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Targeting α7-containing nicotinic receptors on neurons to distal locations

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Abstract

Nicotinic receptors containing the α7 gene product are widely expressed in the nervous system and have a high relative permeability to Ca^{2+}. This permits them to influence a variety of Ca^{2+}-dependent events in neurons. On chick ciliary ganglion neurons, the receptors are concentrated on somatic spines and contribute directly to postsynaptic signaling. Receptors containing the α7 gene product can also be found in the chick sciatic nerve being transported to distal locations. Both motoneurons and dorsal root ganglion neurons are candidate sources of the receptors since both extend processes into the nerve and synthesize α7 protein. Immunoprecipitation assays with subunit-specific monoclonal antibodies and pharmacological comparisons fail to detect differences between sciatic nerve and ciliary ganglion α7-containing receptors. Cell-specific machinery and receptor posttranslational modifications may determine which sites the receptors populate.

Keywords: Nicotinic receptor; Acetylcholine receptor; α-Bungarotoxin; Ciliary ganglion; Sciatic nerve; Neuronal

1. Introduction

Nicotinic acetylcholine receptors are widely expressed in the nervous system and appear to participate in a variety of phenomena. One of the most abundant receptor subtypes is a species containing the α7 gene product. Such receptors have a high relative permeability to Ca^{2+} (Bertrand et al., 1993; Seguela et al., 1993) and influence a diverse array of Ca^{2+}-dependent events. Examples include neurotransmitter release (McGehee et al., 1995; Gray et al., 1996; Coggan et al., 1997), second messenger cascades (Vijayaraghavan et al., 1995), neurite extension (Chan and Quik, 1993; Pugh and Berg, 1994; Fu et al., 1998), and both apoptosis (Berger et al., 1998) and neuronal survival (Messi et al., 1997). The receptors can also contribute directly to postsynaptic signaling (Zhang et al., 1996; Ullian et al., 1997; Frazier et al., 1998). Clearly, receptor location plays a critical role in determining the consequences of activating α7-containing nicotinic receptors.

A second determinant of α7-containing receptor signaling effects is likely to be the particular set of functional properties displayed by the receptors. While most native α7-containing receptors studied to date bind α-bungarotoxin in a pseudo-irreversible manner and rapidly desensitize (Zorumski et al., 1992; Alkondon and Albuquerque, 1993; Zhang et al., 1994; Blumenthal et al., 1997), some bind the toxin in a rapidly reversible manner and are slow to desensitize (Cuevas and Berg, 1998; Cuevas et al., unpublished results). Large differences in the rate of desensitization could produce big consequences for the cell if, for example, all α7-containing receptor species have a high relative permeability to Ca^{2+}. It is not clear whether the functional differences observed in α7-containing receptors reflect different posttranslational modifications of a single homopentameric receptor species or instead are generated by multiple native α7-containing species. In rat, only a single putative homomeric α7-containing receptor species has been found to date (Chen and Patrick, 1997), while in chicken, some α7 protein can be found co-assembled with the highly homologous α8 gene product (Schoepfer et al.,

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1990). Recently, it has been proposed that the α7 subunit may assemble with other gene products in vivo as well (Yu and Role, 1998).

A reasonable hypothesis is that differences in receptor composition may target receptors to different locations on the cell. This is supported by the finding that exchanging the large cytoplasmic loop between the α3 and α7 gene products can re-direct chimeric receptors to different sites (Williams et al., 1998). Two extremes in receptor location would be presynaptic sites on axon terminals vs. postsynaptic sites on the cell body. In the chick ciliary ganglion, α7-containing nicotinic receptors are found preferentially concentrated on somatic spines emanating from ciliary neurons (Shoop et al., 1999) where they generate large synaptic currents and support reliable, synchronized transmission through the ganglion at early developmental times (Chang and Berg, 1999). Similar receptors are also present on the presynaptic calyx engulfing the cells (Coggan et al., 1997). The presynaptic receptors are presumably synthesized in preganglionic neurons of the accessory oculomotor nucleus (Dryer, 1994) and transported distally to the calyx.

The present experiments were undertaken for two reasons. The first was to determine whether α7-containing nicotinic receptors could be detected in peripheral nerves and shown to be in transport to remote sites. The second was to determine whether such receptors could be distinguished immunologically or pharmacologically from postsynaptic α7-containing receptors on cell bodies. The chicken sciatic nerve was chosen for these studies because it was large enough to permit analysis of axonal transport and because it contained motor axons, like the accessory oculomotor nerve, which might present sufficient material for binding studies. Ciliary ganglion α7-containing nicotinic receptors were chosen as a source of postsynaptic receptors for comparison because much was known about their function, composition, and distribution.

2. Materials and methods

2.1. Fluorescence imaging

The procedures, equipment, and reagents used for confocal fluorescence imaging were those previously described (Shoop et al., 1999). Dissociated E15 chick ciliary ganglion neurons were prepared and labeled with Alexa488-α-bungarotoxin for α7-containing nicotinic receptors on the cell surface prior to fixing and permeabilizing, and then were co-labeled with either rhodamine–phalloidin for filamentous actin or an anti-drebrin monoclonal antibody (plus fluorescently labeled secondary antibody) for drebrin.

For conventional fluorescence microscopy, E15 chick dorsal root ganglia and E17/18 chick lumbar spinal cord were dissected and fixed immediately in 4% (v/v) paraformaldehyde overnight at 4°C. The fixed tissues were then incubated 6 h in 30% (w/v) sucrose and embedded in OCT for cryostat sectioning. The sections (20 μm) were permeabilized with 0.1% (w/v) Triton-X100 containing 10% (v/v) horse serum in phosphate-buffered saline (0.15 M NaCl, 0.01 M Na_2HPO_4, pH 7.4) and incubated with goat anti-α7 antibodies overnight at 4°C. After rinsing three times for 10 min with phosphate-buffered saline, the samples were incubated with Cy3-conjugated donkey anti-goat antibodies for 1 h at room temperature, rinsed, and viewed with fluorescence microscopy using a Zeiss Axioskop equipped with a Cooke SensiCam 12.5 MHz CCD camera. Substitution of goat immunoglobulin G for the anti-α7 antibodies served as a negative control.

2.2. Binding experiments

Solid-phase immunoprecipitation assays, the competition binding experiments, and the filter assays were conducted as previously described (Conroy and Berg, 1995, 1998; Pugh et al., 1995). The specificities of the monoclonal antibodies used have been documented previously (see references in Vernallis et al. (1993) and Conroy and Berg (1995)). K_i values were calculated as described previously (Cheng and Prusoff, 1973). Unless otherwise indicated, the results are expressed as the mean ± SEM of the number of experiments indicated by *n*. Statistical significance was judged by applying Student's *t*-test.

2.3. Nerve ligation

Chicks at posthatch day 8 were anesthetized by intrapectoral muscle injections of xylazine (0.1 mg/g body weight), ketamine (0.01 mg/g), and valium (1 mg/kg) according to veterinary protocol for young birds under 100-g weight. After a minimal incision in the mid-thigh region, the sciatic nerve was exposed and ligated with a surgical silk thread. As a sham-operated control, the contralateral sciatic nerve was exposed in a similar manner but not ligated. Great care was taken to avoid vascular damage. After a 24-h period, the operated animals were sacrificed and both the ligated and sham-operated sciatic nerves were dissected. Four sequential 5-mm segments were cut from each nerve, two proximal to the ligation and two distal. Segments at equivalent positions either from several ligated nerves or from an equivalent number of sham-operated control nerves were pooled for analysis of α-bungarotoxin-binding sites in the solid-phase immunoprecipitation assay.

2.4. Materials

White Leghorn chick embryos were purchased locally and maintained at 37°C in a humidified incubator. Rho-

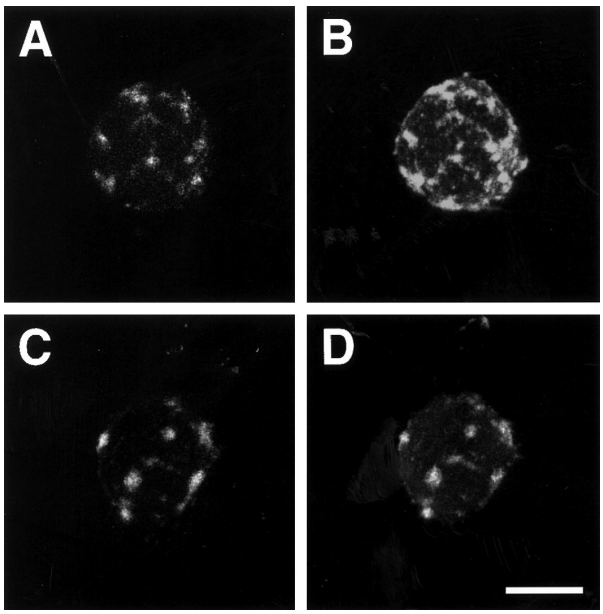

Fig. 1. Confocal immunofluorescence images showing the co-distribution of α7-containing nicotinic receptors with actin filaments and drebrin in spine mats on freshly dissociated E15 chick ciliary ganglion neurons. (A,C) Alexa488-conjugated α-bungarotoxin. (B) Rhodamine–phalloidin staining of cell shown in panel A. (D) Anti-drebrin monoclonal antibody followed by Cy3-labeled secondary antibody staining of cell shown in panel C. Scale bar: 10 μm.

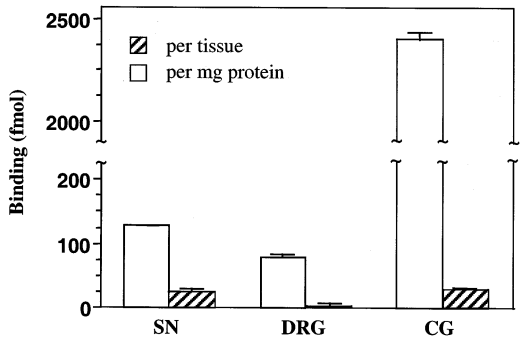

Fig. 3. Comparison of α7-containing receptors levels in tissue extracts using solid-phase immunoprecipitation assays. Assays were performed on extracts of E17/18 sciatic nerve (SN), E15 dorsal root ganglia (DRG), and E17/18 ciliary ganglia (CG) using anti-α7 monoclonal antibodies 318/319 to immunotether α7-containing nicotinic receptors, and ^{125}I-α-bungarotoxin to quantify them. Values represent the mean ± SEM of three or more experiments (triplicate determinations per experiment), and are expressed either per unit tissue (ganglion or nerve, clear bars) or per mg protein (hatched bars).

damine-conjugated phalloidin and Alexa488-conjugated α-bungarotoxin were purchased from Molecular Probes, goat anti-α7 antibodies and goat immunoglobulin G from Santa Cruz Biotech., donkey anti-goat antibodies from Jackson Immunochem., anti-drebrin monoclonal antibody from Medical and Biological Laboratories (Nagota, Japan), and OCT from Miles (Elkhart, IN). All other reagents were purchased from Sigma (St. Louis, MO).

3. Results

3.1. Postsynaptic α7-containing nicotinic receptors in the ciliary ganglion

One of the most abundant sources of α7-containing nicotinic receptors is the chick ciliary ganglion in which the receptors are concentrated on somatic spines folded

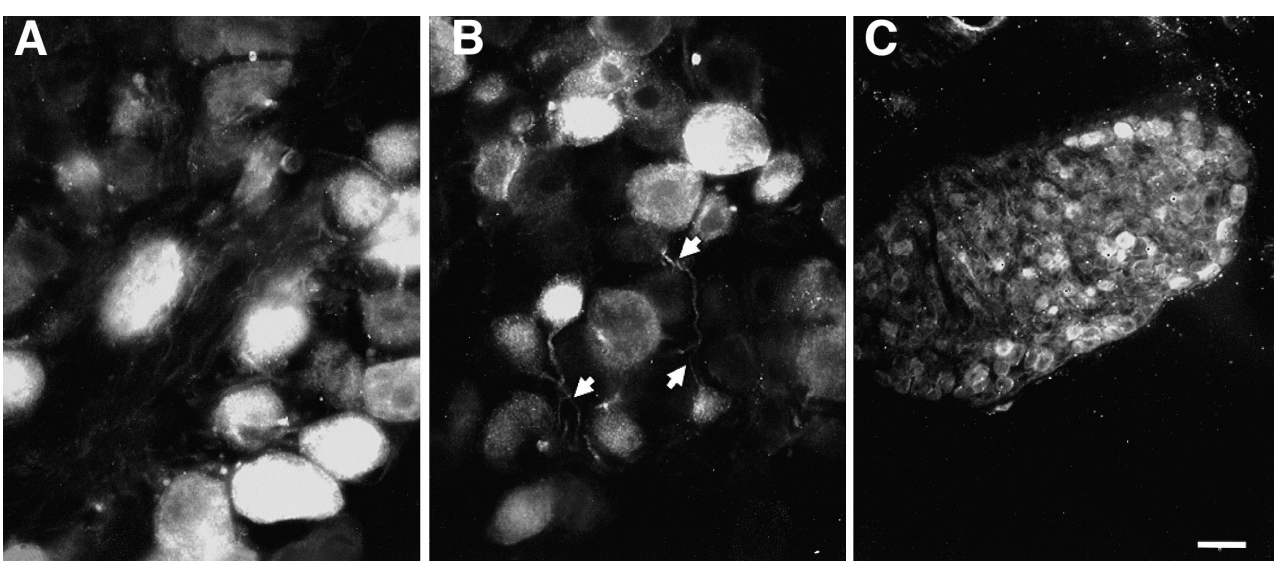

Fig. 2. Immunofluorescence labeling of α7-containing nicotinic receptors in cryostat sections of embryonic chick spinal cord and dorsal root ganglion tissue. Sections were labeled with goat anti-α7 antibodies followed by Cy3-conjugated secondary antibodies and viewed for fluorescence. Substitution of goat immunoglobulin G for the anti-α7 antibodies as a negative control yielded no labeling (not shown). (A,B) E17/18 spinal cord. (C) E15 dorsal root ganglion. Labeled axon segments (arrows) can be seen extending from some of the cell bodies. Scale bar: 25 μm (A,B), 10 μm (C).

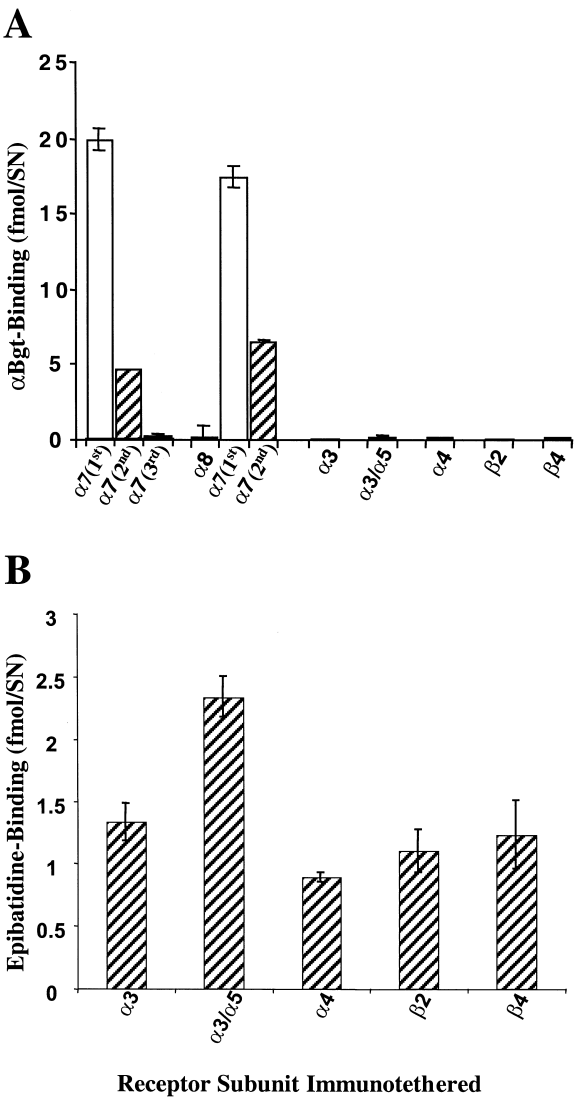

Fig. 4. Subunit composition of sciatic nerve nicotinic acetylcholine receptors determined by immunoprecipitation of detergent-solubilized receptors with subunit-specific monoclonal antibodies. (A) Immunoprecipitation of ^{125}I-α-bungarotoxin binding sites by three sequential rounds with anti-α7 monoclonal antibodies 318/319 (α7(1st, 2nd, 3rd)), or by sequential rounds of first anti-α8 monoclonal antibody 308 and then twice with anti-α7 monoclonal antibodies 318/319 (α8, α7 (1st, 2nd)), or by single rounds with anti-α3 monoclonal antibody 313 (α3), anti-α3/α5 monoclonal antibody 35 (α3/α5), anti-α4 monoclonal antibody 289 (α4), anti-β2 monoclonal antibody 270 (β2), or anti-β4 monoclonal antibody B4-1 (β4). Only the anti-α7 monoclonal antibodies immunoprecipitated α-bungarotoxin binding sites, and the sites were not depleted by the anti-α8 monoclonal antibody. Filter assays revealed no additional α-bungarotoxin-binding sites (not shown). (B) Immunoprecipitation of ^{3}H-epibatidine-binding sites by the non-α7/α8 monoclonal antibodies used in panel A. Significant binding was detected in each case indicating that the antibodies were effective and that the sciatic nerve contains small amounts of non-α7 nicotinic receptors. Values represent the mean ± SEM of results from three separate experiments (triplicate determinations per experiment).

into discrete mats on the ciliary neuron surface (Shoop et al., 1999). This can be seen by labeling α7-containing receptors on freshly dissociated E15 chick ciliary ganglion cells with fluorescently conjugated α-bungarotoxin and then examining the cells with confocal fluorescence microscopy. Bright patches of fluorescence demarcate the spine mats heavily endowed with α7-containing receptors (Fig. 1A and C). Permeabilizing the cells and co-staining them either with rhodamine-conjugated phalloidin to label actin filaments (Fig. 1B) or with an anti-drebrin monoclonal antibody followed by fluorescent secondary antibody to label the actin-associated drebrin (Fig. 1D) demonstrate co-distribution with the receptor labeling and reveal the configuration of spines comprising the mats.

3.2. Transport of α7-containing nicotinic receptors in sciatic nerve

The chick sciatic nerve was tested as a source of α7-containing receptors being transported distally. For much of its length, the sciatic nerve contains both motor and sensory fibers: motor neurons extend axons through the nerve to innervate skeletal muscle while dorsal root ganglion neurons send branches through the nerve to reach

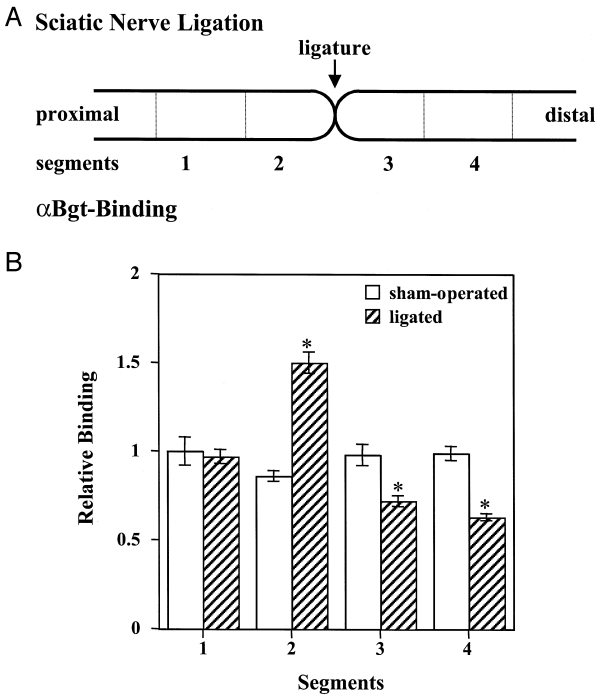

Fig. 5. Ligated sciatic nerve showing accumulation of α7-containing nicotinic receptors on the proximal side of the ligation. (A) Schematic diagram illustrating the positions of dissected segments relative to the ligature. (B) Relative levels of α7-containing receptors present in segment extracts, quantified using the solid-phase immunoprecipitation assay as in Fig. 3. Corresponding segments from several ligated nerves (hatched bars) or from sham-operated contralateral control nerves (open bars) were pooled separately for assay. Values represent the mean ± SEM of results from 16 independent experiments and have been normalized to the values obtained for the initial segment of sham-operated controls, which averaged 26.7 ± 5 fmol/mg protein ($n = 16$). * Different from corresponding sham-operated segment ($P < 0.002$) and different from sham-operated first segment ($P < 0.006$).

their sensory fields in the periphery. Immunofluorescent staining of E17/18 chick spinal cord sections with an anti-α7 polyclonal antibody indicated the presence of α7 protein in large cell bodies and initial axon segments in the ventral horn region (Fig. 2A and B). The location and morphology of the cells were those expected of motor neurons. The same staining procedure applied to E15 dorsal root ganglion sections revealed α7 protein there as well (Fig. 2C).

A solid-phase immunoprecipitation assay was used to quantify the number of α7-containing receptors in the sciatic nerve. The concentration of α-bungarotoxin binding sites (per mg protein) found in sciatic nerve extracts was greater than in dorsal root ganglion extracts but only about 5% of that in ciliary ganglion extracts (Fig. 3). The total amount present in the sciatic nerve was 20-fold greater than that in the dorsal root ganglion due to the difference in tissue mass; it was about twofold greater when expressed per mg protein.

Ligation experiments were performed to determine whether α7-containing receptors detected in sciatic nerve were being transported to the periphery. The sciatic nerve was unilaterally ligated with silk thread in newly hatched chicks, and the contralateral side was exposed in a sham operation as a control. After 24 h, the chick was sacrificed and the nerves were divided into segments for analysis of α7-containing nicotinic receptors in the solid phase assay. Sham-operated control segments have essentially equivalent amounts in all segments, whereas the ligated nerve had about 50% more receptor immediately proximal to the ligation (Fig. 4). Segments immediately distal to the ligation showed decreased receptor levels. This pattern is expected for a component undergoing orthograde transport and indicates that the accumulation of α7-containing receptors proximal to the ligation is unlikely to have resulted from nerve injury per se.

3.3. Comparison of α7-containing nicotinic receptors in sciatic nerve and ciliary ganglia

Two approaches were used to compare the physical properties of α7-containing nicotinic receptors being transported in the sciatic nerve with those present in the ciliary ganglion. The first made use of subunit-specific monoclonal antibodies and immunoprecipitation assays to determine whether other known nicotinic acetylcholine receptor gene products contributed to the receptors. In screening a number of candidates, previous studies found only α7 subunits present in ciliary ganglion α7-containing receptors (Vernallis et al., 1993; Conroy and Berg, 1995). Similar results were obtained here for the sciatic nerve species. Thus, two immunoprecipitations with anti-α7

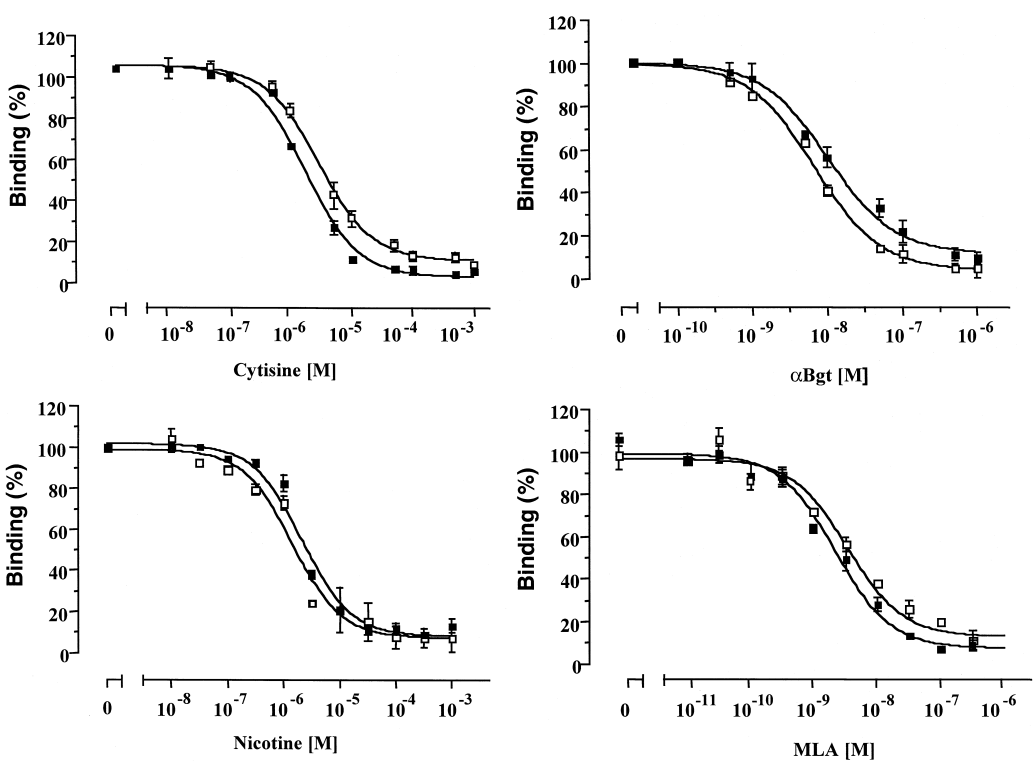

Fig. 6. Comparison of ligand binding properties for α7-containing nicotinic receptors in sciatic nerve and ciliary ganglion extracts. Competition binding experiments were performed with the solid-phase immunoprecipitation assay using ^{125}I-α-bungarotoxin and the indicated agonist (cytisine, nicotine) and antagonists (α-bungarotoxin, methyllycaconitine (MLA)) at the indicated concentrations. No differences were detected between the binding profiles for sciatic nerve (filled symbols) and ciliary ganglion (open symbols) samples. Values represent the mean ± SEM of three to four separate experiments (triplicate determinations per experiment for each concentration).

Table 1
K_i and IC_{50} values for agonists and antagonists competing against ^{125}I-α-bungarotoxin for binding to α7-containing nicotinic receptors from sciatic nerve and ciliary ganglia
Values were obtained from binding experiments like those described in Fig. 6. No differences in K_i values were found for sciatic nerve versus ciliary ganglion α7-containing receptors ($P > 0.39$). The SEMs (not shown) were less than 5% of the values.

	Sciatic nerve		Ciliary ganglion	
	IC_{50}	K_i	IC_{50}	K_i
Nicotine (μM)	2.1	0.7	1.4	0.6
Cytosine (μM)	2.3	0.8	1.4	0.5
Acetylcholine (mM)	0.3	0.1	0.3	0.1
α-Bungarotoxin (nM)	8.7	3.2	5.2	2.1
MLA (nM)	2.5	6.9	10.7	4.1
Tubocurare (μM)	2.4	0.9	3.2	1.3
Strychnine (μM)	21.3	7.8	22.3	8.4

monoclonal antibodies were sufficient to collect all of the α7-containing nicotinic receptors, while none was collected by an anti-α8 monoclonal antibody (#308) or monoclonal antibodies specific for the α3 (#313), α1/α3/α5 (#35), α4 (#289), β2 (#270), or β4 (#B4-1) nicotinic acetylcholine receptor gene products (Fig. 5A). Several of the non-α7 monoclonal antibodies, however, immunoprecipitated a small number of epibatidine-binding sites from sciatic nerve extracts, indicating that the nerve contained other kinds of nicotinic acetylcholine receptors as well (Fig. 5B).

A pharmacological approach was used as the second method of comparing sciatic nerve α7-containing receptors with those in the ciliary ganglion. In these cases, the receptors were solubilized by detergent extraction, tethered with anti-α7 monoclonal antibodies in the solid-phase assay, incubated with ^{125}I-α-bungarotoxin in the presence of competing ligands, and then processed for quantification of bound radioactivity. The binding isotherms both for agonists and antagonists were indistinguishable for sciatic nerve and ciliary ganglion α7-containing receptors (Fig. 6). No significant differences in K_i values were detected between the two classes of receptors for any of the ligands tested (Table 1). Taken together, these experiments found no differences between sciatic nerve and ciliary ganglion α7-containing nicotinic receptors other than their destinations.

4. Discussion

The results reported here indicate that α7-containing nicotinic receptors can be detected in the chick sciatic nerve where they undergo orthograde axonal transport. The final destinations of the receptors are likely to be presynaptic sites at the neuromuscular junction or distal sites on sensory fibers in the periphery. The immunoprecipitation analysis with subunit-specific monoclonal antibodies demonstrated that α-bungarotoxin-binding receptors in the sciatic nerve contain α7 subunits and lack the other nicotinic acetylcholine receptor subunits tested. In this respect and in their pharmacological profile, the receptors are indistinguishable from α7-containing receptors in the ciliary ganglion though the ganglionic receptors undergo a different fate: they are concentrated on somatic spines and play a postsynaptic role.

The immunological tests used to probe for other neuronal nicotinic acetylcholine receptor subunits would have detected α3, α4, α5, α8, β2, and β4 subunits. These include all of the neuronal nicotinic receptor gene products known to be expressed in the periphery and the only other neuronal nicotinic receptor gene product known to bind α-bungarotoxin in chick, namely α8 (Schoepfer et al., 1990). The muscle α1 gene product also binds α-bungarotoxin but it would have been immunoprecipitated by monoclonal antibody 35 in the solid-phase assay used here, had it been present (Conroy and Berg, 1998). If other gene products are present in either the sciatic or ciliary ganglion α7-containing receptors, they are likely to be encoded by genes that have yet to be identified.

The chick sciatic nerve also contains minor populations of epibatidine-binding nicotinic receptors, having a variety of gene products. Epibatidine is an excellent broad-spectrum high affinity ligand for many kinds of neuronal nicotinic receptors lacking the α7 and α8 gene products (see references in Conroy and Berg, 1998). Some of these are known to be expressed by dorsal root ganglion neurons (Boyd et al., 1991) and may be expressed by motoneurons as well. Their functions remain unknown, but may be analogous to that of α7-containing receptors.

Previous studies in mammals demonstrated that α-bungarotoxin-binding proteins could be identified in the sciatic nerve, and ligation experiments suggested they were subject both to orthograde and anterograde axonal transport (Ninkovic and Hunt, 1983; Millington et al., 1985). At least part of the receptors in transport were shown to be dorsal root ganglion in derivation because of their presence in dorsal roots. No evidence for anterograde transport of α7-containing receptors was seen in chick sciatic nerve; it is not clear whether this is a species or techniques difference. The mammalian studies did not look for other kinds of nicotinic receptors in sciatic nerve.

In failing to find differences between sciatic nerve and ciliary ganglion α7-containing receptors, the present study leaves unanswered the question of how different cell types target the receptors to diverse locations. Also intriguing is the question of what functions α7-containing receptors serve in peripheral locations innervated by the sciatic nerve. An obvious possibility is presynaptic modulation of transmitter release at motor axon terminals. This would be consistent with their demonstrated presence on preganglionic terminals (McGehee et al., 1995; Coggan et al., 1997). Another strong possibility is that the receptors also serve a sensory function on dorsal root ganglion processes

in the periphery. Reports that an epibatidine analog can act as an analgesic support the idea of nicotinic receptors mediating sensory functions (Bannon et al., 1998), and knockout mice lacking either the α4 or β2 nicotinic receptor gene product have altered nociception (Marubio et al., 1999). Knockout mice lacking the α7 gene product have also been produced (Orr-Urtreger et al., 1997), but may require additional tests to reveal a role for α7-containing nicotinic receptors in the periphery.

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