Postsynaptic Targets of Purkinje Cell Terminals in the Cerebellar and Vestibular Nuclei of the Rat

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Abstract

The cerebellar and vestibular nuclei consist of a heterogeneous group of inhibitory and excitatory neurons. A major proportion of the inhibitory neurons provides a GABAergic feedback to the inferior olive, while the excitatory neurons exert more direct effects on motor control via non-olivary structures. At present it is not clear whether Purkinje cells innervate all types of neurons in the cerebellar and vestibular nuclei or whether an individual Purkinje cell axon can innervate different types of neurons. In the present study, we studied the postsynaptic targets of Purkinje cell axons in the rat using a combination of pre-embedding immunolabelling of the Purkinje cell terminals by L7, a Purkinje cell-specific marker, and postembedding GABA and glycine immunocytochemistry. In the cerebellar nuclei, vestibular nuclei and nucleus prepositus hypoglossi Purkinje cell terminals were found apposed to GABAergic and glycinergic neurons as well as to larger non-GABAergic, non-glycinergic neurons. In the cerebellar and vestibular nuclei individual Purkinje cell terminals innervated both the inhibitory and excitatory neurons. Both types of neurons were contacted not only by GABAergic Purkinje cell terminals but also by GABA-containing terminals that were not labelled for L7 and by non-GABAergic, non-glycinergic terminals that formed excitatory synapses. Glycine-containing terminals were relatively scarce (<2% of the GABA-containing terminals) and frequently contacted the larger non-GABAergic, non-glycinergic neurons. To summarize, Purkinje cell axons evoke their effects through different types of neurons present in the cerebellar and vestibular nuclear complex. The observation that individual Purkinje cells can innervate both excitatory and inhibitory neurons suggests that the excitatory cerebellar output system and the inhibitory feedback to the inferior olive are controlled simultaneously.

Introduction

The cerebellar and vestibular nuclei form the output of the cerebellum. This complex of nuclei consists of a heterogeneous group of excitatory and inhibitory projection neurons and interneurons. Most of the excitatory neurons are larger and use glutamate and/or aspartate as their neurotransmitter, while the inhibitory neurons are mostly medium-sized or small and use GABA and/or glycine as their neurotransmitter (Nelson and Mugnaini, 1989; Walberg et al., 1990; Kevetter and Hoffman, 1991; Batini et al., 1992; Chen and Hillman, 1993). The excitatory projection neurons in the cerebellar nuclei innervate a wide variety of areas, including the thalamus, mesodiencephalic junction, pontine and reticular nuclei, medulla oblongata and spinal cord (Fukushima et al., 1977; Wilson et al., 1977; De Zeeuw and Ruigrok, 1994; for review see Ito, 1984), while the GABAergic neurons innervate predominantly the inferior olive (De Zeeuw et al., 1989; Nelson and Mugnaini, 1989; Fredette and Mugnaini, 1991). In addition, some GABAergic cerebellar nuclei neurons may give rise to projections to the cerebellar cortex (Batini et al., 1992) or pons (Aas and Brodal, 1990). Even though most of the glycinergic neurons in the cerebellar nuclei have been reported to be GABAergic (Chen

and Hillman, 1993), none of them has been found to project to the inferior olive (De Zeeuw et al., 1994a). The excitatory and inhibitory projection neurons in the vestibular complex innervate the oculomotor complex (Spencer et al., 1989, 1992; Wentzel et al., 1995), inferior olive (Barmack et al., 1989; De Zeeuw et al., 1993) and spinal cord (Shinoda et al., 1992). With regard to the inhibitory vestibular neurons, GABA is employed for the innervation of the oculomotor nucleus and inferior olive, while glycine is probably the inhibitory neurotransmitter for the innervation of the abducens nucleus and spinal cord.

Despite the extensive knowledge on these different types of neurons in the cerebellar and vestibular nuclei, it is not known whether Purkinje cells innervate all types of neurons in these nuclei or whether an individual Purkinje cell axon can innervate different types of neurons. Recently, we made biocytin injections into the rabbit vestibulocerebellum to study the branching and termination patterns of individual Purkinje cell axons (De Zeeuw et al., 1994b). Purkinje cell axons were frequently found to branch and innervate more than one vestibular nucleus or to innervate both a cerebellar and a

vestibular nucleus. Light microscopic analysis of the labelled terminal varicosities of the branches showed that an individual Purkinje cell axon can be apposed to cells with a wide range of sizes and shapes. Since the smaller and larger cells are usually inhibitory and excitatory respectively (Walberg et al., 1990; Fredette and Mugnaini, 1991; Batini et al., 1992; Chen and Hillman, 1993), it seems likely that an individual Purkinje cell axon can target both inhibitory and excitatory neurons. This pattern of innervation is supported by the observation that many Purkinje cell axons from the flocculus give off terminals both in the dorsolateral and central parts of the superior vestibular nucleus (De Zeeuw et al., 1994b), which are believed to contain excitatory and inhibitory second-order vestibular neurons respectively (Highstein, 1973; Ito et al., 1976; Yamamoto et al., 1978; Highstein and Resine, 1979).

To determine whether individual Purkinje cells establish synaptic contacts with both excitatory and inhibitory neurons, we studied the postsynaptic targets of Purkinje cell axons in the cerebellar and vestibular nuclei of the rat at the electron microscope level using a combination of postembedding GABA or glycine immunocyto- chemistry with pre-embedding L7 immunolabelling of Purkinje cell terminals. The antibody against the L7 protein is an ideal marker because L7 occurs in the brain exclusively in Purkinje cells, is expressed by all Purkinje cells, and is distributed in all neuronal components, including axon terminals (Oberdick et al., 1988; Berrebi and Mugnaini, 1992).

Materials and methods

Antisera

Production of the rabbit L7 antiserum used in this study has been described in detail by Oberdick et al. (1988) and Berrebi et al. (1991); it was kindly provided by Dr J. I. Morgan. Briefly, L7 antiserum was raised against a peptide corresponding to amino acids 41–53 of the predicted L7 sequence (MDDQRVTVNSLPG) coupled to bovine thyroglobulin with glutaraldehyde. The GABA and glycine antisera were generously supplied by Drs R. M. Buijs and R. J. Wenthold respectively; the sensitivity and specificity of both antisera have been tested extensively (Seguela et al., 1984; Buijs et al., 1987; Wenthold et al., 1987; De Zeeuw et al., 1989). Immunopress-blot analysis, in which the same antibody concentrations were used as in the immunocytochemistry, indicated that cross-reactivities of the GABA antibody with glycine and of the glycine antibody with GABA were very low. The GABA antibody detected GABA and glycine at concentrations of 1.1 and 1000 ng/μl respectively, while the glycine antibody detected GABA and glycine at concentrations of 1000 and 3.3 ng/μl respectively.

Light microscopy

To reveal the general distribution of L7 in the cerebellar nuclei and vestibular complex, rats were processed for light microscopy. The rats were anaesthetized with Nembutal (60 mg/kg, i.v.), and perfused transcardially with 250 ml 0.9% NaCl. Fixation was accomplished with a zinc–aldehyde fixative composed of 4% formaldehyde (Fisher Scientific), 0.5% zinc dichromate (K & K Rare and Fine Chemicals, ICN), 0.125% glutaraldehyde (TAAB, Marivac) and 0.75% NaCl, pH 4.8 (20 ml, 20°C). One hour after perfusion the brains were dissected from the cranium and cryoprotected in 30% sucrose in 0.9% NaCl for 2 days at 6°C.

Frozen tissue blocks were cut in 30 μm transverse sections on a microtome. After several rinses in 0.5 M Tris–HCl buffer (pH 7.6),

free floating sections were immunoreacted in tissue culture wells using the peroxidase–antiperoxidase procedure according to the following protocol: 1 h incubation in 5% normal donkey serum (NDS) with 0.5% Triton X-100; 40 h incubation at 4°C in the polyclonal anti-L7 antiserum, diluted 1:1000 with 1% normal donkey serum and 0.2% Triton X-100; 1 h incubation in goat anti-rabbit antiserum diluted 1:100, followed by rabbit peroxidase–antiperoxidase antiserum, diluted 1:100, each with 1% normal donkey serum and 0.2% Triton X-100. The antibody–antigen binding sites were revealed by incuba- tion in 0.05% diaminobenzidine and 0.01% H_2O_2. All immunoreagent solutions were diluted in 0.5 M Tris–HCl buffer (pH 7.6), which also served as the rinsing solution between each step of the immuno- reaction. Control sections incubated with preimmune serum instead of primary antiserum were free of immunoreaction product.

The light microscopic distribution of GABAergic and glycinergic neurons and terminals will not be demonstrated in the present paper (for details see Nelson and Mugnaini, 1989; Walberg et al., 1990; Chen and Hillman, 1993).

Electron microscopy

Four adult rats were processed for pre-embedding L7 immuno- cytochemistry and postembedding GABA and glycine immunocyto- chemistry. The rats were anaesthetized with Nembutal (60 mg/kg, i.v.) and perfused transcardially with 100 ml of a calcium-free variant of Ringer solution, saturated with 95% O_2 and 5% CO_2 to pH 7.2, and 1 litre of fixative consisting of 2% glutaraldehyde and 2% paraformaldehyde in 0.12 M sodium phosphate buffer (pH 7.3) at 20°C. One hour after perfusion the brains were removed from the calvaria, sectioned coronally on a Vibratome at 40 μm, and collected in cold 0.12 M sodium phosphate buffer (pH 7.3). The electron microscopic immunocytochemistry protocol for L7 was as for the light microscopy described above, except that (i) the Triton X-100 detergent was omitted; (ii) Tris-buffered saline (295 mOsm) was used as the diluent and rinsing solution; and (iii) incubation in the primary antiserum took place overnight at room temperature.

After the L7 immunoreaction, the sections were rinsed in 0.12 M sodium phosphate buffer (pH 7.3), postfixed for 1 h in 2% buffered osmium tetroxide in 8% D-glucose, rinsed thoroughly in distilled water, stained in 2% aqueous uranyl acetate, rinsed in distilled water, dehydrated in a graded series of ethanols and propylene oxide, and flat-embedded in Araldite.

Subsequently, we prepared pyramids of the cerebellar and vestibular nuclei and nucleus prepositus hypoglossi and cut ultrathin sections from the most superficial part (a layer of ~3 μm) of these pyramids, which contained abundant L7 labelling. The ultrathin sections were mounted on Formvar-coated nickel grids, and processed for GABA or glycine immunocytochemistry (for details see De Zeeuw et al., 1989, 1994a). Briefly, grids were rinsed in a solution of Tris buffer containing 0.9% NaCl and 0.1% Triton X-100 at pH 7.6 (TBST), and alternately incubated overnight in a droplet of GABA (1:1000 in TBST) or glycine (1:250 in TBST) antiserum. The next day the grids were rinsed twice with TBST and stored for 30 min in the rinsing solution. After rinsing with TBST (pH 8.2) the grids were incubated for 1 h in a droplet of goat anti-rabbit IgG labelled with 15 nm gold particles (Janssen Pharm.) diluted 1:25 in TBST. Subsequently, the grids were washed twice with TBST (pH 7.6) and distilled water, counterstained with uranyl acetate and lead citrate, and examined in a Philips electron microscope. A neuronal structure was considered as GABA- or glycine-positive when the number of gold particles overlying it was at least eight times higher than the number of particles overlying an equal surface area of surrounding glial structures
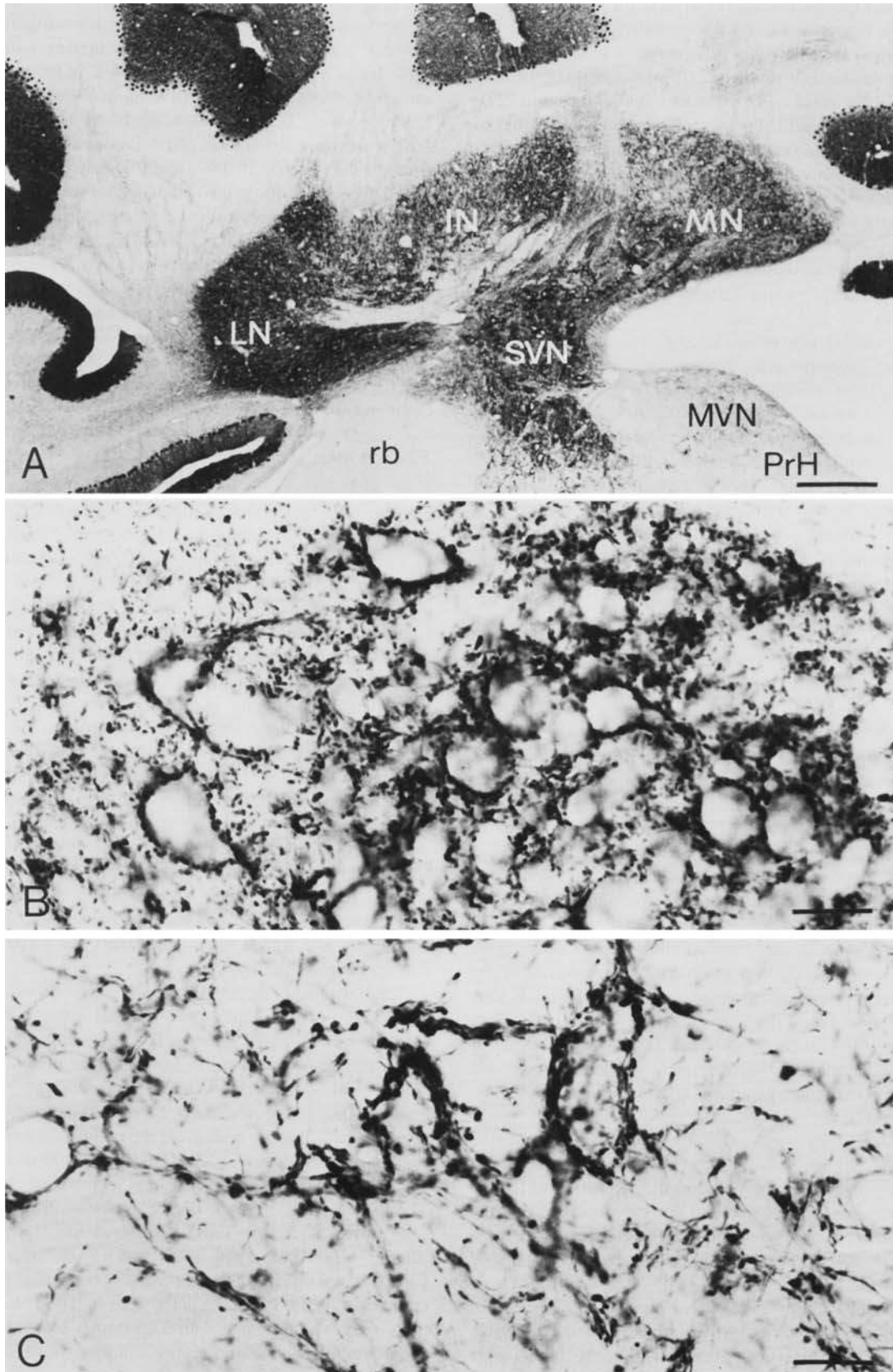
FIG. 1. Micrographs of the cerebellar and vestibular nuclei of sections immunostained for L7. (A) The density of immunoreactive terminals in the lateral cerebellar nucleus (LN), interposed cerebellar nucleus (IN), medial cerebellar nucleus (MN) and superior vestibular nucleus (SVN) is higher than in the medial vestibular nucleus (MVN) and the nucleus prepositus hypoglossi (PrH). (B, C) Examples of L7-labelled boutons in the IN and PrH respectively. Scale bars: A, 390 μm; B, 23 μm; C, 16 μm.
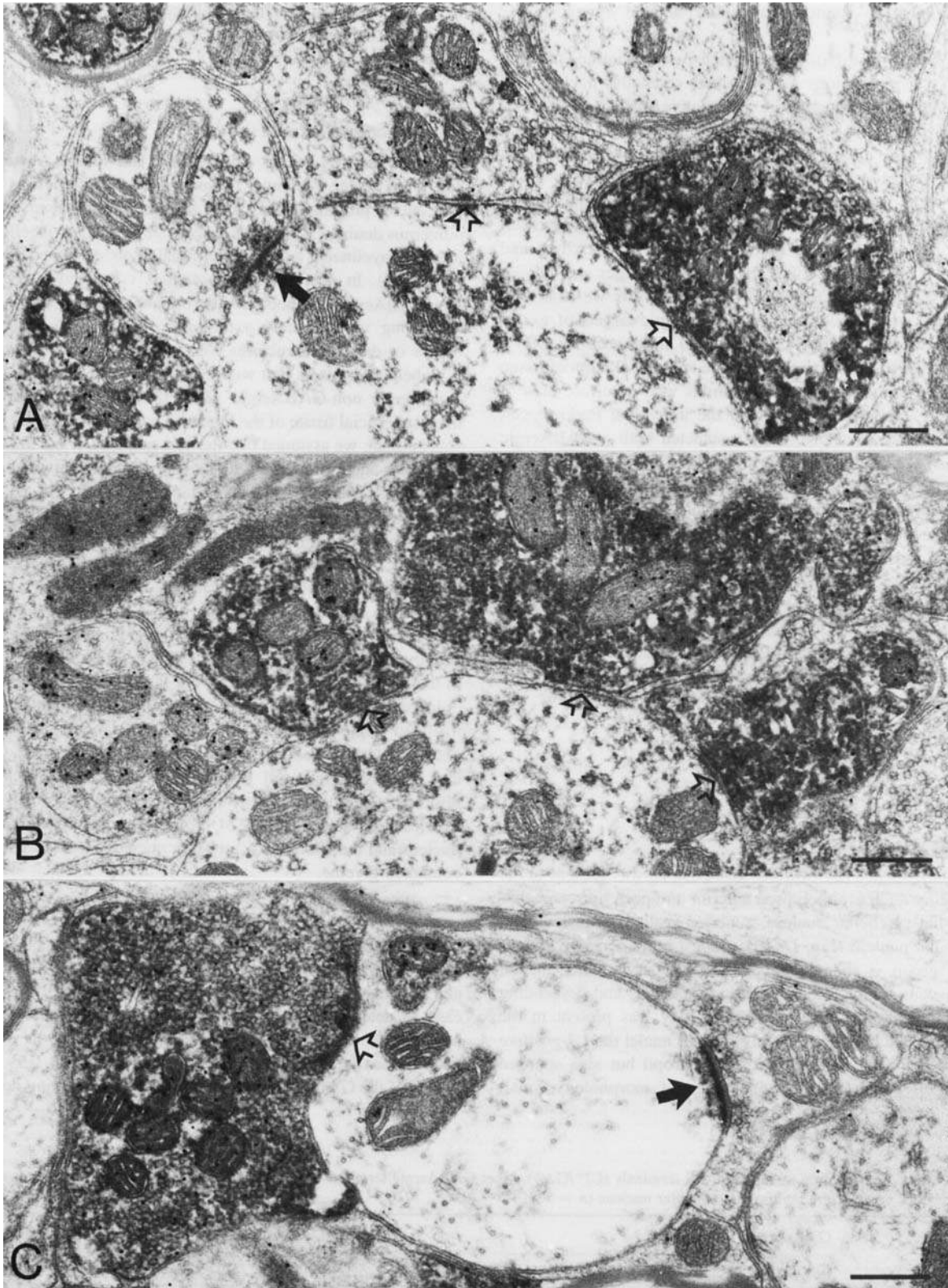
FIG. 2. Electron micrographs of GABAergic, L7-labelled Purkinje cell terminals, GABAergic terminals that are not labelled for L7, and non-labelled terminals in the lateral cerebellar nucleus (A), superior vestibular nucleus (B) and nucleus prepositus hypoglossi (C). Note that the L7-labelled terminals can be readily distinguished from the GABAergic terminals that are not labelled for L7, and that the dendrites in all micrographs are contacted by different types of terminals, indicating that individual neurons in the cerebellar and vestibular nuclei receive both GABAergic terminals from Purkinje cell axons and GABAergic terminals from interneurons or neurons that project to the inferior olive. Note also that the Purkinje cell terminals in these electron micrographs cannot be distinguished from the other GABAergic terminals on morphological criteria. Open arrows indicate symmetrical synapses and filled arrows indicate asymmetric synapses. The asymmetrical synapses frequently showed subsynaptic densities known as Taxi bodies. Scale bars: A, 0.34 μm; B, 0.39 μm; C, 0.28 μm.

(for details about the counting procedure see De Zeeuw *et al.*, 1989, 1994a).

The postsynaptic distribution was extensively quantified in both non-serial and serial sections of the lateral cerebellar nucleus and interposed cerebellar nucleus. In the quantitative analysis of the non-serial sections samples of GABA-containing terminals (i.e. including those labelled for L7) and non-GABAergic terminals were taken from at least three ultrathin sections obtained from at least two tissue blocks from each rat. Distinction was made between terminals that formed synaptic contacts with GABAergic and non-GABAergic cell bodies, proximal dendrites and distal dendrites. The proximal and distal dendrites were characterized by a diameter larger and smaller than 2 μm respectively. In the serial section analysis we examined the GABAergic and non-GABAergic postsynaptic targets of exclusively the L7 labelled terminals. Sets of ~50 serial sections with a silver interference colour were cut from the superficial parts of tissue blocks that were trimmed down to pyramids with a surface area of 0.5–0.5 mm. It was determined for all the individual Purkinje cell axons and terminals what percentage contacted both a GABAergic and a non-GABAergic structure.

In addition, we determined the diameters of cell bodies of the GABAergic, glycinergic and non-labelled neurons in both semithin and ultrathin sections. In this analysis only cell bodies with nucleoli were measured (see also Batini *et al.*, 1992).

Finally, colocalization of GABA and glycine in the cerebellar and vestibular nuclei was studied and observed in serial sections that were processed alternately for GABA and glycine immunocytochemistry. However, since the coexistence of these neurotransmitters in these nuclei has already been firmly demonstrated (Walberg *et al.*, 1990; Chen and Hillman, 1993), no further results on this subject will be reported.

Results

Light microscopy

Dense L7 immunoreactivity of fibres and boutons was present in the lateral cerebellar nucleus, interposed anterior and posterior cerebellar nucleus, medial cerebellar nucleus, superior vestibular nucleus and lateral vestibular nucleus (Fig. 1A, B); modest immunoreactivity was observed in group y, the magnocellular and parvicellular medial vestibular nucleus, caudal medial vestibular nucleus and descending vestibular nucleus, and weak immunoreactivity was present in the nucleus prepositus hypoglossi (Fig. 1C). In all nuclei the L7-positive boutons were not only distributed in the neuropil but also apposed to neuronal cell bodies with a wide range of morphology, small

(5–15 μm in diameter) to large (15–25 μm), spherical or elliptical, triangular or fusiform.

Electron microscopy

Diversity of labelled structures

Ultrathin sections of the superficial 3 μm of the tissue blocks of cerebellar nuclei, vestibular nuclei and nucleus prepositus hypoglossi that were processed for GABA immunocytochemistry included numerous double L7/GABA-labelled, single GABA-labelled and non-labelled myelinated axons, preterminal axon segments, and terminals (Figs 2–5). In addition, these sections contained GABAergic and non-GABAergic cell bodies and proximal dendrites and some GABA-containing unmyelinated axons and peripheral dendrites. No cell bodies or dendritic structures were positive for L7, and none of the L7 labelled terminals that were examined through serial sections was consistently non-GABAergic. Since the analysis was limited to the most superficial tissue of the blocks in which the antibody penetration was optimal, we assumed that the vast majority, if not all, of Purkinje cell axons and terminals would be L7-immunoreactive; i.e. if GABA-containing terminals were L7-negative (e.g. Fig. 2A, B), they were interpreted as terminals that were not derived from Purkinje cells.

Quantitatively, the Purkinje cell boutons dominated the neuropil (Table 1): of the terminals collected from the most superficial sections of the lateral cerebellar nucleus and the interposed cerebellar nucleus, 986 (73%) contained GABA and L7, 215 (16%) contained GABA but were L7-negative, and 149 (11%) were non-GABAergic and L7-negative [Palkovits *et al.* (1977) calculated that 62% of the boutons in the central nuclei of the cat were Purkinje cell terminals].

Ultrathin sections processed for glycine immunocytochemistry included single L7-labelled, single glycine-labelled and non-labelled axons, preterminal axon segments, and terminals (Fig. 5). Glycinergic and non-glycinergic cell bodies and proximal dendrites were also observed. Overall glycine-containing structures were relatively scarce. In a random selection of 374 terminals from the lateral cerebellar nucleus and the interposed cerebellar nucleus, seven (1.9%) terminals contained glycine.

Characteristics of cell bodies

The identity of the cell bodies and proximal dendrites in the cerebellar and vestibular nuclei and nucleus prepositus hypoglossi was usually easy to determine by the presence or absence of the immunogold labelling. Apart from the medial cerebellar nucleus, where glycinergic neurons can be large (see also Chen and Hillman, 1993), the cell bodies of the GABA-containing and glycine-containing neurons were

TABLE 1. Postsynaptic distribution of Purkinje cell terminals (L7$^+$/GA$^+$), other GABAergic terminals (L7$^-$/GA$^+$), and non-GABAergic terminals (L7$^-$/GA$^-$) in the lateral cerebellar nucleus and interposed cerebellar nucleus (*n* = 1350)

	GABAergic			Non-GABAergic		
	Cell bodies	Proximal dendrites	Distal dendrites	Cell bodies	Proximal dendrites	Distal dendrites
L7$^+$/GA$^+$ (*n* = 986)	4%	8%	9%	8%	29%	42%
L7$^-$/GA$^+$ (*n* = 215)	4%	6%	9%	13%	29%	39%
L7$^-$/GA$^-$ (*n* = 149)	3%	9%	10%	1%	20%	57%

Postsynaptic structures are divided into GABAergic and non-GABAergic cell bodies, proximal dendrites and distal dendrites. The percentages make 100% for each row. Percentages within a column cannot be compared unless the sample numbers of the various types of terminals are taken into account. Terminals that formed multiple synapses with both GABAergic and non-GABAergic postsynaptic structures and terminals that formed synapses with an axon hillock are not included in this analysis.

smaller (average diameter ± SD, 11 ± 2.6 μm) than the non-labelled neurons (22.3 ± 4.8 μm). These diameters agree with those of the small GABAergic neurons that project to the olive and with those

of the large non-GABAergic neurons that have been shown both anatomically and physiologically to be excitatory and to project to the mesodiencephalic junction (Ruigrok and De Zeeuw, 1993; De
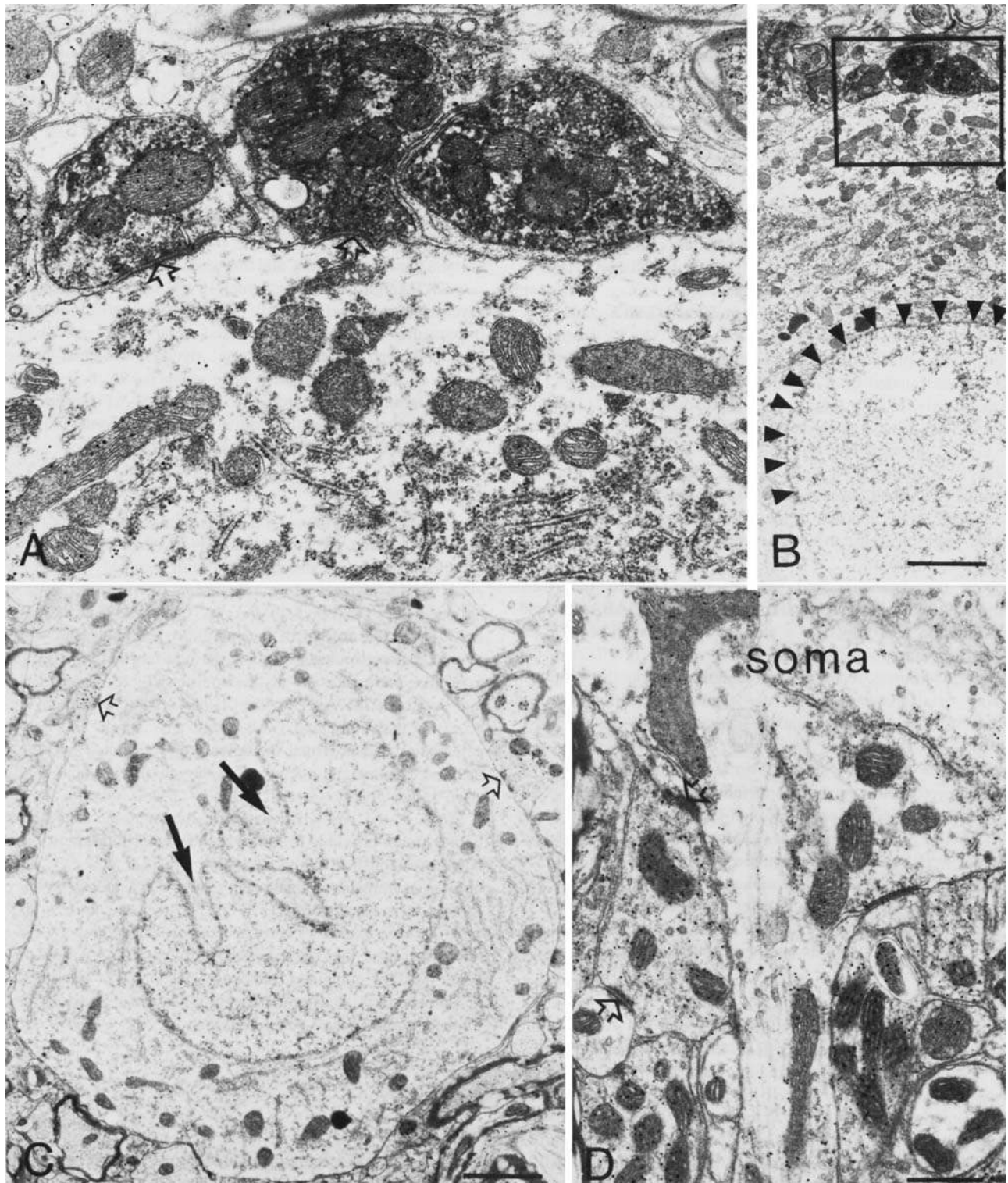

FIG. 3. Electron micrographs of GABAergic cell bodies contacted by GABAergic terminals. The medium-sized GABAergic neurons with smooth nuclei (filled triangles) were often contacted by L7-labelled terminals (A, B), while the somewhat smaller GABAergic neurons with indented nuclei (filled arrows) were only infrequently contacted by GABAergic terminals; relatively few of these GABAergic terminals were L7-positive (C, D). Open arrows indicate symmetrical synapses. Scale bars: B, 8 μm; C, 1.2 μm; D, 0.42 μm.

Zeeuw and Ruigrok, 1994; Teune *et al.*, 1995). Since only a few of the small neurons were GABA-negative and glycine-negative, the number of false-negative cell bodies was probably limited. In contrast to the large neurons, which displayed numerous clumps of Nissl bodies and smooth nuclei, the smaller GABA-containing neurons often contained single cisterns of endoplasmic reticulum in their cytoplasm (see also Sotelo and Angaut, 1973). In general, both the inhibitory and excitatory neurons were rather diffusely distributed in all parts of the cerebellar and vestibular nuclei (for monkey see also Chan-Palay, 1977; cat, Walberg *et al.*, 1990; rat, Batini *et al.*, 1992; Chen and Hillman, 1993; Teune *et al.*, 1995).

Purkinje cell terminals

The L7-labelled terminals ranged in diameter from 0.8 to 3.7 μm, as determined by serial section analysis, and usually included several mitochondria, many pleiomorphic vesicles, some coated vesicles and one or two dense core vesicles (Fig. 2). They established primarily symmetrical synapses; the larger terminals often formed more than one synapse. The Purkinje cell terminals contacted GABA-containing, glycine-containing or non-labelled neurons (Fig. 3A, B); frequently the cell bodies of the large non-labelled neurons were virtually completely surrounded by L7-positive terminals (see also Fig. 1B). In the quantitative analysis of the postsynaptic distribution of Purkinje cells in the lateral and interposed cerebellar nuclei (*n* = 986; see Table 1), we observed that (i) the majority were apposed to non-GABAergic structures (79%), and (ii) that about half of all Purkinje cell terminals contacted distal dendrites (51%). In some cases GABA-ergic terminals were apposed to the axon hillock of GABAergic or non-GABAergic neurons; these few terminals were not included in the quantitative analysis.

In both the cerebellar and the vestibular nuclei, individual Purkinje cell terminals were found to innervate both GABAergic and non-GABAergic neurons (Figs 4 and 6). In non-serial sections of the lateral and interposed cerebellar nuclei 3% (26 of 1012) of the Purkinje cell terminals made synaptic contacts with both structures. Following serial analysis of sets of ~50 ultrathin sections (with a thickness of 50 nm), this percentage increased to 9% (34 of 378). Frequently, we encountered individual Purkinje cell axons that contained more than one terminal in a particular set of serial sections. Therefore, the percentage of individual Purkinje cell axons that contacted both GABAergic and non-GABAergic structures was higher (13%; 49 of 378) than that of individual terminals. Considering that we did serial section analysis on pieces of tissue not thicker than 3 μm and that individual Purkinje cell axons give off varicosities in the cerebellar and vestibular nuclei over hundreds of micrometres in the rostrocaudal plane (De Zeeuw *et al.*, 1994b), it seems likely that most of the Purkinje cell axons innervate both GABAergic and non-GABAergic neurons.

GABAergic terminals not derived from Purkinje cells

The GABA-containing terminals that were not labelled for L7 resembled, apart from the lack of a diaminobenzidine reaction product, morphologically the Purkinje cell terminals. These L7-negative GABA-containing terminals, which must have been derived from interneurons and/or recurrent collaterals of GABAergic neurons projecting to the olive, contacted both GABAergic and non-GABA-ergic cell bodies and dendrites. Frequently, the L7-negative GABA-containing terminals contacted the same GABAergic, glycinergic or non-labelled cell bodies or proximal dendrites as the Purkinje cell terminals and non-labelled terminals did (Figs 2A, B and 5C). In general, we did not find significant differences between the postsyn-

aptic distribution of the L7-negative terminals and the Purkinje cell terminals (Table 1). In some instances, however, small GABA-containing neurons were contacted by relatively few terminals; most of these terminals were immunonegative for L7 despite adequate penetration of the L7 antibody in the uppermost 3 μm of the tissue (Fig. 3C, D). These small GABA-containing neurons frequently contained nuclei that were invaginated, and they may correspond to the small neurons observed by Matsushita and Iwahori (1971) and Van Der Want (1986) and Van Der Want and Voogd (1987) in the cerebellar nuclei of the cat; these neurons receive few axosomatic terminals, some of which are derived from recurrent collaterals from cerebellar projection neurons.

Glycinergic terminals

The glycine-containing terminals were generally of the same size as the Purkinje cell terminals (Fig. 5), but more often they contained clusters of neurofilaments, and they showed a preference for contacting non-GABAergic and non-glycinergic cell bodies and dendrites. The glycinergic terminals usually formed symmetrical synapses, but infrequently they established mixed synapses, as described below. Frequently, individual GABAergic, glycinergic or non-labelled cell bodies or proximal dendrites were contacted by both glycine-containing terminals and Purkinje cell terminals (Fig. 5D). We were unable to demonstrate that individual glycine-containing terminals contacted both an inhibitory and an excitatory cell.

Non-labelled terminals

The non-labelled terminals, i.e. the terminals that did not contain L7, GABA or glycine, showed the morphology characteristic of excitatory terminals: they were usually filled with rounded vesicles and they established asymmetrical synapses, some of which displayed subsynaptic densities (Taxi bodies) (Fig. 2A, C). The size of these terminals was comparable to that of the inhibitory terminals, but with regard to their postsynaptic distribution the non-labelled terminals showed a relative preference for distal dendrites (Table 1; see also Angaut and Sotelo, 1973; Van Der Want, 1986). Even so, in many cases we observed that individual GABAergic, glycinergic and non-labelled cell bodies and proximal dendrites were contacted by both a non-labelled terminal and a terminal of the inhibitory type, i.e. a GABA-ergic and/or glycinergic terminal (Figs 2A, C and 4B).

Some of the non-labelled terminals contacting non-labelled cell bodies and/or proximal dendrites in the medial and superior vestibular nucleus established mixed excitatory synapses, i.e. they established both an asymmetrical chemical synapse and an electrotonic contact formed by a gap junction (Fig. 5A, B) (see also Sotelo and Palay, 1970). These excitatory terminals were usually larger (3–5 μm in diameter) than the non-labelled terminals apposed to the distal dendrites, they contained rounded vesicles and an abundant number of mitochondria, and they frequently showed a web of fine neurofilaments in the centre of the terminal.

Discussion

Light microscopy

The L7 immunoreactivity of terminal profiles was present in all parts of the cerebellar and vestibular nuclei, which agrees with the well known ubiquitous innervation of these nuclei by the Purkinje cells (for reviews see Chan-Palay, 1977; Voogd and Bigaré, 1980). In addition, we observed that L7 was present in the nucleus prepositus hypoglossi. The projection from the flocculus to this nucleus is
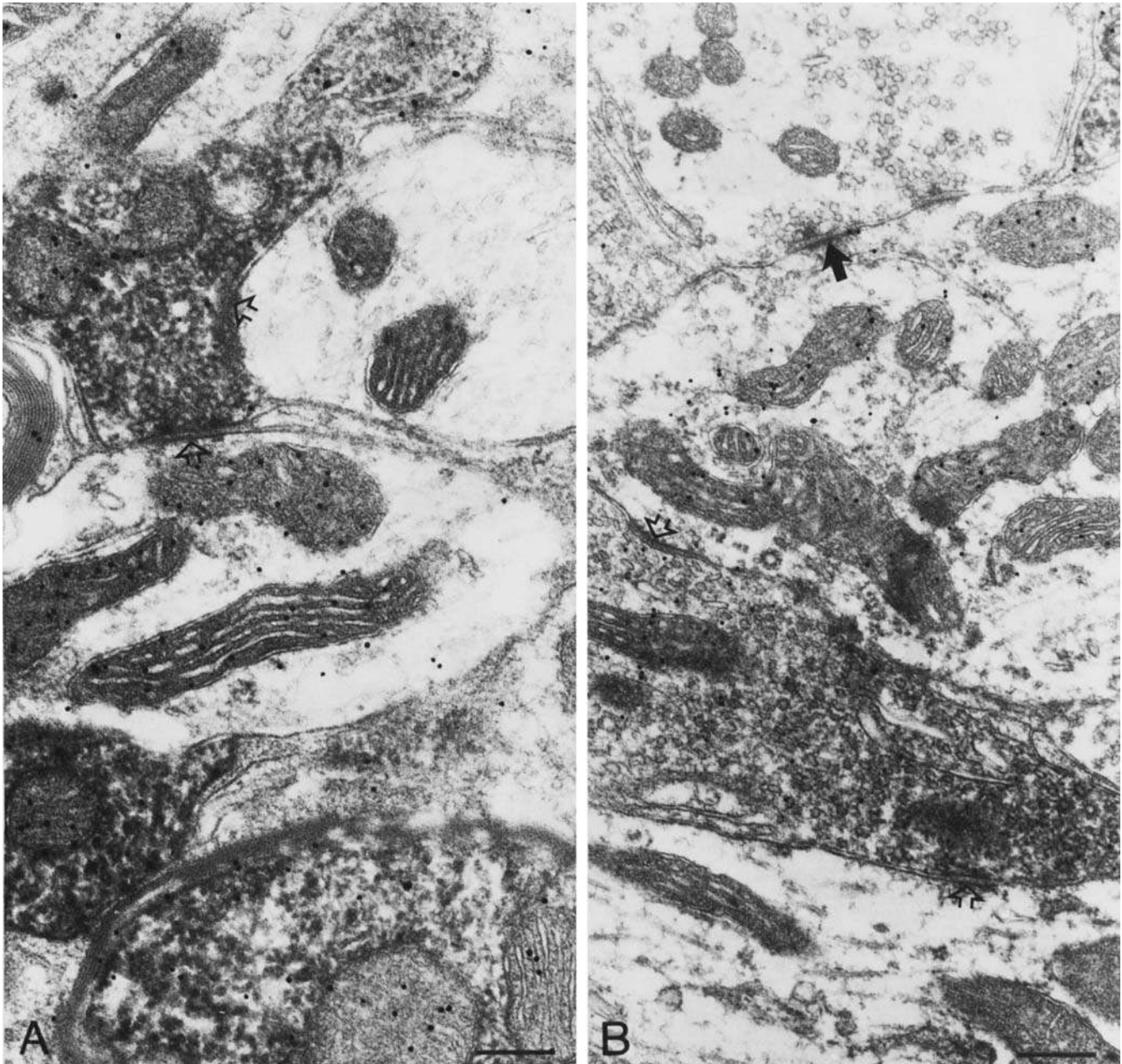
Fig. 4. Both in the vestibular (A) and cerebellar (B) nuclei individual Purkinje cell terminals were found to innervate both GABAergic and non-GABAergic dendrites. Note that the GABAergic dendrite in B is contacted by both an L7-labelled GABAergic dendrite and an excitatory, non-GABAergic terminal with round vesicles and an asymmetrical synapse. Open arrows indicate symmetrical synapses and the filled arrow indicates an asymmetrical synapse. Scale bars: A, 0.19 μm; B, 0.25 μm.

debated (rabbit, Tan et al., 1995; De Zeeuw et al., 1994b; rat, Umetani, 1992; cat, Angaut and Brodal, 1967; Baker and Berthoz, 1975; Yingcharoen and Rinvik, 1983; primates, Haines, 1977) and the projection from folium p of the ventral paraflocculus to the nucleus prepositus hypoglossi has only been demonstrated with the use of retrograde tracing (rabbit, Yamamoto, 1978). Because the medial vestibular nucleus adjacent to the nucleus prepositus hypoglossi also receives a projection from folium p (rabbit, Tan et al., 1995), this latter finding could have been biased. The present study demonstrates that the Purkinje cell input to the nucleus prepositus hypoglossi is sparse but present, and that the Purkinje cell terminals make

synaptic contacts with both the inhibitory and the presumptive excitatory neurons in this nucleus.

Electron microscopy

GABAergic and non-GABAergic targets of Purkinje cells

The major findings of this study are that individual Purkinje cells contact neurons with a wide range of sizes and shapes and that these neurons can be GABAergic and non-GABAergic (Fig. 6). The vast majority of the GABAergic neurons contacted by the Purkinje cells in the cerebellar nuclei are probably neurons projecting to the inferior
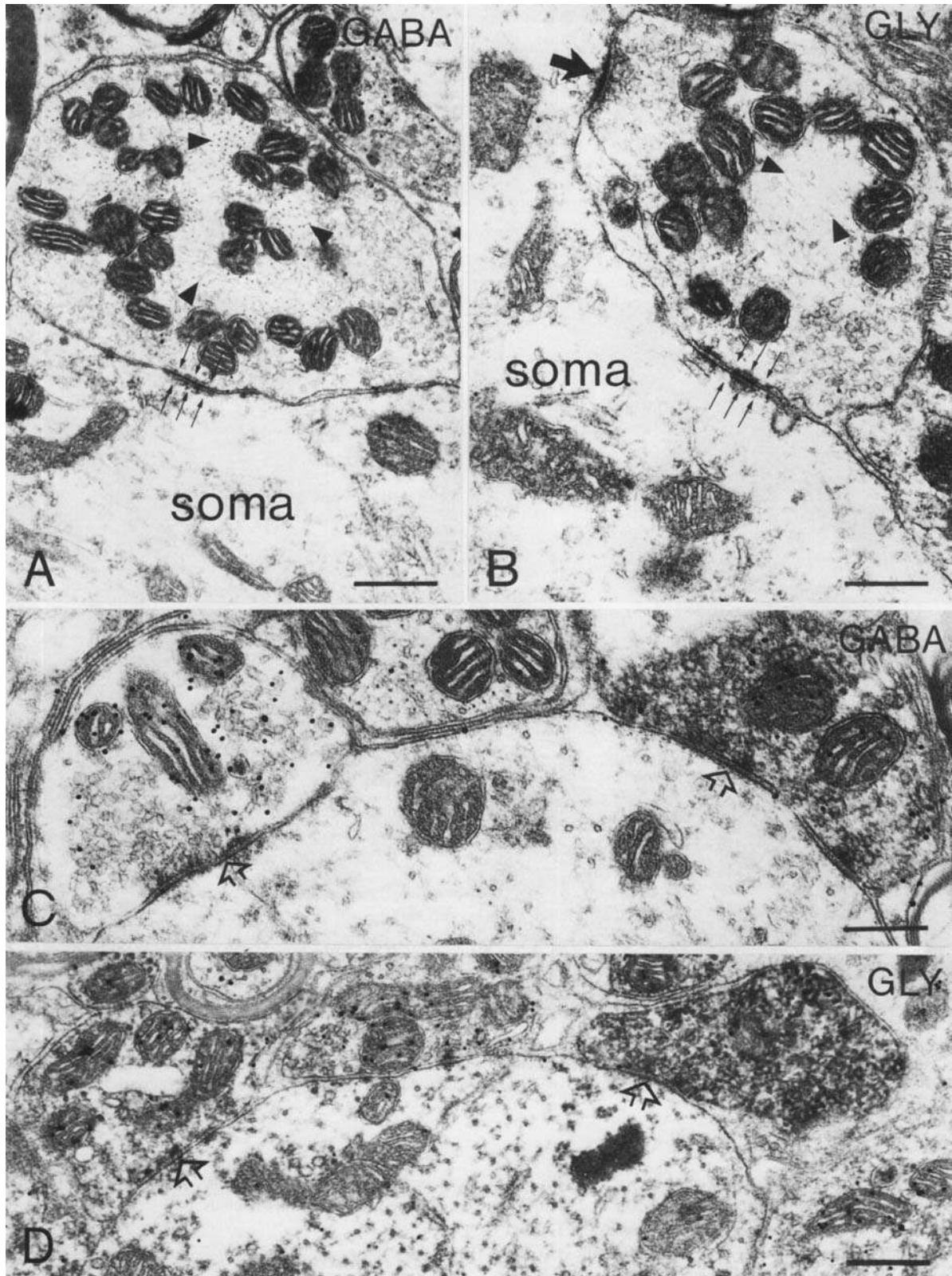
FIG. 5. Electron micrographs of terminals in the medial (A, D) and superior (B, C) vestibular nuclei. Some of the excitatory terminals in these vestibular nuclei had so-called mixed synapses (Sotelo and Palay, 1970), i.e. these terminals contained both asymmetrical synapses (filled arrow) and gap junctions (small arrows). *Frequently the terminals* with mixed synapses contained a cluster of neurofilaments in the centre of the terminal (triangles). The dendrites in the vestibular nuclei were often contacted by L7-labelled terminals, L7-negative GABAergic terminals (C) and glycinergic (GLY) terminals (D). Following serial section analysis the glycinergic 'terminals' in D were found to be part of the same glycinergic fibre (data not shown). Scale bars: A, 0.42 μm; B, 0.39 μm; C, 0.24 μm; D, 0.35 μm.
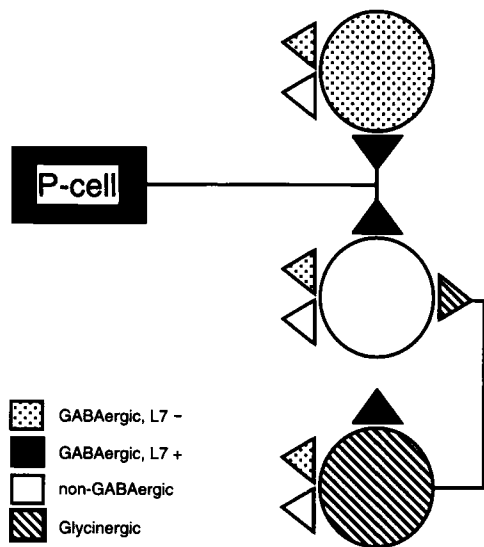
FIG. 6. Diagram of major connections in the cerebellar and vestibular nuclei. Individual Purkinje cell terminals innervated both GABAergic and non-GABAergic neurons. Triangles indicate terminals, circles indicate post-synaptic structures.

olive (De Zeeuw et al., 1989; Nelson and Mugnaini, 1989), since Fredette and Mugnaini (1991) observed that at least 93% of glutamic acid decarboxylase-positive neurons in the cerebellar nuclei are retrogradely labelled following injection of horseradish peroxidase in the inferior olive, and that these neurons are innervated by GABAergic terminals in the same fashion as they are innervated by the L7 terminals. This presumably monosynaptic input from the Purkinje cells onto the olivary projection neurons in the cerebellar nuclei indicates that the olivocerebellar modules described by Voogd and Bigaré (1980) are indeed formed by a three-element loop consisting of a specific olivary subnucleus, a specific cerebellar sagittal zone of Purkinje cells, and a specific cerebellar nucleus. The finding that the Purkinje cell terminals also synapsed onto the GABAergic neurons in group y, the nucleus prepositus hypoglossi, and the descending and medial vestibular nucleus, which are known to project to the ventrolateral outgrowth, dorsal cap, and β-nucleus of the inferior olive respectively (De Zeeuw et al., 1993 and 1994a), suggests that the arrangement of the three-element modules also occurs in the vestibulocerebellum (De Zeeuw et al., 1994b; Tan et al., 1995). The observation that individual Purkinje cells innervate both GABAergic and non-GABAergic neurons in the cerebellar and vestibular nuclei suggests that the Purkinje cells may control simultaneously the inhibitory feedback to the inferior olive, which is part of the closed, three-element, anatomical pathway, and the excitatory cerebellar output system, which is part of an open anatomical pathway through which the motor commands are finally influenced (De Zeeuw et al., 1994b). These combined inhibitory and excitatory effects of individual Purkinje cells may, therefore, be of crucial importance for the role of the cerebellum in controlling the timing of movements (Welsh et al., 1995; De Zeeuw et al., 1995).

Purkinje cell input to glycinergic neurons

Apart from the large glycinergic neurons that occur in the fastigial nucleus and may contain aspartate, most of the glycinergic neurons in the cerebellar nuclei contain GABA as a co-neurotransmitter (Chen

and Hillman, 1993). These glycine-containing neurons are probably not involved in the olivocerebellar modules since glycinergic boutons have not been found in the inferior olive (De Zeeuw et al., 1994a). Because no glycinergic projections from or to the cerebellar nuclei have been described and glycinergic terminals and receptors do occur in the nuclei (Chen and Hillman, 1993; present study), most of the glycine-containing neurons in the cerebellar nuclei may in fact be interneurons. Glycine and GABA could cooperate as inhibitory neurotransmitters that hyperpolarize target neurons by opening chloride channels (Curtis et al., 1968; Ottersen and Storm-Mathisen, 1990). The combination of the rapid inhibitory onset of glycine (Curtis et al., 1968) and the slower onset and longer duration by GABA (Faber and Korn, 1991) probably results in a rapidly evoked but sustained inhibition of postsynaptic targets. As such, activation of the glycinergic, GABAergic interneurons may, even though these neurons only provide a small proportion of the total of inhibitory terminals, result in temporal modulation of inhibitory patterns in the central nuclei neurons. The observations that Purkinje cells project not only to the GABAergic and non-GABAergic neurons but also to the glycinergic neurons, and that the glycinergic neurons were found to project mainly to the larger, presumptive excitatory neurons, may explain why the time course of the inhibition in the excitatory central nuclei neurons after climbing fibre activation does not correspond to the time course of the facilitation of the simple spikes after the climbing fibre pause (Ruigrok et al., 1994).

In the vestibular nuclei, glycine is also colocalized with other neurotransmitters like GABA and aspartate in the same neurons (Walberg et al., 1990). However, in these nuclei glycine is not restricted to interneurons since glycinergic neurons in the medial vestibular nucleus and descending vestibular nucleus have been shown to project to the abducens nucleus and spinal cord respectively (Spencer et al., 1989, 1992; Shinoda et al., 1992).

Non-Purkinje cell terminals with mixed synapses

Some of the non-labelled terminals and some of the glycinergic terminals in the medial and superior vestibular nucleus formed mixed excitatory synapses establishing both an asymmetrical chemical synapse and an electrotonic contact formed by a gap junction with a non-labelled cell body. Similar terminals have been described by Sotelo and Palay (1970) for the ventral part of the lateral vestibular nucleus [currently named the magnocellular medial vestibular nucleus (De Zeeuw et al., 1994b)]. Since we were unable to find terminals with these mixed synapses in the cerebellar nuclei or nucleus prepositus hypoglossi, it is possible that they are in fact terminals of primary vestibular afferents. Morphologically, these terminals would correspond best to those of the irregular primary afferents (Sato and Sasaki, 1993; see also Korte and Friedrich, 1979). If the terminals of the primary afferents indeed make electrotonic contacts with the cell bodies of second-order vestibular neurons, then these axosomatic gap junctions may explain why floccular activity can be evoked by stimulation of the vestibular labyrinths at latencies suggestive of a monosynaptic connection (Shinoda and Yoshida, 1975; Ito, 1984).

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Abbreviations

GABA   γ-amino butyric acid

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