Nicotinic Acetylcholine Receptor Subtypes in the Rat Sympathetic Ganglion: Pharmacological Characterization, Subcellular Distribution and Effect of Pre- and Postganglionic Nerve Crush

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Abstract. Nicotinic acetylcholine receptors (nAChRs) mediate fast synaptic transmission in autonomic ganglia, which innervate and control the activity of most visceral organs. By combining ultrastructural, immunocytochemical, and pharmacological analyses, we characterized the nAChR subtypes in the rat superior cervical ganglion (SCG) and the effect of pre- and postganglionic nerve crush on their number in the ganglion and their distribution at the intraganglionic synapses. Binding with radioactive nicotinic ligands, immunoprecipitation, and immunolocalization experiments revealed the presence of different nAChR subtypes: those containing the α3 subunit associated with β4 and/or β2 subunits that bind 125I-αBungarotoxin. After postganglionic nerve crush, the number of nicotinic receptors and immunopositive intraganglionic synapses for each nAChR subunit strongly decreased. Both the number of nAChRs and immunoreactivity recovered 26 days after injury, when regenerating postganglionic fibers had reinnervated the peripheral target organs, as shown by the restoration of tyrosine hydroxylase immunoreactivity in the iris. This observation and the lack of any effect of preganglionic nerve crush on the number of nicotinic receptors suggest that the peripheral targets affect the organization of intraganglionic synapses in adult SCG.

Key Words: αBungarotoxin; Epibatidine; Immunoelectron microscopy; nAChR subunit-specific antibodies; Superior cervical ganglion; Tyrosine hydroxylase.

INTRODUCTION

Most vertebrate’s visceral organs are innervated by the autonomic nervous system which, by regulating their activity, plays an essential role in the homeostasis of several physiological parameters, that are subject to functional and environmental changes (1–3).

The principal neurotransmitter that generates the fast excitatory postsynaptic potential in autonomic ganglia is acetylcholine, which acts through nicotinic acetylcholine receptors (nAChRs). These receptors are pentameric ligand-gated ion channels, which in the nervous system can be formed by a combination of α and β subunits or by α subunits alone. Twelve distinct neuronal nAChR subunits have so far been identified (α2–α10 and β2–β4). Three of these (α7, α8, and α9) can form homopentameric receptors, whereas the others are co-assembled in heteromeric receptors containing 2 α subunits and 3 β subunits, with many receptor subtypes showing different electrophysiological and pharmacological properties (4–6).

Studies of knockout mice lacking nAChR subunits have revealed the prominent role of these receptors in controlling the structural and functional integrity of organs such as the heart, intestine, and bladder (7). In particular, α3 knockout mice exhibit increased perinatal mortality and severe physiological impairment in the organs innervated by the autonomic nervous system (8). A very similar phenotype has also been described in animals lacking both the β2 and β4 subunits (9), whereas mice lacking only the β4 subunit survived but there was a considerable reduction in nicotine-induced whole cell currents in the superior cervical ganglion (SCG) (7). The β4 knockout animals retained sufficient autonomic function probably because of a certain degree of redundancy or compensation between the β2 and β4 subunits (9).

Both pre- and postganglionic autonomic neurons can regenerate their axons after axonal damage, whereas the neurons of the CNS generally die or atrophy in response to the same stimuli (10). An experimental model extensively used to study neuronal reactions to axotomy is the rodent SCG, whose principal target organs are the iris, pineal gland, salivary glands, heart, and some artery smooth muscles. Axotomy causes functional and structural changes in the cell body of ganglionic neurons (11–13) and leads to dramatic changes in gene expression (14).

The expression of genes coding for nAChR subunits decreases after axotomy: in mouse SCG, there is dramatic decrease in α3 mRNA in the first 2 days (15), and in rat SCG the transcripts for α3, α5, α7, and β4 are strongly downregulated. Only β2 mRNA increases after 3 days.
(16). The protein levels of α3, β4 and α7 subunits also decrease after axotomy (17, 18). No direct evidence is yet available regarding the nAChR subtypes present at the intraganglionic synapses of adult rat SCG, their pharmacological characteristics, or the kinetics of their disappearance-reappearance during the detachment-reattachment of pre- and postsynaptic elements of the intraganglionic synapses induced by postganglionic nerve crush.

In this study, we immunolocalized the α3, β2, β4, and α7 nAChR subunits at the intraganglionic synapses of rat SCG. The presence of differently assembled nAChR subtypes and their pharmacological characteristics were revealed by using ligands specific for different nAChR subtypes and immunoprecipitation with subunit-specific antibodies. The time course of the changes induced by postganglionic nerve crush in the intraganglionic synaptic localization of the nAChR subunits, and on the levels of the nAChR subtypes, was also analyzed. The time course of the degeneration-regeneration of the injured axons was monitored by immunolocalizing the tyrosine hydroxylase (T-OH) enzyme in the iris, one of the target organs of the SCG.

**MATERIALS AND METHODS**

**Animals and Surgical Procedures**

Male Wistar rats (120–190 g body weight) were used (Charles River S.P.A., Calco, Italy). The animals were housed and handled in accordance with the guidelines laid down by the European Communities Council Directive (86/609/EEC) and the American Society for Neuroscience.

The rats were anesthetized by intraperitoneal injection of Rompun (xylazine, 20 mg/ml, 0.5 ml/kg body weight; Bayer, Leverkusen, Germany) and Zoletil (tiletamine and zolazepam, 100 mg/ml, 0.5 mg/kg body weight; Virbac, Carros, France). Postganglionic nerve crush was performed as previously described (19). Briefly, the right SCG was exposed and both the internal and external carotid nerves (the 2 major postganglionic branches) were crushed approximately 1 mm from the ganglion. The wound was sutured and the rats were left to recover from the anesthesia. Ipsilateral ptosis of the eyelid was used as the criterion for a successful operation. Operated rats were then killed at 1, 3, 7, 14, and 26 days after surgery. Preganglionic nerve crush was performed by crushing the right cervical sympathetic nerve trunk proximal to the SCG (about 1 mm from the ganglion).

**Immunocytochemistry**

Two or 3 animals for each time point were killed and the dissected tissue analyzed.

**Light Microscopy:** Control and operated rats were deeply anesthetized with Rompun and Zoletil, as described above, and perfused transcardially with oxygenated Ringer’s solution (pH 7.3) followed by 4% freshly depolymerized paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4). The right iris was removed from both control and operated rats and placed whole free-floating in 0.5 M Tris/HCl (pH 7.6). The specimens were processed for T-OH immunohistochemistry using the peroxidase-anti-peroxidase (PAP) procedure as follows. After inactivation of the endogenous peroxidase with 10% methanol and 3% H2O2 in 0.5 M Tris/HCl (pH 7.6), the specimens were incubated for 1 h at room temperature (RT) with a blocking solution of 5% dry milk, 0.5% Triton X-100 in 0.5 M Tris/HCl, followed by incubation for 36 h, at 4°C, with the primary antibody diluted in 1% dry milk, 0.2% Triton X-100 in 0.5 M Tris/HCl. After several rinses in buffer, the irises were incubated for 1 h at RT in 1:300 goat anti-rabbit IgG (Sternberger Monoclonals Inc., Baltimore, MD), rinsed again and incubated for 1 h at RT in rabbit PAP diluted 1:1,000 (Sternberger Monoclonals Inc.). Antibody binding sites were revealed by incubation with 0.05% 3,3’-diaminobenzidine (DAB) and 0.01% H2O2. After several rinses in buffer and one in cresyl gel, each

**TABLE 1**

Sequences of the Peptides Used as Antigens, Animals Species, and Dilutions Used for Immunocytochemistry

<table>
<thead>
<tr>
<th>Subunit</th>
<th>Sequence</th>
<th>Species</th>
<th>Dilutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>α3</td>
<td>TRPTSNEGNAKPRPLYGAEELSNLNC</td>
<td>Human</td>
<td>1:150 (2.17 μg/ml)</td>
</tr>
<tr>
<td>α7</td>
<td>PSGDPLAKILEEVRVIANRFR</td>
<td>Mouse</td>
<td>1:250 (6.56 μg/ml)</td>
</tr>
<tr>
<td>β2</td>
<td>RQREREALFFRFFAGDSTC</td>
<td>Human</td>
<td>1:130 (2.85 μg/ml)</td>
</tr>
<tr>
<td>β4</td>
<td>VSSHTAGLRDARLSSGFRDELDQEALEGc</td>
<td>Rat</td>
<td>1:40 (13.75 μg/ml)</td>
</tr>
</tbody>
</table>

**Primary Antibodies**

Polyclonal antibodies against the α3, α7, β2, and β4 nAChR subunits were raised in rabbit, affinity-purified, and characterized by immunoblot of mouse SCG extracts as previously described (20, 21). The peptides used as antigens are comprised in the cytoplasmic loop between the M3 and M4 transmembrane regions, which represents the most divergent region within the sequence of nAChR subunits. This reduces the risk of cross-reactivity within antibodies raised against the different receptor subunits. Table 1 shows the sequences of the peptides used as antigens, the animal species to which each sequence refers, and the dilutions used for immunocytochemistry. Immunocytochemical control of each subunit antibody was performed by incubating the sections with the primary antibodies preabsorbed overnight with 40 μg/ml of the respective peptide used as antigen.

By light microscopy, tyrosine hydroxylase was revealed in control and denervated irises using an affinity purified polyclonal antibody produced in rabbit (Chemicon International Inc., Temecula, CA) and diluted 1:100. The same antibody was used for immunoblot.

**Immunoblot**

Control and operated rats using an affinity purified polyclonal antibody produced in rabbit (Chemicon International Inc., Temecula, CA) and diluted 1:100. The same antibody was used for immunoblot.
iris was placed on a subbed glass slide and permanently cov-
serslipped with Eukitt balsam. The specimens were observed and
photographed under a Zeiss Axioshot light microscope.

**Electron Microscopy:** Rats were perfused as described in the
light microscopy section. The SCGs from control and operated
rats were rapidly removed, embedded in 4% agar, and cut on a
Vibratome into 50-μm-thick serial sections and collected free
floating in different wells containing 0.1 M PB (pH 7.4). Each
well was then processed with a different antibody. The sections
were treated as described for light microscopy, with the excep-
tion of the time of incubation of the primary antibodies (18 h
instead of 36 h) and of the buffer used for rinsing and incu-
bation (PB instead of TRIS-HCl). After inactivation of the en-
dogenous peroxidase and before incubation with blocking solu-
tion, the sections were cryoprotected, quickly frozen in liquid
nitrogen-cooled isopentane (2-methylbutane) and then thawed.
The freeze-thaw procedure was repeated 4 times, allowing an-
tibody penetration without the use of tissue detergents. After
the DAB reaction, the immunolabeled sections were rinsed,
osmicated for 1 h, rinsed again, dehydrated in a series of ascend-
ing concentration of ethyl alcohol and propylene oxide, infiltr-
ated, and flat-embedded in Epon 812. During dehydration, the
slices were incubated for 20 min at 4°C with 1% uranyl acetate
dissolved in 70% ethyl alcohol. Ultrathin sections (60–70 nm)
were cut from flat-embedded vibratome slices (chosen random-
ly from each well) on a Reichert-Jung Ultracut E ultramicro-
tome, collected on copper grids, and observed under a Philips
EM 2085 transmission electron microscope operating at 60 kV.
The ultrathin sections were left unstained to avoid shading of the
immunoreaction product.

**Controls:** Control sections from all types of specimen were
obtained by omitting the primary antibody. The sections were
then treated for immuno-light and -electron microscopy as de-
scribed above.

**Quantitative Analysis of Immunopositive**
**Postsynaptic Specializations**

Synapses and postsynaptic specializations of intact synapses,
immunopositive for the α3, α7, β2, and β4 nAChR subunits,
were counted in ultrathin sections of both control and injured
SCGs under an electron microscope at ×28,000 magnification.
For each primary antibody the counts were performed by scan-
ning 20 meshes of the copper grids (each mesh has an area of
9,000 μm²) containing ultrathin sections obtained from at least
2 different control or operated animals on each experimental
day. For the control SCGs, the number of immunopositive post-
synaptic specializations for each nAChR subunit was expressed
as a percentage of the number of postsynaptic specializations
 counted (immunopositive + immunonegative). For operated an-
imals, the number of immunopositive postsynaptic specializa-
tions for each subunit at each experimental time was expressed
as a percentage of the respective controls. The differences in
the percentage between control and injured SCGs at each time
point were evaluated by Student t-test.

In order to reduce the risk of false negatives the counts were
performed at the Epon-tissue interface, well within the range
of antibody penetration. Since we considered only unequivoc-
cally immunopositive postsynaptic specializations, we are
aware that their final numbers may be underestimated. Never-
theless, since the criteria used to identify immunopositive post-
synaptic specializations were identical at every experimental
time considered, this underestimation is constant for both con-
trol and injured ganglia.

**Dissection of SCGs and Irises for Biochemical and**
**Pharmacological Assays**

Rats were anesthetized with ether and killed by decapitation.
The SCGs and the irises were removed, frozen, and stored at
−70°C until use.

**Electrophoresis and Immunoblotting**

Irises were homogenized in RIPA buffer containing 50 mM
Tris/HCl pH 7.4, 150 mM NaCl, 1% sodium deoxycholate sulfate
(SDS), 1% Triton and a cocktail of inhibitors (1 mM PMSF, 10
μg/ml leupeptin, 10 μg/ml pepstatin, 1 mM EDTA, 0.2 mM
Na3VO4, and 0.1 mM NaF) with a ground-lass microhomo-
genizer kept in ice. After centrifugation (15,000 g for 15
min at 4°C), supernatants were divided into aliquots and protein con-
centration determined using the Micro BCA kit (Pierce, Rock-
ford, IL). Proteins were separated by SDS-polyacrylamide gel
electrophoresis (SDS-PAGE) on 10% gel and transferred onto
nitrocellulose membrane. Nonspecific binding sites were
blocked with 5% dry milk in TTBS (20 mM TRIS, 500 mM
NaCl, and 0.05% Tween-20) and then membranes were incu-
bated overnight at 4°C with the anti-T-OH affinity-puriﬁed
polycyonal antibody diluted 1:500. After a thorough wash in
buffer, the membranes were incubated for 1 h at RT with an
anti-rabbit IgG secondary antibody conjugated to horseradish
peroxidase (Promega, Madison, WI), diluted 1:10,000, and then
developed by the use of enhanced chemiluminescence (Pierce,
Rockford, IL). The immunoreacted bands were visualized by
exposure of the membrane to X-OMAT films (Kodak, Cedex,
France).

**3H-Epibatidine and 125I- αBungarotoxin Binding to**
**Homogenated and Intact SCGs**

**Homogenated SCGs:** Rat SCGs were first homogenized using
a Potter homogenizer in an excess of buffer A (50 mM Tris-
HCl pH 7, 120 mM NaCl, 5 mM KCl, 1 mM MgCl2, 2.5 mM
CaCl2, and 2 mM PMSF) and then centrifuged (centrifugation
60 min at 30,000 × g). The pellet (SCG membranes) was rinsed
twice before final suspension in the same buffer containing 20
μg/ml of leupeptin, bestatin, pepstatin A, and aprotinin protease
inhibitors.

Saturation experiments were performed by incubating SCG
membranes overnight with 0.01 to 5 nM 3H-Epibatidine (‘H-
Epi) (Amersham; Piscataway, NJ; specific activity 56 Ci/mmol)
at 4°C, or with 0.01–10 nM 125I- αBungarotoxin (125I- αBgtx)
(Amersham; specific activity 243 Ci/mmol) at 20°C. It is well
known that α3-containing nAChRs bind Epi with picomolar
affinity but not αBgtx, whereas the α7-containing receptors not
only bind αBgtx with nanomolar affinity, but also Epi, albeit
with a much lower affinity than that of the heteromeric recep-
tors (22, 23). In order to prevent the binding of ‘H-Epi to the
αBgtx-binding receptors, the membranes were preincubated
with 2 μM αBgtx and then with ‘H-Epi. In the case of 125I-
αBgtx, 2 mg/ml bovine serum albumin (BSA) was added to the

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[Note: The document appears to be a research report involving neuroscience, detailing methods and results of experiments performed on rat spinal cord ganglia (SCGs) and irises. It includes descriptions of immunohistochemical and electron microscopic analyses, as well as biochemical methods involving electrophoresis and radioligand binding assays.]
suspension buffer. Specific radioligand binding was defined as total binding minus nonspecific binding determined in the presence of 100 nM cold Epi or 1 μM cold αBgtx, respectively.

The inhibition of radioligand binding by cytisine, nicotine, and acetylcholine was measured by preincubating SCG membranes with increasing doses (10 pM–10 mM) of the nicotinic agonists for 30 min at RT, followed by overnight incubation with a final concentration of 0.15 nM 3H-Epi or 0.75 nM 125I-αBgtx, at the same temperatures as those used for the saturation experiments. These ligand concentrations were used for the competition binding experiments because they are within the range of the Kd values of the ligands for the 2 different classes of nAChR.

The experimental data obtained from the 3 saturation and 3 competition binding experiments were analyzed by means of a nonlinear least square procedure using the LIGAND program described by Munson and Rodbard (24). The binding parameters were calculated by simultaneously fitting 3 independent saturation experiments and the Ki values were determined by fitting the data of 3 independent competition experiments. The errors in the Kd and Ki values of the simultaneous fits were calculated using the LIGAND software, and expressed as percentage coefficients of variation.

Intact SCGs: Single frozen SCGs were washed in buffer A, the buffer was then removed after centrifugation at 10,000 RPM in an Eppendorf centrifuge.

3H-Epibatidine Binding: The ganglia were preincubated with 2 μM cold αBgtx in buffer A containing the protease inhibitors as described above for SCG membranes and then with a saturating concentration of 2 nM 3H-Epi. Nonspecific binding was determined in parallel incubations of SCGs carried out in the presence of 100 nM unlabeled Epi. After incubation, the SCGs were washed several times with wash buffer (10 mM Na phosphate, 50 mM NaCl pH 7.4) and the buffer was removed each time after centrifugation at 10,000 RPM in an Eppendorf centrifuge at 4°C. Ganglia labeled with 3H-Epi were dissolved in 2 N NaOH overnight at RT, scintillation liquid (Packard, Meriden, CT) was added to the dissolved SCGs and the samples were then counted in a β counter.

125I-αBgtx Binding: Single frozen SCGs were washed as for 3H-Epi binding and then incubated with 10 nM 125I-αBgtx in buffer A plus 2 mg/ml of BSA. Nonspecific binding was determined in parallel incubations of SCGs carried out in the presence of 1 μM unlabeled αBgtx. Binding was performed overnight at RT. After incubation SCGs were washed as described for ganglia incubated with 3H-Epi and then directly counted in a γ counter.

Immunoprecipitation of nAChRs by Anti-Subunit-Specific Antibodies

The SCG membranes were prepared as described above. Membranes were solubilized by incubating the resuspended pellet in the presence of Triton X-100 at a final concentration of 2% for 3 to 4 h at 4°C. The extract was centrifuged at 60,000 × g for 1.5 h, at 4°C, and the clear supernatant was recovered.

In the case of immunoprecipitation of 3H-Epi receptors the 2% Triton X-100 extracts obtained from SCGs were preincubated with 2 μM αBgtx and then labeled with 2 nM 3H-Epi. For the 125I-αBgtx receptors the 2% Triton X-100 extracts were labeled with 10 nM 125I-αBgtx. The labeled extracts were then incubated overnight with a saturating concentration (20–30 μg) of the antibodies raised against the α2, α3, α4, α5, α6, α7, β2, β3, β4 peptides of the rat nAChR subunits. The antibody specificity and immunoprecipitation capacity were previously reported by Gotti et al (20), Zoli et al (25), and Champtiaux et al (26). The immunoprecipitate was recovered by incubating the samples with bound goat anti-rabbit IgG beads (Tecnogenetics, Milan, Italy). The level of immunoprecipitated 3H-Epi or 125I-αBgtx receptors was expressed as the percentage of the total amount of labeled receptors present in the solution before immunoprecipitation.

RESULTS

Immunolocalization of α3, α7, β2, and β4 nAChR Subunits in Control and Injured Rat SCG

In rat SCG, both neurons and perineuronal satellite cells were immunopositive for the α3, α7, β2, and β4 nAChR subunits. All the nAChR subunits considered showed the same subcellular pattern of immunolabeling.

In neurons the immunoreactivity was found in the cytoplasm, mostly associated with the cisterns of the rough endoplasmic reticulum (RER; Fig. 1A) and, rarely, of the Golgi apparatus (Fig. 1B), and at the cell surface, associated with the postsynaptic specializations of several ganglionic synapses (Fig. 1C). Multivesicular bodies and the rare vacuolar elements of probable endosomic origin were always immunonegative. The percentage of the postsynaptic specializations immunopositive for the α3, α7, β2 and β4 nAChR subunits is shown in Table 2. In glial cells, immunoreaction product was mainly localized in the cell processes surrounding neuronal somata, dendrites, and axons (Fig. 1D).

Postganglionic nerve crush induces a series of alterations to the perikaryon of the injured neurons, among which chromatolysis, known as the axon reaction (11, 12), as well as reversible detachment of preganglionic terminals from the damaged neurons mediated by satellite cell processes (27). At an early stage of the chromatolytic reaction the immunopositivity associated with the RER drastically decreased (data not shown), while the numerous degenerative elements such as multivesicular bodies were often filled with immunoreaction product (Fig. 2A). At 1 and 3 days after postganglionic nerve crush a massive detachment of the preganglionic terminals from the injured neurons was observed. The few morphologically intact synapses still present were either immunonegative (Fig. 2A) or immunopositive (Fig. 2B). Satellite cell processes approaching (Fig. 2B) or intruding (Fig. 2C) between the pre- and postsynaptic elements of intraganglionic synapses were often observed. Twenty-six days after surgery, the latest postoperative time analyzed, the subcellular pattern of immunolabeling for nAChR subunits was back to control levels and signs of the axon reaction were no longer detected (data not shown). No changes in the pattern or intensity of immunostaining for...
**Fig. 1.** Electron micrographs showing immunoreactivity for nAChR subunits in control rat SCG. 

**A:** The immunoreaction product for the β4 subunit is associated with a cistern of the rough endoplasmic reticulum (rer; arrowhead) of a ganglionic neuron. Magnification: ×27,000.

**B:** Some cisterns of the Golgi apparatus (g) are immunolabeled. Magnification: ×33,000.

**C:** The postsynaptic specialization of a synapse established between a preganglionic bouton (b) and a dendrite of a ganglionic neuron (d) is immunopositive for the β2 subunit (arrows). SC, immunopositive perineuronal satellite cell processes. Magnification: ×65,000.

**D:** Process of a perineuronal satellite cell (arrowheads) showing clumps of immunoreactivity for the α7 subunit. The neuronal membrane is clearly immunonegative (arrows). Magnification: ×30,000.

**E:** Control section from a rat SCG obtained by preabsorbing the anti-α3 antibody with the respective peptide. The postsynaptic apparatus (arrowheads) of this synapse established by a presynaptic bouton (b) on a neural dendrite (d) is immunonegative. Magnification: ×65,000.

**TABLE 2**

<table>
<thead>
<tr>
<th>Subunit</th>
<th>Immunopositive synapses (%)</th>
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</thead>
<tbody>
<tr>
<td>α3</td>
<td>57 ± 4</td>
</tr>
<tr>
<td>α7</td>
<td>37 ± 3</td>
</tr>
<tr>
<td>β2</td>
<td>42 ± 5</td>
</tr>
<tr>
<td>β4</td>
<td>45 ± 3</td>
</tr>
</tbody>
</table>

The means ± SEM of the number of postsynaptic specializations immunopositive for α3, α7, β2, and β4 nAChR subunits are reported. Data are expressed as the percentage of the total number of postsynaptic specializations (immunopositive + immunonegative) counted in 20 meshes of the copper grids, as described in Materials and Methods.

The different nAChR subunits were observed in the satellite cells at any postinjury date.

**Time Course of Loss and Recovery of Immunoreactivity for nAChR Subunits at Postsynaptic Specializations of Axotomized Ganglionic Neurons**

Figure 3 shows the number of intact intraganglionic synapses and their postsynaptic specializations immunopositive for the α3, α7, β2, and β4 nAChR subunits as a function of the time following postganglionic nerve crush, expressed as a percentage of the respective controls.

At 1 day after injury a reduction in the numbers of both intraganglionic synapses and subunit-immunopositive postsynaptic apparatus was observed. No statistically significant differences were found within the percentages of α3-, β2-, and β4-immunopositive postsynaptic apparatus remaining (26 ± 4, 37 ± 6 and 30
Fig. 2. Electron micrographs showing immunoreactivity for nAChR subunits in injured rat SCG. A, B: Rat SCG 3 days after postganglionic nerve crush. A: The postsynaptic specialization of an intact synapse is immunonegative for the α3 subunit (arrowheads). A multivesicular body (mb) filled with clumps of α3 subunit immunoreactivity (asterisk) is visible. B: The postsynaptic specialization of a synapse is α7 immunopositive (arrows). A satellite cell process (asterisk) approaches the preganglionic bouton and the dendrite. Magnification: ×52,000. C: Rat SCG 7 days after postganglionic nerve crush. A satellite cell process (asterisk) separates the dendrite from a preganglionic bouton filled with synaptic vesicles. The postsynaptic specialization (arrowheads) is immunonegative. Magnification: ×45,000. Abbreviations: b, preganglionic boutons; d, dendrites; SC, satellite cell.

Effects of Postganglionic Nerve Crush on Tyrosine Hydroxylase Immunoreactivity in the Iris

Tyrosine hydroxylase is the rate-limiting enzyme in norepinephrine biosynthesis (28). In the rodent SCG, this enzyme is localized in the cell bodies, axons, and synaptic terminals ending in the target organs of ganglionic neurons and is an index of their sympathetic innervation.
Fig. 3. Time course of the changes in numbers of intraganglionic synapses and postsynaptic specializations immunopositive for \( \alpha_3, \alpha_7, \beta_2, \) and \( \beta_4 \) nAChR subunits induced by postganglionic nerve crush in rat SCG. For each experimental time, the mean ± SEM of the number of synapses and of postsynaptic specializations immunopositive for each nAChR subunit counted in 20 meshes of the copper grids is reported. Data at each postinjury day (d) are expressed as a percentage of the respective controls. At 1 d the percentage of postsynaptic specializations immunopositive for \( \alpha_7 \), analyzed using the Student \( t \)-test, is not significantly different from that of synapses. On the other hand, the percentages of \( \alpha_3-, \beta_2-, \) and \( \beta_4 \)-immunopositive postsynaptic specializations are significantly lower than that of the remaining synapses (\( p < 0.001, p < 0.01, \) and \( p < 0.001 \), respectively, using Student \( t \)-test).

(29). The level of immunoreactivity for T-OH was determined by both immunolocalization and immunoblot in the iris at different times after postganglionic nerve crush. At 1 day (data not shown), and more evidently at 3 days after surgery, the regular, dense, fine-meshed network of T-OH-positive fibers observed in control irises (Fig. 4A) was drastically reduced, as was the level of T-OH detected by immunoblot (Fig. 4D, lanes 3 and 4; in a longer exposure, faint bands were also observed in these lanes). The recovery of T-OH immunoreactivity, beginning 7 days after surgery (data not shown), was clearly evident at 26 days (Fig. 4C, D). However, different degrees of recovery for T-OH immunoreactivity were observed in irises dissected from different rats sacrificed at the same postinjury day (Fig. 4D, compare lanes 5 and 6). Differences in T-OH levels were also observed within control irises (Fig. 4D, lanes 1 and 2).

Pharmacological Characterization of the Nicotinic Receptors Present in SCG

To follow nicotinic receptor levels in control and injured SCGs we used the binding technique. In preliminary studies we first characterized the number and pharmacological properties of the nicotinic receptors present in SCG membranes (Table 3). We found that 2 classes of nicotinic receptors are present in SCG membranes: one that binds \(^3\)H-Epi with high affinity and another that binds \(^{125}\)I-\( \alpha \)Bgtx with high affinity.

The \(^3\)H-Epi-binding receptors were present as a single class and bound \(^3\)H-Epi with a Kd of 0.15 nM and a Bmax of 190 ± 16 fmol/mg of protein. Pharmacological characterization using nicotinic ligands showed that this class of receptors has a relatively low affinity for nicotinic agonists, with a Ki of 0.14 \( \mu \)M for cytisine, 0.35 \( \mu \)M for nicotine, and 0.45 \( \mu \)M for acetylcholine.

Saturation binding experiments with \(^{125}\)I-\( \alpha \)Bgtx showed toxin binding with a Kd of 0.75 nM and a Bmax of 322 ± 36 fmol/mg of protein. Pharmacological characterization using competition binding experiments showed that this class of receptors has a Ki of 0.45 \( \mu \)M for cytisine, 0.36 \( \mu \)M for nicotine, and 10.3 \( \mu \)M for acetylcholine.

\(^3\)H-Epi- and \(^{125}\)I-\( \alpha \)Bgtx-binding Receptor Levels in SCGs after Postganglionic Nerve Crush

The levels of \(^3\)H Epi-binding receptors present in individual ganglia from operated and control rats were
TABLE 3
Pharmacological Characterization of Nicotinic Receptors Present in Rat SCG

<table>
<thead>
<tr>
<th></th>
<th>'H-Epi-binding receptors</th>
<th>125I-αBgtx-binding receptors</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Kd (nM)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytisine</td>
<td>0.14 (33)</td>
<td>0.45 (12)</td>
</tr>
<tr>
<td>Nicotine</td>
<td>0.35 (33)</td>
<td>0.36 (9)</td>
</tr>
<tr>
<td>Acetylcholine</td>
<td>0.45 (23)</td>
<td>10.3 (35)</td>
</tr>
</tbody>
</table>

The Kd and Ki values were respectively derived from curves of 125I-αBgtx (125I-αBungarotoxin) and 3H-Epi (3H-Epibatidine) saturation and competition binding to SCG membranes as described in Materials and Methods. The curves obtained from 3 to 4 separate experiments were fitted using a nonlinear least squares analysis program. The numbers in parentheses represent the percentage of coefficient of variation (CV).

measured using a saturating concentration (2 nM) of 3H-Epi in the presence of 2 μM cold αBgtx in order to avoid 3H-Epi binding to α7 containing receptors.

We found that the crushing of the 2 major postganglionic nerve trunks of SCGs caused a rapid and dramatic change in the level of 3H-Epi-binding receptors. Figure 5 (upper panel) shows the level of 3H-Epi-binding receptors at different times after surgery. These were already significantly lower than the control value at 1 day and reached the lowest level at 3 days. At day 7, an initial recovery was observed that increased further at 14 days, reaching a value not significantly different from the control at 26 days.

The level of 125I-αBgtx-binding receptors after postganglionic nerve crush was also strongly affected. As shown in Figure 5 (lower panel), a significant decrease was already observed at 1 day and a maximum was reached at 3 days. After that a recovery began at 7 days and was already complete at 14 days, when the number of receptors was not statistically different from that of the controls.

Fig. 4. A–C: Tyrosine hydroxylase immunoreactivity in control and denervated irises. A: Control iris. The tyrosine hydroxylase (T-OH) immunoreactivity reveals a regular, dense, fine-meshed network of sympathetic fibers. B: Iris 3 days (d) after postganglionic nerve crush. A drastic reduction in the extension and intensity of the sympathetic fiber network is observed. C: Iris 26 d after postganglionic nerve crush. Recovery of the extension and intensity of T-OH immunoreactivity is evident, although not yet complete. Magnification: ×130. D: Western blot analysis of T-OH levels in iris extracts from 2 control rats (lanes 1 and 2), 2 rats killed at 3 d (lanes 3 and 4), and 2 killed at 26 d (lanes 5 and 6) after postganglionic nerve crush. Twenty μg of proteins were loaded in each lane. The molecular weight markers are indicated on the right.
**POSTGANGLIONIC NERVE CRUSH**

Fig. 5. $^3$H-Epi- (upper part) and $^{125}$I-αBgtx-binding (lower part) receptor levels in intact SCGs at different times after postganglionic nerve crush. At each indicated postinjury day (d) total binding was performed using 2 nM $^3$H-Epi or 10 nM $^{125}$I-αBgtx and subtracted for the aspecific binding performed in parallel using 2 nM $^3$H-Epi or 10 nM $^{125}$I-αBgtx and 100 nM cold Epi or 1 μM cold αBgtx. For total and aspecific $^3$H-Epi binding, SCGs were always incubated with 2 μM cold αBgtx. The receptor number is expressed as fmol of specifically labeled receptors/intact SCG. The reported values are the mean ± SEM of 4 to 6 SCGs of operated rats for each time point and 12 SCGs for controls. The differences between control (C) and injured SCGs were analyzed using the Student t-test: ***p < 0.001, **p < 0.01.

**PREGANGLIONIC NERVE CRUSH**

Fig. 6. $^3$H-Epi- (upper) and $^{125}$I-αBgtx-binding (lower) receptor levels in intact SCGs at different times after preganglionic nerve crush. The receptor number is expressed as fmol of specifically labeled receptors/intact SCG. The binding experiments were performed as described in Figure 5 and the reported data are the mean ± SEM of 4 to 6 SCGs of operated rats for each postinjury day (d) and 12 SCGs for controls. The differences between control (C) and injured SCGs analyzed using the Student t-test were not significant.

$^3$H-Epi- and $^{125}$I-αBgtx-binding Receptor Levels in SCGs after Preganglionic Nerve Crush

Unlike postganglionic crush, we found that there was no significant decrease in the numbers of $^3$H-Epi- (Fig. 6, upper panel) and $^{125}$I-αBgtx-binding (Fig. 6, lower panel) receptors counted 1, 3, 7, 14, and 26 days after preganglionic nerve crush. However, the small and transient decrease present at day 3 could be related to the axotomy of some ganglionic neurons whose axons run in the preganglionic nerve trunk as described by Bowers and Zigmund (30) and Zochodne et al (31).

Subunit Composition of $^3$H-Epi- and $^{125}$I-αBgtx-binding Receptors

The similar extent and kinetics of decrease observed after injury for the intraganglionic postsynaptic apparatus immunopositive for α3, β2, and β4 subunits strongly suggests that these subunits are co-assembled in the nAChR subtypes. To demonstrate the association of these subunits directly we performed quantitative immunoprecipitation experiments with Triton X-100 solubilized $^3$H-Epi-binding receptors using specific antibodies directed against each of the heteromeric nicotinic subunits. In 4 separate experiments we found that (mean ± SEM) 75% ± 2% of the $^3$H-Epi-binding receptors were immunoprecipitated by anti-α3, 31% ± 2% by anti-α5, 73% ± 4% by anti-β4, and 19% ± 2% by anti-β2, showing the presence of nAChRs containing the α3 subunit associated with α5, β2, and/or β4. The antibodies directed against the α2, α4, α6, and β3 subunits immunoprecipitated less than 2% of the labeled receptors, thus indicating that the $^3$H-Epi-binding receptors containing these subunits are not present in the SCG. The low percentage of $^3$H-Epi-binding receptors immunoprecipitated by anti-α5 and -β2 subunits, or the lack of immunoprecipitation by anti-α2,
-α4, -α6, and -β3 subunits, are not due to their limited immunoprecipitation capacity as they immunoprecipitated a high percentage (75% or more) of receptors purified from various brain areas (20, 26, 27). Therefore, the differences observed within the percentage of 1H-Epi-receptors immunoprecipitated by the antibodies raised against the different nAChR subunits reveal low amounts or absence of these subunits in SCG 1H-Epi-receptors.

Parallel immunoprecipitation of 125I-αBgtx-binding receptors showed that these receptors can only be immunoprecipitated by anti-α7 antibody and no specific immunoprecipitation was obtained using any of the other antibodies.

DISCUSSION

To ensure the homeostasis of parameters that may otherwise be modified by physiological or pathological environmental changes, the activity of a neural circuit is constantly adjusted. This activity is strictly correlated with the efficiency of synaptic transmission, which depends on a high density of postsynaptic neurotransmitter receptors.

We here show that this plastic response is also present in SCG neurons, which undergo a number of morphological, biochemical, and electrophysiological changes after axonal damage (11, 12, 32, 33). Among these changes, the removal of the nAChRs, followed by the detachment of the presynaptic terminals from the injured neurons, is the fastest way of eliminating any presynaptic input that could interfere with the molecular and biochemical events leading to the regeneration of the damaged axon.

By combining ultrastructural, immunocytochemical, and pharmacological assays, we characterized the nAChR subtypes in rat SCG and the changes in their numbers throughout the process of axonal degeneration-regeneration induced by postganglionic nerve crush. We found that rat SCG contains different nAChR subtypes: those containing the α3 subunit associated with the β4 and/or β2 subunits and those containing the α7 subunit. The α3-containing nAChRs have faster kinetics of removal from the intraganglionic synapses undergoing disassembly after postganglionic nerve crush than those containing the α7.

Immunoreactivity for nAChR Subunits in Control and Injured SCGs

Each nAChR subunit was detected by its respective antibody in rat ganglionic neurons and satellite cells with no differences in subcellular distribution pattern of the subunits, as we previously described in mouse SCG (21). Immunopositivity for nAChR subunits has also been described in other glial cell types, i.e. cultured rat cortical astrocytes (34). Postganglionic nerve crush did not change the pattern or intensity of immunoreactivity for any of the nAChR subunits in the satellite cells, but there were differences in the immunolabeling of neuronal cytoplasmic organelles. The immunoreactivity associated with the neuronal RER in the control SCG neurons was greatly reduced in the injured SCGs and the multivesicular bodies that were always immunonegative in control SCGs were filled with immunonegative products. These observations suggest a decrease in nAChR synthesis, as also indicated by the decrease in mRNAs for nAChR subunits (15, 16), and a rapid removal of nAChRs from the postsynaptic membrane by endocytosis.

Immunoreactivity for nAChR Subunits at the Intraganglionic Postsynaptic Apparatus of Rat SCG and Changes Induced by Postganglionic Nerve Crush

In the SCG the nAChR subunits are present in various neuronal and ganglionic compartments, where they are assembled in receptors that primarily function at the postsynaptic apparatus. We found that α3 is the most widely distributed subunit, being localized in 57% of the intraganglionic postsynaptic apparatus, whereas the α7, β2, and β4 subunits were detected in about 40% of synapses.

Together with the immunoprecipitation data, the similar extent and kinetics of the decrease in the α3-, β2-, and β4-immunopositive postsynaptic apparatus observed after postganglionic nerve crush strongly suggests that these subunits associate to form nAChR subtypes containing α3β4 and/or β2. Moreover, unlike the decrease in the α7-immunopositive postsynaptic apparatus, which followed the loss of synapses, the decrease in the α3-, β2-, and β4-immunopositive postsynaptic apparatus preceded synapse disassembly.

The different kinetics of the disappearance of α3- and α7-nAChRs from the disassembling synapses strongly suggests that these 2 nAChR subtypes may be stabilized by different protein complexes, which allow the selective removal of each type of receptor. Differences in the protein complexes stabilizing the various nAChR subtypes have also very recently been shown in chick parasympathetic ciliary ganglia in which different nAChR subtypes are associated with different members of the PSD95/SAP90 family (35).

Although it has previously been shown that both receptor classes participate in synaptic transmission in several autonomic ganglia (7), the α3 nAChRs are the principal agents. Their removal after postganglionic nerve crush, before the detachment of presynaptic terminals from the injured neurons, contributes to the early elimination of any input from the postsynaptic apparatus. However, we cannot exclude the possibility that the α7nAChRs may subserve fast ganglionic transmission to a greater extent, when the number of α3 nAChRs decreases as observed in mdx mouse SCG (21) or as shown here by axotomy. As α7 nAChRs are highly permeable...
to Ca\textsuperscript{2+}, they may primarily be involved in signal transduction pathways (7). More specifically, the fact that they can be detected at the intraganglionic synapses after postganglionic nerve crush, until the completion of synapse disassembly, suggests that they may be involved in the activation of pathways leading to axonal regeneration. The different roles played by the $\alpha_3$ and $\alpha_7$ nAChR subtypes in ganglionic transmission are also strongly suggested by studies of chick ciliary ganglion showing that $\alpha_7$ nAChRs are concentrated on somatic spines (which also have $\alpha_3$ nAChRs), but are absent from postsynaptic densities, in which only $\alpha_3$ nAChRs can be detected (36, 37).

The numbers of synapses, and the percentages of those that were immunopositive for each of the nAChR subunits, reached a minimum 3 days after injury, and no significant recovery was observed until day 14. On the other hand, by day 26, the number of synapses and the percentages of those immunopositive for $\alpha_7$, $\beta_2$, and $\beta_4$ (but not of those immunopositive for $\alpha_3$) were similar to those observed in the control SCGs. At this stage, T-OH immunostaining in the iris, which was lost 3 days after injury and began to recover on day 7, is clearly evident. This indicates that the impairment and recovery of the integrity of intraganglionic synapses is strictly related to the re-innervation of SCG target organs, which are the source of trophic factors such as nerve growth factor, the lack of which has previously been shown to be responsible for many aspects of the axon reaction in SCG neurons (13, 38, 39).

**Pharmacological Characterization and Subunit Composition of nAChRs in Rat SCG**

We quantitatively identified the nAChR subtypes present in the SCG and characterized their subunit composition and pharmacology. To this end, we carefully checked both the specificity and immunoprecipitation capacity of the antibodies used in the present study on purified rat nAChR subtypes as well as in the tissues of knockout animals (20, 25). We found that the large majority of $^3$H-Epi-binding receptors in the SCG contains the $\alpha_3\beta_4$ subunits, but subtypes containing the $\beta_2$ and/or $\alpha_5$ subunits associated with the $\alpha_3\beta_4$ subunits may also be present.

The subunit heterogeneity revealed by the immunoprecipitation of $^3$H-Epi-binding heteromeric receptors was not confirmed by the binding studies because the observed affinity of all 3 agonists used (cytisine, nicotine, acetylcholine) was similar to that previously reported for the heterologously expressed rat $\alpha_3\beta_4$ subtype (40, 41). This is not surprising for receptors containing the $\alpha_5$ subunit, because co-assembly of the $\alpha_5$ subunit with the $\alpha_3\beta_4$ or $\alpha_3\beta_2$ subunits does not change $^3$H-Epi affinity for the receptors (42). However, it suggests that the SCG $\beta_2$ subunit may be associated with the $\alpha_3$ subunit in combination with the $\beta_4$ subunit, as the $\alpha_3\beta_2$ subtype has a much higher affinity for nicotinic agonists (40) than that we observed. Another possibility is that in the SCG the affinities of the different subtypes are so similar that they cannot be distinguished by binding studies.

Our data concerning the subunit composition of heteromeric SCG nAChRs fit nicely with the earlier biochemical characterization of nAChR subtypes in chick ciliary ganglia, in which the large majority of receptors contains the $\alpha_3\beta_4$ subunits and a fraction also contains the $\alpha_5$ and $\beta_2$ subunits (43).

The pharmacological characterization of the $^{125}$I-$\alpha$-Bgtx-binding receptors present in ganglia shows very similar affinity and pharmacological properties to those previously reported for homomeric transfected or native rat $\alpha_7$ receptors (44). The $\alpha_7$ nAChR response has recently been studied in chick sympathetic (45) and rat SCG (46) neurons, in which the application of acetylcholine evoked both a rapid and a slow desensitizing current. Both currents were blocked by $\alpha$Bgtx but the rapidly desensitizing current was blocked almost irreversibly, whereas the slow current was blocked in a reversible manner. The molecular basis of the 2 responses is unknown, but it may depend on a distinct subunit composition, as is also suggested by studies of transfected cells showing that the $\alpha_7$ subunit can co-assemble with the $\beta_2$ subunit and form slowly desensitizing channels in oocytes (47). Our immunoprecipitation experiments on $^{125}$I-$\alpha$-Bgtx-binding receptors did not show any significant association of the $\beta_2$ subunit with $\alpha_7$ receptors. As the receptors solubilized by 2% Triton represent only the 30% to 40% of the total number of $^{125}$I-$\alpha$-Bgtx-binding receptors, we cannot exclude the possibility that some heteromeric receptors present were not solubilized.

**Effect of Postganglionic Nerve Crush on the Number of $^3$H-Epi- and $^{125}$I-$\alpha$-Bgtx-binding Receptors**

After postganglionic nerve crush, the number of $^3$H-Epi- and $^{125}$I-$\alpha$-Bgtx-binding receptors greatly decreased, reaching a minimum after 3 d. This suggests that the loss of immunoreactivity for the nAChR subunits observed at the intraganglionic synapses was due to a reduction in the number of nAChRs and not to a decrease in their subsynaptic concentration as a result of lateral diffusion in the plane of the membrane. Although the postsynaptic apparatus accounts for most nAChRs (48), they are also present in other neuronal subcellular compartments and satellite cells. The number of nAChRs determined by ligand binding therefore accounts for all of the nAChRs present in the SCG. The complete recovery in the number of $^3$H-Epi-binding receptors observed on day 26 (when the percentage of intraganglionic synapses immunopositive for $\alpha_3$ was still lower than that in controls), may be ascribed to $\alpha_3$ nAChRs present in the ganglion but not...
yet inserted in the postsynaptic membrane. This may similarly explain the recovery in the number of $^{125}$I-α-Bgtx-binding receptors observed on day 14, when the percentage of α7 immunopositive synapses was still lower than that in controls.

Our findings concerning the different regulation of the expression of α7 and β4 subunits are in agreement with those reported by Zhou et al (19), who showed by immunoblot that the α7 subunit level dramatically decreased 2 days after axotomy but recovered very quickly, whereas the level of the β4 subunit had not recovered fully even 14 days after axotomy.

Effect of Preganglionic Nerve Crush on the Number of $^3$H-Epi- and $^{125}$I-α-Bgtx-binding Receptors

The greatest regulation of muscle nicotinic receptors occurs after its denervation (49–51), whereas the denervation of the ganglionic neurons induced by preganglionic nerve crush had no effect on the number of $^3$H-Epi- or $^{125}$I-α-Bgtx-binding receptors, as also observed by Zhou et al (19) for α7 and β4 protein levels. This suggests that as previously observed in chick developing interneuronal sympathetic synapses (52), the peripheral target organs in the adult SCG play an essential role in retaining nAChRs at the intraganglionic synapses. This also indicates that all of the changes observed in the SCG, particularly the decrease in nAChRs after postganglionic nerve crush, may not only be the consequence of the direct insult to neuronal integrity, but also due to the interruption of the connections between the injured neuron and its target organs.

In conclusion, our study shows that the rat SCG contains different nAChR subtypes: those containing the α3 subunit and those containing the α7 subunit. The 2 receptor subtypes showed different kinetics of removal from the synapses undergoing disassembling after postganglionic nerve crush, suggesting that different protein complexes may be involved in their stabilization at the postsynaptic apparatus and that they may subserve different functions in the SCG. The rapid removal of the α3 nAChRs, which are mainly involved in fast synaptic transmission, may be functional to the elimination of any input to the damaged neuron that could interfere with axonal regeneration. The α7 nAChRs remain longer at the postsynaptic apparatus of the injured neuron and, because of their high Ca$^{2+}$ permeability, may be involved in activating the pathways that are essential for triggering axonal regeneration. The lack of any effect of preganglionic nerve crush on the α3 and α7 nAChRs suggests that the peripheral target organs of ganglionic neurons play a primary role in controlling the organization of intraganglionic synapses in adult rat SCG.

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