°C. Frozen sections were thaw-mounted onto Superfrost Plus slides (Thermo Fisher Scientific, Waltham, MA), air-dried and stored at −80°C for autoradiography.

Electrochemical Measurement of Dopamine Release. For the fast-scan cyclic voltammetry experiments, carbon fiber microelectrodes were constructed as described previously (Perez et al., 2008a). The electrode potential was linearly scanned from 0 to −400 to 1000 to −400 to 0 mV versus an Ag/AgCl reference electrode at a scan rate of 300 mV/ms (Zhou et al., 2001; Perez et al., 2008a). This triangular wave was repeated every 100 ms at a sampling frequency of 50 Hz. Current was recorded with an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA). Triangular wave generation and data acquisition were controlled by pClamp 9.0 software (Molecular Devices). Electrical stimulation was applied using a bipolar tungsten stimulating electrode (Plastics One, Roanoke, VA) connected to a linear stimulus isolator (WPI, Saratoga, FL) and triggered by a Master-8 pulse generator (A.M.P.I., Jerusalem, Israel). All electrode placements were made in the dorsal striatum with the aid of a stereomicroscope and micromanipulators. Background current was digitally subtracted and the peak oxidation currents were converted into concentration after postexperimental calibration of the carbon fiber electrode with a fresh solution of 1 μM dopamine in experimental buffer.

After a 2-h incubation period, the slice was transferred to a submersion recording chamber (Campden Instruments Ltd., Lafayette, IN), perfused at 1 ml/min with oxygenated physiological buffer at 30°C, and allowed to equilibrate for 30 min. Dopamine release from dorsal striatum was evoked by either a single, rectangular electrical pulse (4 ms) applied every 2.5 min or by a burst of four pulses at 30 or 100 Hz applied every 5 min, with a stimulus intensity that achieved 60% maximal release. The burst stimulation paradigm was chosen based on previous rodent studies, which showed that maximal effects of the drugs on nAChR-modulated responses occur at these frequencies (Rice and Cragg, 2004; Zhang and Sulzer, 2004). The recording sites were always restricted to the same area of the dorsal striatum to ensure consistency of the signals across animals. Total evoked release by both a single and a burst of pulses was first assessed in physiological buffer. NAChR-modulated release was assessed in the presence of 100 nM α-conotoxinMII (α-CtxMII) or 100 μM mecamylamine. These concentrations were chosen based on previous studies showing that they yielded maximal blockade of $\alpha6\beta2^*$

and $\alpha4\beta2$* nAChRs (Exley et al., 2008; Perez et al., 2009). Perfusion of the slice with α-CtxMII resulted in a maximal decrease in release within ~15 min and with mecamylamine by 10 min. Signals remained stable throughout data collection for each experimental condition. The reported effects on release with each antagonist represent the average of those signals obtained once a stable maximal response was established.

Dopamine Transporter Autoradiography. Binding to the dopamine transporter was measured using [^{125}I]RTI-121 (3β-(4-[^{125}I]iodophenyl)tropane-2β-carboxylic acid; 2200 Ci/mmol; PerkinElmer Life and Analytical Sciences, Waltham, MA), as described previously (Quik et al., 2003; Bordia et al., 2007). Thawed sections were preincubated twice for 15 min each at room temperature in 50 mM Tris-HCl, pH 7.4, 120 mM NaCl, and 5 mM KCl and then incubated for 2 h in buffer with 0.025% BSA, 1 μM fluoxetine, and 50 pM [^{125}I]RTI-121. Sections were washed at 0°C four times for 15 min each in buffer and once in ice-cold water, air-dried, and exposed for 2 days to Kodak MR film (PerkinElmer Life Sciences) with ^{3}H microscale standards (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Nomifensine (100 μM) was used to define nonspecific binding.

^{125}I-Epibatidine Autoradiography. Binding of ^{125}I-epibatidine (2200 Ci/mmol) was done as previously reported (Quik et al., 2003; Bordia et al., 2007). Slides were preincubated at 22°C for 15 min in buffer containing 50 mM Tris, pH 7.5, 120 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, and 1.0 mM $MgCl_2$. They were incubated for 40 min with 0.015 nM ^{125}I-epibatidine in the presence of α-CtxMII (300 nM) to define $\alpha4\beta2$* nAChRs. They were then washed, dried, and exposed to Kodak MR film with ^{3}H microscale standards for several days. Nonspecific binding was assessed in the presence of 100 μM nicotine and was similar to the film blank.

^{125}I-α-CtxMII Autoradiography. Binding of ^{125}I-α-CtxMII (specific activity, 2200 Ci/mmol) was done as reported previously (Quik et al., 2003; Bordia et al., 2007). Striatal sections were preincubated at room temperature for 15 min in binding buffer (144 mM NaCl, 1.5 mM KCl, 2 mM $CaCl_2$ 1 mM $MgSO_4$, 20 mM HEPES, and 0.1% bovine serum albumin, pH 7.5) plus 1 mM phenylmethylsulfonyl fluoride. This was followed by 1-h incubation at room temperature in binding buffer also containing 0.5% bovine serum albumin, 5 mM EDTA, 5 mM EGTA, and 10 μg/ml each of aprotinin, leupeptin, and pepstatin A plus 0.5 nM ^{125}I-α-CtxMII. The assay was terminated by washing the slides for 10 min at room temperature, 10 min in ice-cold binding buffer, twice for 10 min in 0.1× buffer at 0°C, and two final 5-s washes in ice-cold deionized water. The striatal sections were air-dried and exposed to Kodak MR (PerkinElmer Life and Analytical Sciences) for 2 to 5 days together with ^{3}H microscale standards (GE Healthcare). Nicotine (100 μM) was used to determine nonspecific binding.

Data Analyses. To evaluate optical density values from autoradiographic films, we used the ImageQuant program (GE Healthcare). To assess specific binding of the radioligands, background tissue levels were first subtracted from total binding to the tissue. The resultant values were converted to fmol/mg tissue using standard curves determined from ^{3}H standards. The ^{3}H standards were calibrated for ^{125}I autoradiography using the corrections described previously, including exposure time, section thickness, and concentration of radioactivity (Artymyshyn et al., 1990). The optical density readings of the samples were always within the linear range of the film.

Statistical Analysis. All curve fittings and statistics were conducted using Prism (GraphPad Software Co., San Diego, CA). Statistical comparisons were performed using analysis of variance (analysis of variance) followed by Newman-Keuls or Bonferroni post hoc tests (Prism). Values of $P < 0.05$ were considered significant. All values are expressed as the mean ± S.E.M. of the indicated number of animals.

Results

Progressive Nigrostriatal Damage with 6-OHDA Lesioning. The purpose of our study was to evaluate the role of the $\alpha4\beta2$* and $\alpha6\beta2$* nAChR subtypes in regulating striatal dopaminergic signaling with different degrees of nigrostriatal damage. To achieve this, rats were unilaterally lesioned with various doses of 6-OHDA (4.0–12.0 μg). Previous work had shown that dopamine transporter levels correlates well with the extent of dopamine denervation (Quik et al., 2003). Dopamine transporter values were therefore determined in the dorsal striatum, the specific striatal area in which the cyclic voltametric measurements were done. Figure 1 shows the animals grouped according to the severity of nigrostriatal damage, with mean dopamine transporter values of 23 ± 1.1 (n = 9), 17.7 ± 0.6 (n = 4), 13 ± 0.7 (n = 4), 6.3 ± 0.8 (n = 4), and 0.8 ± 0.4 (n = 4) nCi/mg tissue for the control, mild, moderate, moderately severe, and severe lesion groups, respectively (Fig. 1).

Behavioral studies were also done to evaluate motor deficits with lesioning, using the forelimb asymmetry or cylinder test (Schallert et al., 2000). The use of the impaired contralateral limb during rearing was significantly decreased by ~45% in the moderately severe group ($p < 0.01$) and by ~75% in the severely lesioned group ($p < 0.001$) compared with control, with no change in mild and moderately lesioned rats (Fig. 2). These data are in agreement with previous studies that demonstrated motor deficits only with more severe nigrostriatal damage (Cenci and Lundblad, 2007).

Decreases in Both Single-Pulse and Burst-Evoked Total Dopamine Release Correlate with Nigrostriatal Damage. Cyclic voltammetry offers the advantage that evoked dopamine release can be assessed with single-pulse and burst stimulation, conditions that may mimic tonic and phasic neuronal firing in vivo (Rice and Cragg, 2004; Zhang and Sulzer, 2004). Endogenous striatal dopamine release was therefore determined in control and lesioned animals in response to a single-pulse stimulus (Fig. 3, top), a burst of four pulses at 30 Hz (Fig. 3, middle) or a burst of 4 pulses at 100 Hz (Fig. 3, bottom). These frequencies were selected because previous work had shown that dopaminergic neurons in vivo fire in a low frequency tonic mode (0.5–10 Hz) interspersed by bursting activity (50–100 Hz) (Rice and Cragg, 2004; Zhang and Sulzer, 2004). Representative traces for dopamine signals obtained from control rats and those with mild, moderate, moderately severe, and severe lesions

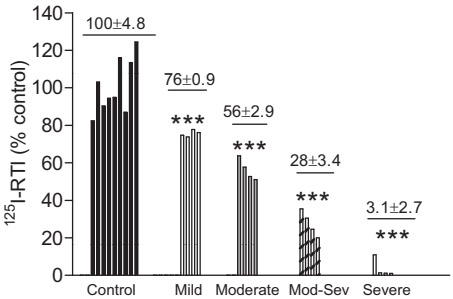


Fig. 1. Striatal dopamine transporter declines with 6-OHDA lesioning. Rats received unilateral 6-OHDA injections at two different sites in the medial forebrain bundle as described under *Materials and Methods*. Various doses of the toxin were injected to achieve different degrees of nigrostriatal damage. Alterations in striatal dopamine transporter expression were assessed using [^{125}I]RTI-121 autoradiography. After quantitative analyses, rats were grouped as shown. The numerical values represent the mean ± S.E.M. of the indicated number of rats. ***, $p <$ 0.001 indicates significance of difference from control using a Newman-Keuls multiple comparisons *post hoc* test.

are shown for each stimulation frequency (Fig. 3 left). Quantitative analyses demonstrate that dopamine release decreased in proportion to lesion size at all stimulus frequencies (Fig. 3, right). Single pulse-stimulated dopamine release significantly decreased by 50 ($p < 0.05$), 66 ($p < 0.01$), 78 ($p < 0.001$), and 98% ($p < 0.001$) in the rats with mild, moderate, moderately severe, and severe lesions, respectively, compared with control rats. Similar declines were observed with the four pulses at 30 Hz and four pulses at 100 Hz stimulation frequencies. The correlation coefficients (r) between lesion size and dopamine release were equal to 0.94, 0.93, and 0.94 for one pulse, four pulses at 30 Hz, and four pulses at 100 Hz, respectively. These data show that there is a decline in both tonic and phasic dopamine release, as might be expected with dopaminergic denervation.

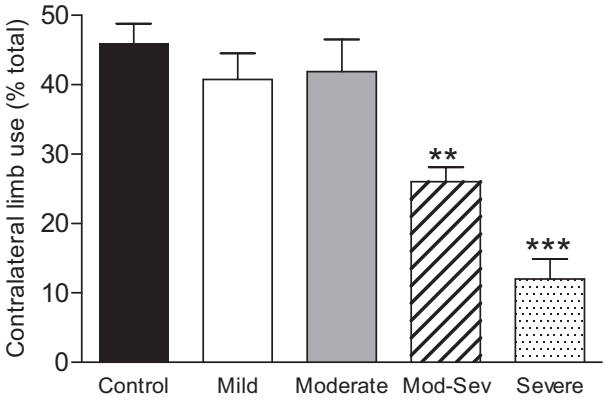


Fig. 2. Motor deficits with progressive nigrostriatal damage. Parkinsonism was assessed using the limb asymmetry or cylinder test. The percentage use of the affected limb was determined during a 5-min rating period for each animal. A statistically significant decrease in the use of the contralateral paw was observed only in rats with moderately severe and severe lesions. The values represent the mean ± S.E.M. of four to nine rats. **, $p < 0.01$; ***, $p < 0.001$ indicate significance of difference from control using a Newman-Keuls multiple comparisons *post hoc* test.

Dopamine Uptake Rate Decreases in Proportion to the Extent of Nigrostriatal Damage. Peak dopamine levels are affected by the balance between dopamine release and uptake. To determine uptake rate constants in slices from control and lesioned animals, the dopamine peaks obtained after stimulation were fitted to one-phase exponential decay analysis, as described previously (Wightman and Zimmerman, 1990; Cragg et al., 2001; John et al., 2006; Perez et al., 2008b). Uptake rate constants were significantly decreased by 26 ($p < 0.01$), 43 ($p < 0.001$), 58 ($p < 0.001$), and 92% ($p < 0.001$) for rats with mild, moderate, moderately severe, and severe lesions, respectively (Fig. 4). Correlation analyses showed a significant decreasing trend in uptake as the size of the lesion increased ($r = 0.90$), as might be expected.

The Effect of nAChR Antagonists on Dopamine Release in Control Rat Striatum. Previous studies in mice, guinea pigs, and monkeys have shown that single-pulse stimulated dopamine release is reduced in the presence of nAChR antagonists (Rice and Cragg, 2004; Zhang and Sulzer, 2004; Exley et al., 2008; Meyer et al., 2008; Perez et al., 2009). The

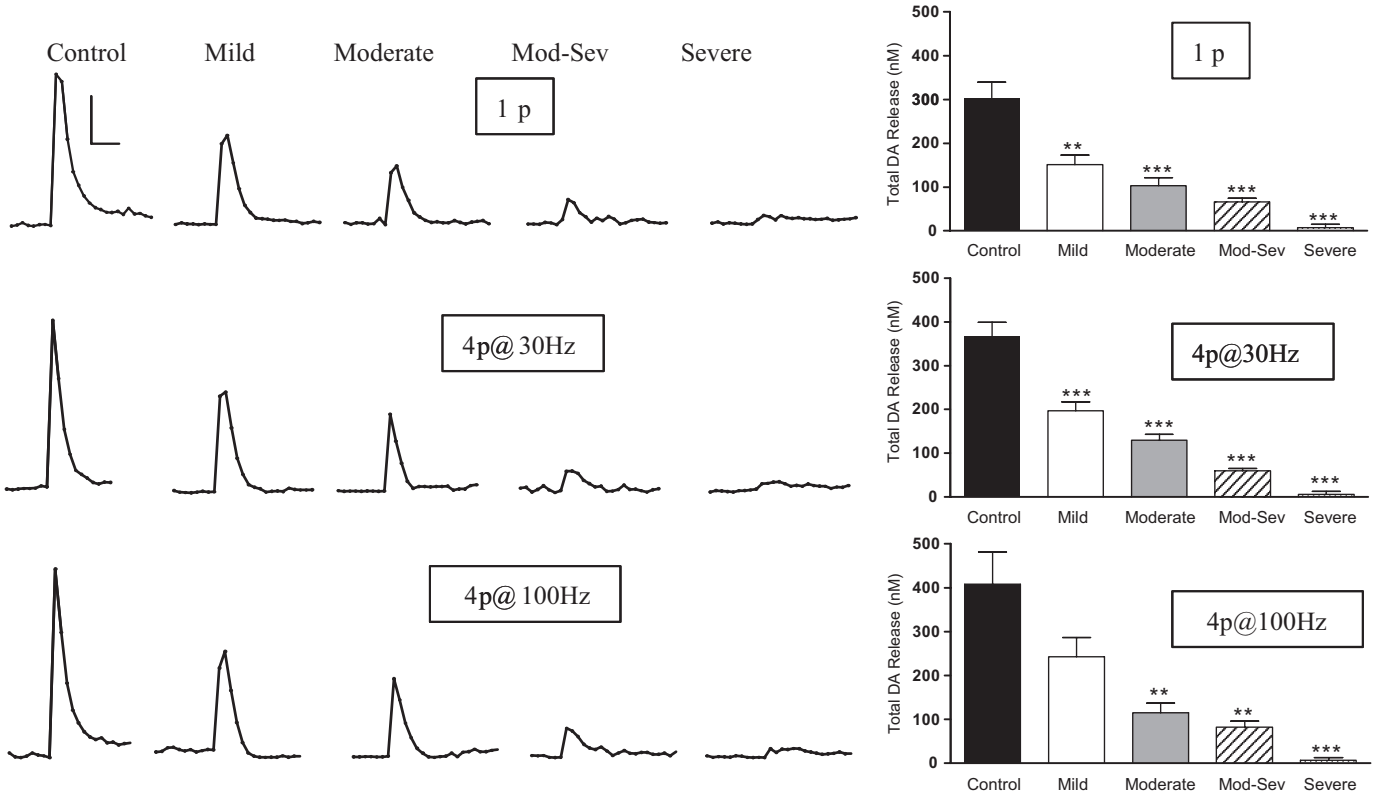


Fig. 3. Decreases in both single and burst-evoked dopamine release correlate with nigrostriatal damage. Evoked endogenous dopamine release across the different range of lesions was measured after one pulse, four pulses at 30 Hz, and four pulses at 100 Hz electrical stimulation. Peak dopamine release decreased in proportion to the extent of lesion regardless of stimulation frequency. Sample traces of dopamine signals measured from a representative animal from each lesion group are shown to the left of the average group data. The scale bar represents 100 nM and 0.5 s. Values represent the mean ± S.E.M. of four to nine rats. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ indicate significance of difference from control using a Newman-Keuls multiple-comparisons *post hoc* test.

present results also demonstrate that single-pulse-stimulated dopamine release was decreased in rat striatal slices by nAChR blockers (Fig. 5). α-CtxMII, a α6β2* nAChR antagonist, significantly decreased release by ~45% ($p < 0.001$). Subsequent perfusion with the α4β2* and α6β2* nAChR antagonist mecamylamine led to an additional 27% decline in evoked release ($p < 0.001$), which was significantly different compared with that with α-CtxMII alone ($p < 0.05$). Thus, the dominant effect of α6β2* nAChRs in modulating single-pulse-stimulated nAChR-mediated dopamine release is evident across species.

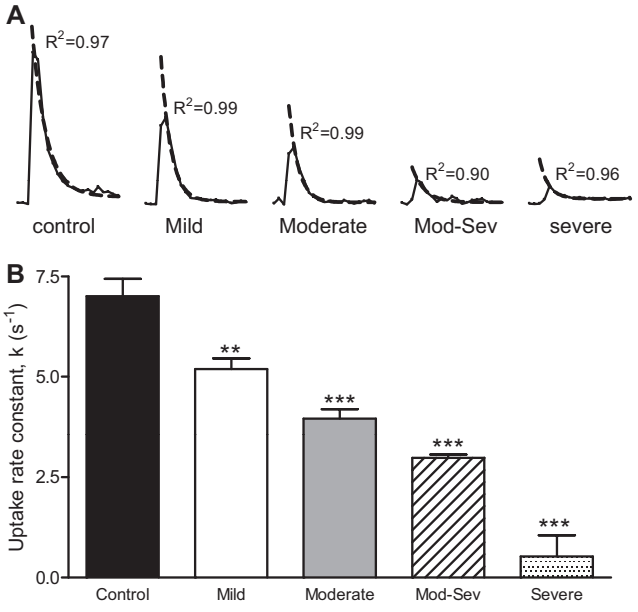


Fig. 4. Uptake rate constants decrease in proportion to the extent of nigrostriatal damage. A, representative traces of dopamine release from each group. Uptake rate constants were calculated by fitting the clearance portion of the curve to one-phase exponential decay ($r > 0.9$) B, uptake rate constant values were significantly decreased in proportion to lesion size. Values represent mean ± S.E.M. of four to nine rats per group. **, $p < 0.01$; ***, $p < 0.001$ indicate significance of difference from control using a Newman-Keuls multiple comparisons *post hoc* test.

In contrast to the effect of nAChR antagonists on single-pulse stimulated dopamine release, burst-evoked dopamine release may be similar to total release (or possibly enhanced) in the presence of antagonists as a result of a relief of short-term depression (Rice and Cragg, 2004; Zhang and Sulzer, 2004; Exley et al., 2008; Meyer et al., 2008; Perez et al., 2009; Zhang et al., 2009b). Our results in rat striatal slices also show that evoked dopamine release with α-CtxMII or with mecamylamine was at control levels with high frequency stimulation (four pulses at 100 Hz; Fig. 5).

Single-Pulse Stimulation Studies Show That Both α6β2* and α4β2* nAChRs Modulate Dopamine Release with Increased Nigrostriatal Damage. We next measured single-pulse–evoked dopamine release in the absence and presence of α-CtxMII or mecamylamine in striatal sections from lesioned animals (Fig. 6). In the data analyses, dopamine release was normalized to total release for each lesioned group. With a mild lesion, α-CtxMII and mecamylamine both still resulted in significant decreases in evoked dopamine release, with a 40% decrease in the presence of α-CtxMII ($p < 0.05$) and a 60% decrease after the application of mecamylamine ($p < 0.05$) (Fig. 6B). These antagonist-induced declines in endogenous release became progressively smaller with increased lesioning. In the moderately lesioned group, there was a nonsignificant 37% decrease in dopamine release with α-CtxMII, whereas mecamylamine significantly decreased release by 60% ($p < 0.05$) (Fig. 6C). However, neither α-CtxMII nor mecamylamine significantly decreased release in the moderately severe and severely lesioned groups (Fig. 6, D and E). There was no significant difference in release in the presence of α-CtxMII compared with that with mecamylamine in any lesioned group (Fig. 6, B–E). These data indicate that there is a reduction in the ability of nAChR to modulate dopamine release with increased lesion size.

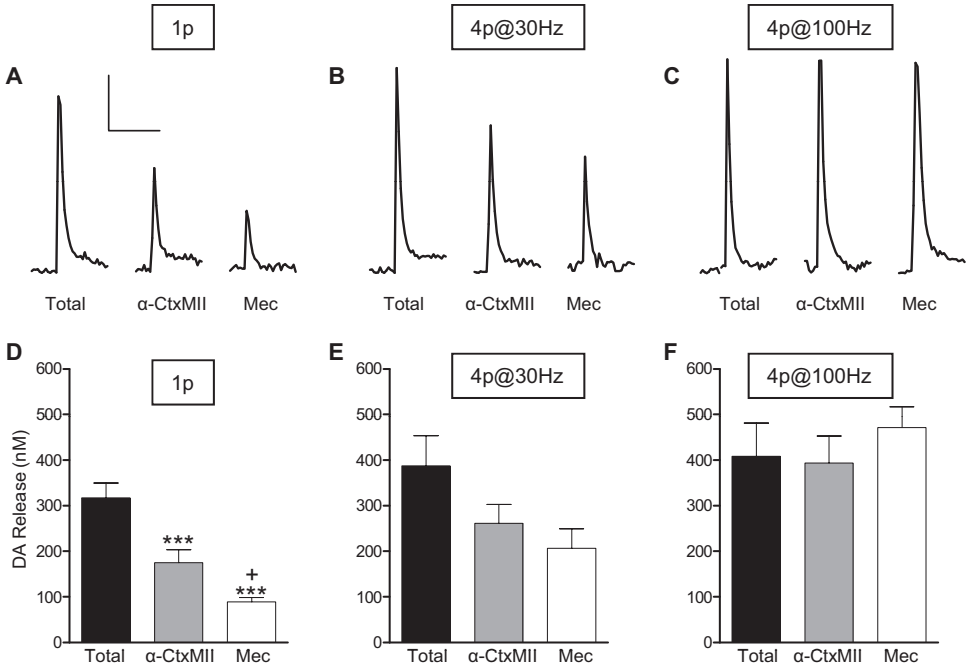
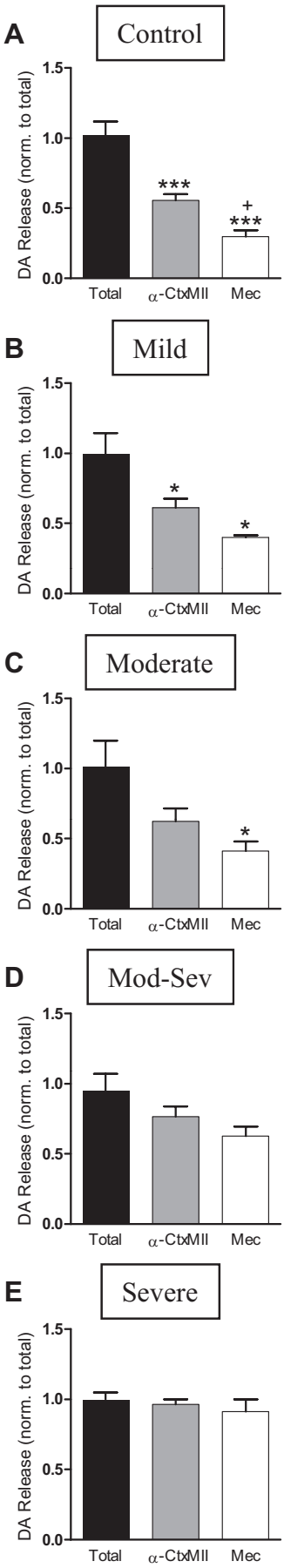


Fig. 5. nAChR blockade decreases dopamine release with single but not burst stimulation in control rat striatum. Dopamine release was measured in the absence (total) and presence of the α6β2* nAChR antagonist α-CtxMII (100 nM) or the general nAChR blocker mecamylamine (100 μM). Sample dopamine signals after a 1 pulse (A), 4 pulses at 30 Hz (B), and 4 pulses at 100 Hz (C) electrical stimulation are shown. The scale bar represents 100 nM and 2.5 s. Quantitative analyses of peak dopamine release show that with a one-pulse stimulus (D), ~45% of endogenous dopamine release is mediated through α6β2* nAChRs whereas ~25% is modulated by α4β2* nAChRs, as evidenced by significant decreases in release in the presence of α-CtxMII or mecamylamine. In contrast, dopamine release-stimulated at higher frequencies was affected by perfusion of neither α-CtxMII nor mecamylamine (E and F). Values represent the mean ± S.E.M. of four to nine rats. ***, $p < 0.001$ indicates significance of difference from total; +, $p < 0.05$ indicates significance of difference from α-CtxMII using a Newman-Keuls multiple comparisons post hoc test.



The results in Fig. 6 were analyzed to evaluate the contribution of the $\alpha4\beta2^*$ and $\alpha6\beta2^*$ nAChR subtypes in modulating evoked dopamine release with increased nigrostriatal damage (Fig. 7). Overall nAChR-mediated release was calculated by subtracting release in the presence of mecamylamine from total release (Table 1). The $\alpha6\beta2^*$ nAChR-mediated component was calculated by subtracting release in the presence of α-CtxMII from total release (Table 1). $\alpha4\beta2^*$ nAChR-mediated release was determined by subtracting release in the presence of mecamylamine from that in the presence of α-CtxMII (Table 1). The results in Fig. 7 show that both $\alpha4\beta2^*$ and $\alpha6\beta2^*$ nAChR-mediated release were significantly decreased in proportion to the extent of dopamine transporter loss ($p < 0.001$). These results would suggest that both nAChR subtypes are important in the regulation of evoked dopamine release throughout the neurodegenerative process.

Effect of Burst Stimulation on Dopamine Release in the Presence of $\alpha6\beta2^*$ and $\alpha4\beta2^*$ nAChR Antagonists with Nigrostriatal Damage. To assess whether nigrostriatal damage modified the effects of nAChR blockade on burst-stimulated release, we measured striatal dopamine release in the presence of α-CtxMII or mecamylamine after a four-pulse stimulus at either 30 or 100 Hz. As mentioned earlier, under control conditions, burst-evoked dopamine release is similar to total release (or possibly enhanced) in the presence of antagonists because of a relief of short-term depression (Rice and Cragg, 2004; Zhang and Sulzer, 2004; Exley et al., 2008; Meyer et al., 2008; Perez et al., 2009; Zhang et al., 2009b). The results show that the frequency dependence of release in the absence and presence of the antagonists was similar in control and lesioned rat striatum. The decreased release in the presence of the antagonists is overcome with burst stimulation in lesioned striatum similar to the results in control striatum, although there was only minimal release with severe lesioning (Table 1, Fig. 8 left and middle column). Normalization of the data to 1 pulse at the same condition for each type of lesion (Fig. 8 right column) more clearly shows the increase in dopamine release with nAChR inhibition. Blockade of $\alpha6\beta2^*$ nAChRs with either α-CtxMII or mecamylamine resulted in a significant increase in the ratio of burst- to single-pulse–induced dopamine release in control rats ($p < 0.01$) and in rats with moderate ($p < 0.01$) and moderately severe ($p < 0.05$) lesions (Fig. 8, right), with similar trends in rats with mild lesions (Fig. 8 right column). The lack of change in the rats with severe lesions may simply represent a floor effect. These findings suggest that $\alpha6\beta2^*$ and $\alpha4\beta2^*$ receptors both regulate burst-evoked dopamine release with nigrostriatal damage. The cellular mechanisms that regulate dopamine release

Fig. 6. Effect of $\alpha6\beta2^*$ and/or $\alpha4\beta2^*$ nAChR blockade on single-pulse stimulated dopamine release with varying nigrostriatal damage. Dopamine release was measured in the absence (total) and presence of the $\alpha6\beta2^*$ nAChR antagonist α-CtxMII (100 nM) or the general nAChR blocker mecamylamine (100 μM). Release was normalized to total release for each lesioned group. NAChR inhibition with either α-CtxMII or mecamylamine significantly decreased dopamine release in rats with mild (B) and moderate (C) lesions, although to a lesser extent than in control rats (A). No significant changes were observed in the rats with moderately severe (D) and severe (E) lesions. Values represent the mean ± S.E.M. of four to nine rats. *, $p < 0.05$; ***, $p < 0.001$ indicate significance of difference from total; +, $p < 0.05$ indicates significance of difference from α-CtxMII using a Newman-Keuls multiple comparisons post hoc test.

with burst firing seem to be retained throughout the neurodegenerative process.

Declines in $\alpha4\beta2$* and $\alpha6\beta2$* nAChR Binding Sites with Nigrostriatal Damage Assessed Using Autoradiography. Experiments were performed to determine the effect of nigrostriatal damage on nAChR binding sites. To identify $\alpha4\beta2$* nAChRs, binding of ^{125}I-epibatidine was done in the presence of α-CtxMII using autoradiography. Significant decreases in $\alpha4\beta2$* nAChRs ($p < 0.001$) were obtained in both rats with the moderately severe lesions and those with severe lesions (Table 2). $\alpha6\beta2$* nAChRs, identified using ^{125}I-α-CtxMII, were more severely affected with a decline in binding at all stages of nigrostriatal damage (Table 2). These differential declines in $\alpha4\beta2$* and $\alpha6\beta2$* sites probably occur because $\alpha4\beta2$* nAChRs are located at both postsynaptic sites (80%) and dopaminergic terminals (20%), with only the latter affected by nigrostriatal damage (Grady et al., 2007; Gotti et al., 2009; Livingstone and Wonnacott, 2009). By contrast, $\alpha6\beta2$* nAChR seem to be present primarily on dopaminergic terminals (Grady et al., 2007; Gotti et al., 2009; Livingstone and Wonnacott, 2009).

Discussion

The current results are the first to investigate the contribution of striatal $\alpha4\beta2$* and $\alpha6\beta2$* nAChRs to tonic and phasic evoked dopamine release with nigrostriatal damage. Fast-scan cyclic voltametric data show that nAChRs have the potential to modulate single-pulse and burst-stimulated dopamine release from striatal slices throughout the neurodegenerative process. These findings have important clinical implications for neurological disorders such as Parkinson's disease because they suggest that drugs targeting $\alpha4\beta2$* and $\alpha6\beta2$* receptor subtypes may both be of therapeutic importance, with a somewhat more prominent and possibly more selective role for the $\alpha6\beta2$* subtype with burst firing.

Dopaminergic neurons communicate with other neuronal systems via tonic or single-pulse firing, as well as via phasic or burst stimulation (Rice and Cragg, 2004; Zhang and Sulzer, 2004; Exley et al., 2008; Meyer et al., 2008; Perez et al., 2008a; Zhang et al., 2009a). Although the precise functional role of these different modes of signaling on behavior remains to be elucidated, current evidence suggests that tonic neuronal firing may exert a pace-making role to maintain basal activity (Heien and Wightman, 2006; Sandberg and Phillips, 2009). The current data demonstrate that nAChRs modulate ~70% of tonic dopamine release, in agreement with previous findings (Rice and Cragg, 2004; Zhang and Sulzer, 2004; Exley et al., 2008; Perez et al., 2008a; Zhang et al., 2009a,b). $\alpha4\beta2$* and $\alpha6\beta2$* nAChRs both modulate single-pulse evoked dopamine release in intact rat striatum, the

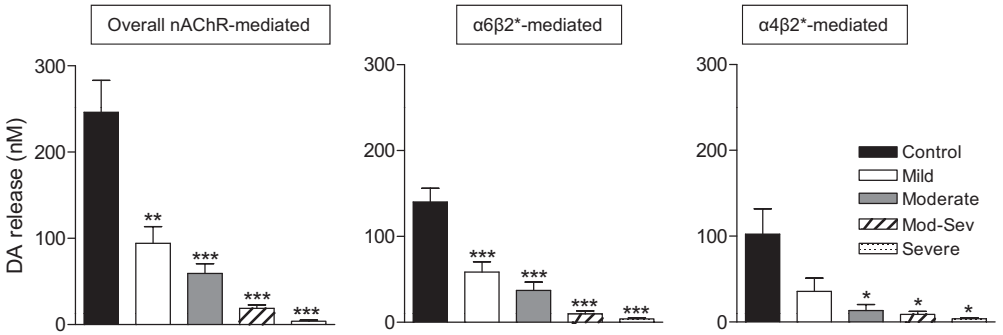


Fig. 7. Both $\alpha6\beta2$* and $\alpha4\beta2$* nAChR-modulated release decline with nigrostriatal damage. NAChR-mediated release was determined by subtracting release in the presence of mecamylamine from total release. $\alpha6\beta2$* mediated release was determined by subtracting release in the presence of α-CtxMII from total release. $\alpha4\beta2$* mediated release was determined by subtracting release in the presence of mecamylamine from that in the presence of α-CtxMII. Quantitative analyses showed a significant decrease in nAChR-mediated release in proportion to the extent of lesioning. This was accompanied by a significant decrease in $\alpha6\beta2$* and $\alpha4\beta2$* mediated release. Values represent the mean ± S.E.M. of four to nine rats. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ indicate significance of difference from control using a Newman-Keuls multiple comparisons post hoc test.

TABLE 1

Blockade of nAChRs does not significantly change burst-stimulated dopamine release in control and lesioned rats

Dopamine release in the absence or presence of α-CtxMII or mecamylamine after a single pulse stimulation or a four pulse stimulus at either 30 or 100 Hz. NAChR inhibition decreased single-pulse stimulated dopamine release while it did not significantly affect burst-induced release regardless of the lesion size. Values represent the mean ± S.E.M. of nine controls and four rats in each lesioned group.

Group	Dopamine Release								
	Total			CtxMII			Mecamylamine		
	One Pulse	Four Pulses		One Pulse	Four Pulses		One Pulse	Four Pulses	
		30 Hz	100 Hz		30 Hz	100 Hz		30 Hz	100 Hz
	nM								
Control	317 ± 32	388 ± 65	408 ± 73	175 ± 28	261 ± 42	393 ± 59**	89 ± 9.4	207 ± 43*	472 ± 45***†
Mild	156 ± 31	195 ± 47	243 ± 44	98 ± 24	104 ± 21	214 ± 72	62 ± 12	150 ± 30	201 ± 43*
Moderate	103 ± 19	115 ± 24	115 ± 22	66 ± 18	88 ± 20	120 ± 23	43 ± 10	84 ± 17	102 ± 23
Moderately severe	66 ± 9.2	65 ± 8.9	82 ± 13	52 ± 11	56 ± 14	102 ± 30	43 ± 9.3	62 ± 15	88 ± 21
Severe	6.1 ± 6.1	7.4 ± 7.4	6.6 ± 6.6	6.9 ± 6.9	6.2 ± 6.2	7.1 ± 7.1	7.2 ± 7.2	4.1 ± 4.1	9.6 ± 9.6

Bonferroni post hoc test was used to calculate significance:
* $P < 0.05$, significantly different from one pulse.
** $P < 0.01$, significantly different from one pulse.
*** $P < 0.001$, significantly different from one pulse.
† $P < 0.001$, significantly different from four pulses at 30 Hz.

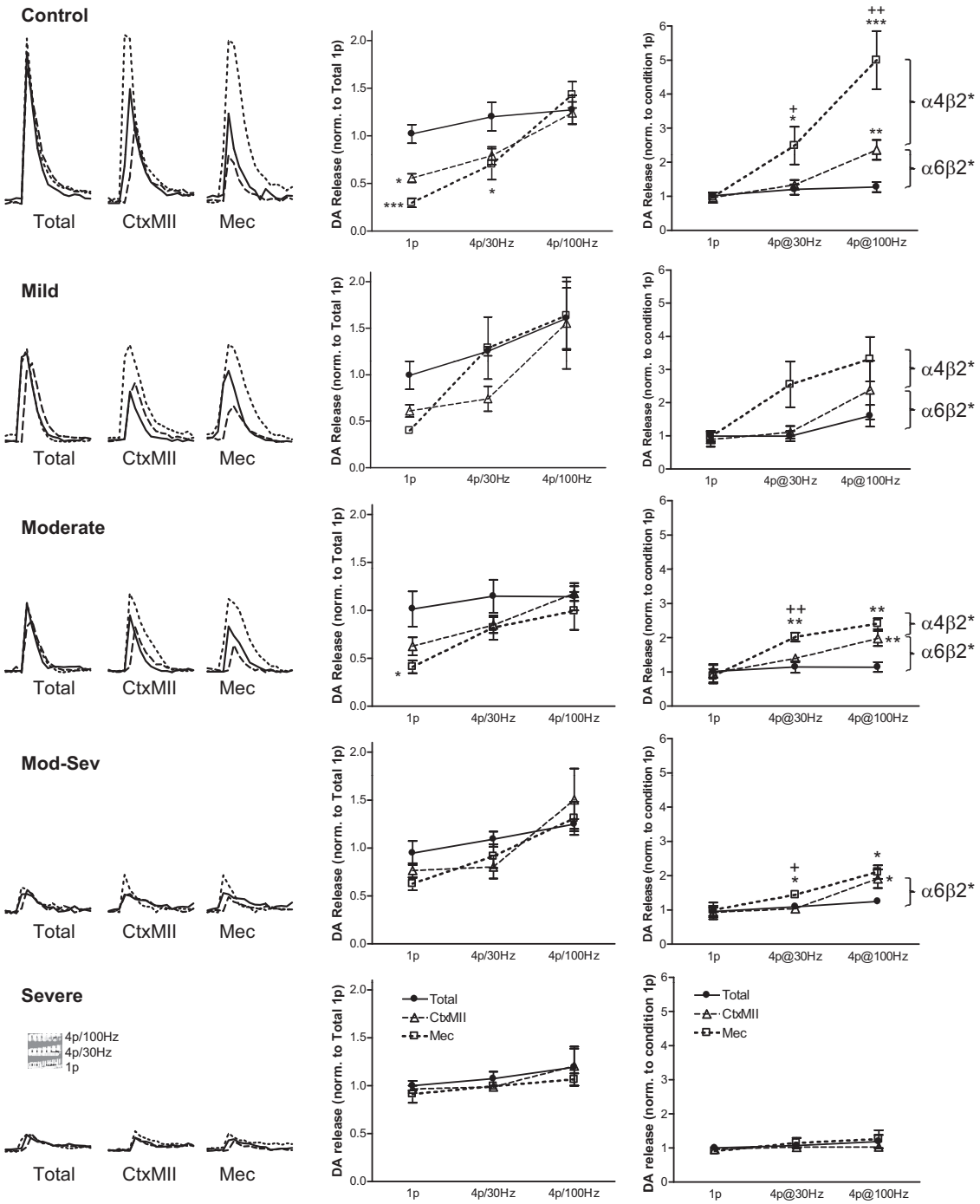


Fig. 8. NAChR antagonism results in similar effects on burst-stimulated dopamine release in control and dopamine-depleted striatum. Left, representative traces of dopamine release in the absence or presence of α-CtxMII or mecamylamine after a single pulse or at 4 pulses at either 30 or 100 Hz. Middle, quantitative analyses of the data for control (n = 9 rats) and lesioned rats (n = 4 rats per lesion group) at varying frequency, as indicated. The frequency dependence of release in the absence and presence of the antagonists was similar in striatum of control and lesioned rats. Thus, the relief of short-term depression with nAChR blockade during burst stimulation is observed in both control striatum and with nigrostriatal damage. *, $p < 0.05$; ***, $p < 0.001$ indicate significance of difference from total release using a Bonferroni post hoc test. Right, normalization of the data to one pulse at the same condition for each lesion paradigm. nAChR antagonism effectively relieves short-term depression at the higher stimulation frequencies, although there was less of an increase with greater nigrostriatal damage. Thus both $\alpha4\beta2$* and $\alpha6\beta2$* nAChRs influence burst-evoked release throughout the neurodegenerative process. Values represent the mean ± S.E.M. of nine control rats and four rats per lesioned group. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ indicate significance of difference from total release; +, $p < 0.05$; ++, $p < 0.01$ indicates significance of difference from release in the presence of α-CtxMII using a Bonferroni post hoc test.

major component of nAChR-modulated release (~65%) being mediated by the $\alpha6\beta2^*$ nAChR. These results are similar to previous data in mice and monkeys, suggesting that the mechanisms whereby nAChRs modulate release are maintained across species (Exley et al., 2008; Meyer et al., 2008; Perez et al., 2008a, 2009). Our current results in lesioned rats show that the total amount of tonically evoked nAChR-modulated dopamine release declines with the extent of neuronal damage but that the ratio of release regulated by the $\alpha6\beta2^*$ and $\alpha4\beta2^*$ nAChRs remained similar (that is, 65 and 35%, respectively). Thus, the contribution of the two subtypes to the regulation of tonic release is unaffected by nigrostriatal damage.

In addition to tonic firing, dopamine neurons also exhibit phasic or burst firing, which has been associated with stimuli leading to the initiation or execution of movement and other behaviors such as reward (Sandberg and Phillips, 2009). Fast-scan cyclic voltametric studies have proved very useful in elucidating the contribution of nAChR subtypes to phasic dopaminergic signaling in intact striatum (Exley et al., 2008; Meyer et al., 2008; Perez et al., 2008a, 2009). In our studies, dopamine release stimulated by higher frequencies seemed to be unaffected by nAChR antagonism. These data can be interpreted to mean that nAChRs are not involved in burst-stimulated dopamine release. However, previous studies assessing paired-pulse release ratios have consistently shown that blockade or desensitization of nAChRs increases the probability of dopamine release at high frequencies by decreasing dopamine release probability at low stimulation frequencies—an effect known as short-term facilitation or relief of short-term depression (Rice and Cragg, 2004; Zhang and Sulzer, 2004; Exley et al., 2008; Perez et al., 2008a; Zhang et al., 2009a,b). Thus, there seems to be an involvement of nAChRs on tonic as well as burst-induced dopamine release, although the contribution of non-nAChR-mediated mechanisms on phasic dopamine release cannot be discarded. Thus far, studies have investigated the effect of dopamine transporter and/or dopamine receptor inhibitors, with neither one affecting the facilitation of burst-induced dopamine release observed with nAChR blockade (Zhang and Sulzer, 2004, 2009a).

Work by Garris and coworkers (Garris et al., 1997; Bergstrom et al., 2001; Sandberg and Phillips, 2009) has shown that nigrostriatal damage reduces phasic dopamine signaling. We obtained similar results and further demonstrate the involvement of the $\alpha4\beta2^*$ and $\alpha6\beta2^*$ nAChR subtypes in phasic signaling with nigrostriatal damage. The results show that the $\alpha6\beta2^*$ nAChR subtype contributes to the regulation of burst-evoked release throughout the neurodegenerative process. By contrast, the influence of the $\alpha4\beta2^*$ nAChR subtype on phasic release seems to decline to a proportionately greater extent with increasing lesion size. Thus, with mild nigrostriatal damage, the contribution of the $\alpha4\beta2^*$ nAChRs to burst-evoked release is similar to that for the $\alpha6\beta2^*$ subtype, with a negligible involvement of the $\alpha4\beta2^*$ receptor in moderate and moderately severe damage. These findings may suggest that the $\alpha6\beta2^*$ subtype plays a greater role with increased dopaminergic denervation. A possible explanation for this finding is that the $\alpha6\beta2^*$ nAChRs that modulate burst-evoked dopamine release are spared until a greater lesion is achieved. The complex modulatory control of dopaminergic function exerted by the $\alpha4\beta2^*$ and the $\alpha6\beta2^*$ nAChR subtypes may play a pivotal role in the functional changes observed with nigrostriatal dopamine degeneration.

Previous work has shown that nicotine protects against nigrostriatal damage in parkinsonian animal models and also improves L-DOPA-induced dyskinesias, a debilitating side effect of dopamine replacement therapy for Parkinson's disease (Quik et al., 2009). Because nicotine stimulates multiple nAChRs, the question arises: which subtypes are important for these behavioral effects? The present studies showing that striatal $\alpha4\beta2^*$ and $\alpha6\beta2^*$ nAChRs modulate evoked dopamine release suggests that both of these populations are important in striatal function with increasing nigrostriatal damage. Earlier receptor work had shown that striatal $\alpha6\beta2^*$ nAChR sites are more susceptible to nigrostriatal degeneration than $\alpha4\beta2^*$ nAChRs, with a complete loss of $\alpha6\beta2^*$ sites with severe nigrostriatal damage (Quik et al., 2001, 2003). By contrast, $\alpha4\beta2^*$ nAChR expression decreased by 30 to 40% under the same experimental conditions. The present data demonstrating similar declines in $\alpha4\beta2^*$ and $\alpha6\beta2^*$ nAChR-modulated function with nigrostriatal damage suggest that the $\alpha4\beta2^*$ and $\alpha6\beta2^*$ nAChRs that influence dopamine release exist on dopaminergic terminals equally susceptible to nigrostriatal damage. The $\alpha4\beta2^*$ nAChRs unaffected by nigrostriatal damage are most likely localized to nondopaminergic neurons such as GABAergic, cholinergic, and other neuronal and/or non-neuronal elements in the striatum.

An interesting issue related to the development of Parkinson's disease is the time lag between the onset of neurodegeneration and the appearance of symptoms, such that motor disabilities are not evident until there is a relatively large loss of dopaminergic neurons (Singh et al., 2007). We also observed this phenomenon in our 6-OHDA-lesioned rat model, with motor impairments arising only with a >70% loss of dopamine terminals. Plasticity in dopamine neurotransmission is thought to play a role in this symptomatic delay. Our studies in primates demonstrated a compensatory increase with moderate nigrostriatal damage in both striatal nicotine-evoked ^{3}H-dopamine release from synaptosomes

TABLE 2

Preferential decrease in striatal $\alpha6\beta2^*$, as compared to $\alpha4\beta2^*$ nAChR sites with nigrostriatal damage

^{125}I-Epibatidine in the presence of α-CtxMII and ^{125}I-α-CtxMII binding assays were done to determine $\alpha4\beta2^*$ and $\alpha6\beta2^*$ nAChR expression, respectively, as described in *Materials and Methods*. Animals were divided into several groups according to dopamine transporter values.

	Control (n = 9 rats)	Mild (n = 4 rats)	Moderate (n = 4 rats)	Moderately Severe (n = 4 rats)	Severe (n = 4 rats)
			% control		
$\alpha4\beta2^*$ nAChRs	100 ± 2.3	92 ± 2.7	90 ± 2.3	75 ± 2.6***	53 ± 3.5***
$\alpha6\beta2^*$ nAChRs	100 ± 4.2	70 ± 7.8**	39 ± 7.7***	19 ± 9.8***	7.0 ± 3.3***

Newman-Keuls post hoc test was used to calculate significance:
** $P < 0.01$.
*** $P < 0.001$.

and in evoked endogenous dopamine release measured using cyclic voltammetry (McCallum et al., 2005, 2006; Perez et al., 2008b). These data in nonhuman primates suggest that an enhanced dopaminergic tone may represent a mechanism underlying dopaminergic compensation during the presymptomatic stages of Parkinson's disease. In contrast to these findings in nonhuman primates, studies in rodent models to investigate the role of the dopaminergic system in compensation seem conflicting. In support of dopaminergic compensation, Zigmond and coworkers (Zigmond et al., 1984, 1990; Snyder et al., 1990) observed enhanced electrically stimulated ^{3}H-dopamine release from striatal slices of 6-OHDA lesioned rats compared with controls. However, Garris and coworkers (Garris et al., 1997; Bergstrom et al., 2001) obtained no enhancement of dopamine release in the same parkinsonian animal model as assessed using cyclic voltammetry. Instead, they proposed that dopamine tone is maintained through passive stabilization or enhanced volume transmission because of the observed decrease in dopamine uptake. Our current data are in agreement with those from the latter studies.

Altogether, our results suggest that $\alpha6\beta2^*$ and $\alpha4\beta2^*$ nAChR modulate evoked dopamine release throughout the neurodegenerative process. Both of these receptor subtypes may thus influence the progressive changes observed in Parkinson's disease. A better understanding of the dynamic control of $\alpha4\beta2^*$ and $\alpha6\beta2^*$ nAChR-modulated dopaminergic function during the course of nigrostriatal damage may facilitate the development of improved therapies for disorders involving nigrostriatal damage, such as Parkinson's disease.

Acknowledgments

We thank Yu Young Lee for excellent technical assistance.

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