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A light and electron microscope study of rat abducens nucleus neurons projecting to the cerebellar flocculus

L. RODELLA, R. REZZANI, G. CORSETTI, C. SIMONETTI, A. STACCHIOTTI AND R. G. VENTURA

Anatomy Section, Department of Biomedical Sciences, University of Brescia, Italy

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ABSTRACT

Injection of horseradish peroxidase (HRP) into the cerebellar flocculus of the rat was employed to identify neurons in the abducens nucleus that project to the flocculus. The number, ultrastructural features and precise localisation of these neurons in the nucleus were examined. They were present bilaterally and represented about 7% of the total neuronal population of each nucleus. They were localised principally in the dorsomedial area of the cranial half of each nucleus and did not display the typical ultrastructural features of motoneurons. It is concluded that the localisation and ultrastructural characteristics of these HRP-positive neurons are useful for distinguishing them from other neuronal populations within the nucleus.

INTRODUCTION

In addition to motoneurons that innervate the lateral rectus muscle of the eye, the abducens nucleus contains interneurons that project to areas of the CNS involved in eye movement, particularly to the contralateral oculomotor nucleus (Baker & Highstein, 1975; Spencer & Sterling, 1977; Steiger & Büttner-Ennever, 1978; Glicksman, 1980; Labandiera et al. 1983; Cabrera et al. 1988). Furthermore, abducens nucleus neurons that project to the cerebellum, most notably to the flocculus, have been reported by a number of authors (Brodal & Brodal, 1985).

The flocculus has been implicated in a variety of mechanisms associated with the control of eye movement. A direct projection from the abducens nucleus to the flocculus was first described by Kotchabhakdi & Walberg (1977) and subsequently by Graybiel (1977), Banks et al. (1983) and Langer et al. (1985). After horseradish peroxidase (HRP) or HRP-wheat germ agglutinin (HRP–WGA) injection into the cerebellum, these authors reported labelled neurons in the abducens nucleus and in other motor nuclei of cranial nerves. To our knowledge abducens neurons projecting to the flocculus have not been investigated systematically, and no ultrastructural data are available. The present study, involving light (LM) and electron (EM) microscope observations of abducens nucleus preparations after injection of free HRP into the flocculus, was undertaken to identify the cytological characteristics of the labelled neurons that could distinguish them from other neurons, and to determine their precise localisation and number.

MATERIALS AND METHODS

A total of 28 male Wistar rats (200–300 g) were used. Group A: 20 animals were injected with HRP in the left flocculus for LM (15 animals) and EM (5 animals) studies; group B: 8 animals were injected in the flocculus on both sides for LM study.

Injection procedure

Under ketamine/xylazine anaesthesia (5 mg/100 g b.w. ketamine, 1 mg/100 g b.w. xylazine) the skull over the cerebellum was opened and, after incising the dura, 0.2–0.3 µl of 20% HRP solution in 0.1 M phosphate buffer, pH 7.4, was injected into the left flocculus, or into the flocculus bilaterally with a glass micropipette attached to a Hamilton microsyringe. After penetration, the micropipette was kept in place for 5 min. HRP was then injected and the micropipette

Correspondence to Prof. Raoul G. Ventura, Anatomy Section, Department of Biomedical Sciences, University of Brescia, Via Valsabbina 19, 25123 Brescia, Italy.

was left in place for a further 5 min before removal. Following injection, the muscle and skin were carefully sutured and the animals allowed to recover for 36 h.

Tissue processing

The animals were killed under chloral hydrate (35 mg/100 g body weight) anaesthesia by perfusion with 1 l of cold saline followed by 1 l of fixative containing 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer pH 7.4. The brains were removed and stored for 12 h at 4 °C in 0.1 M phosphate buffer pH 7.4 containing 10% sucrose. Sections containing the abducens nucleus were cut transversely at 25 μm by cryostat (23 brains) and at 60 μm by vibratome (5 brains) for LM and EM observation respectively. The cerebella of all animals were also sectioned to examine the injection sites. All sections were treated with tetramethylbenzidine (TMB) according to Mesulam's (1978) method. The LM sections of 5 animals (3 group A, 2 group B) were counterstained with 1% neutral red for 1 min. All LM sections were dehydratated in graded alcohol solutions and mounted. The morphology of the nucleus was studied after counterstaining with neutral red. The locations of retrogradely labelled neurons were noted and mapped on a diagram of the nucleus. The numbers of HRP-positive neurons found in all mounted sections were noted. The mean neuronal diameter was measured in the sections counterstained with neutral red and the total number of neurons in each nucleus calculated and corrected for section thickness and cell size according to Königsmark (1970). Means and S.D.s were calculated and the estimated number of neurons for group A and group B were subjected to analysis of variance.

For EM observation, after TMB labelling, the region containing the abducens nucleus was selected and removed under LM and postfixed with 1% osmium in phosphate buffer, pH 6.0, at 30 °C for 2 h, dehydrated in graded solutions of acetone and embedded in Araldite. Thin and ultrathin sections were cut (the latter were not stained with uranyl acetate) and examined.

RESULTS

Analysis of injection sites

As described by Larsell (1970), the rat cerebellar flocculus consists of a single folium ventromedial to the ventral paraflocculus (PFLv). In 25 rats the injection site was confined to and involved most of the flocculus, with small variations in location between animals. In 3 animals (2 group A, 1 group B) the injection site involved most of the flocculus and had also spread to the PFLv.

Light microscope observations

The rat abducens nucleus is situated in the pons, ventral to the genu of the facial nerve, between the genu and the medial longitudinal fasciculus (MLF) (Fig. 1).

In the rats injected in the left flocculus, the HRP-labelled neurons were distributed bilaterally within the dorsal and dorsomedial zone of each nucleus (Figs 2, 3). They had small rounded to elliptical cell bodies (16 ± 3 μm mean diameter). Few HRP-positive dendrites were present in the sections (Figs 3–6). The positive neurons were located principally in the cranial half of the nucleus (Fig. 1) and many were intermingled with HRP-negative neurons (Fig. 4) most of which, from their localisation, were probably abducens motoneurons (Glicksman, 1980; Cabrera et al. 1988).

The population of HRP-positive neurons located in the dorsomedial and medial area of the nucleus merged imperceptibility with the population of HRP-positive neurons of the supragenual nucleus (SG) and MLF respectively (Figs 1, 2).

There were 13 ± 3 HRP-labelled neurons in the abducens nucleus ipsilateral to the injected flocculus and 16 ± 2 in the nucleus contralateral to injection. In animals injected in both flocculi, localisation of HRP-positive neurons in the abducens nucleus did not differ from that in rats injected only in the left flocculus; however the mean number found (28 ± 4) was significantly greater ($P < 0.01$) than when one side only was injected, and represents about 7% of the total number of neurons in each abducens nucleus (mean 410 ± 36) (Rodella et al. 1994). In animals in which HRP had also involved the PFLv, the labelling pattern did not appear to differ from that in rats injected only in the flocculus.

Electron microscope observations

Only animals in which HRP injection was confined to the flocculus were studied by EM. HRP-positive neurons were clearly distinguished by the presence of TMB reaction product in their cell bodies (Figs 7, 8). At high magnification the electron-opaque crystals were needle-shaped and were closely associated with membrane-bound organelles (mostly lysosomes) (Fig. 7). Some smaller aggregates of TMB reaction product

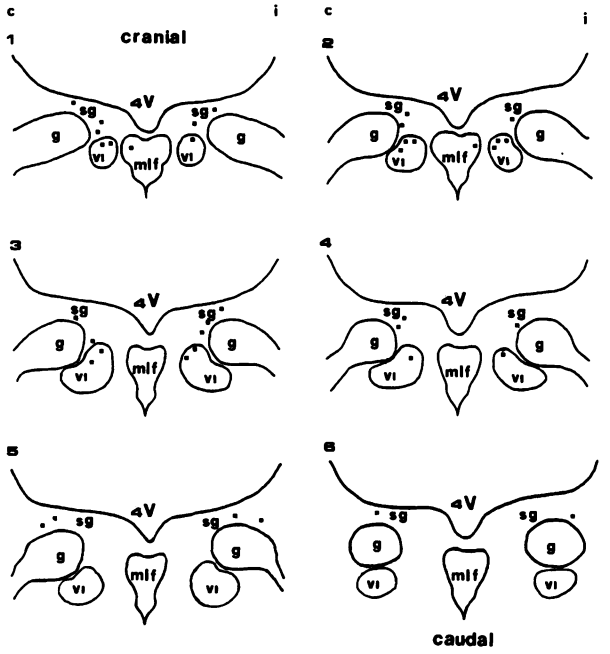

Fig. 1. Locations of labelled neurons in abducens nucleus and adjacent structures after injection of HRP into left flocculus. Drawings are from 25 μm sections taken every 100 μm from the rostral tip (1) to the caudal end (6) of the nucleus. Squares represent positive neurons. 4 V, 4th ventricle; g, genu of facial nerve; sg, supragenual nucleus; mlf, medial longitudinal fasciculus; VI, abducens nucleus; i, ipsilateral to injection site; c, contralateral to injection site.

were observed overlying or adjacent to the Golgi apparatus, and sometimes were not clearly associated with any organelle (Fig. 7).

HRP-positive neurons had irregular rounded or moderately indented nuclei (Fig. 8) and fairly well-developed rough endoplasmic reticulum (RER) consisting of short clusters of tubules scattered throughout the cell soma. Mitochondria and lysosomes were distributed throughout the cytoplasm. Golgi apparatus was not very abundant and consisted of the typical complexes of packets of cisternae and vesicles. The TMB crystals associated with lysosomes were mostly localised at one pole of the neuron (Fig. 8). The membranes of these neurons received few synaptic contacts. Origins of dendrites were observed rarely, and the initial axon segment was never identified. HRP-positive dendrites sometimes receiving synapses were occasionally found (Fig. 7).

Most of the HRP-negative neurons close to positively-stained cells had multipolar, rounded or elongated cell bodies and the typical morphology of motoneurons. They presented rounded nuclei, cytoplasm rich in RER distributed in large clusters, poorly developed Golgi apparatus close to nuclei, and numerous mitochondria and lysosomes. Multiple synapses were present on their cell surfaces.

DISCUSSION

In addition to motoneurons that innervate the lateral rectus muscle, the abducens nucleus contains internuclear neurons with exclusively intracerebral projections. The present LM observations confirm the existence of projections from the rat abducens nucleus to the flocculus, and provide precise information about their localisation within the nucleus. These neurons are a part of a continuum of neurons lying between the fascicles of the MLF and paramedian tract in the caudal pons and medulla, and constitute the rostral part of the classic abducens nucleus (Büttner-Ennever et al. 1989). After injection of the left flocculus, were found that HRP-positive neurons were located in both abducens nuclei, in the dorsomedial part of the cranial half of each, and that they represented about 4% of the total neuronal population ipsilaterally and about 4% of the total neurons contralaterally. Moreover in the animals injected in both flocculi, we found that about 7% of the neurons in each abducens nucleus were HRP-positive, i.e. about twice the number found in rats injected in the left flocculus only. It would seem unlikely that the axons of these neurons have a collateral branch and hence project to both flocculi; the more likely interpretation is that 50% of projections are ipsilateral and 50% are crossed, each projection arising from separate neurons in the abducens nucleus. Our finding that about 7% of abducens neurons project to the flocculus appears consistent with the data of Cabrera et al. (1988). These authors labelled abducens motoneurons and internuclear neurons projecting to the oculomotor nucleus with two different fluorochromes and reported that about 15% of neurons in the nucleus were not stained. It is likely, therefore, that these 15% unstained neurons include those, identified in this study, that project to the flocculus. We further suspect that some of the additional nonstained neurons probably project to areas of the cerebellum other than the flocculus (Kotchabhakdi & Walberg, 1977).

Our ultrastructural observations confirmed the LM localisation of HRP-positive neurons. They showed that TMB crystals were not confined within cytoplasmic organelles that probably contain HRP but exceeded their boundaries (Mesulam, 1982). HRP-positive neurons did not have the typical ultrastructural characteristics of abducens motoneurons (Spencer & Sterling, 1977; our unpublished observations). Motoneurons thus seem to be distinguishable from neurons that project to the flocculus on the basis of their EM characteristics. Furthermore, com-

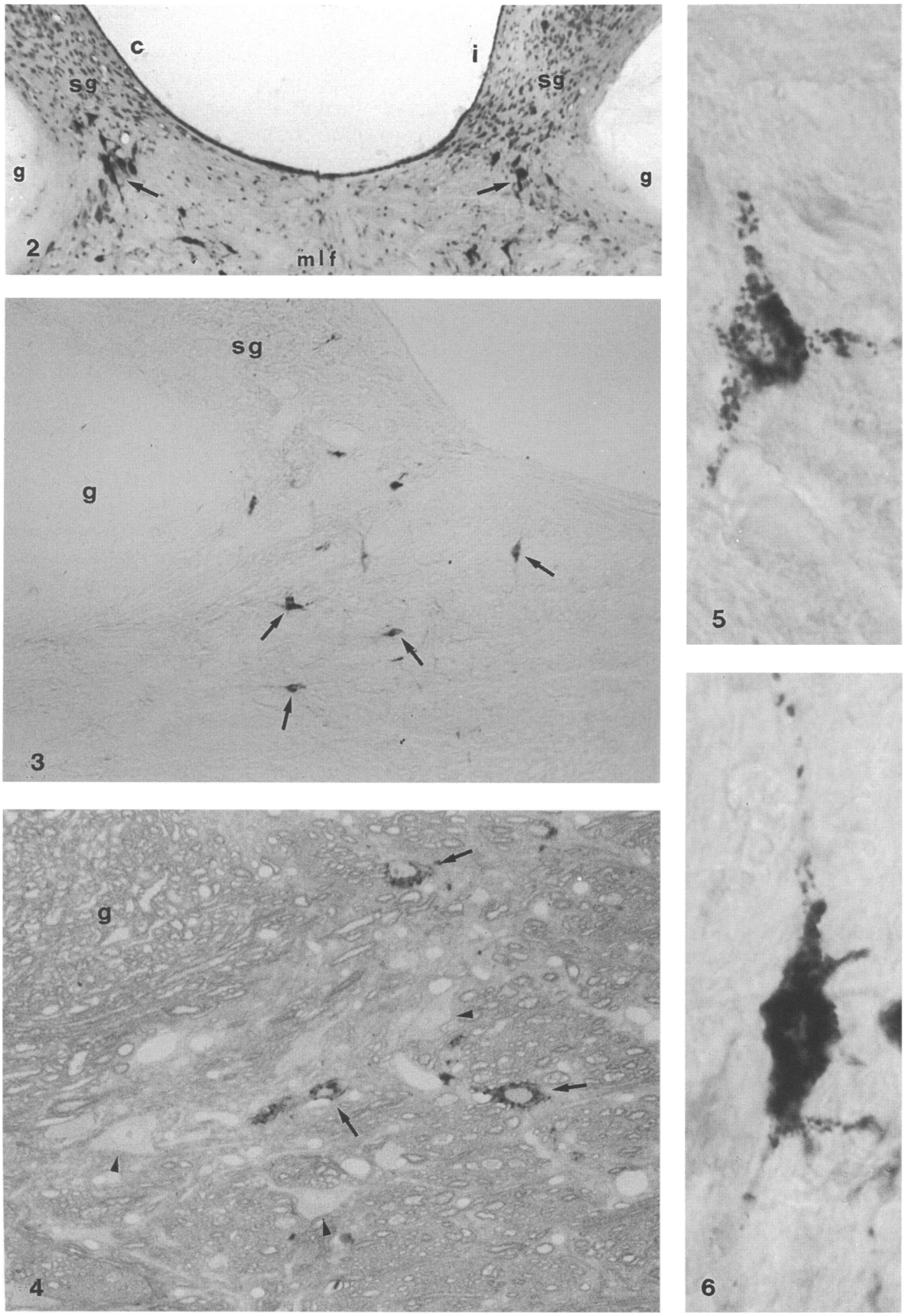

Fig. 2. Transverse 25 μm section counterstained with neutral red showing HRP-positive neurons distributed bilaterally in the abducens nuclei (arrows). (For its level see Fig. 1.1). g, genu of facial nerve; sg, supragenual nucleus; mlf, medial longitudinal fasciculus; i, ipsilateral to injection site; c, contralateral to injection site. × 110.

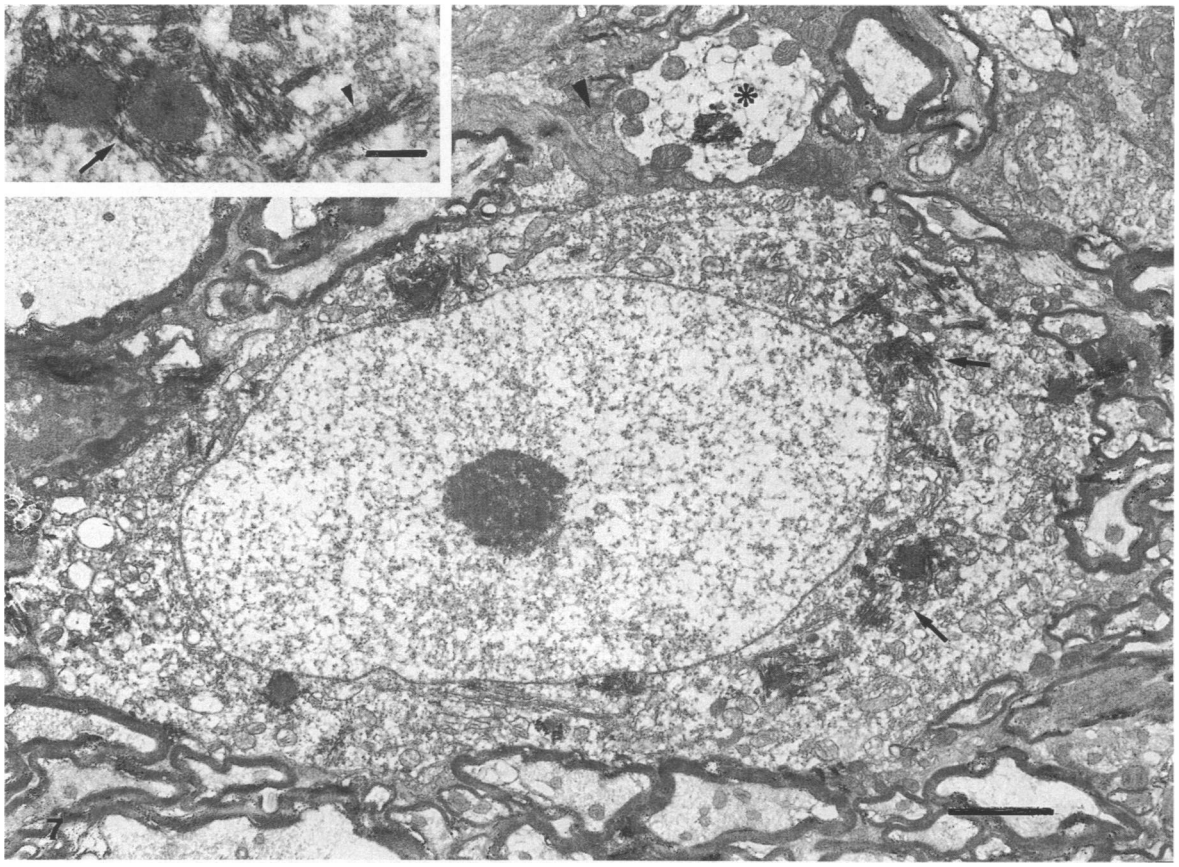

Fig. 7. Electron micrograph of HRP-positive neuron. Reaction product is indicated by arrows. Asterisk indicates a labelled dendrite with an associated bouton (arrowhead). Bar, 2 μm. Inset: HRP-reaction product at high magnification. The TMB crystals are needle-shaped and associated with lysosomes (arrow) and Golgi cisternae (arrowhead). Bar, 0.5 μm.

parison of the number of HRP-positive dendrites present in the LM and EM sections observed in this study with that in sections in which only motoneurons were retrogradely labelled with HRP (Rodella et al. 1994), gave the impression that their number is lower in the abducens nucleus neurons projecting to the flocculus.

On the basis of these considerations and of those reported by Langer et al. (1986) in the monkey, Roste & Dietrichs (1988) and Roste (1989) in other motor nuclei, we conclude that it is highly unlikely that these cerebellar projections originate from collateral axons of motoneurons. We also conclude that the rat abducens neurons that project to the flocculus partially intermingle with motoneurons, and are located separately from the area containing internuclear neurons projecting to the oculomotor nucleus (Langer et al. 1986). The latter in fact constitute the bulk of abducens nucleus interneurons, and are mainly located in the centrolateral zone of the nucleus below the central part of the genu of the facial nerve, where they partially intermingle with motoneurons (Glicksman, 1980; Cabrera et al. 1988).

It is noteworthy that the ultrastructural features of the HRP-positive neurons described in these papers (elliptical cell body, poorly developed granular endoplasmic reticulum and indented nucleus) are shared by a type of internuclear neuron, distinct from motoneurons, observed to project to the oculomotor nucleus in the cat (Spencer & Sterling, 1977). Neurons with the same ultrastructural characteristics are also present in the centrolateral zone of the rat abducens nucleus (unpublished observations). Thus while in normal material for LM it is not possible to

Fig. 3. Transverse 25 μm section showing HRP-positive neurons (arrows) present in the dorsomedial zone of the abducens nucleus (for level of section, see Fig. 1.2). g, genu of facial nerve; sg, supragenual nucleus. × 180.

Fig. 4. Semithin section through abducens nucleus showing HRP-positive neurons (arrows). Note unlabelled neurons (arrowheads). g, genu of facial nerve. × 350.

Fig. 5. High magnification of HRP-positive neuron showing 'rounded' perikaryon, × 1400.

Fig. 6. High magnification of HRP-positive neuron showing elliptical perikaryon. × 1400.

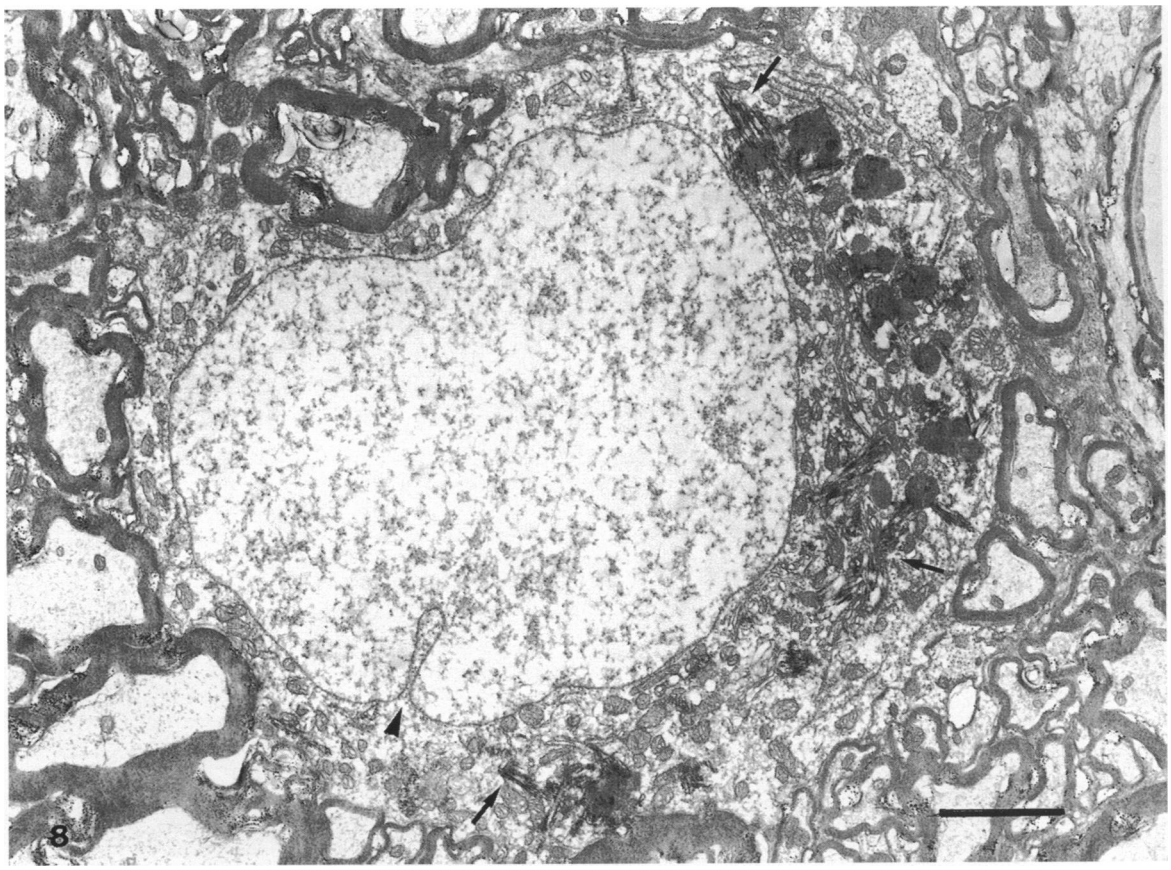

Fig. 8. Electron micrograph of HRP-positive neuron. The arrows indicate reaction product. Note deep indentation of the nuclear envelope (arrowhead). Bar, 2 μm.

distinguish the rat abducens nucleus neurons projecting to the flocculus, their ultrastructure, which distinguishes them from motoneurons, and their localisation are useful for identifying this neuronal population.

Functional considerations

Physiological experiments have provided evidence that the flocculus is involved in the regulation of eye movement (Brodal, 1983; Büttner et al. 1986). It has been shown that this structure is involved in the adaptative vestibulo-ocular reflex (Lisberger & Fuchs, 1978; Langer et al. 1985), in smooth pursuit (Zee et al. 1981) and optokinetic nystagmus (Waespe et al. 1983).

Since it is likely that some of the mossy fibres in the flocculus actually originate from neurons in the abducens nucleus, they may therefore be part of the circuits through which the cerebellum modulates visual activities and, in particular, maintains eye position. They would therefore be part of the continuum of neurons that receive afferents from all areas that contain direct premotor neurons of the oculomotor system (Büttner-Ennever et al. 1989). This type of information is essential for any structure involved in gaze-holding and smooth pursuit eye movements. Further physiological studies are needed to improve our understanding of these circuits.

Our ultrastructural data indicate that synapses on the soma of these neurons are rare. This suggests that afferent inputs are few or concentrated on dendrites, a situation likely to produce a delay between synaptic action and activation of the soma (Rall, 1967), thus acting as a retarding mechanism within the neuronal circuits.

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