Cellular/Molecular

Nicotinic Cholinergic Receptors in the Rat Cerebellum: Multiple Heteromeric Subtypes

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Nicotinic receptors (nAChRs) in the cerebellum have been implicated in the pathology of autism spectrum disorders (Lee et al., 2002; Martin-Ruiz et al., 2004). The subtypes of nAChRs in the cerebellum are not known in any detail, except that, in addition to the homomeric $\alpha7$ subtype, there appears to be one or more heteromeric subtypes consisting of combinations of α and β subunits. To begin to better understand the potential roles of these heteromeric nAChRs in cerebellar circuitry and their potential as targets for nicotinic drugs, we investigated their subunit composition. Using subunit-selective antibodies in sequential immunoprecipitation assays, we detected six structurally distinct heteromeric nAChR populations in the rat cerebellum. Among these were several subtypes that have not been encountered previously, including $\alpha3\alpha4\beta2$ and $\alpha3\alpha4\beta4$ nAChRs. This diversity suggests that nAChRs play multiple roles in cerebellar physiology.

Key words: **nicotinic receptor; acetylcholine; cerebellum; rat; receptor; antibody**

Introduction

Neuronal nicotinic cholinergic receptors (nAChRs) are crucial to acetylcholine neurotransmission in both the CNS and autonomic nervous system. They mediate the fast excitatory signaling found in virtually all autonomic and sensory ganglia. In the CNS, however, these receptors are more often associated with modulation of release of several neurotransmitters including dopamine, norepinephrine, GABA, and glutamate (Wonnacott, 1997; Girod and Role, 2001). nAChRs have been implicated in the pathology and/or treatment of several neurological disorders including Alzheimer's disease, Parkinson's disease, Tourette's syndrome (for review, see Lindstrom, 1997), nicotine addiction (Mansvelder and McGehee, 2002), and recently, in autism disorders (Perry et al., 2001; Lee et al., 2002; Granon et al., 2003).

nAChRs are comprised of α and β subunits that form a pentameric structure surrounding an ion channel. After activation by acetylcholine, the channel opens, allowing passage of sodium and calcium ions into the cell and potassium ions out of the cell. These receptors exist as subtypes based on their subunit compositions. Nine α subunits ($\alpha2$–$\alpha10$) and three β subunits ($\beta2$–$\beta4$) are expressed in vertebrate systems and are relatively well conserved across most species (the $\alpha8$ subunit is an exception). The most frequently encountered nAChRs in the CNS are the heteromeric $\alpha4\beta2^*$ subtype and the homomeric $\alpha7$ subtype, whereas in the autonomic nervous system, the $\alpha3\beta4^*$ subtype is thought to predominate (the * is used to indicate that other, unidentified, subunits may also be incorporated in the receptor). However, other subtypes also play crucial roles in the nervous system. For example, receptors containing $\alpha6$ and $\beta3$ subunits appear to mediate a significant fraction of nicotine-stimulated dopamine and norepinephrine release (Champtiaux et al., 2002; Cui et al., 2003).

In the cerebellum, nAChRs mediate the release of glutamate (Reno et al., 2004), GABA (De Filippi et al., 2001; Rossi et al., 2003), and norepinephrine (O'Leary and Leslie, 2003). Thus, these receptors may significantly influence activity within the cerebellar circuitry, and dysregulation of this activity could contribute to developmental disorders involving the cerebellum. For example, aberrations in the relative distributions of cerebellar nAChRs have been described in autism (Court et al., 2000; Lee et al., 2002; Martin-Ruiz et al., 2004), suggesting that these receptors may play a role in this developmental disorder and/or that they may be potential therapeutic targets.

The subtypes of nAChRs in the cerebellum are not known in any detail except that, in addition to the homomeric $\alpha7$ subtype, there appears to be one or more heteromeric subtypes, consisting of α and β subunits. To begin to better understand the potential roles of these heteromeric nAChRs in cerebellar circuitry, we investigated their subunit composition in the rat cerebellum. To do this, we used subunit-selective antibodies in sequential immunoprecipitation assays. We detected six structurally distinct heteromeric nAChR populations in the rat cerebellum, including several subtypes that have not been encountered previously.

Materials and Methods

Materials. Frozen brains from Sprague Dawley rats (~250 g) were purchased from Zivic Miller Laboratories (Portersville, PA). [^{3}H]Epibatidine ([^{3}H]EB) and [^{125}I]5-iodo-3(2(*S*)-azetidinylmethoxy)pyridine ([^{125}I]A-85380) were obtained from PerkinElmer (Boston, MA). Dihydro-β-erythroidine (DHβE) was from Research Biochemicals International (Natick, MA). Nicotine tartrate, cytisine, A-85380, and other chemicals were purchased from Sigma (St. Louis, MO), unless otherwise

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noted. Rabbit antisera directed at a bacterially expressed fusion protein containing partial sequences of the cytoplasmic domains of nAChR α2, α4, α5, β3, and β4 subunits were kind gifts from Drs. Scott Rogers and Lorise Gahring (University of Utah, Salt Lake City, UT). These antisera have been described previously (Flores et al., 1992; Rogers et al., 1992). An antibody directed at a peptide sequence of the rat nAChR α3 subunit was affinity purified from rabbit serum. This antibody has been described previously (Yeh et al., 2001). A monoclonal antibody (mAb 270) to the chick β2 subunit was made from hybridoma stocks (American Type Culture Collection, Manassas, VA). This mAb was originally developed and characterized by Whiting and Lindstrom (1987). Protein G-Sepharose beads were purchased from Amersham Biosciences (Piscataway, NJ). Protein A (Pansorbin) and normal rabbit serum (NRS) were purchased from Calbiochem (La Jolla, CA). For simplicity, in this paper, we use the term antibody to refer to unpurified antisera, as well as to affinity-purified antiserum and monoclonal antibody.

Receptor binding. Tissues were homogenized in 50 mM Tris HCl buffer, pH 7.4 at 24°C, and centrifuged twice at 35,000 × *g* for 10 min in fresh buffer. The membrane pellets were resuspended in fresh buffer and added to tubes containing [^{3}H]EB or [^{125}I]A-85380 with or without competing drugs. Incubations were performed in Tris buffer at pH 7.4 for 2 h at 24°C with [^{3}H]EB and [^{125}I]A-85380. Bound receptors were separated from free ligand by vacuum filtration over GF/C glass-fiber filters (Brandel, Gaithersburg, MD) that were prewet with 0.5% polyethyleneimine, and the filters were then counted in a liquid scintillation counter. Nonspecific binding was determined in the presence of 300 μM nicotine, and specific binding was defined as the difference between total binding and nonspecific binding.

mRNA measurements. Total cellular RNA was isolated using RNA-STAT-60 (Tel-Test B, Friendswood, TX). DNA templates for antisense riboprobes were prepared as described previously (Xiao et al., 1998). Antisense riboprobes for the α2–α7 and β2–β4 nAChR subunits were generated from DNA templates using T7 RNA polymerase and [α-^{32}P]CTP. The RNase protection assays were performed using the RPA II kit (Ambion, Austin, TX). Total RNA (20 μg) from the tissue samples was hybridized overnight at 42°C with the subunit riboprobes and a riboprobe for rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was used as an internal and loading control. After hybridization, nonprotected fragments were digested with a combination of RNase A and RNase T1 for 30 min at 37°C. The numbers of bases of the full-length probes and the protected fragments of the probe were as follows: α2, 416 and 332; α3, 306 and 230; α4, 496 and 408; α5, 411 and 380; α6, 462 and 396; α7, 450 and 376; β2, 322 and 263; β3, 430 and 394; β4, 252 and 170; and GAPDH, 204 and 135. The protected probe fragments were separated by electrophoresis on a 6% denaturing polyacrylamide gel, and the fragments were visualized on x-ray film or with a phosphorimager.

Immunoprecipitation. Tissue membrane homogenates were prepared as above for binding assays. The receptors were solubilized by incubating the homogenates in 2% Triton X-100 with gentle rotation for 2 h at room temperature. After centrifuging the mixture at 35,000 × *g* for 10 min, aliquots of the clear supernatant (equivalent to 9 mg of original tissue weight) were added to sample tubes containing [^{3}H]EB and either one of the subunit-specific antibodies at a concentration determined in preliminary studies to be optimal for each or an equivalent volume of NRS. The samples were then rotated overnight at 4°C. After the addition of 50 μl of a 50% slurry of protein G-Sepharose beads or a 12% slurry of Pansorbin (source of protein A), the rotation of the samples at 4°C was continued for 1 h. The samples were then centrifuged at ~7000 × *g* for 5 min, and the supernatants were removed and placed on ice for later use in sequential immunoprecipitation studies. The remaining tissue pellets were washed once with 1 ml of 50 mM Tris HCl buffer, pH 7.0, dissolved in 1N NaOH, and then counted in a scintillation counter. After subtracting the number of counts precipitated in tubes containing NRS, the number of [^{3}H]EB-labeled nAChRs immunoprecipitated by each antibody was compared with the total number of labeled receptors, as measured in samples of the solubilized cerebellar membranes before addition of the antibodies.

Sequential immunoprecipitation assays. To determine associations between subunits, we used a sequential immunoprecipitation assay. This or conceptually similar methods have been used previously to determine the predominant nAChR subtypes in several other neuronal tissues including chick ciliary ganglia, retina, and brain (Conroy et al., 1992; Vernallis et al., 1993; Conroy and Berg, 1998; Vailati et al., 2003), rat brain (Flores et al., 1992; Zoli et al., 2002), rat trigeminal ganglia (Flores et al., 1996), rat pineal gland (Hernandez et al., 2004), and most recently, the rat retina (Moretti et al., 2004; Marritt et al., 2005). Thus, the sequential immunoprecipitation approach to determine subunit composition has been found to be useful in a large number of tissues, but each tissue may present its own specific challenges, depending on the number of receptor subunits and subtypes present and their relative abundance.

In this assay, the clear supernatant remaining after immunoprecipitation with the first antibody or NRS was incubated with a different subunit-selective antibody, and the immunoprecipitation steps with protein G or protein A were then repeated, as described above. The rationale for this procedure is that if two subunits are associated in a nAChR, antibodies to either subunit will immunoprecipitate that receptor and the resultant supernatant will contain fewer receptors to be immunoprecipitated by the antibody directed at the second subunit. In control studies, the number of solubilized nAChRs measured with [^{3}H]EB was stable when incubated in the absence of an antibody over the time course of the sequential immunoprecipitation procedure.

Data analysis. Binding data were fit to one- and two-site models using the GraphPad Prism 4.0 software package (GraphPad Software, San Diego, CA). A one-sample *t* test was used in drug binding competition assays to determine whether the Hill coefficients were different from 1 and in immunoprecipitation assays to determine whether residual values were different from 0. The propagation of error method (Bevington, 1969) was used to calculate the SEM for the difference between groups and for comparing the sum of subtypes to the total number of nAChRs in the cerebellum. Statistical analyses of the differences between groups were assessed using one-way ANOVA followed by Bonferroni's multiple comparison test.

Results

Binding of [^{3}H]EB and [^{125}I]A-85380 to nicotinic receptors in the rat and human cerebellum

Saturation binding experiments using [^{3}H]EB and [^{125}I]A-85380 were performed with cerebellum homogenates. Epibatidine is a broad-spectrum nAChR ligand that binds with very high affinity (25–300 pM) to all known heteromeric nAChRs (Houghtling et al., 1995; Parker et al., 1998; Xiao and Kellar, 2004). In contrast, [^{125}I]A-85380 binds selectively to β2-containing nAChRs (Mukhin et al., 2000; Xiao and Kellar, 2004). Therefore, examination of the nAChR binding sites labeled by these two ligands helps to distinguish between the nAChRs that contain β2 subunits from the total population of receptors (i.e., those that that contain β2 and/or β4 subunits). As shown in Figure 1, the density of cerebellar nAChR binding sites measured with [^{125}I]A-85380 is ~67% of the density measured with [^{3}H]EB. These data suggest that approximately two-thirds of the nAChR binding sites in the cerebellum contain β2 subunits (those labeled by both [^{3}H]EB and [^{125}I]A-85380), and one-third contain β4 subunits (those labeled by [^{3}H]EB only).

The inset to Figure 1 shows the relative binding of a single saturating concentration of [^{3}H]EB and [^{125}I]A-85380 in both the rat and human cerebellum. These data indicate that the overall nAChR density is similar in the rat and human cerebellum and also suggest that [^{3}H]EB binds more sites than [^{125}I]A-85380.

Drug competition for [^{3}H]EB binding sites in the cerebellum

In binding competition studies, we used several ligands that can differentiate between nicotinic receptor subtypes that contain β2 and β4 subunits, including A-85380 and I-A-85380. All of the ligands examined here competed for the nAChRs labeled by [^{3}H]EB in the cerebellum (Fig. 2). In all cases, the competition

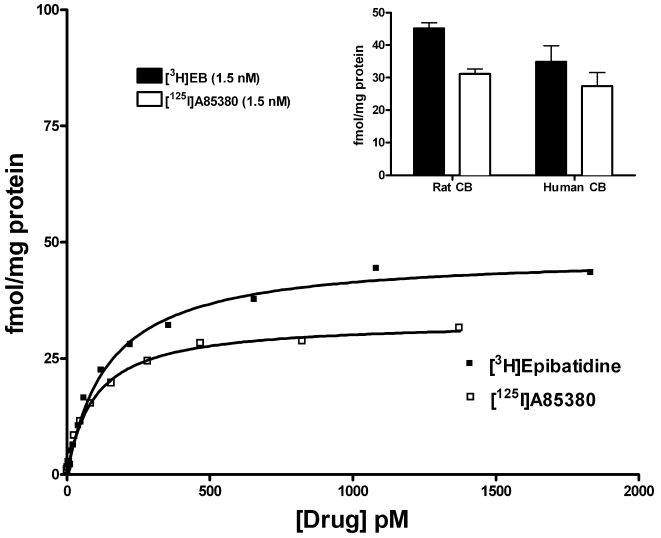

Figure 1. Binding of [^{3}H]EB and [^{125}I]A-85380 to membrane homogenates from rat and human cerebellum. Representative saturation binding curves of [^{3}H]EB and [^{125}I]A-85380 binding to membrane homogenates of rat cerebellum are shown. The B_{max} values for [^{3}H]EB and [^{125}I]A-85380 are 45 ± 2 and 30 ± 1 fmol/mg protein, respectively. The K_d values for [^{3}H]EB and [^{125}I]A-85380 are 119 ± 21 and 96 ± 42 pM, respectively ($n = 3$). Inset, Specific binding of [^{3}H]EB and [^{125}I]A-85380 at a single saturating concentration to membrane homogenates of rat and human cerebellum (CB) to demonstrate the relative densities of the total nAChR population (measured with [^{3}H]EB) and nAChRs containing β2 subunits (measured with [^{125}I]A-85380). Values are the mean ± SEM from four human and four rat samples.

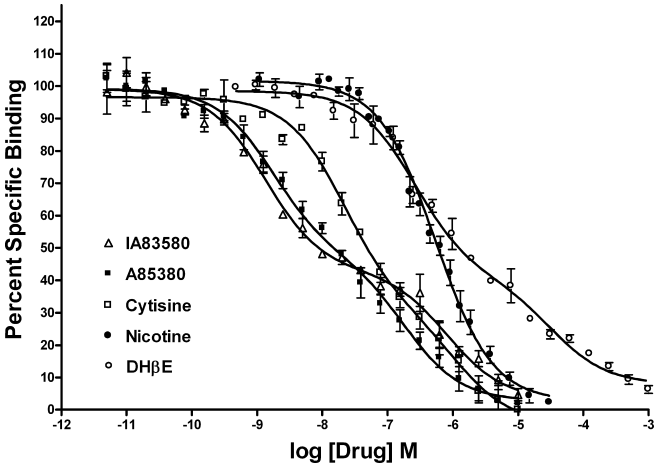

Figure 2. Competition by drugs for [^{3}H]EB-labeled binding sites in membrane homogenates from rat cerebellum. The membrane homogenates were incubated with 1 nM [^{3}H]EB, and the competing drugs were added at the concentrations indicated. All curves were fit best by a two-site binding model. Data shown are the mean ± SEM from three or four experiments. The K_i values, Hill slopes, and fraction of the high-affinity site are provided in Table 1.

curves were shallow (Hill slopes, <1; $p < 0.02$), suggesting the presence of at least two classes of binding sites based on their affinities. Indeed, as summarized in Table 1, the curves fit best to a model for two classes of binding sites with ~60% of the sites in the high-affinity class and 40% in the lower-affinity class. Together, these data indicate that the rat cerebellum contains at least two classes of nAChR binding sites, consistent with the presence of more than one receptor subtype.

Nicotinic receptor subunit expression in the rat cerebellum

We used RNase protection assays to detect the presence of nAChR subunit mRNA in the rat cerebellum. Of the nine nAChR

Table 1. Binding parameters at cerebellar nAChRs for the drugs shown in Figure 2

Ligand	K_i1 (nM)	K_i2 (nM)	Hill slope	Fraction of K_i1	*n*
I-A-85380	0.06	120	0.39 ± 0.05	61 ± 3%	3
A-85380	0.07	24	0.47 ± 0.08	49 ± 5%	3
Cytisine	0.95	240	0.55 ± 0.08	69 ± 5%	4
Nicotine	8	363	0.83 ± 0.02	62 ± 27%	4
DHβE	10	5700	0.41 ± 0.03	63 ± 3%	4

K_i1, K_i for high-affinity site; K_i2, K_i for low-affinity site.

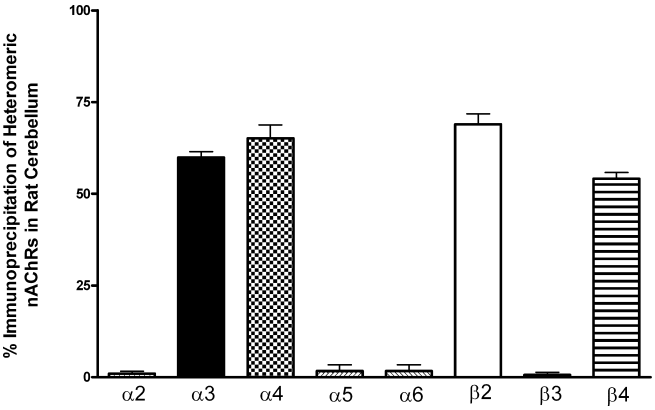

Figure 3. nAChR subunits detected by immunoprecipitation in the rat cerebellum. Rat cerebellum nAChRs were solubilized, labeled with [^{3}H]EB, and incubated with each of the antibodies shown. Nonspecific immunoprecipitation was measured with normal rabbit serum and has been subtracted. Only the antibodies to the α3, α4, β2, and β4 subunits immunoprecipitated [^{3}H]EB-labeled nAChRs from the rat cerebellum, indicating that all heteromeric nAChRs in the cerebellum are composed of combinations of these subunits. Data shown are mean ± SEM of 3–13 independent experiments.

subunits (α2–α7 and β2–β4) examined using a multiplex assay, mRNA was found for only the α3, α4, and β2 subunits (supplemental material, available at www.jneurosci.org). Previous studies have detected these subunits in the rat cerebellum (Winzer-Serhan and Leslie, 1997; Nakayama et al., 1998; Zhang et al., 1998), and, in addition, the β4 subunit was detected by *in situ* hybridization (Winzer-Serhan and Leslie, 1997). Although we did not detect the β4 subunit mRNA transcripts in the cerebellum in our protection assays, we did detect them in parallel studies with the rat pineal gland and PC12 cells (data not shown). It is possible that the level of β4 mRNA within the cerebellum is below the level of detection in our assay. In this regard, other nAChR subunits may also be expressed in low amounts, below our limits of detection.

Immunoprecipitation of nAChRs in the cerebellum

Because nAChR subtypes are defined by their subunit composition, we used highly selective antibodies directed at specific nAChR subunits to immunoprecipitate [^{3}H]EB-labeled receptors solubilized from cerebellum homogenates.

Although we detected mRNA transcripts only for α3, α4, and β2 subunits in the cerebellum, nAChRs might also be on axons originating outside of the cerebellum; therefore, we tested for the presence of nAChRs containing α2–α6 subunits and β2–β4 subunits in our immunoprecipitation assays. The results indicate that the adult rat cerebellum expresses receptors containing α3, α4, β2, and β4 subunits, but any receptors containing α2, α5, α6, or β3 subunits were undetectable (Fig. 3). The α3, α4, β2, and β4 subunits often form two major classes of subtypes, as defined primarily by their pharmacological characteristics: $\alpha3\beta4^*$ and

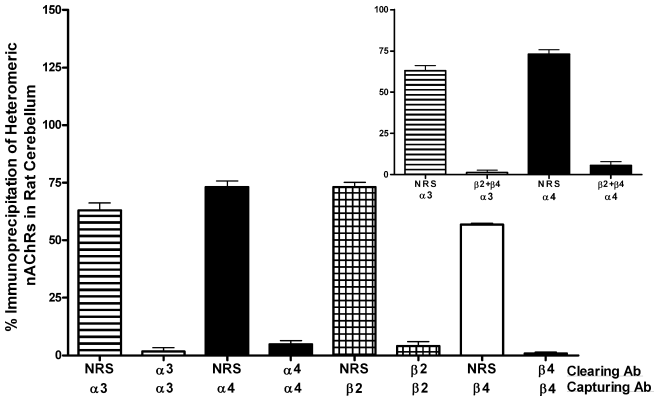

Figure 4. Completeness of immunoprecipitation of nAChRs in rat cerebellum. Sequential immunoprecipitation of cerebellar nAChRs with the antibodies shown was performed using the same antibody in the first (clearing) and the second (capturing) immunoprecipitation. Comparisons were made to samples in which the first immunoprecipitation was performed with NRS followed by immunoprecipitation with each of the antibodies. The bars represent the percentage of the total number of [^{3}H]EB-labeled nAChRs in the cerebellum immunoprecipitated by the capturing antibody and are the mean ± SEM from three or four experiments. This study demonstrates that the concentrations of antibodies used immunoprecipitated essentially all of the nAChRs that contain the cognate subunit. Inset, To test the assumption that all nAChRs contain β2, β4, or both subunits, cerebellum tissue samples were first immunoprecipitated (cleared) with antibodies to both the β2 and β4 subunits in the same test tube, and the resulting supernatant was then subjected to a second immunoprecipitation with a capturing antibody directed at either the α3 or α4 subunit. As shown, the initial immunoprecipitation with the two β subunit antibodies cleared virtually all nAChRs in the sample, demonstrating that all heteromeric nAChRs contain β2 and/or β4 subunits.

$\alpha4\beta2^*$. However, as shown in Figure 3, our immunoprecipitation studies indicated that the percentage of α3- and α4-containing nAChRs (60 and 65%, respectively) and the percentage of β2- and β4-containing nAChRs (70 and 55%, respectively) each exceed 100% of the total number of nAChRs in the cerebellum ($p < 0.01$). This suggests that the cerebellum contains one or more mixed heteromeric receptor subtypes (i.e., receptors containing the two different α and/or the two different β subunits associated in one complex). Sequential immunoprecipitation assays provide a direct method for examining subunit composition.

Basis for sequential immunoprecipitation method

This procedure is based on the observation that [^{3}H]EB binds to assembled α and β subunit combinations, which represent potential heteromeric nAChRs, but it does not bind to α subunits that are not associated with a β subunit partner or vice versa (Xiao et al., 1998; Xiao and Kellar, 2004). Therefore, once the presence of a particular nAChR subunit is established with an antibody in the first immunoprecipitation assay, the subunit(s) it is associated with can be determined by performing a second immunoprecipitation on the resultant supernatant with a different antibody. The rationale for this sequential procedure is that if two (or more) different subunits are part of the same receptor, then initial immunoprecipitation of that receptor with an antibody directed at one subunit (the clearing antibody) will decrease the amount of the receptor available in the remaining supernatant of the sample for immunoprecipitation with a subsequent antibody (the capturing antibody) directed at the second subunit (Flores et al., 1992; Hernandez et al., 2004). Sequential immunoprecipitation can thus demonstrate the direct association of two nAChR subunits.

To test whether the antibodies we used immunoprecipitated all the receptors containing their cognate subunits, we performed sequential immunoprecipitation studies using the same antibody

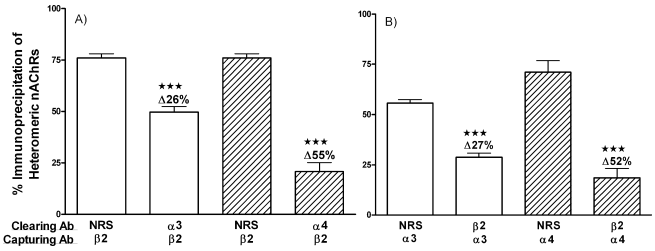

Figure 5. Sequential immunoprecipitation of nAChRs in rat cerebellum: association with β2 subunits. ***A***, Samples were first cleared with NRS as a control or antibodies directed at either the α3 or α4 subunit; the resulting supernatant was then immunoprecipitated with the antibody to the β2 subunit. ***B***, To confirm the results in ***A***, the order of antibodies was reversed. That is, after first clearing the sample with NRS (control) or antibodies to the β2 subunit, the resultant supernatant was immunoprecipitated with antibodies to the α3 or α4 subunits. The bars represent the percentage of the total number of [^{3}H]EB-labeled nAChRs immunoprecipitated by the capturing antibody and are the mean ± SEM of 7–10 experiments. The values above the bars are the percentage reduction of total cerebellar nAChRs resulting from clearing with the indicated antibody compared with the corresponding value after clearing with NRS. Different from corresponding NRS control, $^{***}p < 0.001$.

in both the clearing and capturing steps (e.g., β2 followed by β2) and compared the results to parallel studies in which NRS instead of the specific antibody was used in the clearing step. Incubation of cerebellar extracts with NRS in the clearing step did not affect the immunoprecipitation by any of the antibodies, and each immunoprecipitated a similar percentage of nAChRs as it had in the single immunoprecipitation studies (compare Figs. 3, 4). In contrast, when each antibody was used in the clearing step, it removed nearly all of the nAChRs containing its cognate subunit, so there were essentially no nAChRs containing that subunit remaining in the supernatant to be immunoprecipitated by the same antibody in the capturing step (Fig. 4).

All heteromeric nAChRs are assumed to contain either β2 or β4 subunits or both. We tested this assumption as well as the efficacy of the sequential immunoprecipitation procedure by incubating cerebellar extracts with both the β2 and β4 antibodies simultaneously in the clearing immunoprecipitation. The sequential method predicts that the second round of immunoprecipitation, performed on the resulting supernatant with a capturing antibody directed toward an α subunit, would find no nAChRs remaining in that supernatant. The results from this series of experiments are shown in the inset to Figure 4. After an initial incubation with NRS, the α3 and α4 antibodies immunoprecipitated ~60 and 70%, respectively, of the nAChRs in the cerebellar extracts. In contrast, after concurrent exposure of the extracts to both the β2 and β4 antibodies, no significant immunoprecipitation with either the α3 or α4 antibodies was detected. These results strongly support the assumption that all heteromeric nAChRs in the cerebellum contain a β2 and/or β4 subunit and also demonstrate that the sequential immunoprecipitation method can quantitatively assess associations between different subunits.

nAChR subunit associations in the rat cerebellum

The sequential immunoprecipitation procedure was used to detect and quantify the association between subunits in nAChRs in the cerebellum. We first examined the associations between α and β subunits and then examined associations between the two α subunits and between the two β subunits.

Associations with the β2 subunit

Results from sequential immunoprecipitation studies to examine the subunit associations with the β2 subunit in the cerebellum are shown in Figure 5. Consistent with the studies shown in Figure 4,

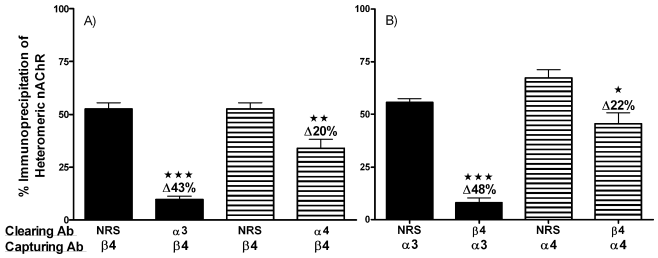

Figure 6. Sequential immunoprecipitation of nAChRs in rat cerebellum: association with $\beta 4$ subunits. ***A***, Samples were first cleared with NRS as a control or antibodies directed at either the $\alpha 3$ or $\alpha 4$ subunit; the resulting supernatant was then immunoprecipitated with the antibody to the $\beta 4$ subunit. ***B***, To confirm the results in ***A***, the order of antibodies was reversed. That is, after first clearing the sample with NRS (control) or the antibody to the $\beta 4$ subunit, the resultant supernatant was immunoprecipitated with antibodies to the $\alpha 3$ or $\alpha 4$ subunits. The bars represent the percentage of the total number of [^{3}H]EB-labeled nAChRs immunoprecipitated by the capturing antibody and are the mean ± SEM of seven to nine experiments. The values above the bars are the percentage reduction of total cerebellar nAChRs resulting from clearing with the indicated antibody compared with the corresponding value after clearing with NRS. Different from corresponding NRS control, $^*p < 0.05$, $^{**}p < 0.01$ $^{***}p < 0.001$. In addition, all of the values for the residual receptors remaining after the capturing antibodies are different from 0 ($p < 0.01$).

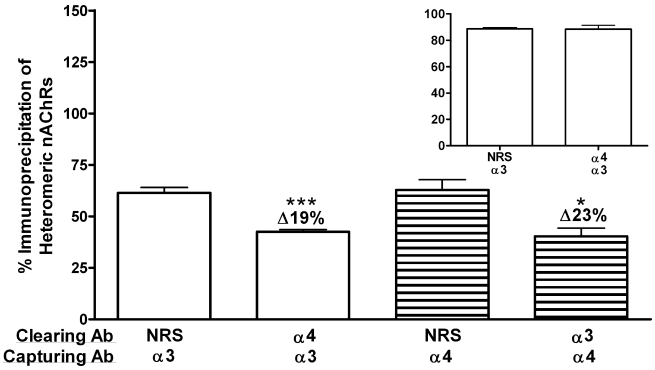

Figure 7. Sequential immunoprecipitation of nAChRs in the rat cerebellum: association of $\alpha 3$ and $\alpha 4$ subunits. After first clearing the sample with NRS as a control or the $\alpha 4$ antibody, the remaining supernatant was immunoprecipitated with the antibody to the $\alpha 3$ subunit. Inset, Clearing with the $\alpha 4$ antibody did not affect immunoprecipitation by the $\alpha 3$ antibody of nAChRs in the pineal gland, which are all $\alpha 3$-containing receptors. This experiment served as an additional control for the sequential immunoprecipitation procedure. To confirm these results, the order of antibodies was reversed. That is, after clearing the sample with NRS or the $\alpha 3$ antibody, the resulting supernatant was immunoprecipitated with the $\alpha 4$ antibody. Bars represent the percentage of the total number of [^{3}H]EB-labeled nAChRs immunoprecipitated by the capturing antibody and are the mean ± SEM of six to nine experiments in the main figure and of three experiments in the inset. The values above the bars are the percentage reduction of total cerebellar nAChRs resulting from clearing with the indicated antibody compared with the corresponding value after clearing with NRS. Different from corresponding NRS control, $^*p < 0.05$, $^{***}p < 0.001$.

antibodies to the $\beta 2$ subunit immunoprecipitated ~75% of the nAChRs in the cerebellum, and this was unaffected by clearing with either NRS (Fig. 5*A*) or with an irrelevant monoclonal antibody (data not shown). In contrast, after the antibody directed at the $\alpha 3$ subunit was used in the clearing step, the $\beta 2$ antibody immunoprecipitated only ~49% of the total heteromeric nAChR population in the cerebellum (Fig. 5*A*), indicating that ~26% of the nAChRs in the cerebellum contain $\alpha 3$ subunits in association with $\beta 2$ subunits (i.e., they are $\alpha 3\beta 2^*$ receptors). Similarly, clearing with the $\alpha 4$ antibody reduced the number of cerebellar heteromeric nAChRs immunoprecipitated with the $\beta 2$ antibody to 20% (Fig. 5*A*), indicating that ~55% are $\alpha 4\beta 2^*$ receptors.

To test the precision of the sequential immunoprecipitation method, we reversed the order of the antibodies; that is, we performed the first immunoprecipitation (the clearing step) with the $\beta 2$ antibody and the second immunoprecipitation (the capture step) with the $\alpha 3$ or $\alpha 4$ antibody. The results of these experiments are shown in Figure 5*B*. After clearing the cerebellar extracts with NRS, the $\alpha 3$ and $\alpha 4$ antibodies immunoprecipitated 56 and 71% of the nAChRs, respectively; whereas, after clearing with the $\beta 2$ antibody, the total number of cerebellar nAChRs immunoprecipitated with the $\alpha 3$ and $\alpha 4$ antibodies was decreased by 27 and 52%, respectively. The consistency between the results obtained in the studies shown in Figure 5, *A* and *B*, reinforces the degree of associations of the $\beta 2$ subunit with $\alpha 3$ and $\alpha 4$ subunits, and together, these results indicate that ~26% of the nAChRs in the cerebellum are an $\alpha 3\beta 2^*$ subtype and ~54% are an $\alpha 4\beta 2^*$ subtype.

Associations with the β4 subunit

Similar sequential studies were performed to examine the subunit associations of the $\beta 4$ subunit with the $\alpha 3$ and $\alpha 4$ subunits. As shown in Figure 6*A*, after clearing the cerebellum extracts with NRS, the $\beta 4$ antibody immunoprecipitated ~52% of the [^{3}H]EB-labeled nAChRs; in contrast, after clearing with the $\alpha 3$ antibody, the $\beta 4$ antibody immunoprecipitated only ~9% of the remaining nAChRs in the extracts. These results indicate that ~43% of the total heteromeric nAChRs in the cerebellum contain $\alpha 3$ subunits in association with $\beta 4$ subunits (i.e., they are $\alpha 3\beta 4^*$ subtypes). Likewise, clearing the cerebellar extracts with the $\alpha 4$ antibody decreased the subsequent immunoprecipitation by the $\beta 4$ antibody to ~32%, indicating that ~20% of the total heteromeric nAChRs in the cerebellum are $\alpha 4\beta 4^*$ subtypes (Fig. 6*A*).

Again, we then examined the implied subunit associations when the order of antibodies was reversed. The results of these studies are shown in Figure 6*B*. After clearing with the $\beta 4$ antibody, the number of heteromeric nAChRs in the cerebellum immunoprecipitated by the $\alpha 3$ and $\alpha 4$ antibodies was decreased by 48 and 22%, respectively. Again, the similarity of the results from the studies shown in Figure 6, *A* and *B*, reinforces the associations of the $\beta 4$ subunit with $\alpha 3$ and $\alpha 4$ subunits and indicates that ~45% of the nAChRs in the cerebellum are an $\alpha 3\beta 4^*$ subtype and ~21% are an $\alpha 4\beta 4^*$ subtype.

Together, the initial experiments shown in Figures 5 and 6 indicate the following subunits associations among the heteromeric nAChRs in the cerebellum: $\alpha 3\beta 2^*$ (~26%), $\alpha 4\beta 2^*$ (~54%), $\alpha 3\beta 4^*$ (~45%), and $\alpha 4\beta 4^*$ (~21%). The total number of these subunit associations equals 146% of the nAChRs in the cerebellum, which is statistically >100% ($p < 0.01$). This obvious discrepancy along with the single immunoprecipitation data in Figures 3 and 4 strongly suggests that the cerebellum expresses mixed heteromeric receptors composed of both α subunits and/or both β subunits. To test this directly, we examined associations between $\alpha 3$ and $\alpha 4$ subunits and $\beta 2$ and $\beta 4$ subunits.

Associations between the α3 and α4 subunits in the cerebellum

Sequential immunoprecipitation studies were performed to determine the extent of association, if any, between the $\alpha 3$ and $\alpha 4$ subunits in nAChRs in the cerebellum. As shown in Figure 7, when the cerebellum extracts were first cleared with NRS, subsequent incubation with the $\alpha 3$ antibody immunoprecipitated ~60% of the [^{3}H]EB-labeled nAChRs. But after clearing with the $\alpha 4$ antibody, the number of nAChRs subsequently immunoprecipitated by the $\alpha 3$ antibody was decreased to ~41% of the total, indicating that ~19% of the heteromeric nAChRs in the cerebellum contain both the $\alpha 3$ and $\alpha 4$ subunits. We then reversed the order of the antibodies to confirm this subunit association and

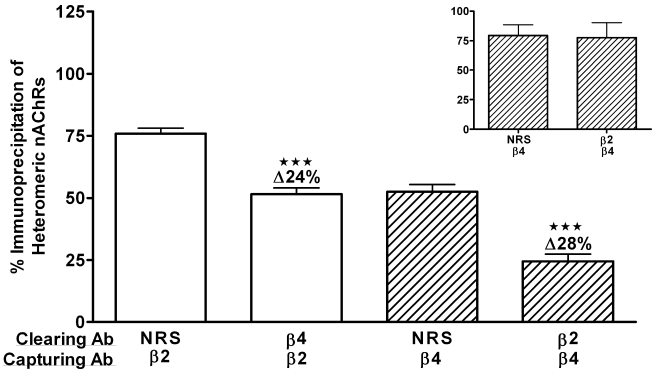

Figure 8. Sequential immunoprecipitation of nAChRs in the rat cerebellum: association of $\beta 2$ and $\beta 4$ subunits. After first clearing the sample with NRS as a control or the $\beta 4$ antibody, the remaining supernatant was immunoprecipitated with the antibody to the $\beta 2$ subunit. To confirm these results, the order of antibodies was reversed. That is, after clearing the sample with NRS or the $\beta 2$ antibody, the resulting supernatant was immunoprecipitated with the $\beta 4$ antibody. Inset, Control experiment showing that clearing with the $\beta 2$ antibody did not significantly affect immunoprecipitation by the $\beta 4$ antibody of nAChRs in the pineal gland, which are all $\beta 4$-containing receptors. The bars represent the percentage of the total number of [^{3}H]EB-labeled nAChRs immunoprecipitated by the capturing antibody and are the mean ± SEM of six to nine experiments in the main figure and of three experiments in the inset. The values above the bars are the percentage reduction of total cerebellar nAChRs resulting from clearing with the indicated antibody compared with the corresponding value after clearing with NRS. Different from corresponding NRS control, $***p < 0.001$.

test the precision of this estimate. Figure 7 shows that clearing with the $\alpha 3$ antibody decreased the number of nAChRs subsequently immunoprecipitated by the $\alpha 4$ antibody by 23% of the total. These data thus support the hypothesis that the cerebellum contains mixed heteromeric nAChRs and indicate that ~21% of the receptors contain both $\alpha 3$ and $\alpha 4$ subunits (i.e., $\alpha 3\alpha 4\beta x^*$).

All of the antibodies used here are highly selective for their cognate subunits, but as an additional control for the sequential immunoprecipitation procedure itself, we also examined the association of these two α subunits in rat pineal gland, in which virtually all of the nAChRs are the $\alpha 3\beta 4$ subtype (Hernandez et al., 2004). As shown in the inset in Figure 7, the sequential immunoprecipitation procedure did not detect an association between $\alpha 3$ and $\alpha 4$ subunits in this tissue, indicating that the association between these two subunits detected in the cerebellum is unlikely to be an artifact of the procedure.

Associations between the β2 and β4 subunits in the cerebellum

Similar studies were performed to test for an association between the $\beta 2$ and $\beta 4$ subunits in cerebellar nAChRs. Clearing with the $\beta 4$ antibody decreased the total number of cerebellar nAChRs immunoprecipitated with the $\beta 2$ antibody by ~24% (Fig. 8), whereas clearing with the $\beta 2$ antibody decreased the total number of receptors immunoprecipitated with the $\beta 4$ antibody by ~28% (Fig. 8). Again, no evidence of an association between the $\beta 2$ and $\beta 4$ subunits was found in the pineal gland (Fig. 8, inset). These data thus also support the hypothesis that the cerebellum expresses mixed heteromeric nAChRs and indicate that ~26% of those receptors contain both $\beta 2$ and $\beta 4$ subunits (i.e., $\alpha x\beta 2\beta 4^*$).

Together, the data in Figures 7 and 8 indicate that the cerebellum expresses nAChR subtypes that contain $\alpha 3$ subunits in association with $\alpha 4$ subunits, as well as subtypes that contain $\beta 2$ subunits in association with $\beta 4$ subunits. The presence of these mixed heteromeric subtypes explains how the percentage of cerebellar nAChRs immunoprecipitated with the $\alpha 3$ and $\alpha 4$ antibodies and the percentage immunoprecipitated with the $\beta 2$ and $\beta 4$ antibodies can exceed 100% of the total nAChRs in the cerebellum.

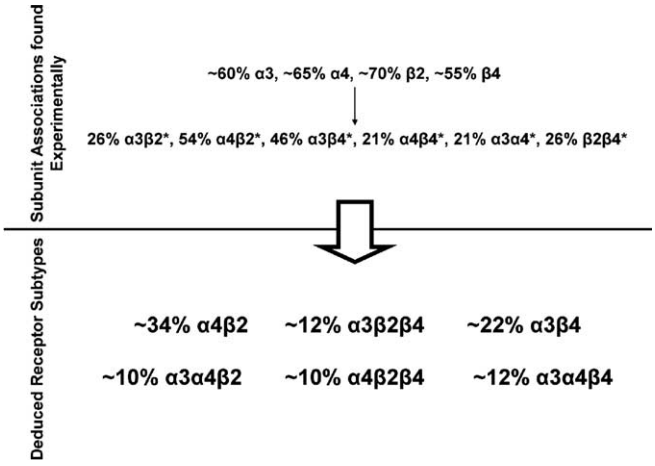

Figure 9. nAChR subunits, subunit associations, and deduced receptor subtypes in the rat cerebellum. Schematic showing experimentally derived data from single immunoprecipitations (row 1) and sequential immunoprecipitations (row 2) and the six deduced receptor subtypes present within the rat cerebellum (see Discussion).

Discussion

These studies show that the rat cerebellum expresses several subtypes of nAChRs, including mixed heteromeric subtypes that contain $\alpha 3$ subunits in association with $\alpha 4$ subunits and $\beta 2$ subunits in association with $\beta 4$ subunits. Although a previous study reported the presence of nAChRs containing $\beta 3$ subunits in association with $\beta 4$ subunits in the rat cerebellum (Forsayeth and Kobrin, 1997), we did not find evidence for the $\beta 3$ subunit mRNA or protein in our studies. It is possible that the $\beta 3$ subunit is expressed but at levels below our detection limits.

Figure 9 provides a summary of the subunit associations determined experimentally in our studies and the proposed nAChR heteromeric subtypes that can be deduced from these data. We arrived at these proposed subtype assignments as follows: the single immunoprecipitation studies (Figs. 3, 4) showed that the number of nAChR subtypes containing $\alpha 3$ and $\alpha 4$ subunits as well as the number containing $\beta 2$ and $\beta 4$ subunits were each significantly >100% of the nAChRs in the cerebellum. This indicates that some of the receptors contain both $\alpha 3$ and $\alpha 4$ subunits and some contain both $\beta 2$ and $\beta 4$ subunits. The sequential immunoprecipitation data in Figures 5 and 6 indicated that the $\alpha 3\beta 2^*$, $\alpha 4\beta 2^*$, $\alpha 3\beta 4^*$, and $\alpha 4\beta 4^*$ subunit associations together add up to ~146% of the total number of heteromeric nAChRs measured with [^{3}H]EB, again supporting the hypothesis that there are mixed heteromeric receptors in the cerebellum. Moreover, the studies that showed an association between $\alpha 3$ and $\alpha 4$ subunits (Fig. 7) and between $\beta 2$ and $\beta 4$ subunits (Fig. 8) provide direct evidence for the existence of mixed heteromeric nAChRs.

Subtypes deduced from the α3α4 subunit associations

Our results indicate that ~21% of the heteromeric nAChRs in the cerebellum contain both $\alpha 3$ and $\alpha 4$ subunits (Fig. 7). Initial immunoprecipitation with the $\alpha 3$ antibody cleared ~43% of the $\beta 4$-containing receptors but left ~10% of the receptors that could still be immunoprecipitated by the $\beta 4$ antibody (Fig. 6*A*). This 10% residual population of $\beta 4$-containing receptors presumably represents $\alpha 4\beta 4^*$ receptors that do not contain $\alpha 3$ subunits. The $\alpha 3\beta 4^*$ and $\alpha 4\beta 4^*$ receptor subtypes together appear to comprise ~67% of the measured heteromeric nAChRs in the cerebellum (Fig. 6), but the $\beta 4$ subunit was found in only ~55% of the nAChRs (Figs. 3, 4). This difference is statistically significant ($p < 0.01$) and therefore

suggests that ~12% of the β4 subunits are associated with both α3 and α4 subunits, yielding an α3α4β4* subtype. Because the α4β4 association also was found in ~21% of the receptors (Fig. 6) and ~12% can be accounted for by the α3α4β4* subtype, the remaining ~9% of the α4β4 association is consistent with the receptors designated as α4β4* with no α3 that were found as the residual in Figure 6*A*. The α3β2* and α4β2* subtypes represent ~80% of the measured nAChRs in the cerebellum (Fig. 5), but the β2 subunit was found in only ~70% of the receptors (Fig. 3). This difference is statistically significant ($p < 0.01$) and thus suggests that ~10% of the β2 subunits are associated with both α3 and α4 subunits, which would yield an α3α4β2* subtype. This subtype would then account for the remaining α3α4 subunit associations. Together, this analysis can account for virtually all of the mixed heteromeric receptors that contain both α3 and α4 subunits and indicates that these appear to be nearly equally divided between α3α4β2 and α3α4β4 subtypes.

Subtypes deduced from the β2β4 subunit associations

Our measurements indicate that ~26% of the heteromeric nAChRs in the cerebellum contain both β2 and β4 subunits (Fig. 8). These β2β4 subunit associations also appear to represent two receptor populations, because after clearing with the β4 antibody, ~10% of the nAChRs could still be immunoprecipitated by the α3 antibody (Fig. 6*B*). This population represents α3β2* receptors that do not contain β4 subunits. All of the α3β2* subtypes together represent ~26% of the heteromeric nAChRs in the cerebellum (Fig. 5), and ~10% of these also contain an α4 subunit (i.e., the α3α4β2* subtype derived above). The α3β2* and α3β4* subtypes together appear to comprise ~72% of the heteromeric nAChRs in the cerebellum (Figs. 5, 6), but the α3 subunit itself was found in only ~60% of the receptors (Figs. 3, 4). Again, this difference is statistically significant ($p < 0.01$). Together, these data suggest that ~12% of the α3 subunits associate with both β2 and β4 subunits, forming an α3β2β4 receptor subtype. The α4β2* and α4β4* subtypes together comprise ~75% of the heteromeric nAChRs in the cerebellum (Figs. 5, 6), but the α4 subunit was found in only ~65% of the receptors (Figs. 3, 4). This suggests that ~10% of the α4 subunit associates with both β2 and β4 subunits, forming an α4β2β4 receptor subtype. Thus, this analysis can account for nearly all of the mixed heteromeric subtypes that contain both β2 and β4 subunits, and these too appear to be nearly equally divided between α3β2β4 and α4β2β4 subtypes.

In these analyses, the mixed heteromeric receptors together accounted for ~44% of the total number of heteromeric nAChRs in the cerebellum; thus, ~56% would be designated as simple heteromeric subtypes. The α4β2* subtypes together comprised ~54% of the heteromeric nAChRs in the cerebellum (Fig. 5), and ~20% of the total population of nAChRs can be accounted for by the two mixed heteromeric subtypes designated as α3α4β2 and α4β2β4; therefore, ~34% of the nAChRs in the cerebellum appear to be the simple heteromeric subtype α4β2. Similarly, the α3β4* subtypes represent ~46% of the nAChRs in the cerebellum (Fig. 6), and ~24% of the total population are accounted for by the two mixed heteromeric subtypes α3α4β4 and α3β2β4; thus, ~22% of the nAChRs in the cerebellum appear to be the simple heteromeric subtype α3β4. Although the subtype containing all four subunits might exist within the cerebellum, based on analysis of residual values, it would represent a small fraction, below our level of detection.

Together, these analyses indicate that the cerebellum expresses at least six different heteromeric nAChRs, including two simple heteromeric subtypes and four mixed heteromeric subtypes. Interestingly, analysis of heteromeric nAChR subtypes in the granule cell layer of the cerebellum using autoradiography with pharmacological masks indicated that 51% of the [^{3}H]EB binding sites are an α4β2* subtype and ~49% are an α3β4* subtype (Perry et al., 2002), values that are close to the combined percentages of simple and mixed heteromeric receptors representing these subtypes found here by immunoprecipitation. Previous data demonstrated that the pharmacology of simple heteromeric nAChR binding sites reflects primarily the presence of the β2 or β4 subunit (Parker et al., 1998; Xiao and Kellar, 2004). The current data suggest that the influence of the β subunits extends to mixed heteromeric receptors that contain both α3 and α4 subunits, but these data do not allow a good assessment of the pharmacology of the nAChRs containing both β2 and β4 subunits.

The two simple heteromeric subtypes α4β2 and α3β4 appear to account for slightly more than one-half the nAChRs in the rat cerebellum. These receptors are found in abundance in other parts of the rat nervous system. In fact, the α4β2 subtype is thought to be the predominant heteromeric nAChR in the rat brain (Whiting et al., 1991; Flores et al., 1992; Perry et al., 2002), and α3β4 subtypes appear to be the predominant nAChR in several autonomic nervous system ganglia (for review, see Wang et al., 2002), as well as in the trigeminal ganglia (Flores et al., 1996) and the pineal gland (Hernandez et al., 2004). In addition, α3β4* subtypes exist in relatively high densities in several regions of rodent brain, including the medial habenula, interpeduncular nucleus, brainstem nuclei, subiculum of the hippocampus, and the cerebellum (Marks et al., 1998; Perry et al., 2002; Gahring et al., 2004). In fact, because the α3β4* subtypes represent nearly one-half of the total heteromeric nAChRs in the cerebellum, they might mediate important functions in this brain region, as they do in the medial habenula (Quick et al., 1999) and hippocampus (Clarke and Reuben, 1996; Alkondon and Albuquerque, 2002).

According to our analyses, the rat cerebellum expresses four mixed heteromeric nAChR subtypes: the α3α4β2, α3α4β4, α3β2β4, and α4β2β4, with each representing ~10–12% of the total population of nAChRs. Moreover, several other mixed heteromeric receptor subtypes have been found by similar methods in other neuronal tissues, but to our knowledge, only the α3β2β4 subtype has been found in another rat tissue, the retina (Marritt et al., 2005). Together, these studies suggest the existence of an impressive diversity of nAChR subtypes in native tissues.

The cerebellum has a rich network of intrinsic GABA and glutamate pathways essential to its major functions. In addition, it receives substantial innervation by cholinergic, noradrenergic, and serotonergic axons originating in brainstem nuclei. If different nAChR subtypes are associated with these different neurotransmitter pathways, it could provide a basis for fine-tuning the cerebellar signals and even of selectively influencing cerebellar function. For example, human cerebellum expresses these four nAChR subunits as well as the α6 subunit (Graham et al., 2002), and recent autopsy evidence points to the possible involvement of cerebellar nAChRs in developmental disorders such as autism (Lee et al., 2002; Martin-Ruiz et al., 2004). This suggests the importance of determining the nAChR subtypes and understanding their physiological roles. These receptors might then provide therapeutic targets for such disorders.

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