Functional contribution of the α5 subunit to neuronal nicotinic channels expressed by chick sympathetic ganglion neurones

C. Ron Yu and Lorna W. Role

Department of Anatomy and Cell Biology in the Center for Neurobiology and Behavior, College of Physicians and Surgeons, Columbia University, 722 West 168th Street, Research Annex Room 807, New York, NY 10032, USA

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1. Heterologous expression studies of the α5 subunit of the neuronal acetylcholine receptor (nAChR) gene family have demonstrated that it can participate in the function of ACh-gated channels if co-expressed with another α- and a β-subunit. Previous studies also indicate prominent expression of α5 in both central and peripheral nervous systems. The participation of α5 in native nAChRs and its functional role in these channels is, however, unknown.

2. In this study, we present evidence that α5 has a role in at least two distinct subtypes of nAChR complexes expressed by embryonic chick sympathetic neurones.

3. α5 contributes not only to agonist but also to antagonist sensitivity of natively expressed nAChR channels. Functional deletion of the α5 subunit by antisense oligonucleotide treatment removes the nAChRs with relatively low affinity to ACh and cytisine. Deletion of α5 also eliminates channels that are blocked by the α7-specific antagonist methyllycaconitine (MLA) while increasing the percentage of current carried by nAChRs that are sensitive to α-bungarotoxin (α-BgTx).

4. Single channel analyses indicate that functional deletion of α5 results in the deletion of both the 'brief' and 'long' open duration, 50 pS subtypes of nAChR channels while increasing the expression of the 18 pS, α-BgTx-sensitive native nAChRs normally detected in sympathetic neurones at later developmental stages.

5. The biophysical and pharmacological profiles of native nAChRs revealed by this study and previous work are discussed in the context of a proposed model of the nAChR channels expressed by chick sympathetic neurones throughout development.

The α5 subunit, like α7, has a chequered past. Cloning and characterization of this neuronal nicotinic receptor subunit gene by Ballivet, Heinemann and their colleagues revealed sequence encoding the N-terminal vicinal cysteine residues, which are generally viewed as a fingerprint of α-type neuronal acetylcholine receptor (nAChR) subunits. As such, the subunit was classified as one of the now eight neuronal α- (or ligand-binding) subunits (Couturier *et al.* 1990; Wada, McKinnon, Heinemann, Patrick & Swanson, 1990). Subsequent attempts to assess the functional properties of the α5 subunit by heterologous expression with other α- or β-subunits, however, failed to yield ligand-gated ion channels. Characterization of the pattern of expression of α5 mRNA also revealed an unusual and restricted distribution, with the most prominent expression detected in central and peripheral nervous system (CNS and PNS, respectively) structures involved in limbic and autonomic functions.

Examination of the α5 sequence reveals several unusual features. First, the N-terminal sequence adjacent to the paired cysteine residues equivalent to the 190 position of the α1 sequence lacks the typical tyrosine residue present in all other α-subunits of the nAChR family. This residue has previously been demonstrated to be essential to high affinity agonist binding (Tomaselli, McLaughlin, Jurman, Hawrot & Yellen, 1991). In addition, the proposed transmembrane (TM)2 and TM4 domains of the α5 sequence include two positively charged residues that, by analogy to studies of the comparable region within α1, may determine ion selectivity and conductance.

These odd features of the α5 sequence as well as the relatively low levels and restricted expression of α5 mRNA (Wada *et al.* 1990) nearly quelled interest in the α5 subunit. Nevertheless, studies of chick ciliary ganglion nAChRs revealed that α5 is co-immunoprecipitated with α3 and β4/β2 subunits (Conroy, Vernallis & Berg, 1992; Conroy & Berg, 1995) and developmental studies demonstrated that the levels of α5 subunit expression are differentially regulated by input and target interactions (Devay, Qu & Role, 1994; Levey, Brumwell, Dryer & Jacob, 1995; P. Devay, D. S. McGehee, C. R. Yu & L. W. Role, unpublished results).

Finally, a potential functional role for α5 was suggested by recent studies demonstrating that heterologous expression of α5 in combination with α4 and β2 or with α3 and β4 subunits yields functional nAChR channels (Ramirez-Latorre, Yu, Qu, Perin, Karlin & Role, 1996; Wang *et al.* 1996; Sivilotti, McNeil, Lewis, Nassar, Schoepfer & Colquhoun, 1997). Furthermore, these nAChRs are characterized by larger conductances and lower apparent affinity for agonist than the α4–β2 or α3–β4 channels. Using α5-targeted antisense oligonucleotide deletion and pharmacological tools, we demonstrate that the α5 subunit is assembled in and is a key functional determinant of specific subtypes of native nAChR channels. In particular, we propose that α5 contributes to the function of two low affinity 50 pS channel subtypes that differ in open kinetics, relative abundance during embryonic development, and, in the inclusion, or the lack thereof, of α7 with α5 in the channel complex.

METHODS

The experimental and analytical procedures used in this study are the same as those given in detail in the preceding paper (Yu & Role, 1998) with the following exceptions. Instead of the α7 sequences we tested two different α5 antisense (AS) sequences, two corresponding 'mismatch' (MM) controls and 1 scrambled sequence, although most experiments employed the following α5 AS and α5 AS mismatch sequences: α5 AS-1, ATG TGA GGA GGA GCA; α5 AS-2, GGC GCG AAG AGG CAT; α5-mismatch, AAG TGT GGA CGA GCA. The externally applied drugs included cytisine, nicotine and DMPP (1,1-dimethyl-4-phenylpiperazinium iodide) and were prepared in external solution.

RESULTS

Our initial tests to determine whether α5 participates in the native nAChR channels expressed by sympathetic neurones employed oligonucleotides designed specifically to target and 'knock out' α5 expression, thereby selectively deleting α5-containing channels. These experiments tested the effect of two different antisense oligonucleotide sequences; both were 15mers targeted at the 5′-end of the α5 sequence and designed to span the translation initiation domain (see Methods in Yu & Role, 1998). Each antisense oligonucleotide alone or in combination (at 10 or 20 μM, respectively) resulted in identical changes in the single channel and macroscopic currents elicited by nicotinic agonists. As neuronal nAChR channels turnover with a half-time ($t_{½}$) of ~22 h and neurones are treated with an irreversible nAChR ligand (bromo-acetylcholine bromide, BAC; see Methods in Yu & Role, 1998; see Gardette, Listerud, Brussaard & Role, 1991) prior to oligonucleotide treatment, the channels assayed after 2–3 days of antisense treatment were primarily from a newly synthesized nAChR pool. Several control oligonucleotides were tested to ensure α5 specificity (see Methods; Listerud, Brussaard, Devay, Colman & Role, 1991; Yu *et al.* 1993).

Contribution of the α5 subunit to apparent agonist affinity of native nAChRs

Heterologous expression studies of the nAChR channels with and without α5 demonstrated that the apparent affinity for agonist was decreased by the inclusion of α5 in the channel complex. One might predict that if a subset of nAChRs expressed by sympathetic neurones includes the α5 subunit, deletion of the α5-containing complexes would enhance the apparent affinity for agonist. Examination of the macroscopic currents elicited by several nicotinic agonists over a 1000-fold range of agonist concentrations confirmed this prediction. The concentration dependence of the cytisine-evoked macroscopic currents in control neurones was compared with the cytisine dose–response curve obtained in neurones treated with α5-specific antisense oligonucleotide (Fig. 1*A*). This 'functional deletion' of α5 resulted in a significant shift of the dose–response curve, revealing an approximately 4-fold increase in the apparent affinity for cytisine in α5 minus neurones compared with control (control *vs.* α5 minus: EC_{50}, 61 ± 18·4 *vs.* 17 ± 8·64 μM). Functional deletion of α5 yielded a qualitatively similar increase in the apparent affinity for ACh (Fig. 1*B*) and nicotine (data not shown). Examination of the concentration dependence of ACh- and nicotine-activated currents in α5 minus neurones reveals a more complex picture; the biphasic dose–response curves obtained when these agonists were assayed probably reflects the expression of more than one type of nAChR. For example, analysis of the EC_{50} values of each of the two components after α5 deletion reveals that one group of residual nAChRs is activated by agonist with ~2-fold higher affinity for ACh than control, and another subset of nAChRs with ~7 times higher ACh affinity than the full compliment of AChRs expressed by control neurones (control *vs.* ACh1 *vs.* ACh2: EC_{50}, 76·5 ± 10·4 *vs.* 47·5 ± 1·47 *vs.* 8·5 ± 2·59 μM; Fig. 1*B*).

Contribution of α5 to apparent agonist efficacy of native nAChRs

Further examination of agonist-gated macroscopic currents revealed that cytisine and ACh differ in the extent of maximal activation of both native nAChRs and nAChRs in α5 minus neurones (i.e. ACh apparently had greater efficacy for nAChR activation than cytisine). Thus, at maximal concentrations of agonist, ACh evoked currents 50 % larger than those elicited by maximal concentration of cytisine (ACh *vs.* cytisine: $I_{peak,max}$, 1398·3 ± 259·1 *vs.* 961·1 ± 313·0 pA; Fig. 2). The functional deletion of α5 further reduced the amplitude of maximal responses to cytisine compared with those in control neurones, suggesting that cytisine-activated currents are composed, at least in part, of α5-containing nAChRs in native neurones (ACh *vs.* cytisine: $I_{peak,max}$, 1753·7 ± 456·1 *vs.* 685·7 ± 62·7 pA; Fig. 2).

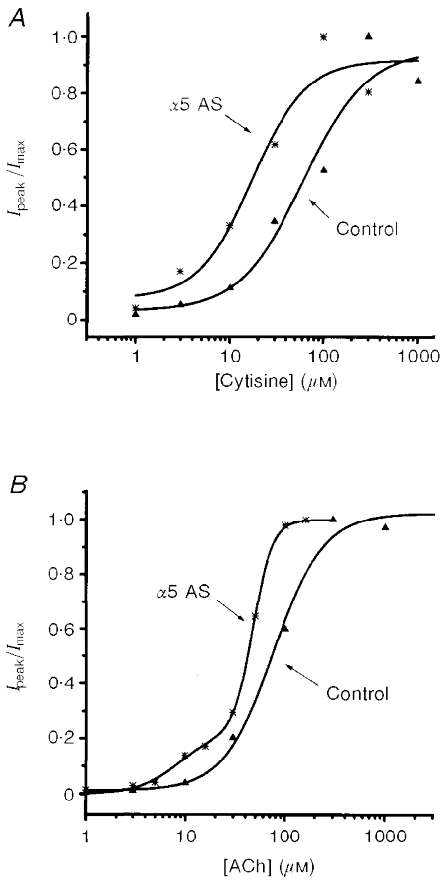

Figure 1. α5 deletion increases the nAChR sensitivity to agonist
The peak amplitude of the currents (I_{peak}) evoked by the indicated concentrations of either ACh or cytisine were measured and normalized to the maximal response (I_{max}) (maximal [ACh], 500 μM; maximal [cytisine], 300 μM). An average of two to seven amplitudes are plotted as a function of the agonist concentration. ▲, data from the control neurones; ✳, data from α5 minus neurones. *A*, the dose–response curve for cytisine in control neurones was fitted as a simple sigmoidal curve indicating an EC_{50} of 61·6 μM and an apparent Hill coefficient of 1·29. In α5 minus neurones the cytisine dose–response curve indicated an EC_{50} of 17·3 μM and an apparent Hill coefficient of 1·57. *B*, dose–response curves to ACh. ▲, data from control neurones; ✳, data from α5 minus neurones. Under control conditions, the concentration dependence of ACh-evoked currents was sigmoidal with an EC_{50} of 76·6 μM and an apparent Hill coefficient of 1·74. Following the deletion of α5, two components of the ACh dose–response curve were resolved: one with an EC_{50} of 8·47 μM and the other with an EC_{50} of 47·48 μM. The apparent Hill coefficient for ACh was 2·63.

Results from the above studies of agonist activation of native nAChRs expressed following the functional deletion of α5 indicate that α5 contributes to the function of at least a subset of the nAChR channels expressed by sympathetic neurones. These results also reveal that the inclusion of α5 in nAChRs yields complexes of generally lower agonist affinity. The observation that α5 deletion differentially affected the efficacy of nAChR activation, dependent on the particular agonist used, stimulates the additional proposal that the inclusion of α5 in nAChR complexes may influence the probability of opening by specific agonist (see below).

We propose that the low efficacy of cytisine in control neurones and its even lower efficacy for α5 minus nAChRs is due to cytisine's preferential activation of α5-containing receptor complexes (i.e. cytisine activates the α5 nAChRs to a greater extent than α5 minus receptors). Finally, the observed increase in the maximal response to ACh in α5 minus neurones compared with control is intriguing and may reveal additional contributions of the α5 subunit to the coupling of agonist binding and nAChR activation. Clearly, single channel recording is required to determine whether ACh-elicited macroscopic currents are smaller in control

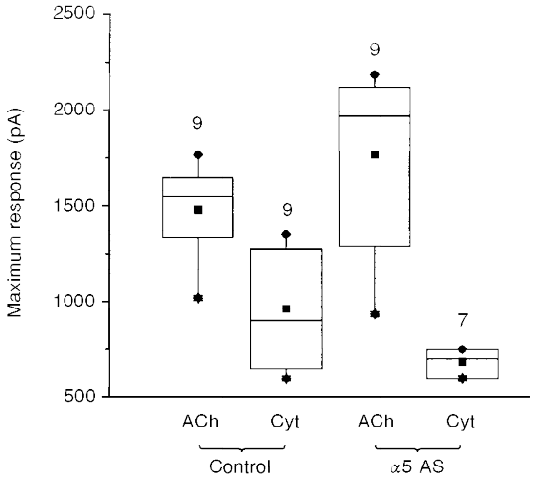

Figure 2. Maximal activation of native nAChRs and α5 minus nAChRs by cytisine is less than that elicited by ACh
The amplitudes of macroscopic currents evoked by ACh and cytisine (Cyt) were compared in control and α5 minus neurones. The amplitudes were analysed by non-parametric techniques and are presented as box plots (see Methods in Yu & Role, 1998). At the maximal concentration of ACh (500 μM) and cytisine (300 μM), macroscopic currents elicited by cytisine were significantly smaller than those elicited by ACh in both untreated neurones ($P \leqslant 0{\cdot}012$) and neurones treated with α5 antisense oligonucleotide ($P \leqslant 0{\cdot}00003$).

neurones than in α5 minus neurones because the latter produce a greater number of native-like nAChR subtypes (i.e. channels of identical subunit composition to native nAChR(s) that do not include α5) or because of the assembly of novel ('mutant') nAChR channels (i.e. complexes whose assembly is not normally favoured) following α5 deletion (see below).

α5 deletion alters the nAChR channel sensitivity to different antagonists

Subsequent comparison of the antagonist profile of nAChRs in control *vs.* α5 minus neurones yielded additional insight into the nature of the nAChR complexes that are activated by ACh but not by cytisine. Specifically, examination of the α7 nAChR-specific antagonists, MLA and αBgTx (see accompanying paper, Yu & Role, 1998) suggested that cytisine, in contrast to ACh, may not activate nAChRs that include the α7 subunit, whether or not these complexes contain α5. Examination of the macroscopic currents activated by cytisine revealed that neither the time course nor the amplitude of the currents was altered by treatment of neurones with MLA (10 nM), α-BgTx (500 nM) or both α7-specific antagonists together (Fig. 3). Particularly striking was the lack of effect of these α7 antagonists on cytisine-elicited currents in α5 minus neurones, where the relative contribution of (only α-BgTx-sensitive) α7-containing nAChRs to the total macroscopic currents activated by ACh was greatly enhanced (see below; Fig. 3). These experiments are consistent with the proposal that although cytisine does activate currents carried by both α5-containing and α5 minus nAChRs, it does not activate either the MLA- or α-BgTx-sensitive α7 nAChRs.

Examination of the antagonist sensitivity of neurones also suggests that, in the absence of α5-containing channels, a greater percentage of the ACh-evoked macroscopic currents is carried by the α-BgTx-sensitive α7 nAChRs (but not the MLA-sensitive α7 nAChRs), compared with control (Fig. 4 and 5; below and accompanying paper, Yu & Role, 1998). Thus, comparison of both the amplitudes and time courses of ACh-elicited currents of control *vs.* α5 minus neurones revealed a significant increase in the α-BgTx-sensitive components in ACh-elicited currents in α5 minus neurones. In fact, the increase in the α-BgTx-sensitive component was sufficiently large that α-BgTx-induced decrease in the peak as well as in the decay rate was readily detected (i.e. I_{peak}: ACh alone, 992 ± 41 pA; ACh alone for α5 minus neurones, 1807 ± 593 pA; ACh + α-BgTx for α5 minus neurones 1139 ± 450 pA; Fig. 4). This observation suggests that the α-BgTx-sensitive channels constituted a greater percentage of the total functional receptor pool following antisense-mediated deletion of the α5 subunits. Single channel studies (below) revealed that this increase in α-BgTx-sensitive nAChRs was largely due to an increased contribution of the 18 pS α7-containing nAChR that is also expressed in normal control neurones rather than the expression of mutant channels.

Functional deletion of α5 also altered the profile of MLA-sensitive ACh-evoked currents. Under control conditions, nanomolar concentrations of MLA significantly decreased the open probability of a 35 pS channel and decreased the amplitude of ACh-evoked macroscopic currents by up to 60% (see accompanying paper, Yu & Role, 1998). In contrast, neither the single channel nor the macroscopic currents elicited by ACh from neurones treated with α5-specific antisense oligonucleotides were sensitive to MLA block (Fig. 5). This result suggests that the α5 subunit is required for the expression of MLA-sensitive nAChR channels. In view of previous demonstrations that the MLA-sensitive nAChRs include α7 (Ward, Cockcroft, Lunt, Smillie & Wonnacott, 1990; Alkondon & Albuquerque, 1993; see accompanying paper, Yu & Role, 1998), we propose that the native nAChRs expressed by sympathetic neurones include nAChR channels that contain both the α5 and α7 subunits.

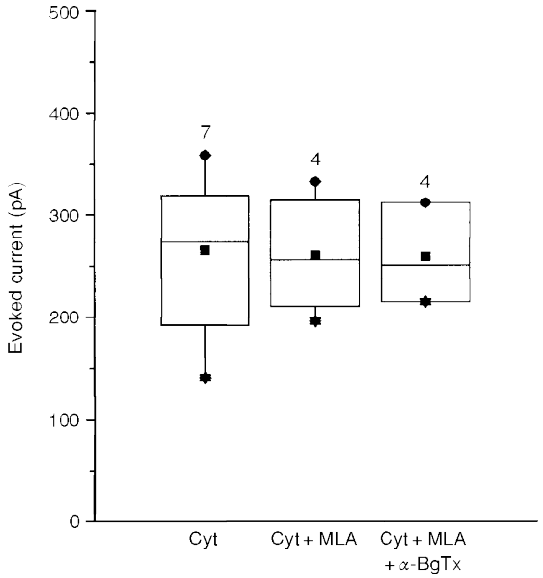

Figure 3. The α5 subunit is required for cytisine gating of α7-containing nAChRs in sympathetic neurones

The amplitudes of cytisine (300 μM)-evoked currents in α5 minus neurones were measured and compared with responses obtained in these neurones following treatment with MLA (10 nM), or with α-BgTx (500 nM) and MLA (10 nM). Whereas α7-containing nAChRs gated by ACh were sensitive to block by α-BgTx and MLA (see Fig. 4 and Fig. 4 in preceding paper, Yu & Role, 1998), currents activated by cytisine in α5 minus neurones were not. Currents elicited by cytisine, following treatment of neurones with α5 antisense oligonucleotides, were unaffected by either α-BgTx or MLA.

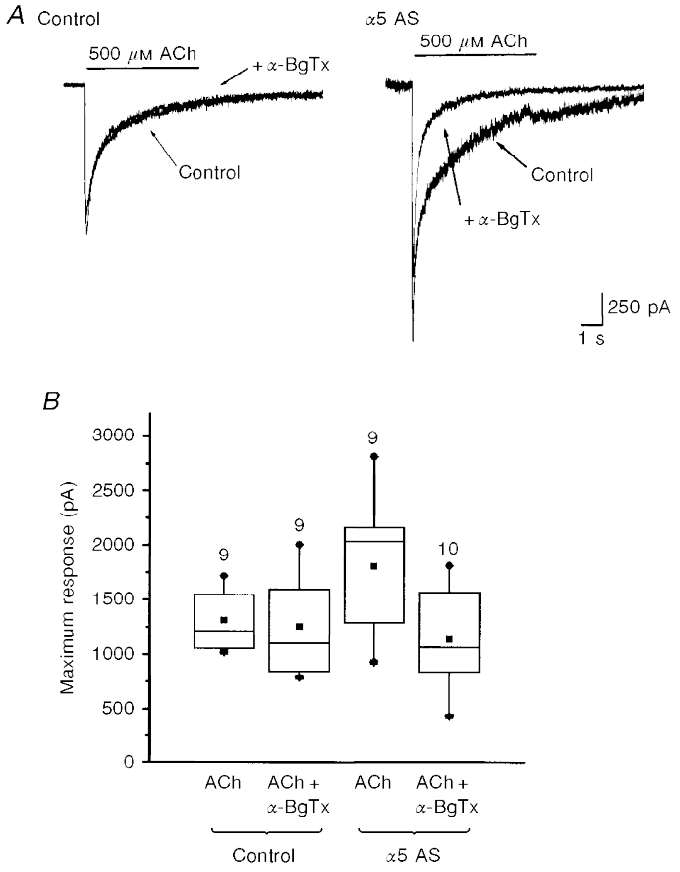

Figure 4. ACh gates larger macroscopic currents characterized by a greater proportion of α-BgTx-sensitive nAChRs in α5 minus neurones compared with control

A, representative macroscopic currents evoked by 500 μM ACh in control *vs.* α5 minus neurones are shown. Macroscopic currents evoked by ACh included only a small α-BgTx-sensitive component, as the amplitudes of currents elicited by maximal concentrations of ACh were not significantly affected by α-BgTx in native sympathetic neurones (see also Fig. 1 of the accompanying paper, Yu & Role, 1998). In contrast, α-BgTx significantly decreased the peak as well as increased the rate of decay of ACh-elicited currents in α5 minus neurones. *B*, the peak amplitude of the many such currents recorded (numbers shown on top of each box) under the conditions detailed are shown as box plots for comparison. The effect of α-BgTx on the amplitude of ACh-evoked currents in α5 minus neurones is statistically significantly ($P \leqslant 0{\cdot}013$).

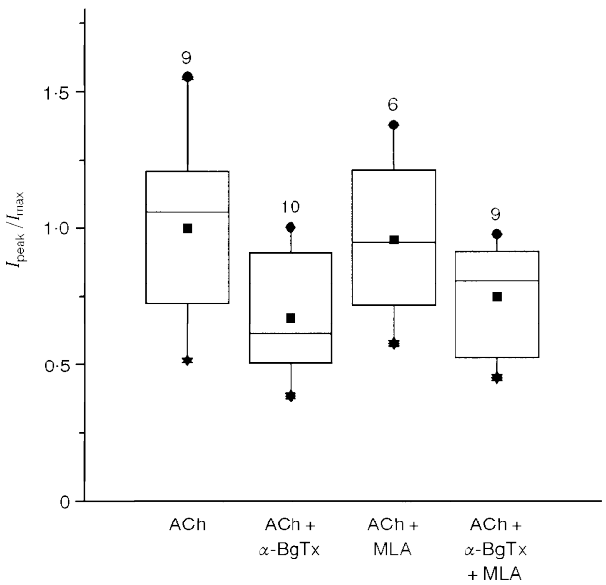

Figure 5. The nAChR sensitivity to MLA is abolished following α5 deletion

Macroscopic currents were elicited by maximal concentrations of ACh and the amplitude and time course determined as described in Methods in Yu & Role (1998). Assessment of the amplitude of the macroscopic currents elicited by ACh following treatment with α-BgTx, or MLA, or with both α-BgTx and MLA revealed that, in contrast to native nAChRs, receptors in α5 minus neurones were not blocked by MLA. One-way ANOVA reveals significant inhibition of peak ACh-evoked currents by α-BgTx alone ($P \leqslant 0{\cdot}005$), and by α-BgTx + MLA ($P \leqslant 0{\cdot}05$) while MLA alone has no significant effect.

Compensatory changes in nAChR profile following subunit-specific deletion

Previous studies (Listerud *et al.* 1991; Yu *et al.* 1993) have revealed that even short term 'knock out' or 'knock down' of specific subunits by antisense oligonucleotides can produce compensatory changes in the profile of receptor expression. Thus the deletion of α3 with antisense oligonucleotides results in a more prominent role for the α7-containing nAChRs. Likewise, experiments involving the deletion of α5 reveal compensatory changes in the channel profile, including enhanced sensitivity of the newly produced α5 minus nAChRs to the α7-specific antagonist α-BgTx (Fig. 4). Taken together, these results suggest that decreasing the contribution of a particular subunit to the assembly pool might increase the extent of incorporation of other subunits not usually 'favoured' for assembly into surface nAChR complexes. The observation that deletion of a particular subunit resulted in the deletion of specific receptor subtypes (as one might predict) but could also involve perhaps compensatory increase in the expression of other channels certainly complicates the analysis of 'knock out' studies. Extensive characterization of the channels is required to determine whether the methods have included the assembly of entirely novel complexes. Channels that persist or are

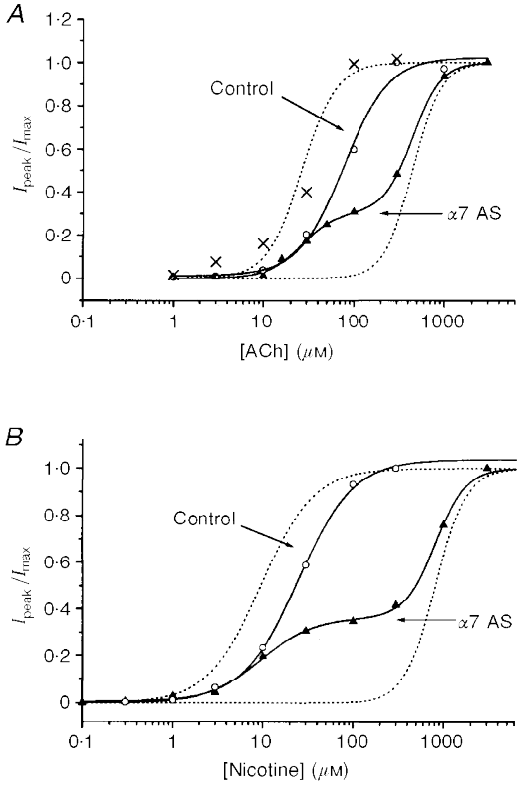

Figure 6. Further examination of the contribution of α5 to agonist–nAChR interactions: increased contribution of α5 nAChRs following α7 deletion

The normalized peak amplitudes of the agonist-evoked responses from control and α7 antisense oligonucleotide-treated, and (α5 + α7) antisense-treated neurones are plotted against the concentration of ACh. *A*, ACh-elicited macroscopic currents in control neurones (○) are fitted by a single sigmoidal curve with an EC_{50} of 76·6 μM, ACh-elicited responses in α7 minus neurones (▲) are fitted by a biphasic curve with apparent affinity for ACh of 26·6 μM and 443·3 μM, respectively. The apparent Hill coefficients of these components are 2·34 and 2·78, respectively. The calculated fits to the two components are indicated by the dotted lines. To test our hypothesis that low agonist affinity nAChRs include the α5 subunit, α5 was deleted from control (see Fig. 1) and α7 minus neurones. Concentration dependence of ACh-evoked responses in neurones treated with both α5 and α7 antisense oligonucleotides (×) is fitted by a single sigmoidal curve with an EC_{50} of 37·8 μM and an apparent Hill coefficient of 2·58. Additional deletion of α5 from the α7 minus neurones removes all the low agonist affinity nAChRs. *B*, the concentration dependence of the nicotine-evoked response in α7 minus neurones also revealed the increased contribution of a low affinity component. Nicotine-elicited currents in control neurones are fitted by a single sigmoidal curve with an EC_{50} of 24·4 μM and an apparent Hill coefficient of 1·44. The biphasic dose–response curve of nicotine-elicited currents from 'α7 minus' neurones indicates a high apparent affinity component with an EC_{50} of 9·52 μM and a very low apparent affinity component with an EC_{50} of 811·9 μM. The apparent Hill coefficients for these components are 1·49 and 2·58, respectively. Dotted curves indicate calculated fits to the two components.

biophysically identical to native nAChRs, as well as mutant channel subtypes, are found. In any case, the upregulation of 'native-like' channels can be exploited to better assess possible functional contributions of the component subunits. Thus, deletion of α5 not only decreases the contribution of MLA-sensitive, α7-containing nAChRs but also increases the proportion of α-BgTx-sensitive nAChRs with the same conductance and kinetic properties as those detected under control conditions, an observation exploited for further study of α7-containing nAChR subtypes (see preceding paper, Yu & Role, 1998). Likewise, experiments from the accompanying study suggest that α7 antisense-treated neurones might provide a similar opportunity to further characterize α5-containing complexes. Analysis of the agonist dose–response characteristics of α7-deleted neurones reveals a biphasic curve that can be fitted by the function:

$$I(x) = 1 - A_1(1 + (x/k_1)^{n_1})^{-1} - (1 - A_1)(1 + (x/k_2)^{n_2})^{-1},$$

where k_1 and k_2 are the apparent affinity constants for the high and low affinity components of the dose–response curve, respectively, and n_1 and n_2 are the Hill coefficients for the corresponding portions. ACh-evoked macroscopic currents in control neurones are characterized by a monophasic (sigmoid) dose–response relationship with an EC_{50} of 77 μM. Analysis of data from α7 minus neurones yields estimated EC_{50} values of $26{\cdot}6 \pm 0{\cdot}06$ and $443 \pm 0{\cdot}36$ μM (Fig. 6*A*, dotted curves). Similar biphasic curves are obtained with analysis of the dose–response data to nicotine. Using the same function, we obtain EC_{50} values of $9{\cdot}52 \pm 1{\cdot}29$ and $811{\cdot}92 \pm 47{\cdot}88$ μM for the two different phases (Fig. 6*B*, dotted curves).

Consideration of these data in conjunction with (a) the effect of α5 deletion on agonist dose dependence, and (b) the effect of heterologous co-expression of α5 with other α- and/or β-subunits, leads us to predict that the low affinity portion of the dose–response is composed of α5-containing receptors (see below). Furthermore, the convergence of studies of α3, α5 and α7 suggests that the nAChRs that underlie the high affinity portion of the agonist dose–response curves include channels composed of only α3 with β4 and/or β2. To test the hypothesis that a 'pure' α3/β nAChR population could account for the relatively high affinity component, we examined the dose dependence of ACh-evoked currents after deletion of both α5 and α7 subunits (Fig. 6*A*, crosses). Following concurrent deletion of α5 and α7, the dose–response relations of the currents were fitted by a single sigmoid curve, with an EC_{50} ($37{\cdot}8 \pm 6{\cdot}0$ μM) similar to that calculated for the high affinity portion of the α7 minus ACh dose–response curve. Note that the low affinity portion of the α7 minus dose–response curve that we attributed to α5-containing nAChRs was, indeed, removed by deletion of the α5 subunit.

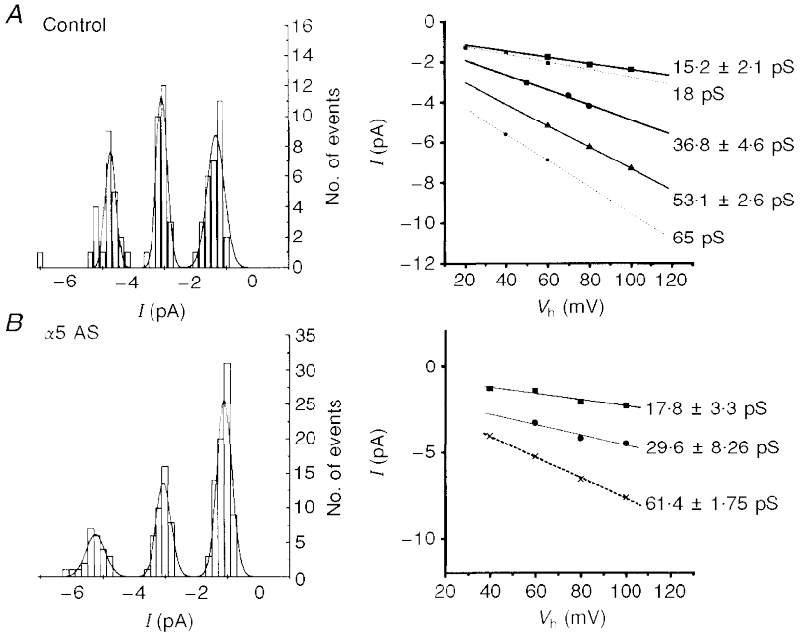

Figure 7. α5 deletion removes all of the native 50 pS conductance channels

Single channel recording from control and α5 antisense oligonucleotide-treated neurones (cell attached configuration; 2·5 μM ACh). *A*, left panel, all point histograms of ACh-gated channels in patches from control neurones ($V_h = +60$ mV). Right panel, determination of the slope conductances of the current amplitude detected from the all point histogram at distinct holding potentials indicates three dominant classes of channels (15, 35 and 50 pS) with less frequent events of ~18 pS channels. *B*, all point histogram (left) and slope conductance determination (right) of ACh-gated channels in patches from α5 minus neurones. The patches are dominated by 18, 35 and 65 pS channels with less frequent openings of the 15 pS class. The 50 pS conductance was never detected in α5 minus neurones.

Single channel recordings suggest the participation of α5 in the 50 pS channels

To examine the functional contribution of α5 to the properties of nAChR channels in detail, we assayed the single channel profile of ACh-evoked currents with and without treatment of neurones with α5-specific antisense oligonucleotides. Under control conditions, the channel profile typically includes 15 and 35 pS channels as well as both the long and short burst kinetic 50 pS channels (Fig. 7). In addition, the 18 and a 65 pS channel are detected, albeit at considerably lower open probability (see accompanying paper, Yu & Role, 1998). In all single channel recording experiments on α5 minus neurones, three prominent subtypes were seen. The usually rare 18 pS channel was reliably detected (slope conductance, 17·8 ± 3·3 pS). A 30 pS channel (rather than the 35 pS MLA-sensitive channel) is also detected in these neurones, reminiscent of channels of the same conductance detected in neurones at early developmental stages. In no cases were the 50 pS channel subtypes (either short or long) detected, although a 60 pS channel not typically seen in control neurones was detected ($n = 6$). These results indicate the inclusion of the α5 subunit in (at least) both the brief and prolonged burst, native 50 pS channels.

DISCUSSION

The foremost conclusion of this study is that the α5 subunit participates in a specific subset of nAChR channels normally expressed by embryonic sympathetic neurones. Treatment of embryonic sympathetic neurones with two different α5 antisense oligonucleotides alters both the biophysical and pharmacological profiles of nAChR; sibling neurones treated with three base mismatch oligonucleotides are unaffected. These results constitute strong support for the participation of the α5 subunit in native nAChR channels and further indicate a major functional role for α5 in nAChRs. Thus, analysis of dose–response curves for ACh, nicotine and cytisine demonstrates that the participation of the α5 subunit decreases the apparent agonist affinity of nAChRs. The latter conclusion is consistent with data obtained from heterologous coexpression of α5 with other α and β nAChR subunits (Ramirez-Latorre *et al.* 1996; Wang *et al.* 1996; Sivilotti *et al.* 1997).

Analysis of the characteristics of the agonist and antagonist dose–response curves in neurones subjected to antisense-mediated deletion of both α5 and α7 yields further insight into how the inclusion of α5 in nAChR complexes modifies the properties of agonist–receptor activation. Thus, deletion of α7 alone yields a biphasic dose–response curve indicative of the expression of two populations of nAChRs, which are distinct in their apparent affinity for agonist and in the derived Hill coefficients of agonist binding. Deletion of α5 from such 'α7 minus' neurones removes the low affinity portion of the dose–response curve, indicating the participation of α5 in a population of low affinity nAChRs.

The magnitude of the macroscopic currents activated by maximal concentration of agonists is also altered following the deletion of α5, indicating that the participation of the α5 subunits in nAChRs alters the efficacy of nAChR activation by agonist. Although the deletion of α5 subunits removes a subset of the native nAChRs (i.e. both the long and short burst 50 pS channels), the macroscopic currents elicited by ACh are, on average, 25% larger in α5 minus neurones compared with control (i.e. mismatch oligonucleotide-treated and native neurones).

Based on comparison of the conductance, kinetics and open probability of nAChRs in control and α5 minus neurones, the increase in magnitude of the ACh-elicited macroscopic currents following α5 deletion is probably due in part to an increase in the number of 18 pS (α-BgTx-sensitive) channels. This nAChR subtype is also detected in native sympathetic neurones, although at lower frequency. Maximal concentrations of cytisine activate substantially smaller macroscopic currents than ACh, in both control and α5 neurones. Whether the lower apparent efficacy of cytisine compared with ACh is due to a relatively low level of cytisine-gating of nAChRs that include α7 and/or a particular β-subunit in a heteromeric complex is less clear. Some studies indicate that activation by cytisine is determined by the β- rather than the α-subunit (Luetje & Patrick, 1991; Papke & Heinemann, 1994), nevertheless, for reasons discussed below, we favour the hypothesis that the cytisine-elicited currents are mediated by α3-, β- and α5-containing nAChRs, as well as by nAChRs without α5, but not by the native heteromeric nAChR complexes that include α7. The principal conclusion we draw from these studies of the function and pharmacological profile of nAChRs is that α5 participates in native nAChRs and that α5 modifies the agonist-dependent activation of the receptors in which it is included.

Our studies of nAChR agonists and antagonists in control and α5 minus neurones as well as our studies with α5 and/or α7 deletion stimulate further proposals relative to the role of α5 and α7 in native nAChRs. First, we propose that α5 participates in two low agonist affinity nAChR channels characterized by a unitary conductance (γ) of ~50 pS that differ in channel open kinetics (γ1, 50 pS: 'brief' or 'fast', $\tau \approx 3$ ms; γ2, 50 pS: 'long' or 'slow', $\tau \approx 12$ ms). The fast 50 pS channel is normally detected in sympathetic neurones from early stages of embryonic development, whereas the slow 50 pS nAChR is detected at later developmental stages. At the intermediate stage of development studied here, both the brief and long 50 pS channels are expressed.

Our second proposal draws from these and our previous studies examining the effects of single α-subunits on the nAChR subtypes expressed by sympathetic neurones (Listerud *et al.* 1991; Yu *et al.* 1993; and accompanying paper, Yu & Role, 1998). Specifically, we propose that all native complexes include α3. They may also include α5 or α7, or both. Finally, we propose that neither the 18 pS

subtype nor the slow, 50 pS subtype is activated by cytisine; these subtypes are blocked by α-BgTx and MLA, respectively. The rationale underlying these proposals and additional tests of the hypotheses are considered in detail below and in the Appendix (see also Table 1 in the Appendix).

Our proposed biophysical profile of the major α5 nAChRs is based primarily on single channel recordings from control *vs.* α5 minus neurones. Thus both the brief and long open duration 50 pS channels that are normally expressed by developing chick sympathetic neurones most probably include α5 subunits since channels of this conductance are never detected in neurones treated with α5-specific antisense oligonucleotides.

The loss of sensitivity to MLA following selective deletion of α5 was a surprise, as MLA has long been considered to be only α7 specific. Since deletion of α5 also abolishes the sensitivity of ACh-elicited currents to nanomolar concentrations of MLA, yet increases the percentage of current blocked by α-BgTx (Fig. 4), α5 may be required for MLA inhibition of α7-containing heteromeric nAChRs. Alternatively, α5 subunits may not participate directly in α7-containing nAChRs, but may rather influence nAChR assembly so that the formation of the MLA-sensitive nAChRs is favoured. Indeed, immunoprecipitation studies by Berg and colleagues did not detect nAChR complexes that include both α5 and α7 subunits although the large intracellular pool of nAChRs could obscure the contribution of such a combination to the functional (surface) pool of nAChRs (Pugh, Corriveau, Conroy & Berg, 1995). Some support for the participation of α5 as well as α7 in MLA-sensitive channels is provided by recent experiments on expression of 'SCAM'-tagged α5 and α7 nAChR channels in *Xenopus* oocytes (Ramirez-Latorre *et al.* 1996; G. Crabtree, J. Ramirez & L. W. Role, unpublished observation). Finally, data presented in this and the accompanying paper (Yu & Role, 1998) indicate that although a 35 pS channel persists following the deletion of either α5 or α7, the MLA-sensitive 35 pS channel in native neurones is eliminated by deletion of either the α5 or the α7 subunit. We therefore suggest that, despite the apparent conflict with biochemical data, native sympathetic neurones express heteromeric nAChRs that include both α5 and α7.

The increase in the magnitude of ACh-evoked currents, as well as the increased contribution of an α-BgTx-sensitive component to the total macroscopic currents after deletion of α5 suggests that α7-containing nAChRs comprise a greater proportion of the nAChRs expressed in α5 minus neurones. Furthermore, single channel recording from α5 minus neurones reveals a significant increase in the number of 18 pS openings as well as the appearance of a 60 pS channel. The kinetic profile of the 18 pS channels in α5 minus and the increase in the α-BgTx-sensitive component of ACh-elicited macroscopic currents, are consistent with an increased level of expression of a channel identical to the native 18 pS α-BgTx-sensitive nAChR subtype. On the other hand, because of the low opening frequencies, we cannot adequately assess and compare the properties of native ~65 pS nAChRs with those of the ~60 pS channels detected after α5 deletion; the latter may be one of the 'mutant' channels, sometimes detected in 'knock out' experiments (see below).

A more prominent contribution of α7 subunit-containing nAChRs could also explain the low efficacy of nAChR activation by cytisine in α5 minus neurones. As shown in Fig. 3, neither α-BgTx nor MLA blocked cytisine-evoked responses, consistent with our proposal that cytisine does not activate the α7-containing heteromeric nAChRs

Our studies also indicate that the nAChR channels that remain following α7 deletion include α5-containing nAChRs. The 50 pS channels that can be detected in a subset of patches following α7 deletion include only the brief events; long duration 50 pS events are absent from all 'α7 minus' patches. The simplest interpretation of this finding in conjunction with the absence of both the 'brief" and the 'long' duration 50 pS channels in α5 minus neurones is that all native 50 pS nAChRs include α5, and that only the long duration 50 pS nAChRs include both α5 and α7.

Finally, as most of our proposals depend heavily on antisense-mediated subunit deletion, we must stress that the specificity of this approach has been assessed under numerous control conditions in each experiment. First, each antisense sequence employed was compared with corresponding sense, missense (scrambled) and/or mismatch (three bases in 15mers) sequences. In addition, neurones treated with the subunit-targeted oligonucleotides as well as the control sequences were screened for non-specific effects including possible changes in neurite outgrowth, cell viability or electrophysiological profiles. Biophysical properties tested include changes in GABA response, voltage-gated sodium, potassium and calcium currents. Several oligonucleotides directed against specific (and distinct) regions of the α5 or α7 subunit genes, as well as multiple control oligonucleotides, yielded consistent results. None of the various control oligonucleotides used (six distinct control sequences including three base mismatch antisense oligonucleotides, random oligonucleotides, or sense orientation oligonucleotides) had any effect on nAChR function or altered any of the other cellular or physiological properties screened.

In addition to these important procedural controls, the interpretation of 'knock out' or 'knock down' type experiments in general, and of antisense oligonucleotide-mediated deletion experiments, in particular, is subject to considerable constraint. The first and most obvious is that the analysis of protein function from any gene deletion technique is only as accurate as the assays used. For example, treatment with α3 or α7 antisense appears to have little effect when only macroscopic responses at relatively high concentrations are examined. Assessment of the functional contribution of each nAChR subunit to native complexes requires analysis of the concentration dependence

of agonist and antagonist sensitivity, as well as examination of the activation, inactivation and desensitization kinetics of both the macroscopic and single channel currents. Another important caveat to the interpretation of antisense 'knock out' (as well as genetic knock out experiments) is the possible contribution of compensatory mechanisms that may alter assembly, synthesis and insertion of related subunits. For example, the deletion of the α7 subunits shifts the agonist dose–response curves. Possible interpretations of this finding are as follows. (a) α7 normally participates in high affinity channels that are deleted by α7 antisense treatment. (b) The deletion of the α7 subunit results in increased synthesis and/or assembly of low affinity nAChRs that are identical to a subset of native nAChRs, i.e. equivalent to nAChRs normally expressed, although at a lower level, in control neurones. (c) The deletion of the α7 subunit results in the synthesis and/or assembly of mutant low affinity nAChRs, i.e. nAChRs that are never detected in untreated (control) neurones. Detailed characterization of receptor profiles is clearly required to distinguish between these possible interpretations. In fact, subsequent analysis of the nAChRs in α7 minus neurones reveals significant contributions of both the native-like (low affinity α5) nAChRs as well as the expression of novel mutant nAChRs. Likewise, the deletion of α5 alone increases the proportion of the ACh-evoked current that is blocked by α-BgTx; subsequent analysis indicates that this largely results from a significant and selective increase in the α-BgTx-sensitive 18 pS channel. In contrast, the MLA-sensitive α7 nAChRs detected in native neurones are entirely absent from α5 minus neurones.

A final word of caution; such 'compensatory' changes may only be evident from functional analyses; we (and others) failed to detect changes in the level of mRNA or protein of any subunit other than the one that was specifically targeted. The mechanism by which the decreased expression of one subunit leads to an increased contribution of others to functional oligomeric complexes is not clear. It is possible that when the synthesis of one subunit is attenuated by targeted deletion, the altered ratios between subunits within a putative 'assembly pool' could alter the profile of nAChRs subsequently produced. In this case, the newly expressed nAChRs may reflect increased assembly of nAChR complexes that are normally expressed (albeit at relatively low level) and/or the assembly of entirely novel, or 'mutant' complexes. Our data, from both CNS and PNS neurones, suggest that both occur (Listerud *et al.* 1991; Yu *et al.* 1993; Brussaard, Yang, Doyle, Huck & Role, 1994).

The goals of the studies described in this and the preceding paper (Yu & Role, 1998) were: (1) to determine whether α5 and/or α7 participate in native nAChRs expressed by embryonic sympathetic neurones, (2) to determine the functional contribution of these subunits to nAChR channels expressed by sympathetic neurones during embryonic development and (3) to further our understanding of the subunit composition of the native nAChRs. Based on our findings, we conclude that both α5 subunits and α7 nAChRs contribute to native nAChRs in sympathetic neurones. We also conclude that the α5 subunit significantly alters the properties of agonist binding and activation of nAChRs in which it is included. Further, we propose that α5 participates in both kinetic classes of the 50 pS channels expressed by developing sympathetic neurones, which are equivalent to the L10 and L17 channels previously reported (Moss *et al.* 1989; Moss & Role, 1993). In addition, we propose that a subset of α5-containing channels are heteromeric complexes that also include α7 and are blocked by MLA (results from this and the preceding paper, Yu & Role, 1998).

Finally, we propose that the biophysical and pharmacological properties of native nAChRs in sympathetic neurones are largely (but not exclusively) determined by the participation of specific subunits in the nAChR complex. The distinct properties of these channel subtypes suggest that physiologically distinct nAChRs play very different roles in synaptic transmission. Thus, the participation of the α5 subunit yields lower affinity but large conductance nAChRs. Both α5 and α7 apparently participate in the 50 pS long burst channel. In this regard it is particularly striking that modelling of synaptic current and the synaptic potential profiles of embryonic neurones indicates that it is the expression of the long burst, 50 pS channel (L17) that exerts the greatest influence on whether a sympathetic neurone will or will not fire an action potential in response to a given presynaptic input (i.e. ACh) (D. McGehee, L. Role & A. B. Brussaard, unpublished results). Immunohistochemical confocal studies (Sargent & Pang, 1989; Horch & Sargent, 1995) as well as studies of synaptic transmission also implicate α5- and α7-containing nAChRs at ciliary ganglion synapses.

APPENDIX

This Appendix presents our proposed model for nAChRs expressed during the embryonic development of chick sympathetic neurones. The model designates the physiological and pharmacological profiles, as well as proposing possible subunit compositions, for each of the native nAChR channel subtypes detected. Although the model is highly speculative, it attempts to integrate current findings with those from numerous other laboratories, with particular focus on previous studies of ganglionic nAChRs (Mathie, Cull-Candy & Colquhoun, 1987, 1991; Margiotta & Gurantz, 1989; Anand, Conroy, Schoepfer, Whiting & Lindstrom, 1991; Halvorsen, Schmid, McEachern & Berg, 1991; Jacob, 1991; Listerud *et al.* 1991; Conroy *et al.* 1992; Anand, Peng, Ballestra & Lindstrom, 1993*a*; Anand, Peng & Linstrom, 1993*b*; Vernallis, Conroy & Berg, 1993; Covernton, Kojima, Sivilotti, Gibb & Colquhoun, 1994; Devay *et al.* 1994; Connolly, Gibb & Colquhoun, 1995; Conroy *et al.* 1995; De Koninck & Cooper, 1995; Mandelzys, De Koninck & Cooper, 1995). The model describes the properties of seven distinct, native nAChR channels previously characterized in these sympathetic neurones at two extremes of synaptic

development (i.e. 'early', S10, M10, L10; 'late', S17, M17, L17 and XL; see Moss *et al.* 1989; Moss & Role, 1993). We propose that the seven channel subtypes detected at the intermediate developmental stages examined in this and previous studies are distinguishable from one another on the basis of their conductance, kinetics, activation, inactivation and desensitization properties as well as by distinct agonist and antagonist pharmacology. In addition, we propose particular subunit combinations (and even go so far as to suggest possible stoichiometries) for each of the nAChR subtypes expressed by embryonic chick sympathetic ganglion neurones.

This model is proposed, first and foremost, as a testable hypothesis for future biochemical, electrophysiological and molecular genetic studies of native nAChR channels. In addition, we present this model to facilitate consideration of the data presented in this and the preceding paper (Yu & Role, 1998) in the context of previous development studies. Our proposals are consistent with most (but not all) of these data and incorporate the following additional considerations.

(a) nAChRs are pentameric and can include two or more α subunits. Although the latter assumption is not consistent with some previous studies of the chick α4–β2 type channels (Anand *et al.* 1991; Cooper, Couturier & Ballivet, 1991), the number of α-subunits present in other heterologously expressed or native nAChRs is not known.

(b) nAChR complexes where the α/β stoichiometry is $>2/3$ are predicted to have greater unitary conductance than those where $\alpha/\beta \leqslant 2/3$. This hypothesis is based on previous studies and proposals relative to the role of specific residues within the M2 sequence of muscle type nAChRs in determining single channel conductance. These key residues are included in all α sequences but not in the M2 domain of either β2 or β4 subunits (Villarroel & Sakmann, 1992; for reviews see also Sargent, 1993; McGehee & Role, 1995).

(c) Our proposal assumes that channels characterized by distinct biophysical and/or pharmacological properties are distinct molecular entities. This assumption is incorporated in the model because each subtype has been detected in isolation without transition to another of the designated subtypes. Furthermore, our analyses have been confined to unitary events with no discernible subconductance transitions. Our model is further constrained by the interpretation of 'distinct molecular entities' as equivalent to differences in subunit composition or stoichiometry. An obvious alternative is that the properties of distinct nAChR channels are determined by differential post-translational modification, as previous studies in many laboratories have demonstrated dramatic effects of post-translational modification on the number, open probability and desensitization of native nAChRs (Magiotta & Gurantz, 1989; Simmons *et al.* 1990). As our model does not address the potential contribution of post-translational mechanisms to the functional characteristics of the nAChR channels, it is, as such, clearly limited.

(d) We also propose that all of the nAChR channels on the somata are heteromeric combinations of α3 and β2 or β4, with or without α5 and/or α7. Although it remains a possibility that α7 homomeric channels are expressed by sympathetic neurones, they have yet to be detected by functional assays (see accompanying paper, Yu & Role, 1998).

(e) Finally, the model assumes that all of the native nAChRs described include α3 in combination with only the α and β subunits delineated in (d), above. That is, the model does not account for the possible inclusion of as yet to be identified nAChR subunits or for the inclusion of α1, α2, α8 or α9 type subunits. Likewise neither α5 homomeric or α5/β nAChRs are proposed. The justification for these additional constraints on the proposed nAChR compositions is as follows. (1) Neither α1, α2 nor α8 mRNA is detected at significant levels in chick sympathetic neurones, even with the use of PCR amplification techniques (Listerud *et al.* 1991; Devay *et al.* 1994). To date, α9 mRNA is reportedly detected only in sensory epithelia (Elgoyhen, Johnson, Boulter, Vetter & Heinemann, 1994), and is therefore considered an unlikely component of nAChRs in sympathetic neurones. (2) Although the potential contribution of as yet unidentified nAChR subunits cannot be excluded, co-ordinate deletion of α3 and α7 eliminates ∼90 % of the ACh-gated currents in chick sympathetic neurones. Examination of the pharmacological and physiological characteristics of the residual ACh-elicited channels (i.e. the small current remaining in 'α3 and α7 minus' neurones) suggests that the current is carried by native nAChR complexes, assembled and/or inserted after elimination of pre-existent surface receptors, but before the antisense-mediated translational block (Listerud *et al.* 1991; Yu *et al.* 1993). (3) nAChRs composed of only α5 subunits or of α5 in combination with β2 and/or β4 are unlikely, as attempts to produce such channels in heterologous systems have been completely unsuccessful (e.g. Ramirez-Latorre *et al.* 1996).

The model is presented in Table 1. The subunit compositions proposed specify the α-, but not the β-, subunit(s) thought to be included in each native nAChR complex, as current and previous functional studies have focused on dissecting the role of the various α-subunits expressed by chick sympathetic neurones (Listerud *et al.* 1991; Yu *et al.* 1993; this and accompanying paper, Yu & Role, 1998). The inclusion of a β-subunit in the hypothetical complexes is therefore designated as β*x*, although the much higher levels of β4 (*vs.* β2) expression in sympathetic neurones may reflect a prominent role of β4. The rationale behind the proposed developmental expression, biophysical and pharmacological properties as well as our justifications for the proposed subunit composition of each channel subtype are detailed below.

The 15 pS channel, with open channel kinetics of ∼1 and 5 ms, is a prominent component of single channel records at early developmental stages, and is equivalent to the subtype previously designated S10 (Moss *et al.* 1989; Moss & Role, 1993). This channel is activated by relatively low

Table 1. Summary of proposed model for nAChR expression in embryonic chick neurones during development

Proposed composition	[1,2]γ (pS)	[1,2]Open duration (ms)	Relative abundance			Pharmacology – relative sensitivity to					Deletion by AS			Prior name
			[1]Early	[2]Int	[1]Late	[1,2]†ACh	[2]Cyt	[1]n-BgTx	[2,3]α-BgTx	[2]MLA	[3]α3	[2]α5	[2]α7	
$(\alpha3)_2(\beta x)_3$	~15	~1 & 5	+++(+)	+++	—	++++	+	+	—	—	y	n	n	S10
$(\alpha3)_1(\alpha7)_1(\beta x)_3$	~18–22	~1 & 5	—	+	++	+++	—	+	+	—	y	n	y	S17
$(\alpha3)_3(\beta x)_2$	~25–30	~1 & 7	+++	++	—	++++	+	+	—	—	y	n	n	M10
$(\alpha3)_1(\alpha5)_1(\alpha7)_1(\beta x)_2$	~35	~1 & 7	—	++++	++++	++	—	+	—/?+	+	y	y	y	M17
$(\alpha3)_2(\alpha5)_2(\beta x)_1$	~50	~3	+	++(+)	*	+	+	+	—	—	y	y	n	L10
$(\alpha3)_1(\alpha5)_2(\alpha7)_1(\beta x)_1$	~50*	~20	—	++	+++	+	—	+	?/—	?	y	y	y	L17*
?	65		—	(+)	(+)	+	?	+	?	?	y	?	?	XI

References: [1]Moss *et al.* (1989) and Moss & Role (1993), [2]This and accompanying paper, Yu & Role (1998) and [3]Listerud *et al.* (1991) and Yu *et al.* (1993). * Note that our previous nomenclature did not distinguish between slow and fast 50 pS channels, i.e. both were designated L17 in ED17 neurones so that the relative abundance of 'L17' refers to both kinetic classes. †The number of +s indicated represent the proposed apparent affinity for ACh with +++ indicating higher affinity than +. The stages of neuronal development are referred to as early (embryonic days (E) 8–10), intermediate (Int, E11–14) and late (>E17). Abbreviations: y, yes; n, no.

concentrations of ACh and nicotine (and is presumed to be activated by cytisine). The 15 pS/S10 channel is sensitive to neuronal bungarotoxin (n-BgTx) but not to α-BgTx. This channel is hypothesized to include only α3 and β subunits as it is deleted by α3-targeted antisense, but not deleted by either α5 or α7 antisense oligonucleotide treatment. The observed gating of this nAChR subtype by low agonist concentrations, its persistence in α5 and/or α7 'minus' neurones, and its lack of susceptibility to block by α7-specific antagonists supports our proposal that the major nAChR expressed by sympathetic neurones early in development is a high agonist-affinity complex composed of only α3 and βx subunits.

The 18–22 pS nAChR subtype also characterized by brief open channel kinetics, is activated by low concentrations of ACh and is more prominent in single channel records at later developmental stages. These features indicate that the 18–22 pS/brief opening channel is equivalent to the previously designated S17 channel, whose expression is regulated by both input and target interactions (Moss *et al.* 1989; Moss & Role, 1993; P. Devay, D. S. McGehee, C. R. Yu & L. W. Role, unpublished results). We propose that 18 pS/S17 channels include α3, α7 and β-subunits, but do not include the α5 subunit, based on macroscopic, single channel and pharmacological experiments in control, α3 minus, α5 minus and α7 minus neurones. The inclusion of α7 in the native complex is supported by the findings that α-BgTx blocks a small, slowly desensitizing, relatively high ACh affinity component of the macroscopic currents as well as the 18 pS channel component of single channel records. The inclusion of α7 in this complex is also consistent with the observed increase in the expression of the 18 pS/S17 channel concomitant with the developmental increase in α7 mRNA and protein (Moss *et al.* 1989; Moss & Role, 1992; Devay *et al.* 1994). The proposal that ACh and nicotine activate this channel, whereas cytisine does not, derives from our observation that cytisine-activated currents do not include an α-BgTx-sensitive component. Finally, the inclusion of α3 but not α5 in the complex is proposed since the 18 pS/S17, α-BgTx-sensitive, nAChRs are eliminated by deletion of α3 and/or α7 but not by the deletion of α5. We note that our proposal for a channel composed of both α3 and α7 does not conform to the results of immunoprecipitation studies of chick autonomic neurones (Pugh *et al.* 1995). This discrepancy is, perhaps, due to the relatively small number of these channels on the neuronal surface; certainly, these complexes do not constitute a significant percentage of the total nAChR pool assayed in biochemical studies, even at later developmental stages (see Table 1, Relative abundance).

We designate the 25–30 pS channels, which are activated by low agonist concentrations and characterized by intermediate burst kinetics (τ, 1 and 7 ms) and are also inhibited by n-BgTx but not blocked by either α-BgTx or MLA, as equivalent to the previously described M10 channel. This designation is consistent with all measured biophysical and pharmacological properties of both channel types as well as the relative abundance of this channel at early *vs.* later stages of development. Our proposed composition for the 25–30 pS/M10 channel is the same as that of the 15 pS/S10 channel, i.e. it includes only α3 subunits in combination with βx. The proposed composition derives from the persistence of this subtype following the deletion of α5 and α7, its removal by α3-targeted antisense oligonucleotides, and the well-known inability of α3 to form homomeric channels. We further postulate, however, that the subunit stoichiometry of the 30 pS/M10 channel is $(\alpha3)_3(\beta x)_2$ and that it is the difference in the ratio of α/β

subunits that accounts for the larger conductance of the M10 (*vs.* S10) subtype. Although the latter postulate is based primarily on theoretical grounds (see (b), above), results of at least some heterologous expression studies are consistent with this idea. Thus, co-injection of various ratios of rat α3 and β4 cRNAs in *Xenopus* oocytes (unlike co-expression of chick α4 with β2: Cooper *et al.* 1991) routinely yields channels of more than one conductance class (Sivilotti *et al.* 1997). Although the results of the heterologous expression studies have frustrated attempts to recapitulate native channels by 'simple' pair-wise expression of α3–β4, these findings may reveal that α3 and β4 subunits can coassemble in complexes of various α:β ratios. Even if one discounts our optimistic interpretation of the heterologous expression data, the distinct biophysical profiles of the 15 *vs.* 30 pS subtypes might best be explained by differences in stoichiometry, as evidence for differences in subunit composition *per se* are lacking, at the moment. Clearly, it is important to test whether differences in post translational modification and/or the inclusion of novel subunits (neither of which are considered in the current model) might better explain the distinct conductance and kinetics of the S10 and M10 subtypes. In addition, we cannot rule out the possibility that the 25–30 pS is only composed of α7 and β*x*, without the participation of α3.

We designate the 35 pS channel, that is characterized by intermediate burst kinetics (τ, 1 and 7 ms) as equivalent to the M17 type channel, that is prominently expressed at later developmental stages (Moss *et al.* 1989; Moss & Role 1993). Other characteristics of the ~35 pS/M17 channel include a relatively low affinity for ACh and nicotine, high affinity block by MLA (yet, a rather puzzling insensitivity to α-BgTx).

Our proposed composition for the 35 pS/M17 channel is that it is a complex of α3, α5, α7 and β*x*, with a putative stoichiometry of $(\alpha3)_1(\alpha5)_1(\alpha7)_1(\beta x)_2$. Although this proposed composition is rather heretical, it best represents the convergence of the physiological studies on embryonic chick sympathetic neurones. The inclusion of the α7 subunit in the 35 pS/M17 channels is based on the removal of this subtype by α7 oligonucleotide treatment as well as the high affinity block by MLA. The inclusion of α3 and α5 in this complex derives from the sensitivity of this channel to n-BgTx, and the deletion of this channel by treatment with either α3 or α5-targeted antisense oligonucleotides. It is difficult to explain the apparent lack of α-BgTx sensitivity of the 35 pS/M17 channel. Experiments examining the effects of α-BgTx on the single channel profile of native nAChRs suggested that the overall frequency of openings of channels in the range of 30–35 pS may be decreased, but the number of events in this conductance range before and after α-BgTx treatment was not sufficient to permit adequate comparison of the conductance and kinetic properties.

Other experiments further distinguish the MLA-sensitive 35 pS channel from the α-BgTx-sensitive 18 pS subtype. Our finding that the MLA-sensitive/35 pS channel was deleted by treatment with α5 (and α7) antisense oligonucleotides was a surprise, particularly in view of the observed increase in the contribution of 18 pS, α-BgTx-sensitive channels following α5 deletion. The simplest interpretation of the differences in conductance, apparent affinity, MLA *vs.* α-BgTx sensitivity, and the deletion (or lack thereof) from α5 minus neurones is that native 35 pS/M17 channels include α5, whereas 18 pS/S17 channels do not. This suggests that in addition to the previously described effects of α5 on single channel conductance and apparent agonist affinity, if α5 is combined with α7 in a nAChR complex it may influence the gating by cytisine, and the susceptibility to block by MLA and α-BgTx. Clearly, the resolution of these questions requires more extensive single channel pharmacology – a proposition sufficient to daunt the most intrepid biophysicists!

The 50 pS nAChR, with burst kinetics described by a single τ of 3–4 ms, matches the properties of the channel subtype seen in about 30% of patches early in development and previously designated L10. The 50 pS/fast τ/L10 type channel was present in most patches at the intermediate developmental stages studied here, typically accounting for ~65% of the 50 pS events (kinetic analysis).

We propose that the 50 pS/fast τ/L10 channels include the α3, α5 and β-subunits, but do not include α7. The inclusion of both α3 and α5 is based on the elimination of this channel subtype by deletion of either of these subunits. The insensitivity of this channel to either MLA or αBgTx, as well as its preservation in neurones treated with α7 antisense oligonucleotides, indicates that α7 is not included in the 50 pS/fast τ/L10 channel. The relatively low agonist affinity of this subtype, manifest in its increased and persistent gating by higher concentrations of ACh in the patch pipette (5–10 *vs.* 2·5 μM), is consistent with the decreased affinity of α5-containing nAChRs (e.g. Ramirez-Latorre *et al.* 1996; Wang *et al.* 1996; Sivilotti *et al.* 1997). The significant increase in the percentage of patches and the number of 50 pS/fast τ/L10 events at later stages of development is also consistent with the relatively low levels of α5 early on and the 5- to 7-fold increase in the mRNA encoding this subunit with innervation and with target contact (Devay *et al.* 1994; P. Devay, D. S. McGehee, C. R. Yu & L. W. Role, unpublished results).

We further hypothesize that the stoichiometry of this channel may be $(\alpha3)_2(\alpha5)_2(\beta x)_1$. The inclusion of two α5 subunits in this complex is proposed to account for the larger conductance and lower agonist affinity of this channel, as compared with the 35 pS/M17 and the 15 and 20 pS (S10 and S17) channels, respectively.

The 50 pS nAChR, with τ of ~12 ms, matches the properties of a channel subtype seen in 40–50% of patches late in development (L17). Although the previous developmental scheme designated both the fast and slow kinetic 50 pS channels as L17, re-examination of these data in light of our current findings suggests that the subtypes

represent distinct molecular entities. Thus, 50 pS channels with a τ of ~12 ms are never detected early in development. In contrast, analysis of all records from neurones at the intermediate and later developmental stages that include 50 pS events revealed a long duration component of the burst distribution, accounting for 30–40% of the 50 pS openings. Most importantly, the slow τ/50 pS events (but not the fast τ 50 pS events) are deleted by treatment with α7 antisense oligonucleotides (see preceding paper, Yu & Role, 1998). Clearly the analysis of the kinetics, as well as the conductance profiles, of channels remaining after α7 deletion is crucial to this proposal. Thus, the burst duration of 50 pS events remaining in approximately 60% of the patches from α7 antisense-treated neurones was fitted by a single exponential with a τ of ~3 ms.

We propose that the 50 pS/slow τ/L17 channels include the α3, α5, and α7 (as well as β-) subunits. The inclusion of α3 and α5 is based on the elimination of this channel subtype by deletion of either of these subunits. The inclusion of α7 is based on the kinetic analysis of 50 pS openings following the deletion of α7 (as described above). Finally, as with the 35 pS channel, our proposal that this complex includes α7, as opposed to the non-α7, non-α1, αBgTx-binding component that co-precipitates with α5 (Pugh *et al.* 1995), is based on the following observations: (a) an α7-specific antisense oligonucleotide deletes the slow τ 50 pS channel, (b) the difficulty in detecting an α7-/α5-containing channel in biochemical assays of total (as opposed to surface) receptor pools, (c) recent demonstrations that α5α7β4-containing channels can form in heterologous expression systems (G. Crabtree, J. Ramirez-Latorre, C. R. Yu & L. W. Role, in preparation) and (d) our failure to demonstrate α-BgTx inhibition of the 50 pS/slow τ channel.

We further hypothesize that the stoichiometry of the 50 pS/slow τ/L17 channel is $(\alpha3)_1(\alpha5)_2(\alpha7)_1(\beta x)_1$ in part because of the features common to both the slow and fast τ 50 pS channels. Thus we suggest that the inclusion of two α5 subunits in both 50 pS complexes accounts for the lower apparent affinity for ACh and the higher conductance of these subtypes relative to the 35 pS/M17 channel.

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Corresponding author

L. W. Role: Department of Anatomy and Cell Biology in the Center for Neurobiology and Behavior, College of Physicians and Surgeons, Columbia University, 722 West 168th Street, Research Annex Room 806, New York, NY 10032, USA.

Email: lwr1@columbia.edu