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TITLE PAGE

α6β2* and α4β2* nicotinic receptors both regulate dopamine signaling with increased nigrostriatal damage; relevance to Parkinson's disease

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Running Title: nAChR-mediated release in lesioned rat striatum

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ABBREVIATIONS: nAChRs, nicotinic acetylcholine receptors; α-CtxMII, α-conotoxinMII; RTI-121, 3β-(4-iodophenyl)tropane-2β-carboxylic acid; BSA, bovine serum albumin; *, the asterisk indicates the possible presence of other nicotinic subunits in the receptor complex.

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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 α6β2* and α4β2* subtypes. Lesion studies show that the α6β2* receptor is decreased to a much greater extent with nigrostriatal damage than the α4β2* 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 α6β2* and α4β2* 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 α4β2* and α6β2* subtypes in release. Single-pulse-stimulated α6β2* and α4β2* 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 α4β2* and α6β2* 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 α6β2* subtype. These data have important therapeutic implications as they suggest that drugs directed to both α4β2* and α6β2* nAChRs may be useful in the treatment of neurological disorders such as Parkinson's disease.

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Introduction

The striatal dopaminergic and cholinergic systems play an overlapping role in regulating CNS 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 co-localization 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, Zhou et al., 2002, Exley and Cragg, 2008, Livingstone and Wonnacott, 2009). A concerted action at these sites is most likely 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. Chronic nicotine administration is neuroprotective against nigrostriatal damage in parkinsonian animal models (Quik et al., 2007b, Picciotto and Zoli, 2008) and also improves L-dopa-induced dyskinesias, a debilitating side effect of dopamine replacement therapy (Quik et al., 2007a, Bordia et al., 2008, Quik et al., 2009).

Nicotine most likely modulates nigrostriatal dopaminergic transmission through an action at nAChRs, with the two major subtypes in the nigrostriatal pathway being the α4β2* and α6β2*

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nAChRs (Grady et al., 2007, Gotti et al., 2009, Livingstone and Wonnacott, 2009, Quik et al., 2009). The α6β2* nAChRs appear to be exclusively expressed on dopaminergic neurons, while α4β2* 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, while 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 voltammetric studies have proved very useful in elucidating the contribution of nAChRs to tonic and phasic dopaminergic signaling. The α6β2* receptor plays a prominent role in tonic dopamine release, controlling ~75% of nAChR-mediated release in striatum, while α4β2* 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, Perez et al., 2009).

The goal of the present study was to understand the role of α4β2* and α6β2* nAChRs in regulating single-pulse and burst stimulated striatal dopamine signaling with progressive nigrostriatal damage. Fast scan cyclic voltammetric data show that the α6β2* and α4β2* 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.

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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 to 12-h light/dark cycle in a temperature-controlled room with free access to food and water. Starting two days after arrival, rats were unilaterally lesioned with 6-hydroxydopamine (6-OHDA) HCl (Sigma-Aldrich, St, Louis, MO) as previously described (Bordia et al., 2008). Briefly, 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 4-12 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 NIH Guide for the Care and Use of Laboratory Animals 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 two and three weeks after the 6-OHDA lesion as previously described 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

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expressed as a percent of total limb use.

Tissue preparation. Rats were killed 4-5 weeks after the 6-OHDA lesion. The brain was quickly removed and chilled in ice-cold, pre-oxygenated (95% O_2/5% CO_2) physiological buffer containing (in mM); 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, 25 mM $NaHCO_3$ (pH 7.4) as previously described (Perez et al., 2008a). Coronal corticostriatal slices (400 μm thick) were cut using a vibratome (Leica VT1000S) 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^{o}C. Sections (8 μm) were prepared using a cryostat (Leica Microsystems, Inc., Deerfield, IL) at –20^{o}C. Frozen sections were thaw-mounted onto Superfrost Plus slides (Fisher, Pittsburgh, PA), 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 previously described (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, Sunnyvale, CA). 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

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micromanipulators. Background current was digitally subtracted and the peak oxidation currents were converted into concentration after post-experimental 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 4 pulses at 30 Hz 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 they yielded maximal blockade of α6β2* and α4β2* 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 (2200 Ci/mmol; Perkin Elmer Life Sciences, Boston, MA, USA),

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as previously described (Quik et al., 2003, Bordia et al., 2007). Thawed sections were pre-incubated 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 hr in buffer with 0.025% BSA, 1 μM fluoxetine and 50 pM ^{125}I-RTI-121. Sections were washed at 0°C for 4 x 15 min each in buffer and once in ice-cold water, air dried, and exposed for 2 d 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 pre-incubated 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 α4β2* 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-ConotoxinMII (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, 2mM $CaCl_2$ 1mM $MgSO_4$, 20mM HEPES and 0.1 % BSA (bovine serum albumin), pH 7.5) plus 1 mM PMSF (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 0.1X buffer at 0^{o}C and two final 5-s

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washes in ice cold deionized water. The striatal sections were air-dried and exposed to Kodak MR (Perkin Elmer Life Sciences, Boston, MA) for 2 to 5 days together with ^{3}H-microscale standards (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Nicotine (100 µM) was used to determine nonspecific binding.

Data analyses. To evaluate optical density values from autoradiographic films, we used the ImageQuant program from GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK. 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 previously described, 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 GraphPad Prism (Graph Pad Software Co., San Diego, CA, USA). Statistical comparisons were performed using analysis of variance (ANOVA) followed by Newman-Keuls or Bonferroni post hoc tests (GraphPad prism). A value of $p < 0.05$ was considered significant. All values are expressed as the mean ± SEM of the indicated number of animals.

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Results

Progressive nigrostriatal damage with 6-OHDA lesioning. The purpose of our study was to evaluate the role of the α4β2* and α6β2* 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 voltammetric measurements were done. Fig. 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 severely 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 (mod-sev) group ($p < 0.01$) and by ~75% in the severely lesioned group ($p < 0.001$) as compared to control, with no change in mild and moderately lesioned rats (Fig. 2). These data are in agreement with previous studies, which demonstrate 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 which may mimic tonic and phasic neuronal firing in vivo (Rice and Cragg, 2004, Zhang and Sulzer, 2004). Endogenous

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striatal dopamine release was therefore determined in control and lesioned animals in response to a single-pulse stimulus (Fig. 3 top panel), a burst of 4 pulses at 30 Hz (4p@30Hz) (Fig. 3 middle panel) or a burst of 4 pulses at 100 Hz (4p@100Hz) (Fig. 3 bottom panel). These frequencies were selected as previous work had shown that dopaminergic neurons *in vivo* fire in a low frequency tonic mode (0.5 to 10 Hz) interspersed by bursting activity (50 to 100 Hz) (Rice and Cragg, 2004, Zhang and Sulzer, 2004). Representative traces for dopamine signals obtained from control, mild, moderately, moderately-severe and severely lesioned rats 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 mild, moderately, moderately-severe and severely lesioned groups, respectively, as compared to controls. Similar declines were observed with the 4p@30Hz and 4p@100Hz stimulation frequencies. The correlation coefficients (r) between lesion size and dopamine release were equal to 0.94, 0.93 and 0.94 for 1p, 4p@30Hz and 4p@100Hz, respectively. These data show that there is a decline in both tonic and phasic dopamine release, as might be expected with dopaminergic denervation.

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 previously described (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 the mildly, moderately, moderately-severe and severely

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lesion groups, 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 had 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 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 to 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.

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 due to 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 (4 pulses@100Hz, 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

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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 non-significant 37% decrease in dopamine release with α-CtxMII while 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. 6D, E). There was no significant difference in release in the presence of α-CtxMII compared to that with mecamylamine in any lesioned group (Fig. 6B to 6E). These data indicate that there is a reduction in the ability of nAChR to modulate dopamine release with increased lesion size.

The results in Fig. 6 were analyzed to evaluate the contribution of the α4β2* and α6β2* 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 α6β2* nAChR-mediated component was calculated by subtracting release in the presence of α-CtxMII from Total release (Table 1). α4β2* 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 α4β2* and α6β2* 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.

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Effect of burst stimulation on dopamine release in the presence of α6β2* and α4β2* 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 due to 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 α6β2* nAChRs with either α-CtxMII or mecamylamine resulted in a significant increase in the ratio of burst to single-pulse induced dopamine release in the control ($p < 0.01$), moderately lesion ($p < 0.01$) and moderately severe ($p < 0.05$) lesioned groups (Fig. 8 right column), with similar trends in the mildly lesioned group (Fig. 8 right column). The lack of change in the severely lesioned group may simply represent a floor effect. These findings suggest that α6β2* and α4β2* receptors both regulate burst-evoked dopamine release with nigrostriatal damage. The cellular mechanisms that regulate dopamine release with burst firing appear to be retained throughout the neurodegenerative process.

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Declines in α4β2* and α6β2 * 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 α4β2* nAChRs, binding of ^{125}I-epibatidine was done in the presence of α-CtxMII using autoradiography. Significant decreases in α4β2* nAChRs ($p < 0.001$) were obtained in both the moderately-severe and severely lesioned groups (Table 2). α6β2* 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 α4β2* and α6β2* sites are most likely due to the fact that α4β2* 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, α6β2* nAChR appear to be primarily present on dopaminergic terminals (Grady et al., 2007, Gotti et al., 2009, Livingstone and Wonnacott, 2009).

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Discussion

The present results are the first to investigate the contribution of striatal α4β2* and α6β2* nAChRs to tonic and phasic evoked dopamine release with nigrostriatal damage. Fast scan cyclic voltammetric 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 as they suggest that drugs targeting α4β2* and α6β2* receptor subtypes may both be of therapeutic importance, with a somewhat more prominent and possibly more selective role for the α6β2* 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 present 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, Zhang et al., 2009b). α4β2* and α6β2* nAChRs both modulate single-pulse evoked dopamine release in intact rat striatum, with the major component of nAChR-modulated release (~65%) mediated by the α6β2* 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, Perez et al., 2009). Our current results in lesioned rats show that the total amount of tonically

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evoked nAChR-modulated dopamine release declines with the extent of neuronal damage, but that the ratio of release regulated by the α6β2* and α4β2* nAChRs remained similar, that is, 65% to 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 also other behaviors such as reward (Sandberg and Phillips, 2009). Fast-scan cyclic voltammetric 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, Perez et al., 2009). In our studies, dopamine release stimulated by higher frequencies appeared not to be affected 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, Zhang et al., 2009b). Thus, there appears 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, Zhang et al., 2009a).

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Work by Garris and coworkers has shown that nigrostriatal damage reduces phasic dopamine signaling (Garris et al., 1997, Bergstrom et al., 2001, Sandberg and Phillips, 2009). We obtained similar results and further demonstrate the involvement of the α4β2* and α6β2* nAChR subtypes in phasic signaling with nigrostriatal damage. The results show that the α6β2* nAChR subtype contributes to the regulation of burst-evoked release throughout the neurodegenerative process. By contrast, the influence of the α4β2* nAChR subtype on phasic release appears to decline to a proportionately greater extent with increasing lesion size. Thus, with mild nigrostriatal damage, the contribution of the α4β2* nAChRs to burst-evoked release is similar to that for the α6β2* subtype, with a negligible involvement of the α4β2* receptor with moderate and moderately-severe damage. These findings may suggest that the α6β2* subtype plays a greater role with increased dopaminergic denervation. A possible explanation for this finding is that the α6β2* 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 α4β2* and the α6β2* nAChR subtypes may play a pivotal role in the functional changes observed with nigrostriatal dopamine degeneration.

Previous work had 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). Since nicotine stimulates multiple nAChRs, the question arises which subtypes are important for these behavioral effects. The present studies showing that striatal α4β2* and α6β2* nAChRs modulate evoked dopamine release suggests that both these populations are important in striatal function with increasing nigrostriatal damage. Earlier receptor work had shown that striatal α6β2* nAChR sites are more susceptible to nigrostriatal degeneration than α4β2* nAChRs, with a

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complete loss of α6β2* sites with severe nigrostriatal damage (Quik et al., 2001, Quik et al., 2003). By contrast, α4β2* nAChR expression decreased by 30-40% under the same experimental conditions. The present data demonstrating similar declines in α4β2* and α6β2* nAChR-modulated function with nigrostriatal damage suggest that the α4β2* and α6β2* nAChRs that influence dopamine release exist on dopaminergic terminals equally susceptible to nigrostriatal damage. The α4β2* 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 and in evoked endogenous dopamine release measured using cyclic voltammetry (McCallum et al., 2005, McCallum et al., 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 pre-symptomatic 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 appear conflicting. In support of dopaminergic compensation, Zigmond and coworkers had observed enhanced electrically-stimulated ^{3}H-dopamine release from striatal slices of 6-OHDA lesioned rats as compared to

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controls (Zigmond et al., 1984, Snyder et al., 1990, Zigmond et al., 1990). However, Garris and coworkers obtained no enhancement of dopamine release in the same parkinsonian animal model as assessed using cyclic voltammetry (Garris et al., 1997, Bergstrom et al., 2001). 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 these latter studies.

Altogether, our results suggest that α6β2* and α4β2* nAChR modulate evoked dopamine release throughout the neurodegenerative process. Both these receptor subtypes may thus influence the progressive changes observed in Parkinson's disease. A better understanding of the dynamic control of α4β2* and α6β2* 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.

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Footnotes

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Figure Legends

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 in 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 ± SEM of the indicated number of rats. ***$p < 0.001$ indicates significance of difference from control using a Newman-Keuls multiple comparisons *post hoc* test.

Fig. 2. Motor deficits with progressive nigrostriatal damage. Parkinsonism was assessed using the limb assymetry or cylinder test. The percent use of the affected limb was determined during a five minute rating period for each animal. A statistically significant decrease in the use of the contralateral paw was only observed in the moderately-severe and severely lesioned groups. The values represent the mean ± SEM of 4-9 rats. **$p < 0.01$; ***$p < 0.001$ indicate significance of difference from control using a Newman-Keuls multiple comparisons *post hoc* test.

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 1 pulse, 4 pulses at 30 Hz, and 4 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

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and 0.5 s. Values represent the mean ± SEM of 4-9 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.

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 ± SEM of 4-9 rats per group. **p < 0.01; ***p < 0.001 indicate significance of difference from control using a Newman-Keuls multiple comparisons *post hoc* test.

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 (100nM) or the general nAChR blocker mecamylamine (100μM). Sample dopamine signals following a (A) 1 pulse, (B) 4 pulses at 30 Hz, and (C) 4 pulses at 100 Hz electrical stimulation are shown. The scale bar represents 100 nM and 2.5 s. Quantitative analyses of peak dopamine release show that with a 1 pulse stimulus (D), ~45% of endogenous dopamine release is mediated through α6β2* nAChRs while ~25% is modulated by α4β2* nAChRs as evidence by significant decreases in release in the presence of α-CtxMII or mecamylamine. In contrast, dopamine release-stimulated at higher frequencies was not affected by perfusion of α-CtxMII nor mecamylamine (E, F). Values represent the mean ± SEM of 4-9 rats. ***p < 0.001 indicates significance of difference from Total; $^{+}$p < 0.05 indicates

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significance of difference from α-CtxMII using a Newman-Keuls multiple comparisons *post hoc* test.

Fig. 6. Effect of α6β2* and/or α4β2* nAChR blockade on single-pulse-stimulated dopamine release with varying nigrostriatal damage. Dopamine release was measured in the absence (Total) and presence of the α6β2* nAChR antagonist α-CtxMII (100nM) or the general nAChR blocker mecamylamine (100μM). Release was normalized to total release for each lesioned group. NAChR inhibition with either α-CtxMII or mecamylamine significantly decreased dopamine release in the mildly (B) and moderately (C) lesioned groups although to a lesser extent than in controls (A). No significant changes were observed in the moderately severe (D) and severely (E) lesioned groups. Values represent the mean ± SEM of 4-9 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.

Fig. 7. Both α6β2* and α4β2* nAChR-modulated release decline with nigrostriatal damage. NAChR-mediated release was determined by subtracting release in the presence of mecamylamine from total release. α6β2* mediated release was determined by subtracting release in the presence of α-CtxMII from total release. α4β2* 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 α6β2* and α4β2* mediated release. Values represent the mean ± SEM of 4-9 rats. *$p < 0.05$; **$p < 0.01$; ***$p <$

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0.001 indicate significance of difference from control using a Newman-Keuls multiple comparisons *post hoc* test.

Fig. 8. NAChR antagonism results in similar effects on burst-stimulated dopamine release in control and dopamine depleted striatum. Left panels: 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 panels: 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 panels: normalization of the data to 1 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 α4β2* and α6β2* nAChRs influence burst-evoked release throughout the neurodegenerative process. Values represent the mean ± SEM of 9 control rats and 4 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.

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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 ± SEM of 9 controls and 4 rats in each lesioned group.

	Dopamine Release (nM)								
Group	Total			CtxMII			Mec		
	1p	4p@30Hz	4p@100Hz	1p	4p@30Hz	4p@100Hz	1p	4p@30Hz	4p@100Hz
Control	317±32	388±65	408±73	175±28	261±42	393±59**	89±9.4	207±43*	472±45^{***,a}
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
Mod-Sev	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

Significance of difference from 1p using a Bonferroni post hoc test: *p < 0.05; **p < 0.01; ***p <0.001. Significance of difference from 4p@30Hz using a Bonferroni post hoc test: ^{a}p < 0.001.

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TABLE 2

Preferential decrease in striatal α6β2*, as compared to α4β2* 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 α4β2* and α6β2* 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)	Mod-Sev (n = 4 rats)	Severe (n = 4 rats)
			(% Control)		
α4β2* nAChRs	100 ± 2.3	92 ± 2.7	90 ± 2.3	75 ± 2.6***	53 ± 3.5***
α6β2* nAChRs	100 ± 4.2	70 ± 7.8**	39 ± 7.7***	19 ± 9.8***	7.0 ± 3.3***

Significance of difference from control using a Newman-Keuls post hoc test: **p < 0.01; ***p < 0.001

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Figure 1

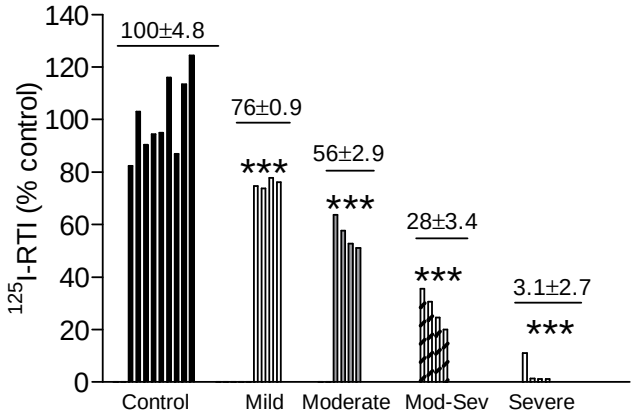

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Figure 2

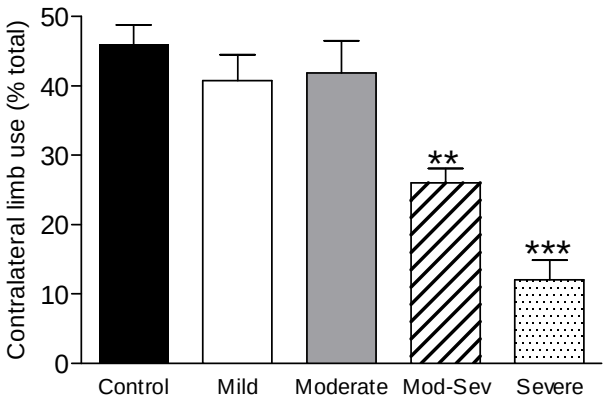

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Figure 3

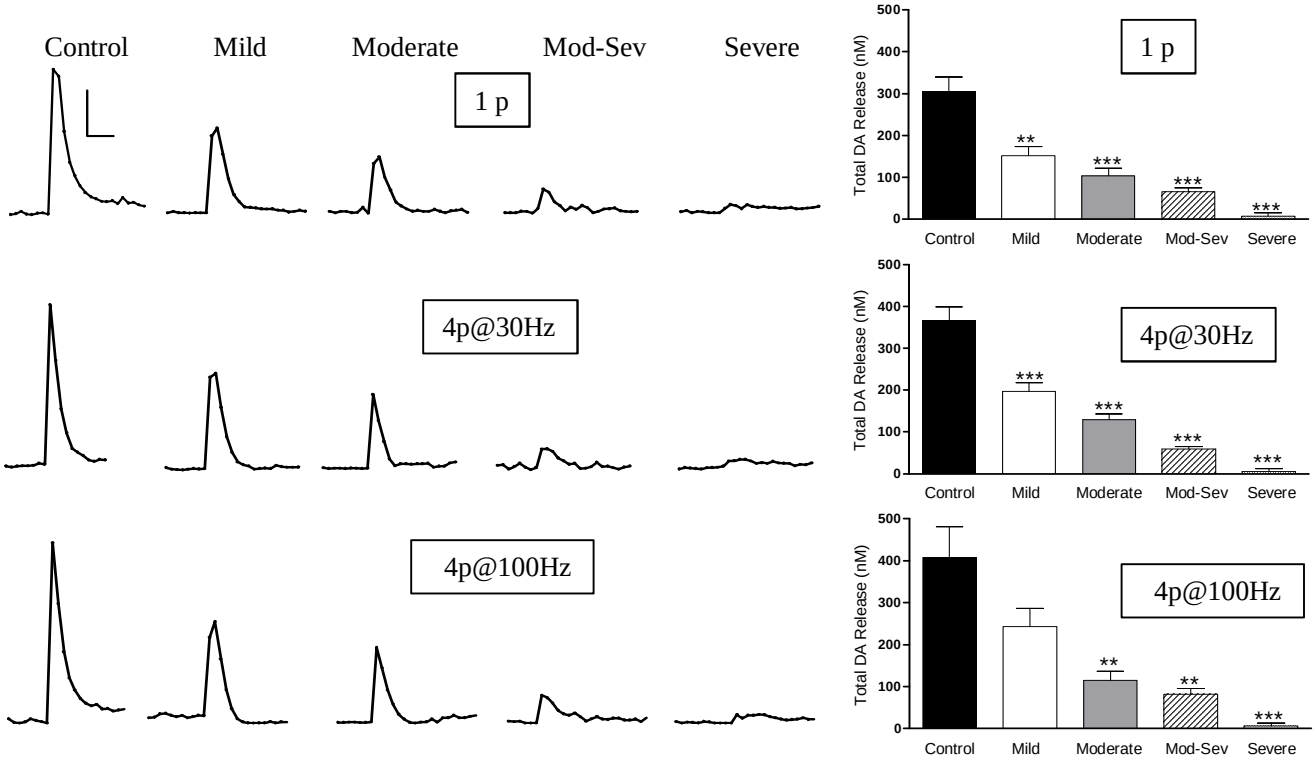

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Figure 4

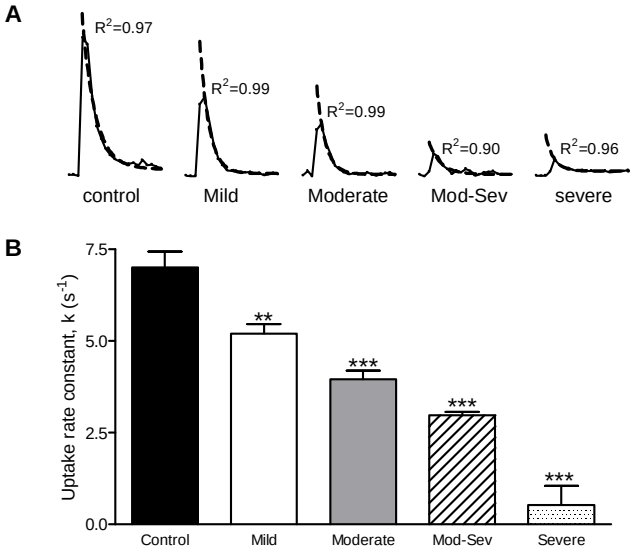

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Figure 5

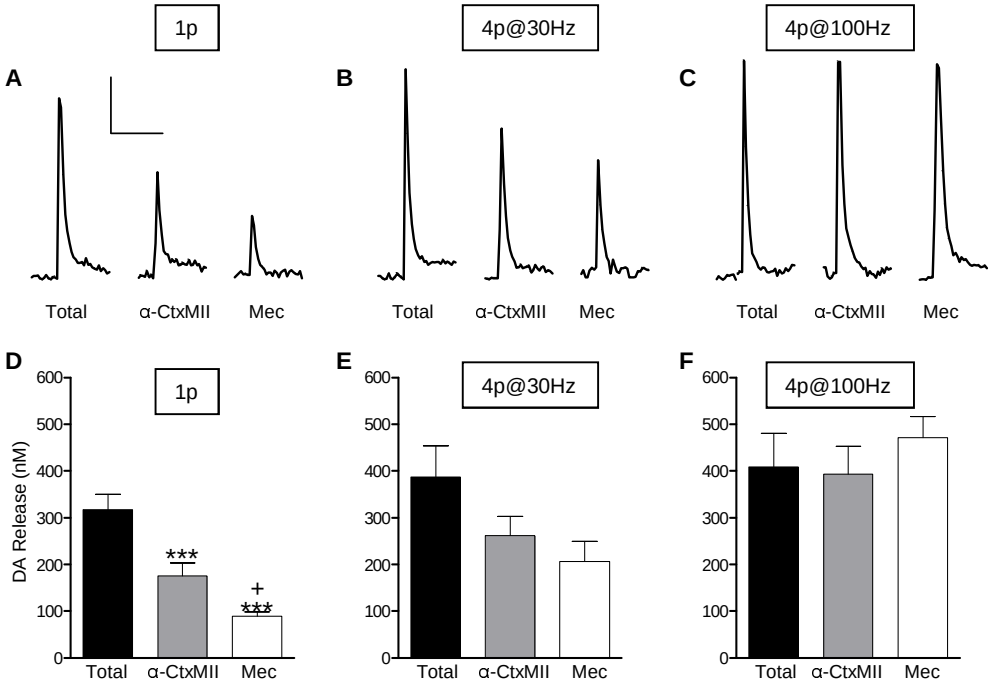

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Figure 6

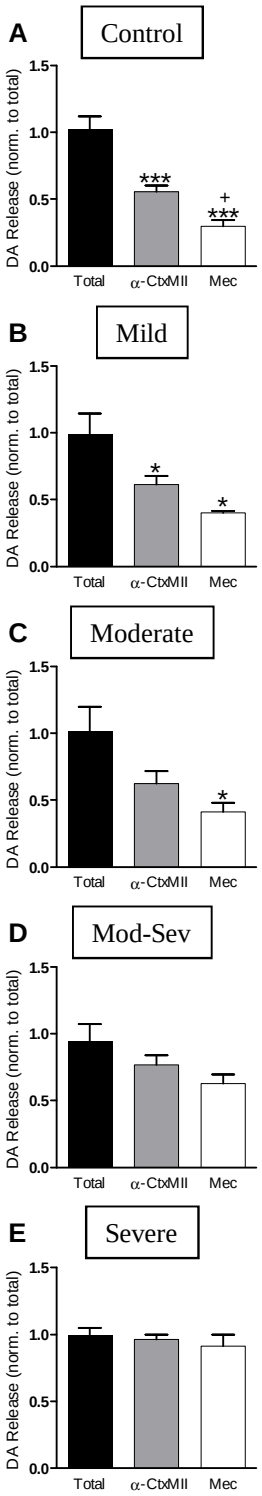

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Figure 7

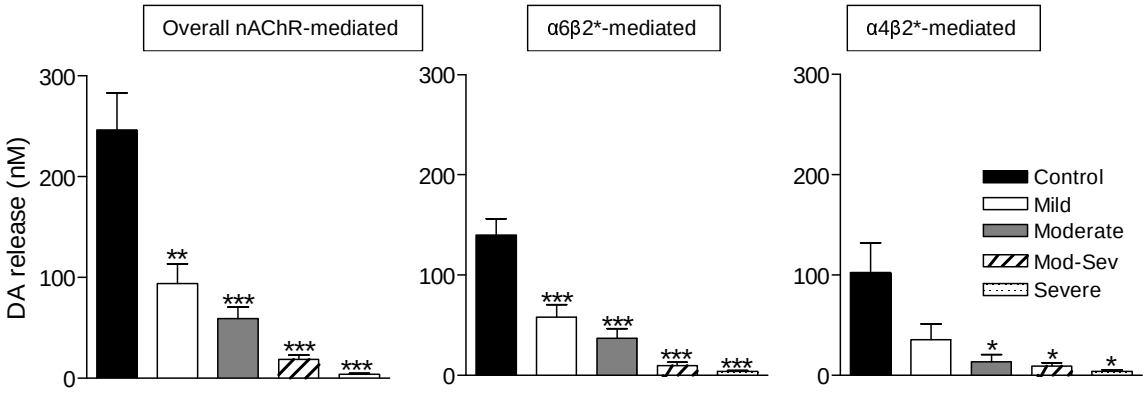

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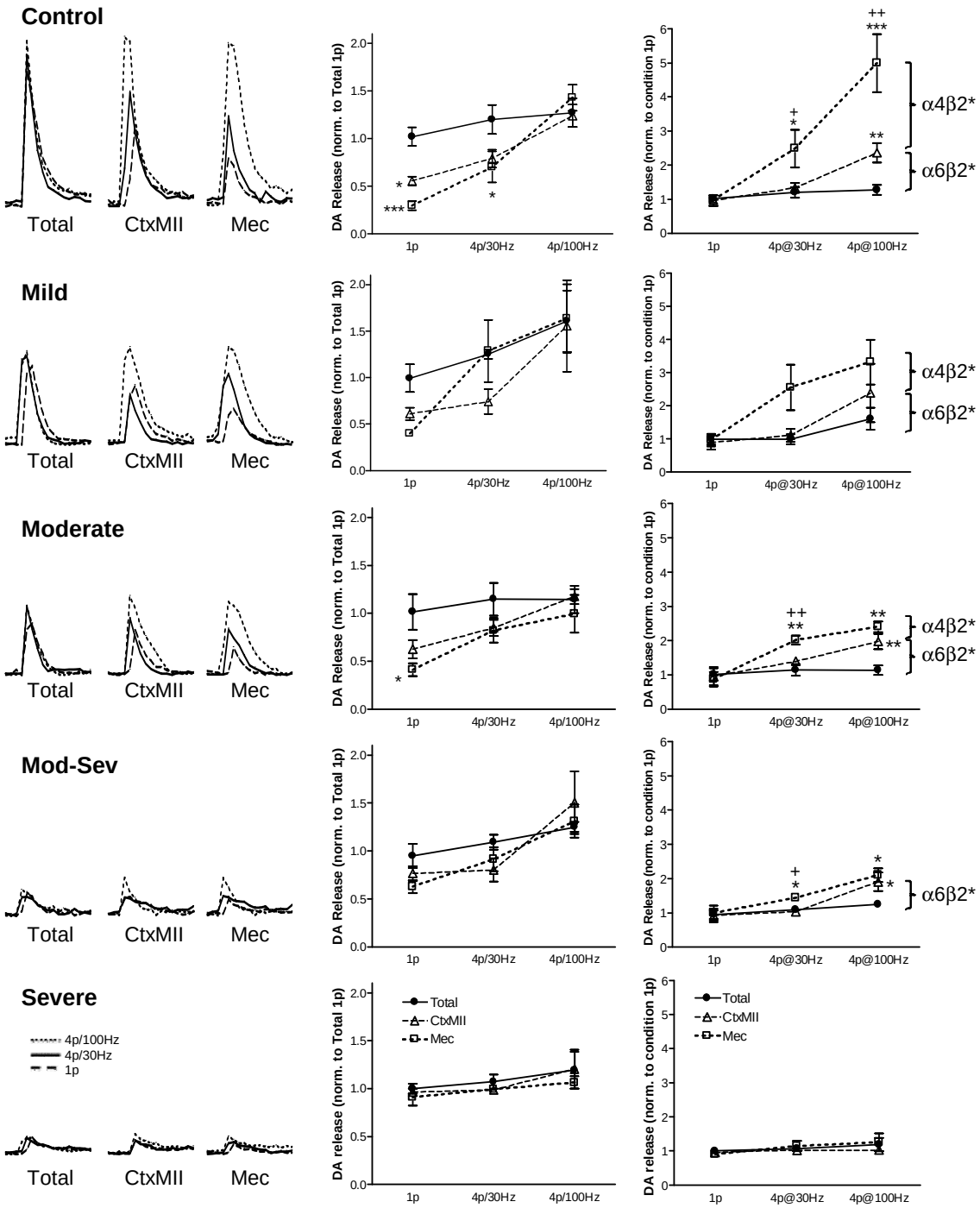
Figure 8



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