ORIGINAL PAPER

Morphological changes in the frog cerebellar cortex after unilateral section of the statoacustic nerve

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SUMMARY

To investigate a possible role of the cerebellum in vestibular compensation that follows a lesion to the vestibular apparatus, the morphological changes of the cerebellar cortex of adult frogs following unilateral statoacustic nerve section was analyzed by means of electron microscopy starting from 3 days after the neurectomy for up to 6 months. On the ipsilateral side, massive abnormality was found in all layers at early postsurgical intervals. This involved both nerve fibers and cell bodies. Fibers often appeared condensed or vacuolated with poorly compacted myelin sheath. Cells had electronlucent and vacuolated cytoplasm to varying extent. Alterations became less conspicuous after 30 days and after 60 days altered nerve cells were no longer present. On the contralateral side, only a few Purkinje and granule cells were affected at early postsurgical stages. This may derive from the fact that, in the frog, some of the vestibular primary afferents reach contralateral cerebellar cortex. At 30 days, alterations had substantially progressed, and at 60 days they involved all the cortical layers. Fiber debris was present in the granular and molecular layers and numerous Purkinje cells were electrondense and shrunken. This lateness in alteration may be a consequence of the prolonged silence of the vestibular nucleus contralateral to the lesion. At 4 and 6 months the tissue architecture was normal.

INTRODUCTION

In all tetrapods, lesions to the vestibular system lead to a typical acute syndrome, which is mainly characterized by head and body twisting and locomotion impairement. At a certain interval after the injury, and depending both on the species and on the extent of the lesion, the animal recovers to near normality. This process is referred to as vestibular compensation (Igarashi, 1984).

The vestibular syndrome and compensation have been object of numerous experimental studies (for a review see: Smith and Curthoys, 1989; Dieringer, 1995; Darlington and Smith, 2000), as a model of neural plasticity. However, the plastic phenomena that are at the basis of vestibular compensation are far from being satisfactorily clarified. Most studies aimed at unraveling the mechanism of vestibular compensation have focused on the vestibular

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nuclei, but morphological analyses of the vestibular nuclei in mammals have shown only degenerative phenomena in the nucleus ipsilateral to the lesion (Mugnaini *et al*., 1967; Gacek *et al*., 1988).

According to some authors (Putkonen *et al*., 1977; Courjon *et al*., 1982), vestibular compensation could be the result of vicarious functioning, i.e., the substitution of labyrinthine with other information from different central nervous system areas. Among these areas, the cerebellum might have a role (Smith and Curthoys, 1989; Darlington and Smith, 2000), as it receives primary and secondary afferents from the vestibular apparatus.

Section of the statoacustic nerve is known to induce the vestibular syndrome (Mugnaini *et al*., 1967; Gacek *et al*., 1988; Cass and Goshgarian, 1990; Kunkel and Dieringer, 1994; Gacek and Schoonmaker, 1997; Darlington and Smith, 2000). In the frog, the vestibular syndrome that follows a lesion to the vestibular apparatus is characterized by alterations of the posture and locomotion. The static symptoms recover within 3 days after the lesion. After 15 days, also the dynamic symptoms begin to disappear, and at 30 days the locomotion is normal (Magherini *et al*., 1981; Dieringer, 1995). In previous studies carried out on the cerebellum of adult frogs after section of the statoacustic nerve, we observed a loss of Purkinje and granule cells at brief intervals from the lesion and, later on, signs of circuitry remodeling (Vignola *et al*., 1992, 1993). To further investigate the role of the cerebellum in the vestibular compensation process and to correlate the recovery of static and dynamic symptoms with tissue changes and neural plasticity, we undertook an ultrastructural study of the ipsi- and contralateral sides of the frog cerebellar cortex after unilateral section of the statoacustic nerve. Changes were monitored from the beginning of the compensatory phase 3 days after neurectomy up to 6 months. We choose these post-surgical intervals to correlate morphological aspects to well characterized physiological data.

The vestibulo-cerebellar system of the frog has been extensively studied (Hillman, 1969; Llinas *et al*., 1969; Precht *et al*., 1974; Sotelo, 1976; Dieringer and Precht, 1979; Cochran *et al*., 1987; Knopfel, 1987; Reichenberger *et al*., 1992; Dieringer, 1995; Hiraoka *et al*., 1995). It consists of primary vestibular fibers that project mainly to the granular layer of auricolar lobe, and secondary vestibular fibers termi-nating in the granular layer of both auricolar lobe and corpus cerebelli, with an occasional branch reaching up to the molecular layer (Honrubia *et al*;, 1985; Kuruvilla *et al*., 1985; Suarez *et al*., 1985; Fuller, 1974). In particular, though the basic circuitry is similar to that of mammals, the cerebellum of the frog is much simpler, thus facilitating the analysis of degenerative changes and synaptic remodeling. In addition, since main efferents from vestibular nuclei project to the corpus cerebelli and plastic phenomena can occur in the vestibular nuclei after hemilabirintectomy (Igarashi, 1984), we have focused our attention mainly on the corpus cerebelli.

MATERIALS AND METHODS

Sixteen adult frogs (*Rana esculenta* L., purchased from a local dealer) were deeply anesthetized by immersion in 0.1% MS222 solution (Sandoz, CH). The roof of the mouth was opened and the right VIII cranial nerve exposed and severed between Scarpa's ganglion and the brainstem. Eight frogs were sham-operated, i.e. the right VIII nerve exposed, but not severed. Additional 8 frogs were used as controls. At days 3, 15, 30 or 60 the animals (4 operated and 2 sham-operated and 2 control frogs per post-surgical stage) were anesthetized again and perfused intracardially (1.5 ml/min for 10 min) with Ringer's solution modified for anurans (composition: 117 mM NaCl; 2.5 mM KCl; 1.2 mM NaHCO$_3$; 0.17 mM NaH$_2$PO$_4$; 1.8 mM CaCl$_2$; 5.5 mM glucose; pH 7.3; Mazzi, 1977) supplemented with heparin (4000 U.I./100 ml). Thereafter, the frogs were perfused for 20 min with a solution containing 1% paraformaldehyde and 1% glutaraldehyde in 0.12 M phosphate buffer, pH 7.3. At the end of the perfusion period, the whole head was isolated and immersed in the same fixative for 2 h. The cerebellum was then removed and divided in two halves. After washing in phosphate buffer and post-fixation in 1% aqueous OsO$_4$ for 2 h at 4°C, the specimens were dehydrated in ethanol, passed through propylen-oxide and embedded in epoxy resin. Four additional frogs were neurectomized as above and killed after 4 months (2 animals) and 6 months (2 animals).

Thin sections of the corpus cerebelli were stained with uranyl acetate and lead citrate and observed with a Zeiss 900 electron microscope. Non-oper-

ated frogs housed identically to the experimental ones were used as controls for the quality of fixation and embedding procedures.

All experiments reported in this article were conducted in accordance with Italian guidelines of the law 116/92 regarding the care and use of laboratory animals.

Cell counting

The percent frequency of altered Purkinje cells was calculated separately in the ipsi- and contralateral corpus cerebelli, by counting normal and abnormal cells in semithin sections in double blind fashion. For counting, an area of 10,000 μm^2 was randomly chosen by means of an eyepiece grid. Two fields in at least 6 semithin sections per animal were considered. The sections were selected on the basis that the same cells were not present in two different sections. Since no significant difference (ANOVA test) was found among animals of the same group, percentages ± SD were calculated from the whole sample data.

RESULTS

Normal frogs

The ultrastructure of the cerebellar cortex in control frogs was as previously described (Hillman, 1969; Sotelo, 1976). A typical Purkinje cell is shown in Fig. 1. No sign of alteration due to inappropriate fixation, like breaks in cell membrane or swelling in endoplasmic reticulum and mitochondria, are present.

Sham-operated frogs

The cerebellum of the sham-operated frogs was largely similar to that of the controls. However, a slight dilation of the endoplasmic reticulum (ER) was sometimes present in Purkinje cells in the ipsilateral side of 3-day-sham-operated animals. At 15 and 30 days, the tissue architecture was completely normal.

Operated frogs: light microscopy and cell counting

Semithin sections of the cerebellar cortex of operated frogs revealed alterations in the Purkinje cell layer ipsi- and contralateral to the lesion (Fig. 2a, b).

In the 3-day-operated animals, in the ipsilateral side (Fig. 2a), Purkinje cells with pale and apparently empty cytoplasm were evenly distributed throughout the layer. Counting of altered Purkinje cells revealed that the 53.06% (Fig. 3) of the population was affected. In 15-day-operated frogs, the pattern was similar (55.63% of the Purkinje cells appeared altered), while 30 days after the injury, the extent of the abnormalities had decreased and only the 8.84% of the Purkinje cells showed cytoplasmic changes. At 60 days, the tissue architecture was as in normal frogs and only sporadical Purkinje cells (1.77%) were altered. After 4 and 6 months the tissue architecture was completely restored.

In the Purkinje cell layer of the contralateral side, we observed cellular changes similar to those described in the ipsilateral side. However, many fewer Purkinje cells (20.34% of the population, Fig.2b, 3) were affected than was the case for the neurectomized side. At 15 days, the frequency of abnormal Purkinje cells decreased to 17.01%. After 30 days, the 29.69% of Purkinje cells appeared altered and at 60 days the affected cells constituted the 43.28% of the population. No changes were apparent at 4 and 6 months.

### Operated frogs, ipsilateral side: electron microscopy

Three days after neurectomy, in the granule cell layer, few fibers showed condensation of the axoplasm and alteration in the myelin sheath, which appeared poorly compacted (Fig. 4a). Some granule cells had vacuolated cytoplasm, with scanty but well preserved mitochondria (Fig. 4b). In the molecular layer, many parallel fibers were swollen and had electronlucent axoplasm. Interneurons showed dilation of the ER and Golgi complex, along with swelling of some of the mitochondria (Fig. 4c). The Purkinje cells showed cytoplasmic changes to varying extent. Some cells had empty, vacuolated, electronlucent cytoplasm, without apparent organization of the few organelles (Fig. 4d). Other cells had electronlucent cytoplasm and, mainly in the peripheral area, the cisternae of both smooth and rough ER were massively distended (Fig. 5). Finally, a few Purkinje cells had undergone dark necrosis (not shown).

Fifteen days after neurectomy the abnormality had progressed further. In the granule cell layer, fibers were surrounded by uncompacted whorls of membranes and many granule cells had empty cytoplasm. There were numerous microglial cells in the process of phagocyting cell debris. Only a few Purkinje cells had a normal healthy appearance. Several
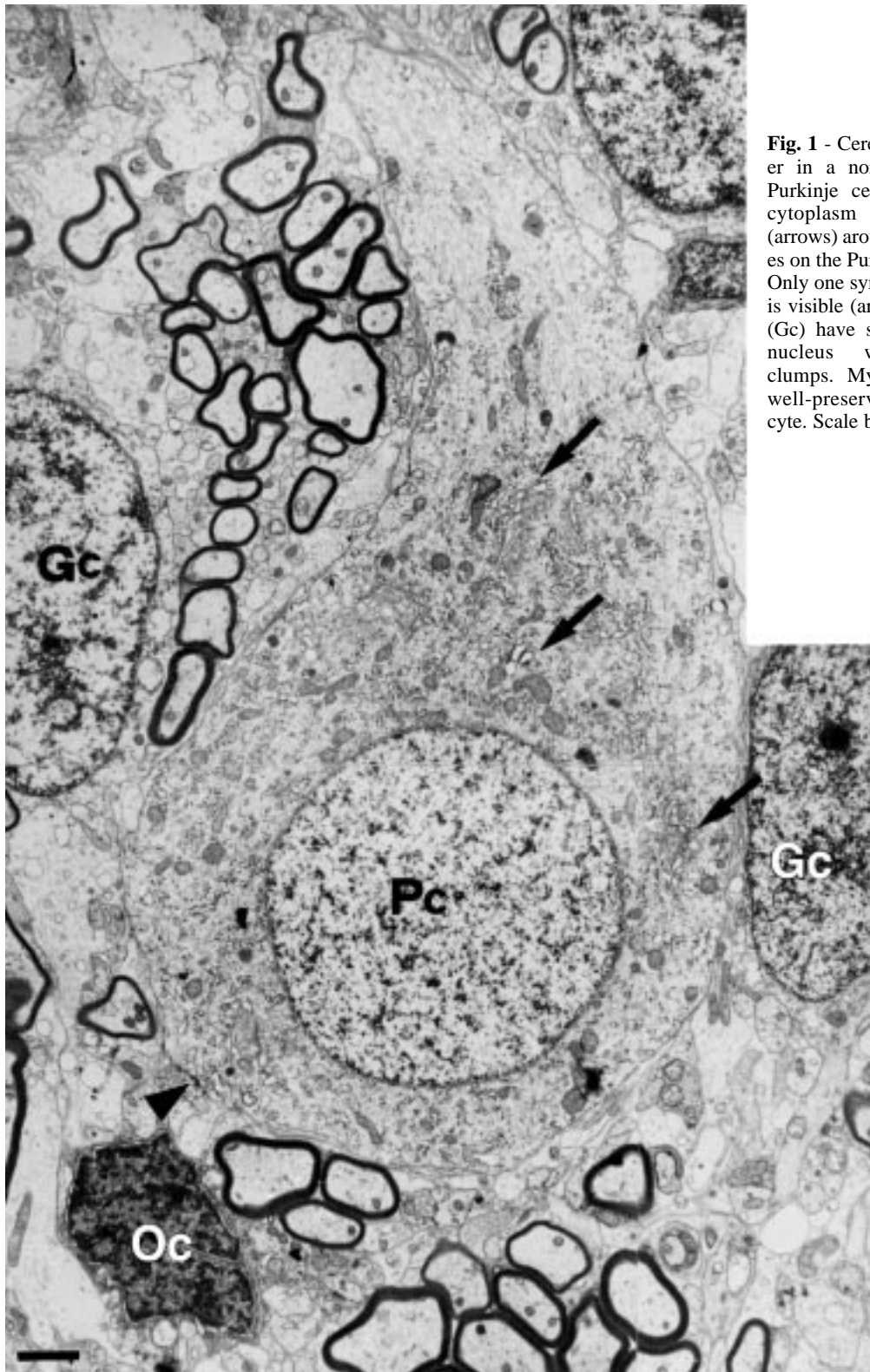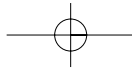
Fig. 1 - Cerebellar Purkinje cell layer in a normal frog. Pear-shaped Purkinje cell (Pc), with abundant cytoplasm and Golgi apparatus (arrows) around the nucleus. Synapses on the Purkinje cell soma are rare. Only one synapse at the axon hillock is visible (arrowhead). Granule cells (Gc) have scanty cytoplasm and a nucleus with heterochromatin clumps. Myelinated fibers appear well-preserved. Oc: oligodendrocyte. Scale bar: 2 µm.
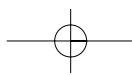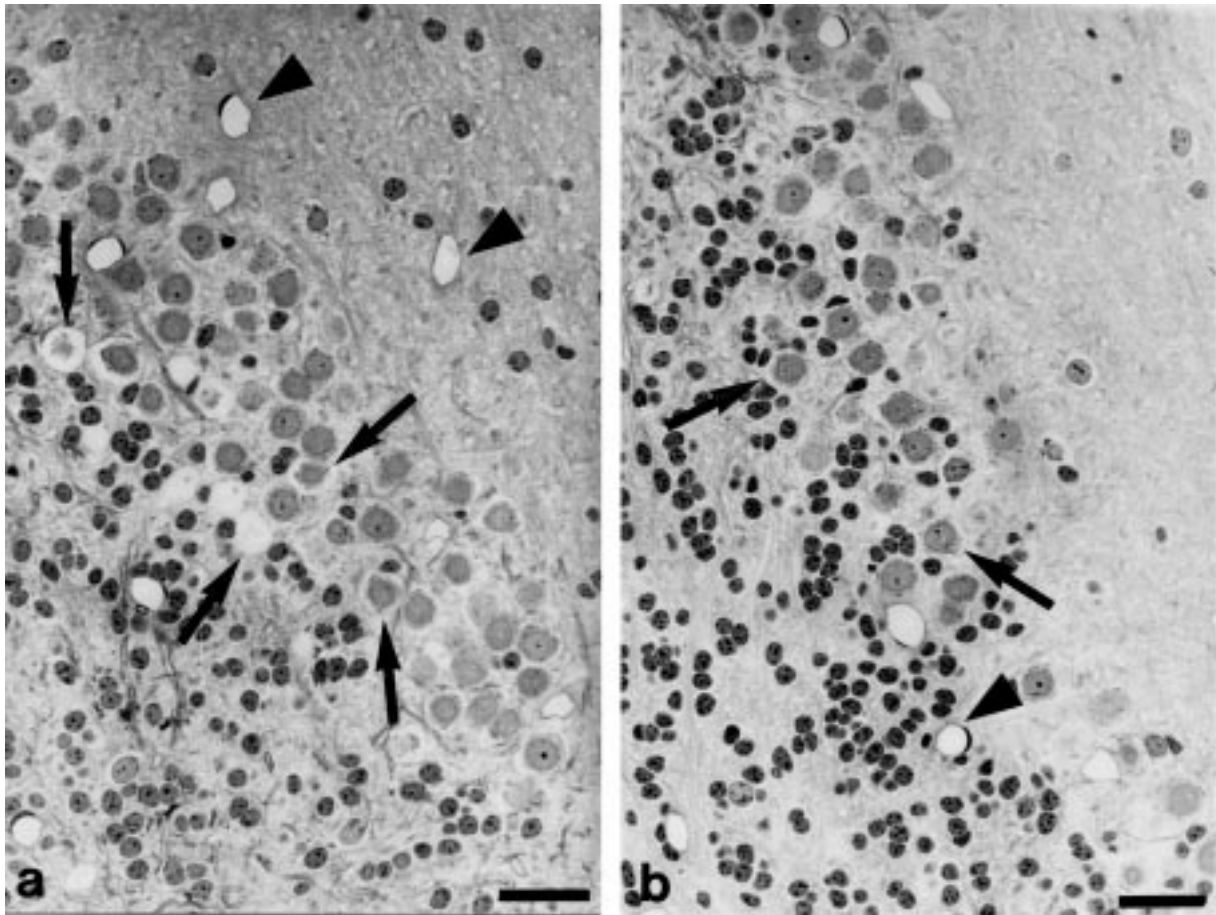
Fig. 2 - Toluidine blue stained semithin section of the cerebellar cortex in a 3-day-operated frog. a) Ipsilateral side. Altered Purkinje cells are evident throughout the layer (arrows). The cytoplasm appears pale and swollen to varying extent. Scale bar: 30 µm. b) Contralateral side. Few Purkinje cells (arrows) are affected. Scale bar: 30 µm. Blood capillaries are indicated (arrowheads).
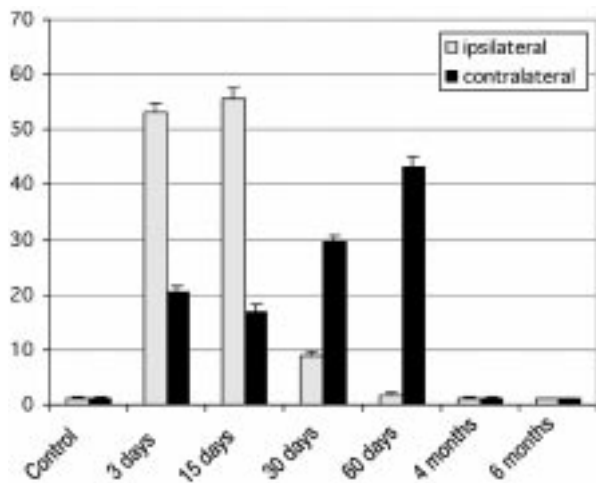

Fig. 3 - Percent frequency of altered Purkinje cells in toluidine blue stained semithin sections.

of them had electrondense cytoplasm (Fig. 6a) with bundles of microtubules. The cisternae of the ER were distended and contained flocculent/proteinaceous material. The contour of the cells was irregular, thus suggesting shrinkage. Around the cells, some glial processes were vacuolated.

Thirty days after injury, the extent of the abnormalities had decreased in the granular and Purkinje cell layers, but not in the molecular layer. The granule cell layer contained fewer altered granule cells and fibers, but an increased incidence of microglial cells. In the molecular layer, many parallel fibers were swollen, and Purkinje cell dendrites were electrondense and contained dilated ER cisternae. Climbing fibers also appeared to be altered (Fig. 6b). Several of the interneurons appeared shrunken, and numerous microglial cells were observed near the altered fibers or cells. Electrondense fibers, and
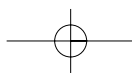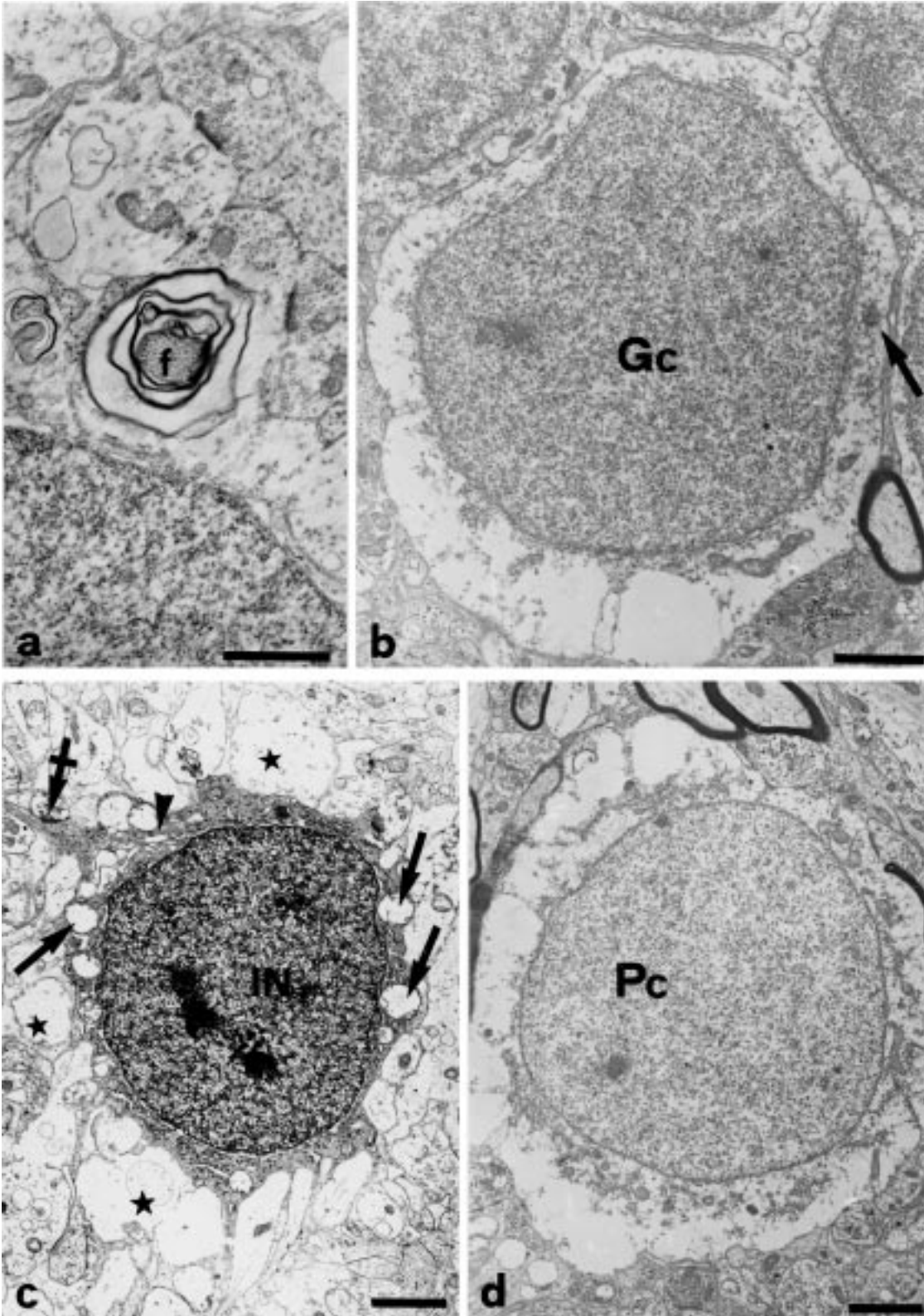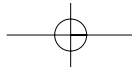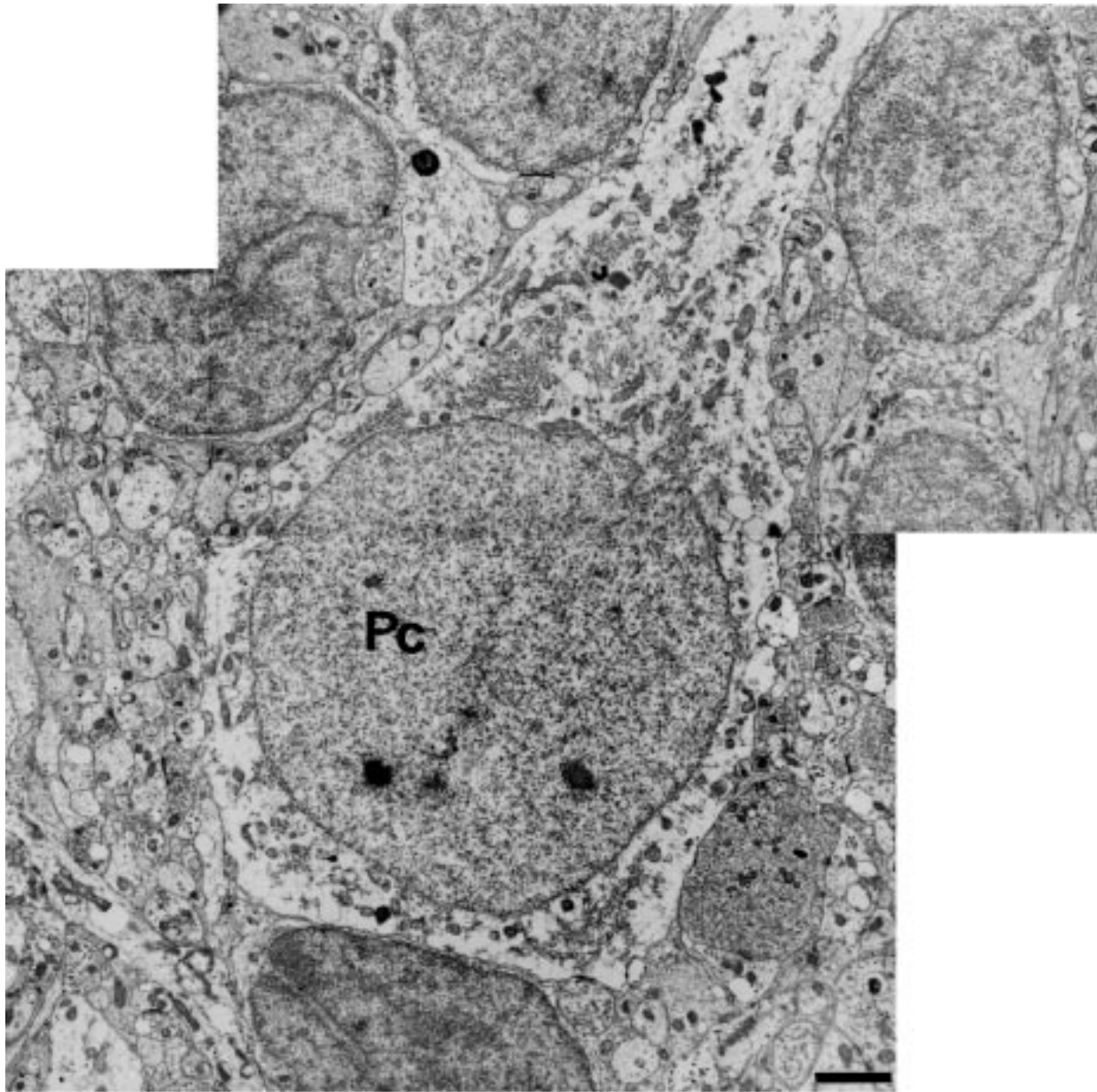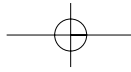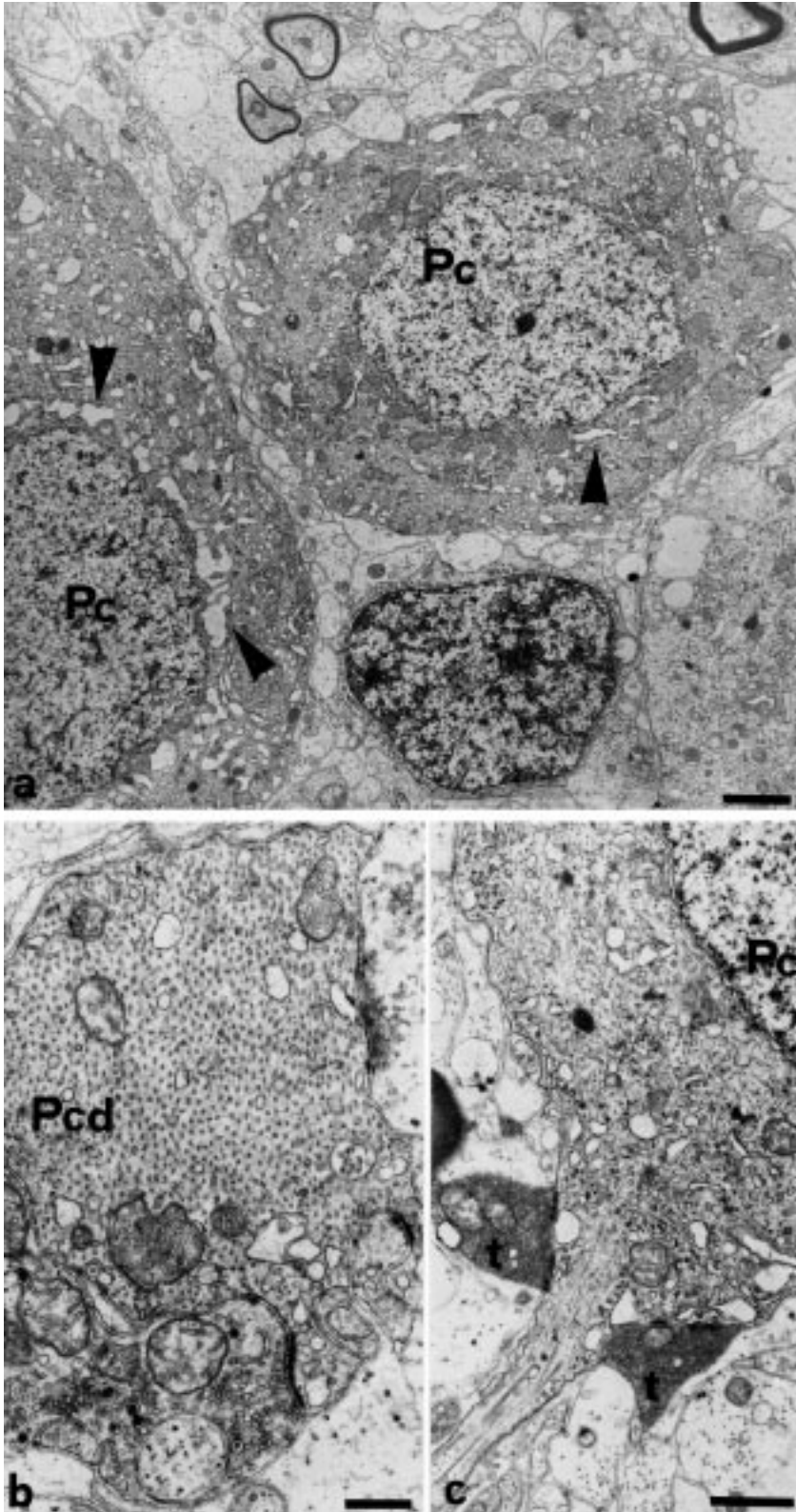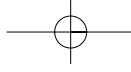
Fig. 5 - 3-day-operated frog, ipsilateral cerebellum. A Purkinje cell (Pc) in which the cytoplasm is electronlucent, with scarce organelles. Granule cells in its surround appear normal. Scale bar: 2 µm.

Fig. 4 - 3-day-operated frog, ipsilateral cerebellum. a) A fiber (f) in the granule cell layer with dense axoplasm and poorly compacted myelin sheath. Scale bar: 1 µm. b) Severely swollen granule cell (Gc) with rare but well-preserved mitochondria (arrow). Portions of normal granule cells are included at the top of the microphotograph. A fiber with an intact myelin sheath is also visible in the lower right. Scale bar: 2 µm. c) In the molecular layer, numerous parallel fibers (stars) are swollen and/or vacuolated. An interneuron (IN) contains electrondense nucleus and has cytoplasm with dilated Golgi apparatus and ER cisternae (arrowhead). The mitochondria are also swollen (arrows). A synapse with a parallel fiber is marked by crossed arrow. Scale bar: 2 µm. d) A Purkinje cell (Pc) with watery cytoplasm, in which only a few organelles are visible. Note intact fibers at the top of the photograph. Scale bar: 3 µm.
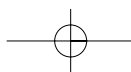
Fig. 6 - Operated frogs, ipsilateral cerebellum. a) 15-day-operated frog. Purkinje cells (Pc) appear to be electrondense, with distended ER cisternae (arrowheads). Scale bar: 2 µm. b) 30-day-operated frog. In the molecular layer, a Purkinje cell dendrite (Pcd) contains swollen ER tubules and cytoplasmic matrix of higher electrondensity than normal. The dendritic spine is contacted by a climbing fiber with swollen mitochondria. Scale bar: 0.5 µm. c) 30-day-operated frog. Degenerating, electrondense nerve cell terminals (t) containing pleomorphic synaptic vesicles contact the emerging axon of a Purkinje cell (Pc). Scale bar: 1 µm.
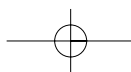
boutons containing pleomorphic vesicles were visible in contact with or close to the initial segment of Purkinje cell axon (Fig. 6c).

At 60 days from neurectomy, the tissue architecture was almost completely restored. Abnormal Purkinje cells and fibers were observed only sporadically.

In 4- and 6-month-operated frogs tissue abnormality was no longer present. Cells, in particular Purkinje neurons, and fibers appeared well preserved.
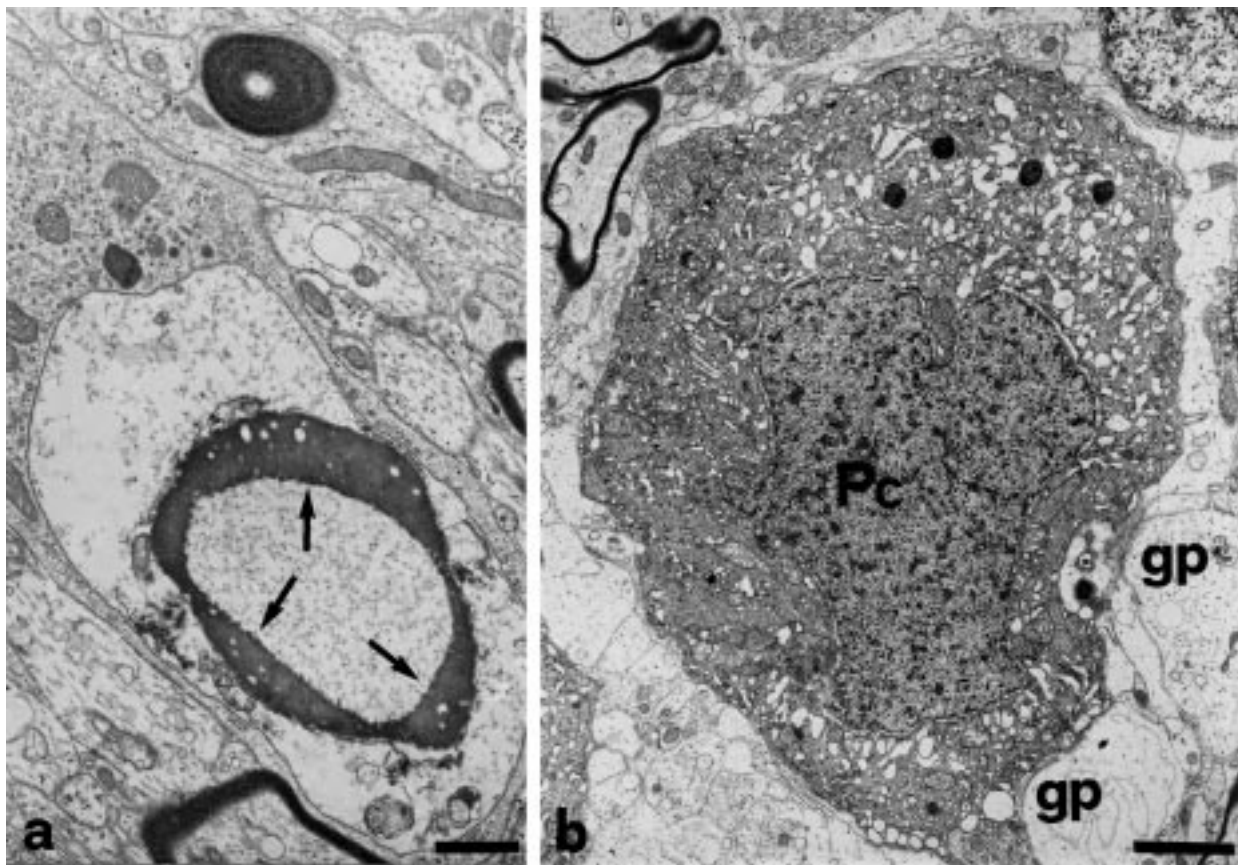
Operated frogs, contralateral side: electron microscopy

In the cerebellar contralateral side of the 3-day-operated frogs, the nerve fibers in the granule cell layer, were normal, but apoptotic cells with peripheral margination of nuclear chromatin and increased cytoplasmic lucency (hydropic changes) were found (Fig. 7a). Microglial cell with cytoplasmic inclusions are present nearby and sometimes engulfanting apoptotic cells (Fig. 7a). In the molecular layer, parallel fibers and interneurons were normal. Although Purkinje cells had dilation of the ER at the cytoplasm periphery, neurons with clear sign of degeneration or necrosis were invariably absent.

At 15 days, the changes were similar to those observed after 3 days, i.e. the situation appeared to be stationary.

In contrast to the ipsilateral side, the contralateral part of the cerebellum of 30-day-operated frogs showed progressing alterations. Some fibers of the granule cell layer had degenerated, and had been engulfed by microglial cells. Although the molecular layer showed a normal appearance, the Purkinje cell layer showed normal cells interspersed with several shrunken and electrondense cells (Fig. 7b).

Fig. 7 - Operated frogs, contralateral cerebellum. a) 3-day-operated frog. In the granule cell layer, a small cell presumably granule cell, which shows electronlucent degenerating cytoplasm and margination of chromatin (arrows) in the nucleus, is engulfed by a microglial cell. Scale bar: 1 μm. b) 30-day-operated frog. Electrondense Purkinje cell (Pc) with dilation of the ER and perinuclear cisterna. The cell is surrounded by swollen glial processes (gp). Scale bar: 3 μm.

At 60 days, all cerebellar layers surprisingly showed clearly greater damage than that previously observed. Fiber debris was present in the granular and molecular layers, and several Purkinje cells appeared to be electrondense and shrunken, or had dilated ER cisternae and Golgi apparatus, as observed at the previous post-surgical interval. Glial processes around cells had a watery appearance.

In 4-month- and 6 month-operated frogs, the patterns were similar to those in the ipsilateral side and normal frogs.

DISCUSSION

The cerebellar side ipsilateral to the neurectomy was mainly affected by changes that suggested degenerative events. These were mostly evident at early post-surgical intervals, and involved all the cerebellar layers and both nerve cells and fibers. At the earliest post-surgical interval considered in this work, only a few altered fiber terminals could be observed. This is in part in contrast with the observation of other authors who described many degenerating pre-terminal fibers in the granular layer 3 days after vestibular nerve section (Hillmann, 1969; Gregory, 1972). It must be considered that our observations are predominantly limited to the corpus cerebelli which receives rare primary vestibular fibers (Fuller, 1974). In addition, the post-surgical interval of 3 days might be excessive for detecting the most of degenerating primary mossy fiber terminals (Kunkel and Dieringer, 1994), which are soon phagocyted. At the same time, the 3-day interval was probably insufficient for the detection of secondary fiber degeneration. Moreover a difference in the technique used may be responsible for the discrepancy of results. In fact, while our analysis was morphological, earlier studies used the Fink-Heimer method, which specifically labels degenerating fibers.

It is likely that granule cells are the first neurons to be involved in damage, and that their involvement is secondary to the degeneration of primary mossy fibers. The alteration of Purkinje cells might be secondary to the partial deafferentation due to the loss of parallel fibers and, therefore, starts later. In agreement with previous light microscopy data (Vignola *et al*., 1992), the present results demonstrate that the number of altered Purkinje cells on the ipsilateral

side reached a peak (about 50% of the population) 15 days after neurectomy. After 30 days, only a few Purkinje cells showed alteration, though the total number of cells was greatly decreased (Vignola *et al*., 1992), thus indicating that the alteration observed at early intervals from the lesion was irreversible. The occasional Purkinje cells with cytoplasmic changes we observed at this late interval were probably due to alteration of the molecular layer interneurons, most of which were shrunken and electrondense. Dark axonal profiles, probably belonging to molecular layer interneurons, were observed in the vicinity of Purkinje cell bodies and initial axon segments.

Data reported after hemilabyrinthectomy showed that in the frog, the postural disturbances are recovered in as little as 3 days from the injury, and that the locomotory symptoms begin to disappear after 15 days. At 30 days, locomotion is normal (Magherini *et al*., 1981; Smith and Curthoys, 1989; Dieringer, 1995). The normalization of posture is probably achieved by means of a silencing effect on the contralateral vestibular nucleus neurons and, consequently, on the cerebellum (Igarashi, 1984). It should be noted that the commissural system between the paired vestibular nuclei in the frog is, unlike the equivalent mammal system, mainly excitatory in nature (Will *et al*., 1988; DeWaele *et al*., 1995). This factor probably explains the fast postural recovery, in that neural activity in the nucleus contralateral to the lesion is probably depressed rather than increased (Markham and Yagi, 1984).

The above interpretation does not consider the role of the cerebellar side contralateral to the lesion. Here, the results obtained in this study are intriguing. The pattern of abnormality opposed that of the ipsilateral side. As previously found at the light microscopic level (Vignola *et al*., 1992), at short post-surgical intervals the percentage of altered Purkinje cells (about 20% of the population) was lower than in the ipsilateral side. These early changes may be due to the degeneration of some afferent fibers, since the frog VIII cranial nerve fibers reach not only the ipsilateral side, but also the contralateral side, albeit in less number (Gregory, 1974; Nieuwenhuys and Opdam, 1976; Montgomery, 1988). In addition, the contralateral changes may be secondary to degeneration of granule cells in the ipsilateral side, which extend their parallel fibers along the entire width of the cerebellum (Llinas *et al*., 1969; Sotelo, 1976). The contralateral cerebellar

damage is, at least apparently, rapidly restored, since at post-operative day 15, changes in comparison with the day 3 stage were not detected. However, observation of the cerebellar contralateral side 30 and, above all, 60 days after the VIII nerve section produced the unexpected finding of distinct tissue abnormality, which extended to all the cerebellar layers. This late cerebellar alteration is not easy to explain. It might be part of the strategy to restore and re-balance neural activity. It is intriguing that this phenomenon occurs after both the vestibular compensation and recovery of the dynamic symptoms are completed. In the cerebellar side contralateral to the lesion, after 4 months, the tissue architecture is normal.

ACKNOWLEDGEMENTS

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