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Involvement of the α3 Subunit in Central Nicotinic Binding Populations

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The α3 subunit gene was one of the first neuronal nicotinic acetylcholine receptor (nAChR) subunits to be cloned (Boulter et al., 1986), but direct evidence of α3 subunit contributions to mammalian central nAChR populations has not been presented. The studies reported here used mice engineered to contain a null mutation in the α3 nAChR subunit gene (Xu et al., 1999) to examine the involvement of the α3 subunit in central nAChR populations. Heterologously expressed α3β2 and α3β4 nAChRs are pharmacologically similar to native [^{125}I]α-conotoxin MII (α-CtxMII)-binding and 3-(2(*S*)-azetidinylmethoxy)pyridine dihydrochloride (A85380)-resistant [^{125}I]epibatidine-binding nAChR subtypes, respectively. The hypothesis that both native sites are α3-subtype nAChRs was tested using quantitative autoradiography in α3-null mutant mice. Somewhat surprisingly, deletion of the α3 nAChR subunit gene did not affect expression of the great majority of [^{125}I]α-CtxMII-binding sites, indicating that they do not correspond to heterologously expressed α3β2 nAChRs. The only exception to this was observed in the habenulointerpeduncular tract, where α3-dependent [^{125}I]α-CtxMII binding was observed. This finding may suggest the presence of an additional, minor nicotinic population in this pathway. In contrast, most A85380-resistant [^{125}I]epibatidine-binding nAChRs were dependent on α3 gene expression, suggesting that they do indeed correspond to an α3 nAChR subtype. However, widespread but lower levels of α3-independent A85380-resistant [^{125}I]epibatidine binding were also seen. Again, this may indicate the existence of an additional, minor population of non-α3 A85380-resistant sites.

Key words: nicotinic acetylcholine receptor; α3 subunit; A85380-resistant binding; α-conotoxin MII; autoradiography; α3 subunit-null mutant

Nicotinic acetylcholine receptors (nAChRs) are involved in normal CNS functions, including analgesia, cognition, reward, and motor control (Decker et al., 1995), and have been implicated in many of the diverse behavioral effects of nicotine in mammals (Stolerman, 1990). It is generally accepted that nAChRs are homopentameric or heteropentameric assemblies of homologous subunits and that different combinations of subunits produce distinct nAChR subtypes with diverse biophysical and pharmacological properties (Lindstrom et al., 1996). The potential to form functionally distinct pentamers, combined with differential expression of nAChR subtypes across the brain (Wada et al., 1989; Marks et al., 1992; Whiteaker et al., 2000a), underlies the rich variety of effects and roles attributed to nAChRs.

Epibatidine is a nicotinic agonist with high (picomolar) affinity at many mammalian nAChR subtypes (Badio and Daly, 1994; Houghtling et al., 1995; Perry and Kellar, 1995; Flores et al., 1996; Davila-Garcia et al., 1997; Marks et al., 1998; Whiteaker et al., 2000a,b). The majority of high-affinity epibatidine-binding sites are potently inhibited by the nicotinic agonist cytisine (cytisine-sensitive sites; $K_i = 0.29$ nM) (Marks et al., 1998). These cytisine-sensitive sites primarily correspond to the same α4β2-subtype nAChR that was identified by other agonist ligands such as (−)-[^{3}H]nicotine and [^{3}H]cytisine (Whiting and Lindstrom, 1987; Flores et al., 1992; Picciotto et al., 1995; Marubio et al., 1999). The remaining high-affinity epibatidine-binding sites exhibit much lower cytisine affinity (cytisine-resistant sites; $K_i > 10$ nM) (Marks et al., 1998; Whiteaker et al., 2000a,b). Some cytisine-resistant sites are highly sensitive to the nicotinic antagonist α-conotoxin MII (α-CtxMII) and may be directly identified using [^{125}I]α-CtxMII (Whiteaker et al., 2000b). A second population of cytisine-resistant sites that is unaffected by high (3 μM) concentrations of α-CtxMII and is unusually resistant to inhibition by the nicotinic agonist 3-(2(*S*)-azetidinylmethoxy)pyridine dihydrochloride (A85380) (Abreo et al., 1996) has also been identified (Whiteaker et al., 2000a). Their low A85380 affinity may be used to isolate them pharmacologically from other cytisine-sensitive and -resistant epibatidine-binding sites (A85380-resistant sites). The distribution and pharmacology of both cytisine-resistant populations are suggestive of α3* nAChRs (Whiteaker, 2000a,b), but concrete evidence of α3 involvement has not been provided in either case.

The purpose of this study was to investigate the involvement of the α3 nAChR subunit in the expression of the two cytisine-resistant populations described above using mice engineered to lack the α3 nAChR subunit gene (Xu et al., 1999).

MATERIALS AND METHODS

Animals. Mice engineered to contain a null mutation in the α3 nAChR subunit gene (Xu et al., 1999) were bred at the Division of Neuroscience, Baylor College of Medicine, in accordance with procedures approved by the local Animal Care and Utilization committee.

Materials. [^{125}I]Epibatidine (specific activity, 2200 Ci/mmol) was ob-

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tained from DuPont NEN (Boston, MA). (−)-Nicotine bitartrate was bought from BDH Chemicals (Poole, UK). A85380 was supplied by Research Biochemicals (Natick, MA). α-CtxMII was synthesized as described previously (Cartier et al., 1996), as was [^{125}I]α-conotoxin MII (Whiteaker et al., 2000a) (specific activity, 2200 Ci/mmol). Hyperfilm β-max and ^{125}I autoradiography microscales (original activity, 1.2–650 nCi/mg) were purchased from Amersham Biosciences (Mt. Prospect, IL). All other supplies were purchased from Sigma (St. Louis, MO).

Quantitative autoradiography of [^{125}I]α-CtxMII and [^{125}I]epibatidine binding. Quantitative autoradiography procedures were similar to those described previously (Pauly et al., 1989; Whiteaker et al., 2000a). Mice (8 d of age) of each α3 genotype ($\alpha3^{+/+}$, $\alpha3^{+/-}$, and $\alpha3^{-/-}$) were decapitated, and each brain was removed from the skull and rapidly frozen by immersion in isopentane (−35°C, 10 sec). Tissue was collected from mice from a single litter (2× $\alpha3^{+/+}$, 6× $\alpha3^{+/-}$, and 2× $\alpha3^{-/-}$). Tissue sections (10 μm thick) were prepared from frozen, unfixed tissue using a Leica (Nussloch, Germany) CM1850 cryostat/microtome and were thaw-mounted onto gelatin/poly-L-lysine-subbed glass microscope slides (Richard Allen, Richland, MI).

Before exposure to [^{125}I]α-CtxMII (0.5 nM), sections were incubated in binding buffer (144 mM NaCl, 1.5 mM KCl; 2 mM $CaCl_2$, 1 mM $MgSO_4$, 20 mM HEPES, 0.1% w/v BSA, pH 7.5) plus 1 mM PMSF (to inactivate endogenous serine proteases) at 22°C for 15 min. For all [^{125}I]α-CtxMII-binding reactions, the standard binding buffer was supplemented with 5 mM EDTA, 5 mM EGTA, and 10 μg/ml each of aprotinin, leupeptin trifluoroacetate, and pepstatin A to protect the ligand from endogenous proteases. A separate series of sections from each mouse was used to determine nonspecific [^{125}I]α-CtxMII binding (in the presence of 1 μM unlabeled epibatidine). After incubation with [^{125}I]α-CtxMII, the slides were washed as follows: Thirty seconds in binding buffer plus 0.1% w/v BSA (22°C), 30 sec in binding buffer plus 0.1% w/v BSA (0°C), 5 sec in 0.1× binding buffer plus 0.01% w/v BSA (twice at 0°C), and twice at 0°C for 5 sec in 5 mM HEPES, pH 7.5.

Sections for use in [^{125}I]epibatidine binding were rehydrated in binding buffer at 22°C for 15 min, followed by incubation with 100 pM [^{125}I]epibatidine for 2 hr at 22°C. Three series of adjacent sections were used from each mouse to measure total [^{125}I]epibatidine binding (no competing ligand), [^{125}I]epibatidine binding in the presence of 20 nM cytisine, and [^{125}I]epibatidine binding in the presence of 10 nM A85380. Concentrations of unlabeled drugs were chosen on the basis of results obtained in previous studies (Whiteaker et al., 2000a,b) and confirmed in pilot experiments. Nonspecific [^{125}I]epibatidine binding was defined using a separate series of sections in the presence of 1 mM (−)-nicotine tartrate. Slides were washed by sequential incubation in the following buffers (all steps at 0°C): Five seconds in binding buffer (twice), 5 sec in 0.1× binding buffer (twice), and 5 sec in 5 mM HEPES, pH 7.5 (twice).

Sections were initially dried with a stream of air and subsequently by overnight storage (22°C) under vacuum. Mounted, desiccated sections were apposed to Amersham Hyperfilm β-Max. Because large variations in ligand binding, and thus signal intensity, were observed, several film exposures were made for each binding condition to ensure that all measurements could be made within the accurate recording range of the film (3–7 d for [^{125}I]α-CtxMII-labeled sections; 5–96 hr for [^{125}I]epibatidine-labeled sections). To allow quantification, each film was also exposed to ^{125}I autoradiography microscale standards of defined specific activity.

After the films had been exposed to the sections for an appropriate length of time, they were developed and signal intensity in selected brain regions was measured by digital image analysis. Films were illuminated using a Northern Light (Ontario, Canada) light box, and autoradiographic images of the sections and standards were captured using a CCD imager camera. Signal intensity was determined using NIH Image 1.61 software. Where possible, six independent measurements from different tissue sections were made for each brain region, under each incubation condition, for each mouse. For each subject, the absorbance measurements from each brain area were used to calculate the degree of labeling by reference to the relevant standard curve, and labeling values were used to determine regional labeling in each mouse.

Data processing. All calculations and graph preparation were performed using SigmaPlot for Windows, version 5.0 (Jandel Scientific, San Rafael, CA). Statistical analysis (one-way ANOVA) was performed using SPSS PC+ (Jandel Scientific). Duncan's *post hoc* test was used to test for within-region differences.

RESULTS

Effect of α3-null mutation on [^{125}I]α-CtxMII-binding nAChRs

[^{125}I]α-CtxMII (0.5 nM) was used to detect binding sites with a high affinity for α-CtxMII (Whiteaker et al., 2000b). The distribution of [^{125}I]α-CtxMII-binding sites in the 8 d animals was very similar to that observed in adult animals (Whiteaker et al., 2000b), with the greatest amounts seen in optic tract-associated nuclei (superior colliculus, olivary pretectal nucleus, and the ventrolateral and dorsolateral geniculate nuclei) and the occulomotor nerve and more moderate expression throughout the dopaminergic tracts (substantia nigra, ventral tegmental region, striatum, nucleus accumbens, and olfactory tubercles) (Fig. 1, *fourth column*). Some developmental differences were evident, however: the amounts of [^{125}I]α-CtxMII binding observed in the younger animals were generally higher than those seen in more mature animals (Whiteaker et al., 2000b). Relative amounts also differed between some regions. (For instance, in mature animals, the dorsolateral geniculate nucleus contained 5.0 ± 0.2 fmol/mg protein and the ventrolateral geniculate nucleus contained 5.8 ± 0.2 fmol/mg protein of [^{125}I]α-CtxMII-binding sites, whereas in the 8 d animals, the specific binding was 10.9 ± 0.4 and 6.7 ± 0.6 fmol/mg protein, respectively.)

Specific [^{125}I]α-CtxMII binding was assessed in $\alpha3^{+/+}$, $\alpha3^{+/-}$, and $\alpha3^{-/-}$ mice. In most regions in which [^{125}I]α-CtxMII binding was observed, deletion of the α3 nAChR subunit had no effect, as determined by one-way ANOVA (Fig. 2). [^{125}I]α-CtxMII binding in the medial habenula (MH) and fasciculus retroflexus was eliminated, however, in $\alpha3^{-/-}$ mice ($F_{(2,3)} = 20.60$, $p = 0.0177$ and $F_{(2,3)} = 50.58$, $p = 0.0049$, respectively). In addition, [^{125}I]α-CtxMII binding was approximately halved in the interpeduncular nucleus (IPN) of $\alpha3^{-/-}$ mice (from 4.29 ± 0.54 to 1.98 ± 0.74 fmol/mg protein) (Fig. 2), although this effect was not statistically significant ($F_{(2,3)} = 4.89$; $p = 0.11$). Because these regions are small, represent only 3 of 18 regions with detectable binding, and contain only modest amounts of [^{125}I]α-CtxMII binding even in the $\alpha3^{+/+}$ animals, the great majority of [^{125}I]α-CtxMII-binding nAChRs apparently do not require expression of the α3 subunit, as demonstrated by the high correlation between regional [^{125}I]α-CtxMII binding in $\alpha3^{+/+}$ and $\alpha3^{-/-}$ animals (Fig. 2*A*; *y*-intercept, −0.25 fmol/mg protein; slope, 0.98; $r = 0.98$).

Effect of α3-null mutation on A85380-resistant [^{125}I]epibatidine-binding nAChRs

Resistance to inhibition by the agonist A85380 has been used previously to isolate a population of [^{125}I]epibatidine-binding nAChRs with low affinity for both cytisine and α-CtxMII (Whiteaker et al., 2000a). The highest levels of A85380-resistant [^{125}I]epibatidine binding were detected in the medial habenula–fasciculus retroflexus–IPN tract of $\alpha3^{+/+}$ animals (Figs. 1 and 3). Indeed, the A85380-resistant binding sites in these regions were by far the most densely expressed nAChR population measured in the present study. Both of these findings are consistent with those reported for A85380-resistant binding in adult animals (Whiteaker et al., 2000a), although the accessory olfactory bulbs of the younger animals used in this study were almost devoid of A85380-resistant [^{125}I]epibatidine binding (2.3 ± 0.4 and 1.0 ± 0.2 fmol/mg protein in the glomerular and mitral layers, respectively), unlike those of adult animals (82 ± 9 and 38 ± 3 fmol/mg, respectively).

In contrast to [^{125}I]α-CtxMII-binding nAChRs, the A85380-resistant nAChR population displayed notable sensitivity to the

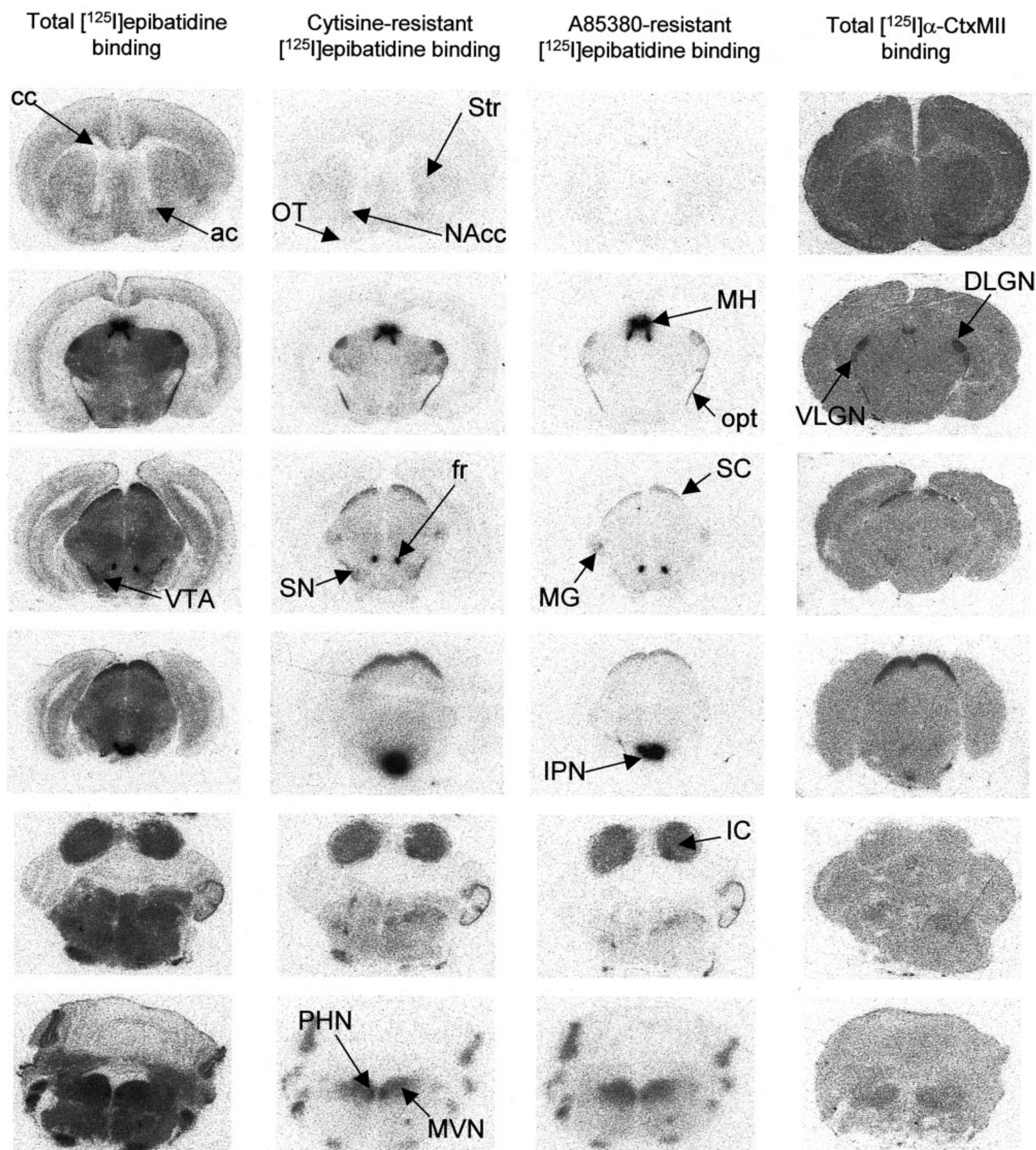

Figure 1. Illustration of total, cytisine-resistant, and A85380-resistant [^{125}I]epibatidine-binding and [^{125}I]α-CtxMII-binding patterns in an 8 d wild-type mouse brain. Sections (10 μM) from $\alpha 3^{+/+}$ mouse brains were incubated in the presence of 100 pM [^{125}I]epibatidine alone (*first column*), [^{125}I]epibatidine plus 20 nM cytisine (*second column*), 100 pM [^{125}I]epibatidine plus 10 nM A85380 (*third column*), and 0.5 nM [^{125}I]α-CtxMII (*fourth column*). *Panels* are digital images of autoradiograms. *ac*, Anterior commissure; *cc*, corpus callosum; *DLGN*, dorsolateral geniculate nucleus; *fr*, fasciculus retroflexus; *IC*, inferior colliculus; *MG*, medial geniculate nucleus; *MVN*, medial vestibular nucleus; *NAcc*, nucleus accumbens; *opt*, optic tract; *OT*, olfactory tubercle; *PHN*, prepositus hypoglossal nucleus; *SC*, superior colliculus; *SN*, substantia nigra; *Str*, striatum; *VLGN*, ventrolateral geniculate nucleus; *VTA*, ventral tegmental area.

loss of α3 subunit expression, as illustrated in Figure 3 and detailed in Table 1. As shown in Figure 3 (*first column*), deletion of the α3 nAChR subunit decreased the amount of A85380-resistant [^{125}I]epibatidine binding detected in the inferior colliculus, medial habenula, and fasciculus retroflexus by at least 90% ($F_{(2,3)} = 10.5$, $p = 0.044$; $F_{(2,3)} = 176$, $p = 0.0008$; and $F_{(2,3)} = 62.2$, $p = 0.0036$, respectively). A85380-resistant [^{125}I]epibatidine binding in the IPN of mutant animals was significantly reduced ($F_{(2,3)} = 15.3$; $p = 0.0268$). Interestingly, however, this binding in IPN was still 24% of wild-type levels. In contrast, A85380-

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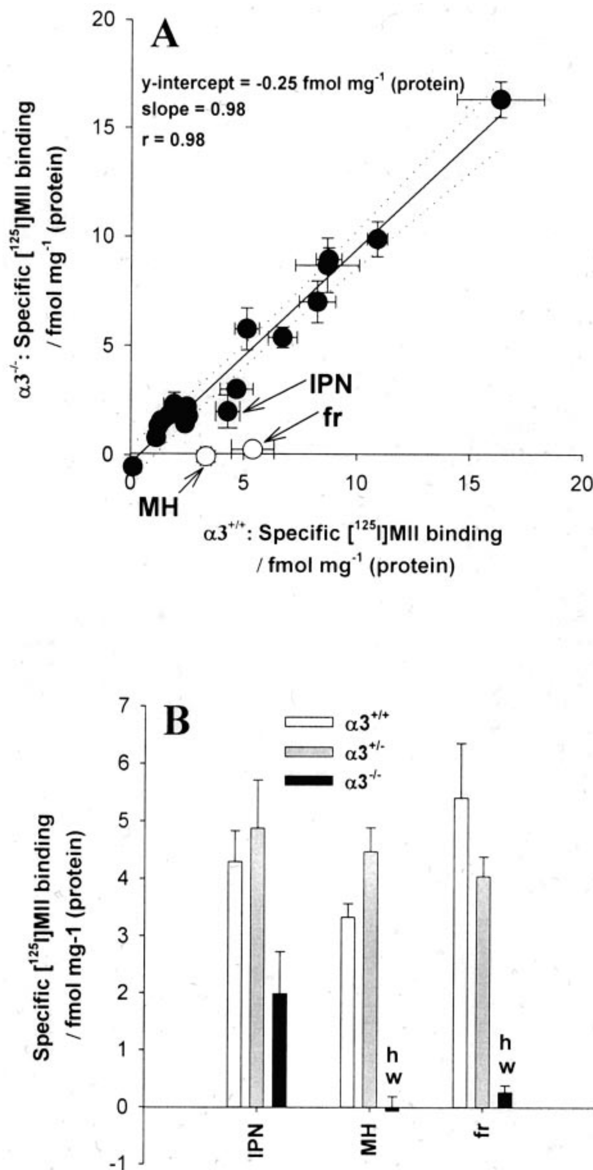

Figure 2. Effect of nAChR α3 subunit-null mutation on mouse brain regional [^{125}I]α-CtxMII (0.5 nM) binding. Levels of specific [^{125}I]α-CtxMII (0.5 nM) binding were determined in the brains of $\alpha3^{+/+}$, $\alpha3^{+/-}$, and $\alpha3^{-/-}$ genotype mice and compared in 18 different brain regions. *A*, Only the medial habenula and fasciculus retroflexus (*open circles*) showed significant loss of specific [^{125}I]α-CtxMII binding in $\alpha3^{-/-}$ mice compared with their $\alpha3^{+/+}$ counterparts (as determined by one-way ANOVA; medial habenula, $F_{(2,3)} = 20.60$, $p = 0.0177$; fasciculus retroflexus, $F_{(2,3)} = 50.58$, $p = 0.0049$). Loss of specific [^{125}I]α-CtxMII binding in the IPN (the remaining habenulo-IPN tract region) approached, but did not attain, significance ($F_{(2,3)} = 4.89$; $p = 0.11$). The regression line (*solid line*) was fit to all regions that were not significantly different between genotypes; also shown are 95% confidence intervals (*dotted lines*). *B*, Within-region differences were tested for in the habenulo-IPN tract using Duncan's *post hoc* test. Significant differences are denoted *h* (different from heterozygous null-mutant) or *w* (different from wild type). *fr*, Fasciculus retroflexus.

resistant binding in mutant animals was only 9.0 and 5.5% of that seen in wild-type mice in the fasciculus retroflexus and medial habenula, respectively. Outside the dorsal cortex of the inferior colliculus and the MH–IPN tract, only the medial vestibular and prepositus hypoglossal nuclei contained substantial amounts (>10 fmol/mg protein) of A85380-resistant [^{125}I]epibatidine binding (Fig. 3, *fourth column*). Deletion of the α3 subunit also produced a significant loss of A85380-resistant [^{125}I]epibatidine binding in these regions. Low (<10 fmol/mg protein) A85380-resistant [^{125}I]epibatidine-binding signals were observed in all of the other regions surveyed. In contrast to the more densely expressed A85380-resistant [^{125}I]epibatidine-binding populations, the α3-dependence of these sites was generally low, with the only exceptions being found in the anterior thalamic and medial geniculate nuclei (Fig. 3). With one exception (glomerular layer of the olfactory bulb), A85380-resistant [^{125}I]epibatidine binding in $\alpha3^{+/-}$ mice was indistinguishable from that in $\alpha3^{+/+}$ mice. No other examples of binding differences between $\alpha3^{+/-}$ and $\alpha3^{+/+}$ mice were seen, suggesting that loss of one copy of the α3 gene has very little effect on nAChR expression.

DISCUSSION

α3 was the first mammalian neuronal nAChR subunit cloned (Boulter et al., 1986) and one of the first to be studied in an heterologous expression system (Boulter et al., 1987). The immunochemical study by Flores et al. (1996) established the existence of α3β4 nAChRs in the rat trigeminal ganglion, but functional (Zoli et al., 1998; Quick et al., 1999; Grady et al., 2001), immunohistochemical (Yeh et al., 2001), and binding (Whiteaker et al., 2000a) studies have provided only circumstantial evidence for central expression of α3* nAChRs. The findings of the present study provide the first direct evidence for α3* nAChR expression in the mammalian CNS.

Expression of [^{125}I]α-CtxMII-binding sites is independent of α3 nAChR subunit expression in 16 of the 18 regions in which these sites were identified (Fig. 2). These data indicate that [^{125}I]α-CtxMII-binding nAChRs are largely not α3-dependent, despite being found in regions (the optic tract and its associated nuclei and dopaminergic terminal regions) in which α3 protein might be expected to be expressed, based on the detection of α3 mRNA (Wada et al., 1989; Whiteaker et al., 2000b). This result was very surprising, because α-CtxMII was originally isolated by virtue of its selectivity for heterologously assembled α3β2-subtype nAChRs (Cartier et al., 1996), and both α-CtxMII and [^{125}I]α-CtxMII display similar affinities at native [^{125}I]α-CtxMII-binding nAChRs and artificially expressed α3β2-subtype nAChRs (Whiteaker et al., 2000b). The lack of effect of α3 subunit deletion on [^{125}I]α-CtxMII-binding nAChRs argues strongly against a role for the α3 nAChR subunit in this binding site.

Recently, it has been established that α6-containing nAChRs can bind α-CtxMII with high (nanomolar) affinity (Vailati et al., 1999; Kuryatov et al., 2000; Klink et al., 2001). In addition, the α6 nAChR subunit is widely coexpressed with α3 (LeNovère et al., 1996), and the two subunits exhibit considerable sequence homology (LeNovère and Changeux, 1995). Together, these findings suggest that the majority of mouse brain [^{125}I]α-CtxMII-binding nAChRs may in fact be of an α6-containing rather than α3-containing subtype as originally suspected. A component of nAChR-stimulated striatal dopamine release is sensitive to inhibition by α-CtxMII (Kulak et al., 1997; Kaiser et al., 1998; Grady et al., 2001), establishing that α-CtxMII-sensitive nAChRs are a functional mammalian neuronal nAChR subtype. In addition, striatal α-CtxMII-sensitive sites are dependent on β2 nAChR subunit expression (Grady et al., 2001), indicating that mouse brain [^{125}I]α-CtxMII-binding nAChRs require β2 subunit expression. Despite their likely physiological significance, [^{125}I]α-

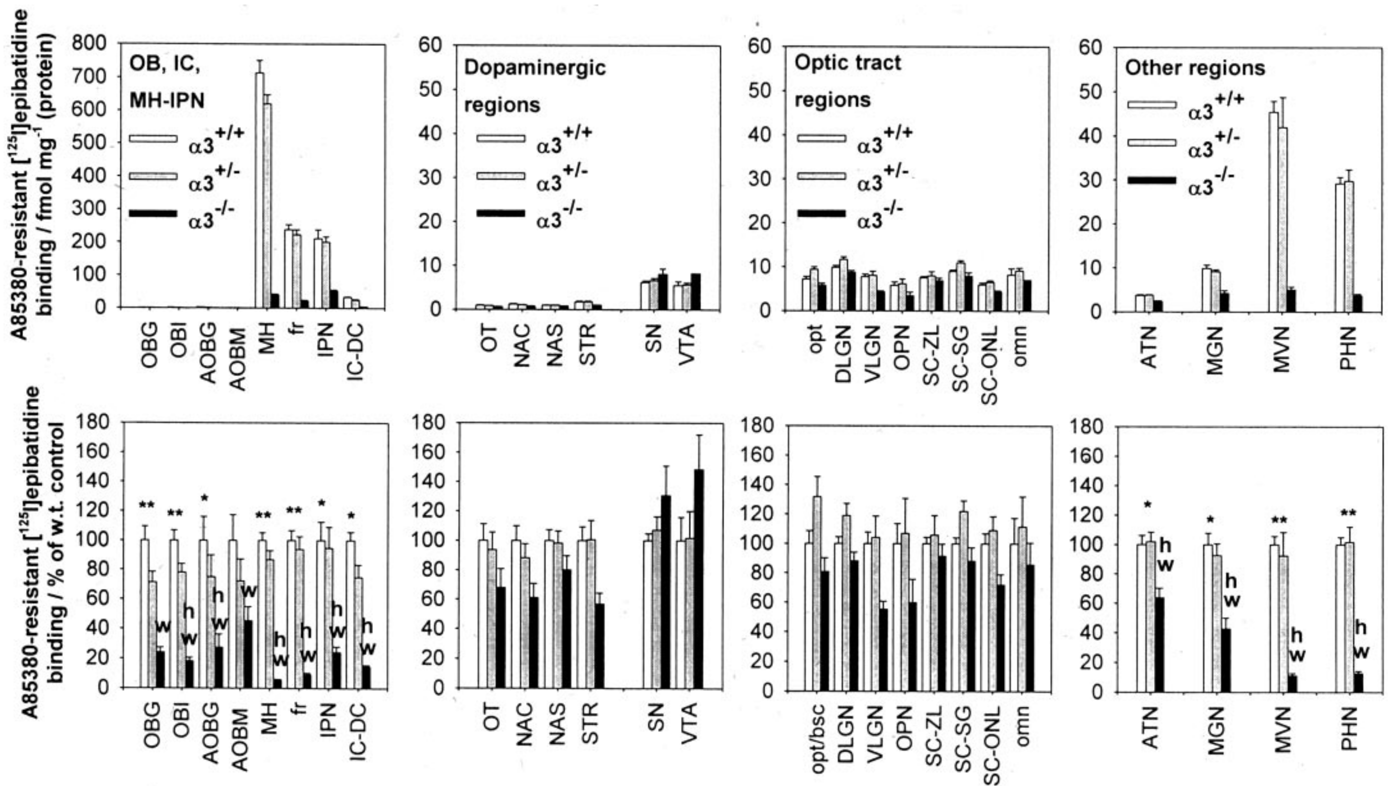

Figure 3. Effect of nAChR $\alpha3$ subunit-null mutation on mouse brain regional A85380-resistant [^{125}I]epibatidine (100 pM) binding. Levels of specific A85380-resistant [^{125}I]epibatidine binding were compared in 26 different brain regions for $\alpha3^{+/+}$, $\alpha3^{+/-}$, and $\alpha3^{-/-}$ genotype mice. *Top row*, Regional distribution of A85380-resistant [^{125}I]epibatidine binding measured in femtomoles per milligram of protein. Because binding levels were so high in the habenulo-IPN tract, this group is displayed on a different scale from the remaining regions. *Bottom row*, Regional effects of $\alpha3$ null-mutation on A85380-resistant [^{125}I]epibatidine (100 pM) binding, presented as percentage of change from wild-type binding. Regions exhibiting significant effects of $\alpha3$ null-mutation on [^{125}I]epibatidine binding were determined with one-way ANOVA; $^*p < 0.05$ ($F_{(2,3)} > 9.55$); $^{**}p < 0.01$ ($F_{(2,3)} > 30.8$). Within-region differences were tested using Duncan's *post hoc* test. Significant differences are denoted *h* (different from heterozygous null-mutant) or *w* (different from wild type). *AOBG*, Accessory olfactory bulb (glomerular layer); *AOBM*, accessory olfactory bulb (mitral cell layer); *ATN*, anterior thalamic nucleus; *bsc*, brachium of the superior colliculus. *DLGN*, dorsolateral geniculate nucleus; *fr*, fasciculus retroflexus; *IC-DC*, inferior colliculus (dorsal cortex); *MGN*, medial geniculate nucleus; *MH*, medial habenula; *MVN*, medial vestibular nucleus; *NAC*, nucleus accumbens (core); *NAS*, nucleus accumbens (shell); *OBG*, olfactory bulb (glomerular layer); *OBI*, olfactory bulb (internal plexiform layer); *omn*, occulomotor nerve; *OPN*, olivary pretectal nucleus; *opt*, optic tract; *OT*, olfactory tubercle; *PHN*, prepositus hypoglossal nucleus; *SC-ONL*, superior colliculus (optic nerve layer); *SC-SG*, superior colliculus (superficial gray); *SC-ZL*, superior colliculus (zonal layer); *SN*, substantia nigra; *STR*, striatum; *VLGN*, ventrolateral geniculate nucleus; *VTA*, ventral tegmental area.

CtxMII-binding nAChRs are relatively rare. For instance, [^{125}I]α-CtxMII-binding sites are outnumbered by both cytisine-sensitive and A85380-resistant [^{125}I]epibatidine-binding sites in the terminal regions of the substantia nigra/ventral tegmental area dopaminergic projections. It is likely, however, that their concentration on dopaminergic termini strengthens their influence over this important pathway (Kulak et al., 1997; Kaiser et al., 1998; Grady et al., 2001; Quik et al., 2001).

Although the $\alpha3$ subunit is not required for the expression of [^{125}I]α-CtxMII-binding sites in most brain regions, [^{125}I]α-CtxMII binding in the medial habenula and fasciculus retroflexus was dramatically reduced in $\alpha3^{-/-}$ versus $\alpha3^{+/+}$ mice (Fig. 2), indicating that in these regions, [^{125}I]α-CtxMII-binding sites may be $\alpha3\beta2^*$ nAChRs. Alternatively, [^{125}I]α-CtxMII binding in these regions may result from low occupancy of a large nicotinic binding population with a lower affinity for α-CtxMII. In either case, the regions containing $\alpha3$-dependent [^{125}I]α-CtxMII-binding receptors are small, as are the amounts of binding observed in these regions. Thus, the great majority of nAChRs identified using [^{125}I]α-CtxMII (0.5 nM) do not appear to require $\alpha3$ nAChR subunit expression.

The agonist A85380 has been used to isolate the population of [^{125}I]epibatidine-binding nAChRs with low affinity for both cytisine and α-CtxMII (Whiteaker et al., 2000a). The distribution of A85380-resistant [^{125}I]epibatidine-binding sites was very different from that of [^{125}I]α-CtxMII-binding nAChRs, demonstrating that two different populations of sites were being identified (Fig. 1). In the regions containing the highest levels of A-85380-resistant binding (medial habenula–fasciculus retroflexus–IPN tract, dorsal cortex of the inferior colliculus, and medial vestibular and prepositus hypoglossal nucleus), binding was dramatically reduced in $\alpha3$-null mutant animals. Although most A85380-resistant [^{125}I]epibatidine-binding sites require expression of the $\alpha3$ nAChR subunit, a substantial population of A85380-resistant [^{125}I]epibatidine-binding sites was retained in the IPN of $\alpha3^{-/-}$ mice. Thus, the IPN may express a novel, $\alpha3$-independent, A85380-resistant nAChR subtype.

An important role for the $\alpha3$ subunit in A85380-resistant [^{125}I]epibatidine-binding sites is consistent with previous findings. First, A85380-resistant [^{125}I]epibatidine-binding sites have a distribution and binding pharmacology suggestive of $\alpha3\beta4^*$ nAChRs (Whiteaker et al., 2000a). Second, the activation and

Table 1. Regional distribution of specific mouse brain A85380-resistant [^{125}I]epibatidine binding: effects of α3 genotype

	$\alpha3^{+/+}$ [^{125}I]Epibatidine binding (fmol/mg protein)	$\alpha3^{+/-}$ [^{125}I]Epibatidine binding (fmol/mg protein)	$\alpha3^{-/-}$ [^{125}I]Epibatidine binding (fmol/mg protein)
Olfactory bulbs, inferior colliculus, habenulointerpeducular tract			
Olfactory bulb, glomerular layer	1.10 ± 0.10	0.79 ± 0.03	0.27 ± 0.02
Olfactory bulb, internal plexiform layer	1.65 ± 0.11	1.29 ± 0.04	0.30 ± 0.03
Accessory olfactory bulb, glomerular layer	2.27 ± 0.36	1.71 ± 0.19	0.62 ± 0.18
Accessory olfactory bulb, mitral cell layer	1.02 ± 0.18	0.74 ± 0.09	0.46 ± 0.06
Medial habenula	713 ± 37	620 ± 28	39.6 ± 3.1
Fasciculus retroflexus	238 ± 16	223 ± 15	21.5 ± 2.5
Interpeduncular nucleus	211 ± 27	200 ± 17	51.2 ± 3.5
Inferior colliculus, dorsal cortex	33.0 ± 1.8	24.7 ± 2.5	4.72 ± 0.25
Dopaminergic regions			
Olfactory tubercle	0.94 ± 0.11	0.88 ± 0.05	0.64 ± 0.10
Nucleus accumbens, core	1.25 ± 0.12	1.10 ± 0.05	0.76 ± 0.09
Nucleus accumbens, shell	1.04 ± 0.08	1.02 ± 0.04	0.83 ± 0.08
Striatum	1.77 ± 0.16	1.78 ± 0.17	1.01 ± 0.09
Substantia nigra	6.20 ± 0.30	6.65 ± 0.46	8.10 ± 1.20
Ventral tegmental area	5.53 ±0.88	5.62 ± 0.46	8.20 ± 0.20
Optic tract regions			
Optic tract	7.16 ± 0.62	9.43 ± 0.56	5.79 ± 0.44
Dorsolateral geniculate nucleus	9.86 ± 0.45	11.7 ± 0.6	8.69 ± 0.43
Ventrolateral geniculate nucleus	7.75 ± 0.6	8.07 ± 0.93	4.28 ± 0.26
Olivary pretectal nucleus	5.81 ± 0.79	6.20 ± 1.10	3.47 ± 0.80
Superior colliculus, zonal layer	7.55 ± 0.33	7.98 ± 0.95	6.91 ± 0.55
Superior colliculus, superficial gray	9.01 ± 0.36	10.98 ± 0.47	7.95 ± 0.74
Superior colliculus, optic nerve layer	6.01 ± 0.41	6.56 ± 0.33	4.33 ± 0.30
Occulomotor nerve	8.21 ± 1.42	9.14 ± 0.63	7.04 ± 0
Other regions			
Anterior thalamic nucleus	3.72 ± 0.23	3.79 ± 0.09	2.37 ± 0.19
Medial geniculate nucleus	9.92 ± 0.76	9.17 ± 0.40	4.23 ± 0.67
Medial vestibular nucleus	45.38 ± 2.55	41.9 ± 6.9	5.01 ± 0.65
Prepositus hypoglossal nucleus	29.29 ± 1.44	29.8 ± 2.7	3.68 ± 0.4

Sections (10 μM) from $\alpha3^{+/+}$, $\alpha3^{+/-}$, and $\alpha3^{-/-}$ mouse brains were incubated in the presence of [^{125}I]epibatidine (100 pM) plus A85380 (10 nM). A85380-resistant [^{125}I]epibatidine binding to the sections was detected autoradiographically using β-max film. Radioligand binding was quantified by digital densiometry of the autoradiographic images and reference to ^{125}I standards. Specific binding was calculated by subtraction of nonspecific from total binding and is presented in terms of femtomoles of [^{125}I]epibatidine bound per milligram of tissue protein. Measurements were made six times in each region, in each mouse, where possible. Values are the mean ± SEM of binding measured in two to three different mice.

binding pharmacology of receptors that persist in the habenulointerpeduncular tract of β2-null mutant mice also resemble that of α3β4* nAChRs (Zoli et al., 1998; Grady et al., 2001). Third, central α3 subunit immunoreactivity is concentrated in habenulointerpeduncular tract nuclei (Yeh et al., 2001). Thus, the past and present findings strongly suggest that A85380-resistant epibatidine-binding sites correspond to a functional CNS α3β4 nAChR subtype similar to that previously identified in the periphery (Flores et al., 1996).

Widely distributed but low-density (<10 fmol/mg protein) A85380-resistant [^{125}I]epibatidine (100 pM) binding was observed (Figs. 1, 3, Table 1). In particular, A85380-resistant sites were found in the optic tract and associated nuclei and in the cell body regions of the dopaminergic tract. However, these additional [^{125}I]epibatidine-binding sites exhibited much lower α3 dependence than those in the more densely expressing regions. Given the generally lower affinity of A85380 at β4- versus β2-containing nAChR subtypes (Parker et al., 1998), it is possible that these sites represent a combination of β4 with a non-α3 subunit.

Loss of α3 expression results in a syndrome with features suggestive of widespread autonomic dysfunction that is lethal within weeks of birth (Xu et al., 1999). This dysfunction might theoretically induce global alterations in nAChR expression. However, the minor effects of α3-null mutation on [^{125}I]α-CtxMII and total [^{125}I]epibatidine-binding sites (Fig. 4) strongly argue against a generalized disruption of nAChR expression. In addition, the early demise of the $\alpha3^{-/-}$ mice necessitated the use of relatively young mice (8 d of age) in this study. It was anticipated that these mice might display major developmental differences in the distribution of nAChR subtypes compared with the more mature 60- to 90-d-old animals used in previous studies from our laboratory. In fact, each of the subtypes measured previously was found in the younger subjects used in this study. Regional distribution of the sites was qualitatively similar to that of adult mice, although some quantitative differences were seen.

In summary, this study has determined the α3 dependence of two previously identified nicotinic binding populations and has provided evidence for the possible existence of additional minor populations. Studies performed with transfected oocytes indicated that α3β2 nAChRs have a high affinity for α-CtxMII.

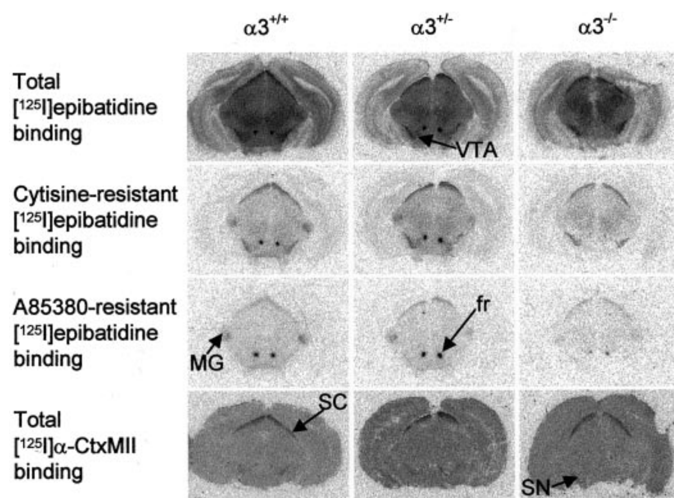

Figure 4. Effects of nAChR α3 subunit-null mutation on nicotinic binding populations illustrated at the level of the substantia nigra/ventral tegmental region. *First row*, The majority of mouse brain [^{125}I]epibatidine-binding sites are of the cytisine-sensitive type and do not show global alterations after loss of α3 subunit gene expression. *Second row*, Cytisine-resistant [^{125}I]epibatidine binding has a more restricted distribution than cytisine-sensitive binding. The effects of α3 null mutation vary between regions, with loss of subunit expression dramatically reducing cytisine-resistant binding in the fasciculus retroflexus and medial geniculate nucleus but having minimal effect on this measure in the substantia nigra and ventral tegmental area. *Third row*, A85380-resistant [^{125}I]epibatidine binding is a subset of cytisine-resistant [^{125}I]epibatidine binding and is strongly affected by α3 null mutation. *Fourth row*, [^{125}I]α-CtxMII-binding nAChRs are a second subset of the cytisine-resistant [^{125}I]epibatidine-binding population and are largely unaffected by the loss of α3 subunit expression. All *panels* are digital images of autoradiograms. *fr*, Fasciculus retroflexus; *MG*, medial geniculate nucleus; *SC*, superior colliculus; *SN*, substantia nigra; *VTA*, ventral tegmental area.

Unexpectedly, [^{125}I]α-CtxMII-binding nAChRs showed almost no dependency on α3 expression (Figs. 2, 4, *fourth row*), strongly arguing against a role for the α3 subunit in native [^{125}I]α-CtxMII-binding nAChRs. The α3 dependence of [^{125}I]α-CtxMII binding in the habenulointerpeduncular tract suggests that these sites may have a different (α3-dependent) composition from the majority of [^{125}I]α-CtxMII-binding nAChRs. A85380-resistant [^{125}I]epibatidine-binding sites were suspected be α3β4 nAChRs (Whiteaker et al., 2000a) and do indeed show strong α3 dependence (Fig. 4, *third row*). In this case, the persistence of A85380-resistant binding in $\alpha3^{-/-}$ mice (particularly in the IPN) may indicate the presence of a novel nicotinic population in this nucleus. This is currently under investigation.

Note added in proof. During the preparation of this manuscript, Champtiaux et al. (2002) confirmed the α6 nAChR subunit dependence of [^{125}I]α-conotoxin MII binding sites using an α6 subunit null mutant mouse model.

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