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# The β3 Nicotinic Receptor Subunit: A Component of α-Conotoxin MII-Binding Nicotinic Acetylcholine Receptors that Modulate Dopamine Release and Related Behaviors

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**Nigrostriatal dopaminergic neurons express many nicotinic acetylcholine receptor (nAChR) subunits capable of forming multiple nAChR subtypes. These subtypes are expressed differentially along the neuron and presumably mediate diverse responses. β3 subunit mRNA has restricted expression but is abundant in the substantia nigra and ventral tegmental areas. To investigate the potential role(s) of nicotinic receptors containing the β3 subunit in dopaminergic tracts, we generated mice with a null mutation in the β3 gene. We were thereby able to identify a population of β3-dependent α-conotoxin MII-binding nAChRs that modulate striatal dopamine release. Changes were also observed in locomotor activity and prepulse inhibition of acoustic startle, behaviors that are controlled, in part, by nigrostriatal and mesolimbic dopaminergic activity, respectively, suggesting that β3-containing nAChRs modulate these behaviors.**

*Key words:* **β3 neuronal nicotinic receptor; gene knock-out; striatal dopamine release; α-conotoxin MII; locomotor activity; prepulse inhibition of acoustic startle**

## Introduction

Nicotinic acetylcholine receptor (nAChR) subtypes are composed of distinct combinations of subunits that exhibit unique pharmacological and physiological properties. With 11 neuronal subunits (α2–α7, α9–α10, and β2–β4) identified in mammalian brain (Lindstrom et al., 1996; Elgoyhen et al., 2001), the potential for subtype diversity is quite large; however, restrictions imposed by regional expression of subunit mRNAs and subunit assembly limit this diversity. In the substantia nigra of rodents, mRNA of many nAChR subunits is expressed (Marks et al., 1992; Clarke, 1993; Charpantier et al., 1998; Klink et al., 2001; Azam et al., 2002), suggesting that the pathway projecting from the substantia nigra to striatum may possess complex populations of nAChRs. The function of these various subtypes has yet to be established definitively.

Presynaptic nAChRs mediate the release of many neurotransmitters in the brain, including dopamine from striatum (Wonnacott, 1997). Indeed, functional, ligand-binding, immunoprecipitation, and single-cell RT-PCR experiments have uncovered a considerable number of nAChR subtypes expressed in the nigrostriatal pathway (Charpantier et al., 1998; Quik et al., 2000; Reuben et al., 2000; Tsuneki et al., 2000; Jones et al., 2001; Klink et al., 2001; Azam et al., 2002; Grady et al., 2002; Zoli et al., 2002). Pharmacological studies indicate that striatal dopamine release is regulated by more than one nAChR subtype (Kulak et al., 1997; Kaiser et al., 1998; Nayak et al., 2001; Grady et al., 2002). β3 nAChR subunit mRNA is highly expressed in dopaminergic neurons of the substantia nigra and ventral tegmental areas (Deneris et al., 1989) and can assemble into complex nAChRs (Forsayeth and Kobrin, 1997; McIntosh et al., 2000; Vailati et al., 2000; Zoli et al., 2002), leading to the postulate that this subunit is an important component of nigrostriatal nAChRs. We investigated the significance of the β3 subunit in nigrostriatal nAChRs by generating mice with a null mutation in the β3 gene. Analysis of β3 null mutant mice demonstrated that a population of nAChRs containing the β3 subunit and sensitive to inhibition by α-conotoxin MII modulates striatal dopamine release. Deletion of the β3 subunit also altered locomotor activity, a behavior that is regulated in part by nigrostriatal dopaminergic neurotransmission, and prepulse inhibition (PPI) of acoustic startle response, which is regulated in part by mesolimbic dopaminergic transmission. These data suggest that β3-containing nAChRs are involved in modulating these behaviors.

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## Materials and Methods

*Generation of β3 knock-out mice.* Exon V of the β3 gene (mouse genomic clone B327) was interrupted by insertion of an internal ribosome entry site (IRES)-tau-lacZ (gene encoding β-galactosidase) cassette (Mombaerts et al., 1996) and a LoxP-neo$^r$-LoxP cassette into a *Bcl*I site. Diphtheria toxin A coding fragment, from plasmid pMC1DT-3 (Yagi et al., 1993), was engineered at the 3′ end of the targeting construct for the negative selection. Transfection of 129Svj embryonic stem (ES) cells was performed by electroporation with the linearized targeting construct, and homologous recombination was analyzed by Southern blot. Positive ES cell colonies were injected into C57BL/6 blastocysts. Germ-line transmission was determined by Southern blot analysis of tail DNA digested with *Hind*III and assessed using the 3′ probe. Although the targeted allele contained the IRES-tau-LacZ fusion, β-galactosidase could not be detected by conventional or enhanced staining methods, leading us to hypothesize possible problems associated with the translation of LacZ, which is initiated by ribosomal binding to the IRES. Mice were maintained at the Salk Institute and the University of Colorado, with a 12 hr light/dark cycle (6 A.M.-6 P.M.) in the vivarium and *ad libitum* access to food and water. All procedures were approved by the Animal Care and Utilization Committee at the Salk Institute and the University of Colorado. Mice were maintained on a 129Svj × C57BL/6 background by heterozygous matings.

In situ *hybridization.* Coronal sections (14 μm) were used for *in situ* hybridization with a protocol identical to that described previously (Marks et al., 1992). Riboprobes complementary to mRNA encoding the intracellular domain of the β3 subunit (downstream of the IRES-tau-LacZ-neo$^r$ insertion) were synthesized by *in vitro* transcription. The construct was linearized with *Bgl*II, and the probe was synthesized using T7 RNA polymerase. Mouse α3, α4, α6, α7, β2, β3, and β4 cDNA clones were also used for the *in situ* hybridizations. The mouse nicotinic receptor α7 subunit cDNA was isolated from a C57BL/6 whole-brain library as described previously (Stitzel et al., 1996). The α7RR construct used for *in situ* hybridization contains the 5′-most 597 bp of the α7 cDNA (Stitzel et al., 1996). The mouse nicotinic receptor α6 subunit cDNA was a generous gift from the laboratory of Dr. Jean-Pierre Changeux (Institut Pasteur, Paris, France). All remaining mouse nicotinic receptor subunit cDNAs were generated by PCR using either Advantage HFII DNA polymerase (Invitrogen, Carlsbad, CA) (α4 and β2) or pfu DNA polymerase (Roche, Indianapolis, IN) (α3, β3, and β4). The α4 (1953 bp) and β2 (1548 bp) cDNAs were amplified from whole-brain total RNA isolated from the long-sleep selected mouse line. The α3 (1612 bp), β3 (1546 bp), and β4 (1578 bp) cDNAs were amplified from DBA/2Ibg adrenal gland total RNA, long-sleep selected line ventral tegmental area total RNA, and P19 teratocarcinoma cell total RNA, respectively. Primers used for cDNA amplification were as follows: α3 (5′-GCTTAGCTGTGCTTCGGTGGTG-3′, 5′-CTTTCATCAGCACAGGTGAGC-3′), α4 (5′-CCATGGAGATCGGGGGCTCCG-3′, 5′-GTGGGTGGACTGACGAGTCGC-3′), β2 (5′-CTCCGCGCTATGCAGGCGGC-3′, 5′-GAGGAGCTGCAAATGAGAGAC-3′), β3 (5′-CGTCTCTCTGAAGCAGACGTC-3′, 5′-GGCTCCCAGGACAAGGCAAGGGCACC-3′), and β4 (5′-CATTGTGGGGTGACCGGCAGC-3′, 5′-GTGGGATGATATGAGCAGCC-3′).

Mouse α2 and α5 were not available, so rat clones HYP16rev and Aα5 (Marks et al., 1992) were used. Plasmids were linearized and cRNA was synthesized with the appropriate RNA polymerase. α-$^{35}$S-UTP was used as the sole source of UTP. Full-length transcripts were hydrolyzed to ~500 base length before hybridization. After wash and dehydration, slides were initially apposed to Cyclone Phosphorimaging screens (Packard). Tissue homogenate standards containing known amounts of $^{35}$S were included on each screen to allow quantitation of signal intensity using a Cyclone Phosphorimager (Packard). Groups were compared by one-way ANOVA. Slides were then apposed to Kodak MR film to obtain photographic images.

*Autoradiography.* Coronal tissue sections (14 μm) were collected at 1.3–1.4 mm from bregma (to visualize binding in dopaminergic terminal fields). Autoradiography procedures for [$^3$H]nicotine (20 nM), [$^3$H]epibatidine (500 pM), and [$^{125}$I]α-bungarotoxin (2 nM) have been described previously (Whiteaker et al., 2000c). Procedures for [$^{125}$I]α-conotoxin MII (0.5 nM) binding were similar to those described previously (Whiteaker et al., 2000a), with altered wash conditions. Sections were washed twice (22°C) for 4 min in binding buffer (144 mM NaCl, 1.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 20 mM HEPES, pH 7.5) plus 1 mg/ml BSA, 5 mM EDTA, 5 mM EGTA, and 10 μg/ml each of aprotinin, leupeptin trifluoroacetate, and pepstatin A, and then twice each at 0°C for 5 sec in 0.1× binding buffer and 5 mM HEPES, pH 7.5. For each set of experiments, an adjacent series of sections from each mouse was used to measure nonspecific binding defined by the addition of 1 mM (−)-nicotine bitartrate. Binding densities were compared between genotypes in each region by one-way ANOVA.

[$^{125}$I]epibatidine membrane binding. Striata were dissected from mice of each genotype, and membranes were prepared by homogenization and repeated washing as described previously (Marks et al., 1998; Whiteaker et al., 2000b). Saturation binding was determined using eight [$^{125}$I]epibatidine concentrations (2–400 pM). Blanks were determined by including 100 μM (−)-nicotine in the incubation. Samples (30 μl final volume) were incubated at 22°C for 3 hr before filtration and washed as described previously (Whiteaker et al., 2000b). Saturation binding data were fit to a single-site Hill equation. Inhibition of the binding of 200 pM [$^{125}$I]epibatidine was measured using 10 cytisine concentrations (0.1–3000 nM). Inhibition curves were fit to a two-site model as described previously (Marks et al., 1998; Whiteaker et al., 2000b). Binding parameters were compared using a one-way ANOVA followed by Duncan's *post hoc* test.

*Dopamine release.* The perfusion method used to examine the release of [$^3$H]dopamine from mouse striatal synaptosomes has been described previously (Grady et al., 2002). Previous studies have demonstrated that under these experimental conditions, uptake of [$^3$H]dopamine occurs exclusively via the dopamine transporter in mouse striatum (Grady et al., 2002). Experiments were conducted at room temperature with nomifensine added to the perfusion buffer (10 μM) to prevent reuptake. Synaptosomes were perfused at 1 ml/min for 10 min before exposure to agonist for 18 sec. Individual animals were assayed for (−)-nicotine-stimulated dopamine release at 8 (−)-nicotine concentrations.

Fifteen fractions were collected; baseline was calculated from fractions collected before and after agonist exposure. Stimulated release was calculated as a fraction of unstimulated baseline (units of release). Fractions with units of release >0.1 were summed to determine total units of release. To determine the α-conotoxin MII-resistant portion of dopamine release, aliquots of synaptosomes were perfused for 5 min with 50 nM α-conotoxin MII immediately before the 18 sec nicotine exposure. The α-conotoxin MII-sensitive portion of dopamine release was calculated by difference from release stimulated by nicotine from synaptosomes not treated and those treated with α-conotoxin MII. Each individual mouse was assayed for α-conotoxin MII-sensitive and –resistant dopamine release at each concentration of nicotine. $EC_{50}$ values and maximum release ($R_{max}$) were calculated from the Hill equation using a nonlinear curve-fitting algorithm and assessed for statistical significance by one-way ANOVA.

*Behavioral testing.* β3$^{+/+}$, β3$^{+/-}$, and β3$^{-/-}$ mice between 80 and 90 d of age were tested between 9 A.M. and 6 P.M. (approximately equivalent number of males and females). Locomotor activity was assessed in an illuminated open field arena (Marks et al., 1986) for 60 min. Acoustic startle as a measure of sensorimotor reactivity and prepulse inhibition of acoustic startle as a measure of sensorimotor gating (Bullock et al., 1997) were assessed in separate animals. After a 3 min acclimation period of 70 dB white noise, mice were exposed to a sequence of pure acoustic trials interspersed with PPI trials in pseudorandom order. Each sequence consisted of one acoustic trial (90, 95, 100, 105, 110, 115, or 120 dB) and one PPI trial (acoustic prepulse of 75, 80, 85, 90, or 95 dB given 120 msec before a 120 dB acoustic stimulus). Each trial was separated by an intertrial interval of 10 or 15 sec in pseudorandom order, and the entire sequence was repeated four times. The average of all four presentations of each trial was used, and a ratio of startle response with prepulse to response without prepulse was used. Results were assessed using repeated measures multiple ANOVA (MANOVA) (SPSS, Chicago, IL). Analyses were collapsed on sex because no interaction of sex with genotype was detected for any variable examined.

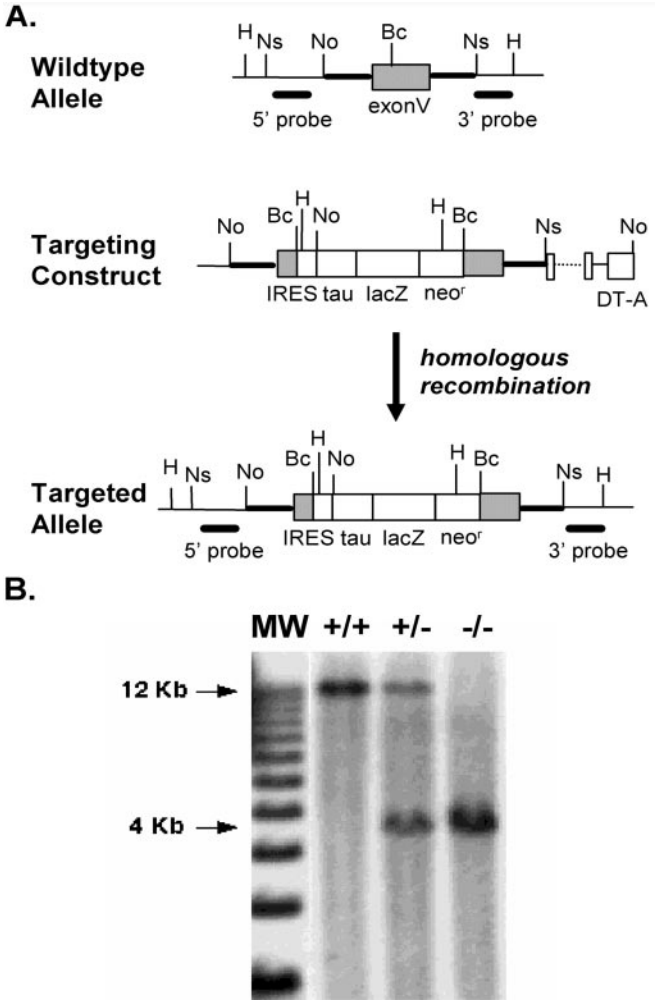


**Figure 1.** Targeted disruption of the β3 gene. *A*, Diagram of exon V and the surrounding intronic regions of the wild-type β3 gene, targeting construct, and resulting targeted allele. Exon V of the β3 gene was disrupted by insertion of a cassette containing an IRES, tau-lacZ fusion, and the neomycin resistance gene (neo$^r$) via homologous recombination. Diphtheria toxin A gene fragment (DT-A) was included in the targeting vector as a negative selection marker. Restriction sites: Bc, *Bcl*I; H, *Hind*III; No, *Not*I; Ns, *Nsi*I. *B*, Southern blot analysis of tail DNA digested with *Hind*III and assessed using the 3′ probe shown above identified appropriate restriction fragments for mice of all genotypes (MW, molecular weight marker; +/+, β3 wild type; +/−, β3 heterozygous; −/−, β3 homozygous null mutant).

## Results

### Generation of β3 null mutant mice

β3 null mutant mice were generated using homologous recombination techniques (Cappechi, 1989) to disrupt the β3 gene by inserting a cassette containing an internal ribosome entry site (IRES), a tau-lacZ fusion, and a neomycin resistance gene into exon V, upstream of the sequence encoding the first transmembrane domain (Fig. 1*A*). Analysis of tail DNA by Southern blot confirmed the incorporation of the targeting cassette (Fig. 1*B*). As compared with wild-type ($\beta3^{+/+}$) or heterozygous ($\beta3^{+/-}$) littermates, null mutant mice ($\beta3^{-/-}$) are of normal body weight, capable of reproduction, and display no gross physical or developmental deficits.

### *In situ* hybridization

Expression of β3 mRNA in the brain was examined using *in situ* hybridization. Using a riboprobe complementary to the intracellular loop of β3 (encoded by exon V downstream of the gene disruption site), strong expression of β3 mRNA was detected in the substantia nigra, ventral tegmental area, and medial habenula, with weaker labeling in the superior colliculi and a subset of the medial vestibular nuclei of $\beta3^{+/+}$ mice (Fig. 2*A*), consistent with our report of the initial cloning of the β3 gene (Deneris et al., 1989). Decreased expression was observed in $\beta3^{+/-}$ mice, whereas no specific labeling was detected in $\beta3^{-/-}$ mice, verifying disruption of the β3 gene. Similar expression patterns were observed with a full-length mouse β3 cDNA (hydrolyzed) probe (Fig. 2*B*). Semiquantitative analysis of the data shown in Figure 2*B* demonstrated that disruption of exon V reduced, but did not eliminate, β3 mRNA hybridization measured with the full-length cRNA (Fig. 2*C*). This result contrasts with that obtained with the probe directed toward the disrupted sequence (see Discussion).

*In situ* hybridization studies were also conducted for other neuronal nAChR subunits to investigate any compensatory expression in the cell body and terminal regions of the substantia nigra and ventral tegmental area after deletion of the β3 subunit (Fig. 3). No significant difference in mRNA expression was detected at the level of the dopaminergic cell bodies in substantia nigra or ventral tegmental areas for α3, α4, α5, α6, α7, β2, or β4 after deletion of the β3 subunit (α2 mRNA levels were below the level of detection in these regions). α7 and β2 mRNA were also detected in striatum, olfactory tubercles, and nucleus accumbens (dopaminergic terminal areas). Disruption of the β3 gene did not affect expression of mRNA for these subunits (data not shown).

### Ligand binding

To investigate incorporation of the β3 subunit into the various striatal nAChR populations, ligand-binding autoradiographic studies were conducted using four well characterized nicotinic ligands: [$^{125}$I]α-conotoxin MII, (−)-[$^3$H] nicotine, [$^{125}$I]α-bungarotoxin, and [$^3$H]epibatidine. [$^{125}$I]α-conotoxin MII (a radiolabeled derivative of a peptide originally isolated from the venom of the predatory marine snail, *Conus magus*) binds in discreet regions of mouse brain, including regions that are rich in dopaminergic terminals, such as striatum, olfactory tubercle, and nucleus accumbens (Whiteaker et al., 2000a). Furthermore, 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP) lesions in monkeys reveal that striatal [$^{125}$I]α-conotoxin MII-binding nAChRs are found exclusively on substantia nigra–ventral tegmental area dopaminergic projections (Quik et al., 2001). The effect of β3 deletion on the binding of [$^{125}$I]α-conotoxin MII to terminal fields of dopaminergic neurons is shown in Figure 4*A* (top row), with quantitated results in Figure 4*B*. At this level of $\beta3^{+/+}$ mouse brain, specific binding was confined to striatum, nucleus accumbens, and olfactory tubercles. In $\beta3^{-/-}$ mice, [$^{125}$I]α-conotoxin MII binding was nearly abolished (8.0% of wild-type levels remaining in striatum, one-way ANOVA, $p = 0.0003$; 3.9% of wild-type in nucleus accumbens, $p = 0.0007$; and 3.2% of wild-type in olfactory tubercles, $p < 0.0001$). At the level of the dopaminergic cell bodies, the loss of [$^{125}$I]α-conotoxin MII binding was similar, although less severe, with 31.5% of wild-type levels remaining in substantia nigra ($p < 0.0001$) and 43.7% of wild type in ventral tegmental area ($p = 0.0093$). Binding densities in $\beta3^{+/-}$ were intermediate between those of $\beta3^{+/+}$ and $\beta3^{-/-}$ mice in each region.

[$^3$H]nicotine binds with high affinity predominantly to nAChRs containing α4 and β2 subunits (Whiting and Lindstrom, 1988; Flores et al., 1992; Picciotto et al., 1995; Marubio et al., 1999). At the level of the striatum, $\beta3^{+/+}$ animals displayed intermediate levels of [$^3$H]nicotine binding in the olfactory tubercles and ventrolateral parts of the striatum and lower densities

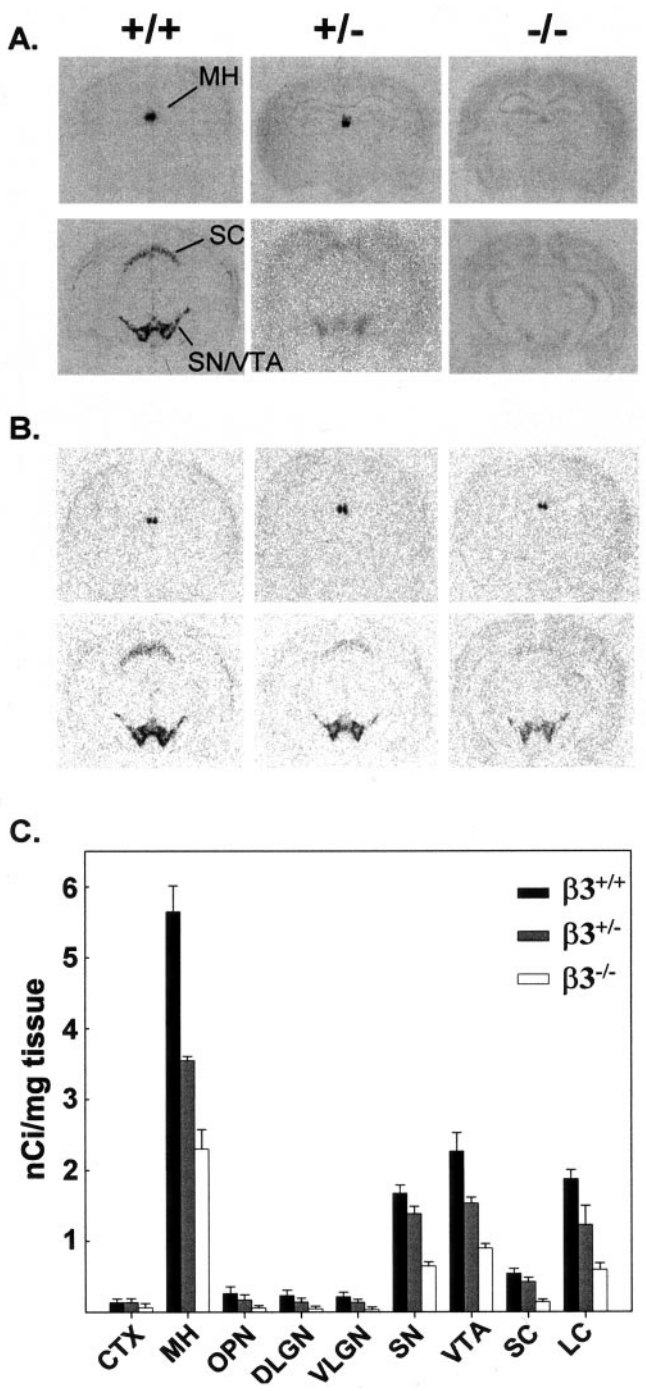


**Figure 2.** Expression of β3 mRNA across genotypes using *in situ* hybridization. *A*, *In situ* hybridization conducted using an antisense riboprobe complementary to the β3 cytoplasmic loop (downstream of inserted IRES-tau-lacZ-neo$^r$ cassette). Specific labeling of β3 mRNA expression was observed in medial habenula (MH), superior colliculus (SC), and substantia nigra–ventral tegmental (SN/VTA) areas in β3 wild-type (+/+) mice. Slightly lower levels were observed in β3 heterozygous mice (+/−), whereas all specific labeling was lost in β3 null mutants (−/−). *B*, *In situ* hybridization using a full-length mouse cDNA probe (hydrolyzed). Specific labeling of β3 mRNA was detected in the same regions as above. Samples from $\beta3^{-/-}$ mice demonstrated reduced labeling relative to those from $\beta3^{+/+}$ mice, but some residual labeling was retained even in $\beta3^{-/-}$ samples (*C*). *C*, Semiquantitative analysis of *in situ* hybridization data obtained using the full-length mouse cDNA (hydrolyzed) probe. β3 mRNA was reduced by ~70% from wild-type values in every region examined. CTX, Cortex; MH, medial habenula; OPN, olivary pretectal nucleus; DLGN, dorsolateral geniculate nuclei; VLGN, ventrolateral geniculate nuclei; SN, substantia nigra; VTA, ventral tegmental area; SC, superior colliculi; LC, locus coeruleus.

in the dorsal striatum and nucleus accumbens (Fig. 4*A*, second row, *C*). Outside these dopaminergic regions, [$^3$H]nicotine binding was also detected in the septum and the intermediate layers of the cortex. No significant differences in [$^3$H]nicotine binding density were detected among β3 genotypes.

[$^{125}$I]α-bungarotoxin binds predominantly, if not exclusively, to a subtype of nAChRs containing the α7 subunit (Schoepfer et al., 1990; Séguéla et al., 1993; Orr-Urtreger et al., 1997). The pattern observed for [$^{125}$I]α-bungarotoxin binding was distinctly different from that observed for either [$^{125}$I]α-conotoxin MII or [$^3$H]nicotine. Within the dopaminergic terminal field, the highest levels of [$^{125}$I]α-bungarotoxin binding were seen in the ventral and lateral striatum, with relatively low levels in the nucleus accumbens and olfactory tubercles (Fig. 4*A*, third row, *D*). Outside these dopaminergic regions, [$^{125}$I]α-bungarotoxin binding was also detected in the intermediate layers of the cortex. No significant effect of β3 genotype was observed on the distribution or density of [$^{125}$I]α-bungarotoxin binding.

[$^3$H]epibatidine binds with high affinity to several different nAChR subtypes, including those labeled by [$^3$H]nicotine and [$^{125}$I]α-conotoxin MII (above) (Marks et al., 1998; Whiteaker et al., 2000b). The binding pattern for [$^3$H]epibatidine was similar to that for [$^3$H]nicotine (Fig. 4*A*, fourth row, *E*). Again, no difference in [$^3$H ]epibatidine binding was observed among animals of different β3 genotypes at the level of the brain examined. Because a subset of striatal [$^3$H]epibatidine binding sites interact with α-conotoxin MII, the failure to detect significant changes in $\beta3^{-/-}$ mice may reflect the difficulty of measuring this small subset (<15% of the total binding sites) (Whiteaker et al., 2000a). Alternatively, loss of β3 subunits may result in an increase in other [$^3$H]epibatidine-binding nAChR subtypes, or loss of α-conotoxin MII sensitivity in receptors previously containing the β3 subunit.

To test these alternative possibilities, the effect of β3 genotype on [$^{125}$I]epibatidine binding was measured in striatal membrane homogenates (Fig. 5). Saturation binding analysis (Fig. 5*A*) demonstrated that β3 deletion results in a small decrease in the density of striatal [$^{125}$I]epibatidine binding sites [$\beta3^{+/+}$ $B_{max}$ = 140 ± 8 fmol/mg (protein); $\beta3^{+/-}$ $B_{max}$ = 125 ± 7 fmol/mg (protein); $\beta3^{-/-}$ $B_{max}$ = 113 ± 3 fmol/mg (protein)] that was not quite statistically significant (one-way ANOVA; $p = 0.075$). $K_d$ values were similar among genotypes ($\beta3^{+/+}$ $K_d$ = 60.1 ± 2.0 nM; $\beta3^{+/-}$ $K_d$ = 46.3 ± 3.8 nM; $\beta3^{-/-}$ $K_d$ = 47.3 ± 3.5 nM), as were Hill coefficients ($\beta3^{+/+}$ $n_H$ = 0.89 ± 0.02; $\beta3^{+/-}$ $n_H$ = 0.91 ± 0.01; $\beta3^{-/-}$ $n_H$ = 0.87 ± 0.02). Cytisine inhibition of [$^{125}$I]epibatidine (200 pM) binding to striatal membranes was also measured in mice of each genotype to define the pharmacology of the β3-dependent [$^{125}$I]epibatidine binding sites (Fig. 5*B*). Cytisine inhibited [$^{125}$I]epibatidine binding to striatal homogenates from $\beta3^{+/+}$ mice biphasically. Most [$^{125}$I]epibatidine binding to $\beta3^{+/+}$ membranes was highly sensitive to cytisine [cytisine-sensitive binding; $B_{max}$ = 80.1 ± 11.2 fmol/mg (protein); $K_i$ = 0.41 ± 0.04 nM], but a smaller population with lower affinity for cytisine was also observed [cytisine-resistant binding; $B_{max}$ = 20.9 ± 3.3 fmol/mg (protein); $K_i$ = 10.5 ± 3.7 nM]. This observation closely matches previous observations (Whiteaker et al., 2000a). Although β3 gene disruption did not significantly affect the binding parameters measured for the cytisine-sensitive population in $\beta3^{-/-}$ mice [$B_{max}$ = 79.4 ± 1.9 fmol/mg (protein); $K_i$ = 0.48 ± 0.02 nM], no cytisine-resistant population could be measured in striatal homogenates prepared from $\beta3^{-/-}$ mice. The inhibition binding parameters measured in $\beta3^{+/-}$ mice were intermediate to those derived from $\beta3^{+/+}$ and $\beta3^{-/-}$ mice

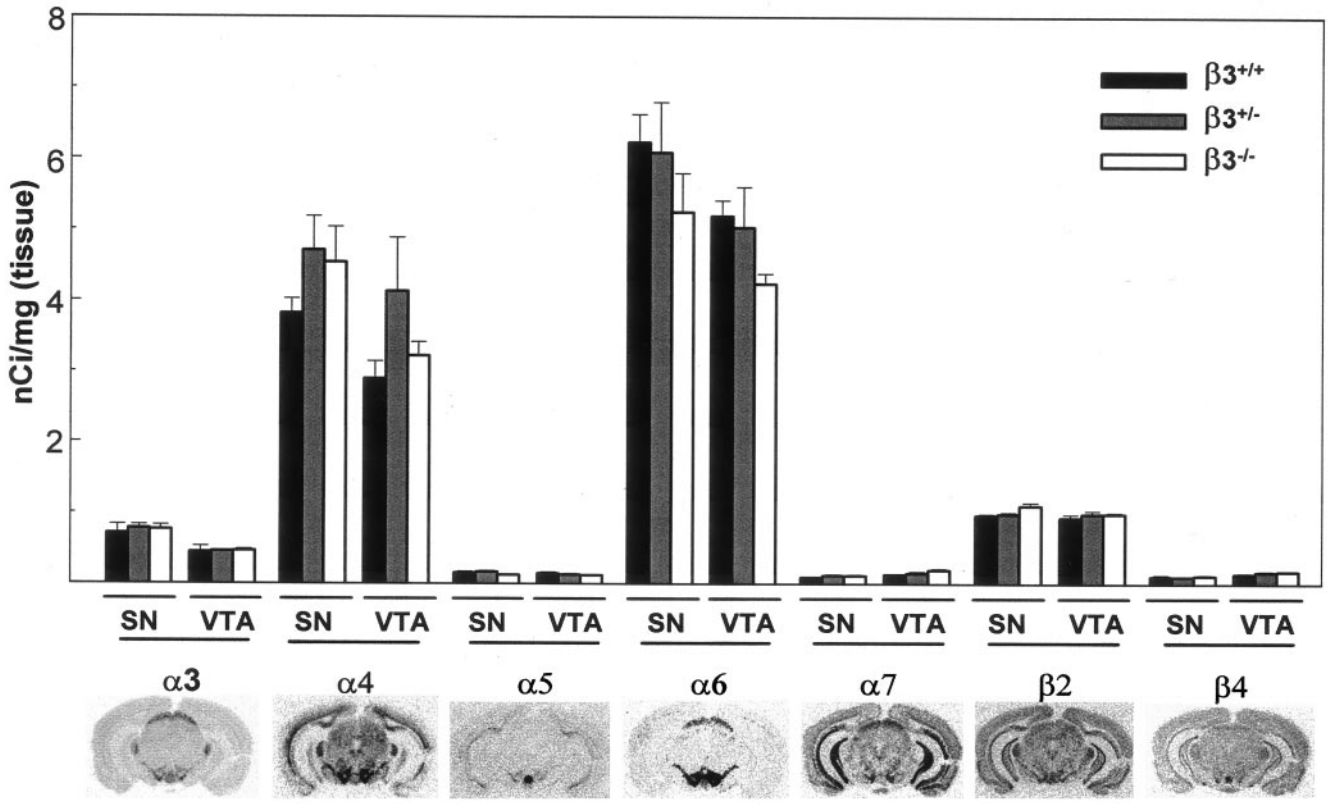


**Figure 3.** Effect of β3 deletion on expression of remaining neuronal nAChR subunits. *In situ* hybridization was conducted to examine mRNA expression of neuronal nAChR subunits with deletion of β3. Adjacent brain slices were hybridized with riboprobes complementary to α2, α3, α4, α5, α6, α7, β2, or β4 subunits. Average values obtained from quantitative analysis of *in situ* hybridization ± SEM are shown for substantia nigra (SN) and ventral tegmental (VTA) areas ($n = 3$ per genotype; data for α2 expression are not shown, because α2 hybridization could not be detected above background at this level of the brain). Representative slices are shown for each subunit to illustrate specific labeling at the level of dopaminergic cell bodies in substantia nigra–ventral tegmental areas. No significant differences in mRNA expression between genotypes were detected for any subunit.

[cytisine-sensitive binding; $B_{max} = 87.6 \pm 5.5$ fmol/mg (protein), $K_i = 0.42 \pm 0.03$ nM; cytisine-resistant binding; $B_{max} = 8.5 \pm 2.1$ fmol/mg (protein); $K_i = 34.7 \pm 17.2$ nM]. Thus, β3 deletion results in a gene-dose-dependent loss of cytisine-resistant striatal [$^{125}$I]epibatidine binding sites (one-way ANOVA; $p < 0.002$) but has no effect on the density of cytisine-sensitive sites ($p > 0.6$). Because most cytisine-resistant [$^{125}$I]epibatidine binding sites in mouse striatum are sensitive to inhibition by α-conotoxin MII (Whiteaker et al., 2000a), this result is consistent with the selective loss of α-conotoxin MII binding sites (Fig. 4*A*).

Thus, after deletion of the nAChR β3 subunit, expression of [$^{125}$I]α-conotoxin MII-binding nAChRs was significantly reduced in the dopaminergic cell bodies and virtually abolished at the level of the terminal fields. No significant changes could be observed in nAChR populations labeled by [$^3$H]nicotine, [$^3$H]epibatidine, or [$^{125}$I]α-bungarotoxin as measured with quantitative autoradiography; however, more detailed analysis using filtration binding techniques demonstrated a selective loss of cytisine-resistant (presumably α-conotoxin MII sensitive) [$^{125}$I]epibatidine binding sites.

### Nicotine-stimulated dopamine release

Next, nAChR-mediated neurotransmitter release was used to evaluate the effects of β3 gene disruption on receptor function. The application of nicotine to striatal synaptosomes stimulates the release of dopamine. Several nAChR subtypes mediate nicotine-stimulated [$^3$H]dopamine release, including a subtype that is sensitive to inhibition by α-conotoxin MII (Kulak et al., 1997; Kaiser et al., 1998; Nayak et al., 2001; Grady et al., 2002). We anticipated, therefore, that the loss of this toxin-sensitive subtype (as indicated by the loss of [$^{125}$I]α-conotoxin MII binding sites, above) would result in a subsequent decrease in the component of nicotine-stimulated [$^3$H]dopamine release mediated by the α-conotoxin MII-sensitive population of nAChRs. Figure 6*A* presents dose–response curves for α-conotoxin MII-sensitive nicotine-stimulated dopamine release from striatal synaptosomes prepared from the $\beta3^{+/+}$, $\beta3^{+/-}$, and $\beta3^{-/-}$ genotypes. As anticipated, the α-conotoxin MII-sensitive component of dopamine release is dramatically reduced. (Calculated $R_{max}$ values are $\beta3^{+/+}$ 2.86 ± 0.07 U; $\beta3^{+/-}$ 3.32 ± 0.44 U; $\beta3^{-/-}$ 0.56 ± 0.23 U; $EC_{50}$ values are $\beta3^{+/+}$ 0.81 ± 0.12 μM; $\beta3^{+/-}$ 2.67 ± 1.16 μM; $\beta3^{-/-}$ 0.42 ± 0.83 μM.) Unexpectedly, dopamine release mediated by nAChRs that are not antagonized by α-conotoxin MII (α-conotoxin MII-resistant dopamine release) (Fig. 6*B*) increased in the $\beta3^{-/-}$ mice. (Calculated $R_{max}$ values are $\beta3^{+/+}$ 8.15 ± 0.23 U; $\beta3^{+/-}$ 10.78 ± 0.29 U; $\beta3^{-/-}$ 15.76 ± 0.41 U; $EC_{50}$ values are $\beta3^{+/+}$ 6.05 ± 0.56 μM; $\beta3^{+/-}$ 7.40 ± 0.74 μM; $\beta3^{-/-}$ 7.68 ± 0.65 μM.) Total nicotine-stimulated dopamine release (sum of the two components) is slightly higher in the $\beta3^{-/-}$ genotype (Fig. 6*C*). Small but significant increases in both $R_{max}$ ($\beta3^{+/+}$ 11.39 ± 0.20 U; $\beta3^{+/-}$ 14.37 ± 0.81 U; $\beta3^{-/-}$ 16.09 ± 0.56 U) and $EC_{50}$ values ($\beta3^{+/+}$ 4.01 ± 0.32 μM; $\beta3^{+/-}$ 5.98 ± 1.23 μM; $\beta3^{-/-}$ 6.79 ± 0.8 μM) were observed in the $\beta3^{-/-}$ mice compared with the $\beta3^{+/+}$ mice. Whether this increase in the α-conotoxin MII-resistant dopamine release represents the expression of a novel receptor subtype or increased

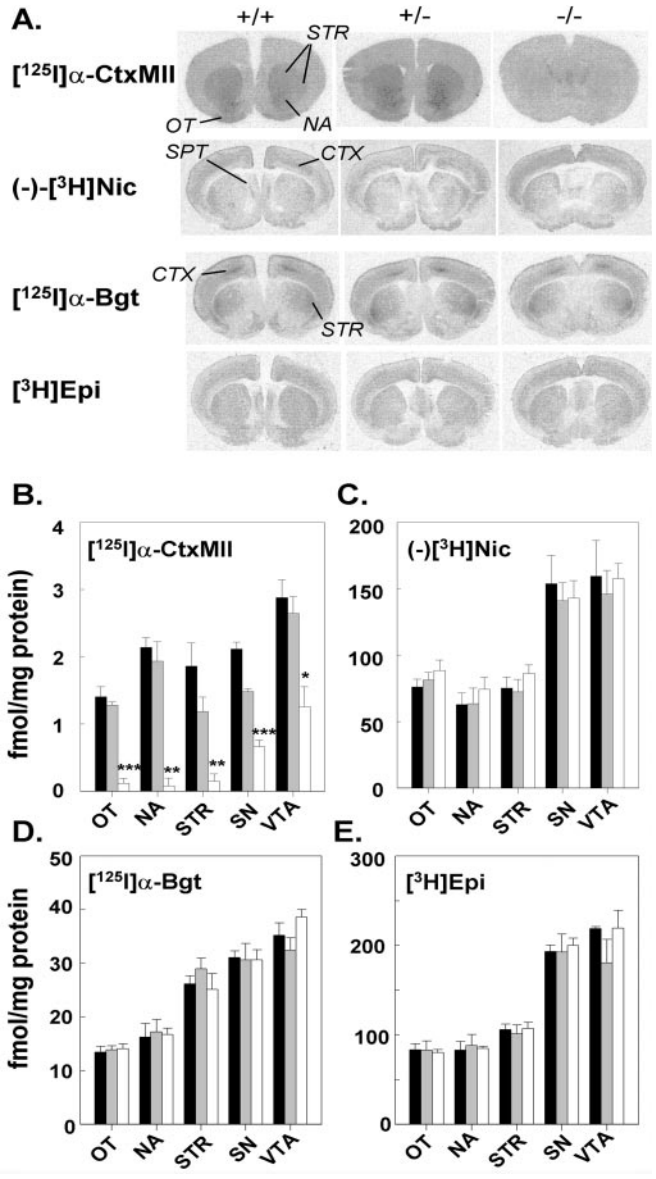


**Figure 4.** Autoradiographic analysis of nAChR populations at the level of the striatum across β3 genotypes. The binding of four nicotinic ligands that bind with high affinity to different subtypes of nAChRs was examined. *A*, Representative slices demonstrating binding at the level of the dopaminergic terminal fields in striatum (STR), nucleus accumbens (NA), and olfactory tubercles (OT). [$^{125}$I]α-conotoxin MII ([$^{125}$I] α-Ctx) binding (0.5 nM) was observed in $\beta3^{+/+}$ and $\beta3^{+/-}$ mice; however, nearly all [$^{125}$I]α-conotoxin MII binding sites were abolished in $\beta3^{-/-}$ mice. No difference in the binding of (−)-[$^3$H]nicotine ([$^3$H]Nic) (20 nM), [$^{125}$I]α-bungarotoxin ([$^{125}$I]α-Bgt) (2 nM), or [$^3$H]epibatidine ([$^3$H]Epi) (0.5 nM) was observed across genotypes. CTX, Cortex; SPT, septum. Quantitative analysis of ligand binding is shown (*B–E*) for regions containing dopaminergic terminal fields [olfactory tubercle (OT), nucleus accumbens (NA), and striatum(STR)] and cell bodies [substantia nigra (SN) and ventral tegmental area(VTA)]. Black bars = $\beta3^{+/+}$; gray bars = $\beta3^{+/-}$; white bars = $\beta3^{-/-}$; $n = 3$ per genotype. *$p < 0.05$.

function of a subtype already present in striatum remains to be determined. No difference was observed in samples stimulated with 20 mM potassium, indicating that the general responsiveness of the dopamine release process was unaffected by deletion of the β3 subunit.

### Behavioral analysis

Locomotor activity and prepulse inhibition of acoustic startle are both behavioral tasks that involve dopaminergic pathways

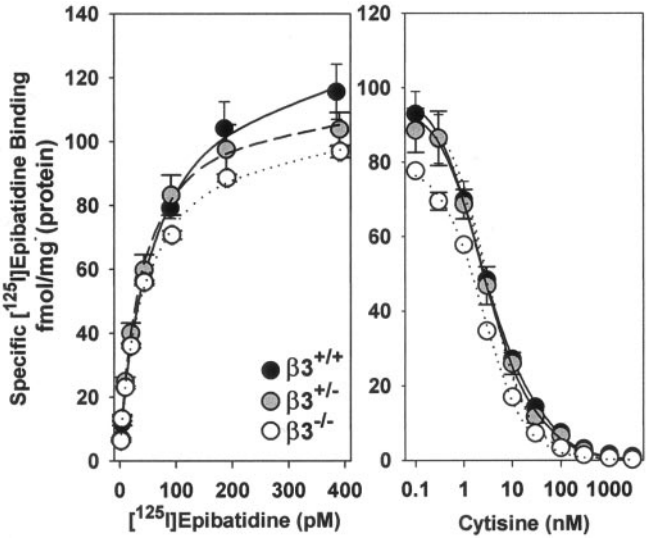


**Figure 5.** Saturation binding and cytisine inhibition of striatal [$^{125}$I]epibatidine binding. Saturation binding of [$^{125}$I]epibatidine (left panel) and cytisine inhibition of [$^{125}$I]epibatidine (200 pM) binding (right panel) were measured for three male mice of each β3 genotype. Points represent mean ± SEM, and curves represent best nonlinear least squares fit of the data as described in Materials and Methods. In the right panel, solid lines represent two-site curve fits, and dotted lines represent single-site fits to the data. Two sites could not be discerned in the $\beta3^{-/-}$ tissue.

(Ralph et al., 2001) and are modulated by nAChRs (Marks et al., 1986; Bullock et al., 1997). Therefore, these tests were selected to further investigate potential roles of β3-containing nAChRs. Locomotor activity, which is initiated in part by dopaminergic transmission in the nigrostriatal pathway and significantly affected after nigrostriatal degeneration in Parkinson's patients (Rinne, 1993) was assessed using the open field arena (Fig. 7*A*). Overall, $\beta3^{-/-}$ mice demonstrated significantly higher levels of activity than $\beta3^{+/+}$ littermates over the 60 min testing period (repeated measures MANOVA; $p < 0.03$), with the largest differences observed during the first 30 min of testing and a decline to wild-type levels by 60 min. These findings suggest that β3-containing nAChRs modulate locomotor activity. In another test, the startle response to an acoustic stimulus (acoustic startle) and the inhibition of acoustic startle by administration of a tone before the acoustic stimulus (prepulse inhibition of startle) were examined. Prepulse inhibition of startle has previously been shown to be mediated by mesolimbic dopaminergic pathways (Ralph et al., 2001). Deletion of the β3 subunit did not affect the acoustic startle response (Fig. 7*B*). $\beta3^{-/-}$ mice, however, demonstrated significantly less overall prepulse inhibition of startle (repeated measures MANOVA; $p < 0.05$) (Fig. 7*C*). The changes observed in locomotor activity and prepulse inhibition of startle in the $\beta3^{-/-}$ mice provide evidence that these behavioral tasks involving dopaminergic pathways are modulated by nAChRs containing the β3 subunit.

## Discussion

### High-affinity α-conotoxin MII site contains the β3 subunit

Our analysis of the β3 null mutant mice revealed that the β3 subunit is an essential component of α-conotoxin MII-sensitive nAChRs on dopaminergic nerve terminals. This finding expands on those of previous immunochemical, lesioning, and gene deletion studies, which demonstrated that high-affinity α-conotoxin MII binding sites in rodent brain may be a complex mixture of α6β2* nAChR subtypes, with contributions from α4 or β3 sub-

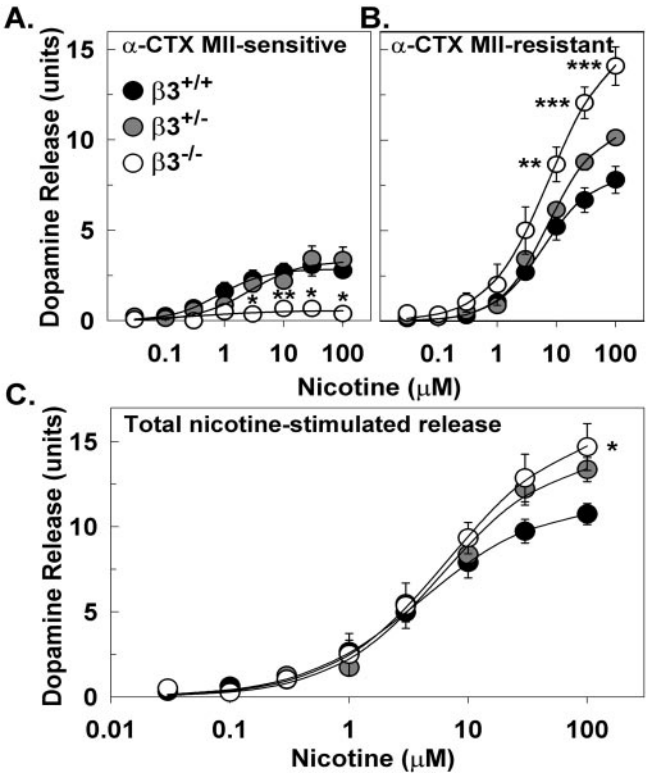


**Figure 6.** β3-containing nAChRs mediate striatal dopamine release. Individual aliquots of striatal synaptosomes loaded with [$^3$H]dopamine were stimulated with nicotine (0.3–100 μM) with or without a 5 min exposure to 50 nM α-conotoxin MII. Units of release are stimulated release as a fraction of baseline. *A*, α-Conotoxin MII-sensitive nicotine-stimulated [$^3$H]dopamine release. The portion of [$^3$H]dopamine release inhibited by α-conotoxin was determined by difference from individual aliquots of synaptosomes with and without α-conotoxin MII treatment. *$p < 0.05$; **$p < 0.01$ different from $\beta3^{+/+}$ by one-way ANOVA followed by least significant difference (LSD) *post hoc* test ($n = 5$–7). *B*, α-Conotoxin MII-resistant nicotine-stimulated [$^3$H]dopamine release. Nicotine-stimulated [$^3$H]dopamine release was assessed after a 5 min exposure to α-conotoxin MII (50 nM). **$p < 0.01$; ***$p < 0.001$ different from $\beta3^{+/+}$ by one-way ANOVA followed by LSD *post hoc* test ($n = 5$–7). *C*, Total nicotine-stimulated [$^3$H]dopamine release. *$p < 0.05$ different from $\beta3^{+/+}$ by one-way ANOVA followed by LSD *post hoc* test ($n = 5$–7). Genotype had no effect on release stimulated by 20 mM potassium, nor did α-conotoxin MII treatment affect baseline release or potassium-stimulated controls (data not shown).

units, or both (Vailati et al., 2000; Whiteaker et al., 2000c; Champtiaux et al., 2002; Zoli et al., 2002). Deletion of either the β2 (Whiteaker et al., 2000c) or the α6 (Champtiaux et al., 2002) subunit in mouse brain resulted in a loss of high-affinity [$^{125}$I]α-conotoxin MII binding sites. In addition, α-conotoxin MII-sensitive nicotine-stimulated [$^3$H]dopamine release (as well as the α-conotoxin MII–resistant component) was abolished in β2 null mutant mice (Grady et al., 2002; Whiteaker et al., 2000c), further implicating the β2 subunit in α-conotoxin MII-sensitive nAChRs in rodent brain. Finally, denervation induced by the neurotoxins 6-hydroxydopamine and MPTP nearly eliminates α-conotoxin MII-sensitive nAChRs in rodent striatum, indicating that this receptor subtype is expressed selectively on striatal dopaminergic neurons (Zoli et al., 2002; Quik et al., 2003). These reports, together with the data presented in this paper, indicate that β3 may assemble with α4, α6, and β2 subunits to form functional α-conotoxin MII-sensitive nAChRs in the mouse nigrostriatal pathway. Comparisons between the responses reported here and nAChRs expressed *in vitro* are difficult, because few studies have reported successful heterologous expression of β3-containing nAChRs, and those that have (Kuryatov et al., 2000; McIntosh et al., 2000) did not study the subunit combination(s) of interest here.

Interestingly, although α-conotoxin MII binding was nearly abolished at the level of the dopaminergic nerve terminals in striatum of $\beta3^{-/-}$ mice, only a partial decline in binding sites was observed in the dopaminergic cell bodies in substantia nigra. Given the subunit complexity of nAChRs in this pathway (Vailati et al., 2000; Zoli et al., 2002), it is possible that several populations of α-conotoxin MII-sensitive nAChRs are expressed on the cell bodies in substantia nigra and ventral tegmental areas, including a non-β3-containing α-conotoxin MII-sensitive nAChR, whereas in the nerve terminals projecting into striatum, β3-containing α-conotoxin MII-sensitive nAChRs are expressed almost exclusively.

### Effect of β3 deletion on α6 expression

Because nAChRs that interact with α-conotoxin MII in rodent brain have been shown to require both β2 and α6 nAChR subunits (Champtiaux et al., 2002: Grady et al., 2002; Zoli et al., 2002), it is possible that alteration of the expression of these subunits after β3 gene disruption may have confounded interpretation of the role of the β3 subunit. Alteration of α6 expression could have arisen as a consequence of inserting an IRES-tau-lacZ cassette in close proximity to the α6 gene (Chrna6 and Chrnb3 in mice are located as a cluster on a region of mouse chromosome 8 (8A2–8A3) that is syntenic with the region of human chromosome 8 (8P11.2–8P11.3) that contains CHRNA6 and CHRNB3. In mice, Chnrb3 is ~29,500 bp long and Chrna6 is ~10,800 bp long, with ~7000 bp intragenic region. The two genes are in opposite orientation with their 3′ ends in closest proximity. Although β3 gene disruption may have resulted in a small (<10%) decrease in α6 mRNA transcription (Fig. 3), the dramatic changes in receptor expression observed for $\beta3^{-/-}$ mice are unlikely to result from this putative, comparatively minor change in α6 mRNA expression.

Some residual labeling of β3 mRNA was observed in *in situ* hybridization experiments using the full-length mouse β3 cDNA probe. The lack of any specific labeling of β3 mRNA with the probe complementary to the downstream of the IRES-tau-*LacZ*-neo$^r$ cassette insertion site demonstrates the disruption of the β3 gene. The residual labeling observed with the full-length β3 probe is attributable to the detection of the region upstream of the cassette insertion site. We do not expect that translation of this upstream region would produce any β3 protein capable of assembling into nAChRs, because it only encodes the sequence before the first transmembrane domain. Additionally, the insertion cassette contains an SV40 polyadenylation signal (Mombaerts et al., 1996), which prevents the translation of the remainder of the β3 gene.

### Presynaptic β3 nAChRs modulate dopamine release

Our data also demonstrate that the β3 subunit is an integral component of a subtype of nAChRs that normally modulates dopaminergic neurotransmission in the nigrostriatal pathway. Approximately 30% of nicotine-stimulated dopamine release is mediated by α-conotoxin MII-sensitive nAChRs in wild-type mice (Kulak et al., 1997; Kaiser et al., 1998; Nayak et al., 2001; Grady et al., 2002). Although the α-conotoxin MII-sensitive population of nAChRs is virtually eliminated in $\beta3^{-/-}$ mice, there is a corresponding increase in the function of the α-conotoxin MII-resistant population. As a result, overall nicotine-stimulated [$^3$H]dopamine release is actually increased in $\beta3^{-/-}$ mice, sug-

gesting enhanced striatal dopaminergic transmission after cholinergic stimulation. The loss of the α-conotoxin MII-sensitive population may have further ramifications, because the agonist affinity for the remaining α-conotoxin MII-resistant population is considerably lower (sixfold) (Fig. 6) than for the α-conotoxin MII-sensitive subtype lost with deletion of the β3 subunit. The increased overall function is not matched by a measurable increase in nicotinic binding sites, perhaps suggesting that the remaining receptors are coupled more effectively to dopamine release. Although unexpected, this physiological compensation in $\beta3^{-/-}$ mice subunit does not negate the demonstration that β3-containing nAChRs normally mediate a component of this response.

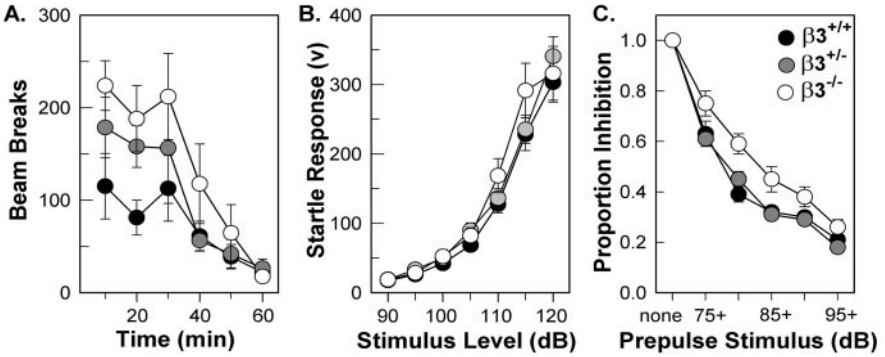


**Figure 7.** Behavioral analysis of β3 null mutant mice. *A*, Open field activity. Mice were tested in an illuminated open field arena for 60 min. $\beta3^{-/-}$ mice demonstrated significantly higher levels of activity than $\beta3^{+/+}$ littermates over the testing period (repeated measures ANOVA; $*p < 0.03$; Bonferroni *post hoc* test) ($n = 3–6$). Acoustic startle response (*B*) and prepulse inhibition of startle (*C*) were tested during the same session. Although acoustic startle was unaffected by genotype, $\beta3^{-/-}$ mice displayed significantly less prepulse inhibition of startle than $\beta3^{+/+}$ littermates (repeated measures MANOVA; $*p < 0.05$) ($n$: $\beta3^{+/+} = 38$, $\beta3^{+/-} = 51$, $\beta3^{-/-} = 25$).

### Behavioral effects of β3 deletion

Deletion of the β3 nAChR subunit had significant behavioral effects. β3 null mutant mice displayed increased activity in the open field arena and decreased prepulse inhibition of acoustic startle response. Given that deletion of the β3 subunit altered nAChR-mediated dopamine release, it is tempting to ascribe the changes in behavior to the changes in dopaminergic function. Indeed, a large body of evidence implicates dopaminergic systems in the regulation of locomotion (for review, see Jaber et al., 1996), including the observation that deletion of the dopamine transporter (and thereby hyperdopaminergic activity attributable to increased presence of dopamine extracellularly) increases locomotor activity (Giros et al., 1996). It would be premature, however, to conclude that the increase in locomotor activity is a direct result of altered dopaminergic activity as a consequence of deletion of the β3 subunit. Locomotor activity in the open field is a combination of exploration and anxiety and is influenced by many factors, including but not limited to lighting, apparatus size, previous experience of the mouse in the apparatus, hunger, and thirst (Kelley et al., 1996). Indeed, the activity that a rat or mouse exhibits in a test box is influenced by as many as 10 genes (Flint et al., 1995). In contrast, prepulse inhibition of acoustic startle response appears to be a less complex behavior that is also modulated by dopaminergic systems; the response is disrupted by dopaminergic agonists and enhanced by dopaminergic antagonists (Geyer et al., 2001). The fact that deletion of the β3 subunit altered two independent behaviors thought to be mediated by dopaminergic systems is consistent with the proposal that alterations of dopaminergic systems observed in the null mutants underlie these changed responses. Nevertheless, alternative explanations for the effect of β3 subunit deletion on these behaviors are clearly possible.

### Conclusions

The data presented here demonstrate that many of the α-conotoxin MII-sensitive nAChRs expressed on substantia nigra–ventral tegmental area dopaminergic neurons are β3 dependent. In turn, these β3-containing nAChRs have a physiologically significant role in dopaminergic neurotransmission. Furthermore, locomotor activity and prepulse inhibition of startle, both mediated at least in part by dopaminergic pathways, were altered after β3 subunit deletion. This suggests a potential role for β3-containing nAChRs in the modulation of these behaviors.

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