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Role for $\alpha 6$ nicotinic receptors in L-dopa-induced dyskinesias in parkinsonian mice

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Abstract

L-Dopa-induced dyskinesias are a serious side effect that develops in most Parkinson's disease patients on dopamine replacement therapy. Few treatment options are available to manage dyskinesias; however, recent studies show that nicotine reduces these abnormal involuntary movements (AIMs) in parkinsonian animals by acting at nicotinic acetylcholine receptors (nAChRs). Identification of the nAChR subtypes that mediate this reduction in AIMs is important as it will help in the development of nAChR subtype selective drugs for their treatment. Here we investigate the role of $\alpha 6\beta 2^*$ nAChRs, a subtype selectively present in the nigrostriatal pathway, using $\alpha 6$ nAChR subunit null mutant ($\alpha 6(-/-)$) mice. Wildtype and $\alpha 6(-/-)$ mice were lesioned by unilateral injection of 6-hydroxydopamine (3 $\mu\text{g}/\mu\text{l}$) into the medial forebrain bundle. They were then given L-dopa (3 mg/kg) plus benserazide (15 mg/kg) 2-3 wk later. L-dopa-induced AIMs developed to a similar extent in $\alpha 6(-/-)$ and wildtype mice. However, AIMs in $\alpha 6(-/-)$ mice declined to ~50% of that in wildtype mice with continued L-dopa treatment. Nicotine treatment also decreased AIMs by ~50% in wildtype mice, although not in $\alpha 6(-/-)$ mice. There were no effects on parkinsonism under any experimental condition. To conclude, the similar declines in L-dopa-induced AIMs in nicotine-treated wildtype mice and in $\alpha 6(-/-)$ mice treated with and without nicotine indicate an essential role for $\alpha 6\beta 2^*$ nAChRs in the maintenance of L-dopa-induced AIMs. These findings suggest that $\alpha 6\beta 2^*$ nAChR drugs have potential for reducing L-dopa-induced dyskinesias in Parkinson's disease.

Keywords

$\alpha 6$; dyskinesia; L-dopa; nicotine; 6-hydroxydopamine; Parkinson's disease

1. Introduction

The dopamine precursor L-dopa is the gold standard for treating the motor deficits in Parkinson's disease, a neurodegenerative disorder characterized by a progressive loss of

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nigrostriatal dopaminergic neurons (Halliday et al., 2011; Rascol et al., 2011; Schapira and Jenner, 2011; Wichmann et al., 2011). Although L-dopa ameliorates the rigidity, tremor and bradykinesia associated with Parkinson's disease, long term treatment results in a unique repertoire of abnormal involuntary movements or dyskinesias that can be very debilitating (Halliday et al., 2011; Rascol et al., 2011; Schapira and Jenner, 2011; Wichmann et al., 2011). Numerous studies over the last few decades have attempted to identify the molecular mechanisms that underlie L-dopa-induced dyskinesias. Converging evidence suggests a primary role for the D1 dopaminergic system (Fisone and Bezard, 2011). In addition, molecular, cellular and drug behavioral studies indicate an involvement of numerous other neurotransmitter systems including the serotonergic, glutamatergic, adrenergic, opioid and adenosine systems (Brochie and Jenner, 2011; Carta and Bezard, 2011; Fisone and Bezard, 2011).

The nicotinic cholinergic system also appears to contribute to L-dopa-induced dyskinesias. This contention is based on studies showing that nicotine administration reduced these abnormal movements in parkinsonian monkeys, rats and mice (Bordia et al., 2008; Huang et al., 2011a; Quik et al., 2007). This effect of nicotine is most likely due to an interaction at nicotinic acetylcholine receptors (nAChRs) since nAChR agonists also decrease L-dopa-induced AIMs (Huang et al., 2011b). The nAChR antagonist mecamylamine also attenuated AIMs, suggesting that nicotine and nAChR agonists induce their effect by desensitizing or inactivating nAChRs (Bordia et al., 2010). This possibility is consistent with nicotine's mechanisms of action in other disorders, such as depression, nicotine addiction and alcoholism (Dopico and Lovinger, 2009; Mineur and Picciotto, 2010).

Current work shows that the majority of nAChRs in the nigrostriatal pathway contain the $\beta 2^*$ subunit ($\beta 2^*$), with the asterisk denoting the presence of other subunits in the receptor complex (Gotti et al., 2010; Quik and Wonnacott, 2011). These $\beta 2^*$ nAChRs are pentameric ligand-gated cation channels that may also contain other α or ligand binding subunits ($\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 6$ and $\alpha 7$) and β or structural subunits ($\beta 2$ and $\beta 4$), as well as accessory subunits ($\beta 3$ and $\alpha 5$). The most abundant $\beta 2^*$ subtypes in the striatum have been subdivided into $\alpha 4\beta 2^*$ and $\alpha 6\beta 2^*$ nAChRs, based on the ability of these receptors to interact with α -conotoxinMII (α -CtxMII), a snail toxin that selectively binds to the $\alpha 6$ nAChR subunit (Quik et al., 2011). $\alpha 4\beta 2^*$ nAChRs are widely expressed throughout the brain, including the nigrostriatal pathway. By contrast, $\alpha 6\beta 2^*$ nAChRs are restricted to a limited number of brain regions, including the nigrostriatal system. Functionally, both the $\alpha 4\beta 2^*$ and $\alpha 6\beta 2^*$ nAChRs play a critical role in regulating striatal activity and behaviors mediated through the dopaminergic system (Quik and Wonnacott, 2011; Threlfell and Cragg, 2011).

With respect to an involvement in behavior, our previous data had shown that $\beta 2^*$ nAChRs play an essential role in the antidyskinetic effect of nicotine (Huang et al., 2011a). Evidence for this stemmed from studies showing that L-dopa-induced AIMs were about 40% lower in $\beta 2^{-/-}$ mice compared to their wildtype littermates. Moreover, this reduction was similar to the nicotine-mediated decline in L-dopa-induced AIMs in wildtype mice. The present studies were done to determine whether select $\beta 2^*$ nAChR subtypes might be important for the occurrence of L-dopa-induced dyskinesias. To approach this, mice with a genetic deletion of the $\alpha 6$ subunit were lesioned with 6-hydroxydopamine (6-OHDA), administered L-dopa and subsequently treated with or without nicotine. The results indicate that $\alpha 6\beta 2^*$ nAChRs primarily mediate the antidyskinetic effect of nicotine. These data suggest that drugs targeting $\alpha 6\beta 2^*$ nAChRs may be useful in the management of L-dopa-induced dyskinesias in Parkinson's disease.

2. Methods

2.1. Animals and treatment

$\alpha 6(-/-)$ C57BL/6 mice (Champtiaux et al., 2002) were originally obtained from Uwe Maskos (Pasteur Institute, Paris, France). $\alpha 6(-/-)$ C57BL/6 mice and their wildtype littermates were bred by heterozygous matings, raised and genotyped at the University of Colorado, as previously described (Salminen et al., 2004). The adult mice (20-25g) were then shipped to SRI for the behavioral and biochemical studies. Upon arrival, the mice were given food and water *ad libitum*. They were group housed in a room with controlled temperature and humidity, and a 12 h light/dark cycle. One wk later, they were lesioned by unilateral administration of 6-OHDA (Sigma-Aldrich Co., St. Louis, MO) into the right medial forebrain bundle, as previously described (Huang et al., 2011a). The mouse was placed in the stereotaxic frame, with the head aligned such that bregma and lambda were leveled, that is, with the incisor bar set at zero. 6-OHDA (3 μ g free base/ μ l in 0.9% saline containing 0.02% ascorbic acid) was stereotactically injected under isofluorane anesthesia at the following site: anteroposterior, -1.2; lateral, -1.2; ventral, 4.75, relative to the bregma. The cannula was slowly positioned into the target area and drug delivery was done over a 2 min period. The cannula was maintained at the injection site for an additional 2 min, followed by a 2 min removal. Buprenorphine (0.3 mg/kg subcutaneously) was immediately injected postoperatively, as well as 10 ml/kg saline to minimize dehydration. Following surgery, a 20% sucrose solution containing ground food pellets was placed at the bottom of the cage to assist feeding for 1 to 2 wk, as necessary. All procedures were approved by the Institutional Animal Care and Use Committee in accordance with the NIH Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to *in vivo* techniques, if available.

2.2. Forelimb use asymmetry test

Two wk after the lesion, the mice were tested for nigrostriatal damage associated motor deficits using the forelimb use asymmetry (cylinder) test. The mice were placed in a transparent container and evaluated for exploratory activity by a blinded rater over a 3-min period, as previously described (Brooks and Dunnett, 2009; Huang et al., 2011a; Schallert et al., 2000). Wall exploration was expressed as % use of the impaired (contralateral) forelimb to the total number of limb use movements.

2.3. Measurement of L-dopa-induced AIMs

Mice were subcutaneously administered 3 mg/kg L-dopa methyl ester plus 15 mg/kg benserazide, an aromatic amino acid decarboxylase inhibitor given to inhibit the breakdown of L-dopa in the periphery (both drugs from Sigma-Aldrich Co., St. Louis, MO). They were injected once daily for 2 wk until they were stably dyskinetic as assessed using a previously described mouse AIMs rating scale (Cenci and Lundblad, 2007; Huang et al., 2011a). Briefly, mice were placed in separate transparent containers, and scored individually for 1 min every 15 min over a 2 h period by a blinded rater. Each AIM subtype (oral, axial and forelimb) was scored on a frequency scale ranging from 0 to 4 (0 = no AIMs; 1 = occasional AIMs displayed <50% of the observation time; 2 = sustained AIMs for >50% of the observation time; 3 = continuous AIMs; 4 = continuous AIMs not interruptible by external stimuli). Each of the AIM subtypes was also scored for amplitude designated as A or B, with "A" representing oral AIMs without tongue protrusion, forelimb AIMs without shoulder involvement, and axial AIMs with body twisting <60°. "B" represents oral AIMs with tongue protrusion, forelimb AIMs with shoulder involvement or axial AIMs with body twisting >60°. The total score per mouse at any time point was calculated as follows; 1A = 1, 1B = 2, 2A = 2, 2B = 4, 3A = 4, 3B = 6, 4A = 6, 4B = 8, with a score for any one component (oral, axial or forelimb) ranging from 0 to 8. Therefore, the maximum possible

score for each mouse was 192 (max score per session = 24, with 8 sessions over the 2 h period).

2.4. Nicotine treatment

Mice were given nicotine in drinking water containing 2% saccharin to mask the bitter taste of nicotine. Saccharin alone was first administered for 2 d to acclimate the mice to the drinking solution, after which nicotine was added. The nicotine dose (free base) was started at 25 µg/ml for 2 d and then gradually increased over 2 to 3 d in 50 to 100 µg/ml increments (nicotine titration) to 300 µg/ml (Fig. 1), as previously described (Huang et al., 2011a; Pietila and Ahtee, 2000; Sparks and Pauly, 1999). Several wk later the dose was increased to 400 µg/ml as plasma cotinine levels were relatively low at the 300 µg/ml dose.

2.5. Plasma cotinine measurement

Plasma nicotine metabolite levels were measured as they provide a relatively good marker of nicotine intake (Matta et al., 2007). We assayed plasma cotinine, a primary metabolite of nicotine with an 18-h half-life. Blood (~0.2 ml) was drawn from the retro-orbital sinus under isoflurane anesthesia at wk 2 and wk 5 after the nicotine titration phase when the mice were receiving 300 and 400 µg/ml nicotine in the drinking water, respectively. Plasma cotinine was then measured using an EIA kit from Orasure Technologies, Bethlehem, PA.

2.6. Tissue preparation

Mice were killed by cervical dislocation 45 min after L-dopa administration. The brains were quickly removed and coronally bisected at the level of the mid striatum. The striatum from the anterior portion of the brain was immediately dissected for preparation of synaptosomes for the ³H-dopamine release studies. The posterior portion of the brain was quick frozen in isopentane on dry ice and stored at -80°C. When required, 8 µm sections were cut at -20°C in a cryostat (Leica Microsystems Inc., Deerfield, IL), thaw mounted onto poly-L-lysine coated slides, dried, and stored at -80°C.

2.7. Dopamine transporter autoradiography

¹²⁵I-RTI-121 (specific activity, 2200 Ci/mmol; PerkinElmer Life and Analytical Science, Boston, MA, USA) autoradiography was performed as previously described (Huang et al., 2011a). Sections were preincubated in buffer (50 mM Tris-HCl, pH 7.4, 120 mM NaCl, 5 mM KCl) twice for 15 min and then incubated for 2 h in the same buffer also containing 20 pM ¹²⁵I-RTI-121, 0.025% bovine serum albumin (BSA) and 1 µM fluoxetine. Nonspecific binding was determined in the presence of the dopamine uptake inhibitor nomifensine (100 µM). Binding was terminated by washing of the sections 4 × 15 min in ice cold buffer and then for 10 s in ice cold deionized water. The sections were dried and exposed to Kodak MR film (Easterman Kodak Co., Rochester, NY, USA) for several days along with ³H-microscale standards (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK).

2.8. Nicotinic receptor autoradiography

Binding of ¹²⁵I-epibatidine (specific activity, 2200 Ci/mmol; PerkinElmer Life and Analytical Science, Boston, MA, USA) was done as described (Huang et al., 2011a). Slides were pre-incubated for 15 min at room temperature in buffer containing 50 mM Tris, pH 7.0, 120 mM NaCl, 5 mM KCl, 2.5 mM CaCl₂, and 1.0 mM MgCl₂, followed by a 40 min incubation with 0.015 nM ¹²⁵I-epibatidine with or without α-CtxMII (100 nM). Nonspecific binding was determined in the presence of 100 µM nicotine. Binding was terminated by washing the slides 2 × 5 min in buffer at 4°C and 10 s in ice-cold deionized water. They were then dried and exposed to Kodak MR film with ³H-microscale standards for several days.

^{125}I - α -CtxMII (specific activity, 2200 Ci/mmol) binding to striatal section was done as reported previously (Quik et al., 2003). Slides were preincubated for 15 min at room temperature in binding buffer (144 mM NaCl, 1.5 mM KCl, 2mM CaCl_2 , 1mM MgSO_4 , 20mM HEPES and 0.1% BSA, pH 7.5) plus 1 mM PMSF (phenylmethylsulfonyl fluoride), followed by 1 h incubation in the same buffer also containing 0.5% BSA, 5 mM EDTA, 5 mM EGTA and 10 $\mu\text{g/ml}$ each of aprotinin, leupeptin and pepstatin A, plus 0.15 nM ^{125}I - α -CtxMII. Nicotine (100 μM) was used to determine nonspecific binding. The assay was terminated by washing the slides for 10 min at room temperature in binding buffer, 10 min in ice-cold binding buffer, twice for 10 min in 0.1x 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 film for several days with ^3H -microscale standards.

2.9. ^3H -Dopamine release

A crude synaptosomal fraction was prepared as described (Quik et al., 2003). Briefly, tissue was homogenized in 2 ml ice-cold 0.32 M sucrose buffered with HEPES 50 mM (pH 7.5) and centrifuged at 12,000 g for 20 min. The resuspended synaptosomes were incubated in 37°C buffer (128 mM NaCl, 2.4 mM KCl, 1.2 mM KH_2PO_4 , 3.2 mM CaCl_2 , 1.2 mM MgSO_4 , 25 mM HEPES, pH 7.5, 10 mM glucose, 1 mM ascorbic acid and 0.01 mM pargyline) for 10 min. They were then incubated with 100 nM ^3H -dopamine (3,4-[ring-2,5,6- ^3H]; 30-60 Ci/mmol, Perkin Elmer Life Sciences, Boston, MA, USA) (4 μCi for 0.8 ml synaptosomes) for 5 min. Eighty μl synaptosomal aliquots (0.5 to 2 mg tissue) were next pipetted onto 5 mm A/E glass-fiber filters (Gelman, Inc, Ann Arbor, MI) on polypropylene platforms. Synaptosomes were perfused for 10 min with perfusion buffer (uptake buffer plus 0.1% BSA and 10 μM nomifensine) at a rate of 1 ml/min. Synaptosomal ^3H -dopamine release was evoked by an 18 s exposure to nicotine (0.3 μM to 100 μM). α -CtxMII (50 nM) was perfused for 3 min before nicotine exposure to distinguish between $\alpha 4\beta 2^*$ and $\alpha 6\beta 2^*$ nAChR-mediated release. Twelve 18 s fractions were collected, and radioactivity determined using liquid scintillation counting. Release was determined using a curve-fitting algorithm of SigmaPlot 5.0 for DOS (Jandel Scientific, San Rafael, CA) as previously described (Quik et al., 2003). Release was normalized to the tissue wet weight to obtain units of cpm/mg tissue.

2.10. Data analyses

For quantitation of the autoradiograms, optical density measurements were assessed using the ImageQuant system (GE Healthcare, Little Chalfont, Buckinghamshire, UK). These values were converted to fmol/mg tissue using standard curves generated from ^3H standards as previously described (Artymyshyn et al., 1990). The optical density readings of the samples fell within the linear range of the standards. Data analyses were done with GraphPad Prism® (GraphPad Software, Inc, San Diego, CA) using analysis of variance (ANOVA) followed by the appropriate post hoc test. A level of 0.05 was considered significant. Values are the mean $\pm$ S.E.M. of the indicated number of mice.

3. Results

3.1. Loss of the $\alpha 6\beta 2^*$ nAChR does not influence the development of L-dopa-induced AIMs

Our previous data had shown that L-dopa-induced AIMs were reduced in $\beta 2(-/-)$ mice compared to their wildtype littermates (Huang et al., 2011a), suggesting that $\alpha 4\beta 2^*$ and/or $\alpha 6\beta 2^*$ nAChRs play a role in their occurrence. To better understand the contribution of the $\alpha 6\beta 2^*$ receptor population, we investigated the effect of genetic deletion of the $\alpha 6$ subunit on L-dopa-induced AIMs. For these studies, $\alpha 6(-/-)$ mice and their wildtype littermates were first unilaterally lesioned with 6-OHDA, as described in Methods. They were then given L-dopa plus benserazide 2-3 wk later, as described in Methods and outlined in Fig. 1. L-dopa

treatment led to the development of AIMs in both wildtype and $\alpha 6(-/-)$ mice, with no significant difference in scores between the groups either 3 or 4 wk later. Three wk after the start of L-dopa treatment, AIMs were 59.48 ± 10.71 ($n = 23$) for $\alpha 6(-/-)$ mice and 66.18 ± 10.03 ($n = 22$) for wildtype littermates. The values for 4 wk after L-dopa-treatment, that is, immediately prior to the nicotine titration phase, were 56.6 ± 9.7 ($n = 23$) in the $\alpha 6(-/-)$ mice and 64.7 ± 9.7 ($n = 22$) in their wildtype littermates. These latter scores are depicted on the y-axis in Fig. 2. Thus the absence of $\alpha 6^*$ nAChRs does not significantly affect the development of L-dopa-induced AIMs.

3.2. Loss of the $\alpha 6\beta 2^*$ nAChR results in a decline in the maintenance of L-dopa-induced AIMs

The $\alpha 6(-/-)$ and the wildtype mice were each randomly divided into two groups, with one group given saccharin only in drinking water and the other given saccharin plus nicotine. Plasma cotinine levels were similar in $\alpha 6(-/-)$ and wildtype mice (Table 1) and corresponded to those observed with moderate smoking (Matta et al., 2007). There was no detectable cotinine in the plasma of saccharin-treated mice.

A time course analysis shows that nicotine treatment reduced L-dopa-induced AIM scores in the wildtype mice (Fig. 2), consistent with previous work (Huang et al., 2011a), with a significant main effect of treatment ($p < 0.001$) using two-way ANOVA. Unexpectedly, L-dopa-induced AIMs gradually decreased in saccharin-treated $\alpha 6(-/-)$ mice compared to saccharin-treated wildtype mice over a 3-4 month period (Fig. 2), with a significant main effect of genotype ($p < 0.001$) using two-way ANOVA. There was no further decline in AIM scores in $\alpha 6(-/-)$ mice treated with nicotine as compared to the ones treated with saccharin (Fig. 2). There was thus a decline in L-dopa-induced AIMs in $\alpha 6(-/-)$ mice irrespective of nicotine treatment. Interestingly, similar changes were observed in L-dopa-induced AIMs with time and nicotine treatment in a study using $\beta 2(-/-)$ mice (Huang et al., 2011a), as depicted in the inset in Fig. 2. The current data suggest that the $\alpha 6\beta 2^*$ nAChR subtype may be the one important for the maintenance of L-dopa-induced AIMs.

We next assessed whether the decrease in L-dopa-induced AIMs in the $\alpha 6(-/-)$ mice is due to a selective decline in their onset and/or intensity. The pattern of the daily time course of L-dopa-induced AIM scores in $\alpha 6(-/-)$ and wildtype mice treated with and without nicotine at wk 3, 10 and 14 is shown in Fig. 3. At all 3 time points, L-dopa treatment to saccharin-treated wildtype mice led to an increase in AIMs which was maximal by 20 to 60 min. At the 3 wk time point, a decrease in AIM scores was observed in nicotine-treated wildtype mice and in both sets of $\alpha 6(-/-)$ mice, with a significant main effect of genotype ($p < 0.05$), although not nicotine treatment. By 10 wk, there were declines in AIM scores in the nicotine-treated wildtype mice and in $\alpha 6(-/-)$ mice treated with and without nicotine, with significant main effects of both nicotine ($p < 0.01$) and genotype ($p < 0.01$). At wk 14, the decline in AIM scores was maintained in the nicotine-treated wildtype mice and in $\alpha 6(-/-)$ mice treated with and without nicotine, with significant main effects of nicotine ($p < 0.001$) and genotype ($p < 0.001$). There was no significant interaction at any time point, indicating that the daily pattern of total L-dopa-induced AIM scores was similar among the different groups.

3.3. Decline in L-dopa-induced AIMs in nicotine-treated wildtype mice and in $\alpha 6(-/-)$ mice was most pronounced in oral and axial AIMs

The results in Figs. 2 and 3 depict the effect of nicotine treatment and genotype on total L-dopa-induced AIMs, a measure representing the sum of three AIM components, as detailed in Methods. Effects on the separate AIM subtypes, that is, oral, axial and forelimb AIMs, at 3, 10 and 14 wk after the initiation of nicotine treatment are shown in Fig. 4. Oral AIMs are

most sensitive to the effect of nicotine treatment and the absence of $\alpha 6\beta 2^*$ nAChRs, with significant reductions as early as 3 wk after the start of nicotine treatment. These decreased levels were maintained with time and were even more pronounced by wk 14. Significant decreases were also observed in axial AIMS in saccharin-treated $\alpha 6(-/-)$ mice at 3, 10 and 14 wk; however, the effect of nicotine on this AIM measure was very variable, with no significant differences between any treatment groups including vehicle-treated $\alpha 6(-/-)$ mice. By contrast, no significant changes were observed in forelimb AIMS. These selective effects of genotype and nicotine on specific AIM measures suggest that distinct mechanisms are linked to oral and axial, as compared to forelimb AIMS, with the former two involving $\alpha 6\beta 2^*$ nAChRs.

In addition to significant effects of both nicotine and genotype, there were also significant interactions between nicotine treatment and genotype for total and AIM subtypes at the different time points. At week 3, there was a significant interaction for oral ($p < 0.001$) and axial ($p < 0.05$) AIMS; at week 10, for total ($p < 0.05$), oral ($p < 0.001$) and axial ($p < 0.05$) AIMS; and at wk 14, for total ($p < 0.01$), oral ($p < 0.001$) and axial ($p < 0.01$) AIMS. These data indicate there are differential effect of nicotine in the wildtype and $\alpha 6(-/-)$ mice.

3.4. Striatal dopamine transporter levels are reduced after 6-OHDA lesioning in wildtype and $\alpha 6(-/-)$ mice treated with or without nicotine

Striatal dopamine transporter levels were measured on the intact and lesioned side of wildtype and $\alpha 6(-/-)$ mice treated with or without nicotine (Fig. 5, top panel) to evaluate the extent of striatal damage after 6-OHDA lesioning. Nicotine treatment did not modulate dopamine transporter levels in wildtype mice, consistent with previous results (Huang et al., 2011a), nor did it affect dopamine transporter levels in striatum of $\alpha 6(-/-)$ mice. However, there was a ~90% decrease in striatal ^{125}I -RTI-121 binding after 6-OHDA administration in wildtype mice, as predicted (Quik et al., 2003). There was a similar decline in $\alpha 6(-/-)$ mice, indicating that genetic deletion of the $\alpha 6$ subunit did not influence the extent of nigrostriatal damage. The finding that dopamine transporter levels were similar in the lesioned striata of $\alpha 6(-/-)$ and wildtype mice treated with and without nicotine suggests that changes in the extent of nigrostriatal damage did not underlie the changes in L-dopa-induced AIM scores in the different treatment groups.

3.5. Striatal nAChR levels in wildtype and $\alpha 6(-/-)$ mice treated with or without nicotine

Experiments were then done to measure nAChR levels in striata of wildtype and $\alpha 6(-/-)$ mice (Fig. 5). As expected, the most prominent result was a lack of ^{125}I - α -CtxMII binding, that is, a loss of $\alpha 6\beta 2^*$ nAChRs, in the striatum of $\alpha 6(-/-)$ mice. These data confirm that the null mutation was complete and indicate that $\alpha 6\beta 2^*$ nAChRs are necessary for the partial maintenance of L-dopa-induced AIMS. There was also a large reduction (~85%) in $\alpha 6\beta 2^*$ nAChR binding in wildtype mice with nigrostriatal damage, as previously shown (Quik et al., 2003). In wildtype mice, nicotine treatment reduced ^{125}I - α -CtxMII binding in the intact striatum by ~30%, consistent with previous findings (Lai et al., 2005; Perry et al., 2007). However, nicotine treatment did not further reduce $\alpha 6\beta 2^*$ nAChR levels on the lesioned side compared to saccharin-treated mice. Since the results with the $\alpha 6(-/-)$ indicate an essential requirement for $\alpha 6\beta 2^*$ nAChRs in the maintenance of AIMS, this would suggest that nicotine reduces AIMS in wildtype mice through a desensitization block of residual $\alpha 6\beta 2^*$ nAChRs.

$\alpha 4\beta 2^*$ nAChR levels were determined by measuring ^{125}I -epibatidine binding in the presence of α -CtxMII to block binding to $\alpha 6\beta 2^*$ nAChRs (Fig. 5). Nicotine treatment increased $\alpha 4\beta 2^*$ nAChRs in the intact and lesioned striatum of wildtype mice, as well as in the lesioned striatum of $\alpha 6(-/-)$ mice, irrespective of the levels of L-dopa-induced AIMS.

These data suggest that changes in $\alpha 4\beta 2^*$ nAChR levels do not underlie the occurrence of L-dopa-induced AIMs.

3.6. nAChR-mediated striatal dopamine release in wildtype and $\alpha 6(-/-)$ mice with and without nicotine treatment

We next did experiments to determine how the declines in L-dopa-induced AIMs correlated with changes in $\alpha 4\beta 2^*$ and $\alpha 6\beta 2^*$ nAChR-mediated function. To approach this, we measured nicotine-evoked ^3H -dopamine release from striatal synaptosomes in the presence and absence of the $\alpha 6\beta 2^*$ nAChR antagonist α -CtxMII. Release in the presence of α -CtxMII was defined as $\alpha 4\beta 2^*$ nAChR-mediated, while $\alpha 6\beta 2^*$ nAChR-mediated release was assessed by subtracting $\alpha 4\beta 2^*$ nAChR-mediated release from total release.

Consistent with the receptor studies, there was no $\alpha 6\beta 2^*$ -nAChR evoked ^3H -dopamine release in the striatum of $\alpha 6(-/-)$ mice. In wildtype mice, nicotine treatment reduced $\alpha 6\beta 2^*$ -nAChR evoked ^3H -dopamine release in the intact striatum by ~50% (Fig. 6), consistent with previous findings (Lai et al., 2005; Perry et al., 2007). There was also a large reduction (~85%) in $\alpha 6\beta 2^*$ nAChR binding with nigrostriatal damage, as previously shown (Quik et al., 2003). There was no difference in $\alpha 6\beta 2^*$ -nAChR evoked ^3H -dopamine in the lesioned striatum of wildtype mice treated with and without nicotine, in agreement with the results of the receptor studies described above. Since the experiments with the $\alpha 6(-/-)$ mice point to an essential role for $\alpha 6\beta 2^*$ nAChRs in the maintenance of AIMs, these combined data support the idea that the nicotine-mediated decline in L-dopa-induced AIMs is due to a desensitization block of the remaining $\alpha 6\beta 2^*$ nAChRs. This block may not be evident in the ^3H -dopamine release experiments because of resensitization during the *in vitro* phase of this assay.

The data for $\alpha 4\beta 2^*$ nAChR-mediated release in wildtype and $\alpha 6(-/-)$ mice treated with and without nicotine is shown in Fig. 6. Despite an increase in $\alpha 4\beta 2^*$ nAChR binding in wildtype mice with nicotine treatment, release was significantly decreased in the intact striatum, with a significant main effect of treatment ($p < 0.001$), in agreement with previous data (Lai et al., 2005). A similar decline in $\alpha 4\beta 2^*$ nAChR-mediated ^3H -dopamine release was observed in the intact striatum of $\alpha 6(-/-)$ mice ($p < 0.01$). Although there was a large decrease in release with 6-OHDA lesioning, there were no changes in release in the lesioned striatum of either wildtype or $\alpha 6(-/-)$ mice with nicotine treatment. Thus alterations in $\alpha 4\beta 2^*$ nAChR-mediated dopamine release do not appear to correlate with AIM scores in either wildtype or $\alpha 6(-/-)$ mice, suggesting that $\alpha 4\beta 2^*$ nAChR-mediated release does not contribute to the occurrence of L-dopa-induced AIMs.

3.7. No worsening of parkinsonism in lesioned wildtype and $\alpha 6(-/-)$ mice treated with or without nicotine

The forelimb use asymmetry (cylinder) test was done 2 wk after lesioning as a measure of parkinsonism (Table 2). Lesioning induced similar functional declines in wildtype and $\alpha 6(-/-)$ mice suggesting that the loss of $\alpha 6\beta 2^*$ nAChRs did not modulate motor activity linked to parkinsonism. Forelimb use asymmetry was also evaluated during the nicotine treatment regimen (Table 2). No significant differences were observed in nicotine-treated lesioned wildtype mice as compared to vehicle, consistent with previous results (Huang et al., 2011a). There were also no differences in forelimb use in $\alpha 6(-/-)$ mice treated with or without nicotine, or between wildtype and $\alpha 6(-/-)$ mice.

4. Discussion

The present results are the first to show that $\alpha 6\beta 2^*$ nAChRs influence the maintenance of L-dopa-induced AIMs in a parkinsonian mouse model, with ~ 50% decline in AIM scores in $\alpha 6(-/-)$ compared to wildtype mice. The reduced AIM scores in $\alpha 6(-/-)$ mice were similar to those in nicotine-treated wildtype mice. Notably, nicotine treatment did not further decrease L-dopa-induced AIMs in $\alpha 6(-/-)$ mice. These combined results suggest that chronic nicotine administration reduces L-dopa-induced AIMs by acting at $\alpha 6\beta 2^*$ nAChRs. Such findings support the idea that drugs that interact at $\alpha 6\beta 2^*$ receptors may be useful for the treatment of L-dopa-induced dyskinesias in Parkinson's disease.

The $\alpha 6\beta 2^*$ nAChR-mediated reduction in L-dopa-induced AIMs did not appear to be due to an effect on their initial development as scores were similar in wildtype and $\alpha 6(-/-)$ mice during the first few weeks of L-dopa treatment. Instead, it seemed to be due to a failure to maintain AIMs in $\alpha 6(-/-)$ mice since there was a decline in their levels with continued L-dopa treatment (3-4 months). These observations suggest that distinct mechanisms contribute to the development and maintenance of L-dopa-induced AIMs, with $\alpha 6\beta 2^*$ nAChRs involved in the persistence of these abnormal movements. Other neurotransmitter systems previously linked to the presence of AIMs may regulate their development, including the serotonergic, glutamatergic, adrenergic, cholinergic, opioid, adenosine and various peptidergic systems (Calabresi et al., 2010; Carta and Bezard, 2011; Duty, 2010; Rascol et al., 2011). How these systems initiate and regulate the occurrence of L-dopa-induced AIMs is an area under intense investigation.

The current studies demonstrate that a complete loss of $\alpha 6\beta 2^*$ nAChRs is associated with a reduction in L-dopa-induced AIMs. They also show that nicotine treatment decreased AIMs in wildtype mice to the same level as in $\alpha 6(-/-)$ mice, even though there were detectable striatal $\alpha 6\beta 2^*$ nAChR binding sites. Nicotine treatment is well known to desensitize $\alpha 4\beta 2^*$ nAChRs (Picciotto et al., 2008) and has more recently been shown to desensitize $\alpha 6\beta 2^*$ nAChRs (Capelli et al., 2011; Grady et al., 2012; Kuryatov and Lindstrom, 2011). Thus one explanation for our findings is that nicotine treatment to wildtype mice results in a desensitization block of the remaining $\alpha 6\beta 2^*$ nAChRs. This interpretation, coupled with previous data showing that the nAChR blocker mecamylamine reduces L-dopa-induced AIMs (Bordia et al., 2010), provides further support for the idea that nicotine decreases AIMs through a desensitization block.

As an approach to investigate receptor function, we measured $\alpha 4\beta 2^*$ and $\alpha 6\beta 2^*$ nAChR-evoked ^3H -dopamine release from striatal synaptosomes of saccharin- and nicotine-treated $\alpha 6(-/-)$ and wildtype mice. Chronic nicotine exposure decreased total ^3H -dopamine release in the intact striatum of wildtype mice as compared to saccharin treatment, with a decline in both $\alpha 4\beta 2^*$ and $\alpha 6\beta 2^*$ nAChR-mediated release, in agreement with previous studies (Lai et al., 2005). Nicotine dosing also reduced total ^3H -dopamine release in the intact striatum of $\alpha 6(-/-)$ mice as compared to saccharin treatment, due to an effect on $\alpha 4\beta 2^*$ nAChR-mediated release. However, nAChR-evoked ^3H -dopamine release was similar in the lesioned striata of wildtype mice treated with and without nicotine. Thus, alterations in dopamine release mechanisms, as measured using synaptosomal ^3H -dopamine release, do not appear to underlie the development of dyskinesias. However, these data do not rule out the possibility that the receptors may have been desensitized *in vivo* and then resensitized during the course of the release assay because of a lack of nicotine in the various wash buffers. *In vivo* studies may help resolve the question whether changes in dopamine release underlie the occurrence of L-dopa-induced AIMs.

A question that arises is whether a specific $\alpha 6\beta 2^*$ subtype is important for the maintenance of L-dopa-induced AIMs, that is, the $\alpha 6\alpha 4\beta 2\beta 3$ or $\alpha 6\beta 2\beta 3$ nAChR population. Previous studies had shown that nigrostriatal damage leads to a complete loss of $\alpha 6\alpha 4\beta 2\beta 3$ but only a partial decline in $\alpha 6\beta 2\beta 3$ nAChRs (Bordia et al., 2007). This would suggest that the ability of nicotine to reduce L-dopa-induced AIMs in lesioned mice is due to an action at $\alpha 6\beta 2\beta 3$ nAChRs since these are the ones remaining after nigrostriatal damage.

The long half-life of the primary nicotine metabolite cotinine raises the question whether the L-dopa-induced decline in AIMs with nicotine administration may be due to this primary nicotine metabolite (Matta et al., 2007). Further rationale for this suggestion stems from the results of studies showing that cotinine stimulates nAChRs (Dwoskin et al., 1999; O'Leary et al., 2008) and that it has similar effects as nicotine on acoustic startle and attention, as well as on neuroprotection (Buccafusco et al., 2009; Buccafusco and Terry, 2003). However, our previous studies in rats showed that cotinine administration did not reduce L-dopa-induced AIMs in rats even with plasma cotinine levels five times higher than those obtained with nicotine dosing (Bordia and Quik, 2010).

A comparison of our earlier studies in $\beta 2(-/-)$ mice to the current results in $\alpha 6(-/-)$ mice shows that the decline in the maintenance of L-dopa-induced AIMs in the two sets of animals is similar. These observations further support the idea that $\alpha 6\beta 2^*$ nAChRs are primarily involved in the maintenance of L-dopa-induced AIMs. The $\alpha 6(-/-)$ mice lack the $\alpha 6\alpha 4\beta 2\beta 3$ and $\alpha 6\beta 2\beta 3$ nAChRs while the $\beta 2(-/-)$ mice lack the $\alpha 6\alpha 4\beta 2\beta 3$, $\alpha 6\beta 2\beta 3$, $\alpha 4\beta 2$ and $\alpha 4\alpha 5\beta 2$ nAChRs. If one assumes that the similarity in the pattern of L-dopa-induced AIMs in $\alpha 6(-/-)$ mice and $\beta 2(-/-)$ mice is due to a common receptor decline, this suggests that the $\alpha 6\alpha 4\beta 2\beta 3$ and $\alpha 6\beta 2\beta 3$ nAChR subtypes are involved. However, as mentioned above, $\alpha 6\alpha 4\beta 2\beta 3$ are lost with nigrostriatal damage (Bordia et al., 2007). Thus, $\alpha 6\beta 2\beta 3$ nAChRs may primarily be involved in the nicotine-mediated reduction in L-dopa-induced AIMs.

Another question is whether compensatory alterations in $\alpha 4\beta 2^*$ nAChR populations play a role in the maintenance of L-dopa-induced AIMs. Our data show that $\alpha 4(\text{non}\alpha 6)\beta 2$, that is, $\alpha 4\beta 2$ and $\alpha 4\alpha 5\beta 2$, nAChR expression is elevated in lesioned wildtype and $\alpha 6(-/-)$ mice with nicotine treatment. Thus, increases in these subtypes may be important. However, $\alpha 4(\text{non}\alpha 6)\beta 2$ nAChRs are not increased in lesioned saccharin-treated $\alpha 6(-/-)$ mice, which also have reduced AIM scores. These inconsistencies, as well as discrepancies between changes in $\alpha 4(\text{non}\alpha 6)\beta 2$ nAChR-mediated ^3H -dopamine release and L-dopa-induced AIM scores, suggest that $\alpha 4(\text{non}\alpha 6)\beta 2$ nAChRs do not play a role, although desensitization may complicate interpretation of these findings. It is also possible that $\alpha 4\beta 2^*$ and $\alpha 6\beta 2^*$ nAChRs have opposing roles on L-dopa-induced AIMs such that $\alpha 4\beta 2^*$ nAChRs exert a tonic inhibitory effect on the $\alpha 6\beta 2^*$ nAChR-mediated induction of AIMs.

Previous studies had shown that nicotine more effectively reduced L-dopa-induced AIMs in animals with lower AIM scores, with the increased scores most likely due to more severe striatal damage (Huang et al., 2011a). A similar analysis of the current data did not yield such differences probably because the mice were less severely lesioned. This may be due to inherent genetic differences between the mice in the previous and current study, the lot of 6-OHDA and/or other experimental variables.

In both wildtype mice receiving nicotine and in $\alpha 6(-/-)$ mice with or without nicotine, the declines were most pronounced for oral and axial AIMs, with no effect on forelimb AIMs. This differential effect of nicotine on various AIM components most likely relates to the complexity of basal ganglia circuitry, which regulates these movements. Numerous interconnected subcortical nuclei including the striatum, the globus pallidus, the subthalamic

nucleus and the substantia nigra are involved, as well as multiple inputs to and outputs from the basal ganglia to different cortical areas and the thalamus (Gerfen and Surmeier, 2011; Guigoni et al., 2005; Obeso et al., 2008). Selective effects of nicotine on distinct AIM components may have arisen because unique basal ganglia circuits underlie the occurrence of oral, axial and forelimb AIMs, and these are differentially affected by the molecular and cellular changes associated with chronic nicotine dosing. The idea that different behaviors are associated with different neuronal circuits is readily evident from an extensive literature showing that the neuronal pathways whereby L-dopa improves the bradykinesia, tremor, and rigidity observed in Parkinson's disease are unique from those through which it induces L-dopa-induced dyskinesias, with the former most likely mediated primarily by the indirect and the latter by the direct dopaminergic pathway (Gerfen and Surmeier, 2011; Guigoni et al., 2005; Obeso et al., 2008). Similarly, the present results also show that nicotine did not affect parkinsonism although it did reduce L-dopa-induced abnormal movements.

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Abbreviations

AIMs	abnormal involuntary movements
ANOVA	analysis of variance
BSA	bovine serum albumin
α-CtxMII	α -conotoxinMII
nAChRs	nicotinic acetylcholine receptors
CNS	central nervous system
6-OHDA	6-hydroxydopamine
^{125}I-RTI-121	125 I-3 β -(4-iodophenyl)tropane-2 β -carboxylic acid isopropyl ester
*	denotes the possible presence of other subunits in the receptor complex
(-/-)	null mutant

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Highlights

- ▶ Abnormal movements developed to a similar extent in wildtype and $\alpha 6(-/-)$ mice.
- ▶ Abnormal movements declined in $\alpha 6(-/-)$ mice with continued L-dopa treatment.
- ▶ $\alpha 6\beta 2^*$ nAChRs play a critical role in the maintenance of L-dopa-induced dyskinesias.
- ▶ $\alpha 6\beta 2^*$ nAChR drugs may be useful in the treatment of dyskinesias.

Fig. 1.

Treatment timeline to investigate the role of $\alpha 6^*$ nAChRs in L-dopa-induced AIMs in parkinsonian mice. $\alpha 6^{-/-}$ mice and their wildtype littermates were lesioned unilaterally by an injection of 6-OHDA into the right medial forebrain bundle at wk 0. Parkinsonism was evaluated 2 wk after lesioning using the forelimb use asymmetry or cylinder test. L-dopa (3 mg/kg) plus benserazide (15 mg/kg) was then administered subcutaneously once a day, 5 d a week throughout the study. At wk 6 the mice were given a 2% saccharin solution for 2-3 d. Nicotine treatment was then initiated in the drinking water starting at 25 $\mu\text{g/ml}$ with the dose of nicotine increased gradually to 300 $\mu\text{g/ml}$ over 10 d (nicotine titration). The numbers in the white squares in the timeline denote the number of wk after the nicotine titration phase. At 4 wk after titration, nicotine was increased to 400 $\mu\text{g/ml}$ and subsequently maintained at that dose until the end of the study. AIMs were rated throughout the study. An additional cylinder test was performed at wk 9 after the nicotine titration phase. Mice were euthanized from wk 15 to 19 to allow time for the dopamine release studies; L-dopa and nicotine treatments were continued until the time of death.

Fig. 2.

$\alpha 6\beta 2^*$ nAChRs are required for the maintenance of L-dopa-induced AIMs. Weekly time course of the decline in total L-dopa-induced AIMs in $\alpha 6(-/-)$ mice and in wildtype (WT) mice treated with nicotine. Two and a half wk after 6-OHDA lesioning, L-dopa (3 mg/kg) plus benserazide (15 mg/kg) and nicotine treatments were initiated as described in Methods. Animals were then rated for oral, axial, and forelimb AIMs for 1 min every 15 min over a 2 h period, with total AIMs representing the sum of these three components at the indicated time points. The scores on the y-axis represent AIM levels prior to the nicotine titration phase. The x-axis represent the number of weeks after nicotine titration. Nicotine treatment reduced total L-dopa-induced AIMs in wildtype mice over the 14 wk period. There was also a decline in AIM scores in saccharin-treated $\alpha 6(-/-)$ mice, with no further effect of nicotine in these mice. The inset shows that similar changes were observed in L-dopa-induced AIMs with time and nicotine treatment in a study using $\beta 2(-/-)$ mice (Huang et al., 2011a). Values are the mean $\pm$ S.E.M. of 11 to 12 mice per group. Significance of difference of nicotine-treated wildtype from saccharin-treated wildtype mice: $*p < 0.05$; of saccharin-treated $(-/-)$ from saccharin-treated wildtype mice: $^+p < 0.05$; of nicotine-treated $(-/-)$ from saccharin-treated wildtype mice: $^{\#}p < 0.05$. There was a significant main effect of nicotine treatment between nicotine-treated wildtype and saccharin-treated wildtype mice ($p < 0.01$); of genotype between saccharin-treated $(-/-)$ and saccharin-treated wildtype mice ($p < 0.001$); of nicotine treatment and genotype between nicotine-treated $(-/-)$ and saccharin-treated wildtype mice ($p < 0.001$).

Fig. 3.

Daily time course of the decline in total L-dopa-induced AIMs in $\alpha 6(-/-)$ and wildtype (WT) mice treated with and without nicotine. Two wk after 6-OHDA lesioning, L-dopa (3 mg/kg) plus benserazide (15 mg/kg) and nicotine treatments were initiated as described in Methods. Animals were then rated for oral, axial, and forelimb AIMs for 1 min every 15 min over a 2 h period, with total AIMs representing the sum of these three components at the indicated time points. The data shown are for wk 3, 10 and 14 after the nicotine titration period. There was a decline in AIM scores in nicotine-treated wildtype mice, as well as in saccharin- and nicotine-treated $\alpha 6(-/-)$ mice. Values are the mean $\pm$ S.E.M. of 11 to 12 mice per group. Significance of difference of nicotine-treated wildtype from saccharin-treated wildtype mice: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; of saccharin-treated $\alpha 6(-/-)$ from saccharin-treated wildtype mice: + $p < 0.05$, ++ $p < 0.01$, +++ $p < 0.001$; of nicotine-treated $\alpha 6(-/-)$ from saccharin-treated wildtype mice: # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$. There was a significant main effect of genotype at wk 3 ($p < 0.05$), at wk 10 ($p < 0.01$), and at wk 14 ($p < 0.001$); of nicotine treatment at wk 10 ($p < 0.01$) and at wk 14 ($p < 0.001$). Data were analyzed by two-way ANOVA followed by a Bonferroni post hoc test.

Fig. 4.

Changes in L-dopa-induced AIMs in nicotine-treated wildtype (WT) mice and in $\alpha 6(-/-)$ mice are most pronounced in oral and axial AIMs. Lesioned mice were treated with L-dopa (3 mg/kg) plus benserazide (15 mg/kg) and nicotine as described in Methods. They were then rated for oral, axial, and forelimb AIMs. Values are the mean $\pm$ S.E.M. of 11 to 12 mice per group. Significance of difference of nicotine-treated wildtype from saccharin-treated wildtype mice: * $p < 0.05$, *** $p < 0.001$; of saccharin-treated $\alpha 6(-/-)$ from saccharin-treated wildtype mice: + $p < 0.05$, ++ $p < 0.01$, +++ $p < 0.001$. There was a significant main effect of genotype (# $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$). Data were analyzed by two-way ANOVA followed by a Bonferroni post hoc test.

Fig. 5.

Striatal dopamine transporter and nAChR levels in wildtype (WT) and $\alpha 6(-/-)$ mice treated with or without nicotine. The top panel shows striatal dopamine transporter levels measured using ^{125}I -RTI-121. The middle panel depicts striatal $\alpha 6\beta 2^*$ nAChR levels assessed using ^{125}I - α -CtxMII, with no detectable binding in $\alpha 6(-/-)$ mice as expected. The bottom panel shows striatal $\alpha 4\beta 2^*$ nAChR levels measured using ^{125}I -epibatidine in the presence of α -CtxMII. Changes in the striatal dopamine transporter and nAChR levels do not appear to correlate with the presence of dyskinesias. Values are the mean $\pm$ S.E.M. of 11 to 12 mice per group. Significance of difference from own saccharin-treated group: ** $p < 0.01$, *** $p < 0.001$; from saccharin-treated wildtype: + $p < 0.05$. There was a significant main effect of genotype (# $p < 0.05$). Data were analyzed by two-way ANOVA followed by a Bonferroni post hoc test.

Fig. 6.

$\alpha 6\beta 2^*$ and $\alpha 4\beta 2^*$ nAChR-mediated striatal dopamine release in $\alpha 6(-/-)$ and wildtype (WT) mice with and without nicotine treatment. Mice were treated as depicted in the timeline in Fig. 1. At the end of the study they were killed, and striatal synaptosomes prepared as described in Methods. Total ^3H -dopamine release was evoked by exposure of synaptosomes to 0.3, 1.0, 3.0, and 100 μM nicotine. $\alpha 4\beta 2^*$ nAChR-mediated (α -CtxMII-resistant) release represents release in the presence of α -CtxMII (50nM). $\alpha 6\beta 2^*$ nAChR-mediated release was determined by subtracting $\alpha 4\beta 2^*$ nAChR-mediated release from total nAChR-mediated release. The left panels demonstrate that nicotine treatment and lesioning decreased $\alpha 6\beta 2^*$ nAChR-mediated release in the intact striatum of wildtype mice, with no detectable $\alpha 6\beta 2^*$ nAChR-mediated release in $\alpha 6(-/-)$ mice, as expected. The middle and right panels show that nicotine treatment and lesioning decreased $\alpha 4\beta 2^*$ nAChR-mediated release in the intact striatum of both wildtype and $\alpha 6(-/-)$ mice. Values are the mean $\pm$ S.E.M. of 8 mice per group. Significance of difference from own saccharin-treated group: * $p < 0.05$, ** $p < 0.01$. There was a main effect of nicotine on the intact side of wildtype mice ($p < 0.001$) and $\alpha 6(-/-)$ mice ($p < 0.01$). Data were analyzed by two-way ANOVA followed by a Bonferroni post hoc test.

Table 1

Plasma cotinine levels were similar in lesioned wildtype and $\alpha 6(-/-)$ mice receiving nicotine. Blood was drawn from the retro-orbital sinus under isofluorane anesthesia at wk 2 and wk 5 after the nicotine titration phase. Cotinine levels were not detectable in saccharin-treated mice. The values represent the mean $\pm$ S.E.M. of the indicated number of mice.

Nicotine dose ($\mu\text{g/ml}$)	Time of testing (wk)	Group	# Mice	Cotinine level (ng/ml)
300	2	Wildtype	11	250 $\pm$ 52
		$\alpha 6(-/-)$	12	171 $\pm$ 70
400	5	Wildtype	11	291 $\pm$ 55
		$\alpha 6(-/-)$	12	330 $\pm$ 74

Table 2

No difference in parkinsonism in lesioned wildtype and $\alpha 6(-/-)$ mice treated with and without nicotine. Two wk after lesioning, mice were rated for parkinsonism using the forelimb use asymmetry (cylinder) test. They were again tested for parkinsonism 9 wk after lesioning while being treated with nicotine. The values represent the mean $\pm$ S.E.M. of the indicated number of mice.

Time of testing	Genotype	Group	# Mice	Impaired forelimb use (%)
2 wk post lesion	Wildtype	Pre-saccharin	11	26.9 $\pm$ 4.9
		Pre-nicotine	11	24.2 $\pm$ 5.3
	$\alpha 6(-/-)$	Pre-saccharin	11	25.1 $\pm$ 6.1
		Pre-nicotine	12	30.3 $\pm$ 5.5
9 wk post nicotine titration	Wildtype	Saccharin	11	29.9 $\pm$ 4.3
		Nicotine	11	25.3 $\pm$ 3.4
	$\alpha 6(-/-)$	Saccharin	11	26.2 $\pm$ 3.8
		Nicotine	12	21.7 $\pm$ 3.8