ULTRASTRUCTURAL ALTERATIONS INDUCED IN QUAIL CILIARY NEURONS BY POSTGANGLIONIC NERVE CRUSH AND BY RICINUS TOXIN ADMINISTRATION, SEPARATELY AND IN COMBINATION

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Abstract—The response to postganglionic nerve crush and Rcinus toxin administration by the ciliary neurons of the quail ciliary ganglion was investigated at the ultrastructural level. The toxin was either applied at the crush site on the postganglionic nerves or injected into the anterior eye chamber without any other operative intervention. Crush of postganglionic nerves without toxin administration and saline injection into the anterior eye chamber served as controls for the two toxin administration procedures. Postganglionic nerve crush caused a distinct chromatolytic reaction, accompanied by massive detachment of the preganglionic axon terminals from the ciliary neurons and loss of most of the synapses, both chemical and electrical. This process does not induce cell death and is reversible. Saline injection in the anterior eye chamber caused a moderate retrograde reaction in some of the ciliary neurons, presumably as a consequence of paraesthesiae. The changes consisted mainly of an increase of perikaryal neurofilaments with, at most, a minor detachment of the preganglionic boutons from a small portion of the cell body at the nuclear pole. Rcinus toxin administration induced neuronal degeneration following a pattern common to both delivery modes. The degenerative process consisted of disruption and detachment of polyribosomes from the rough endoplasmic reticulum, an increase of smooth cisterns and tubules, a dramatic increase of neurofilament bundles, compartmentalization of the cytoplasmic organelles and, finally, karyorrhexis and cell lysis. The final stages of Rcinus toxin degeneration involve a progressive accumulation of extracellular flocculo-filamentous material and cell lysis. After administration of Rcinus toxin to the crush site, ricin-affected neurons showed withdrawal of the preganglionic boutons from a portion of the ciliary neuron, especially at the nuclear pole. After Rcinus toxin injection into the anterior eye chamber, however, the bouton shell surrounding the affected ciliary neurons remained intact in the early stages of degeneration. Detachment of the preganglionic terminals and disruption of the cell junctions, therefore, is the consequence of nerve crush and not of the toxin itself.

This study demonstrates that quail ciliary neurons are a suitable model for experimental neuropathology and neurotoxicology.

The avian parasympathetic ciliary ganglion has proven to be a useful model for cellular and developmental neurobiology.55 It contains only two neuronal populations, the ciliary and choroid neurons. These cells have distinct morphological and chemical phenotypes and innervate separate targets in the eye bulb, the iris and ciliary body and the choroidal coat, respectively.53,54,13,28,41,45,47,58 The fine structure of the avian ciliary ganglion has been amply documented at both adult and embryonic stages,53,54,13,28,41,45 but relatively few studies have utilized this subject as a model for cellular neuropathology.53 Ultrastructural studies on motoneurons have shown that axonal crush induces detachment of the presynaptic boutons impinging upon their cell bodies and proximal dendrites.3,30,32,43 A similar phenomenon of synaptic detachment has also been observed in the peripheral nervous system (rat sympathetic ganglion cells,41 guinea-pig sympathetic ganglion cells,56 ciliary ganglion cells of the newly hatched chick,53).

The Rcinus toxin (RCA-60) is a ribosome inactivating protein6,42 composed of two subunits: a lectin, the B subunit, which binds galactoside residues on the plasma membrane allowing toxin internalization,7,49,50,62,65,66 and the A subunit, which exerts the toxic effect by inhibiting protein synthesis through irreversible ribosome inactivation.6,17,20,51 In the nervous system, RCA-60, applied to crushed or cut nerves or in the proximity of their terminals, kills the neurons, reaching the perikarya via retrograde transport, and causes neuronal death with a highly variable rate.11,13,23,32,70 In most of these experiments, the mode of the toxin application engendered the

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Abbreviations: RCA-60, Rcinus toxin; RER, rough endoplasmic reticulum; SER, smooth endoplasmic reticulum.
possibility that both the retrograde axon reaction in the parent cell bodies and the toxin injury to the synthetic machinery of the neurons combine to produce neuronal alterations, thus blurring the respective aspects of cellular neuropathology.

Therefore, we decided to utilize the avian ciliary ganglion, and in particular quail ciliary neurons, in an attempt to clearly differentiate the ultrastructural alterations caused by an axonal lesion from those due to the RCA-60 toxicity. In selecting the quail ciliary neurons as the experimental model we were guided by several considerations. (1) Quails are smaller than chickens and afford easier husbandry; quails reach the adult condition three to four months after hatching, and the ciliary ganglion is fully developed at this age. (2) While the choroid neuron has preganglionic terminals sparsely distributed on the surface of the perikaryon and its short dendrites, the ciliary neuron is densely innervated on the perikaryon by preganglionic boutons developed during the first few weeks after hatching from the fragmentation of an individual, caliciform ending. In the adult chicken, this cap only covers the aspect of the cell opposite the nucleus, the hilar pole, from which the postganglionic axon arises. The synaptic relations as seen in random electron micrographs, therefore, sharply differ depending on the plane of section of the individual ciliary neurons. By contrast, in the adult quail the bouton cap covers the ciliary cell body almost entirely, thus facilitating detection of bouton detachment following postganglionic nerve crush without the need of complex neuronal reconstructions from serial ultrathin sections or elaborate sampling for statistical analysis. (3) Ciliary neurons recover from postganglionic nerve crush, but are killed by RCA-60, while choroid neurons die after either treatment.

In the hope of separating the effects of nerve damage and RCA-60 toxicity, we applied the toxin either at the site of a nerve crush or, without nerve crush, in the proximity of the target tissue, i.e. by injection into the anterior chamber of the eye. This second mode of administration was based on the assumption that the latter procedure is surgically non-traumatic with respect to the axons of the ciliary neurons that innervate the striated muscle of the iris. Control experiments were performed by crushing postganglionic nerves without toxin application, and by injecting saline into the anterior eye chamber.

**EXPERIMENTAL PROCEDURES**

Adult quails (Coturnix coturnix japonica) three to four months old, were used for this study. All surgical and drug treatment procedures were performed unilaterally.

The quails were anesthetized by injection in the pectoral muscles of 13 mg/100 g body weight of ketamine hydrochloride (Fort Dodge Laboratories Inc., Fort Dodge, IA) and 1 mg/100 g body weight of xylazine (Ben Venue Laboratories, Bedford, OH).

All the studies reported in this paper were carried out in compliance with the regulations laid down in Italian Law (decreto legislativo no. 116 of 27 January 1992), as well as with the guidelines proposed by the European Community (No. 86/609/CEE of 24 November 1986) and the American Society for Neuroscience.

The quails were subdivided into the following experimental groups.

**Postganglionic nerve crush**

In four quails, under deep anesthesia, the skin overlying the inferior orbit was cut with an electrocautery. To expose the postganglionic nerves at their exit from the ciliary ganglion, it was necessary to collapse the eyeball with an approximately 1-2 mm long incision in its posterior part. The postganglionic nerves were then crushed with no. 5 Dumont tweezers at 1-2 mm from their exit from the ganglion, producing a constriction of the nerves that was visible under the dissecting microscope, but without separating the nerve trunks. The incision of the eyeball was left untreated, while the skin wound was sutured with metal clips and treated with local antibiotics. In all cases, the animals showed a dilated pupil. The birds were killed, in groups of two, three and six days after operation. The contralateral eye was used as intraspecimen control. The effects on the ciliary neuron population of collapsing the eyeball without nerve crush were not analysed in the present study (but see Ref. 11).

**Postganglionic nerve crush and Ricinus toxin treatment**

In four quails, under deep anesthesia, a 5 μl drop of RCA-60 (Calbiochem-Behring, La Jolla, CA) solution, 1 mg/ml saline, was applied immediately over the crush site on the postganglionic nerve trunk with the tip of an unbevelled needle mounted on a Hamilton syringe. The ganglion is covered by a thick capsule and we assumed that the toxin applied at the crush site reached the ciliary neurons by retrograde axonal transport. The birds were killed, in groups of two, three and six days after treatment. The contralateral eye was used as intraspecimen control.

**Saline injection into the anterior eye chamber**

In three quails, under deep anesthesia, 1 μl of 0.9% NaCl solution was injected into the anterior chamber of the eye by means of a 5-μl Hamilton syringe, to which was attached a 33-gauge needle. The needle was inserted through the cornea, next to the distal border of the iris, with gentle pressure and rotation, and carefully moved toward the center of the eye chamber, in correspondence with the pupil, without piercing the iris. The injection time was about 10 s. The needle was then slowly withdrawn and the seepage of aqueous humor from the anterior chamber was minimized by applying a slight pressure on the needle hole with a bulbous tipped glass pipette. The birds were killed three and six days after treatment. The contralateral eye was used as intraspecimen control.

**Ricinus toxin injection into the anterior eye chamber**

In three quails, under deep anesthesia, 1 μl of the toxin (1 mg/ml saline) was injected into the anterior eye chamber following the procedure described above. By trials, this toxin dosage appeared to be the highest that could be used without causing death of the animals. The toxin batch used in this experiment was different from the one used for administration at the site of the nerve crush and may have been more potent. Alternatively, the toxin may gain easier access to the blood when injected into the anterior eye chamber than when applied to the crushed nerve. After the injection, the needle was held in place for 10 min before careful removal as specified above. The birds were killed three, six and eight days after treatment. The contralateral eye was used as intraspecimen control.
Electron microscopy

Under deep anesthesia, all quails were perfused transcardially, by means of a pump, with 400 ml of 4% formaldehyde, 2% glutaraldehyde and 0.005% CaCl₂, in 0.1 M cacodylate buffer, pH 7.4, at 37°C, or with 400 ml of 2% formaldehyde and 1.25% glutaraldehyde in 0.12 M phosphate buffer, pH 7.3, at 37°C. Both ciliary ganglia from each quail were removed. In the quails injected with RCA-60 in the anterior chamber, we also obtained a sample from the iris of the treated eyes in order to ascertain the extent of the damage eventually caused by the toxin. Tissues were postfixed for 24 h at 4°C in the perfusion fixative. All the specimens were then rinsed in buffer, osmicated for 1 h, rinsed again, dehydrated in a series of ethyl alcohols and propylene oxide, and embedded in Epon 812. Ultrathin sections were cut on an ultramicrotome with a diamond knife, collected on copper grids, contrasted with uranyl acetate and lead citrate, and observed under an electron microscope operated at 80 kV.

A schematic representation of the different experimental treatments described in this section is shown in Fig. 1.

RESULTS

Ciliary neurons were differentiated from choroid neurons by sets of criteria specified elsewhere. Figure 2 recapitulates the features of the ciliary neuron in normal adult quails. In the untreated eye of all experimental animals, ciliary neurons conformed to this basic observation and were therefore deemed unaffected by the various procedures. The nucleus of the normal ciliary neuron is located at one pole of the cell (nuclear pole) and the Nissl substance is preferentially distributed in a perinuclear ring. Microtubules and neurofilaments are sparsely distributed in the perikaryon and are not individually discernible at low magnification. The preganglionic fiber reaches the ciliary neuron at the hilar pole, near the origin of the axon, and splits into branches that form a characteristic shell of boutons which completely, or at least almost completely, surrounds the cell body. Although in some of the cells small segments of the neuronal perimeter are directly contacted by satellite cell processes, the neuronal surface devoid of preganglionic boutons usually accounts for no more than 5% of the entire cell body perimeter. Preganglionic boutons form cell junctions with the ciliary neuron, that at high magnification are classified into three types: asymmetric chemical synapses, gap junctions and puncta adherentia. Satellite cells and their lamellar processes surround the bouton shell and form regions of compact and loose myelin (labeled my in Fig. 2) along the cell perimeter. The outer border of the satellite cell sheath is marked by a basal lamina.

Effects of postganglionic nerve crush on ciliary neurons

In both three and six day survival groups, most, if not all, the ciliary neurons were affected by crush of postganglionic nerves, at least to some extent. Some of the cells were globose (Fig. 3A), while others had a crenated perimeter (Fig. 4), suggesting a moderate amount of swelling or shrinkage, respectively. Compared to normal quails, the nucleus was slightly more displaced towards the cell surface at the nuclear pole; the cisterns of the rough endoplasmic reticulum (RER) formed assemblies that were scattered throughout the perikaryon (Figs 3A, 4), rather
Fig. 2. Normal quail ciliary neuron. The nucleus (N) is located at one pole of the cell and the Nissl substance (ns) is preferentially organized in the perinuclear area. The preganglionic terminals (some of which are labeled b) almost completely surround the cell body, forming a bouton shell. A small interruption of the bouton shell is indicated by an open triangle. The arrow points to a spinous appendage that forms a crest synapse with two boutons (for details see De Stefano et al.14). A satellite cell sheath envelops the neuron. The satellite cell processes form compact myelin (my) at points (for high resolution details see Ref. 14). The outer border of the satellite cells is flanked by a basal lamina. SC, satellite cell body. × 4600.
than being collected in large masses in a ring-like region of the central cytoplasm; the neurofilaments increased slightly in number and in most cells formed small but discrete bundles (Figs 3A, B, 4). The more conspicuous effect of the treatment, however, involved the contacts between ciliary neurons and their preganglionic nerve endings. The region of apposition between the cell body and the bouton shell was markedly reduced, essentially as a consequence of the retraction of the boutons from the nuclear pole. This portion of the cell surface appeared smooth and tightly adjacent to the satellite cell sheath (Figs 3A, 4). Neuronal alterations became more striking after six days (Fig. 5A). In the most severely affected ciliary neurons, shrinkage was conspicuous and the RER assemblies were small. The increase in neurofilaments, however, remained moderate. The hilar pole showed numerous finger-like protrusions (Fig. 5A), ranging from 1 to 5 μm in length and from 0.3 to 0.5 μm in diameter. These evaginations often showed synaptic contacts, both along their shafts and at their tips (Fig. 5B). At the sharply crenated surface of the most severely affected neurons, direct contact with the boutons was often limited to some of the puncta adherentia and chemical synaptic junctions (Fig. 5B, C). Postsynaptic sites, marked by the postsynaptic densities and disjoined from the native partners, were also present (Fig. 5D). Gap junctions were not observed. The intercellular spaces were extremely dilated over most of the neuronal perimeter and between the boutons (Fig. 5A). The satellite cell sheath did not show visually detectable alterations (Fig. 5A).

**Effects of Ricinus toxin administration at the site of postganglionic nerve crush**

Although a number of the ciliary neurons seemed unchanged at all survival times (see also Ref. 1), ultrastructural changes were already evident in some of the ciliary neurons three days after application of RCA-60 at the site of the nerve crush. The cells that showed varying degrees of alteration presumably represented progressive steps in a continuous process of degeneration leading to cell death. The characteristic effects of the toxin treatment, i.e. detachment of the polyribosomes from the RER and dissolution of the polyribosomal chains with formation of masses of ribosomal debris, were marked in some of the ciliary neurons (Figs 6A, B, 7A). There was a conspicuous increase in the profiles of smooth endoplasmic reticulum (SER). A number of these SER profiles presumably represented granular cisterns that had lost their complements of ribosomal rosettes (Figs 6A, 7A). In some of the affected neurons these changes were focally distributed, and the co-existence of normal and altered cytoplasmic areas within the same neuron was a characteristic lesion at this stage. In addition to damage of the RER and the polyribosomes, another effect of the toxin treatment was readily apparent; namely, a dramatic increase of neurofilaments. These were often arranged in streams oriented at different angles (Figs 6A, B, 7A). Furthermore, in some of the cells, the cytoplasmic organelles appeared distinctly segregated into large compartments creating three main regions: a peripheral region containing masses of ribosomes and SER cisterns separated by small bundles of neurofilaments and scattered mitochondria, an intermediate region occupied primarily by large bundles of neurofilaments, with rare SER cisterns and few microtubules, and a central region containing groups of closely packed mitochondria and dense bodies (Figs 6A, 7A). A large proportion of the neuronal perimeter was not covered by boutons (Figs 6A, 7A). However, compared to the situation after postganglionic nerve crush without toxin administration, the majority of the neurons did not show the crenated perimeter that accompanies shrinkage, and the relation between the neuronal surface and the shell of boutons showed relatively minor changes: preganglionic terminals were absent from the nuclear pole (Fig. 7B), but still present over portions of the hilar pole (Fig. 6A); the intercellular spaces were less dilated and finger-like protrusions at the hilar pole were rare or absent (Fig. 6A). Gap junctions were rarely observed.

The dying cells showed high compartmentalization of the organelles, a diffusely granular cytoplasm and numerous vacuoles and autophagosomes (Fig. 7B). Cell death was characterized by darkening of both cytoplasm and nucleoplasm, dilation of the SER and karyorrhexis (Fig. 7B). Dense granules in the mitochondrial matrix, probably representing calcium deposits, were occasionally observed (inset to Fig. 7B). At the periphery of some of the affected neurons in advanced degenerative state, an electron-dense floccular and filamentous material filled the spaces between the neuronal cell body, the boutons and the satellite cells (Fig. 8A). Extracellular floccular and/or filamentous materials and scattered, small (~110 nm) membrane-bound bodies were also observed in zones vacated by degenerated neuronal cell bodies, but containing cell debris (Figs 8B, 9A, B). Individual and bundled filaments, oriented at different angles, were clearly distinguishable in the extracellular material (Figs 8A, B, 9B). The filaments were mostly of two kinds: thin filaments (~5 nm), isolated or in masses, and thicker filaments (5–10 nm) that formed small, electron-dense bundles. Depending on the plane of section, the filament bundles showed a poorly defined periodical substructure (with a repeat of 11–14 nm) which appeared in register from one filament to another (boxed area in Fig. 9B). Electron-dense material was present among the filaments and at points it seemed to establish cross-bridges. Whether the periodicity is due to a helical structure of individual thicker filaments, to cross-bridges or to twisting of two paired subunits could not be resolved. In cross-section (inset to Fig. 9B) the filamentous subunits seemed to be organized in tubular structures, joined.
Fig. 4. Ciliary neuron at an intermediate stage of chromatolytic reaction (survival: three days). Cell shrinkage and fragmentation of the Nissl substance (ns) have begun. The asterisks indicate discrete bundles of neurofilaments scattered in the cell body. The nucleus (N) is separated from the plasmalemma by a thin rim of cytoplasm. Retraction of the boutons (b) from the nuclear pole is complete. The hilar pole appears somewhat ruffled and extracellular spaces are widened. SC, satellite cell bodies; my, compact myelin. × 6700.

Fig. 3-5. Electron micrographs showing the chromatolytic events occurring in quail ciliary neurons after crush of the postganglionic nerves.

Fig. 3. (A) Ciliary neuron at the earliest stage of chromatolytic reaction (survival: three days). The cell appears slightly swollen. The nucleus (N) is located eccentrically. The Nissl bodies (ns) appear of normal size but are distributed throughout the cytoplasm and are separated from each other by discrete bundles of neurofilaments (asterisks). The preganglionic boutons (b) have almost completely withdrawn from the nuclear pole and have partially detached from the hilar pole. Myelin (my) is evident at points along the cell perimeter. × 3300. (B) Higher magnification of neurofilament bundles (asterisks) interspersed among the Nissl bodies (ns) in the neuronal perikaryon. × 39,500.
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Fig. 5. Survival: six days. (A) Ciliary neuron at advanced stage of chromatolytic reaction. The neuron has been sectioned at the hilar pole. The neuronal surface appears highly indented, forming several finger-like appendages (block arrows). Most of the presynaptic boutons (b) have detached from the neuronal cell body and only few of them (b) still bear synaptic contacts (arrows). Wide extracellular spaces (stars) are present among the presynaptic boutons, and between the neuron and the satellite cells (SC). The satellite cell sheath completely surrounds the reactive neuron and its preganglionic terminals. ×5,600. (B) Finger-like appendages and smooth portions of the ciliary cell body (CN) are contacted by six boutons (b), which form chemical synapses (arrows) and adherent junctions (arrowheads). Three other boutons (b) are in the vicinity of the neuron but they do not contact the cell body. × 16,500. (C) Complex of four puncta adherentia (arrowheads) between a bouton (b) and a ciliary neuron (CN) without intervening gap junctions. × 30,750. (D) Presumed postsynaptic density (curved arrow) on a ciliary neuron (CN) without immediately apposed bouton. Membranous debris (open block arrow) and boutons (b) are present in the vicinity of the neuron. × 21,000.

Fig. 6. 9. Electron micrographs showing the degenerative events occurring in quail ciliary neurons after RCA-60 administration on crushed postganglionic nerves.

Fig. 6. (A) Ciliary neuron at early stage of degeneration (survival: three days). The polyribosomes have completely detached from the endoplasmic reticulum and the cell body is occupied by numerous smooth cisterns (some of which are indicated by thin arrows), preferentially distributed in the peripheral cytoplasm. Clusters of mitochondria (m) are separated by bundles of neurofilaments (asterisks) and masses of ribosomal debris (r). Preganglionic boutons (b) have withdrawn from part of the cell perimeter, which is now in direct contact with the satellite cell processes (sp) (bottom left), but they remain in contact with the cell body at top right. my, compact myelin. The boxed area is shown in detail in B. × 6,000. (B) Higher magnification of two clusters of ribosomal debris (r) and three small bundles of neurofilaments (asterisks) from the cell shown in A. × 44,300.

Fig. 7. Ciliary neurons in advanced stages of degeneration after application of toxin to site of nerve crush (survival: six days). (A) The cytoplasmic organelles are compartmentalized. Large and compact streams of neurofilaments are indicated by asterisks. m, groups of mitochondria; my, compact myelin; r, masses of ribosomal debris; se, areas predominantly occupied by smooth endoplasmic reticulum. No preganglionic boutons are present on this portion of the cell. × 8500. (B) A ciliary neuron shows a highly crenated nucleus (N) with a darkened nucleoplasm, and a dense cytoplasm occupied by vacuoles (v), dense bodies (db) and clusters of mitochondria (m). The satellite cell processes (sp) appear unaffected, my, compact myelin. × 6700. (Inset) Detail from a similar cell showing dense granules (double block arrows), presumably representing calcium deposit, in the mitochondrial (m) matrix. × 20,700.

Fig. 8. Details of ciliary neurons in the final stage of degeneration after application of toxin to site of nerve crush (survival: six days). (A) Extracellular flocculo-filamentous material (f) has accumulated between a bouton (b), still attached to the neuronal cell body (CN), and the satellite cell processes (sp). Individual filaments and aggregated filaments (double arrows) are scattered within the flocculent material. The arrowheads indicate a punctum adherens and the open curved arrow a large coated pit. × 34,000. (B) Flocculo-filamentous material (f), intruding satellite cell processes (sp), cellular debris (cd) and preganglionic boutons (b) fill the area previously occupied by a ciliary neuron. Double arrows point to bundled extracellular filaments. Other boutons and preterminal axonal branches (ax) are enveloped by the satellite cells (SC) that lie at the periphery of the field. my, compact myelin. × 6800.

Fig. 9. (A) Detail from the periphery of a degenerated ciliary neuron showing accumulation of flocculo-filamentous material (f) and preganglionic boutons (b) surrounded by myelin lamellae (my). Survival: six days after application of toxin to site of nerve crush. × 22,750. (B) Higher magnification of bundled filaments (double arrows) and membrane-bound spherical bodies (triangles) embedded in the flocculent material (f) at a degeneration site. One of the bundles at the top right of the picture shows a poorly defined periodical structure among the filaments (boxed area). × 65,500. (Inset) Bundles of filaments cut in cross-section. The punctiform profiles appear linked by dense material that may represent cross-bridges. × 150,000.

Fig. 10. Ciliary neurons six days after saline injection into the anterior eye chamber. (A) This abnormal ciliary neuron contains conspicuous bundles of neurofilaments (asterisks). Nissl substance (ns) is scattered in the cell body. The bouton shell still covers the entire neuronal perimeter. Some of the preganglionic boutons are labeled b. N. nucleus; my, compact myelin. × 6100. (B) Higher magnification of the neurofilament bundles (asterisks) in the cell body of a reactive ciliary neuron. Microtubules are indicated by double open block arrows. × 34,800. (C) Gap junction (double arrowheads) between a preganglionic bouton (b) and a reactive ciliary neuron (CN). × 112,200.

Fig. 11. Ciliary neurons six days after RCA-60 injection into the anterior eye chamber. (A) This swolen ciliary neuron shows numerous profiles of the endoplasmic reticulum (some of them indicated by arrows, and appearing free of ribosomes at higher magnification) scattered in the cytoplasm among conspicuous bundles of neurofilaments (asterisks) oriented at different angles. Two clusters of ribosomal debris (r) are situated adjacent to the nuclear membrane. Preganglionic boutons (some of which are labeled b) completely envelop the neuronal cell body. N, nucleus; my, compact myelin; SC, satellite cells. × 4000. (B) Cluster of ribosomal debris (r) surrounded by neurofilament streams (asterisks) in the perikaryon of an affected ciliary neuron. × 39,500. (C) Two puncta adherentia (arrowheads) between a preganglionic bouton (b) and an affected ciliary neuron (CN). × 112,200.
together in small groups by electron-dense material. The chemical characterization of these extracellular materials remains to be ascertained by special fixation procedures and immunocytochemistry. After dissolution of the nerve cell bodies, the preganglionic boutons became partially or completely engulfed by satellite cell processes (Figs 8B, 9A).

Effects of saline injection into the anterior eye chamber

Injection of saline into the anterior eye chamber produced a moderate reaction in some of the ciliary neurons. The affected cells were scattered throughout the ganglion and presumably represented ciliary neurons innervating the iris (see Ref. 54). The cellular reaction resembled that provoked by postganglionic nerve crush, although it was much more modest in extent. The affected neurons primarily showed a moderate spreading of the Nissl bodies and accumulation of discrete bundles of neurofilaments (Fig. 10A, B). In some of the affected cells the bouton shell appeared intact (Fig. 10A), while in others (not shown) there was a partial detachment of the preganglionic boutons from part of the nuclear pole. The bouton shell at the hilar pole, however, appeared normal in all affected cells. Interestingly, unlike after crush of postganglionic nerve fibers, the preganglionic boutons still formed all three types of cell junctions, including the gap junctions (Fig. 10C), present in normal animals.

Effects of Ricinus toxin injected into the anterior eye chamber

RCA-60 injected into the anterior chamber of the eye, and thus in the immediate vicinity of the iris (Fig. 1), which is the target of some of the ciliary neurons, produced effects on the ciliary neurons that were only partly similar to those observed after toxin application on the crushed postganglionic nerves. As expected, the affected cells represented only a small percentage of this cell population, since the ciliary body, another target of ciliary neurons, is faced by the vitreous humor in the posterior eye chamber (Fig. 1). The degenerating ciliary neurons, three to six days after toxin injection into the anterior eye chamber, showed neurofilamentous hypertrophy, detachment of the ribosomes from the RER cisterns and formation of masses of ribosomal debris. An increase in the SER was also noted (Fig. 11A, B), but it was not determined whether this was balanced by the decrease in RER. In the most severely affected cells (not shown), segregation of the cell organelles into three main cytoplasmic regions was also observed. Notably, many of the affected neurons were completely (Fig. 11A), or almost completely, surrounded by the preganglionic bouton shell. Even in cells completely devoid of polyribosomes and with a great part of the perikaryon occupied by large streams of neurofilaments, the signs of a well advanced degeneration process, the boutons appeared normal and formed

the three types of cell junctions observed in the normal ganglion, including the gap junctions (inset to Fig. 11B).

The iris tissue was severely affected by the toxin (not illustrated). As early as three days after the injection, the iris appeared pale, the pupillary portion was highly indented and during the dissection the tissue was soft and friable. Eight days after the injection, the iris was no longer a compact structure. Electron microscopically, degenerating muscle cells were observed throughout the iris; axons appeared dystrophic, and infiltrating white cells and phagocytes were present among the cell debris.

DISCUSSION

We have demonstrated that postganglionic nerve crush and administration of RCA-60 have conspicuous and distinct effects on the ciliary neurons of the adult quail. The characteristic cytological and synaptic features of the quail ciliary neurons facilitated the analysis of these alterations, which are represented schematically in Fig. 12. Ciofi Luzzatto and colleagues1 demonstrated previously that in these neurons postganglionic nerve crush induced chromatolytic alterations clearly detectable in the light microscope, and that these changes appeared reversible within the time frame of their experiment (40 days). We have shown here that this chromatolytic reaction involves a moderate increase in neurofilaments and is accompanied by a severe detachment of preganglionic nerve terminals from the nerve cell bodies and disappearance of the gap junctions without any apparent involvement of the satellite cells. The ciliary neurons affected by RCA-60 delivered to the crushed postganglionic nerves show a characteristic alteration of the polyribosomal arrays and a striking increase of neurofilaments, culminating in the formation of large filamentous masses, segregation of the cytoplasmic organelles into separate compartments and ultimately extracellular accumulation of flocculo-filamentous material and cell death. It also appeared that treatment with ricin combined with postganglionic nerve crush may have reduced the shrinkage of the neuronal cell body usually seen in the advanced stages of postganglionic nerve damage.

In the following, we will briefly analyse the effects of the experimental conditions on the quail ciliary neurons in relation to findings in analogous experimental models.

Postganglionic nerve crush

The chromatolytic reaction and the detachment of synaptic terminals from ciliary neurons subjected to axonal damage are in accordance with several previous observations not only on motoneurons,1,10,22,36,63,65,89 but also on peripheral neurons.36,42,44,56 Detachment of presynaptic terminals from axotomized nerve cells was observed by
Fig. 12. Schematic diagram of the main features of quail ciliary neurons in the normal state (A), after postganglionic nerve crush (B) with prominent chromatolytic reaction, after saline injection into the anterior eye chamber (C) with moderate chromatolytic reaction, and at early stage of degeneration after RCA-60 administration in the anterior eye chamber (D), with disruption of polyribosomes, neurofilament hypertrophy, and organelle compartmentalization. See text for further details. PRE and POST, pre- and postganglionic axons; IAS, initial axon segment; AxH, axon hillock; Gl, satellite cell; N, nucleus of the ciliary neuron; ns, Nissl substance; r, ribosomal debris; SER, smooth profiles of endoplasmic reticulum.
Matthews and Nelson\textsuperscript{46} and Purves\textsuperscript{47} in the superior cervical ganglion of adult rats and guinea-pigs, respectively. Brenner and Johnson\textsuperscript{1} and Brenner and Martin\textsuperscript{48} studied the effects of axotomy in the ciliary ganglia of newborn to nine-day-old chicks, and observed that synaptic transmission was initially depressed and then suppressed, the synaptic contacts were partially lost and the morphological maturation of the presynaptic structures was impaired. We now know that, in normal newly hatched chicks, the entire perikaryon of the ciliary neuron is covered by the preganglionic calyciform ending.\textsuperscript{45} and it is probable that Brenner underestimated the extent of the detachment of the calyx and the consequent structural damage to the synaptic junctions and the gap junctions (mixed synapses), which have already formed between the ciliary neuron and the calyx at the developmental stage that he analysed. The suppression of synaptic transmission measured by electrophysiological techniques, therefore, may be more tightly correlated with the morphology than previously assumed. In the adult quail ciliary neurons, all types of cell junctions were severely affected after axonal crush. Because mixed synapses are more frequent in adult birds than in hatchlings,\textsuperscript{49} we suppose that, in our model, axonal damage severely affects both chemical and electrical synaptic transmission. That electrical transmission at the ciliary neurons is almost completely abolished by axotomy is also suggested by an electrophysiological study of Pilar (personal communication) in one-week-old chicks. Further studies on the chick ciliary ganglion have demonstrated that a main factor of the transmission failure is a massive (10-fold) decrease in the postsynaptic nicotinic acetylcholine receptors, both on the cell surface and in the intracellular pool of the ganglionic neurons.\textsuperscript{50,51} In addition, changes affecting some constituents of the cell surface essential for synaptic contact (e.g. adhesion molecules) could be associated with the loss of acetylcholine receptors. These changes, which may be produced by a similar mechanism, could contribute to the bouton detachment, together with the observed shrinkage of the ciliary neurons. Furthermore, there are reports of alterations of membrane conductance in axotomized motoneurons,\textsuperscript{52,53} indicating a general perturbation of membrane proteins.

An active role of the surrounding glial cells has been advocated to help explain loosening of the synaptic attachments in axotomized mammalian central neurons.\textsuperscript{54,55} In this respect, it is interesting to note that, in our model, satellite cell processes were apparently not involved in the detachment process. On the contrary, they seemed to remain in contact with the boutons in the neuronal surroundings. This phenomenon probably facilitates reattachment of the preganglionic boutons to the neuronal cell body during the recovery process. All these aspects indicate that ciliary neurons of adult quail, with their simple and unique innervation and lack of true dendrites, may be worthy of a careful microelectrode and cytochemical investigation after axotomy and during recovery.

Several studies have indicated that gene expression and protein synthesis are modified after axotomy.\textsuperscript{34,56-62} This response, which may be part of an attempt to regenerate the axon, includes an increase of actin and tubulin synthesis\textsuperscript{15,16} and transport,\textsuperscript{56} and a decrease in neurofilament synthesis.\textsuperscript{17,149,157,122,131} A decrease in the synthesis of these cytoskeletal components may appear to contrast with the formation of discrete filament bundles in the perikaryon of the quail ciliary neurons after nerve crush and after perturbation of the target structures in the eyeball observed in this study. The formation of these neurofilament bundles, however, might be the consequence of a rearrangement of pre-existing neurofilaments and/or a premature phosphorylation on the amino- and/or carboxyl-terminal tails of the neurofilaments\textsuperscript{20-24} that leads to their accumulation in the cell body. In fact, it has been demonstrated that axonal injury alters the pattern of distribution of phosphorylated and non-phosphorylated neurofilament subunits in the damaged neurons and leads to the appearance of phosphorylated epitopes in their cell bodies, both in motoneurons\textsuperscript{16,40,59} and sympathetic ganglion neurons.\textsuperscript{40} This event might promote the inappropriate interaction of the neurofilaments with other cytoskeletal components and prevent their transport along the axon.\textsuperscript{20,24} Phosphorylation of neurofilaments could be indirectly induced by influx of Ca\textsuperscript{2+} in the damaged neurons.\textsuperscript{35} Entrance of Ca\textsuperscript{2+} at the lesion site could promote the activation of protein kinase C, either directly or through other kinases via a "cascade" mechanism.\textsuperscript{20} Moreover, because neurofilament proteins become heavily phosphorylated at their carboxyl-terminal tails after neurofilaments enter the axon,\textsuperscript{7,46} the protein kinases involved in this process may principally reside in the axon itself.\textsuperscript{29} Thus, abundant neurofilament phosphorylation in the soma may imply that axotomy also redistributes activated protein kinases in the neuronal perikaryon.\textsuperscript{29} Neurofilament phosphorylation in axotomized neurons is a reversible process and the capability of the neurons to control the status of neurofilament phosphorylation might be a prerequisite for axonal regeneration.\textsuperscript{60}

\textit{Saline injection into the anterior eye chamber}

Although saline injection into the anterior chamber of the eye was done as control for the toxin injection at this site, this treatment by itself unexpectedly produced moderate alterations in some of the ciliary neurons. These changes consisted of an increase of perikaryal neurofilaments accompanied, in some of the cells, by retraction of the synaptic boutons from portions of the nuclear pole of the ciliary neuron, which represents the distalmost reach of the preganglionic fiber terminals (Fig. 12). These changes took place without any apparent surgical damage to
the ciliary axons; they were reminiscent of those observed after postganglionic nerve crush, although they appeared much slighter. There are no previous experimental findings in birds that may explain this novel observation. A study by Raviola on the effects of paracentesis in the macaque eye, however, may offer a clue to the possible mechanism. She reported that, when the anterior chamber is opened and drained, blood flows back from the episcleral veins into Schlemm’s canal and its endothelial lining develops gaps large enough to allow passage of plasma protein into the juxtanaculcular connective tissue, trabecular meshwork and anterior chamber. The composition of the aqueous humor, therefore, becomes substantially altered.

Although in our experiment we exerted special care not to drain the aqueous humor, puncture of the cornea certainly produced some fluid escape after withdrawal of the injection needle, with consequent drop of pressure in the anterior chamber. One may therefore assume that paracentesis of the bird eye has the same effects as in the primate eye, and that the composition of the aqueous humor becomes altered. The moderate, and presumably reversible, reaction observed in some of the ciliary neurons in our saline injection “controls”, may thus have been caused by a combination of pressure alteration and chemical insult of the ciliary axons innervating the iris, which is not covered by epithelial barriers. A similar, albeit more drastic, phenomenon was unrecorded in a previous study, where it was shown that up to 34% of the choriocaroid neurons die after piercing the posterior aspect of the eye bulb, including the choriocaroid coat. These results serve to re-emphasize the delicate balance existing within the eye bulb. The inescapable conclusion is that it is not possible to produce a perfect control for the effects of toxin administration into the anterior chamber of the eye. The experiment was also complicated by the damage caused to the iris tissue by the ricin toxin, which killed the target cells of the tributary ciliary neurons.

**Ricinus toxin treatment**

The block of protein synthesis induced by RCA-60 is slow and irreversible, and the toxin has to enter the cell to exert its effect. It has been shown that the process of internalization and processing of RCA-60 in various cell types is highly complex. It is very difficult, therefore, to know the amount of toxin that has reached the cell body with any mode of administration. Although one or a few toxin molecules may be sufficient to produce cell death, a large number of toxin molecules would conceivably exert a faster effect. In previous experimental studies, the toxin was usually applied on a crushed or cut nerve, thus combining the toxin effect with that of axonal damage.

In this study, we applied the toxin either on crushed postganglionic nerves or in the proximity of their terminals, by injecting it into the anterior chamber of the eye. One can surmise that in both procedures not all the neurons take up the same amount of toxin and that some of the neurons may have escaped the treatment altogether. This can explain the observed death of less than 50% of the ciliary neurons and the varying degree of morphological alterations among the ciliary neurons in the same ganglion (this study). Ciliary neurons affected by the toxin were clearly identifiable by the detachment of the ribosomes from the RER cisterns and by fragmentation of the polyribosomal rosettes, which represents the typical damage induced by this toxin, as also demonstrated in cultured Vero cells. This ribosomal alteration is the morphological counterpart of the block of protein synthesis. These events were accompanied by a massive accumulation of neurofilaments in the cell body, followed by compartmentalization of the cytoplasmic organelles. Conceivably, during the time required to reach complete block of protein synthesis, neurofilament proteins may polymerize into abnormal neurofilaments that are not transported down the axon or may undergo post-translational changes that prevent their moving down the axon and, perhaps, lead to their hyperphosphorylation in the cell body. Another possibility is that, at the onset of the protein synthesis block, the cell might selectively shut off the synthesis of certain proteins, for instance phosphatases, which are normally involved in the removal of phosphorylated groups from the newly synthesized neurofilament subunits in the cell body, or the proteins involved in the mechanism of slow axonal transport, thus impairing export of neurofilaments.

Increase of bundled perikaryal neurofilaments has also been observed after ricin treatment in superior cervical ganglion of the rat and hamster, in spinal and vagal motoneurons of the rat and in nodose ganglion of the hamster. These increases, however, were moderate and in no way comparable in extent to those found in our experimental conditions. A similar alteration was instead observed in rat spinal motoneurons treated with adriamycin, a lectin which acts at the transcriptional level by blocking protein synthesis with a slower rate than ricin. Thus, the severe neurofilament accumulation in RCA-60-treated quail ciliary neurons is at variance with previous studies on mammalian neurons. It would be interesting to ascertain whether RCA-60 treatment alone induces aberrant Ca influx and activation of protein kinases in the ciliary neurons.

The terminal stages of cell degeneration produced by RCA-60 in the quail ciliary neurons mimic certain features observed in human neurodegenerative disorders. The neurofilamentous skeins illustrated in Fig. 7A are reminiscent of intracellular neurofibrillary tangles, although they do not include paired helical filaments. The accumulation of extracellular floucculo-filamentous material (Figs 8A, B, 9A, B), probably a combination of proteins shed by
the degenerating neurons, and/or the satellite cells and plasma exudate, may bear some resemblance to the formation of amyloid plaques in Alzheimer's disease. The accumulation of a filamentous extracellular material has never been observed in previous studies that utilized RCA-60, and represents an interesting pattern of neuronal degeneration in the avian ciliary ganglion. These phenomena could be studied further in a tissue culture model, where the medium composition is controlled and all ciliary ganglion cells are exposed to the toxin.

After RCA-60 administration at the crush site, almost all affected neurons showed withdrawal of the preganglionic terminals from the nuclear pole, while the boutons contacting the hilar pole were less perturbed. In the early stages of degeneration after RCA-60 injection into the anterior eye chamber, by contrast, the affected ciliary neurons presented an intact bouton shell all around the cell bodies or, at worst, showed a moderate withdrawal of the preganglionic terminals from part of the cell body. From these data we can surmise that RCA-60 per se does not induce detachment of the preganglionic terminals before cell lysis occurs. Presumably, the alteration in the synaptic organization observed after postganglionic nerve crush and RCA-60 administration is a response to axonal injury; the moderate alteration of the preganglionic bouton shell observed in some of the ciliary neurons after injection of RCA-60 into the anterior eye chamber could be attributed to paracentesis and toxin damage of the muscle fibers of the iris.

CONCLUSIONS

The uniquely structured and peculiarly innervated quail ciliary neurons represent a valuable model for cellular neuropathology. The cells respond to axonal perturbation with a reversible, moderate degree of chromatolysis, but with a severe detachment of preganglionic terminals from most of the cell body. The ciliary neurons respond to RCA-60 treatment in the usual way with disruption of the polyribosomes, but also with a severe accumulation of neurofilaments resembling that found in numerous neurological diseases. The quail ciliary ganglion, therefore, lends itself to further studies of the molecular mechanisms involved in the axon reaction and the organization and perturbation of the neuronal cytoskeleton.

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