Partial Glycinergic Denervation Induces Transient Changes in the Distribution of a Glycine Receptor–associated Protein in a Central Neuron

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The effect of partial glycineric denervation on the cellular distribution of the 93 kDa peripheral polypeptide associated with the glycine receptor was studied at the level of the teleost Mauthner cell, an identified neuron of the goldfish (Carassius auratus). Previous studies using monoclonal antibodies raised against purified glycine receptors and immunoperoxidase staining have shown that these proteins are localized in clusters on the entire surface of this neuron. Specifically, the 93 kDa polypeptide was situated only on the cytoplasmic side of the postsynaptic membrane facing active zones.

Unilateral electrolytic lesions of the vestibular complex caused the degeneration of some glycineric afferents to this neuron. When the first signs of this response appeared, 3 d after the surgery, there was also a change in the ultrastructural distribution of the 93 kDa polypeptide in the deafferented cell. The synaptic protein presumed to degenerating axons did not spread onto adjacent extrasynaptic membranes, and it disappeared a few hours after the disruption of its presynaptic element. At the same time, a cytoplasmic immunoreactivity appeared as randomly distributed clusters in the deafferented Mauthner cell; these aggregates, not seen in control preparations, were never found inside membrane-bound organelles. In some preparations these clusters were localized along arrays at a relatively constant distance from the plasma membrane. The intracellular immunoreaction product was found in the soma and the initial part of the dendrites, gradually decreasing in number and intensity toward the extremities of these processes. At later postoperative stages, 10–15 d after surgery, the 93 kDa immunoreactivity remained only at postsynaptic membranes facing intact terminals. Similar alterations following denervation were observed in reticular neurons, at the level at which degenerating presynaptic terminals were also detected.

In contrast, continuous 3-d blockade of synaptic transmission by strychnine, an antagonist of the glycine receptor, had no effect on either the distribution of the surface receptor clusters, or the 93 kDa peripheral protein linked to these receptors.

Taken together, our results suggest that the ultrastructural distribution of the glycine receptor complex is regulated by "trophic" factors rather than by transmitter-evoked synaptic activity.

At most chemical synapses, cytoplasmic proteins are associated with receptor molecules. The best-documented case is that of the 43 kDa protein that is linked to the nicotinic receptor at the neuromuscular junction (Froehner et al., 1981; St.John et al., 1982; Sealock et al., 1984; Bridgman et al., 1987). It has been recently confirmed that this protein induces the clustering of ACh receptors when coexpressed with it in oocytes (Froehner et al., 1990), or in transfected fibroblast cell lines (Phillips et al., 1991; see also Cartaud et al., 1981; Rousselet et al., 1982; Burden et al., 1983; Peng and Froehner, 1985; Bloch and Froehner, 1987), implying that its function does not require the presence of nerve inputs per se. However, despite extensive studies of the effects of denervation on peripheral nicotinic receptor (Jacob and Berg, 1987, 1988; Sargent and Pang, 1988; for reviews, see also Fambrough, 1979; Schuetze and Role, 1987), nothing is known about the possible influence of nerve injury on the cellular distribution of the 43 kDa–associated protein. Such is also the case for anchoring proteins in the CNS.

Until recently, morphological data following selective denervations have been difficult to obtain in the vertebrate brain, for two reasons. One is that central pathways are intermingled, and few of them can be severed in isolation. The second is the lack of specific antibodies, which are required for detailed immunocytochemical studies and ultrastructural analysis of central synapses. An exception is the Mauthner (M)-cell inhibitory network, part of which belongs to the vestibulo-vestibular commissural pathway (Zottoli and Faber, 1980; Triller and Korn, 1981), that has been characterized electrophysiologically (Faber and Korn, 1973; Korn and Faber, 1976). Its terminals are glycinergic (Faber and Korn, 1980, 1988), and their effect on the M-cell is blocked by strychnine (Faber and Korn, 1988).

The glycine receptor (GlyR) is a ligand-gated ion channel that mediates CI-dependent inhibitory currents in the CNS (Korn et al., 1990). It is antagonized by strychnine (reviewed in Korn and Faber, 1990). This property has been used to purify the receptor complex from the mammalian spinal cord and to demonstrate that the protein contains three polypeptides of 48, 58, and 93 kDa. The first two are transmembrane polypeptides.
tors, have been presented briefly in a previous communication (Seitanidou et al., 1990). The antibodies raised against the purified rat GlyR, the 4a mAb specifically binds to the 48 kDa or α-subunit, while the 5a and 7a ones bind to the 93 kDa protein (Pfeiffer et al., 1984).

The 48 kDa subunit, which bears the antagonistic binding site (Pfeiffer et al., 1984), and the 93 kDa subunit have been detected at restricted sites facing presynaptic active zones in the adult brains of the rat (Triller et al., 1985; Altschuler et al., 1986; Van den Pol and Gorcs, 1988) and goldfish (Triller et al., 1986; Seitanidou et al., 1988, 1991). Furthermore, Western blot analysis has shown that the antibodies recognize only antigens of similar molecular weight in the rat and goldfish brain and that they are colocalized regionally with strychnine-binding sites (Becker et al., 1991).

Taking advantage of these probes, we have investigated the role of innervation on the ultrastructural distribution of the M-cell postsynaptic 93 kDa polypeptide. Our results indicate that even a mild denervation results in striking alterations of this protein’s cytoplasmic localization that are different from those reported in the neuromuscular junction. In contrast, a chronic and massive block of synaptic activity has no detectable effect on the localization of the 4a mAb or of the 4a subunit. In this manner, 7 of them were used to visualize the localization of the 93 kDa polypeptide with electron microscopy, and 8 were used to analyze the α-subunit distribution. The remaining 9 were used for electrophysiological recordings in order to determine if in those conditions the antagonist had reduced or suppressed the M-cell responses to synaptically released glycine. Three of them were investigated 5-6 hr after a unique dose, the remaining 4 (two of which were recorded on both M-3 and M-4) were subjected to a chronic treatment for 3 d before physiological analysis.

Electrophysiological recordings. Fish treated with strychnine as described above were anesthetized with MS222 and immobilized with Flaxedil (1 μg/ml body weight). The preparation and basic physiological techniques were similar to those employed before (Korn and Faber, 1976). The M-cell was identified on the basis of its stereotyped response to antidromic stimulations of its axon in the spinal cord (Furushpan and Furukawa, 1962), and extra- or intracellular activities were monitored with local-resistance (2-5 MΩ) microelectrodes. The latter were filled with K-acetate or with 3 M KCl in order to maximize and therefore detect possible inhibitory postsynaptic potentials (IPSPs) since in this neuron resting membranes at Cl– equilibrium potentials are close to each other (Furukawa and Furushpan, 1963). At the end of some recording sessions, the resting membrane conductance was measured on current pulses evoked by the mAb using the technique of hyperpolarizing current injection by the mAb and depolarizing current injection by the mAb.

Materials and Methods

Our experiments were based on the notion that glycinerergic commissural interneurons innervate both Mauthner cells in the goldfish brainstem (Triller and Korn, 1981).

Electrolytic lesions. Adult goldfish (Carassius auratus) 13–15 cm in body length were used. They were anesthetized with 0.30% 3-aminobenzoic acid ethyl ester (Sigma). When gill movement ceased, they were transferred to a surgical chamber where 0.2% of the same anesthetic was recirculated through the mouth and over the gills during surgery. Once a fish was immobilized, the cerebellum and the anterior part of the vagal lobe were exposed. The cerebellar stalk was pushed forward and the dura mater over the fourth ventricle was removed. During this procedure, both otic capsules remained intact, but the semicircular canals of the lesioned site were often disrupted. An insulated tungsten electrode (tip diameter, <1 μm) was then introduced in the medulla, at the most anterior part of the right cerebellar crest. A positive current of 0.005–0.05 mA was applied for 15–30 sec, with the expectation that lesions produced by similar anodal intensities are reproducible in size and shape (Moore, 1981). The electrode was removed and the cerebellum was returned to its original position. A piece of paraffin slightly larger than the size of the hole was placed on the skull, sealed around its edge with acrylic glue, and covered with dental cement. The fish was then resired continuously with tap water for 15 min and returned to the aquarium. After variable survival times, the location of the lesion and its extent were verified in transverse thick sections (80 μm) embedded in araldite (see below).

As summarized in Table 1, brains from 31 lesioned fish, 10 intact ones, and 5 sham-operated control animals were processed for electron microscopy. The ultrastructural distribution of the 93 kDa protein was analyzed after different postoperative intervals of 2–15 d, as listed in Table 1.

Chronic block of glycine receptors by strychnine. As in previous studies (Furushpan and Korn, 1982, 1988), glycinerenic receptors were functionally blocked by injecting intramuscularly 5 μg/gm body weight of strychnine sulfate diluted in 0.9% saline solution. The animals received a dose of strychnine every 6 hr for 1–3 d. A total of 24 adult goldfish were treated in this manner. 7 of them were used to visualize the localization of the 93 kDa polypeptide with electron microscopy, and 8 were used to analyze the α-subunit distribution. The remaining 9 were used for electrophysiological recordings in order to determine if in those conditions the antagonist had reduced or suppressed the M-cell responses to synaptically released glycine. Three of them were investigated 5–6 hr after a unique dose, the remaining 4 (two of which were recorded on both M-3 and M-4) were subjected to a chronic treatment for 3 d before physiological analysis.

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### Table 1

| Condition       | Number of Animals | Survival Time (d) |
|-----------------|-------------------|-------------------|
| Intact          | 10                | 15                |
| Sham-operated   | 5                 |                   |
| Lesioned        | 31                | 2-15              |

The Journal of Neuroscience, January 1992, 12(1) 117
Results

Behavioral changes in operated animals

Immediately after recovery from anesthesia, all fish with unilaterally damaged vestibular nuclei were unable to swim along a straight line. They swam with disordered movements in an arc bent toward the lesion. When still, they usually remained on the affected side, near the bottom of the tank. Two days after surgery, they exhibited normal posture and equilibrium at rest, and they began to swim normally at 4 d. Finally, their swimming and resting behavior had completely recovered by the tenth day after the operation.

Sham-operated control fish had normal swimming responses even though they remained immobile at the bottom of the aquarium during the first postoperative days.

Unilateral lesion of vestibular complex and degeneration pattern

In every operated animal selected for this study (Table 1), the lesion was restricted to the vestibular complex and affected all four vestibular nuclei. Its maximum dimensions never exceeded 800 μm in the longitudinal and 600 μm in the dorsoventral and lateromedial axes.

A representative photomicrograph of a transverse thick section at the level of the lesion obtained 3 d after surgery is shown in Figure 1A. Its shape was ovoid, extending up to 640 μm in the rostrocaudal and 450 μm in the horizontal and vertical axes. The central cavity, where the tissue was completely destroyed, was no more than 200 μm in diameter (in this and other preparations as well). Damaged tissue, encircled by a narrow rim of gliosis, was present 360 μm posterior to the M-cell’s lateral dendrite, disappearing at the level of the tip of its ventral dendrite. In all material selected for the present study, the lesion was located in the vestibular complex, either just above the secondary gustatory and descending trigeminal tracts (Fig. MB) or, more medially, between the ventricle and the outer limit of the brain (not shown).

As schematized in Figure 1B, the damaged area was situated at the entrance of the eighth nerve above the descending trigeminal tract and close to the lateral dendrite of the M-cell. Thus, it is possible that in some experiments, the current had reached the extremity of this process, which penetrates into the vestibular nuclei in this region. In order to ensure that any morphological changes following surgery were due to causes other than (1) the degeneration of the M-cell itself or (2) the disappearance of the eighth nerve endings, one of the major excitatory inputs of the ipsilateral M-cell, we have restricted our study to the M-cell contralateral to the lesion. However, in three preparations with intact ipsilateral M-cells, identical results were obtained from each command neuron, on either side of the brain.

Three days after the operation, there was an anterograde degeneration of approximately 10% of the presynaptic afferents at the level of the M-cell soma. As depicted in Figure 1C, this phenomenon concerned crossed second-order neurons whose axons project ipsi- and contralaterally, since commissural neurons project bilaterally onto the soma and within the axon cap of both M-cells, to the reticular formation and to the contralateral vestibular complex.

The morphology of the investigated M-cell was compared with that of the unoperated side within those of control animals.
Figure 1. Extent of electrolytic lesions. A, Photomicrograph of a frontal thick (80 μm) section along the medial line of the brain, at the level of the vestibular nuclei, showing the lesion (broken line) produced by a cathodal current. Its boundaries are designated by a marked scar (arrows). Scale bar, 500 μm. B, Transverse section through the same lesion (stippled area), indicating its relation to adjacent structures at the level of the Mauthner cell. C, Drawing of commissural glycinergic vestibular neurons synapsing on both M-cells. Vestibular complexes ipsi- [N. Vest (ipsi)] and contralateral [N. Vest (contra)] to the lesion are surrounded by dotted circles. Note that efferent processes (solid lines) terminating on both M-cells [M cell (ipsi, contra)] and in the reticular nuclei (N. Ret) can be affected by a lesion of the vestibular nucleus. Axons issued by vestibular neurons contralateral to the lesion (broken lines) are also shown. AC, axon cap; CB, cerebellum; DT, descending trigeminal tract; EG, eminentia granularis; MC, Mauthner cell; MLF, medial longitudinal fasciculus; RN, reticular nuclei; TGS, secondary gustatory tract; Vv, fourth ventricle.
Figure 2. Distribution of degenerating profiles on the contralateral M-cell following short postoperative intervals. A, Somatic membrane of M-cell from unoperated animals with its typical investment by afferent terminals. Note the labeled postsynaptic differentiations (arrows) of two synaptic contacts. B, Diagram of the distribution of degenerating profiles (shaded area) at the peripheral part of the axon cap, on the soma (S) and on small ventral dendrites (sDV). C, Electron micrographs of the M-cell axon cap, 3 d after a unilateral lesion of the vestibular complex. The peripheral part (PP) of the axon cap is occupied by necrotic debris of fibers (single arrowheads), some of which are surrounded by a lamina of glia (double
on semithin sections. The shape, size, position, and orientation of its dendrites and axon remained unaltered after denervation. The cell nucleus was always located centrally and somewhat dorsomedially, close to the axon hillock. The nuclear outline was smooth and slightly infolded with no sign of chromatolysis (referred in Zottoli et al., 1984). At the EM level, the perikaryal and dendritic cytoplasm appeared normal, containing their usual supply of organelles.

**Ultrastructural distribution of degenerating axons and fate of synaptic GlyR-associated 93 kDa protein on the M-cell**

Previously, in nonoperated fish, the 93 kDa immunoreactivity (93kd-IR) was only detected on synaptic membranes, in apposition to presynaptic release sites (Seitanidou et al., 1988). The immunoreactive product was contiguous to SVBs at the soma (Fig. 2A) and dendrites, and to almost all unmyleinated club endings (UCEs) in the peripheral part of the axon cap.

In control animals (n = 4), 38.5 ± 3.1% of the somatic terminals presynaptic to the M-cell were apposed to 93 kDa-stained postsynaptic densities. This number decreased 3 d after lesion (n = 3), when only 31.1 ± 3.1% of intact endings were in front of immunolabeled membrane. The afferent inputs on the two main dendrites remained unaffected.

As shown in Table 1, degenerating profiles were either in direct contact with their target, or slightly detached from the plasmalemma of the postsynaptic cell. After the third postoperative day, the profiles were rarely still attached to the M-cell. However, scarce degenerating fibers could be observed in the synaptic bed until 7 d after surgery. The distribution of degenerated terminals was analyzed at 3 d after lesion (n = 3), when 31.1 ± 3.1% of intact endings were in front of immunolabeled membrane. The afferent inputs on the two main dendrites remained unaffected.

At this time, many dark axonal profiles surrounded by astrocytic processes were found in the peripheral part of the axon cap (Fig. 2C), but never in its central core. The lesioned dendrites were occasionally in contact with cap dendrites present in this region. As shown in Figure 2D, they were also present at the somatic level of the M-cell, either in direct contact with it, or in its synaptic bed at some distance (2–5 μm) from the plasmalemma. Dark terminals, still attached to the M-cell (Fig. 2D), were considered as an early sign of degeneration, which preceded complete enwrapping by glial processes.

To determine if the affected afferents were normally apposed to the 93 kDa protein, tissues were immunolabeled with the 7a mAb. Again, two distinct types of degenerating profiles were observed in the synaptic bed: (1) electron-dense boutons, attached to the M-cell and engulfed by glial cell processes (Fig. 2D), their corresponding postsynaptic membrane being often immunolabeled (Fig. 2D inset); the staining in front of the degenerating or intact presynaptic endings never outflanked the extrasynaptic membranes; and (2) dark fibers (Fig. 2D) in the synaptic bed, disconnected from the M-cell and completely enwrapped by a lamina of reactive astrocytic cells with numerous cisternae. For a very brief period of time, labeled membrane patches were left deprived of any presynaptic element, apposed to glial processes or to necrotic debris. Randomly distributed glial processes, next to intact terminals, which themselves were apposed to a 93kd-IR site, were directly in contact with the M-cell somatic membrane over long distances, a feature never observed in nonoperated animals.

**Intracellular 93kd-IR**

A striking modification of the 93 kDa protein’s expression took place in the cytoplasm of the denervated M-cell (Fig. 3A; see also Table 1) under the form of an immunoreactivity on the third day that persisted up to the seventh postoperative day. This staining was concentrated at discrete loci, where it had a circular or oval shape. The cytoplasmic electron-dense precipitate was never observed inside membrane-limited structures such as mitochondria. Golgi and Golgi vesicles, rough and smooth reticulum, or lysosomes. Rather, as revealed by high magnification (Fig. 3C), the oxidized DAB at the periphery of the cluster appeared to be related to the cytoskeletal domain and had the form of small dots. Cisternae of agranular endoplasmic reticulum or small vesicles found at the border of the immunoreaction product were stained along their entire perimeter or just a part of it. In this case, the reaction product was associated only with the cytoplasmic surface of their membrane.

Most often clusters of the intracellular 93 kDa protein were randomly distributed in the cytoplasm of the M-cell, in front of any type of presynaptic ending, at varying distances from the plasma membrane, including a juxtaposition (Fig. 3C). However, in three preparations obtained 3 d or 4 d postoperatively, they were aligned at 1–5 μm from the somatic membrane (Fig. 4). This ordered distribution was almost parallel to the plasmalemma for at least one-third of its perimeter.

In the principal dendrites of the M-cell, where degenerating fibers were not encountered, the cytoplasmic immunoreactivity was nevertheless present (data not shown), although less pronounced than that at the somatic level. In the lateral and in the ventral dendrite of deafferented M-cells, the occurrence of precipitate diminished progressively with distance from the soma and was absent at their extremities. In these processes, the cytoplasmic staining was dispersed within the cytoskeletal elements and not associated with the membranes of the endoplasmic reticulum.

The effect of presynaptic deprivation was also studied 10–15 d after surgery. During this late postoperative interval, degenerating terminals were never present close to the M-cell. The presynaptic bed was comparable to that of the control animals. Nevertheless, terminal-free regions were apparent, with afferent fibers spaced farther apart in the extracellular space. The only antigenic determinant recognized by the 7a mAb during this period was that of membranes postsynaptic to afferent processes filled with pleiomorphic vesicles.

As already described, the cytoplasmic 93kd-IR was almost exclusively localized on nonmembranous structures. However

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*arrowheads.* In contrast, the central core is unaffected. D, Degenerating axonal profiles (arrowheads) in the synaptic bed of and apposed to (arrows) the M-cell soma. Same survival time as in C. Note stained glycine receptors (crossed arrow) in front of a terminal bouton undergoing dark degeneration, still in contact with the M-cell. *Inset.* Higher magnification. A, axon; CC, central core; DL, lateral dendrite; DV, ventral dendrite; n, nucleus. Scale bars: A, C, and D, 2 μm; inset, 500 nm.
in a few instances, it was found near the Golgi apparatus or associated with the cytoplasmic side of peripheral vesicles of the Golgi complex.

**Effect of denervation on the reticular neurons**

Cells lying close to the medial and distal parts of the M-cell's ventral dendrite were examined since they are also targets of the commissural vestibular interneurons (Triller and Korn, 1981). The 93 kDa polypeptide was affected, as described above. At early stages after surgery, the immunoreactivity was present not only at synaptic membranes in front of intact endings, but also in the form of clusters, randomly distributed within the somatic cytoplasm (Fig. 5) and the initial part of the dendrites. Layered astrocytic processes occupied the extracellular space and were occasionally in contact with the noninnervated plasmalemma, interspersed between afferent terminals. Some neurons were di-
rectly in contact with electron-dense presynaptic endings, undergoing a degenerative process and facing immunolabeled postsynaptic membranes. The evolution in this transient cytoplasmic staining was identical to that of the M-cell.

Extent of 93 kDa labeling on postsynaptic membranes
In order to establish if the length of the synaptic labeling was modified after lesions, the extent of the corresponding immunoprecipitate was compared from data collected in six control and six operated animals. As indicated by the histograms (Fig. 6A, B, C), obtained from random EM sections of lesioned animals, the mean length of labeled specialized membranes was 0.452 µm (SD = 0.149 µm; n = 75) on the cap dendrites, 0.761 µm (SD = 0.313 µm; n = 74) on the ventral and lateral dendrites, and 0.653 µm (SD = 0.265 µm; n = 104) on the soma. In unoperated animals (Fig. 6A, B, C), the extent of the staining was about the same. More specifically, it averaged 0.447 µm (SD = 0.137 µm; n = 62), 0.728 µm (SD = 0.229 µm; n = 130), and 0.656 µm (SD = 0.290 µm; n = 182) at the corresponding regions of the M-cell.

In contrast, the length of patches linked to degenerating somatic terminals was reduced to 0.504 µm (SD = 0.157 µm; n = 25) (Fig. 6D), that is, 77% that of the control. These data were obtained from stained postsynaptic densities in apposition to electron-dense endings still attached to the M-cell, one of which is shown in Figure 6E.

Block of the GlyR complex by strychnine
Swimming behavior of animals injected with strychnine was severely affected. At approximately 3 min after the first injection, the fish was stationary in a straight posture. The startle response did not habituate and 10 responses in series could be elicited at brief intervals by taps on the aquarium. After the second injection, the animals were lying on one side, on the bottom of the aquarium. At this stage, the stimulus triggered a strong contraction and a tremor of the muscles, but the animals were unable to swim.

Block of synaptic responses by strychnine
Intracellular recordings demonstrated that chronic injections of strychnine significantly impeded the M-cell responses to activation of the recurrent collateral network that otherwise remained essentially unaltered. The experimental design (Fig. 7A) and the basic principles used to reach this conclusion were as follows.

First, after ortho- or antidromic firing of the M-cell, the presynaptic volleys in the terminals of collateral interneurons appear, in the axon cap, as a positive potential (Fig. 7B), the so-called extrinsic hyperpolarizing potential or EHP (Furukawa and Furshpan, 1963). In normal conditions, this volley evokes the glycine-mediated postsynaptic inhibition of the M-cell (referenced in Faber and Korn, 1982) and both disappear at stim-
Figure 5. Partially denervated reticular neuron, at early (3 d) postoperative intervals: antigenicity to the 7a mAb at normal synaptic contacts (arrows) and in the cytoplasm (triangles). Scale bar, 1 µm.

ulation rates of >4/sec (Fig. 7B,) due to a marked frequency sensitivity of the synapses intercalated along the collateral pathway (Faber and Korn, 1978). Second, the peak postsynaptic inhibitory conductance change, $G_{\text{IPSP}}$ (or $G_{\text{coll.}}$, in the case of the inhibitory conductance underlying the collateral IPSP), can be determined by measuring its shunting effect on the height of a testing antidromic spike. Briefly, treating the M-cell membrane as having a resting conductance $G_0$, in parallel with the inhibitory channels and in series with the driving force for chloride, led to the expressions

$$G(\text{IPSP}) = (r/1 - r)G_0,$$

where $r$ is the fractional reduction of the spike, and $V$ and $V'$ are the amplitudes of the control and test spikes, respectively (for derivations and justifications, see Faber and Korn, 1982).

In practical terms, $V$ and $V'$ can be assessed from the onset to the peak of the corresponding action potentials (Fig. 7B, B,).

At all M-cells subjected to strychnine injections for 6 hr ($n = 3$) or 3 d ($n = 6$), the waveform and amplitude of the EHP were normal (Fig. 7B,), indicating that the presynaptic volley reached the endings of the collateral neurons and that, as expected, it vanished during high-frequency stimulation rates (Fig. 7B,). In contrast, and although the antidromic spike evoked by threshold spinal stimulation was normal (Fig. 7B,), the collateral IPSP and its underlying conductance change were blocked by the drug, as shown by (1) the absence of Cl$^-$-dependent voltage change after firing of the M-cell, in recordings obtained with KCl electrodes (not shown), and (2) estimates of the parameter $r$, which was almost identical in a given cell, at slow (Fig. 7B,) and at high (Fig. 7B,) frequencies of antidromic stimulations, that is, when the reduction of the test height spike could be attributed to refractoriness alone. Also, when the strength of the spinal stimulus was increased, the test spike could summate with added EPSPs (Fig. 7B,), a situation never encountered in the case of composite excitatory and inhibitory responses (Korn and Faber, 1975).

Pooled ($n = 9$) mean values of $r$ in absence or in presence of collateral input were almost identical, $0.13 \pm 0.07$ (±SD; range, 0.03–0.23) and $0.15 \pm 0.06$ (±SD; range, 0.05–0.24), respectively; the latter value indicates considerably less reduction in the test spike amplitude, at the peak of the anticipated collateral IPSP, than the 50–70% in normal conditions, as shown, for comparison, in Figure 7C, C, (see also Fukami et al., 1965; Korn and Faber, 1976).

Morphological observations

The distribution of the $\alpha$-subunit of the GlyR was examined with CSLM, 2 d ($n = 2$ fish) or 3 d ($n = 6$) after the beginning of strychnine treatment. The immunoreactivity pattern of the surface receptors (Fig. 8A,) did not differ from that of control ($n = 10$) animals (Fig. 8B; see also Triller et al., 1990). Fluo-
LENGTH OF 93 KD-IR APPOSED TO INTACT TERMINALS

**A.1** DENERVATED
- **axon-cap**
  - $m = 0.452 \pm 0.149$
  - $n = 75$

**A.2** CONTROL
- $m = 0.447 \pm 0.137$
- $n = 62$

**B.1**
- **dendrites**
  - $m = 0.761 \pm 0.313$
  - $n = 74$

**B.2**
- $m = 0.728 \pm 0.229$
- $n = 130$

**C.1**
- **soma**
  - $m = 0.653 \pm 0.265$
  - $n = 104$

**C.2**
- $m = 0.656 \pm 0.290$
- $n = 182$

LENGTH OF 93 KD-IR APPOSED TO DEGENERATING TERMINALS

**D**
- **soma**
  - $m = 0.504 \pm 0.157$
  - $n = 25$

**E**
rescent clusters separated by unlabeled membrane patches were present in the entire M-cell plasmalemma. The high-resolution CSLM pictures allowed us to demonstrate that the size of each individually labeled microdomain remained unchanged. As illustrated in Figure 8A and B, the mean surface area of somatic clusters was 0.53 μm² (SD = 0.41 μm²; n = 516) in strychnine-injected animals and 0.51 μm² (SD = 0.43 μm²; n = 970) in controls. These means were not significantly different (p < 0.05, Student’s t test).

At the ultrastructural level, the localization of the 93 kDa protein at synaptic contacts persisted the first (n = 1), the second (n = 2), and the third (n = 4) day of strychnine application (Fig. 8C). The overall shape and the number (39.3 ± 1.71%; n = 4) of labeled synapses were identical to those of control preparations (see Fig. 2A). Furthermore, the average diameter of the stained somatic contacts (mean ± SD = 0.639 ± 0.232 μm; n = 105) remained unchanged. In contrast to the denervation effect of the distribution on the 93 kDa peripheral protein, intracellular antigenicity was absent in strychnine-treated M-cells. Finally, the distribution of the GlyR complex on reticular neurons was comparable to that in the M-cell.

Discussion
The major findings of this study, summarized in Table 1, were the following: (1) the short persistence of the postsynaptic 93 kDa protein at degenerating terminals and its transient appearance as clusters in the cytoplasm of the deafferented neurons, and (2) the absence of significant modifications of the GlyR and
A2  B2

Figure 8. Unaltered cellular distribution of the GlyR complex on the M-cell, after chronic application of strychnine. A and B. Effect of blockade of glycineric transmission on the 48 kDa polypeptide. A, and B,, Confocal fluorescent images of the α-subunit on the soma (arrows) or on dendrites (crossed arrows), in treated (A,) and in control (B,) animals. A, and B,, Frequency histograms of the surface areas of stained clusters obtained from the M-cell somatic region. Note that the means were not significantly different, as confirmed by a Student’s t test. C, Electron micrograph of somatic synaptic contacts (arrows) stained by the 7a mAb, with normal expression of the 93 kDa protein. Note the absence of immunoprecipitate in the cytoplasm. Mc, M-cell soma. Scale bars: A, and B,, 6 µm; C, 725 nm.
of the 93 kDa protein distribution after chronic block of synaptic transmission. Thus, our data suggest that factors, other than glycine, associated with the afferent nerve endings control the expression of these synaptic molecules.

Glycinergic nature of the degenerating endings
Different types of degenerative effects may coexist, such as those described in the axotomized M-cell, where terminal profiles display reactive deafferentation, but with no signs of degeneration (Wood and Faber, 1986). However, immunolabeling of the 93 kDa protein requires a fixative containing a lower concentration of glutaraldehyde (0.1%) than the 2.5% used by those authors. In order to avoid dubious interpretations of our data, we considered as degenerated fibers only those containing a dark electron dense material.

The unilateral destruction of the vestibular nuclei caused the degeneration of no more than 10% of the terminal boutons on the soma of the M-cell. All the morphological criteria used to classify the afferents of the M-cell (Nakajima, 1974) could not be applied to these altered terminals; yet, their location on the M-cell already suggested that they belonged to UCEs in the axon cap, and SVBs when in contact with the soma, outside this region. Excitatory second-order vestibular neurons that cross the midline have been reported in other species (Graf and Ezure, 1986), but excitatory endings, containing gap junctions and identified as "club endings" (Nakajima, 1974), were never observed on the contralateral deafferented M-cell. Furthermore, the degenerating fibers were found in regions where the 93 kDa polypeptide is expressed, and where activation of the crossed vestibular pathway produces a strong chloride-dependent inhibition that is mediated by glycine (Korn and Faber, 1976; Triller and Korn, 1981; Faber and Korn, 1982). However, electrolytic lesions could have caused also retrograde damage to contralateral neurons, some branches of which innervate the M-cells. The influence of terminals other than glycinergic ones on the distribution of the 93 kDa polypeptide during the early stage after lesion is unlikely, although heterologous regulation of neurotransmitter receptors has been reported in the CNS. In the rat brain, serotonergic deprivation results in an increase of α-adrenergic receptors (Rappaport et al., 1985) and non-dopaminergic fibers modulate a D₃ receptor denervation supersensitivity (Hervé et al., 1989).

 Fate of postsynaptic sites and of synaptic 93kd-IR
The 93kd-IR facing dark degenerating terminals remained behind the apposed synaptic contacts. Its average length was only slightly diminished compared with the control animals, 3 d after disruption of the crossed inhibitory input. We never observed changes in the density and/or small fragmentations of the synaptic 93 kDa clusters, although low quantities of this polypeptide at extrasynaptic loci may have remained undetected with our immunohistochemical technique.

A persistence of postsynaptic specializations has been reported after primary deafferentation in the ventral cochlear nucleus of the rat (Gentschev and Sotelo, 1973), and after sectioning the preganglionic fibers in frog sympathetic ganglia (Sotelo, 1968). In our material, unoccupied stained postsynaptic densities that persisted only during the early phase of the degenerative process seem to have shorter life spans. Reoccupation of vacant postsynaptic sites by a sliding process from adjacent intact terminals (Gentschev and Sotelo, 1973) was never observed at early or late postoperative intervals. Thus, it is unlikely that reinervation of preexisting labeled membrane patches due to collateral sprouting of proximal axons takes place during the first 2 weeks following our lesions, which is the longest time period covered by this study.

Comparison with the nicotinic receptor-associated 43 kDa protein
Several lines of evidence suggest that membrane proteins are involved in the anchoring and/or the maintenance of the receptor clusters (Bloch and Froehner, 1987; Schmitt et al., 1987; Kordeli et al., 1989) via interactions with the cytoskeleton. No conclusive data are yet available on the role of innervation on the distribution of the 43 kDa protein. Accumulation and clustering of the 43 kDa protein can occur in the absence of afferent nerve (Peng and Froehner, 1985; Bloch and Froehner, 1987; Kordeli et al., 1989), and a diffuse cytoplasmic form has been detected in developing electrocytes (Kordeli et al., 1989). Other studies have shown that denervation causes a threefold increase in the amount of the mRNA for the 43 kDa protein in a leg muscle of the adult mouse (Fraill et al., 1989).

Possible function of the cytoplasmic 93 kDa polypeptide
The transient appearance of the 93kd-IR in the cytoplasm of the denervated cells may result from (1) a decrease in the rate of its transport to the plasma membrane, (2) an increased rate of its synthesis, or (3) an alteration in any posttranslational modification that would affect the expression of the epitope. It may also be the consequence of the 93 kDa protein recycling from the deafferented synapses. A number of other receptors such as those of NGF or epidermal growth factor are known to be recycled with their ligands (for review, see Greene and Shooter, 1980; Goldstein et al., 1985). However, internalization seems unlikely in our experiments, since the only labeled vesicles observed occasionally were close to the Golgi apparatus, that is, far from the somatic membrane.

A different mechanism for internalization of the components of the postsynaptic site has been reported in the ventral cochlear nucleus of the rat (Gentschev and Sotelo, 1973), where entire free postsynaptic sites were eliminated by engulfment into the cytoplasm. In the M-cell, unstained internalized postsynaptic densities were never observed. Furthermore, in the labeled cytoplasmic cluster of the 93 kDa protein, membrane remnants were never visualized and intracellular clusters were detected in the principal dendrites where degenerating profiles were not found.

The pattern of the cytoplasmic staining of the 93 kDa protein described in this work completely differs from that of the nicotinic receptor in the chicken ciliary ganglia (Jacob et al., 1986; Jacob and Berg, 1987) and of the GABA receptor in the rat cerebellum (Somogyi et al., 1989). In these studies, the intracellular immunoreactivity was only associated with membrane-bound organelles like the endoplasmic reticulum or the Golgi apparatus, or the nuclear envelope. This difference was expected since transmembrane subunits, like those of glycine, ACh, and GABA receptors, are thought to be synthesized and modified in the rough endoplasmic reticulum and sorted out in the Golgi apparatus (Rothman and Fine, 1980). Conversely, the 93 kDa polypeptide is a nonglycosylated peripheral membrane protein and should not follow the same biosynthetic or regulatory pathway; it is most probably synthesized by free polyribosomes. The staining of the Golgi by the 7a mAb might result from an early association at this level of the cytoplasmic 93 kDa polypeptide.
to the glycine receptor transmembrane subunits, before their translocation and their subsequent insertion into the plasma membrane. It is also possible that proximal vesicles and Golgi membranes, or even ribosomes, are stained, due to absorption of translocated DAB precipitate during the enzymatic immunocytchemistry.

Since intracellular clusters of 93kd-IR were not apparent in the axon hillock or the M-cell axon, passive diffusion seems unlikely to account for their transport in the dendrites. Rather, their location at a fixed distance from the plasma membrane (Fig. 4) suggests the involvement of the cytoskeleton. An example of a similar mechanism of a nonvesicular transport of molecules by the cytoskeleton was reported in cultured hippocampal neurons where newly synthetized mRNA is transported to the dendrites (Davis et al., 1987). Such an active transport may contribute to selectively direct and segregate proteins at specialized regions, such as surface microdomains of central neurons. A subplasmalemmal synaptic molecule, like the 93 kDa polypeptide, should be only localized at restricted areas of the plasma membrane, associated with the appropriate receptor, and functionally match with its corresponding presynaptic element. Along this line, in multinucleated muscle fibers from mature animals, ACh receptors are preferentially transcribed and synthesized by a few nuclei at synaptic areas (Merlie and Sanes, 1985; Fontaine et al., 1988).

**Chronic block of synaptic responses**

It has long been shown that strychnine blocks the collateral IPSP (Furukawa et al., 1963), a property extensively used to demonstrate that, although GABA receptors are present on the M-cell (Diamond and Roper, 1973; Diamond et al., 1973; Faber and Korn, 1980), glycine is the major transmitter operant at the somatic level (Mazliah and Werman, 1974; Faber and Korn, 1982), including at synapses issued by the commissural interneurons (Faber and Korn, 1988). Also, despite suggestions that in other systems, strychnine may affect responses mediated by GABA (Davidoff et al., 1969), the latter are not changed in this neuron by doses of the drug that block the collateral IPSP (Diamond et al., 1973).

Recovery of synaptic potentials from a block by strychnine is exceptional during acute experiments and was only observed when low amounts of the antagonist were injected iontophoretically in the axon cap to verify that the background synaptic noise is inhibitory in the M-cell (Korn and Faber, 1990), yet prolonged exposure of neurons to strychnine has not been attempted in vivo for behavioral and/or physiological studies of its long-term effects that in the present study remained confined to the GlyRs. Indeed, spike amplitudes (30-50 mV) and resting membrane conductance with little influence on the presynaptic releasing parameters n and p (Faber and Kom, 1988). Whatever its origin, our data indicate that a larger fraction of glycineric junctions was involved by prolonged applications of strychnine than after denervation.

**Absence of morphological effects of the "functional" denervation**

The specificity of strychnine's action on the glycineric postsynaptic sites has been recognized (Young and Snyder, 1973, 1974; Faber and Korn, 1988), and its effects on other receptor systems occur at concentrations above its kᵦ at the inhibitory GlyR by a factor of 100-50 × 10⁶ (Baron and Guth, 1987). Thus, although there is a GlyR less sensitive to strychnine in the spinal cord of cats (Ryall et al., 1972), in neonatal rats (Becker et al., 1988; Hoch et al., 1989), in primary cultures of fetal motoneurons (Hoch et al., 1989), and in medulillary neurons (Lewis et al., 1989), it is unlikely that their presence accounts for the failure of the antagonist to reproduce the morphological effects of denervation, since the M-cell somatic IPSPs and inhibitory postsynaptic currents are completely abolished by low doses of strychnine (Faber and Korn, 1988). Furthermore, the staining of the GlyR 48 kDa channel-forming polypeptide was not hampered by strychnine. This was probably due to the fact that the epitope of this immunoglobulin is located within the first 100 amino acids of the 48 kDa chain and is different from the antagonist binding site (Schröder et al., 1991).

As for the neuronal nicotinic receptor, denervation of the cardiac ganglion neurons causes a decrease of the total number of the surface ACh receptors (Sargent and Pang, 1988) and that of the chick ciliary ganglion produces a decline in the number of intracellular receptors, leaving unaffected the postsynaptic ones (Jacob and Berg, 1987, 1988).

These data, and our results, contrast with those obtained at the neuromuscular junction or on the electrocytes, where denervation (Bourgeois et al., 1973, 1978a; Fambrough, 1974; Loring and Salpeter, 1980), as well as a postsynaptic blockade of the receptors by d-tubocurarine (Berg and Hall, 1975) or α-bungarotoxin (Chang et al., 1975), and presynaptic inhibition by botulinum toxin (Chang et al., 1975; Bourgeois et al., 1978b) all caused the appearance of extrajunctional ACh receptors over the entire myofiber surface. These treatments were reported to accelerate the turnover of the junctional ACh receptors (Loring and Salpeter, 1980; Aviña et al., 1989, Fumagalli et al., 1990). However, their number revealed after labeling with ¹²⁵I-α-bungarotoxin remained intact 18 d after denervation (Porter and Barnard, 1975). Several factors that interfere with the synthesis or clustering of ACh receptors have been recently identified, including the calcitonin gene–related peptide (Lauffer and Changgeux, 1987), or agrin, that is released from the presynaptic nerve ending (Godfrey et al., 1984; Nitkin et al., 1987) and whose staining intensity decreases after denervation (Reist et al., 1987).
We observed that the denervated 93 kDa–immunostained synapses do not persist and a transient intracellular 93kd-IR appears after denervation; these changes were absent after functional blockade of the glycineergic synaptic transmission. Furthermore, the distribution of the α-subunit of the GlyR remained unaltered after strychnine treatment. This implies that the interruption of GlyR activation is not sufficient to trigger the redistribution of postsynaptic protein. Other types of interactions may be involved between the pre- and the postsynaptic cell. These could include the corelease of other molecules together with glycine or the structural contact of the intact afference with the postsynaptic element.

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