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Mouse Striatal Dopamine Nerve Terminals Express $\alpha 4\alpha 5\beta 2$ and Two Stoichiometric Forms of $\alpha 4\beta 2^*$ -Nicotinic Acetylcholine Receptors

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Abstract

Wild-type and $\alpha 5$ null mutant mice were used to identify nicotinic cholinergic receptors (nAChRs) that mediate α -conotoxin MII (α -CtxMII)-resistant dopamine (DA) release from striatal synaptosomes. Concentration–effect curves for ACh-stimulated release (20 s) were monophasic when wild-type synaptosomes were assayed but biphasic with synaptosomes from the $\alpha 5$ null mutant. Deleting the $\alpha 5$ gene also resulted in decreased maximal ACh-stimulated α -CtxMII-resistant DA release. When a shorter perfusion time (5 s) was used, biphasic curves were detected in both wild-type and $\alpha 5$ null mutants, indicative of high- and low-sensitivity (HS and LS) activity. In addition, DH β E-sensitive (HS) and DH β E-resistant (LS) components were found in both genotypes. These results indicate that α -CtxMII-resistant DA release is mediated by $\alpha 4\alpha 5\beta 2$, ($\alpha 4$)₂($\beta 2$)₃ (HS), and ($\alpha 4$)₃($\beta 2$)₂ (LS) nAChRs.

Keywords

Neuronal nicotinic acetylcholine receptors; Synaptosomes; subunit null mutation; α -Conotoxin MII

Introduction

Many studies have attempted to identify the nicotinic cholinergic receptor (nAChR) subtypes that mediate nicotinic agonist-induced release of dopamine (DA) from striatal synaptosomes of the mouse and rat. Early results suggested that this process is mediated by a single receptor, but the finding that agonist-stimulated DA release from rat (Kulak et al. 1997) and mouse (Grady et al. 2001) striatal synaptosomes can be separated into two components that differ in sensitivity to inhibition by α -conotoxin MII (α -CtxMII) indicated that at least two subtypes may exist. Two recent studies (Champtiaux et al. 2003; Salminen et al. 2004) that used nAChR subunit null mutant (gene knockout) mouse strains and a double null mutant strain helped establish that $\alpha 4\alpha 6\beta 2\beta 3$, $\alpha 6\beta 2\beta 3$, and low levels of $\alpha 6\beta 2$ mediate the α -CtxMII-sensitive component of DA release from striatal synaptosomes. The α -CtxMII-resistant component is eliminated by $\alpha 4$ and $\beta 2$ gene deletion and $\alpha 5$ gene deletion resulted in a marked reduction in agonist efficacy with no change in potency, indicating that $\alpha 4\beta 2$ and $\alpha 4\alpha 5\beta 2$ nAChRs mediate the α -CtxMII-resistant DA release. The finding that $\alpha 5$ gene deletion altered agonist efficacy is consistent with studies in expression systems that have shown that adding $\alpha 5$ subunits to $\alpha 3\beta 2$, $\alpha 3\beta 4$, and $\alpha 4\beta 2$ nAChRs increases agonist efficacy (Gerzanich et al. 1998; Kuryatov et al. 2008).

Several studies with $\alpha 4\beta 2$ -type nAChRs expressed in oocytes indicate that two $\alpha 4\beta 2$ subtypes [$(\alpha 4)_2(\beta 2)_3$ and $(\alpha 4)_3(\beta 2)_2$] can be formed by manipulating $\alpha 4$ and $\beta 2$ messenger RNA levels (Zwart and Vijverberg 1998; Nelson et al. 2003; Khiroug et al. 2004; Moroni et al. 2006). The $(\alpha 4)_2(\beta 2)_3$ subtype is referred to as high sensitivity (HS) and the $(\alpha 4)_3(\beta 2)_2$ is called low sensitivity (LS) because they differ in potency (EC_{50} values) for agonist activation (Moroni et al. 2006). We have developed and characterized an assay that measures a biphasic agonist-stimulated $^{86}\text{Rb}^+$ efflux from mouse brain synaptosomes (Marks et al. 1999). Both HS and LS responses are clearly mediated by $\alpha 4\beta 2^*$ nAChRs, given that all response is absent from synaptosomes obtained from $\alpha 4$ (Marks et al. 2007) and $\beta 2$ (Marks et al. 2000) null mutant mice. Some of the HS response is also mediated by the $\alpha 4\alpha 5\beta 2$ -nAChR, given that $\alpha 5$ null mutation results in a decrease in agonist efficacy for the higher sensitivity efflux (Brown et al. 2007).

While we have successfully measured a biphasic response with the $^{86}\text{Rb}^+$ flux assay (Marks et al. 1999, 2007; Gotti et al. 2008), we have not seen this with DA release (Salminen et al. 2004, 2007). As a result, it is not clear whether both HS and LS $\alpha 4\beta 2$ -type nAChRs modulate DA release. A major difference between the DA release and $^{86}\text{Rb}^+$ flux experiments is that we have used a broader range of agonist concentrations for the ion flux experiments. This facilitates measurement of the low sensitivity $(\alpha 4)_3(\beta 2)_2$ nAChR. The studies reported here evaluated the effects of $\alpha 5$ gene deletion on DA release using a broad range of concentrations of ACh, an agonist that has equal efficacy at the HS and LS forms of $\alpha 4\beta 2$ -type nAChRs (Marks et al. 1999). We used two agonist exposure durations (20 and 5 s) as well as DH β E, a selective HS antagonist, to resolve HS and LS activity at dopaminergic terminals. The results obtained indicate that the α -CtxMII-resistant component of dopamine release is mediated by $\alpha 4\alpha 5\beta 2$, $(\alpha 4)_2(\beta 2)_3$, and $(\alpha 4)_3(\beta 2)_2$ nAChRs.

Materials and Methods

Mice

All mice were bred and maintained at the Institute for Behavioral Genetics. The care and experimental procedures were in accordance with the Animal Care and Utilization Committee of the University of Colorado, Boulder, CO. Dr. Arthur Beaudet (Baylor College of Medicine, Houston, TX) generously provided the original breeding population of $\alpha 5$ -subunit null mutant mice (Salas et al. 2003). These mice were bred onto the C57BL/6 strain for six generations and genotyped by previously published methods (Salminen et al. 2004).

Synaptosome preparation and DA uptake

The procedures used in these experiments are described in detail in Salminen et al. (2007). Crude synaptosomes were prepared by homogenizing striatal tissue in cold 0.32 M sucrose buffered with 5 mM HEPES (pH 7.5) and centrifugation at 12,000 $\times g$ for 20 min. Pellets were resuspended (1.6 ml/ mouse) in uptake buffer [NaCl (128 mM), KCl (2.4 mM), CaCl₂ (3.2 mM), KH₂PO₄ (1.2 mM), MgSO₄ (1.2 mM), glucose (10 mM), ascorbic acid (1 mM), pargyline (1.6 mM), and HEPES (25 mM), pH 7.5]. The resuspended synaptosomes were then incubated for 10 min at 37° before addition of 100 nM [³H]-dopamine (DA) (Perkin-Elmer, specific activity=40–60 Ci/mmol) and incubated 5 min longer. A cholinesterase inhibitor, diisopropylfluorophosphate (10 μ M), was added to the buffer during uptake for those experiments where ACh was used as the agonist.

DA release

Synaptosomal aliquots (80 μ l) were pipetted onto filters and perfused with the same buffer as was used for uptake with additions of 0.1% bovine serum albumin, nomifensine (1 μ M) to block the reuptake of released DA, and atropine (1 μ M) to block muscarinic receptors. Samples were perfused with buffer (0.7 ml/ min) for 10 min before fraction collection started. Fractions were collected every 10 s (~0.1 ml) using a Gilson FC204 fraction collector adapted for a 96-well plate. Radioactivity in each fraction was measured using a 1450 MicroBeta Trilux scintillation counter (Perkin Elmer).

To assess only $\alpha 4\beta 2^*$ -mediated DA release, α -CtxMII (50 nM) (synthesized at University of Utah by the methods of Cartier et al. 1996) was used to block activity of $\alpha 6\beta 2^*$ -nAChRs and was added to the perfusion buffer for 5 min before stimulation by agonists. The time of exposure and concentration used were chosen to produce maximal inhibition (Salminen et al. 2004).

Data analysis

Fractions obtained before agonist application and after return to basal levels were used to estimate baseline release by fitting the data to a single-phase exponential decay. Data obtained following stimulation were divided by the calculated baseline to generate “response units” (one unit is twice baseline) and fractions in the evoked peak area above 0.1 units were summed. Concentration–effect data were plotted as units released vs concentration of agonist and fit (by SigmaPlot DOS or SigmaPlot 8.0) to the single-site two-parameter hyperbolic equation $f = VS/(K + S)$ or to the two-site four-parameter hyperbolic equation $f =$

$VS/(K + S) + cS/(K + S)$, where S is agonist concentration, V and v are maximum response for high and low-sensitivity components, respectively, and half-maximal agonists concentrations for high- and low-sensitivity components are represented by K and k , respectively.

Results

Figure 1 depicts the results of experiments that determined the effects of a broad range (0.1 μ M to 3 mM, 20 s exposures) of ACh on the α -CtxMII-resistant component of DA release. The concentration–effect curve for ACh-induced DA release is of the inverted U type with maximal release (24.6 ± 2.7 units) produced by stimulation with 300 μ M. When higher concentrations were tested, reduced release was seen. A single site model (maximum release = 22.7 ± 0.79 units, $EC_{50} = 0.79 \pm 0.08$ μ M) provides the best fit for the data obtained with wild-type mice when the results obtained using the ascending limb of the concentration–effect curves were analyzed. ACh also elicited a concentration-dependent increase in DA release from synaptosomes obtained from $\alpha 5$ null mutants. The concentration–effect curve was also of the inverted U type; the maximal effect (15.0 ± 3.6 units) was produced by the 300- μ M concentration. A two-site model provided the best fit of the null mutant data when ascending limb results were analyzed. Curve fit parameters are as follows: HS maximum release = 4.82 ± 0.28 units, $EC_{50} = 0.35 \pm 0.05$ μ M; LS maximum release = 8.20 ± 0.25 , and $EC_{50} = 18.8 \pm 2.1$ μ M. The $\alpha 5$ wild-type EC_{50} value (0.79 ± 0.08 μ M) was significantly higher than the EC_{50} value for the $\alpha 5$ null mutant HS component (0.35 ± 0.05 μ M) ($P < 0.001$). Deleting the $\alpha 5$ subunit also produced a significant ($P < 0.001$) decrease in total maximal release.

Measuring the LS component of the DA release process is complicated by the inverted U concentration–effect curves. Receptor desensitization should become more important at high concentrations; this might explain the decrease in release seen at high concentrations. Consequently, the experiment was repeated with the modification that agonist exposure was limited to 5 s. Figure 2 shows the results of experiments using the shorter (5 s) exposure to ACh. The curves still exhibit a strong inverted U shape above 100 μ M ACh, but both wild-type and $\alpha 5$ null mutant data are best fit by a two-site model. As expected, the amount of release produced by ACh is less than that elicited by a 20-s exposure, and the EC_{50} values are shifted to higher values (see Table 1). The results of the biphasic curve fit indicate that $\alpha 5$ deletion results in a significant decrease in HS maximal release ($P < 0.05$); the LS component is not significantly changed.

An alternate method that has been used to resolve HS and LS activity in $^{86}\text{Rb}^+$ efflux assays is the selective antagonism of HS activity using DH β E with IC_{50} values of ~ 0.3 and 10 μ M, respectively (Marks et al. 1999). For this approach, we perfused the synaptosomes with a concentration of DH β E (1 μ M) that blocks most of the HS form, with minimal effect on the LS form. The agonist exposure period used was short enough so that the DH β E block of the HS form(s) is not reversed. The results of these experiments are shown in Fig. 3. Panel a presents the HS activity measured in the absence of DH β E with the activity measured in the presence of DH β E subtracted. Panel b shows the activity remaining in the presence of DH β E. Note that, at 3 μ M ACh, there is no DH β E-resistant (LS) activity; consequently, only

the higher concentrations of ACh in Fig. 3a are corrected for LS activity. All of data depicted in Fig. 3 were best fit by a single site. Table 1 presents the EC₅₀ and maximal release values calculated from these data. Deleting the $\alpha 5$ subunit produced significant ($P < 0.001$) decreases in maximal release for both the HS and LS components as determined by *t* test.

Discussion

The results of the experiments reported here replicate the finding that $\alpha 5$ gene deletion results in a decrease in maximal ACh-stimulated dopamine release (Salminen et al. 2004). They also provide the first evidence that the α -CtxMII-resistant component of agonist-induced dopamine release is mediated by both the HS and LS stoichiometric forms of $\alpha 4\beta 2$ -nAChR. We have not seen biphasic agonist-induced DA release in any of our earlier studies, the first dating back to 1992 (Grady et al. 1992), and we did not detect biphasic activity in the experiments reported in Fig. 1 in wild-type mice. However, biphasic curves better fit the data from $\alpha 5$ null mutant mice. In addition, when the stimulation time was reduced from 20 to 5 s, a biphasic response was seen with both wild-type and $\alpha 5$ null mutant striatal synaptosomes (Fig. 3). The finding that DH β E-sensitive and DH β E-resistant components of ACh-induced DA release can be detected and that both are changed by $\alpha 5$ deletion provides added support for the assertion that the α -CtxMII-resistant component of ACh-stimulated DA release is mediated by three nAChR subtypes: $\alpha 4\alpha 5\beta 2$ and both the $(\alpha 4)_2(\beta 2)_3$ (HS) and $(\alpha 4)_3(\beta 2)_2$ (LS) forms of $\alpha 4\beta 2$ -type receptors.

The conclusion that $\alpha 4\alpha 5\beta 2$, $(\alpha 4)_2(\beta 2)_3$ (HS), and $(\alpha 4)_3(\beta 2)_2$ (LS) are found in mouse striatal synaptosomes is totally consistent with conclusions drawn by Brown et al. (2007) from a study that measured ACh-stimulated $^{86}\text{Rb}^+$ efflux. The concentration–effect curves for agonist-stimulated $^{86}\text{Rb}^+$ efflux are biphasic in both wild-type and $\alpha 5$ null mutant mice and $\alpha 5$ gene deletion results in a significant decrease in maximal agonist-induced ion flux for the HS component of the $\alpha 4\beta 2$ -mediated response. The concentration–effect curves for ACh-stimulated [^3H]-GABA release from mouse striatal synaptosomes are also biphasic (McClure-Begley et al. 2009). GABA release is eliminated by both $\alpha 4$ and $\beta 2$ gene deletion, and the HS component of the response is reduced by $\alpha 5$ null mutation. Thus, three studies using three different assays, have yielded results that indicate that mouse striatum expresses $\alpha 4\alpha 5\beta 2$, $(\alpha 4)_2(\beta 2)_3$ (HS), and $(\alpha 4)_3(\beta 2)_2$ (LS) nAChRs and that adding or deleting the $\alpha 5$ subunit results in changes in maximal activity of the HS component of the $\alpha 4\beta 2$ -mediated response, indicating that $\alpha 4\alpha 5\beta 2$ is an additional HS form at synaptosomal sites. The effects of $\alpha 5$ on agonist efficacy are consistent with those reported for $\alpha 5$ in expression system studies (Gerzanich et al. 1998; Kuryatov et al. 2008).

We are reasonably confident that $\alpha 5$ gene deletion also elicits a decrease in the LS component of ACh-stimulated DA release. This result is somewhat unexpected, however, because $\alpha 5$ gene deletion does not affect the LS component of either ACh-stimulated $^{86}\text{Rb}^+$ efflux (Brown et al. 2007) or [^3H]-GABA release (McClure-Begley et al. 2009). We cannot explain this result based on changes in receptor binding because null mutation of the $\alpha 5$ gene does not result in detectable changes in a component of [^{125}I]-epibatidine binding (Brown et al. 2007) that absolutely requires $\alpha 4$ and $\beta 2$ subunits (Marks et al. 2007), or in

[¹²⁵I]-α-CtxMII binding that absolutely requires β2 subunits and partially requires α4 subunits (Salminen et al. 2005). The [¹²⁵I]-epibatidine binding assay does not, however, discriminate between the HS- and LS-type α4β2 or α4α5β2-nAChRs (Marks et al. 2007). Thus, it appears that α5 gene deletion results in changes in activity of HS and LS nAChRs, at dopaminergic terminals and is perhaps influenced by the increase in α6β2*-nAChR activity in the α5 null mutant (Salminen et al. 2004).

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Figure 1.

Extended concentration response curve for ACh. Striatal synaptosomes from wild-type ($n=7$) and $\alpha 5$ null mutant mice ($n=7$) were assayed for α -CtxMII-resistant ($\alpha 4\beta 2^*$ -nAChR) DA release by 20 s exposures to ACh (0.1–1,000 μ M). Single-site curve fits are shown for both data sets, and, in addition, a biphasic fit is shown for the null mutant

Figure 2.

Extended concentration response curve for ACh with 5 s exposure time. Striatal synaptosomes from wild-type and $\alpha 5$ null mutant mice ($n=3$ each genotype) were assayed for α -CtxMII-resistant ($\alpha 4\beta 2^*$ -nAChR) DA release by 5 s exposures to ACh (0.1–3,000 μ M). Monophasic and biphasic curve fits are shown for both data sets. The biphasic curves better fit the data for both genotypes. Curve fit parameters are given in Table 1

Figure 3.

Resolution of HS and LS activity by DH β E. Striatal synaptosomes from wild-type and $\alpha 5$ null mutant mice ($n=6$ for both genotypes) were assayed for HS and LS activity by use of the selective antagonist DH β E. HS activity is shown in a, LS activity in b. For details of protocol see “Results.” All data were fit to a single-site equation; parameters are in Table 1

Table 1Effects of $\alpha 5$ gene deletion on parameters of ACh-stimulated dopamine release

	Wild-type maximum release (units)	Wild-type EC ₅₀ (μ M)	$\alpha 5$ null Maximum release (units)	$\alpha 5$ null EC ₅₀ (μ M)
HS biphasic fit	9.7 $\pm$ 1.5	2.02 $\pm$ 0.49	2.8 $\pm$ 0.7 *	0.78 $\pm$ 0.45
HS DH β E-sensitive	13.8 $\pm$ 0.4	2.20 $\pm$ 0.27	5.3 $\pm$ 0.2 **	2.99 $\pm$ 0.48
LS biphasic fit	16.5 $\pm$ 2.0	74.1 $\pm$ 39.6	12.0 $\pm$ 2.3	82.2 $\pm$ 45.8
LS DH β E-resistant	11.0 $\pm$ 0.8	142 $\pm$ 41	4.8 $\pm$ 0.2 **	148 $\pm$ 23

* $P < 0.05$, significantly different from wild type** $P < 0.001$, significantly different from wild type