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Functional Contribution of Neuronal AChR Subunits Revealed by Antisense Oligonucleotides

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Abstract

Although multiple related genes encoding nicotinic acetylcholine receptor (AChR) subunits have been identified, how each of these subunits contributes to AChRs in neurons is not known. Sympathetic neurons express four classes of AChR channels and six AChR subunit genes ($\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 7$, $\beta 2$, and $\beta 4$). The contribution of individual subunits to AChR channel subtypes in these neurons was examined by selective deletion with antisense oligonucleotides. An $\alpha 3$ antisense oligonucleotide decreased the number and altered the properties of the normally expressed ACh-activated channels. The remaining AChR channels have distinct biophysical and pharmacological properties that indicate an important functional contribution of the $\alpha 7$ subunit.

Ligand-gated ion channels are multimeric protein complexes with component subunits encoded by homologous genes (1). The neuronal nicotinic AChRs that mediate neurotransmission at many central and peripheral synapses comprise two subunit types (α and β) encoded by at least 11 related genes (2). Expression of particular combinations of the subunit RNAs in oocytes yields biophysically and pharmacologically distinct channels (3), reminiscent of the multiple AChR channel types detected in embryonic sympathetic neurons (4). However, neither the identity nor the functional contribution of the individual α and β subunits in native AChR channel subtypes is known.

To determine which AChR subunit genes are expressed by sympathetic neurons, we used the polymerase chain reaction (PCR) (5). Primers to the nonconserved regions of the α and β subunit genes were chosen to amplify specific cDNA target sequences (Fig. 1A). Appropriate-sized products were generated in the $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 7$, and β reactions, but not in the $\alpha 2$ reaction (Fig. 1B) (6). Restriction enzyme digestion of the amplified products confirmed their identity. RNA blots of polyadenylated [poly(A)⁺] RNA from sympathetic ganglia under stringent hybridization and washing conditions revealed hybridization to $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 7$, $\beta 2$ (n- α), and $\beta 4$ (n- $\alpha 3$) probes but not to the $\alpha 2$ probe (Fig. 1C) (7). AChR gene expression ranged from undetectable ($\alpha 2$) and low ($\alpha 4$, $\alpha 5$, $\beta 2$) to relatively high ($\alpha 3$, $\alpha 7$, $\beta 4$).

Because sympathetic neurons express multiple types of α and β subunit genes, the AChR channel subtypes observed (4) might result from different subunit combinations. To determine the role of individual AChR subunits in sympathetic neurons, we examined changes in the properties of AChR channels after functional deletion of a particular subunit with antisense oligonucleotides.

The contribution of preexistent surface AChRs was removed by treatment with bromoacetylcholine (BAC), which covalently binds to and inactivates AChRs (8). BAC-treated

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neurons regain ACh sensitivity through synthesis of new AChRs (9). ACh sensitivity was <15% of control at 3 hours and completely restored within 48 hours of BAC treatment (9). Sympathetic neurons were incubated with oligonucleotide immediately after BAC treatment, and the ACh-activated single channel currents were measured after 48 hours (10). Analysis of the number of channel openings and the averaged single channel current from all patches in $\alpha 3$ antisense–oligonucleotide treated neurons indicates a >90% decrease from the control levels of AChR channel activity typically evoked by 2.5 μM ACh (Fig. 2) (11). Although the number of AChR channel openings under these conditions was too low for slope conductance determinations, the residual activity included openings at amplitudes consistent with the characterized AChR subtypes (designated S, M, L, and XL) (4). An $\alpha 4$ antisense (Fig. 2) as well as several control oligonucleotides (10) did not affect AChR channel opening frequency, mean current, conductance, or desensitization kinetics. In view of these results and the lack of effect on other ligand or voltage-gated channels (10), it seems likely that $\alpha 3$ antisense reduces AChR channel activity by a specific functional deletion of the $\alpha 3$ subunit.

The observed decrease in AChR channel activity gated by 2.5 μM ACh is apparently due to both an increase in the ACh concentration required for channel activation and a decrease in the number of functional AChRs (Figs. 3 and 4). In patches from control neurons, higher concentrations of ACh (25 μM) gate all four AChR channel classes (4), but these channels desensitize rapidly (Fig. 3, A through C). In contrast, in patches from neurons treated with $\alpha 3$ antisense, 25 μM ACh elicited persistent AChR single channel activity (Fig. 3F). Analysis of AChR openings in $\alpha 3$ antisense–treated neurons revealed two conductance classes similar to those in control neurons and, in addition, a conductance class of ~ 115 pS (Fig. 3 D, E). All of the channels in $\alpha 3$ antisense–treated neurons differed from control in requiring a higher concentration of ACh for their activation and in their slower desensitization in the continued presence of ACh.

Examination of macroscopic currents evoked by maximal concentrations of ACh reveals a significant decrease in the total number of functional AChRs in $\alpha 3$ antisense–treated neurons compared with control neurons ($\sim 60\%$ decrease in the average peak current; Fig. 4, A and C). This analysis underestimates the decrease in AChRs, however, because of significant slowing of AChR desensitization after $\alpha 3$ antisense treatment (12). High concentrations of ACh normally evoke currents that decay with a biphasic time course (Fig. 4A). In contrast, in neurons treated with $\alpha 3$ antisense, macroscopic currents evoked by the same concentration of ACh desensitized at a slower rate reflected in a significant increase in both the fast and slow time constants of desensitization (Fig. 4, A and D).

We next sought to identify which α subunit might contribute to the new, slowly desensitizing currents evoked by ACh in neurons where $\alpha 3$ had been functionally deleted. Because the $\alpha 5$ subunit gene fails to yield functional AChR channels when coexpressed in oocytes with either $\beta 2$ or $\beta 4$ genes (13), $\alpha 7$ seemed the best candidate for the ligand-binding subunit in the novel AChRs expressed by neurons treated with $\alpha 3$ antisense. The role of $\alpha 7$ in the AChRs was tested two ways. The $\alpha 7$ subunit, unlike $\alpha 3$, is blocked by α -bungarotoxin (α -BGT) (14). If $\alpha 7$ is an important component of the AChRs, then the slowly desensitizing current in neurons treated with $\alpha 3$ antisense should be eliminated by α -BGT. Similarly, oligonucleotide-mediated deletion of $\alpha 7$ should have quantitatively the same effect as α -BGT treatment, if $\alpha 7$ participates in these AChRs. Both these predictions are supported by the experiments in Fig. 4.

ACh-activated currents in control neurons were unaffected by α -BGT. However, in $\alpha 3$ antisense–treated neurons the toxin decreased the peak of macroscopic currents by 60 to 70% (Fig. 4, A and C). The residual, α -BGT insensitive current was blocked by neuronal bungarotoxin (nBGT) and desensitized rapidly, which are features characteristic of control AChR currents (Fig. 4D) (15). Treatment with an $\alpha 7$ antisense decreased control ACh-evoked

currents by <25%. However, like α -BGT, the $\alpha 7$ antisense decreased the ACh-evoked currents by 60 to 70% in $\alpha 3$ antisense-treated neurons (Fig. 4, B and C). The residual current (15) also resembled control ACh-evoked currents: it desensitized with kinetics equivalent to control, exhibited insensitivity to α -BGT (Fig. 4D), and was blocked by nBGT. Thus, the slowly desensitizing AChRs expressed in $\alpha 3$ antisense-treated neurons are blocked by α -BGT and dependent on the expression of $\alpha 7$. This indicates that $\alpha 7$ may be the principal ligand-binding subunit of the AChR channels expressed after functional deletion of $\alpha 3$.

Treatment of embryonic chick sympathetic neurons with antisense oligonucleotides against $\alpha 3$, $\alpha 4$, or $\alpha 7$ subunit sequences suggests specific functional contributions of these subunits to neuronal AChRs. The $\alpha 3$ subunit appears to play an important role in all four AChR channel classes normally expressed by these neurons. The functional deletion of the $\alpha 3$ subunit decreases the number of openings of all channel subtypes, alters the concentration of ACh sufficient to gate channel activity, alters AChR desensitization, and results in the expression of a new, larger conductance AChR channel. Biochemical and molecular studies have also indicated that the $\alpha 3$ subunit is a component of peripheral neuronal AChRs (16).

After functional deletion of $\alpha 3$, a new class of channels emerges that appears to be dominated by the $\alpha 7$ subunit. Because treatment with $\alpha 7$ antisense alone significantly decreased the control ACh-evoked current, $\alpha 7$ is likely a component subunit of the AChR channels normally expressed in sympathetic neurons. However, α -BGT, which blocks $\alpha 7$ subunit homooligomers in the oocyte (14), does not decrease the control ACh-evoked currents (Fig. 4). Thus, the AChRs in neurons that include the $\alpha 7$ subunit or subunits must include other subunits that are α -BGT-resistant ($\alpha 3$) or that influence the α -BGT sensitivity of the $\alpha 7$ subunit (17). Furthermore, because macroscopic ACh-evoked currents, which are mediated by $\alpha 7$ homooligomers in the oocyte, desensitize rapidly (14), our findings suggest that other factors [for example, different subunit combinations or posttranslational modification (18)] are important determinants of AChR desensitization kinetics in neurons. Homooligomers of $\alpha 7$ mediate a large ACh-evoked macroscopic current in the oocyte (14) consistent with our finding an unusually large conductance channel. Although $\alpha 4$ is expressed in sympathetic neurons, treatment of cells with an $\alpha 4$ antisense had no effect on AChR-activated currents. If the $\alpha 4$ antisense functionally deletes $\alpha 4$, then expression of a given AChR subunit gene may not indicate participation in functional AChR channels.

These studies also provide a general approach for the dissection of receptor function in primary neurons that complements studies in heterologous cell expression systems. The use of antisense oligonucleotides to unravel the functional contribution of individual subunits is applicable to the analysis of AChRs in other regions of the nervous system (19) and should permit the molecular dissection of the contribution of individual subunits to other ligand-gated ion channels within their native cellular environment.

REFERENCES AND NOTES

1. Stroud RM, McCarthy MP, Shuster M. *Biochemistry* 1990;29:11009. [PubMed: 1703009]
2. Deneris ES, Connolly J, Rogers SW, Duvoisin R. *Trends Pharmacol. Sci* 1991;12:34. [PubMed: 2006540]
3. Papke RL, Boulter J, Patrick J, Heinemann S. *Neuron* 1989;3:589. [PubMed: 2484342] Luetje C, Patrick J. *J. Neurosci* 1991;11:837. [PubMed: 1705971]
4. Moss B, Schuetze S, Role L. *Neuron* 1989;3:597. [PubMed: 2642012]
5. Sambrook, J.; Fritsch, EF.; Maniatis, T. *Molecular Cloning, A Laboratory Manual*. ed. 2. Cold Spring Harbor Laboratory; Cold Spring Harbor, NY: 1989.
6. Polymerase chain reaction (PCR) assay: RNA purified from ED10 or ED17 sympathetic ganglia by the guanidium-CsCl centrifugation method (5). RQ1 DNase-treated total RNA served as template for

first strand synthesis (AMV reverse transcriptase). Aliquots (0.5 µg of RNA or cDNA) were then submitted to PCR in a Perkin-Elmer Cetus DNA Thermal Cycler: 35 cycles of 94°C, 0.5 min; 40°C, 0.5 min; 75°C, 0.75 min. Reverse transcriptase was omitted from the cDNA synthesis reaction before amplification in controls (–). The same AChR subunits were expressed in ED10 and ED17 ganglia. The α2 target can be amplified from genomic chick DNA (digested with Eco RI and Bgl II) with the appropriate primers. Sense primers: α3, α5: CTCCTCTTCAC(C/T)ATGATATTTGT; α2, α4 CTCTTCACCATGAT(A/C)TTTG; α7: ATGCCAGCAACATCTGATTCT and β TGGT(A/G) CTGGTGACCTTCTC. Antisense primers: (reverse complement of sequences specified by number) α2: 1367 to 1386; α3: 1305 to 1325; α4: 1201 to 1221; α5: 1164 to 1184; α7: TACTACAGAGGCTGCAAACTT and β: ACCATGGC(A/C)AC(A/G)TACTT.

7. We ran 2.5 µg of poly(A)⁺ RNA from ED10 sympathetic ganglia per lane on a formaldehyde (1%) agarose gel and blotted it onto Nytran (Schleicher and Schuell) for hybridization, according to the manufacturer's instructions. Probes generated by random primer synthesis (Promega) to a specific activity of $\geq 5 \times 10^8$ cpm/µg. Following the lack of hybridization to α2 probe, the lane was rehybridized to other probes to confirm RNA retention on the blot.
8. Silman I, Karlin A. *Science* 1969;164:1420. [PubMed: 5783718]
9. Gardette R, et al. *Dev. Biol* 1991;147:83. [PubMed: 1652527]
10. Leprince P. *Biochemistry* 1983;22:5551. [PubMed: 6652075] Methods: E10 and 11 chick lumbar sympathetic ganglion neurons were prepared as described (4) and plated in dishes with a 13-mm sunken well of ~150 µl. BAC bromide treatment was modified from (9)]. Oligonucleotide treatment: Functional block oligonucleotides: α3 sense and antisense –77 to –63; α3 mismatch antisense CGACCGTAAAACAAC; α4 antisense –69 to –55; α7 antisense GCATCAGCGCCCGGA. Oligos made on Applied Biosystems Automated DNA Synthesizer (380B) and purified by polyacrylamide gel electrophoresis and reversed-phase chromatography (5). No known chick AChR sequences are complementary to these 15-mers (optimal complementarity with other AChR sequences $\leq 72\%$). Oligonucleotides in growth media (4) were added to neurons within culture wells and maintained in a humidified chamber (48 hours). Additional antisense controls: neither inclusion of α3 antisense in the recording pipette nor treatment of neurons with oligonucleotides similar to α3 in size, charge, or sequence (three base mismatch) had any effect on AChR function. K⁺-channel activity and GABA-evoked currents were unaltered in antisense-treated neurons.
11. Snedecor, GW.; Cochran, WG. *Statistical Methods*. ed. 6. Iowa State Univ. Press; Ames, IA: 1967. p. 329 Single channel and macroscopic current analysis. Data acquisition and analysis used BASIC 23-based software developed by S. Schuetze (4) or S. Siegelbaum. Slope conductance determined from recordings at –30, –50, and –70 mV relative to rest. We obtained mean single channel current activity in individual patches by integrating $t(\text{open})/t(\text{total})$ for the first 3 min (cell-attached patch) or for 10 s after agonist application (outside-out). The decay time course of macroscopic currents was fit by the sum of two exponential functions (fast and slow time constants τ_1 and τ_2). Presentation and statistics. Mean single channel current and macroscopic current decay time constant data were not normally distributed and therefore are presented in box plots. The central box shows the middle 50% of the data (25th through 75th percentiles); the horizontal line indicates the median, and the whiskers indicate the range of data values (10th through 90th percentiles). Outliers, if present, are plotted as asterisks. Mean current and decay constants of control groups were normalized to permit statistical comparison between corresponding experimental groups. The values obtained were normally distributed following log transformation [Kolmogorov-Smirnov test NPAR and MGLH modules of SystatTM]. Subsequent analysis of variance (ANOVA) was followed by a test for multiple comparisons between pairs of group means based on unequal sample sizes
12. AChR number in α3 antisense-treated neurons: ACh dose response data indicate that 500 µM evokes a maximal response in both control and α3 antisense-treated neurons. Mathematical subtraction of the contribution of slower desensitization indicates that α3 antisense decreased the number of activatable AChRs by >65% (rather than 58%). This correction is still an underestimate because it assumes comparable single-channel conductances in α3 antisense-treated and control neurons whereas larger conductance channels predominate in α3 antisense-treated neurons. (L versus α3 AS-L: 66% versus 89% of patches; XL versus α3 AS-Giant: 37% versus 52% of patches).
13. Couturier S, et al. *J. Biol. Chem* 1990;265:17560. [PubMed: 1698777]
14. Couturier S, et al. *Neuron* 1990;5:847. [PubMed: 1702646]

15. Loring R, Zigmond R. Trends Neurosci 1988;11:73. [PubMed: 2465603]The residual current in neurons treated with $\alpha 3$ antisense plus $\alpha 7$ antisense or α -BGT resembles control in desensitization kinetics and in its complete block by nBGTThis current may be composed of native receptors assembled from the internal pool or due to delayed or incomplete antisense-mediated deletion
16. Boyd RT, et al. Neuron 1988;1:495. [PubMed: 3272177]Schoepfer R, et al. FEBS Lett 1989;257:393. [PubMed: 2583285]
17. Blount P, Merlie JP. Neuron 1989;3:349. [PubMed: 2642001]
18. Huganir RL, Greengard P. 1990;5:555. *ibid.*
19. Brussaard, A.; Doyle, J.; Role, L. in preparation
20. We thank R. Axel, T. Jessell, A. Karlin, and S. Siegelbaum (Columbia College of Physicians and Surgeons) for discussions, reagents, and extensive comments on the manuscript; M. Ballivet and collaborators from the University of Geneva for sharing clones and sequence information before publication; and D. King, A. Roy, A. Dolorico, A. Witty, and E. Hubel for technical assistance. Supported by awards from NSF, North Atlantic Treaty Organization, Muscular Dystrophy Association, NIH (NS27680, NS 29071), and the Council for Tobacco Research.

Fig. 1. AChR subunit gene expression in sympathetic neurons. **(A)** Diagram of neuronal AChR cDNA sequences. Proposed transmembrane-spanning domains are indicated by boxes; arrows indicate the regions used for PCR primers; bold lines indicate the fragments used to generate probes for Northern (RNA) blots, and asterisks near the start sites indicate the region used for functional block oligonucleotides (6,10). **(B)** PCR assay of embryonic day 10 sympathetic ganglia RNA with (+) or without (-) previous cDNA synthesis (6). **(C)** Northern (RNA) analysis of ED 10 sympathetic ganglia poly A⁺ RNA (7). Approximate sizes: $\alpha 3$: 2.0 kb, $\alpha 4$: 2.4 kb, $\alpha 5$: 2.9 kb, $\alpha 7$: 2.3 and 5.0 kb, $\beta 2$: 1.8 and 1.5 kb, $\beta 4$: 1.9 kb.

Fig. 2.

AChR channel activity is decreased in $\alpha 3$ antisense-treated neurons (10,11). (A) Nonconsecutive traces from cell-attached patches on control and $\alpha 3$ antisense-treated neurons (2.5 μ M ACh, V_h : -50 mV relative to rest, 5 pA $\times$ 5 ms). (B) Mean current versus time for patches in (A). Average mean current values over 3 min were 0.0323 (Con) and 0.0031 ($\alpha 3$ AS). (C) Box plot representation of mean current determinations. Control: $n = 30$, $\alpha 3$ antisense: $n = 27$, $\alpha 4$ antisense: $n = 9$, $\alpha 3$ sense: $n = 18$. The $\alpha 3$ antisense group (a) is significantly different ($P \leq 0.001$) from other groups. Examination of channel opening frequency yielded identical results.

Fig. 3.

$\alpha 3$ antisense-treated neurons express novel AChR channels (10,11). High concentrations of ACh activate three AChR subtypes in $\alpha 3$ antisense-treated neurons. (A) Nonconsecutive records of channels activated by 25 μ M ACh in control neurons. S, M, L, XL subtypes with $\gamma \pm$ SD of 17 ± 5.1 , 31 ± 5.9 , 49 ± 4.7 , and 65 ± 4.6 were detected. (B) Amplitude histogram of channels (Con) with summed Gaussian fit superimposed. (D) Nonconsecutive records of channels gated by 25 μ M ACh from $\alpha 3$ antisense-treated neurons. Three AChR subtypes designated $\alpha 3$ AS-S (small), $\alpha 3$ AS-L (large), and $\alpha 3$ AS-G (giant) with $\gamma \pm$ SD of 24 ± 9.5 , 53 ± 6.0 , and 114 ± 13.0 , respectively, are seen. (E) Amplitude histogram of channels ($\alpha 3$ AS) with summed Gaussian fit superimposed. All openings required ACh and were blocked by

hexamethonium (500 μ M). (Cell-attached patches; V_h : -50 mV relative to rest for (A) and (B); 5 pA $\times$ 5 ms). (C) and (F): Desensitization of AChR openings analyzed by comparison of mean single-channel current over two consecutive 10-s periods of ACh application to outside-out patches from control and $\alpha 3$ antisense-treated neurons (V_h : -100 mV; ACh: 25 μ M). Boxplots of data from all patches indicate persistent ACh-gated single channel activity in $\alpha 3$ antisense-treated neurons compared with control (a and b pair, $P \leq 0.001$).

Fig. 4.

Both α -bungarotoxin and $\alpha 7$ antisense alter ACh-evoked currents in $\alpha 3$ antisense-treated neurons (10,11). **(A)** Macroscopic currents from control and $\alpha 3$ antisense-treated neurons before and after (*) α -BGT application (ACh: 500 μ M; α -BGT: 500 nM, 10 min) and **(B)** (top) Macroscopic currents from control and $\alpha 7$ antisense-treated neuron ($\blacklozenge$), and (bottom) macroscopic currents from control versus $\alpha 3$ antisense-treated neurons (+) versus neuron treated with both $\alpha 3$ antisense and $\alpha 7$ antisense (+ and $\blacklozenge$; V_{hold} : -50 mV; 500 pA $\times$ 1 s). nBGT completely blocked ACh-evoked currents in control and in $\alpha 3$ plus $\alpha 7$ antisense-treated neurons. **(C)** Summary of peak macroscopic current data (mean $\pm$ SEM) **(D)** Summary of desensitization time course data. Fast (τ_1) and slow (τ_2) time constants of the biexponential decay of the macroscopic currents are displayed with boxplots. Control: $n = 27$; + α -BGT: $n = 10$; $\alpha 3$ antisense-treated: $n = 26$; $\alpha 7$ antisense-treated: $n = 7$, $\alpha 3$ antisense-treated + α -BGT: $n = 15$; $\alpha 3$ antisense + $\alpha 7$ antisense-treated: $n = 8$. In **(C)** the comparisons (a) were significantly different ($p \leq 0.001$) for control versus $\alpha 3$ AS, at $P \leq 0.023$ for control versus $\alpha 7$ AS; (b) indicates $p \leq 0.004$ for $\alpha 3$ AS versus $\alpha 3$ AS + α -BGT; (c) indicates $P \leq 0.041$ for $\alpha 3$ AS versus $\alpha 3$ AS + $\alpha 7$ AS; $p \leq 0.001$ for $\alpha 7$ AS versus $\alpha 3$ AS + $\alpha 7$ AS. In **(D)**, (a) indicates differences at $P \leq 0.004$ to 0.001 for $\alpha 3$ AS versus all other groups. The relative contribution of the fast and the slow time constant did not differ among groups. α -BGT did not affect either peak current or decay in $\alpha 4$ antisense-treated neurons.