Disassembly of the Cholinergic Postsynaptic Apparatus Induced by Axotomy in Mouse Sympathetic Neurons: The Loss of Dystrophin and β-dystroglycan Immunoreactivity Precedes that of the Acetylcholine Receptor

M. LETIZIA ZACCARIA, MS, M. EGLE DE STEFANO, PhD, FRANCESCA PROPERZI, MS, CECILIA GOTTI, PhD, TAMARA C. PETRUCCI, PhD, AND PAOLA PAGGI, PhD

Abstract. In mouse sympathetic superior cervical ganglion (SCG), cortical cytoskeletal proteins such as dystrophin (Dys) and β1Σ2 spectrin colocalize with β-dystroglycan (β-DG), a transmembrane dystrophin-associated protein, and the acetylcholine receptor (AChR) at the postsynaptic specialization. The function of the dystrophin-dystroglycan complex in the organization of the neuronal cholinergic postsynaptic apparatus was studied following changes in the immunoreactivity of these proteins during the disassembly and subsequent reassembly of the postsynaptic specializations induced by axotomy of the ganglionic neurons. After axotomy, a decrease in the number of intraganglionic synapses was observed (t1/2 8 h 45'), preceded by a rapid decline of postsynaptic specializations immunopositive for β-DG, Dys, and α3 AChR subunit (α3AChR) (t1/2 3 h 45', 4 h 30' and 6 h, respectively). In contrast, the percentage of postsynaptic densities immunopositive for β1Σ2 spectrin remained unaltered. When the axotomized neurons began to regenerate their axons, the number of intraganglionic synapses increased, as did that of postsynaptic specializations immunopositive for β-DG, Dys, and α3AChR. The latter number increased more slowly than that of Dys and β-DG. These observations suggest that in SCG neurons, the dystrophin-dystroglycan complex might play a role in the assembly-disassembly of the postsynaptic apparatus, and is probably involved in the stabilization of AChR clusters.

Key Words: Axotomy; β1Σ2 spectrin; Dystroglycan; Dystrophin; Immunoelectron microscopy; Mouse sympathetic superior cervical ganglion; Neuronal nicotinic acetylcholine receptor.

INTRODUCTION

The neuronal postsynaptic apparatus is characterized by the presence of the postsynaptic density (PSD), an electron-dense structure linked to the inner face of the postsynaptic membrane, which may play a role in the stabilization of neurotransmitter receptor clusters (1, 2). Many of the mechanisms and related proteins involved in the formation of the postsynaptic apparatus have been investigated at the neuromuscular junction (NMJ). Here, clustering of acetylcholine receptors (AChRs) is triggered by agrin, a protein secreted by the nerve and anchored to the basal lamina (3, 4). There is evidence that AChR clusters are stabilized by interacting with cytoskeletal proteins (5). One component of the cortical cytoskeletal network is dystrophin (Dys), a 427 kDa molecular weight protein encoded by a gene located on the X chromosome and defective in the muscle and brain of patients affected by Duchenne muscular dystrophy (DMD) (6, 7), as well as of genetically dystrophic mdx mice (8). Dys and the dystrophin-related protein utrophin, which in skeletal muscle is restricted to the NMJ, bind to F-actin and to β-dystroglycan (β-DG), a component of a glycoprotein complex that spans the sarcolemma, linking the extracellular matrix to the inner cell cytoskeleton (9). At the NMJ, β-DG colocalizes with rapsyn (10), the 43 kDa membrane-associated protein that clusters AChRs (11). Moreover, in the Torpedo electric organ, β-DG has been shown to bind rapsyn directly (12), thus suggesting that the dystrophin-dystroglycan complex may be involved in the stabilization of AChR clusters. Other cytoskeletal proteins, such as β-spectrin isoform, which is specifically localized at the NMJ (13), may be important in this process. However, the role played by individual cytoskeletal proteins in the stabilization of AChR clusters is not understood.

Even less is known about the proteins and events involved in the assembly of the neuronal postsynaptic apparatus. In central nervous system (CNS), synapses are highly heterogeneous in terms of neurotransmitter receptors, thus suggesting that the molecules and mechanisms involved in the organization of postsynaptic specializations with different receptors may also differ.

In a previous study, we showed that several Dys isoforms are present in the mouse sympathetic superior cervical ganglion (SCG), and that Dys immunoreactivity is associated with specialized cell-cell contacts, including adherens junctions and PSDs of the intraganglionic synapses (14). Moreover, immunoreactivity for β-DG and for the somato-dendritic spectrin isoform (β1Σ2 spectrin) (15, 16) was detected in the mouse SCG and colocalized with Dys at the PSDs (unpublished data). Here we investigate the possible role of the dystrophin-dystroglycan
complex in the assembly of the cholinergic postsynaptic apparatus of intraganglionic synapses.

The mouse SCG contains a limited variety of nerve cells originating from the neural crest, the catecholaminergic neurons, and satellite cells. The preganglionic cholinergic fibers synapse the ganglionic neurons that express the neuronal nicotinic acetylcholine receptors (nAChRs)—a class of ligand-gated cation channels with a pentameric structure—that mediate fast synaptic transmission. Several genes have been identified as codifying for the different nAChR subunits (α2-α9, β2-β4), which are variously distributed in different areas of the nervous system and autonomic ganglia (reviewed in 17, 18, 19). In rat SCG neurons, mRNAs for the α3, α5, β2, and β4 nAChR subunits, as well as those for the α7 subunit, which is probably incorporated in the α-bungarotoxin-sensitive nAChRs also present in these neurons, have been identified (20–22). Moreover, it has been shown that the expression of the α3 subunit is rate-limiting for the appearance of functional nAChRs, and the increase in its mRNA correlates well with the increase in acetylcholine-evoked currents (21).

An insight into the role played by cytoskeletal proteins at the postsynaptic apparatus may be obtained by altering the structural and functional organization of this specialized neuronal compartment. Therefore, the intraganglionic synapses, and the postsynaptic apparatus where Dys, β-DG, β1Σ2 spectrin, and α3 nicotinic AChR subunit (α3AChR) are located, were altered by postganglionic axotomy or preganglionic denervation. These experimental conditions have been widely used in rodent SCG to study the mechanisms involved in the genesis and maintenance of interneuronal synapses (23, 24). Using electron microscopy, we followed changes in the immunoreactivity for Dys, β-DG, β1Σ2 spectrin, and the α3AChR at the postsynaptic specialization of the intraganglionic synapses at different times after either axotomy or decentralization of the ganglionic neurons.

MATERIALS AND METHODS

C57BL/10 young adult mice (18–20 g body weight) (Charles River Italia SPA, Calco, Italy) were used. The mice were housed and handled in accordance with the guidelines laid down by the European Communities Council Directive (86/609/ EEC of 24 November 1986) and the American Society for Neuroscience.

Surgical Procedures

Mice were anesthetized by intraperitoneal injection of chloral hydrate (400 mg/Kg body weight) (Fluka), and their right SCG was exposed. Ganglionic neurons were axotomized by crushing both the internal and external carotid nerves, the 2 major postganglionic branches, by means of a n. 5 Dumont tweezer, approximately 1 mm from the ganglion. Alternatively, neurons were decentralized by cutting approximately 0.5–1 mm of the cervical sympathetic trunk. Care was taken to minimize damage to the blood supply to the ganglion. The wound was sutured and the animals were allowed to recover from anesthesia. The ipsilateral ptosis of the eyelid was used as a criterion for a successful operation. The recovery was slow and a full open eyelid was observed around 20 days after surgical manipulation, suggesting regeneration of the peripheral nerve fibers. No other signs or particular symptoms were observed. Mice recovered perfectly from anesthesia and the wound healed rapidly. The time frame during which the damaged ganglionic neurons undergo chromatolysis and progressive recovery was estimated by standard electron microscopy. Animals were sacrificed 7 hours (h), 15 h, 3 and 6 days (d) after postganglionic axotomy and 15 h after decentralization.

Primary Antibodies

The polyclonal antibody H12 (courtesy of Dr P. Strong, Hammersmith Hospital, London, UK) (diluted 1:1000) is directed against the C-terminal region of the Dys rod domain (25), which is shared by the full-length Dys (427 kDa) and its isoforms (26) and does not recognize utrophin (25). In mouse SCG, H12 specifically reveals the full-length Dys and its shorter isoforms (14).

The polyclonal antibody against β-DG, P20 (diluted 1:500), was raised against the last 20 amino acids of the β-DG cytoplasmic tail (27).

The antibody against the β1Σ2 spectrin (15, 16) was produced in rabbit, using as antigen the β1Σ2 -COOH terminal fragment obtained by thrombin cleavage of GST-β1Σ2 -COOH terminus fusion protein (courtesy of Dr J. S. Morrow, Yale University) (28). Affinity purified β1Σ2 spectrin antibody was used at 1:100 dilution (13.6 μg/ml).

The polyclonal antibody against human α3AChR was produced as previously described (29, 30). The α3 peptide TRPTSNEGNAQPRPLYGAELSNLNC, used as antigen, is comprised in the cytoplasmic loop between the M3 and M4 transmembrane regions and represents the most divergent region within the sequence of human nAChR subunits. The titer and specificity of the antiserum were evaluated by means of ELISA, Western blotting, and immunoprecipitation of 3H-epibatidine-labeled receptor (30). The affinity purified α3 antibody did not recognize peptides obtained from the cytoplasmic loop of other nAChR subunits (30). Immunoprecipitation experiments showed that anti-α3 immunoprecipitated 90% of the high-affinity 3H-epibatidine-labeled receptor (unpublished data), but not the 125I-α-Bungarotoxin-labeled α7-containing receptor (30). Affinity purified α3 antibody was diluted 1:150 (2.17 μg/ml). Immunocytochemical control of the α3 antibody was performed by incubating the sections with the primary antibody pre-adsorbed overnight with 40 μg/ml of the α3 peptide used as antigen.

Immunocytochemistry

Normal and operated mice were deeply anesthetized with chloral hydrate and perfused transcardially with an oxygenated Ringer's solution, pH 7.3, followed by 4% freshly depolymerized paraformaldehyde in 0.1 M phosphate buffer (PB) pH 7.4. After perfusion, ganglia were dissected and cut on a vibratome in 40-μm-thick serial sections, collected free floating in 2 or 3 different wells (the sections in each well were either 80 or 120 μm apart).
Each well was then processed with a different antibody as described elsewhere (14). Briefly, after inactivation of the endogenous peroxidase with 10% methanol and 3% H2O2 in 0.1 M PB, the sections were cryoprotected with a solution of 10% DMSO and 1% glycerol (10 minutes [min]) followed by 20% DMSO and 2% glycerol (2 × 10 min) at 4°C, then quickly frozen in liquid nitrogen-cooled isopentane (2-methylbutane) and thawed. The freeze-thaw process was repeated 3 or 4 times; it allowed antibody penetration without the use of tissue detergents. The slices were incubated for 1 h in a blocking solution containing 1% BSA in 0.1 M PB and then incubated overnight at 4°C, with the primary antibodies diluted with 1% BSA in 0.1 M PB. Some of the sections were incubated by omitting the primary antibodies and used as a negative control. Following several rinses, the sections were incubated for 1 h at RT in 1:300 of goat anti-rabbit IgG (Stemberger Monoclonal Inc., Baltimore, Md), rinsed again, and incubated for 1 h at RT in 1:1000 of rabbit peroxidase-anti-peroxidase (Stemberger Monoclonal Inc., Baltimore, Md). Antibody binding sites were revealed by 3,3’-diaminobenzidinie (DAB)-H2O2. After the DAB reaction, the sections were processed as for standard electron microscopy and flattened in Epon 812. Ultrathin sections (60–70 nm) were cut on a Reichert ultramicrotome, collected on copper grids, and observed under a Phillips 400T transmission electron microscope operated at 60 kV. The ultrathin sections were left unstained to avoid shading of the immunoreaction product. Contralateral ganglia and ganglia from unoperated animals were used as controls.

Quantitative Analysis

The numbers of synapses and of postsynaptic specializations of morphologically intact synapses immunopositive for Dys, β-DG, β1Σ2 spectrin, and α3AChR were counted in sections of both control and injured SCGs by scanning, with the electron microscope, 20 mesh of the copper grids (for a total area of 260,000 μm2) at a magnification of ×28,000. Each primary antibody was tested on vibratome ganglionic sections from 3 different animals on each experimental day. At least 2 immunoreacted vibratome sections per ganglion were cut in 2 series of ultrathin sections, and 1 section from each series was used for the counting. 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 unequivocally immunopositive postsynaptic specializations, we are aware that their final numbers may be underestimated. Nevertheless, since the criteria used to identify immunopositive postsynaptic specializations were identical at every experimental time considered, this underestimation is constant for both control and injured ganglia.

RESULTS

Effects of Postganglionic Nerve Crush on Dystrophin, β-distroglycan, and β1Σ2 spectrin Immunoreactivity at Postsynaptic Specializations of Intraganglionic Synapses

Seven and 15 h after postganglionic nerve crush, a massive detachment of preganglionic terminals from the ganglionic neurons was observed. A few morphologically intact synapses were still present (Fig. 1A, B) and showed either Dys-immunopositive (Fig. 1A) or Dys-immunonegative (Fig. 1B) PSDs. When pre- and postsynaptic elements were detached, usually trough the mediation of interfering satellite cell processes (synaptic stripping) (Fig. 1B), PSDs were always Dys-immunonegative (Fig. 1B). A PSD from a ganglionic section incubated by omitting the primary antibody is shown (Fig. 1C).

The percentage of Dys-immunopositive PSDs belonging to morphologically intact synapses decreased drastically as early as 7 h after postganglionic nerve crush, and a further decrease was observed within 15 h (Table 1). Three and 6 d after axonal damage, when the axotomized neurons had begun to regenerate their axons, the number of synapses increased and a parallel rise in the percentage of Dys-immunopositive PSDs was observed (Table 1).

The cysteine-rich and C-terminal regions of Dys bind β-DG, a transmembrane glycoprotein that links Dys with the plasma membrane (31, 32). Since a decrease in dystrophin-associated glycoproteins has been found in the muscle of DMD patients (33) and mdx mice (34), both of which lack the full-length Dys, we monitored the β-DG immunoreactivity at the postsynaptic membranes of axotomized ganglionic neurons. Three postoperative times, 7 h, 15 h, and 3 d, were chosen, according to the changes observed for Dys immunoreactivity, would eventually allow us to determine the rate of loss and subsequent recovery of β-DG immunoreactivity. In control mouse SCG, β-DG immunoreactivity was present at the postsynaptic specializations of numerous intraganglionic synapses (Fig. 2A). Seven h after postganglionic nerve crush, only a few postsynaptic membranes of still morphologically intact synapses were β-DG, immunopositive (Table 1). Postsynaptic elements in the process of separating from their presynaptic terminals, as indicated by the presence of satellite cell processes approaching the synapse, showed β-DG-immunonegative postsynaptic membranes (Fig. 2B). In Table 1, the percentage of β-DG-immunopositive postsynaptic specializations in control ganglia is compared with that observed 7 h, 15 h, and 3 d after postganglionic nerve crush. The decrease in β-DG immunoreactivity observed after 7–15 h was followed by a recovery that was detectable 3 d after nerve injury.

The β1Σ2 spectrin isoform, another cortical cytoskeletal protein previously localized at PSDs of rodent CNS (15, 16), was immunodetected at the PSDs of the ganglionic neurons (Fig. 2C). After postganglionic nerve crush, dendrites partially (not shown) or completely (Fig. 2D) detached from their presynaptic terminals showed PSDs still immunopositive for β1Σ2 spectrin, although not for Dys and β-DG. It is interesting that, up to 15 h after nerve injury, when the greatest decrease in Dys immunoreactivity was observed, no change in the percentage of β1Σ2 spectrin-immunopositive PSDs was found (Table 1).
TABLE

Percentage of Intraganglionic Postsynaptic Specializations Immunopositive for Dystrophin, β-dystroglycan, β1Σ2 spectrin, and α3 AChR Subunit in Control and Injured Superior Cervical Ganglia

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>7 h</th>
<th>15 h</th>
<th>3 d</th>
<th>6 d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dys</td>
<td>67</td>
<td>23</td>
<td>10</td>
<td>14</td>
<td>34</td>
</tr>
<tr>
<td>β-DG</td>
<td>60</td>
<td>10</td>
<td>8</td>
<td>37</td>
<td>nd</td>
</tr>
<tr>
<td>β1Σ2</td>
<td>47</td>
<td>nd</td>
<td>44</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>α3AChR</td>
<td>77</td>
<td>58</td>
<td>38</td>
<td>43</td>
<td>46</td>
</tr>
</tbody>
</table>

The percentage of postsynaptic specializations immunopositive for dystrophin (Dys), β-dystroglycan (β-DG), β1Σ2 spectrin (β1Σ2), and α3 AChR subunit (α3AChR) was obtained by counting more than one hundred synapses in sections from control or injured ganglia. nd = not determined.

Preganglionic Denervation did not Affect Dystrophin Immunoreactivity at Postsynaptic Densities of Intraganglionic Synapses

The observed loss of Dys immunoreactivity at PSDs after postganglionic nerve crush could have been caused by the disconnection of pre- and postsynaptic elements. This was not the case. In fact, 15 h after decentralization, numerous degenerating presynaptic boutons were observed facing intact postganglionic elements, bearing PSDs still immunopositive for Dys (Fig. 3A). Dys immunoreactivity was also present at several PSDs lacking the presynaptic partner (Fig. 3B).

Effects of Postganglionic Nerve Crush on α3 AChR Subunit Immunoreactivity at Postsynaptic Specializations of Intraganglionic Synapses

At the NMJ, the dystrophin-dystroglycan complex is thought to be involved in the stabilization of junctional AChRs (10). Moreover, it is well known that the loss of AChRs is one of the events occurring after axotomy of cholinergic neurons (23, 35). To investigate whether the loss of Dys and β-DG immunoreactivity observed at postsynaptic specializations after postganglionic nerve crush was associated with the loss of nAChRs, we looked for possible changes in nAChR immunoreactivity at the postsynaptic membranes of axotomized ganglionic neurons. Neuronal nicotinic AChRs were tracked by monitoring the immunoreactivity for their α3 subunit, which

Fig. 1. Electron micrographs showing dystrophin immunoreactivity in mouse SCG 7 h after postganglionic nerve crush. A: The PSD of a synapse established between a preganglionic bouton and a dendrite of a ganglionic neuron is immunopositive (arrows). B: The PSD of an intact synapse is dystrophin immunonegative (arrowheads). A satellite cell process (asterisk) intrudes between a preganglionic bouton and a dendrite, mediating the detachment of the presynaptic element from the postsynaptic element. The remaining PSD is dystrophin immunonegative (double block arrows). SC, immunopositive perineuronal satellite cell process. C: control section obtained by omitting the primary antibody. The PSD of this intact synapse is not labeled (arrowheads). SC, satellite cell process. b, preganglionic boutons; d, dendrites. ×61,600.
Fig. 2. Electron micrographs showing β-dystroglycan and β1Σ2 spectrin immunoreactivity in control and injured mouse SCG. A: β-dystroglycan immunoreactivity in control mouse SCG. The postsynaptic membrane of a synapse established between a preganglionic bouton and a dendrite is strongly immunopositive (arrows). Immunoreaction product (double arrowheads) is present in the dendritic cytoplasm. B: β-dystroglycan immunoreactivity in mouse SCG 7 h after postganglionic nerve crush. A satellite cell process (asterisk) approaches a morphologically intact synapse. The postsynaptic membrane is immunonegative (arrowheads). C: β1Σ2 spectrin immunoreactivity in control mouse SCG. The PSD of a synapse established between a preganglionic bouton and a dendrite is immunopositive (arrows). Some immunoreaction product (double arrowhead) is clustered in its vicinity. D: β1Σ2 spectrin immunoreactivity in mouse SCG 15 h after postganglionic nerve crush. A dendrite, bearing a PSD deprived of its presynaptic partner and facing a satellite cell process, is shown. The PSD is strongly immunopositive (block arrows). B, preganglionic boutons; d, dendrites; SC, satellite cell processes. ×61,600.

is a component of the neuronal nAChRs preferentially located at postsynaptic regions (36).

In the mouse SCG, α3AChR immunoreactivity was detected at postsynaptic membranes of ganglionic neurons (Fig. 4A). A decrease in α3AChR-immunopositive postsynaptic membranes of morphologically intact synapses was observed 7 and 15 h after axonal damage (Table 1). Postsynaptic elements lacking the presynaptic terminals or completely separated from these by satellite cell processes were α3AChR immunonegative (not shown). Sometimes, clumps of α3AChR-immunoreactivity were observed in the vicinity of patches of electron-dense material associated with the plasma membrane and suggestive of disorganized postsynaptic specialization (Fig. 4B). The percentage of α3AChR-immunopositive postsynaptic membranes increased at later stages, when the axotomized neurons had begun to regenerate their axons (Table 1).

Time Course of Loss and Recovery of Dystrophin, β-dystroglycan, and α3 AChR Subunit Immunoreactivity at Postsynaptic Specializations of Axotomized Ganglionic Neurons

Figure 5 shows the number of morphologically intact synapses and postsynaptic specializations immunopositive for Dys, β-DG, and α3AChR as a function of the time following postganglionic nerve crush. The number
Fig. 3. Electron micrographs showing dystrophin immunoreactivity in mouse SCG 15 h after decentralization. A: A degenerating preganglionic bouton (b), emptied of its synaptic vesicles, still synapses a dendrite. The PSD is immunopositive (arrows). B: A vacant PSD is strongly Dys immunopositive (block arrows). d, dendrites; Sc, immunopositive satellite cell processes. x61,600.

Fig. 4. Electron micrographs showing α3 AChR subunit immunoreactivity in control and injured mouse SCG. A: Control mouse SCG. The postsynaptic membrane of a synapse established between a preganglionic bouton and a dendrite of a ganglionic neuron is immunopositive (arrows). B: Mouse SCG 7 h after postganglionic nerve crush. Patches of electron-dense material, suggestive of a disorganized postsynaptic specialization, are indicated (double block arrows). In the vicinity, clumps of α3 AChR subunit immunoreactivity (double arrowheads) are visible. Satellite cell processes (asterisks) separate the dendrite from a preganglionic bouton filled with synaptic vesicles. b, preganglionic boutons; d, dendrites. x61,600.

of synapses established between preganglionic axons and axotomized ganglionic neurons decreased with a half-time of 8 h and 45 min. This loss was preceded by a reduction in the number of postsynaptic specializations immunopositive for β-DG, Dys, and α3AChR with a half-time of 3 h and 45 min, 4 h and 30 min, and 6 h, respectively. When axotomized ganglionic neurons began to regenerate their axons, the number of intraganglionic synapses recovered slowly, being significant 3 d after axotomy. Concomitantly, an increment in the number of β-DG, Dys, and α3AChR-immunopositive postsynaptic specializations was also observed. Up to day 3, the recovery in the number of synapses preceded that of postsynaptic specializations immunopositive for Dys and α3AChR and was parallel to that observed for β-DG-immunopositive postsynaptic specializations. Three and 6 d after postganglionic nerve crush, the number of synapses and Dys-immunopositive PSDs increased at the same rate, and faster than that evaluated for α3AChR-immunopositive postsynaptic membranes. Interestingly,
Fig. 5. Time course of the decrease and recovery in the number of intraganglionic synapses (white box) and postsynaptic specializations immunopositive for dystrophin (white rhombus), β-dystroglycan (black rhombus), and α3 AChR subunit (black circle) after postganglionic nerve crush. For each experimental time, the mean ± SEM of the number of synapses and of postsynaptic specializations immunopositive for each protein counted in 20 mesh of copper grids is reported. Data are expressed as a percentage of the respective controls.

at least up to day 3 the number of postsynaptic specializations immunopositive for β-DG increased faster than those that were immunopositive for Dys.

Effects of Postganglionic Nerve Crush on Dystrophin Immunoreactivity in Cytoplasmic Organelles of Ganglionic Cells

Following postganglionic nerve crush, the ganglionic neurons showed a series of perikaryal alterations, known as chromatolysis (37, 38), occurring early after axonal lesion and developing over days and weeks. When compared with control neurons (Fig. 6A), chromatolytic neurons showed a general decrease in Dys immunoreactivity 7 and 15 h after postganglionic nerve crush (Fig. 6B). Only small and rare lateral portions of the rough endoplasmic reticulum were Dys-immunopositive (Fig. 7A), and the cytoplasmic aggregates of Dys immunoreactivity, sometimes found in control neurons (Fig. 6A), were not observed. On the contrary, multivesicular bodies were still numerous and, as in control neurons, some of them were filled with immunoreaction product (Figs. 6B, 7B).

Three and, more clearly, 6 d after nerve crush, when postganglionic axons had begun to regenerate, a gradual increase in cytoplasmic Dys immunoreactivity was observed. Discrete patches of immunoreaction product were found both beneath the plasma membrane and scattered throughout the cell cytoplasm (Fig. 7C), as well as at the rough endoplasmic reticulum, already reorganized in cisterns arranged in parallel (Fig. 7D).

During the postoperative time frame considered, from 7 h to 6 d, the cytoplasm and processes of the perineuronal satellite cells (Figs. 1B, 6B, 7A, C) and of the Schwann cells (not shown) were always found to be strongly Dys immunopositive.

DISCUSSION

When a peripheral nerve fiber is injured, its distal segment degenerates and the cell body undergoes several morphological, biochemical, and electrophysiological changes (23, 38, 39, 40). Some of these changes, which are strictly related to the events leading to axonal regeneration and the re-establishment of neuronal circuits, are absent in neurons destined to die (41). One of the consequences of the observed changes—detachment of pre-synaptic boutons, morphological disappearance of PSDs, decrease in the number of AChRs and e.p.s.p. amplitude (23)—is the elimination from the injured neuron of any presynaptic inputs that could interfere with the molecular and biochemical events leading to axonal regeneration.

In this paper we show that the synaptic disassembling induced by postganglionic nerve crush in the mouse SCG was preceded by a significant decrease in the immunoreactivity for several proteins involved in the organization of the postsynaptic apparatus. This is the case of dystrophin. The time course of the disappearance of Dys immunoreactivity strongly suggests that the loss of Dys may not be the direct consequence of the disassembly of the postsynaptic apparatus, but rather an event that con-
Fig. 6. Electron micrographs showing dystrophin immunoreactivity in control and injured mouse SCG. A: Control mouse SCG. The cytoplasm of the ganglionic neuron is immunolabeled. Clumps of immunoreactivity (double triangles) are scattered throughout the cytoplasm. B: Mouse SCG 7 h after postganglionic nerve crush. In the cytoplasm of the chromatolytic neuron, dystrophin immunoreactivity is almost exclusively located in some multivesicular bodies (arrows). n: nuclei; SC, strongly immunopositive perineuronal satellite cell processes. ×4,600.
Fig. 7. Electron micrographs showing dystrophin immunoreactivity in injured mouse SCG. A, B: Mouse SCG 7 h after postganglionic nerve crush. A: only 2 small portions of the cisterns of the rough endoplasmic reticulum (rer) are Dys immunopositive (block arrows). SC, immunopositive perineuronal satellite cell process. ×20,000. B: Two multivesicular bodies (mb) are filled with immunoreaction product. Another (mb), located in their vicinity, is dystrophin immunonegative. ×42,000. C, D: Mouse SCG 6 d after postganglionic nerve crush. C: Aggregates of immunoreactivity are associated with the inner face of the neuronal plasma membrane (arrowhead) and scattered throughout the cytoplasm (double triangles). Some cisterns of the rough endoplasmic reticulum are immunolabeled (block arrows). SC, immunopositive perineuronal satellite cell processes. ×16,000. D: dystrophin immunoreactivity (block arrows) associated with portions of cisterns of the rough endoplasmic reticulum (rer) is shown at high magnification. ×42,000.

tributes to its disorganization. The disappearance of Dys immunoreactivity at the postsynaptic specializations is not just related to the structural and functional alteration to the contacts between the pre- and post-synaptic elements. In fact, preganglionic denervation, which causes degeneration of the preganglionic nerve terminals, but leaves the postsynaptic apparatus morphologically unaltered for a long time, does not affect Dys immunoreactivity at the PSDs. Postganglionic nerve crush also induced the disappearance of Dys immunoreactivity associated with neuronal cytoplasmic organelles. However, the pattern of Dys immunoreactivity associated with
satellite and Schwann cells was unaltered, thus suggesting that the removal of Dys is strictly associated with the changes induced in neurons by axotomy.

The polyclonal H12 Dys antibody used here is directed against the C-terminal region of the Dys rod domain, shared by the full-length Dys and its shorter isoforms, which are also present in the mouse SCG (14). For this reason, we were not able to discern whether the disappearance of Dys in the PSDs of axotomized ganglionic neurons is due to the full-length Dys and/or its shorter isoforms. However, we can infer that both the full-length Dys and Dys isoforms were affected by nerve injury. This is supported by the finding that 15 h after postganglionic nerve crush, only 10% of the remaining PSDs were Dys immunopositive, a value well below that found in both normal mouse SCG (about 70%) and mdx mouse SCG (about 20%) (14). Dys isoforms do not have the actin binding domain (42), but all of them share the 427 kDa Dys the β-DG binding domains (26). After postganglionic nerve crush, β-DG immunoreactivity at the postsynaptic specializations changed and its loss and subsequent recovery preceded those of Dys. This finding is in agreement with the early loss of β-DG, compared with that of Dys, observed in rat skeletal muscle during a controlled cycle of degeneration and regeneration induced by injection of a snake venom (43).

Several mechanisms may account for the loss of Dys and β-DG immunoreactivity in axotomized ganglionic neurons. The possibility that protein epitopes recognized by the antibodies we used might be modified after postganglionic nerve crush seems unlikely. In fact, the antibodies still labeled the multivesicular bodies, where proteins to be degraded are sequestered. In particular, the presence of numerous Dys-immunopositive multivesicular bodies, especially 7 h after axotomy, suggests that Dys is removed from the sites where it is normally located in control neurons. Besides the endo-lysosomal system, cytoplasmic proteases such as calpains may be involved in Dys degradation. In fact, Dys, like other cytoskeletal proteins including spectrin, is very sensitive to calpains (44). The activation of the calpain system has been described in injured neurons (45). However, the enzymatic removal of Dys from the PSDs before their disassembly appears to be specific since, unlike Dys, the percentage of β1Σ2 spectrin-immunopositive PSDs does not change after axotomy (15 h). After axotomy, the observed disappearance of Dys immunoreactivity associated with the rough endoplasmic reticulum suggests that Dys synthesis might also be impaired and, as occurs for neurofilament proteins (46), axotomy may decrease the level of Dys by interfering with its gene expression. We are currently carrying out RT/PCR experiments to evaluate the level of mRNA for Dys together with β-DG in SCG after postganglionic nerve crush.

Evidence has been collected of an involvement of Dys and dystrophin-dystroglycan complex in the organization of AChRs at the end plate (10, 47). In muscle, Dys underlies the entire sarcolemma and, at the NMJ, it is essentially located in the depths of the folds, which are devoid of AChRs (48). This observation, as well as the correct localization of AChR at the NMJ of mdx mice (which lack full-length Dys), suggests that Dys is not essential for AChR localization. On the contrary, utrophin, the dystrophin-related protein, is present only at the NMJ located at the crest of the folds, where AChRs are also localized (48). Utrophin-deficient mice show only a few muscle defects such as lower AChR density and fewer junctional folds (49). In contrast, mice lacking both Dys and utrophin show several signs typical of DMD in humans (50), suggesting that these molecules have complementing roles for correct muscle development. Recently, Salpeter et al (47), studying the turnover of AChRs, have shown that, at the NMJ of mdx mice, the degradation of adult-type AChRs resembles that occurring in denervated muscle. They concluded that the organization of AChRs is strictly related to the integrity of the cortical cytoskeletal complex and not just to those proteins colocalizing with AChRs. Moreover, at the NMJ, β-DG colocalizes with rapson clusters (10), and at the Torpedo electric end plate, a direct interaction between β-DG and rapson has been shown (12), thus supporting the hypothesis of a role for the dystrophin-dystroglycan complex during synaptogenesis and synaptic stabilization. In addition, it has been reported that chick ciliary ganglion neurons contain transcripts coding for AChR-associated protein at the synapses (rapson), thus suggesting that multiple rapson-like molecules are involved in clustering the distinct AChRs expressed by muscle fibers and neurons (51).

In the CNS, the localization of Dys at the PSDs (52) suggests its possible involvement in the organization of the postsynaptic apparatus, where neurotransmitter receptors are clustered. In mouse SCG, AChR subunit composition and location are unknown. We found that the α3AChR was present at the postsynaptic apparatus of ganglionic neurons and was affected by postganglionic nerve crush. In chick ciliary ganglion, the α3 subunit has been reported to be a component of AChRs mainly located at the synaptic regions (36). Moreover, in rat SCG it has been shown that the α3 subunit is rate-limiting for the appearance of functional nAChRs, and the increase in its mRNA correlates well with the increase in acetylcholine-evoked currents (21). These findings indicate that changes in α3AChR immunoreactivity may represent changes in AChR. The decline in the percentage of α3AChR-immunopositive postsynaptic specializations reported here is in agreement with the reduction in both the e.p.s.p. amplitude and AChR density previously described in the guinea pig SCG (23). The disappearance

of α3AChR immunoreactivity from the postsynaptic specializations of morphologically intact synapses of axotomized ganglionic neurons occurred later than that of Dys and β-DG, thus suggesting that the removal of the dystrophin-dystroglycan complex might facilitate the mobilization of nAChRs.

Our data support the hypothesis that, in mouse SCG neurons, the dystrophin-dystroglycan complex may play an essential role in assembly-disassembly of the postsynaptic apparatus and is probably involved in the stabilization of nAChR clusters.

ACKNOWLEDGMENTS

The authors would like to thank Gianfranco Macchia for technical assistance. MEDS was recipient of a Postdoctoral Research Fellowship from Istituto Pasteur-Fondazione Cenci Bolognetti.

REFERENCES

36. Vernallis AB, Conroy WG, Berg DK. Neurons assemble acetylcholine receptors with as many as three kinds of subunits while man-

Received January 26, 1998
Revision received May 7, 1998
Accepted May 7, 1998