Heterogeneity of Nicotinic Cholinergic Receptors in Rat Superior Cervical and Nodose Ganglia

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ABSTRACT

Nicotinic cholinergic receptors (nAChRs) are present in ganglia in the peripheral nervous system. In autonomic ganglia, they are responsible for fast synaptic transmission, whereas in the sensory ganglia and sensory neurons, they may be involved in modulation of neurotransmission. The present study measured nAChRs in several rat autonomic ganglia: the superior cervical ganglia (SCG), sensory nodose ganglia, stellate ganglia, and pelvic ganglia. The densities of the heteromeric nAChRs determined by receptor binding assay in those four ganglia are 481, 45, 9, and 11 fmol/mg protein, respectively. Immunoprecipitation studies with subunit-specific antibodies showed that a majority of the nAChRs in the SCG and nodose ganglia contain the $\alpha3$ and $\beta4$ subunits, but a significant percentage of the nAChRs in these ganglia also contain $\alpha5$ and $\beta2$ subunits. A small percentage of the nAChRs in nodose ganglia also contain $\alpha2$ and $\alpha4$ subunits. Sequential immunoprecipitation assays indicated that in the SCG, all $\alpha5$ subunits are associated with $\alpha3$ and $\beta4$ subunits, forming the mixed heteromeric $\alpha3\beta4\alpha5$ subtype. A receptor composed of $\alpha3$, $\beta2$, and $\beta4$ subunits in the SCG was also detected. In rat SCG, we found the following distribution of nAChRs subtypes: 55 to 60% simple $\alpha3\beta4$ subtype, 25 to 30% $\alpha3\beta4\alpha5$ subtype, and 10 to 15% $\alpha3\beta4\beta2$ subtype. These findings indicate that the nAChRs in SCG and nodose ganglia are heterogeneous, which suggests that different receptor subtypes may play different roles in these ganglia or may be activated under different conditions.

Neuronal nicotinic cholinergic receptors (nAChRs) are ligand-gated ion channels present throughout the central and peripheral nervous systems. In the autonomic nervous system, they mediate the fast synaptic transmission crucial to the functions of both sympathetic and parasympathetic ganglia. In addition, they are found in sensory ganglia and sensory neurons (Mandelzys et al., 1990, 1994; Flores et al., 1996), in which they may modulate neurotransmission (Cooper, 2001). These receptors are pentameric structures composed of α and β subunits. Nine α ($\alpha2$–$\alpha10$) and three β ($\beta2$–$\beta4$) subunits have been identified in the vertebrate nervous system. The heteromeric nAChRs are composed of at least one type of α subunit and one type of β subunit. For example, the $\alpha3\beta4*$ and $\alpha4\beta2*$ are the predominant heteromeric subtypes in autonomic ganglia and the central nervous system, respectively. By convention, the asterisk denotes the possibility that one or more unidentified subunits are present

in those receptors. For example, although expression of $\alpha3$ and $\beta4$ subunits or $\alpha3$ and $\beta2$ subunits results in functional nAChRs in heterologous expression systems (Covernton et al., 1994; Wang et al., 1996; Sivilotti et al., 1997), the autonomic ganglia that have been examined also express the $\alpha5$ subunit (Vernallis et al., 1993; Mandelzys et al., 1994; Del Signore et al., 2004). Moreover, in chick ciliary ganglia, some nAChRs appear to contain four subunits (i.e., the $\alpha3\alpha5\beta2\beta4$ subtype) (Conroy and Berg, 1995).

The $\alpha5$ subunit does not form functional nAChRs when expressed alone or with any other α or β subunit (Ramirez-Latorre et al., 1996; Fucile et al., 1997; Gerzanich et al., 1998). In fact, it probably does not even contribute directly to an agonist binding site when expressed with β subunits because it lacks two tyrosine residues implicated in agonist binding in other α subunits (Abramson et al., 1989; Cohen et al., 1991; Tomaselli et al., 1991; Conroy et al., 1992). However, the incorporation of the $\alpha5$ subunit into heterologously expressed $\alpha3\beta2$ and $\alpha3\beta4$ receptors affects the conductance and desensitization properties of the channels, and it may also exert effects on their pharmacological properties (Wang et al., 1996; Fucile et al., 1997; Gerzanich et al., 1998).

ABBREVIATIONS: nAChR, neuronal nicotinic acetylcholine receptor; A-85380, 3–2(S)-azetidinylmethoxy)pyridine; EB, epibatidine; NRS, normal rabbit serum; SCG, superior cervical ganglia; Ab, antibody; NRS, normal rabbit serum; NGF, nerve growth factor.

Moreover, studies of chick sympathetic ganglionic neurons after antisense oligonucleotide treatment to knockdown $\alpha 5$ subunits (Ramirez-Latorre et al., 1996; Yu and Role, 1998) and recent studies on cultured superior cervical ganglion (SCG) neurons from $\alpha 5$ knockout mice (Fischer et al., 2005) suggest that this subunit can significantly influence the functional characteristics of nAChRs in sympathetic ganglia.

In the present study, we measured heteromeric nAChRs in four autonomic ganglia from rats: the sympathetic superior cervical ganglia (SCG) and the stellate ganglia, the sensory/parasympathetic nodose ganglia, and the mixed pelvic ganglia. We then used subunit-selective antibodies in immunoprecipitation assays to compare the nAChR subunits present in the SCG and nodose ganglia, quantify the nAChR subtypes present in the SCG, and determine the subunit composition of the subtype that incorporates the $\alpha 5$ subunit.

Materials and Methods

Materials. Frozen SCG, nodose ganglia, stellate ganglia, and pelvic ganglia from adult Sprague-Dawley rats were purchased from Zivic Miller laboratories (Portersville, PA). [^{3}H]Epibatidine ([^{3}H]EB; 55 Ci/mmol) was obtained from PerkinElmer Life Sciences (Boston, MA). ^{125}I-Epibatidine (^{125}I-EB; ~545 Ci/mmol) and ^{125}I-A-85380 (~700 Ci/mmol) were synthesized and purified as described previously (Musachio et al., 1997; Horti et al., 1999). Rabbit antisera directed at bacterially expressed fusion proteins containing partial sequences of the cytoplasmic domains of nAChR $\alpha 2$, $\alpha 4$, $\alpha 5$, $\beta 3$, and $\beta 4$ 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 the C-terminal peptide sequence of the rat nAChR $\alpha 3$ subunit was affinity-purified from rabbit serum. This antibody has been described previously (Yeh et al., 2001). A monoclonal antibody (monoclonal antibody 270) to the chick $\beta 2$ subunit was made from hybridoma stocks (American Type Culture Collection, Manassas, VA). This monoclonal antibody was originally developed and characterized by Whiting and Lindstrom (1987). The specificity of most of the antibodies in the immunoprecipitation procedures was reported previously (Hernandez et al., 2004; Marritt et al., 2005; Turner and Kellar, 2005) Additional studies to assess the specificity of the $\alpha 5$ antibody have been carried out and are included in another study (D. Mao and K. J. Kellar, unpublished data). Protein G Sepharose beads were purchased from (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Protein A (Pansorbin) and normal rabbit serum (NRS) were purchased from Calbiochem (San Diego, CA). For simplicity, in this article, we use the term "antibody" to refer to unpurified antisera and to affinity-purified antiserum and monoclonal antibody.

Measurement of nAChR Binding Sites. Tissues were homogenized in Tris buffer (50 mM Tris-HCl buffer, pH 7.4, at 24°C), centrifuged twice at 35,000g for 10 min in buffer, and the membrane pellets were resuspended in fresh buffer. nAChR binding sites in the membrane homogenates from the ganglia were then measured with ~2 nM [^{3}H]EB, 2 nM ^{125}I-EB, or 1 nM ^{125}I-A-85380, as indicated in the figures. (Binding with [^{3}H]EB and ^{125}I-EB gave similar results, but less tissue was needed when using ^{125}I-EB.) Incubations were carried out in 200 μl of Tris buffer for 2 to 4 h at 24°C. Bound receptors were separated from free ligand by vacuum filtration over GF/C glass-fiber filters 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.

Immunoprecipitation Assays. Homogenates of SCG and nodose ganglia were prepared as above. The receptors were solubilized by incubating the homogenates in 2% Triton X-100 with gentle rotation for 3 h at 4°C. After centrifuging the mixture at 35,000g for 10 min, aliquots of the clear supernatant from SCG (equivalent to two ganglia) were added to sample tubes containing ~1.5 nM [^{3}H]EB. Aliquots of the clear supernatant from nodose ganglia (equivalent to three ganglia) were added to sample tubes containing ~2 nM ^{125}I-EB. One of the subunit-specific antibodies at an optimal concentration, which had been determined previously, or an equivalent volume of NRS was added to each sample tube. The final volume of the assay was 150 μl. The samples were then rotated gently overnight at 4°C. After the addition of 50 ml 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 another hour. The samples were then centrifuged at 12,000g for 1 min, and the supernatants were removed and filtered over GF/B glass-fiber filters that had been prewet with 0.5% polyethyleneimine. The filters were then counted in a liquid scintillation counter. Alternatively, the supernatants were removed and transferred to fresh tubes on ice for later use in sequential immunoprecipitation assays. The remaining pellets were washed by resuspension in 1.2 ml of 50 mM Tris-HCl buffer, pH 7.0, followed by centrifugation at 12,000g for 1 min. The pellets were then dissolved in 200 μl of 0.1 N NaOH, and the radioactivity was quantified in a scintillation counter. The counts precipitated in tubes containing NRS, which was used as control for nonspecific precipitation, were subtracted and the calculated number of radiolabeled nAChRs immunoprecipitated by each antibody was compared with the total number of radiolabeled receptors, as measured in both the supernatants and the pellets after immunoprecipitation. Data are presented as the percentage of the total nAChRs immunoprecipitated.

Sequential Immunoprecipitation Assays. To determine associations between subunits, we carried out sequential immunoprecipitation assays. 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 and the following quantification have been provided in detail previously (Marritt et al., 2005; Turner and Kellar, 2005). In brief, if two subunits are associated in an nAChR, antibodies to either subunit will immunoprecipitate that receptor, and the resulting supernatant will contain fewer receptors to be immunoprecipitated by the antibody directed at the other subunit.

Data Analysis. The propagation of error method (Bevington, 1969) was used to calculate the S.E.M. for the difference between groups. A one-sample t test was used to determine whether values in immunoprecipitation assays were different from 0. Statistical analyses of the differences between group means were assessed using Student's t test or one-way analysis of variance followed by Bonferroni's multiple comparison test.

Results

Relative Density of Heteromeric nAChRs in Rat SCG, Nodose, Stellate, and Pelvic Ganglia. The nAChRs in the SCG and nodose ganglia were measured with ~2 nM [^{3}H]EB, and those in the stellate and pelvic ganglia were measured with ~2 nM [^{3}H]EB or ^{125}I-EB. These are near-saturating concentrations at all known heteromeric nAChRs; therefore, the number of binding sites measured closely approximates the density of the receptors in these ganglia. As shown in Fig. 1, the density of nAChRs in the rat SCG is ~480 fmol/mg protein, which is ~10 times higher than in the nodose ganglia and 40 to 50 times higher than in stellate and pelvic ganglia. In fact, the density of nAChR binding sites in the SCG is one of the highest we have measured in any rat tissue.

To begin to determine whether SCG and nodose ganglia contain diverse populations of heteromeric nAChRs, we compared ^{125}I-A-85380 binding with [^{3}H]EB in the SCG and the nodose ganglia. At the 1 nM concentration used here, ^{125}I-A-85380 saturates nAChR subtypes containing $\beta2$ subunits and is highly selective for these subtypes (Mukhin et al., 2000; Xiao and Kellar, 2004). Figure 1 (inset) shows that a small population of the nAChRs in both SCG ($\sim$ 4% of the total) and nodose ganglia ($\sim$15% of the total) are labeled by ^{125}I-A-85380. This indicates that $\beta2$-containing nAChRs are present in those ganglia, but the large majority of the nAChRs contains $\beta4$ subunits but not $\beta2$ subunits.

The Subunit Profiles of nAChRs in SCG and Nodose Ganglia. The high density of the nAChRs in the SCG and nodose ganglia allowed quantitative assessment of the subunits present. To do this, we used antibodies directed at the $\alpha2$, $\alpha3$, $\alpha4$, and $\alpha5$ subunits and at the $\beta2$ and $\beta4$ subunits to immunoprecipitate nAChRs labeled with [^{3}H]EB or ^{125}I-EB in these ganglia. In the SCG (Fig. 2A), more than 90% of the nAChRs were immunoprecipitated by the antibodies directed at the $\alpha3$ and $\beta4$ subunits. In addition, 34% of the receptors were immunoprecipitated by the antibody directed at the $\alpha5$ subunit and 14% by the $\beta2$ subunit antibody. No receptors were immunoprecipitated with antibodies to the $\alpha2$ or $\alpha4$ subunits in the SCG. This subunit profile is similar to that found previously in rat SCG (Del Signore et al., 2004) and to the profile in chick ciliary ganglia (Conroy et al., 1992; Vernallis et al., 1993). It indicates that at least half and as many as two thirds of the $\alpha3\beta4$* receptors in the rat SCG are the simple heteromeric subtype $\alpha3\beta4$.

In the nodose ganglia (Fig. 2B), the $\alpha3$ antibody immunoprecipitated virtually all of the heteromeric nAChRs, and the

$\beta4$ antibody immunoprecipitated more than 80% of the receptors. The $\alpha5$ antibody immunoprecipitated $\sim$29% of the receptors, and the $\beta2$ antibody immunoprecipitated $\sim$22% of the receptors in the nodose ganglia. In addition, the $\alpha2$ and $\alpha4$ antibodies immunoprecipitated 9 and 10% of the receptors, respectively. Although these lower percentages were statistically significant [i.e., they were different from 0 ($p <$ 0.05)], the values for the $\alpha2$ subunit in particular were more variable than the other values. These data indicate that the major nAChRs in the nodose ganglia are $\alpha3\beta4$* subtypes, but the presence of multiple α and β subunits clearly suggests heterogeneity of the receptor subtypes within the ganglia, including mixed heteromeric subtypes containing the $\alpha5$ subunit along with other α subunits, which represent $\sim$29% of the receptors.

The nAChRs in the Rat SCG that Contain $\alpha5$ Subunits. The [^{3}H]EB-labeled nAChRs immunoprecipitated by the $\alpha5$ antibody represent mixed heteromeric nAChRs containing $\alpha5$ subunits associated with one or more other α and β subunits. The reasons for this conclusion are: 1) [^{3}H]EB does not label individual subunits (Xiao et al., 1998; Xiao and Kellar, 2004), so its binding represents, at a minimum, pairs of α and β subunits; and 2) because the $\alpha5$ subunit lacks two tyrosine residues implicated in ligand binding in other α subunits (Abramson et al., 1989; Cohen et al., 1991; Tomaselli et al., 1991), it probably cannot form an agonist binding site even when paired with β subunits only (Conroy et al., 1992).
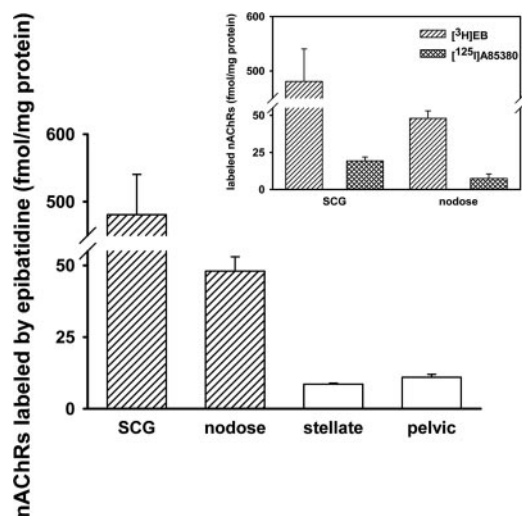
Fig. 1. nAChR density in peripheral ganglia. The total number of heteromeric nAChR binding sites in membranes from the SCG and nodose ganglia were measured with 2 nM [^{3}H]EB, and those in membranes from the stellate and pelvic ganglia were measured with 2 nM [^{3}H]EB or ^{125}I-EB. The binding site density (in femtomoles per milligram of protein) for the four ganglia are the following: SCG, 481 ± 53; nodose ganglia, 48 ± 4; stellate ganglia, 9 ± 0.3; and pelvic ganglia, 11 ± 1. Inset, comparison of 1 nM ^{125}I-A-85380 binding to nAChRs containing $\beta2$ subunits to the total population of heteromeric nAChRs labeled by [^{3}H]EB in the SCG and nodose ganglia. The [^{3}H]EB binding data are from the main figure and are shown here for comparison. ^{125}I-A-85380 binding site densities in SCG and nodose ganglia are 19 ± 2 and 7 ± 3 fmol/mg protein, respectively. Data are mean ± S.E.M. from five to seven experiments.
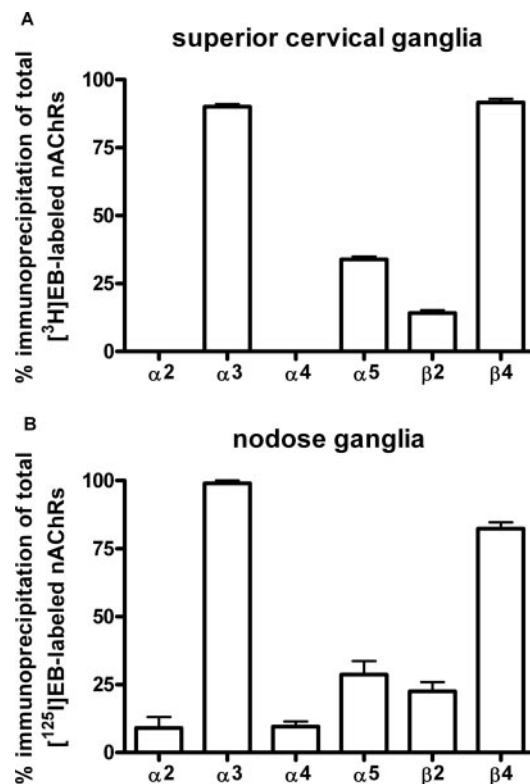
Fig. 2. Subunit profiles of the heteromeric nAChRs in rat SCG and nodose ganglia. nAChRs from rat SCG (A) and rat nodose ganglia (B) were solubilized, labeled with [^{3}H]EB and ^{125}I-EB, respectively, and immunoprecipitated with each of the subunit-specific antibodies shown. Nonspecific immunoprecipitation was measured with normal rabbit serum and has been subtracted. Data are mean ± S.E.M. from 3 to 8 experiments for SCG and for 3 to 10 experiments for nodose ganglia.

To determine the subunit compositions of the nAChRs that incorporate α5 subunits, we carried out sequential immunoprecipitation assays of the [³H]EB-labeled receptors. These assays provide good quantitative assessments of nAChR subunit associations (Flores et al., 1992, 1996; Zoli et al., 2002; Marritt et al., 2005; Turner and Kellar, 2005). We focused these studies on the SCG because its high density of nAChRs makes these kinds of studies feasible. But in addition to that practical issue, the nAChRs in the SCG play a crucial role in regulating autonomic functions in the eye, salivary glands, and pineal gland (Xu et al., 1999; Wang et al., 2002a). Moreover, these receptors have served as an index for changes in the SCG after sympathetic nerve injury and during recovery (Zhou et al., 1998; Yeh et al., 2001; Del Signore et al., 2004).

[³H]EB-labeled nAChRs solubilized from the SCG were immunoprecipitated first with either NRS or the antibody to the α5 subunit (the clearing antibodies). The supernatants from that first immunoprecipitation were then subjected to a second immunoprecipitation with an antibody against each of the other subunits in the SCG (the capturing antibodies). Results from that experiment are shown in Fig. 3A. After initial immunoprecipitation (clearing) with NRS, both the α3 and the β4 antibodies still immunoprecipitated >90% of the receptors, and the β2 antibody immunoprecipitated ~15% of the receptors in the SCG. These percentages are close to those measured in the single immunoprecipitations (Fig. 2A), indicating that initial immunoprecipitation with NRS did not affect nAChRs containing any of those three subunits. In comparison, when the initial immunoprecipitation was carried out with the α5 antibody to clear receptors containing the α5 subunits, the number of nAChRs subsequently immunoprecipitated by the α3 antibody was decreased by 28%, and the number immunoprecipitated by the β4 antibody was decreased by 29%. In contrast, the initial immunoprecipitation with the α5 antibody did not significantly affect the number of nAChRs subsequently immunoprecipitated by the β2 antibody (Fig. 3A), indicating that no receptors contain both α5 and β2 subunits.

To confirm these results, the order of the antibodies was reversed—we cleared with the antibodies to the α3, β4, and β2 subunits before capturing with the α5 antibody. As shown in Fig. 3B, after an initial immunoprecipitation with NRS, the α5 antibody immunoprecipitated ~30% of the nAChRs in the SCG, which again is similar to the value found in the single immunoprecipitation with the α5 antibody (Fig. 2A). After clearing with antibodies to the α3 or β4 subunits, however, the α5 antibody immunoprecipitated less than 5% of the nAChRs in the SCG. Again, in contrast, clearing with the β2 antibody did not decrease the number of nAChRs captured with the α5 antibody (Fig. 3B). Taken together, the results shown in Fig. 3 indicate that in the rat SCG, the α5 subunit is associated exclusively with α3β4 nAChRs; moreover, they indicate that ~28% of the total heteromeric nAChRs are the α3β4α5 subtype.

nAChR Subtypes Containing α3, β2, and β4 Subunits in the SCG. The absence of an association between α5 and β2 subunits implies that the rat SCG does not express a nAChR containing all four subunits. Therefore, studies were carried out to determine what percentage of the receptors in the rat SCG are simple α3β4 and α3β2 subtypes and whether the mixed heteromeric receptor α3β4β2 is present. As shown in Fig. 4A, clearing SCG extracts with the β4 antibody de-

creased the amount of nAChRs that were subsequently immunoprecipitated with the α3 antibody by ~85%, and, conversely, clearing with the α3 antibody removed essentially all receptors that could be immunoprecipitated by the β4 antibody. These results are consistent with the large majority of nAChRs in the SCG being an α3β4* subtype.

As shown in Fig. 4B, clearing the SCG extracts with the β2

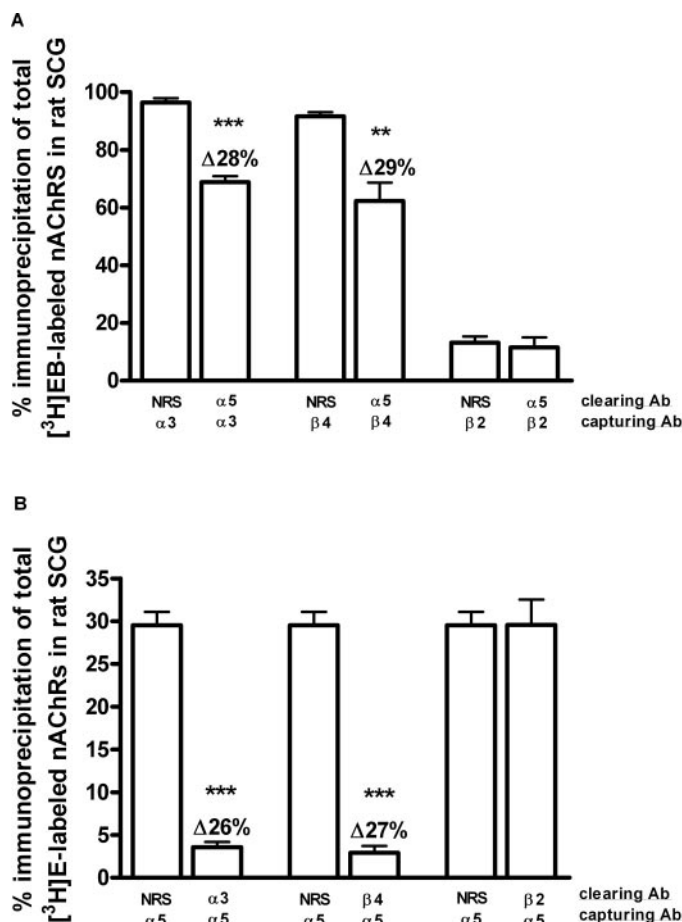**Fig. 3.** The α5 subunit associations in SCG nAChRs measured by sequential immunoprecipitation. A, SCG extracts were first immunoprecipitated with NRS as a control or the antibody to the α5 subunit (indicated as the clearing Ab). The resulting supernatants were then immunoprecipitated with the antibody to the α3, β4, or β2 subunit (indicated as the capturing Ab). Each bar represents the percentage of the total number of [³H]EB-labeled nAChRs immunoprecipitated by the capturing antibody. The values above the bars are the percentage reduction of total [³H]EB-labeled nAChRs calculated as the difference between the percentage captured after clearing with NRS and the indicated antibody. Data are mean ± S.E.M. from six or seven experiments. Different from the corresponding NRS control, **, $p < 0.01$; ***, $p < 0.001$, Student's t test. B, To confirm the results in A, the order of antibodies was reversed. That is, the SCG extracts were first cleared with NRS as a control or with the antibody to α3, β4, or β2 subunit (indicated as the clearing Ab). The resulting supernatants were then immunoprecipitated with the antibody to α5 subunit (indicated as the capturing Ab). Each bar represents the percentage of the total number of [³H]EB-labeled nAChRs immunoprecipitated by the capturing antibody. The values above the bars are the percentage reduction of total [³H]EB-labeled nAChRs, calculated as the difference between the percentage captured after clearing with NRS and the indicated antibody. Data are the mean ± S.E.M. from five experiments. (Data from the control experiment in which NRS was followed by the α5 antibody are plotted repeatedly side by side with data from the corresponding sequential immunoprecipitation assay to allow easier visual comparison.) Different from the corresponding NRS control: ***, $p < 0.001$, one-way analysis of variance followed by Bonferroni's multiple comparison test.
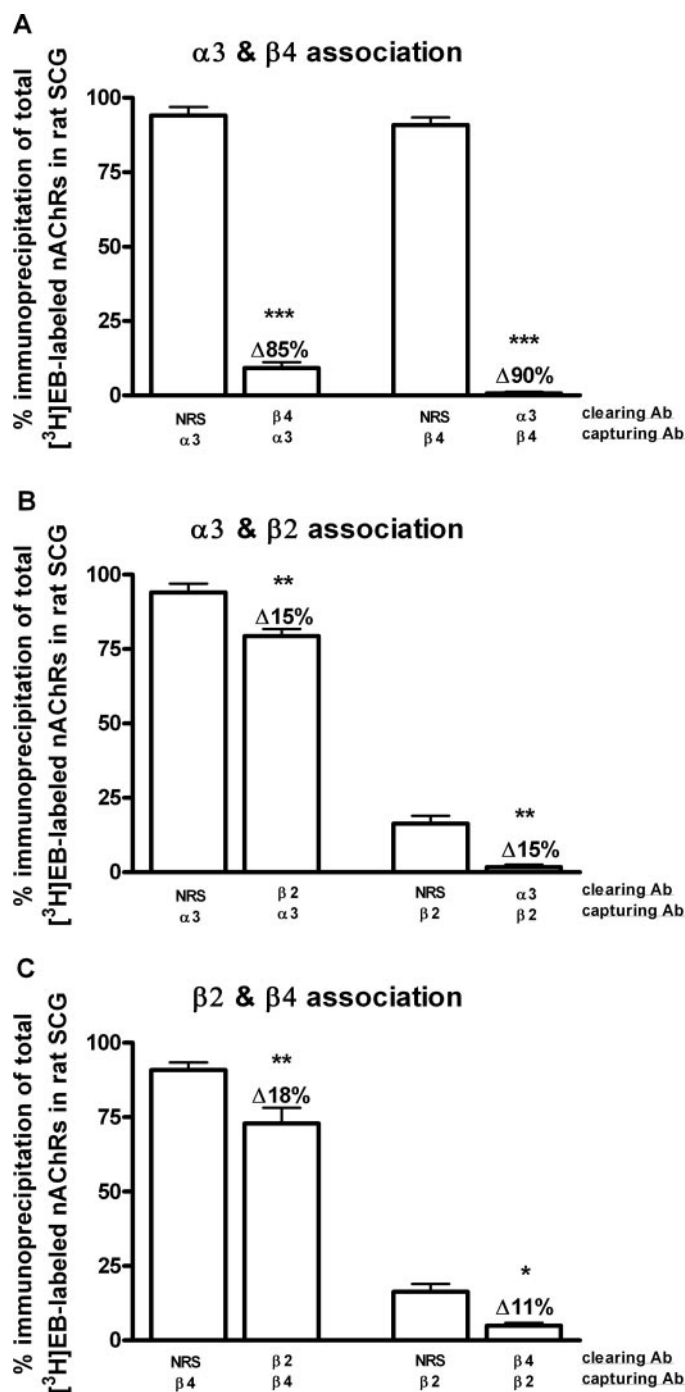
Fig. 4. Associations between the $\alpha3$, $\beta2$, and $\beta4$ subunits in SCG measured by sequential immunoprecipitation. A, association between $\alpha3$ and $\beta4$ subunits. Left pair of bars, SCG extracts were first immunoprecipitated with NRS as a control or the antibody to the $\beta4$ subunit (the clearing Ab), and the resulting supernatants were then immunoprecipitated with the antibody to the $\alpha3$ subunit (the capturing Ab). Right pair of bars, to confirm the results shown on the left, the order of the antibodies was reversed: rat SCG extracts were first immunoprecipitated with NRS or the antibody to the $\alpha3$ subunit (the clearing Ab), and the resulting supernatants were then immunoprecipitated with the antibody to the $\beta4$ subunit (the capturing Ab). Each bar represents the percentage of the total number of [^{3}H]EB-labeled nAChRs immunoprecipitated by the capturing antibody. B, association between $\alpha3$ and $\beta2$ subunits. The same procedures described in A were carried out using antibodies to the $\alpha3$ and $\beta2$ subunits. C, association between $\beta2$ and $\beta4$ subunits. The same procedures described in A were carried out using antibodies to the $\beta2$ and $\beta4$ subunits. The values above the bars are the percentage of reduction of total [^{3}H]EB-labeled nAChRs calculated as the difference between the

antibody decreased the nAChRs subsequently immunoprecipitated by the $\alpha3$ antibody by 15%, whereas clearing with the $\alpha3$ antibody removed virtually all of the $\beta2$ subunits. These results are consistent with ~15% of the nAChRs in the SCG being an $\alpha3\beta2*$ subtype. Next, we determined whether any of these $\alpha3\beta2*$ receptors also contain $\beta4$ subunits. As shown in Fig. 4C, clearing the SCG extracts with the $\beta2$ antibody decreased the receptors immunoprecipitated by the $\beta4$ antibody by ~18%, whereas clearing with the $\beta4$ antibody removed most of the nAChRs that could be precipitated by the $\beta2$ antibody. These results indicate that most of the $\beta2$-containing nAChRs in the SCG are part of an $\alpha3\beta4\beta2$ mixed heteromeric subtype.

Discussion

These studies demonstrate that all four of the rat autonomic ganglia examined, including the sympathetic SCG and stellate ganglia, the sensory nodose ganglia, and the mixed sympathetic/parasympathetic pelvic ganglia, express nAChRs. This is not unexpected, but the large difference in receptor density between the SCG and the other ganglia is striking. The density of nAChRs in the SCG is ~10 times higher than in nodose ganglia and ~50 times higher than in stellate or pelvic ganglia. The high density of nAChR in the SCG might reflect the influence of nerve growth factor (NGF) on these ganglia. In vivo, the sources of NGF are certain target organs, from which it moves by retrograde transport up nerve axons to cell bodies of neurons that have trkA receptors; SCG axons innervate the salivary glands, one of the richest sources of NGF. After axotomy or placing the SCG in organ culture, mRNA transcripts for the $\alpha3$, $\alpha5$, and $\beta4$ subunits as well as the density of the $\alpha3$ and the $\beta4$ subunit protein decrease markedly within a few days; NGF partially prevents and can even reverse these changes (Zhou et al., 1998, 2001; Yeh et al., 2001). NGF also influences nAChRs in cultured nodose neurons (Mandelzys et al., 1990). However, these effects of NGF on nAChRs have not been established in vivo, and other factors may also be involved in regulating nAChRs in vivo.

In contrast to the other autonomic ganglia, all of which receive a prominent cholinergic innervation from preganglionic sympathetic or parasympathetic axons, there is no known cholinergic input to the nodose ganglia; therefore, the physiological functions of the nAChRs in the sensory nodose ganglia are not yet clear. It is possible that these receptors are assembled in cell bodies of nodose neurons and are then transported via vagal afferent axons to their terminations in the nucleus of the solitary tract of the brainstem (Cooper, 2001). In this case, these nAChRs would be in a position to modulate the release of neurotransmitters at this brainstem center, which heavily influences autonomic functions in the cardiovascular, respiratory, and gastrointestinal systems (Zhuo et al., 1997).

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percentage captured after clearing with NRS and the indicated antibody. Data are the mean ± S.E.M. from three to four experiments. (Data from the control experiments in which NRS was followed by $\alpha3$, $\beta4$, or $\beta2$ antibody were plotted repeatedly side by side with data from the relevant corresponding sequential immunoprecipitation assay to allow easier visual comparison.) Different from the corresponding NRS control: $*$, $p < 0.05$; $**$, $p < 0.01$; $***$, $p < 0.001$, one-way analysis of variance followed by Bonferroni's multiple comparison test.

Consistent with studies of nAChR subunit mRNA in rats (Mandelzys et al., 1994; De Koninck and Cooper, 1995; Zhou et al., 1998; Cooper, 2001), we found that both SCG and nodose ganglia express $\alpha3$, $\alpha5$, $\beta2$, and $\beta4$ nAChR subunit proteins. The profile of subunits in the SCG reported here is similar to that in a previous report that also used immunoprecipitation methods (Del Signore et al., 2004), but it differs from a study that found $\alpha4$ subunits by immunocytochemical methods in ~50% of cultured rat SCG neurons (Skok et al., 1999). This difference most likely reflects the different methods used in the studies.

In the nodose ganglia, in contrast to the SCG, we consistently detected nAChRs containing $\alpha4$ subunits. nAChRs containing $\alpha4$ subunits were also found in the trigeminal ganglia (Flores et al., 1996), another sensory ganglion. Moreover, mRNA encoding the $\alpha4$ subunit was found in sensory neurons from chick dorsal root ganglia (Boyd et al., 1991), and currents resembling those from $\alpha4\beta2*$ and $\alpha3\beta4*$ receptors were recorded in small populations of dorsal root ganglion neurons from rats (Genzen et al., 2001). Together, these data suggest that sensory ganglia may express small populations of nAChRs containing $\alpha4$ subunits, perhaps $\alpha3\alpha4\beta2*$ as well as $\alpha3\beta4*$ receptors.

Both the SCG and the nodose ganglia have relatively small but measurable populations of nAChRs that bind the $\beta2$-selective ligand ^{125}I-A-85380, indicating the presence of receptors that contain at least one agonist binding site formed by an interface between α and $\beta2$ subunits. The fact that in both ganglia the percentage of receptors labeled by ^{125}I-A-85380 is lower than the percentage of receptors immunoprecipitated by the $\beta2$ antibody might indicate that in some nAChRs, the $\beta2$ subunit is inserted in the position that does not form an agonist binding interface. For example, in the case of the SCG, 14% of the receptors contain $\beta2$ subunits, which allows them to be immunoprecipitated by the $\beta2$ antibody, but ^{125}I-A-85380 binding sites represent only 4% of the [^{3}H]EB sites. This suggests that less than one third of the $\beta2$ subunits (4%/14%) present form an agonist-binding interface. In the case of the nodose ganglion, this fraction is approximately two thirds (15%/22%).

The high density of nAChRs in the SCG allowed us to directly assess the subunit composition of the nAChR subtypes present in the ganglia and the percentage that contains $\alpha5$ subunits. Our data distinguish three main subtypes of nAChRs in the rat SCG: 55 to 60% $\alpha3\beta4$, 25 to 30% $\alpha3\beta4\alpha5$, and 10 to 15% $\alpha3\beta4\beta2$ (our results leave room for a small number of $\alpha3\beta2$ receptors but probably not more than 5% of the total). Our data do not take into account differences in subunit stoichiometry; thus, for example, receptors with stoichiometries of $\alpha3(\beta4)_2(\beta2)_1$ and $\alpha3(\beta4)_1(\beta2)_2$ would be counted together.

Studies based on comparisons to nAChRs heterologously expressed in oocytes suggested that nAChR-mediated responses in rat SCG most closely resemble those of an $\alpha3\beta4$ subunit combination (Covernton et al., 1994). Our direct measurements of the subunit compositions of the nAChR subtypes in the rat SCG support this suggestion because we found that more than 90% of the receptors are an $\alpha3\beta4*$ subtype. Consistent with this, nicotine-stimulated currents in SCG neurons from $\beta2$ knockout mice resemble those of the wild-type, whereas the currents in SCG neurons from $\beta4$ knockout mice are reduced by >95% (Xu et al., 1999). It is interesting that despite the large disparity in the currents

mediated by the $\beta2$- versus the $\beta4$-containing nAChRs, the phenotypes of the two knockout models are, at least superficially, both remarkably similar to the wild type (Xu et al., 1999). This suggests that nAChRs with either β subunit can function sufficiently to support basic ganglionic functions, at least under nonperturbed physiological and environmental conditions.

The presence of the $\alpha5$ subunit exclusively in the $\alpha3\beta4\alpha5$ nAChR subtype in the rat SCG is similar to results from studies of the parasympathetic ciliary ganglia in chick (Vernallis et al., 1993; Conroy and Berg, 1995), although the percentage of the chick $\alpha3\beta4*$ nAChRs that contain the $\alpha5$ subunit could not be determined at that time. A second similarity in the nAChRs in these ganglia from the two species is the presence of an $\alpha3\beta4\beta2$ subtype. We found that this subtype represents 10 to 15% of the receptors in the rat SCG. One apparent difference between the ganglia, however, is that we did not detect a receptor containing both the $\alpha5$ and $\beta2$ subunits; thus, we did not find evidence for a receptor containing all four subunits. This could reflect a difference in the types of ganglia and/or the species. Nevertheless, the repertoires of subtypes in these two autonomic ganglia that function in the sympathetic and parasympathetic nervous systems of rat and chick seem to be more similar than different.

The heterogeneity of nAChR subtypes in the autonomic ganglia offers the possibility that different subtypes might serve different roles in those ganglia. For example, in heterologous expression systems, the addition of the $\alpha5$ subunit to $\alpha3\beta4$ nAChRs increased channel conductance but also increased the rate and extent of receptor desensitization (Wang et al., 1996; Fucile et al., 1997; Sivilotti et al., 1997; Gerzanich et al., 1998; Nelson and Lindstrom, 1999). In addition, a decrease in receptor affinity for certain agonists, including acetylcholine, was observed after incorporation of the $\alpha5$ subunit in some studies (Ramirez-Latorre et al., 1996; Fucile et al., 1997). In native nAChRs also, the presence of the $\alpha5$ subunit seems to affect the receptor characteristics. For example, in chick sympathetic ganglia neurons, antisense oligonucleotide treatment to decrease the incorporation of $\alpha5$ subunits into nAChRs eliminated a class of channels with high conductance and also the low-affinity component of the response to ACh and cytisine (Ramirez-Latorre et al., 1996; Yu and Role, 1998). The same knockdown treatment also decreased efficacy of cytisine, suggesting that the $\alpha5$-containing nAChRs contribute to the cytisine-sensitive responses (Yu and Role, 1998). In addition, $\alpha5$ knockout mice seemed to have impaired cardiac parasympathetic ganglionic transmission when challenged with high-frequency vagal stimulation (Wang et al., 2002a). It is interesting, however, that both nicotinic agonist-stimulated calcium signals and norepinephrine release were increased in SCG neurons from $\alpha5$ knockout mice (Fischer et al., 2005).

The $\alpha5$ subunit seems to incorporate as a structural component of nAChRs rather than participate directly in the binding site for agonists; nevertheless, as described above, its presence can affect the functional and pharmacological characteristics of nAChRs (Yu and Role, 1998; Wang et al., 2002b). How the $\alpha5$ subunit exerts influence and what advantage it confers are not known. It might affect nAChR channel kinetics or calcium permeability to make the responses more compatible with that neuron's functions under

particular circumstances. In addition, it might influence the surface expression and/or trafficking of nAChRs within the neuron (Conroy and Berg, 1995; Fischer et al., 2005). Therefore, knowing the nAChR subtypes expressed in ganglia and the percentage that contain α5 subunits is potentially important in understanding differences among ganglia and even differential functioning within ganglia.

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