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

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Running Title:

nAChRs in Rat SCG and Nodose Ganglia

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Abbreviations: A-85380, 3-2(S)-azetidinylmethoxy)pyridine; EB, epibatidine; mAb, monoclonal antibody; nAChR, neuronal nicotinic acetylcholine receptor; NRS, normal rabbit serum; SCG, superior cervical ganglia

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Abstract

Nicotinic receptors (nAChRs) are present in ganglia in the peripheral nervous system. In autonomic ganglia, they are responsible for fast synaptic transmission, while 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 α3 and β4 subunits, but a significant percentage of the nAChRs in these ganglia also contain α5 and β2 subunits. A small percentage of the nAChRs in nodose ganglia also contain α2 and α4 subunits. Sequential immunoprecipitation assays indicated that in the SCG all α5 subunits are associated with α3 and β4 subunits, forming the mixed heteromeric α3β4α5 subtype. A receptor comprised of α3, β2 and β4 subunits in the SCG was also detected. In rat SCG, we found the following distribution of nAChRs subtypes: 55-60% simple α3β4 subtype, 25-30% α3β4α5 subtype, 10-15% α3β4β2 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.

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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; Mandelzys et al., 1994; Flores et al., 1996), where they may modulate neurotransmission (Cooper, 2001). These receptors are pentameric structures comprised of α and β subunits. Nine α (α2-α10) and three β (β2- β4) 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 α3β4* and α4β2* are the predominant heteromeric subtypes in autonomic ganglia and the CNS, respectively. By convention, the asterisk denotes the possibility that one or more unidentified subunits are present in those receptors. For example, although expression of α3 and β4 subunits or α3 and β2 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 α5 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 α3α5β2β4 subtype (Conroy and Berg, 1995).

The α5 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 α5 subunit into heterologously expressed α3β2 and α3β4 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). Moreover, studies of chick sympathetic ganglionic neurons after antisense oligonucleotide treatment to knockdown α5 subunits (Ramirez-Latorre et al., 1996; Yu and Role, 1998) and recent studies on cultured superior cervical ganglion (SCG) neurons from α5 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 α5 subunit.

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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 Perkin Elmer 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 α2, α4, α5, β3 and β4 subunits were kind gifts from Drs. Scott Rogers and Lorise Gahring (University of Utah, Salt Lake City). 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 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). 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 α5 antibody have been carried out and are included in another study (D Mao and KJ Kellar, unpublished data). Protein G Sepharose beads were purchased from Amersham Biosciences Corporation (Piscataway, NJ). Protein A (Pansorbin) and normal rabbit serum (NRS) were purchased from Calbiochem (San Diego, CA). For simplicity,

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in this paper we use the term antibody to refer to unpurified antisera, as well as 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 35,000 x g for 10 minutes 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]-A85380, 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 Tris buffer for 2 to 4 hrs at 24°C. Bound receptors were separated from free ligand by vacuum filtration over GF/C glass-fiber filters that were pre-wet 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 three hours at 4°C. After centrifuging the mixture at 35,000 x g for 10 minutes, aliquots of the clear supernatant from SCG (equivalent to 2 ganglia) were added to sample tubes containing ~1.5 nM [^{3}H]EB. Aliquots of the clear supernatant from nodose ganglia (equivalent to 3 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 normal rabbit serum (NRS) was added to each sample tube. The final volume of the assay was 150 ul. The samples were then rotated gently 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 another hour. The samples were then centrifuged at 12,000 x g for 1 minute and the supernatants were removed and filtered over GF/B glass-fiber filters that had been pre-wet 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 50 mM Tris HCl buffer (pH 7.0), followed by centrifugation at 12,000 x g for 1 minute. The pellets were then dissolved in 200ul of 0.1N 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 to the total number of radiolabeled receptors, as measured in both the supernatants and the pellets after immunoprecipitation. Data are presented as the percent 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). Briefly, 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 other subunit.

Data Analysis. The propagation of error method (Bevington, 1969) was used to calculate the SEM for the difference between groups. A one-sample *t*-test was used to determine if 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.

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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 figure 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 40-50 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 if 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 β2 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 (~ 4% of the total) and nodose ganglia (~ 15% of the total) are labeled by [^{125}I]A-85380. This indicates that β2-containing nAChRs are present in those ganglia, but the large majority of the nAChRs contains β4 subunits but not β2 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 α2, α3, α4, and α5 subunits, as well as the β2 and β4 subunits to immunoprecipitate nAChRs labeled with

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[^{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 α3 and β4 subunits. In addition, 34% of the receptors were immunoprecipitated by the antibody directed at the α5 subunit and 14% by the β2 subunit antibody. No receptors were immunoprecipitated with antibodies to the α2 or α4 subunits in the SCG. This subunit profile is similar to that found previously in rat SCG (Del Signore et al., 2004), as well 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 α3β4* receptors in the rat SCG are the simple heteromeric subtype, α3β4.

In the nodose ganglia (Fig. 2B), the α3 antibody immunoprecipitated virtually all of the heteromeric nAChRs, and the β4 antibody immunoprecipitated more than 80% of the receptors. The α5 antibody immunoprecipitated ~29% of the receptors and the β2 antibody immunoprecipitated ~22% of the receptors in the nodose ganglia. In addition, the α2 and α4 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 α2 subunit in particular were more variable than the other values. These data indicate that the major nAChRs in the nodose ganglia are α3β4* 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 α5 subunit along with other α subunits, which represent ~29% of the receptors.

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The nAChRs in the rat SCG that contain α5 subunits. The [^{3}H]EB-labeled nAChRs immunoprecipitated by the α5 antibody represent mixed heteromeric nAChRs containing α5 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 α5 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 can not form an agonist binding site even when paired with β subunits only (Conroy et al., 1992).

To determine the subunit compositions of the nAChRs that incorporate α5 subunits we carried out sequential immunoprecipitation assays of the [^{3}H]EB-labeled receptors. These assays provide good quantitative assessments of nAChR subunit associations (Flores et al., 1992; Flores et al., 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).

[^{3}H]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

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capturing antibodies). Results from that experiment are shown in figure 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 (see Fig. 2A), indicating that initial immunoprecipitation with NRS didn't 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—i.e., we cleared with the antibodies to the α3, β4 and β2 subunits before capturing with the α5 antibody. As shown in figure 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 (see 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 figure 3 indicate that in the rat

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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 figure 4A, clearing SCG extracts with the β4 antibody decreased 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 figure 4B, clearing the SCG extracts with the β2 antibody decreased the nAChRs subsequently immunoprecipitated by the α3 antibody by 15%; whereas, clearing with the α3 antibody removed virtually all of the β2 subunits. These results are consistent with ~15% of the nAChRs in the SCG being an α3β2* subtype. Next, we determined whether any of these α3β2* receptors also contain β4 subunits. As shown in figure 4C, clearing the SCG extracts with the β2 antibody decreased the receptors immunoprecipitated by the β4 antibody by ~18%; whereas clearing with the β4 antibody removed most of the nAChRs that could be precipitated by the β2 antibody. These results indicate that most of the β2-containing nAChRs in the SCG are part of an α3β4β2 mixed heteromeric subtype.

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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 trk A receptors; and 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 α3, α5 and β4 subunits, as well as the density of the α3 and the β4 subunit protein decrease markedly within a few days; NGF partially prevents and can even reverse these changes (Zhou et al., 1998; Yeh et al., 2001; Zhou 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 then transported

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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 release of neurotransmitters at this brainstem center, which heavily influences autonomic functions in the cardiovascular, respiratory and gastrointestinal systems (Zhuo et al., 1997).

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 α3, α5, β2 and β4 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 α4 subunits by immunocytochemical methods in ~ 50% of cultured rat SCG neurons (Skok et al., 1999). This difference most likely reflects the different methods employed in the studies.

In the nodose ganglia, in contrast to the SCG, we did consistently detect nAChRs containing α4 subunits. nAChRs containing α4 subunits were also found in the trigeminal ganglia (Flores et al., 1996), another sensory ganglion. Moreover, mRNA encoding the α4 subunit was found in sensory neurons from chick dorsal root ganglia (Boyd et al., 1991), and currents resembling those from α4β2* and α3β4* receptors were recorded in small populations of DRG neurons from rats (Genzen et al., 2001). Together, these data suggest that sensory ganglia may express small populations of nAChRs containing α4 subunits, perhaps α3α4β2*, as well as α3β4* receptors.

Both the SCG and the nodose ganglia have relatively small but measurable populations of nAChRs that bind the β2-selective ligand [^{125}I]A-85380, indicating the

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presence of receptors that contain at least one agonist binding site formed by an interface between α and β2 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 β2 antibody might indicate that in some nAChRs the β2 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 β2 subunits, which allow them to be immunoprecipitated by the β2 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 β2 subunits (4%/14%) present form an agonist-binding interface. In the case of the nodose ganglion, this fraction is about two-thirds (15%/22%).

The high density of nAChRs in the SCG allowed us to directly assess the subunit composition of the nAChRs subtypes present in the ganglia and the percentage that contains α5 subunits. Our data distinguish three main subtypes of nAChRs in the rat SCG: 55-60% α3β4, 25-30% α3β4α5 and 10-15% α3β4β2 (our results leave room for a small number of α3β2 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 α3β4 subunit combination (Covernton et al., 1994). Our direct measurements of the subunit compositions of the nAChR subtypes in the rat SCG support this suggestion since we found that more than 90% of the receptors are an α3β4* subtype. Consistent with this, nicotine-stimulated currents in SCG neurons from β2 knockout mice resemble those

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of the wild-type, whereas the currents in SCG neurons from β4 knockout mice are reduced by >95% (Xu et al., 1999). Interestingly, despite the large disparity in the currents mediated by the β2- versus the β4-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 non-perturbed physiological and environmental conditions.

The presence of the α5 subunit exclusively in the α3β4α5 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 α3β4* nAChRs that contain the α5 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 α3β4β2 subtype. We found that this subtype represents 10-15% 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 α5 and β2 subunits; thus, we did not find evidence for a receptor containing all four subunits. This could reflect either a difference in the types of ganglia and/or in 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 appear to be more similar than different.

The heterogeneity of nAChRs 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 α5 subunit to α3β4 nAChRs increased channel conductance but also increased the rate and extent of receptor

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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 α5 subunit in some studies (Ramirez-Latorre et al., 1996; Fucile et al., 1997). In native nAChRs also, the presence of the α5 subunit appears to affect the receptor characteristics. For example, in chick sympathetic ganglia neurons, antisense oligonucleotide treatment to decrease incorporation of α5 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 α5-containing nAChRs contribute to the cytisine-sensitive responses (Yu and Role, 1998). In addition, α5 knockout mice appeared to have impaired cardiac parasympathetic ganglionic transmission when challenged with high frequency vagal stimulation (Wang et al., 2002a). Interestingly, however, both nicotinic agonist-stimulated calcium signals and norepinephrine release were increased in SCG neurons from α5 knockout mice (Fischer et al., 2005).

The α5 subunit appears 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 α5 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

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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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Footnotes

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Figure Legends

Figure 1. **nAChR density in peripheral ganglia**. The total number of heteromeric nAChR binding sites in membranes from the superior cervical ganglia (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 (fmol/mg protein) for the four ganglia are: SCG, 481 ± 53; nodose ganglia, 48 ± 4; stellate ganglia, 9 ± 0.3; pelvic ganglia, 11 ± 1. Inset: Comparison of 1 nM [^{125}I]A-85380 binding to nAChRs containing β2 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 density (fmol/mg protein) in SCG and nodose ganglia are: 19 ± 2 and 7 ± 3, respectively. Data are mean ± SEM from 5 to 7 experiments.

Figure 2. **Subunit profiles of the heteromeric nAChRs in rat SCG and nodose ganglia.** nAChRs from **A**) rat SCG and **B**) rat nodose ganglia were solubilized, labeled with [^{3}H]EB and [^{125}I]EB, respectively, and immunoprecipitated with each of the subunit-specific antibodies shown. Non-specific immunoprecipitation was measured with normal rabbit serum and has been subtracted. Data are mean ± SEM from 3 to 8 experiments for SCG, and 3 to 10 experiments for nodose ganglia.

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Figure 3. **The α5 subunit associations in SCG nAChRs measured by sequential immunoprecipitation**. **A)** SCG extracts were first immunoprecipitated with normal rabbit serum (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 percent of the total number of [^{3}H]EB-labeled nAChRs immunoprecipitated by the capturing antibody. The values above the bars are the percent reduction of total [^{3}H]EB-labeled nAChRs calculated as the difference between the percent captured after clearing with NRS and the indicated antibody. Data are mean ± SEM from 6 or 7 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 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 percent of the total number of [^{3}H]EB-labeled nAChRs immunoprecipitated by the capturing antibody. The values above the bars are the percent reduction of total [^{3}H]EB-labeled nAChRs, calculated as the difference between the percent captured after clearing with NRS and the indicated antibody. Data are the mean ± SEM from 5 experiments. (Data from the control experiment in which NRS was followed by 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 ANOVA followed by Bonferroni's multiple comparison test.

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Figure 4. A**ssociations between the α3, β2 and β4 subunits in SCG measured by sequential immunoprecipitation. A**) Association between α3 and β4 subunits. Left pair of bars: SCG extracts were first immunoprecipitated with NRS as a control or the antibody to the β4 subunit (the clearing Ab), and the resulting supernatants were then immunoprecipitated with the antibody to the α3 subunit (the capturing Ab). Right pair: To confirm the results shown on the left, the order of the antibodies was reversed, *i.e.* rat SCG extracts were first immunoprecipitated with NRS or the antibody to the α3 subunit (the clearing Ab), and the resulting supernatants were then immunoprecipitated with the antibody to the β4 subunit (the capturing Ab). Each bar represents the percent of the total number of [^{3}H]EB-labeled nAChRs immunoprecipitated by the capturing antibody. **B**) Association between α3 and β2 subunits. The same procedures described in A) were carried out using antibodies to the α3 and β2 subunits. **C**) Association between β2 and β4 subunits. The same procedures described in A) were carried out using antibodies to the β2 and β4 subunits. The values above the bars are the percent reduction of total [^{3}H]EB-labeled nAChRs, calculated as the difference between the percent captured after clearing with NRS and the indicated antibody. Data are the mean ± SEM from 3 to 4 experiments. (Data from the control experiments in which NRS was followed by α3, β4 or β2 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 ANOVA followed by Bonferroni's multiple comparison test.

Figure 1

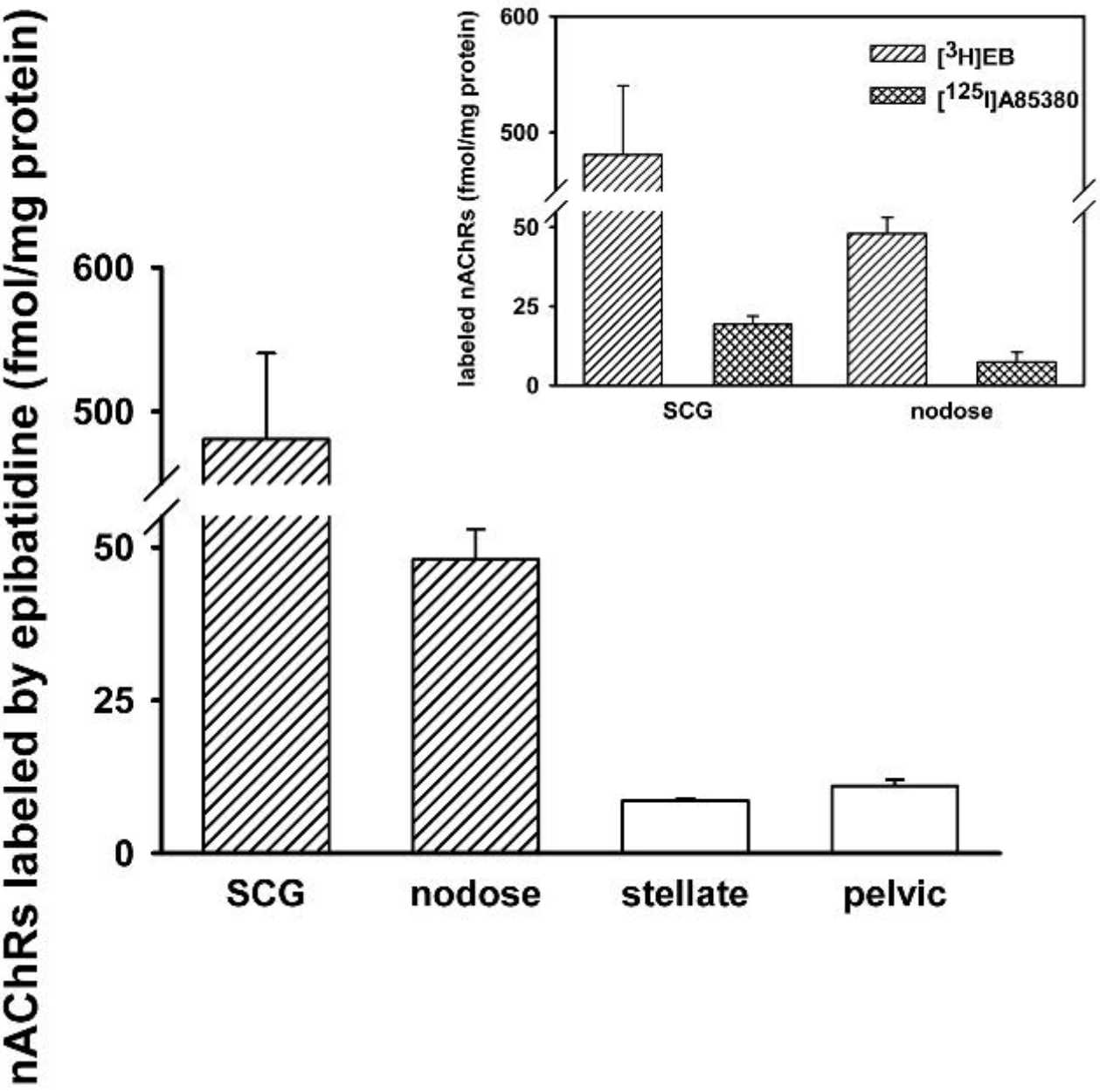

nAChRs labeled by epibatidine (fmol/mg protein)
600
500
50
25
0
SCG
nodose
stellate
pelvic
labeled nAChRs (fmol/mg protein)
600
500
50
25
0
[3H]EB
[125I]A85380
SCG
nodose

Figure 2

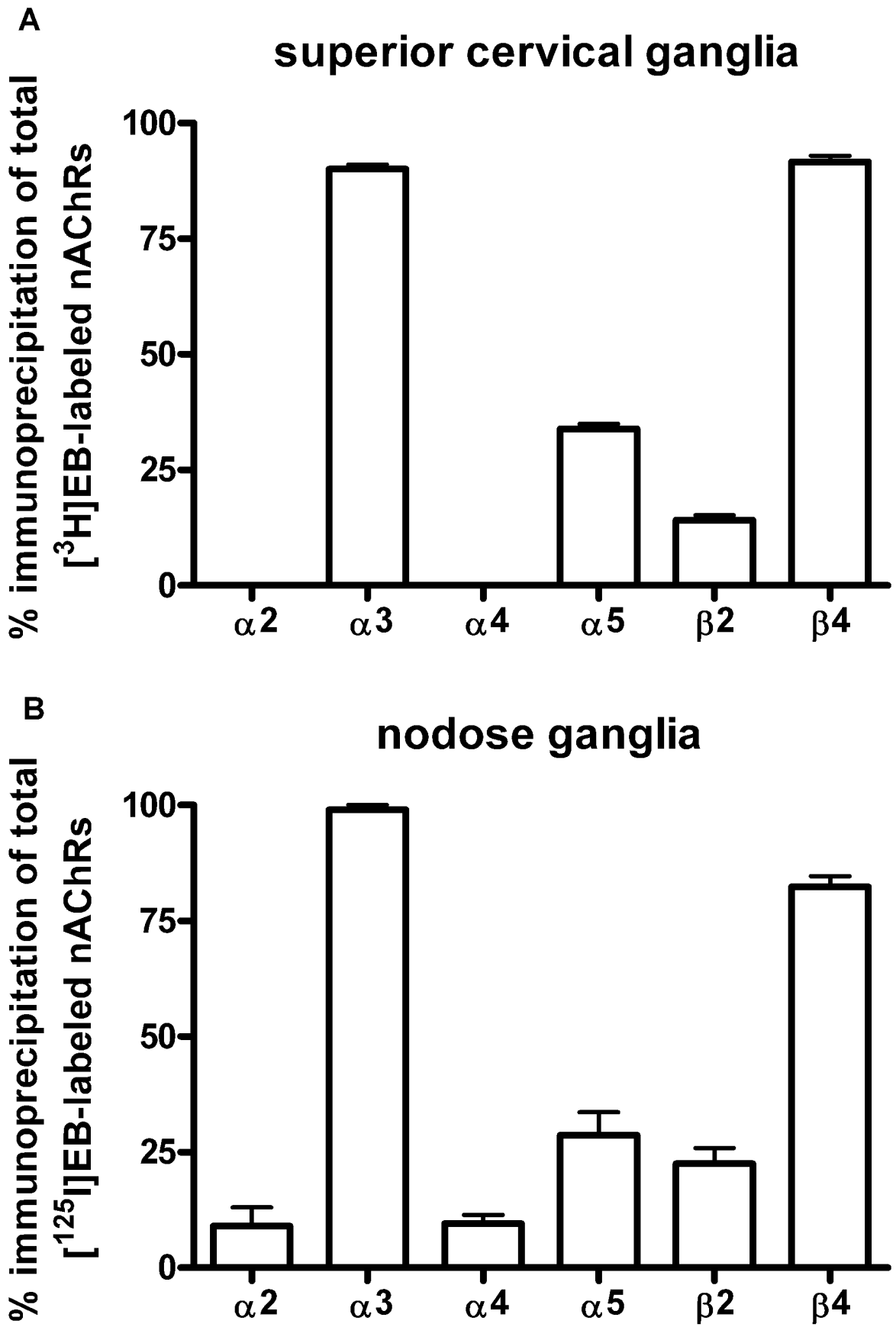
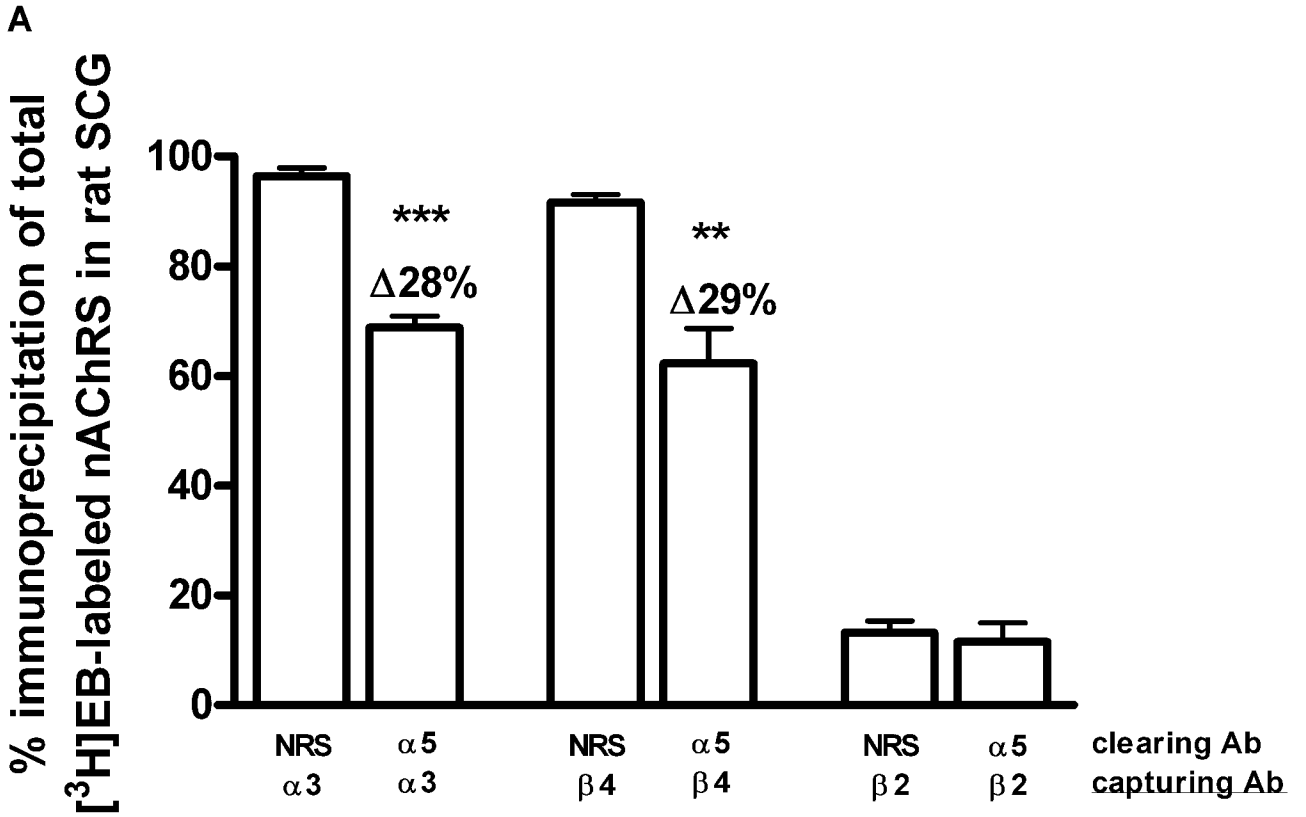

A
superior cervical ganglia
% immunoprecipitation of total [3H]EB-labeled nAChRs
100
75
50
25
0
α2
α3
α4
α5
β2
β4
B
nodose ganglia
% immunoprecipitation of total [125I]EB-labeled nAChRs
100
75
50
25
0
α2
α3
α4
α5
β2
β4

Figure 3


A
% immunoprecipitation of total [3H]EB-labeled nAChRS in rat SCG
100
80
60
40
20
0
***
Δ28%
**
Δ29%
NRS α3
α5 α3
NRS β4
α5 β4
NRS β2
α5 β2
clearing Ab
capturing Ab

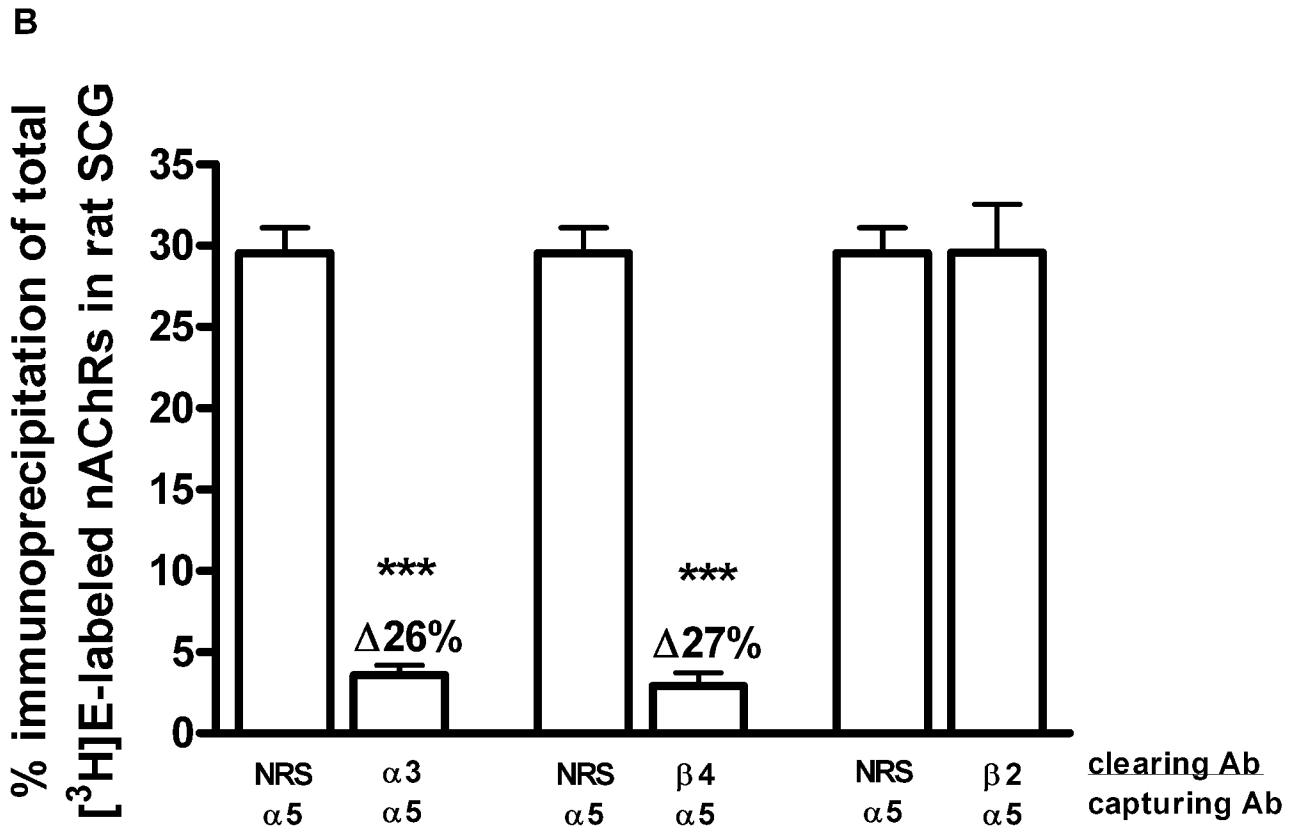
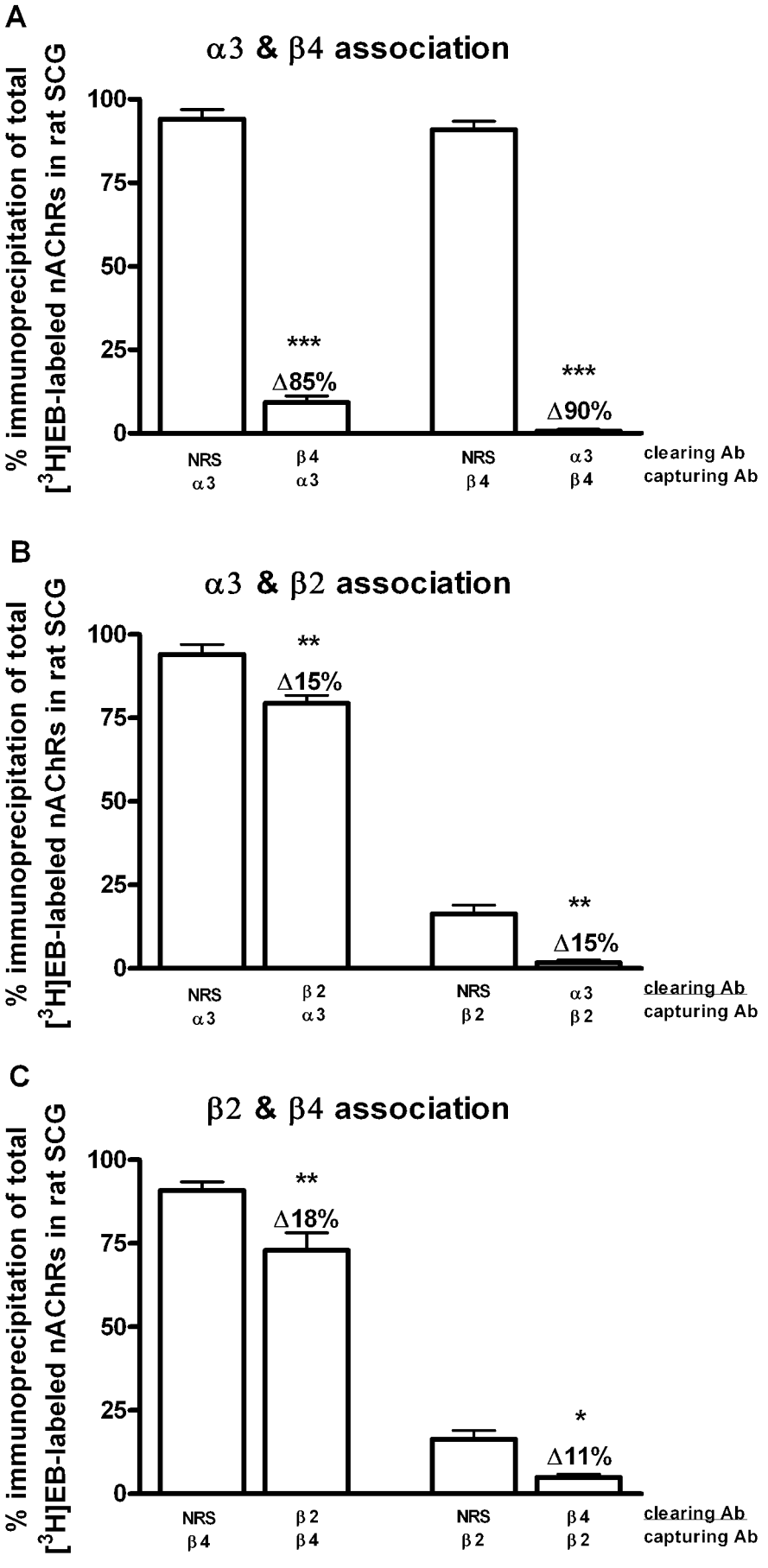

B
% immunoprecipitation of total [3H]E-labeled nAChRs in rat SCG
35
30
25
20
15
10
5
0
***
Δ26%
***
Δ27%
NRS α5
α3 α5
NRS α5
β4 α5
NRS α5
β2 α5
clearing Ab
capturing Ab

Figure 4


A
α3 & β4 association
% immunoprecipitation of total [3H]EB-labeled nAChRs in rat SCG
100
75
50
25
0
***
Δ85%
***
Δ90%
NRS α3
β4 α3
NRS β4
α3 β4
clearing Ab
capturing Ab
B
α3 & β2 association
% immunoprecipitation of total [3H]EB-labeled nAChRs in rat SCG
100
75
50
25
0
**
Δ15%
**
Δ15%
NRS α3
β2 α3
NRS β2
α3 β2
clearing Ab
capturing Ab
C
β2 & β4 association
% immunoprecipitation of total [3H]EB-labeled nAChRs in rat SCG
100
75
50
25
0
**
Δ18%
*
Δ11%
NRS β4
β2 β4
NRS β2
β4 β2
clearing Ab
capturing Ab