Nicotinic Acetylcholine Receptors Containing $\alpha 7$ Subunits Are Required for Reliable Synaptic Transmission *In Situ*

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Nicotinic acetylcholine receptors containing $\alpha 7$ subunits are widely expressed in the nervous system. The receptors are cation-selective, relatively permeable to calcium, and avid binders of α -bungarotoxin. Although the receptors can act both pre- and postsynaptically, their physiological significance is unclear. Using whole-cell patch-clamp analysis of chick ciliary ganglion neurons *in situ*, we show that the receptors are required for reliable synaptic transmission early in development. Stimulation of the presynaptic nerve root elicited a biphasic synaptic current, including a large rapidly decaying component generated by $\alpha 7$ -containing receptors. Selective blockade of $\alpha 7$ -containing receptors by perfusing the ganglion with α -bungarotoxin induced failures in synaptic transmission. One-half of the ciliary neurons that were tested failed when stimulated synaptically at 1 Hz, and two-thirds failed at 25 Hz. Failing cells missed, on average, 80% of the trials during a test train of stimuli. The ability to fire synaptically evoked action potentials

after toxin treatment was correlated positively with the amplitude of the remaining synaptic current, suggesting that $\alpha 7$ -containing receptors were needed to augment synaptic responses. Consistent with patch-clamp analysis, toxin blockade reduced the amplitude of the synaptically evoked compound action potential in the postganglionic nerve; it also desynchronized the firing of the remaining units. Methyllycaconitine, another antagonist of $\alpha 7$ -containing receptors, mimicked α -bungarotoxin blockade. Toxin blockade had less impact on transmission in ganglia at the end of embryogenesis. The ability of the receptors to synchronize and sustain population firing, together with their ability to deliver calcium, may influence early developmental events such as target innervation and neuronal survival.

Key words: nicotinic; receptors; acetylcholine; ciliary; synaptic; transmission; neuronal; $\alpha 7$; α -bungarotoxin; patch clamp; ganglion; calyx; postsynaptic

Acetylcholine receptors containing the $\alpha 7$ gene product ($\alpha 7$ -nAChRs) are among the most abundant nicotinic receptors in the nervous system (Couturier et al., 1990; Schoepfer et al., 1990; Anand et al., 1993; Conroy and Berg, 1998). They are thought in most cases to be homopentamers (Couturier et al., 1990; Anand et al., 1993; Chen and Patrick, 1997) having a high relative permeability for calcium (Bertrand et al., 1993; Seguela et al., 1993) and high affinity for α -bungarotoxin (α Bgt; Schoepfer et al., 1990; Anand et al., 1993; Vernallis et al., 1993). At the cellular level the receptors produce multiple effects. Presynaptically they can modulate neurotransmitter release (McGehee et al., 1995; Gray et al., 1996; Guo et al., 1998; Li et al., 1998). Postsynaptically they can generate depolarizing currents (Frazier et al., 1998). In cell culture they can influence neurite outgrowth (Pugh and Berg, 1994; Fu and Liu, 1997) and activate second messenger systems (Vijayaraghavan et al., 1995). Physiologically, however, their significance remains unclear.

A promising system for examining the role of $\alpha 7$ -nAChRs *in situ* is the chick ciliary ganglion. It has two classes of neurons in approximately equal numbers: large ciliary neurons that innervate striated muscle in the iris and ciliary body, and small choroid neurons that innervate smooth muscle in the choroid layer (Landmesser and Pilar, 1974). Both neuronal populations express $\alpha 7$ -

nAChRs, averaging 10^6 per cell at the end of embryogenesis (Chiappinelli and Giacobini, 1978; Corriveau and Berg, 1994). The receptors can generate large rapidly desensitizing currents when activated *in situ* by stimulating the preganglionic nerve (Zhang et al., 1996; Ullian et al., 1997). In addition, $\alpha 7$ -nAChRs are present on the preganglionic terminals where they can influence neurotransmitter release (Coggan et al., 1997).

Previously, $\alpha 7$ -nAChRs were thought to be unnecessary for ganglionic transmission. Electron microscopic analysis and confocal microscopy suggested that the receptors were confined to extrasynaptic clusters that excluded postsynaptic densities (Jacob and Berg, 1983; Wilson Horch and Sargent, 1995). Moreover, incubating the ganglion in α Bgt to block $\alpha 7$ -nAChRs did not block ganglionic transmission, as indicated by the monitoring of synaptically evoked compound action potentials (APs; Chiappinelli, 1983; Loring et al., 1984). Instead, transmission appeared to be mediated by receptors containing $\alpha 3$, $\beta 4$, $\alpha 5$, and $\beta 2$ subunits (Vernallis et al., 1993; Conroy and Berg, 1995). These latter receptors ($\alpha 3^*$ -nAChRs) were present both in postsynaptic densities and extrasynaptic clusters (Jacob et al., 1984; Loring and Zigmond, 1987; Wilson Horch and Sargent, 1995).

Recent electron microscopic analysis of ciliary neurons with the use of immunogold labeling and tomographic reconstruction demonstrates that the $\alpha 7$ -nAChR clusters represent receptors concentrated on groups of folded somatic spines (Shoop et al., 1999). The spines are engulfed by presynaptic structures packed with synaptic vesicles. These results, together with the fact that $\alpha 7$ -nAChRs can generate large synaptic currents *in situ* (Zhang et al., 1996), motivated a reexamination of receptor roles in ganglionic transmission. We report here that whole-cell patch-clamp

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recording *in situ* demonstrates that $\alpha 7$ -nAChRs are required in a population of ciliary neurons for reliable synaptic transmission and for tightly synchronized firing early in development.

MATERIALS AND METHODS

Tissue preparation. For whole-cell patch-clamp recordings, ciliary ganglia with nerve roots attached were dissected from embryonic day (E) 13 or E14 chicks and prepared as previously described (Zhang et al., 1996). Briefly, the connective sheaths covering the ganglia were softened with collagenase (5 mg/ml; type I, Worthington Biochemical, Lakewood, NJ) applied through a glass pipette (15–35 μ m in diameter) and then removed with fine forceps. Ganglia were cleaned further by focal application of an enzyme solution containing collagenase (2 mg/ml), protease (5 mg/ml; type XIV, Sigma, St. Louis, MO), and dispase (8 mg/ml; type II, Boehringer Mannheim, Indianapolis, IN) and delivered through a glass pipette with gentle pressure; then fine forceps were used to remove debris and loosened tissue. For extracellular compound AP recordings, E13/E14 and E18 ciliary ganglia with attached pre- and postganglionic nerve trunks were exposed to focally applied collagenase (5 mg/ml) to loosen the outer connective covering and improve drug access. Ganglia were perfused with recording medium [(in mM): 120 NaCl, 4 KCl, 10 glucose, 30 NaHCO₃, 1 MgSO₄, 1 NaH₂PO₄, and 2 CaCl₂ plus 100 nM atropine, pH 7.4, gassed with 95% O₂/5% CO₂] at a rate of ~3 ml/min throughout enzyme treatment and subsequent recording. In a few cases atropine was omitted from the extracellular bath solution; no effect was noted on the recordings.

Whole-cell patch-clamp recording. Conventional whole-cell patch-clamp recording from neurons in intact ganglia was performed as previously described (Yawo and Chuhma, 1994; Zhang et al., 1996; Ullian et al., 1997). Patch pipettes were pulled from borosilicate glass (1.5 mm outside diameter; Drummond Scientific, Broomall, PA). The electrodes had resistances of 3–5 M Ω when filled with intracellular solution. Intracellular solution contained (in mM): 140 KCl, 10 HEPES, pH 7.2, 5 glucose, 2 EGTA-KOH, and 1 MgCl₂. Series resistances averaged 8.9 ± 0.2 M Ω (mean $\pm$ SEM; $n = 35$ cells), similar to values reported previously (Yawo and Momiyama, 1993; Coggan et al., 1997); $\leq 80\%$ series resistance compensation was applied during the recording of evoked synaptic currents.

In some cases perforated patch recording, using amphotericin B (Sigma), was performed on neurons in dissected ganglia. Stock solutions of amphotericin B were made fresh as previously described (Rae et al., 1991). Briefly, a 60 mg/ml stock solution was made immediately before recording by dissolving 1 mg of amphotericin B powder in 16.67 μ l of DMSO. From this stock solution, 9 μ l was added to 1 ml of intracellular recording solution containing (in mM): 75 K₂SO₄, 55 KCl, 5 MgSO₄, and 10 HEPES, pH 7.2, with *N*-methyl-D-glucamine. The solution was vortexed, filtered, stored at 4°C in the dark, and used within 3 hr to backfill electrodes. The electrodes had resistances ranging from 1 to 2 M Ω . Series resistance averaged 15.3 ± 0.3 M Ω ($n = 14$) for ciliary neurons; $\leq 80\%$ series resistance compensation was applied.

For both conventional and perforated whole-cell patch-clamp recordings, the cells were held routinely at -70 mV in voltage-clamp mode unless otherwise indicated. In current-clamp mode a hyperpolarizing current usually was injected to shift the resting potential to -60 mV. Resting potentials were measured in the absence of injected current both at the beginning and end of recording sessions in current-clamp mode to ensure a value of at least -50 mV. Synaptic responses were elicited by stimulating the oculomotor nerve root with a suction pipette. Stimulations of 5–40 V (10–300 μ sec) were delivered with a Grass stimulator (model S88, West Warwick, RI). For experiments in which synaptic transmission was examined in the same cell before and after the addition of α Bgt, the stimulation paradigm used the following steps: (1) in voltage-clamp mode, synaptic currents were recorded while the nerve root was stimulated 10 times, first at 0.05 Hz and then at 50 Hz; (2) in current-clamp mode, first a depolarizing current pulse (200 pA, 200 msec) was injected into the cells to assess their intrinsic ability to fire APs, and then the preganglionic nerve root was stimulated 10 times at 1 Hz (10 sec), 25 Hz (400 msec), and sometimes 50 Hz (200 msec) with 1 min intervals to determine whether any of the stimulations failed to elicit APs faithfully in the cell; (3) in voltage-clamp mode, 50 nM α Bgt was perfused into the recording chamber while the synaptic currents elicited by single stimulation of the preganglionic nerve root were monitored at 20 sec intervals for at least 5 min or until maximal blockade by α Bgt was achieved; (4) after being switched back to current-clamp, current injection and nerve

root simulations were performed as in step 2; (5) again, evoked synaptic currents were monitored in voltage-clamp mode as described in step 1.

Cells were rejected if they had resting potentials more positive than -50 mV in current-clamp mode (in the absence of hyperpolarizing current) or showed signs of a regenerative component in voltage-clamp mode. Cells also were rejected if they failed to fire APs repeatedly when current was injected directly into the cell, if they had peak synaptic currents <1 nA before α Bgt blockade, if they failed on any occasion to fire an AP in response to presynaptic stimulation before α Bgt blockade, or if they failed to repolarize completely between synaptically driven APs at 1 Hz before α Bgt blockade. These criteria were chosen to insure the adequacy of the clamp and health of the cell during the recording period. Of the cells that were clamped long enough to permit complete experiments, approximately one-fifth had to be excluded from the final data set because of the selection criteria outlined.

In some experiments the effects of α Bgt were assessed by comparing the responses of cell populations treated with toxin to those treated with vehicle alone. In these cases the toxin-treated cells were prepared by incubating ganglia in 50 nM α Bgt for at least 5 min before recording and then replenishing toxin at the same concentration in the perfusion every 15 min to sustain blockade. This was more than adequate to block receptors on cells near the ganglion perimeter (those normally selected for patch-clamp recording), because previous experiments showed that no recovery of the toxin-sensitive response was obtained in such cells after 15 min of rinse with toxin-free solution (Zhang et al., 1996). The toxin-treated ganglia were subjected to the first two steps of the stimulation paradigm described above, although the 50 Hz stimulation in voltage-clamp mode usually was omitted. Otherwise, the same criteria described above were used to determine which cells were to be included in the data set. The same procedure was applied to control cells except that toxin was omitted from the solution perfusing the ganglia.

Extracellular recording of compound APs. Suction electrodes were used to stimulate the preganglionic nerve root of E13/E14 and E18 ciliary ganglia and to record the resulting compound AP traveling in the postganglionic nerve. At 10 min intervals the presynaptic nerve was stimulated either at 1 Hz for 10 sec or at 25 Hz for 400 msec, yielding 10 pulses per train. Responses in the postganglionic nerve root were monitored for 30 min to obtain a stable baseline, before the addition of either 200 nM α Bgt or 40 nM methyllycaconitine citrate (MLA).

All preparations were viewed with an upright microscope (Zeiss Axioskop, Thornwood, NY) via a 40 $\times$ water-immersion objective. For whole-cell patch-clamp recordings, currents and APs were amplified with an Axopatch 1C amplifier (Axon Instruments, Foster City, CA). For extracellular recordings, compound APs were amplified with a differential amplifier. All data, including both patch-clamp and suction electrode recordings, were stored on videotape (Sony VCR; VR-10B A/D converter, Instrutech, Great Neck, NY) as well as collected on computer hard drive with Clampex (pClamp 6.0, Axon Instruments). Data were filtered at 2 kHz (-3 dB; eight-pole Bessel filter, Frequency Devices, Haverhill, MA), digitized with Digidata 1200 (Axon Instruments), and acquired at 10–14 kHz with Clampex 6.0 (Axon Instruments). Responses were analyzed with Clampfit (pClamp 6.0, Axon Instruments) and Axograph software (Axograph 3.0, Axon Instruments); statistical analyses were performed with SigmaPlot. Current responses were fit by using the method of maximum likelihood as described previously (Zhang et al., 1996).

Competition binding assays. Membrane suspensions of E13/E14 ciliary ganglia were prepared by homogenizing the tissue in (in mM): 50 Na₂HPO₄, pH 7.4, 5 EDTA, 5 EGTA, and 1 phenylmethylsulfonyl fluoride. Aliquots were incubated with either α Bgt (0.05–1.0 μ M) or MLA (0.005–10 μ M) in triplicate and incubated at room temperature for 30 min. Then ³H-epibatidine at 1 nM was added, and the incubation continued for an additional 40 min at room temperature in the presence (nonspecific binding) or absence (total binding) of excess nicotine. Membrane fragments were collected and washed by repeated centrifugation (four times), solubilized in 200 μ l of 2% SDS, mixed with 5 ml of scintillation fluid (Ecoscint H, National Diagnostic, Atlanta, GA), and submitted to scintillation counting for a determination of bound radioactivity. Specific ³H-epibatidine binding was calculated as the difference between total and nonspecific binding and was taken to represent the number of sites associated with $\alpha 3^*$ -nAChRs in the sample (Vernallis et al., 1993; Conroy and Berg, 1998). No difference was observed in the ability of 50 nM α Bgt (that shown to be specific in the whole-cell patch-clamp recording experiments) and 200 nM α Bgt (that used for the extracellular recording experiments) to block the epibatidine binding

($n = 3$ experiments). Similarly, 40 nM MLA had no effect on epibatidine binding, although very high concentrations of MLA (10 μ M) did produce inhibition ($n = 2$ experiments). The binding results indicate that the effects of α Bgt and MLA in the extracellular recording experiments cannot be attributed to the inhibition of $\alpha 3^*$ -nAChRs.

Materials. White Leghorn chick embryos were obtained locally and maintained at 37°C in a humidified incubator. α Bgt was purchased from Biotoxins (St. Cloud, FL). Toxin was dissolved in extracellular solution and perfused into the recording chamber by gravity feed. All other drugs were purchased from Sigma. All experiments were performed at room temperature (20–23°C).

RESULTS

Selection of ciliary neurons for α Bgt tests

The role of $\alpha 7$ -nAChRs in ganglionic transmission was examined by using α Bgt to block the receptors and determining the impact on synaptic signaling. To preserve normal synaptic connections for such tests, we dissected whole E13/E14 chick ciliary ganglia with nerve roots intact, and we used whole-cell patch-clamp recording techniques to monitor postsynaptic responses elicited by stimulating the presynaptic nerve root with a suction electrode. Recordings were performed alternately in voltage-clamp and current-clamp modes so that both postsynaptic currents and resulting APs could be monitored directly.

Ciliary neurons were selected for these tests because previous experiments indicated that a large fraction of the postsynaptic current in them was generated by $\alpha 7$ -nAChRs (Zhang et al., 1996; Ullian et al., 1997). The cells were distinguished from choroid neurons in the ganglion by their location (Marwitt et al., 1971; Pilar et al., 1980), their large size (≥ 20 μ m) (Ullian et al., 1997), and their single innervation (Dryer and Chiappinelli, 1987; Ullian et al., 1997). This latter feature was demonstrated by varying the stimulus intensity to the presynaptic nerve root and determining whether the postsynaptic current fluctuated in an all-or-none manner. Ciliary neurons are innervated by a single large presynaptic calyx at this stage, whereas choroid neurons are multiply innervated by discrete synaptic boutons. Choroid neurons, therefore, can be distinguished by finding multiple components making up the postsynaptic response when the presynaptic stimulus intensity is varied.

Effects of $\alpha 7$ -nAChR blockade on synaptic signaling

Conventional patch-clamp recording was used in most experiments to compare the efficacy of synaptic transmission in ciliary neurons before and after α Bgt blockade of $\alpha 7$ -nAChRs. Stringent criteria were imposed to ensure the health of the cell throughout the test period; such criteria involved the magnitude of the unclamped resting potential, the ability to fire APs repeatedly, and the size of the synaptic response before toxin application (see Materials and Methods). After examining these features, we applied α Bgt at 50 nM for a 5 min period; previous experiments indicated that this is sufficient for a blockade of neurons located near the ganglion surface and likely to be selected for testing (Zhang et al., 1996). Only a single neuron could be examined in this manner per ganglion because the toxin blockade was long-lasting.

Synaptic responses elicited by stimulating the preganglionic nerve root were examined in voltage-clamp mode. Evoked synaptic currents occurred after a delay of 2–4 msec, as reported previously (Zhang et al., 1996). In control neurons the evoked currents were biphasic (Fig. 1*A*, *left*). The decay phase was best fit with the sum of two exponentials having average decay constants of ~ 2 and 19 msec (Table 1). Both components of the response were nicotinic because they both were blocked reversibly by 20 μ M

D-tubocurarine (D-TC; $n = 3$; data not shown). Exposure to α Bgt selectively eliminated the large rapidly decaying component (Fig. 1*A*, *right*). The small toxin-resistant component had a decay phase that usually could be fit adequately with a single exponential having a decay constant of ~ 18 msec (Table 1). The decay constants are similar in value to those reported previously (Zhang et al., 1996).

Of 14 neurons stimulated presynaptically, two-thirds showed failures at both 1 Hz (Fig. 1*B*, *right*) and 25 Hz (Fig. 1*C*, *right*) after being exposed to α Bgt. The severity of the effect varied among cells: approximately one-half of the failing cells were unable to fire any synaptically evoked APs at 1 Hz as in the example shown, whereas others failed as few as three times during the train of 10 stimuli. In contrast, none of 11 control neurons showed failures either at 1 or at 25 Hz when tested after a 5 min exposure to vehicle instead of α Bgt. All cells remained able to fire APs repeatedly when injected with depolarizing current through the patch-clamp electrode (Fig. 1*D*), and all had resting potentials of at least -50 mV at the end of the recording period in the absence of injected hyperpolarizing current. The results indicate that a portion of the ciliary neurons requires $\alpha 7$ -nAChRs to sustain repeated firing in response to synaptic stimulation.

In some experiments the amphotericin B perforated patch recording technique was used to avoid the intracellular dialysis that accompanies conventional whole-cell patch-clamp recording. Intracellular dialysis might have removed intracellular components such as second messengers or calcium-buffering components that could influence the postsynaptic response or firing capacity of the neurons. Using the perforated patch technique to compare responses from the same neurons before and after toxin treatment as described above yielded results indistinguishable from those obtained with conventional patch-clamp recording (Fig. 2*A*). Application of α Bgt to cells induced failures in five of seven ciliary neurons when challenged by synaptic stimulation at 25 Hz, although none had failed before the toxin treatment. The extent of failure (number of failures per 10 stimulus train) again varied considerably among the cells, with some being severely impaired (Fig. 2*B*) whereas others missed only two or three APs. In addition, the toxin treatment increased the time to peak for the AP, measured from the beginning of the postsynaptic response (Fig. 2*B*, compare *insets*). This presumably reflects the fact that synaptic activation of $\alpha 7$ -nAChRs normally is rate-limiting for reaching threshold to fire an AP.

Other experiments compared the responses of neurons in control ganglia with those in ganglia treated with α Bgt. Of 34 control neurons tested with conventional patch-clamp recording, only one failed to fire consistently at 1 Hz and only two failed at 25 Hz. In contrast, of 10 α Bgt-treated neurons tested, nearly one-half failed to fire at least once during the 10 pulse trial at 1 Hz and approximately two-thirds failed at least once during the 25 Hz trial. Similarly, perforated patch-clamp recording indicated that three of four α Bgt-treated cells tested in this way displayed failures at 25 Hz. In contrast none of the 14 control cells that were examined displayed failures.

Pooling the data obtained by all of the methods yielded a value of 66% for the proportion of ciliary neurons in which $\alpha 7$ -nAChRs appeared to be necessary to sustain repeated firing at 25 Hz (Table 1). For cells that failed, the failure rate within a test train was $\sim 80\%$ overall both at 1 and 25 Hz (Table 1). No significant difference was noted in the proportion of failures occurring on the

Figure 1. Responses of a neuron before and after $\alpha 7$ -AChR blockade. Shown are conventional whole-cell patch-clamp recordings from a ciliary neuron synaptically stimulated before (*left column*) and after (*right column*) a 5 min exposure to 50 nM α Bgt. *A*, Recordings in voltage-clamp mode (-70 mV) showing that the toxin treatment blocked the large rapidly decaying synaptic current caused by $\alpha 7$ -nAChRs. *B*, Evoked APs recorded under current clamp during 10 pulses of presynaptic stimulation at 1 Hz. *C*, Evoked APs under current clamp during 25 Hz presynaptic stimulation. The toxin treatment abolished synaptically evoked APs at both stimulation frequencies. The small residual synaptic current, produced by $\alpha 3^*$ -AChRs, was inadequate to generate any APs in this neuron. *D*, Repetitive APs elicited by constant current injection, showing that the toxin treatment did not alter the excitability of the neuron.

first stimulus of a train ($\sim 80\%$) as compared with failures at subsequent stimuli. Another consequence of the α Bgt treatment was a fourfold increase, on average, in the time to peak for the AP as measured from the beginning of the postsynaptic potential (Table 1). An α Bgt-dependent delay in the rise time of the postsynaptic AP also was seen previously with intracellular recording (Zhang et al., 1996). The results indicate that $\alpha 7$ -nAChRs are necessary for rapid and reliable synaptic transmission in a portion of the ciliary neurons at this developmental stage. Differences in the time to threshold among cells in α Bgt could also, in effect, desynchronize firing within the population.

Mechanisms by which $\alpha 7$ -AChR blockade causes transmission failures

The fact that $\alpha 7$ -nAChRs are present both on the presynaptic calyx and on somatic spines emanating from the postsynaptic cell, together with the high relative calcium permeability of the receptors, suggests several mechanisms by which the receptors might influence the efficacy of synaptic transmission during a train of stimuli. Perhaps the simplest possibility is that the receptors are necessary to generate sufficient postsynaptic current for bringing the cell to threshold reliably. In this case, one might expect a positive correlation between the reliability of synaptic transmis-

Table 1. Effects of α Bgt on signaling properties of ciliary neurons

	Peak synaptic current (nA)	Decay time τ (msec)	AP rise time (msec)	Cells failing at 1 Hz (%)	Cells failing at 25 Hz (%)	APs fired by failing cells at 1 Hz (%)	APs fired by failing cells at 25 Hz (%)
Control ($n = 59$)	-3.2 ± 0.3	2.3 ± 0.1 18.7 ± 1.5	0.95 ± 0.04^a	2	3	—	—
After α Bgt ($n = 35$)	-0.31 ± 0.1	18.3 ± 1.4	$4.0 \pm 0.8^{a,b}$	54 ^c	66	20 ± 2	21 ± 6

^aAction potential rise times were determined by measuring the time between 10 and 90% peak value of the first evoked action potential (starting from the foot of the postsynaptic potential) observed during 1 Hz presynaptic stimulation.

^b $n = 15$ cells. Only 15 cells of the 24 cells tested at 1 Hz fired evoked action potentials.

^c24 cells were tested.

Figure 2. α Bgt effects on ciliary responses monitored with perforated patch-clamp recording. Shown are synaptically evoked responses in a ciliary neuron before (left column) and after (right column) exposure to 50 nM α Bgt (50 nM) for 5 min, recorded with the amphotericin B perforated patch-clamp method. *A*, Synaptic currents, voltage-clamp mode. *B*, Synaptically evoked APs at 25 Hz, current-clamp mode. *Insets*, Individual AP with preceding postsynaptic potential on an expanded time scale. The toxin exposure substantially reduced the reliability of synaptic transmission, as also seen with conventional patch-clamp recording, and increased the time to threshold for firing an AP.

sion that follows α Bgt treatment and the size of the α Bgt-resistant synaptic current. To assess such a correlation, we combined and graphed data from both the conventional and amphotericin recordings, showing for each cell the proportion of successful trials obtained within a train versus the α Bgt-resistant current density.

At 1 Hz, most cells having an α Bgt-resistant current density <15 pA/pF displayed severe impairment in their ability to generate synaptically driven APs (Fig. 3A). For responses below 5 pA/pF the failures were often complete, i.e., no APs resulted from the stimulus train. Conversely, all cells having an α Bgt-resistant response >15 pA/pF faithfully followed the stimulus train and displayed no failures at 1 Hz. At 25 Hz stimulation the correlation

was qualitatively similar (Fig. 3B). Again most cells having an α Bgt-resistant response <15 pA/pF showed failures in producing synaptically driven APs, with the proportion of failures during the test train varying widely among cells. Many cells with responses below 5 pA/pF failed completely; all but one cell with α Bgt-resistant responses >15 pA/pF generated APs without failure when stimulated at 25 Hz. Interestingly, there may be some quantitative differences between the patterns at 1 and 25 Hz; at the higher frequency only approximately one-half as many cells failed severely, i.e., fired $<20\%$ of the time. No significant difference was found in the time course of decay for α Bgt-resistant synaptic currents between cells that fired reliably and those that failed [mean decay constants of 16.1 ± 1.9 msec ($n = 23$), and

Figure 3. Positive correlation between the reliability of synaptically evoked APs and the synaptic current density remaining in the presence of α Bgt. The proportion of times an individual cell successfully fired a synaptically driven AP (from a train of 10 stimuli) was graphed as a function of peak synaptic current density (pA/pF). Results were pooled from conventional and perforated patch-clamp recordings performed on cells after toxin treatment. Presynaptic stimulation was delivered either at 1 Hz (*A*) or 25 Hz (*B*). The results indicate a strong correlation between the likelihood of reliable firing and the size of the postsynaptic current after α Bgt treatment.

19.1 ± 2.4 msec ($n = 12$), respectively]. The primary result is a clear correlation between the amplitude of the α Bgt-resistant synaptic current and the ability to fire synaptically driven APs in the absence of functional $\alpha 7$ -nAChRs. The implication is that $\alpha 7$ -nAChRs promote reliable transmission by enhancing the synaptic current.

Evidence against a significant contribution from presynaptic $\alpha 7$ -nAChRs in the present instance comes from the observation noted above that failures were just as likely to occur in α Bgt on the first trial as on succeeding trials of a given train either at 1 or 25 Hz. If the effects of α Bgt observed here on transmission

involved primarily presynaptic receptors, one might expect the most dramatic impact later in the train, particularly at 25 Hz when activation of the receptors would be most assured by ACh accumulating from sequential release events. Additional evidence comes from comparing the peak amplitude of the synaptic current after toxin treatment ($\alpha 3^*$ -nAChR response) with that calculated for the $\alpha 3^*$ -nAChR portion of the total synaptic current before toxin application. Normally, the decrement in peak synaptic current resulting from rundown during the test period is $13 \pm 3\%$ (mean $\pm$ SEM; $n = 14$ cells) for total synaptic current and is $11 \pm 2\%$ ($n = 11$) for the peak current attributed specifically to $\alpha 3^*$ -nAChRs (slowly desensitizing current with $\tau = 19$ msec; Table 1) in the absence of toxin. These values are not significantly different from the decrement seen in the $\alpha 3^*$ -nAChR response for toxin-treated cells in which the peak response remaining after α Bgt treatment is only $16 \pm 3\%$ ($n = 21$) smaller than that calculated for the $\alpha 3^*$ -nAChR portion of the total synaptic current before toxin treatment. Accordingly, we conclude that the influence of presynaptic $\alpha 7$ -nAChRs on transmitter release during an isolated stimulation event as measured by the size of the $\alpha 3^*$ -nAChR response must be small and cannot by itself account for the failures seen in synaptically evoked APs caused by the toxin.

Effects of $\alpha 7$ -nAChR blockade on the synaptically evoked compound AP

Extracellular recording from postganglionic nerve roots has indicated previously that α Bgt does not block ganglionic transmission when tested on ganglia taken from E18–E20 chick embryos (Chiappinelli, 1983; Loring et al., 1984). Accordingly, it seemed important to determine whether a different answer might be obtained when the extracellular recording methods were applied to the younger ganglia used in the present studies. The whole-cell patch-clamp analysis predicted two effects of α Bgt treatment in this case. First, transmission failures caused by α Bgt should score as a decrease in the mean amplitude of the synaptically driven compound AP observed in the postganglionic nerve because a portion of the axons would not be firing in any given trial. Second, the shape of the compound AP should be broadened because of asynchronous firing of neurons brought on by the slower and more variable rate at which α Bgt-resistant synaptic currents drive cells to threshold.

E13/E14 ciliary ganglia dissected with nerve roots intact were stimulated via the preganglionic nerve at 10 min intervals at 1 and 25 Hz (10 stimuli per train in each case) while the elicited compound AP was monitored in the postganglionic nerve. After a 30 min test period to ensure stable and reproducible responses, α Bgt was perfused over the ganglion and the monitoring continued at 10 min intervals. A toxin concentration of 200 nM was used, rather than the 50 nM used for patch-clamp experiments, because complete penetration of the ganglion was sought. Within 40 min a substantial decrement was observed in the mean peak amplitude of the compound AP (Fig. 4). Similar results were obtained when 40 nM MLA was perfused for 50 min, an antagonist thought to be specific for $\alpha 7$ -nAChRs when applied in the nanomolar range (Ward et al., 1990; Alkonon et al., 1992). Neither blockade reversed when unbound antagonist was removed by perfusion. No decrement in amplitude or change in time course was observed over the same test period when ganglia were perfused with vehicle alone (Fig. 4).

Blockade of $\alpha 7$ -nAChRs also delayed initiation of the compound AP and increased the rise time. After the toxin and MLA

Figure 4. Effects of $\alpha 7$ -nAChR blockade on synaptically evoked compound APs in the postganglionic nerve of E13/E14 ganglia. *A*, Extracellular recording of compound APs in the ciliary nerve root before and after perfusion of the ganglion with vehicle alone (*left*), 200 nM α Bgt for 40 min (*middle*), or 40 nM MLA for 50 min (*right*). α Bgt and MLA each decreased the peak amplitude of the compound AP and increased its duration. In the *right panel* a small fast electrical component is observed before the onset of the chemical component of the synaptic response; the electrical component was not decreased either by 100 μ M D-tubocurarine or by 40 nM MLA. *B*, Time dependence of α Bgt and MLA effects on the compound AP. The peak amplitude of the synaptically evoked compound AP was measured at 10 min intervals for ganglia perfused with vehicle alone (*filled diamonds*), with 200 nM α Bgt (*open circles*), or with 40 nM MLA (*filled squares*). α Bgt and MLA perfusion was initiated after 30 min and continued as indicated; results were normalized to those obtained 30 min after the initiation of perfusion, immediately before the addition of toxin or MLA. Values represent the mean $\pm$ SEM of four ganglia for each condition at each time point. Baseline for the compound AP was taken as the point immediately before the rising phase of the chemical response in each case.

treatments the time to peak was $166 \pm 19\%$ ($n = 4$) and $135 \pm 5\%$ ($n = 4$), respectively, of that seen beforehand, whereas perfusion with vehicle alone over the same time period produced no change ($98 \pm 4\%$; $n = 4$). Blockade desynchronized the remaining active units, as seen by a broadening of the compound AP. Measuring the width at half-height showed the compound AP after toxin and MLA treatments to be $147 \pm 7\%$ and $134 \pm 11\%$, respectively, of that seen before treatment, whereas ganglia perfused with vehicle alone showed no change ($97 \pm 2\%$). In each respect the results

were qualitatively consistent with those predicted from the patch-clamp recording experiments.

A second method of assessing the number of units recruited during stimulation is to measure the area under the positive phase of the compound AP. This measurement should be proportional to the total number of units activated and should not be corrupted by a desynchronization of firing among units. Desynchronization would tend to broaden the peak at the expense of height. Comparing the area under the peak of the compound APs indicated

that perfusing ganglia 40 min with 200 nM α Bgt produced an $18 \pm 4\%$ reduction ($p < 0.03$). A 50 min perfusion with 40 nM MLA produced a $21 \pm 1\%$ reduction ($p < 0.004$). In two experiments in which perfusion with 200 nM α Bgt was continued for 70 min, the average decrease was 40%. No decrement was observed for ganglia perfused 120 min with vehicle alone ($n = 4$). Binding experiments confirmed that the concentrations of α Bgt and MLA used for the experiments were specific for $\alpha 7$ -nAChRs and did not occupy $\alpha 3^*$ -nAChRs (see Materials and Methods). The results indicate that inhibition of $\alpha 7$ -nAChRs in E13/E14 ganglia does block a portion of the ganglionic transmission.

In view of the results with E13/E14 ganglia, we decided to perform similar experiments on older ganglia. E18 ciliary ganglia were prepared for recording synaptically evoked compound APs as described above. After allowing 30 min to ensure stable and reproducible responses, we perfused the ganglia with 200 nM α Bgt. Within 80 min the toxin treatment caused an $18.8 \pm 1.5\%$ (mean $\pm$ SEM, $n = 5$) reduction in the peak amplitude of the compound AP. The total area under the positive phase of the compound AP was reduced by $8.4 \pm 2.0\%$. Control E18 ganglia perfused for the same time period in the absence of toxin showed nominal increases in the peak amplitude ($112 \pm 7\%$; $n = 3$) and the area under the positive phase of the compound AP (105 ± 1 ; $n = 3$). Both the toxin effect on amplitude and on area under the curve were significant ($p < 0.04$ and $p < 0.02$, respectively). α Bgt treatment also increased the time to peak for the compound AP ($137 \pm 8\%$, $n = 5$) but had no significant effect on the peak width measured at half-height ($106 \pm 3\%$; $n = 5$; $p = 0.12$). These changes suggest a small but significant effect of α Bgt on the efficacy of synaptic transmission in E18 ganglia, decreasing the reliability of transmission as it does in younger ganglia; the effect, however, is less pronounced than at E13/E14.

DISCUSSION

The principal finding reported here is that $\alpha 7$ -nAChRs are necessary to ensure reliable and synchronous firing of synaptically evoked APs in ciliary neurons. The requirement appears relatively early in ganglionic development and is not limited to high-stimulation frequencies. This is, to our knowledge, the first demonstration of an obligatory role for $\alpha 7$ -nAChRs *in situ*. Interestingly, the requirement revealed here may be primarily transitory, because it appears to play a less prominent role later in development although the neurons acquire even greater numbers of $\alpha 7$ -nAChRs and maintain them throughout adulthood. The implication is that $\alpha 7$ -nAChRs perform other functions as well, particularly in the mature ganglion.

Each of the patch-clamp methods used to assess the efficacy of synaptic transmission gave the same result. The advantage of the population analysis, i.e., comparing toxin-treated neurons with control neurons, was that responses could be measured almost immediately after the patch-clamp access was established. The advantage of recording from the same neurons before and after toxin treatment was that one could be sure that the neurons were capable of fault-free firing before toxin treatment. The advantage of the perforated patch technique, despite the added difficulties posed in terms of achieving adequate patches, was that it prevented intracellular dialysis of components that might have influenced the results. In each case, stringent criteria were used to ensure the competence of the neuron and the synaptic connection.

Recording the synaptically evoked compound AP in the ciliary nerve confirmed the patch-clamp results, namely that blockade of

$\alpha 7$ -nAChRs reduced the number of neurons capable of firing APs in response to stimulation of the preganglionic nerve. The appeal of experiments measuring the compound AP is that the entire population of neurons can be sampled with little intervention. The disadvantage is that complete penetration of the ganglion by the blocking agent is required for maximal effect. This required higher toxin concentrations and longer incubation times than used routinely for the blockade of individual cells on the ganglion surface. Nonetheless, both the competition binding studies performed here and physiological analysis performed previously (Ullian et al., 1997) indicated that the conditions used for toxin blockade in the ganglion specifically blocked $\alpha 7$ -nAChRs and not $\alpha 3^*$ -nAChRs.

The amount of blockade inferred from decrements in the compound AP was consistent with the proportion of ciliary neurons predicted to fail from single cell analysis. Thus at 1 Hz approximately one-half of the ciliary neurons showed some failures, and the mean failure rate among them per stimulus was $\sim 80\%$. Consequently, a 40% failure rate would be expected for the ensemble on a given trial if the cells sampled by patch-clamp recording were representative of the entire ciliary population. In fact, the overall failure rate inferred from the compound AP measurements approached 40% whether measured as a decrement in peak height after 40 min of toxin exposure or measured as area under the peak at 70 min. Even then full blockade may not have been achieved because the effect was still increasing slowly at the end of the toxin incubation. The choroid population did not contribute to the compound AP measurements probably because no choroid branches were apparent among those selected for recording; had they been present, choroid compound APs would have had a longer delay and smaller amplitude than was observed for ciliary responses (Dryer, 1994). Although electrical synapses form on ciliary neurons at later developmental stages and generate APs with no synaptic delay (Dryer, 1994), they rarely are detected at E13/E14 (Yawo and Momiyama, 1993; Coggan et al., 1997) and did not contribute to the compound AP measurements.

The most likely mechanism by which $\alpha 7$ -nAChRs support reliable transmission through the embryonic ciliary ganglion is by contributing directly to the total synaptic current. This is supported by the positive correlation between the success rate for synaptically evoked APs in the presence of α Bgt and the amplitude of the α Bgt-resistant synaptic current. The presynaptic calyx on ciliary neurons contains α Bgt-sensitive nAChRs thought to be $\alpha 7$ -nAChRs (Coggan et al., 1997), but presynaptic nAChRs are unlikely to account for much of the α Bgt effect reported here. Toxin-induced failures in ganglionic transmission occurred as frequently on the first trial of the 10 stimulus test train as at any other position. Were the toxin effect being exerted via presynaptic receptors, one would expect the incidence of failures to be most pronounced later in the test train because that is when transmitter buildup should have been greatest and produced the greatest activation of presynaptic nAChRs. Moreover, no significant difference was found in the amplitude of the $\alpha 3^*$ -nAChR response before and after toxin treatment, indicating that presynaptic $\alpha 7$ -nAChRs did not have a significant impact on transmitter release within a single trial. The physiological significance of presynaptic $\alpha 7$ -nAChRs in the ganglion has yet to be determined.

The kind of ganglionic dependence on $\alpha 7$ -nAChRs seen here at E13/E14 appears to be partly transient during development, as noted above. By E18, α Bgt treatment caused only a small decrease in the amplitude and no significant change in the width of the synaptically evoked compound AP. Apparently, the toxin-

induced desynchronization of firing seen at E13/E14 is much attenuated by E18. The relatively small effect on amplitude at this later stage probably accounts for previous failures to detect a role for $\alpha 7$ -nAChRs in the synaptically evoked compound AP (Chiappinelli, 1983; Loring et al., 1984).

Previous recordings from E13/E14 ciliary neurons with sharp electrodes indicated that toxin blockade of $\alpha 7$ -nAChRs did not abolish qualitatively the synaptically evoked APs in the neurons per se but did increase the time required for successful transmission (Zhang et al., 1996), as seen here with patch-clamp recording. Sharp electrode recording was not used then to quantify the reliability of transmission because of concern that calcium leak around the electrode might alter neuronal excitability and confound the results, as inferred previously for rat intracardiac ganglion neurons (Cuevas et al., 1997). Preliminary experiments with sharp electrode recording did suggest, however, that α Bgt-treated neurons might be less capable than control neurons at firing faithfully in response to trains of presynaptic stimulation (Z.-w. Z. and D. K. B., unpublished data); those findings helped to motivate the present analysis.

The ability of a neuronal population to fire reliable and synchronized APs early in development may have important consequences. Increasing evidence indicates that spontaneous activity is common in developing circuits, that it often depends on chemical transmission, and that it helps to shape the final pattern of connections formed (Ding et al., 1983; Thompson, 1985; O'Donovan and Landmesser, 1987; Galli and Maffei, 1988; Shatz and Stryker, 1988; Dahm and Landmesser, 1991; Mooney et al., 1996; Ruthazer and Stryker, 1996; Weliky and Katz, 1997; Penn et al., 1998). In several cases spontaneous bursting activity recorded *in situ* during development has been shown to depend on or be modulated by nicotinic transmission (Feller et al., 1996; Catsicas et al., 1998; Penn et al., 1998).

Two important developmental processes occur in the ciliary ganglion between E9 and E14, a time when synaptically driven APs in ciliary neurons depend in part on $\alpha 7$ -nAChRs. Ciliary ganglion neurons innervate postsynaptic muscle tissue in the eye at this time, and cell death removes one-half of the neurons in the ganglion (Dryer, 1994). Both of these processes may be influenced by $\alpha 7$ -nAChR-dependent transmission. Indeed, daily administration of α Bgt *in ovo* between E7 and E14 rescues neurons that would have been lost via naturally occurring cell death, but it is unclear whether the toxin effects are caused by the blockade of ganglionic $\alpha 7$ -nAChRs or nAChRs in the postsynaptic muscle (Meriney et al., 1987). It is noteworthy that $\alpha 7$ -nAChRs also have a high relative calcium permeability (Bertrand et al., 1993; Seguela et al., 1993). This, and their ability to synchronize synaptically evoked APs in ciliary neurons, may equip the receptors uniquely to influence maturation of the circuit.

Almost certainly $\alpha 7$ -nAChRs also contribute to ganglionic transmission in other ways. The receptors are concentrated on somatic spines in close proximity to presumed sites of transmitter release (Shoop et al., 1999), and the receptors are maintained in abundance even on adult neurons. Moreover, even at E13/E14 some ciliary neurons do not appear to require the receptors for reliable transmission. What other functions might they serve? Because iris and ciliary body muscle in birds becomes striated between E8 and E17 (Link and Nishi, 1998), it represents fast-twitch muscle in large part and requires prolonged high-frequency stimulation to sustain contraction. Transmission through the adult ganglion is capable of providing such stimulation, driving ciliary neurons at rates in excess of 100 Hz with few

failures (Dryer, 1994). Although $\alpha 7$ -nAChRs displayed only subtle frequency-dependent effects in the present experiments, this does not preclude either pre- or postsynaptic $\alpha 7$ -nAChRs from being essential in some manner for sustaining the higher frequency rates likely to be encountered in adult birds. A motor system with such demands well may require ongoing regulatory activity at multiple levels, including short-term regulation of voltage-gated channels, mid-term regulation of receptor function, and long-term regulation of synaptic structure and gene expression. Each of these can be calcium-dependent and, therefore, possible targets of control by repetitively activated $\alpha 7$ -nAChRs.

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