Developmental Changes in the Nicotinic Responses of Ciliary Ganglion Neurons

EDWARD M. BLUMENTHAL, RICHARD D. SHOOP, AND DARWIN K. BERG
Department of Biology, 0357, University of California, San Diego, La Jolla, California 92093

Blumenthal, Edward M., Richard D. Shoop, and Darwin K. Berg. Developmental changes in the nicotinic responses of ciliary ganglion neurons. J. Neurophysiol. 81: 111–120, 1999. The accumulation of functional neurotransmitter receptors by neurons during development is an essential part of synapse formation. Chick ciliary ganglion neurons express two kinds of nicotinic receptors. One is abundant, contains the $\alpha_7$ gene product, rapidly desensitizes, and binds $\alpha$-bungarotoxin. The other is less abundant, contains multiple gene products ($\alpha_3$, $\beta_4$, $\alpha_5$, and $\beta_2$ subunits), slowly desensitizes, and binds the monoclonal antibody mAb 35. Rapid application of agonist to freshly dissociated neurons elicits responses from both classes of receptors. Between embryonic days 8 and 15, the whole cell response of $\alpha_3$-containing receptors increases fivefold in peak amplitude and, normalized for cell growth, 1.7-fold in current density. In addition, the response decays more slowly in older neurons, suggesting a developmental decrease in the rate of desensitization. The whole cell response of $\alpha_7$-containing receptors increases 10-fold in peak amplitude over the same period and 3-fold in current density. No change in the rate of desensitization was apparent for $\alpha_7$-containing receptors with developmental age, but analysis was limited by overlap in responses from the two kinds of receptors. Indirect immunofluorescence measurements on dissociated neurons showed that the relative levels of $\alpha_7$-containing receptors on the soma increased during development to the same extent as the whole cell response attributed to them. In contrast, the relative levels of $\alpha_3$-containing receptors increased more during the same time period than did the whole cell response they generated. The immunofluorescence analysis also showed that both classes of receptors become distributed in prominent clusters on the cell surface as a function of developmental age. The results indicate that during this period of synaptic consolidation on the neurons, the two major classes of functional nicotinic receptors undergo substantial upregulation; $\alpha_3$-containing receptors as a class may undergo changes in receptor properties as well.

Like vertebrate muscle, autonomic neurons rely on AChRs to mediate primary synaptic input from preganglionic neurons in the CNS. A variety of studies indicate that cell-cell interactions regulate the number and properties of AChRs expressed by the neurons (Arenella et al. 1993; Boyd et al. 1988; Devay et al. 1994; Gardette et al. 1991; Jacob and Berg 1987; Levey and Jacob 1996; Levey et al. 1995; Margiotta and Gurantz 1989; Moss and Role 1993; Moss et al. 1989). Recently it has been shown that an isoform of neuregulin can enhance the number of functional AChRs expressed by sympathetic neurons in culture (Yang et al. 1998).

One of the most interesting and abundant neuronal AChRs in both the peripheral and central nervous systems is a species that binds $\alpha$-bungarotoxin ($\alpha$Bgt) with high affinity and is thought to be a homopentamer containing $\alpha_7$ subunits (Anand et al. 1993; Chen and Patrick 1997; Couturier et al. 1990a; Conroy and Berg 1998; Schoepfer et al. 1990). The receptor ($\alpha_7$-AChR) has a high relative permeability for calcium and rapidly desensitizes (Alkondon and Albuquer-2000; Bertrand et al. 1993; Seguela et al. 1993; Zhang et al. 1994). It modulates transmitter release from presynaptic sites both in the central and peripheral nervous systems (Coggan et al. 1997; Gray et al. 1996; McGehee et al. 1995) and can influence developmental events at the growth cone (Fu and Liu 1997; Pugh and Berg 1994). The developmental regulation of functional $\alpha_7$-AChRs has not been examined.

Chick ciliary ganglion (CG) neurons express large numbers of $\alpha_7$-AChRs (Chiappinelli and Giacobini 1978; Vernallis et al. 1993) and concentrate them at extrasynaptic or perisynaptic sites (Jacob and Berg 1983; Loring et al. 1985; Wilson Horch and Sargent 1995). The neurons express a smaller number of AChRs that bind the monoclonal antibody mAb 35 and contain $\alpha_3$, $\beta_4$, $\alpha_5$, and, sometimes, $\beta_2$ subunits (Conroy and Berg 1995; Vernallis et al. 1993). These latter receptors ($\alpha_3^*$-AChRs) are concentrated at postsynaptic densities as well as extra/perisynaptic sites on the neurons (Jacob et al. 1984; Loring and Zigmond 1987; Wilson Horch and Sargent 1995). Both $\alpha_7$-AChRs and $\alpha_3^*$-AChRs contribute importantly to synaptic transmission elicited by stimulating the preganglionic nerve (Ullian et al. 1997; Zhang et al. 1996).

All CG neurons receive functional nicotinic innervation by embryonic day (E)7 (Landmesser and Pilar 1974). Between E8 and E15, the number of $\alpha_3^*$-AChRs in the ganglion, quantified by $^{125}$I-mAb 35 binding, increases ~10-fold (Corrierveau and Berg 1994; Smith et al. 1985) while the number of $\alpha_7$-AChRs, quantified by $^{125}$I-$\alpha$Bgt binding, increases 6-fold (Chiappinelli and Giacobini 1978; Corrierveau and Berg 1994). These increases occur despite a 50%
The dissociation medium contained (in mM) 140 NaCl, 3 KCl, 10 glucose, and 10 HEPES, pH 7.4 (With NaOH). The neurons have been demonstrated previously (Jacob et al. 1984; Cruz Biotechnology, Santa Cruz, CA) were incubated with collagenase (Boehringer Mannheim, Indianapolis, IN) for 20 min at 37°C. The concentration of collagenase varied with the age of the embryo: 0.1 mg/ml, E6; 0.2, E8; 0.25, E9; 0.3, E10; 0.6, E11; 0.75, E12; 1.0, E13–E14; 1.25, E15.

The dissociated CG neurons were prepared from E6 to E15 chick ciliary ganglia using a modification of methods previously described (Margiotta and Gurantz 1989). The age of the embryo was confirmed before dissection using the staging method of Hamburger and Hamilton (1951). The ganglia were dissected from the embryo, hemisected (except for ganglia from the youngest embryos), and incubated with collagenase (Boehringer Mannheim, Indianapolis, IN) for 20 min at 37°C. The concentration of collagenase varied with the age of the embryo: 0.1 mg/ml, E6; 0.2, E8; 0.25, E9; 0.3, E10; 0.6, E11; 0.75, E12; 1.0, E13–E14; 1.25, E15.

The dissociation medium contained (in mM) 140 NaCl, 3 KCl, 10 glucose, and 10 N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES), pH 7.4 (with NaOH). After collagenase treatment, the ganglia were transferred to culture medium made up of Eagle’s minimal essential medium (GIBCO, Grand Island, NY) supplemented with 10% (vol/vol) heat-inactivated horse serum (Gemini, Cannan Falls, MN) and 3% (vol/vol) embryonic eye extract described in the preceding text. The ganglia were transferred to culture medium made up of Eagle’s minimal essential medium (GIBCO, Grand Island, NY) supplemented with 10% (vol/vol) heat-inactivated horse serum (Gemini, Cannon Falls, MN) and 3% (vol/vol) embryonic eye extract (Nishi and Berg 1981). The cells were dispersed by trituration with a fire-polished Pasteur pipette and plated on tissue culture dishes (Falcon) that had been coated with poly-D-lysine (1 mg/ml). Dissociated cells were used within 1–5 h of plating and were kept in a tissue culture incubator at 37°C until use.

Electrophysiology

Currents were recorded using the whole cell patch-clamp configuration controlled by an Axopatch 200A amplifier (Axon Instruments, Foster City, CA) as previously described (Hamill et al. 1981; Zhang and Berg 1995). All experiments were carried out at room temperature. The extracellular solution contained (in mM) 140 NaCl, 3 KCl, 2 MgCl2, 2 CaCl2, 10 glucose, and 10 HEPES, pH 7.4 (with NaOH). The pipette solution contained (in mM) 140 CsCl, 1 MgCl2, 10 ethylene glycol-bis(β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid, 10 glucose, and 10 HEPES, pH 7.2 (with CsOH). Electrodes were pulled from 1.5-mm-OD borosilicate glass (Drammond, type N51, Broomali, PA) and had resistances of ~3 MΩ. Series resistance was always <6 MΩ and was 80% compensated. Cells were clamped at −60 mV, and agonist was rapidly applied as previously described (Zhang and Berg 1995). Solutions were delivered from a linear array of glass tubing (370 μm ID, 470 μm OD; Polymicro Technologies, Phoenix, AZ) mounted on a piezobimorph element (Morgan-Matroc, Bedford, OH). Solution flow was gravity fed and controlled by a set of solenoid valves (General Valve, Fairfield, NJ). The valves and the bimorph were controlled by a Master-8 programmable stimulator (A.M.P.I., Jerusalem, Israel). Using this system, the time of solution exchange at an open pipette was 0.5–3 ms as measured by the change in junction potential. Data were filtered at 1 kHz and digitized at either 0.7 or 1.5 kHz with the pCLAMP software (Axon Instruments). Currents were analyzed using Axograph software (Axon Instruments).

For recordings from αBgt-treated cells, the toxin (Biotoxins Inc.) was applied at 60 nM for >1 h at 37°C, and 18 nM αBgt also was included in the recording solution. When ACh was used as the agonist, 100 nM atropine was included in the bath and agonist solution. Tetrodotoxin was omitted from the bath because previous experiments showed it to be unnecessary when recording nicotinic responses in the neurons with voltage clamp techniques (Zhang et al. 1994).

Immunofluorescence

Freshly dissociated cells were labeled by incubating with either biotinylated αBgt (Molecular Probes, Eugene, OR; 1:500 dilution) or mAb 35 (1:1,000 dilution) in 10 mM HEPES-buffered medium with 3% egg extract for 1–2 h at 4°C. The neurons then were rinsed three times with HEPES-buffered medium and fixed in 4% paraformaldehyde in 0.15 M sodium phosphate, pH 7.4, for 30 min. After rinsing with 0.10 M sodium phosphate (SP), pH 7.5, toxin-labeled neurons were incubated in a 1:1,000 dilution of Cy3-conjugated streptavidin (Jackson Laboratories, Bar Harbor, ME) for 30 min. After rinsing with 0.10 M sodium phosphate (SP), pH 7.5, toxin-labeled neurons were incubated in a 1:1,000 dilution of Cy3-conjugated donkey anti-rat antibody (Jackson Laboratories) in SP with 5% normal donkey serum (Sigma, St. Louis, MO). The specificities of these probes for immunofluorescent detection of AChRs on ciliary ganglion neurons have been demonstrated previously (Jacob et al. 1984; Wilson Horch and Sargent 1995). In some experiments, α7-AChRs were labeled with indirect immunofluorescence by fixing the cells as described above, permeabilizing with 0.1% (wt/vol) Triton X-100 in SP, and then labeling with anti-α7-AChR antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). In a few experiments, α3*-AChRs were labeled by first fixing the cells and then labeling as described in the preceding text.

Images were visualized either with a Noran ODYSSEE confocal laser scanning microscope or with a Sensys CCD camera (Photometrics, Tucson, AZ) and Zeiss Axioskop. The confocal images represent reconstructions of the neuron surface achieved by assembling 80–120 contiguous individual optical sections taken through an individual cell. An indication that the optical reconstruction did not distort the patterns observed is that computer rotation of the reconstructions in three-dimensional space indicated that fluorescently labeled receptor clusters on the sides of the cells were not significantly different in shape or labeling intensity from those on the top or bottom of the cells, though the former were generated by reconstruction from multiple optical sections while the latter often were contained principally within one or two optical sections. Confocal images of E8 and E15 cells were adjusted individually for brightness and contrast to optimize visualization.

For quantitative comparisons of fluorescence signals, the CCD camera was used. The linearity of fluorescence detection by the camera over the operating range used here was confirmed by calibration experiments. For this purpose, dissociated E8 and E15 ciliary ganglion neurons were incubated with biotinylated αBgt diluted with known concentrations of underivatized αBgt and then carried through the labeling procedure as described. The specific fluorescence signal associated with a cell was determined by mea-
suring the total fluorescence emanating from a circle enclosing the cell and then subtracting the background fluorescence from an equivalent circle lacking a cell in the same field of view. Background values determined in this way were not significantly different from those determined by including an excess of underivatized αBgt or nicotine in the initial binding reaction to block specific binding of the biotinylated toxin. Measurements of specific fluorescence then were used to construct calibration curves that showed the expected dependence on toxin dilution both for E8 and for E15 neurons, corroborating the manufacturer’s calibration of linearity supplied with the camera.

Because the optical image captured by the CCD camera represented a slice through the cell rather than the whole cell, a correction for cell size was necessary when comparing relative fluorescence signals for E8 and E15 neurons. E15 neurons were found on average to be 35% larger in diameter than E8 neurons. Accordingly, when calculating the relative levels of whole cell specific fluorescence associated with E15 versus E8 neurons, a 35% positive correction was applied to the calculated E15:E8 ratio. The fact that identical E15:E8 fluorescence ratios were obtained when measurements for an experiment were made with either a 40 or a 63 oil immersion objective indicates that the results were independent of the thickness of the optical section over the range employed.

Materials

White Leghorn chick embryos were obtained locally and maintained at 37°C in a humidified incubator. αBgt was purchased from Biotoxins (St. Cloud, FL). All other reagents were purchased from Sigma unless otherwise indicated.

Results

α3*-AChR responses

Developmental changes in nicotinic responses between E6 and E15 were examined in freshly dissociated CG neurons using whole cell patch-clamp recording techniques. Initial studies focused on α3*-AChRs because their responses could be studied in isolation. This was achieved by using αBgt to block the α7-AChRs. Rapid application of 1 mM ACh to the neurons in the continued presence of αBgt (and atropine to block muscarinic receptors) elicited currents wholly attributable to α3*-AChRs.

Figure 1 illustrates representative responses obtained from E6 (A and C) and E15 (B and D) neurons. The induced currents increase over several milliseconds on agonist delivery and then diminish in the continued presence of agonist. Both the amplitude of the peak response and the kinetics of decay change during development. The decay phases are shown fit with the sums of three exponentials (Fig. 1, C and D), which change during development (see below, this section). Between E6 and E15, the peak current amplitude increases ~15-fold (Fig. 2A). Much of this is due to neuronal growth: normalizing for cell capacitance (Fig. 2B) yields a value for current density that increases just over threefold between E6 and E15 (Fig. 2C). The peak amplitude and the calculated current density both increase smoothly during the 10 days of development examined.

The observed changes in peak amplitude did not result from a developmental shift in agonist affinity. The concentration of ACh used (1 mM) was equally effective at both early and late developmental times. This can be seen by comparing the responses of individual cells to 1 and 0.1 mM ACh: the ratio of the peak amplitudes elicited by the two concentrations was 2.1 ± 0.7 at E8/9 (27 cells; mean ± SD) and 1.9 ± 0.3 at E14/15 (30 cells). Moreover, normalizing peak amplitudes to those induced by 0.1 mM ACh showed that 0.5 and 1 mM ACh elicited equivalent responses both at E9 (ratios of 1.6 ± 0.2 and 1.7 ± 0.3 for 0.5 and 1 mM vs. 0.1 mM ACh, respectively) and at E15 (ratios of 1.8 ± 0.4 and 1.8 ± 0.2, respectively).

To characterize the developmental change in decay kinetics and accompanying receptor desensitization, we first measured the integral of each current trace over the first second of the recording and normalized the integral to the peak amplitude. The resulting value (desensitization index) gives a rough measure of desensitization, ranging from zero for a current that desensitizes instantaneously to 1.0 for a non-desensitizing current. Almost all of the change in the desensitization index occurred between E8 and E10 and represented a shift from a more rapidly desensitizing early response to a more slowly desensitizing later response (Fig. 3A).

Analysis of the falling phase of the responses indicated that three exponentials were necessary to fit adequately the vast majority of the records. Time constants of 30 ± 2 and 176 ± 20 ms (n = 14 cells) were calculated for the fast and intermediate components, respectively, at E6; these increased to 44 ± 1 and 238 ± 11 ms (n = 64 cells) by E15. The increases were statistically significant (P < 0.001). The time constant of the slow component exceeded the length of the recordings (2 s) and could not be measured accurately. Nonetheless, the amplitude of the slow component could be measured and was found to account for most of the change in the desensitization index between E8 and E10 because it contributed an increased proportion of the total response at the later time (Fig. 3B). The desensitization index, as calculated, arithmetically emphasizes slow components. Together, the three exponentials provided a good fit of the falling phase of the response (Fig. 1, C and D).

We considered whether some of the slowdown in desensitization seen with increasing cell age might result from an increase in mean cell size between E6 and E15. Larger cells presumably impede access of transmitter to the far side of the cell more than smaller cells do and thereby would increase the time over which their receptors are activated and desensitize. Support for this comes from the finding of a positive correlation between cell capacitance and desensitization index among cells of different sizes in the population of E12–E15 neurons (R² = 0.17 for linear regression; n = 139 cells; P = 0.001). Although the two measures are correlated significantly, the correlation coefficient indicates that most of the variability in the desensitization index is not caused by differences in cell size. Moreover, mean cell size changes gradually between E6 and E15 but most of the change in the desensitization index occurs between the narrow window of E8 and E10. In addition, much of the change in the desensitization index occurs between the narrow window of E8 and E10. In addition, much of the shift in the desensitization index, as pointed out above, is due to the relative increase in the amplitude of the slowly decaying component, and it is difficult to see how changes in cell size could account for such a shift.

α7-AChR responses

Nicotine was the agonist of choice for activating α7-AChRs because it produced significant temporal separation of the α7-
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FIG. 1. Whole cell currents activated by 1 mM acetylcholine (ACh) in the presence of αBgt from embryonic day 6 (E6; A and C) and E15 (B and D) ciliary ganglion (CG) neurons. Agonist was applied to the cells for the time indicated by the horizontal bar. C and D are replicates of A and B, respectively, with superimposed dashed lines that indicate the sum of 3 exponentials used to fit the decay phase of the response in each case.

AChR and α3*-AChR peak responses. This was deduced from preliminary experiments, which showed that nicotine activates α7-AChRs much more rapidly than it does α3*-AChRs; ACh produces a much smaller time differential between the two kinds of responses. In fact, all of the rapidly desensitizing response elicited by 20 μM nicotine in both E8 and E14 neurons can be attributed to αBgt-sensitive α7-AChRs (Fig. 4, A–D). Only slowly inactivating currents, indicative of α3*-AChR responses, are observed when nicotine is applied to neurons treated with αBgt (Fig. 4, E and F).

The peak amplitude of the αBgt-sensitive nicotinic response increased ~10-fold between E8 and E15 (Fig. 5A). It was not possible to quantify the αBgt-sensitive component of the nicotinic responses in younger neurons (E6) because it could not be reliably distinguished from αBgt-resistant components, and so those data have been excluded. After correcting for cell size by normalizing the peak response for capacitance, the net increase in current density between E8 and E15 for the αBgt-sensitive response was found to be ~3.5-fold (Fig. 5B). This is greater than the increase in current density seen for the α3*-AChR response over the entire developmental window of E6–E15 and is twice as great as that for the equivalent period of E8–E15 (Fig. 2C).

The developmental increase in peak amplitude of α7-AChR responses could not be ascribed to a shift in the dose-response curve of the receptors for nicotine. Although 20 μM nicotine does not elicit a maximal response, the ratio of peak responses evoked by 100 and 20 μM nicotine was unchanged over development: 2.1 ± 0.7 at E9 (n = 16 cells); 1.9 ± 0.7 at E14 (16 cells).

No developmental change was apparent in the desensitization kinetics of α7-AChRs. Exponential fits of the rapidly decaying nicotine-induced current were not possible because the slow αBgt-resistant current usually contaminated much of the falling phase of the αBgt-sensitive response (Fig. 4, C and D). The extent of desensitization during the initial few milliseconds of the recordings was comparable for neurons from young and late developmental stages (data not shown), but only large differences would have been detected in this way.

Relative increases in α7- and α3*-AChRs during development

In view of past results suggesting that a large number of nonfunctional α3*-AChRs appear at late developmental times (Margiotta and Gurantz 1989), it seemed important to compare changes in the number of α3*-AChRs on the neurons at early and late times with those seen in the whole cell α3*-AChR response under the same conditions. Previous quantification of CG AChRs during development usually measured total α3*- or α7-AChRs in ganglion extracts (Chippinelli and Giacobini 1978; Corriveau and Berg 1994; Smith et al. 1985). It is clear now that such measurements would have included a substantial number of intracellular AChRs and AChRs on neuronal processes (Corriveau and Berg 1994; Jacob and Berg 1988; Stollberg and Berg 1987; R. Shoop and D. Berg, unpublished studies). In addition, at least some of the α7-AChRs present in ganglion extracts are likely to have been presynaptic in origin where they acted presynaptically to modulate neurotransmitter release (Coggan et al. 1997; McGehee et al. 1995).

Indirect immunofluorescence was used in the present experiments to compare the numbers of receptors at early and
α7-AChRs, dissociated neurons were incubated first with biotinylated αBgt and then Cy3-conjugated streptavidin before viewing with confocal laser microscopy. Serial reconstruction of labeled optical sections was performed to generate a composite image of the whole neuron. When viewed in this way, E8 neurons had low levels of labeling distributed among small clusters while E15 neurons had large and prominently labeled receptor clusters (Fig. 6, A and B). Such clusters have been seen previously on neurons in situ late in development and have been described as being perisynaptic because they often are adjacent to but never overlapping with presumed presynaptic sites of transmitter release defined immunocytochemically (Wilson Horch and Sargent 1995). The α7-AChR labeling was judged specific because it was eliminated by using native αBgt to block the binding.

![Figure 2](image2.png)

**FIG. 2.** Developmental change in response of CG neurons to 1 mM ACh in the presence of αBgt. A: peak current amplitudes (pA); B: cell capacitance (pF); C: current density (pA/pF). Values represent the means ± SE of 16–172 cells per point.

late developmental times. This approach allowed us to focus exclusively on cell surface AChRs confined to the cell soma and enabled us to prepare the cells in the same manner as those used for electrophysiological analysis. To visualize

![Figure 3](image3.png)

**FIG. 3.** Developmental change in the desensitization kinetics of α3*-AChRs. A: change in the integral of the current response for the first second of the recording, normalized for the peak current to create an index of relative desensitization (desensitization index) as a function of developmental age. B: sum of 3 exponentials was required to fit adequately the decay phase of the response. Shown here is the developmental change in the fraction of the peak amplitudes contributed by the slowest component. Fractional currents were calculated by extrapolating back to the beginning of the rising phase. Both the desensitization index and the contribution of the slowest component increase most dramatically between E8 and E10. Values represent the means ± SE of 14–64 cells per point.
of biotinylated αBgt to the cells (Fig. 6C). Similar labeling patterns were obtained if the cells first were fixed and permeabilized and then labeled with anti-α7 antibodies (data not shown). CCD images of labeled cells with conventional microscopy was used to more accurately depict the relative levels of fluorescence on E8 versus E15 neurons (Fig. 6, D and E; see following paragraphs).

Using a similar strategy to image α3*-AChRs, dissociated neurons were incubated first with mAb 35 and then with Cy3-conjugated secondary antibody. Reconstructed confocal images revealed low levels of labeling at E8, and much more pronounced levels at E15 when receptor clusters were again readily apparent (Fig. 6, F and G). Omission of the primary mAb in the binding reaction eliminated the fluorescence signal. The antibody incubations were routinely carried out at 4°C to prevent antibody-induced redistribution of receptors. The adequacy of the low-temperature approach was confirmed by fixing cells before incubation with antibody and finding that similar patterns of labeling were obtained, albeit with higher levels of background staining (data not shown). CCD images of labeled cells with conventional microscopy again were used to obtain better estimates of the relative levels of labeling for E8 versus E15 neurons (Fig. 6, H and I).

The CCD images were used for quantitative comparisons of fluorescence labeling at early and late developmental stages. For α7-AChRs, E15 neurons were found to have ~10-fold more whole cell specific fluorescence than did E8 neurons (Fig. 7A). This value is indistinguishable from the 10-fold increase between E8 and E15 in whole cell nicotinic response attributed to α7-AChRs (Fig. 5). For α3*-AChRs, E15 neurons were found to have ~12-fold greater whole cell fluorescence than did E8 neurons (Fig. 7B). This is significantly greater than the approximately fivefold increase between E8 and E15 in whole cell ACh-induced response attributed α3*-AChRs (Fig. 2A).

**DISCUSSION**

We examined the nicotinic responses of CG neurons during the embryological period E6–E15, which encompasses the major milestones of CG development. These include innervation of the ganglion by neurons from the accessory oculomotor nucleus, innervation of postsynaptic target muscle in the iris, ciliary body, and choroid layer by CG neurons, and programmed cell death in the ganglion. Patch-clamp recordings from freshly dissociated neurons reported here show large increases in the whole cell response produced both by α7-AChRs and by α3*-AChRs over this time frame and demonstrate further that a substantial portion of the increase in each case represents a developmental change in the current density generated by the receptors. For α7-AChRs immunofluorescence measurements show that the increases
in whole cell responses are matched by increases in receptor number on the cells; for \( \alpha 3 ^ {+} - \text{AChRs} \), the increases in receptor number exceed the increases in whole cell response. The distribution of receptors also changes during this time frame and increasingly takes on the appearance of discrete receptor clusters reminiscent of the distribution on adult neurons in situ.

Previous developmental studies of functional nicotinic receptors did not include \( \alpha 7 - \text{AChRs} \) apparently because the methods used for agonist application did not permit resolution of their responses. Only \( \alpha Bgt \)-resistant currents, attributable to \( \alpha 3 ^ {+} - \text{AChRs} \), were recorded from freshly dissociated CG neurons in response to puffer-applied ACh (Margiotta and Gurantz 1989; Margiotta et al. 1987a). In those studies, values of 14- and 4.5-fold were obtained for the increases in peak amplitude and current density, respectively, between E8 and E15. Values of 5- and 1.7-fold were obtained in the present studies for the same parameters of the \( \alpha Bgt \)-resistant response. The differences result from the larger responses obtained from young neurons in the present studies when agonist was applied rapidly. The developmental shift reported here in the desensitization kinetics of \( \alpha 3 ^ {+} - \text{AChR} \) responses toward longer times at later developmental stages is the primary explanation: a greater proportion of the signal would have been lost through receptor desensitization in young neurons when agonist was applied by puffer pipette.

A similar explanation may account for the previous report of a developmental shift in agonist affinity (Margiotta and Gurantz 1989). If desensitization at high agonist concentrations caused a truncation of the dose-response curve when using the puffer pipette and was most pronounced at early developmental times, it could have produced an apparent shift with development in the measured EC50 values. No evidence for a developmental shift in agonist affinities was found in the present studies, but it was not examined directly. Dose-response experiments were designed here to test whether the concentrations being used were sufficient to generate maximal responses.

The slower desensitization of the \( \alpha 3 ^ {+} - \text{AChR} \) response in older neurons is the opposite of what one might have expected from muscle AChRs where the mature form of the receptor (\( \alpha 1 \beta 1 \epsilon \delta \)) produces more rapidly decaying responses than does the embryonic form (\( \alpha 1 \beta 1 \gamma 6 \)) (Naranjo and Brehm 1993). It is unlikely that the slower desensitization observed here results primarily from the effects of size differences between E8 and E15 neurons. Although the increase in cell diameter would extend the time required for agonist to access receptors on the far side of the cell (thereby extending the apparent duration of the response at the cost of peak amplitude), the actual amounts of time required for access are, at most, a few milliseconds (Zhang et al. 1994). Further, most of the slowdown in desensitization occurs between E8 and E10 though cell size increases almost monotonically between E6 and E15. Instead, much of the change in the desensitization index can be attributed to a developmental shift in the proportion of the \( \alpha 3 ^ {+} - \text{AChR} \) response generated by the most slowly desensitizing component at the expense of the most rapidly desensitizing component, and this is unlikely to have been influenced by changes in cell size.

Previous studies came to the conclusion that only a small fraction of the \( \alpha 3 ^ {+} - \text{AChRs} \) present on CG neurons at late developmental stages was functionally competent (Margiotta and Gurantz 1989). The determination made use of single-channel analysis combined with measurements of the whole cell response for comparison with levels of \( 125^I \)-mAb 35 binding on freshly dissociated cells. The total number of \( \alpha 3 ^ {+} - \text{AChRs} \) on dissociated neurons at E9 (assuming 2 mAb 35 binding sites per receptor) exceeded the number of functional AChRs by a factor of two. At E15, however, the total number exceeded the functional receptors by a factor of eight. The levels of mAb 35 binding increased in parallel with the whole cell response over this time period, but developmental changes in the ensemble of single channel events led to the increasing disparity as a function of age (Margiotta and Gurantz 1989). The disparity was interpreted as a developmentally increasing population of functionally silent receptors.

Using the new data on current amplitudes obtained here and the single channel data and open time probabilities reported previously (Margiotta and Gurantz 1989) produces a value of \( \sim 5,500 \) for the total number of functional \( \alpha 3 ^ {+} - \text{AChRs} \) on E9 neurons. This compares closely with the

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**FIG. 5.** Developmental change in the response of CG neurons to 20 \( \mu M \) nicotine. A: peak current amplitudes (pA); B: current density (pA/pF). Values represent the means \( \pm \) SE of 16–127 cells per point.
~5,000 mAb 35 binding receptors inferred to be on the cells previously, again assuming two mAb sites per receptor (Margiotta and Gurantz 1989). The results clearly argue against nonfunctional α3*-AChRs at early stages. By E14, however, the present whole cell responses, together with previous single channel data, yield a value of 6100 for the number of functional α3*-AChRs on the neurons. This is to be compared with 25,000 for the total number of α3*-AChRs calculated from previous mAb 35 binding data (Margiotta and Gurantz 1989).

The binding assay used by Margiotta and Gurantz (1989) had the advantages of measuring only AChRs on the cell surface and providing readily quantifiable values. A potential disadvantage was that the binding results might have included receptors present on membrane debris from lysed cells or axon fragments. The fluorescence assay used here measured only surface receptors on intact cells. Reassuringly, the two approaches yielded similar results. Thus the original binding results indicated an increase of ~11-fold in mAb 35 binding levels between E8 and E15 while the fluorescence measurements reported here showed an increase of ~12-fold. Notably the developmental increase between E8 and E15 in whole cell response attributed to α3*-AChRs was only fivefold. This, together with the previously reported changes in single channel properties, supports the contention that a population of functionally silent α3*-AChRs may appear late in development and constitute as much as three-fourths of the total α3*-AChRs.

![Figure 6](image)

**FIG. 6.** Distributions of α7-AChRs and α3*-AChRs on CG neurons during development. Dissociated CG neurons were labeled either with biotinylated αBgt followed by Cy3-conjugated streptavidin (A–E) for α7-AChRs or with mAb 35 followed by Cy3-conjugated donkey anti-rat antibodies (F–I) for α3*-AChRs and visualized either with confocal laser microscopy (A–C, F, and G) or with a CCD camera and conventional fluorescence microscopy (D, E, H, and I). E8 neurons: A, D, F, and H; E15 neurons: B, C, E, G, and I. Cell in C received a large excess of underivatized αBgt to block binding of biotinylated αBgt and serve as a control for non-specific labeling. Both kinds of imaging used ×63 oil immersion objectives. Scale bar: 10 μm.

![Figure 7](image)

**FIG. 7.** Relative levels of α7-AChRs and α3*-AChRs on CG neurons at early and late developmental stages. Dissociated CG neurons labeled as described in Fig. 6 for α7-AChRs (A) and α3*-AChRs (B) were visualized with a CCD camera and conventional fluorescence microscopy to measure relative fluorescence levels as described in METHODS. Scale was adjusted to give optimum sensitivity in the linear range of the camera; arbitrary units are shown. Values represent the means ± SE for a total of 31–32 cells in A and a total of 44–46 cells in B compiled from 3 separate experiments in each case.
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Activation. The consequence would be a diminished peak a large uncertainty into calculations of the maximal whole larger cells impede transmitter access to some extent and, because the receptors desensitize so rapidly. This latter feature introduces a large uncertainty into calculations of the maximal whole cell response produced by α7-AChRs. It will be important in future work to examine the single channel properties of α7-AChRs and to determine whether their function is regulated separately from receptor number during development.

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Present address of E. M. Blumenfeld, Dept. of Biology, University of Virginia, Charlottesville, VA 22903.

Address for reprint requests: D. K. Berg, Dept. of Biology, 035, University of California, San Diego, 9500 Gilman Dr., La Jolla, CA 92039.

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