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Divergent Axon Collaterals from Rat Cerebellar Nuclei to Diencephalon, Mesencephalon, Medulla Oblongata and Cervical Cord

A Fluorescent Double Retrograde Labeling Study*

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Summary. The existence of divergent axon collaterals of neurons in the deep cerebellar nuclei has been investigated in rat by means of the fluorescent retrograde double labeling technique. The results have led to the following conclusions.

A. Many of the neurons in the lateral, the interpositus as well as the caudal half of the medial nucleus project to the diencephalon. Some of these neurons distribute divergent axon collaterals to the superior colliculus, but few neurons project only to the latter structure.

B. Some of the deep cerebellar neurons located laterally, i.e. in the dorsomedial part of the lateral nucleus, as well as some others located medially, i.e. in the medial part of the interpositus nucleus and the adjoining part of the medial nucleus, distribute divergent axon collaterals to the diencephalon and the spinal cord.

C. Deep cerebellar neurons located laterally: in the cell group of the dorsolateral hump (Dlh) and in the adjoining lateral part of the interpositus nucleus, as well as some others located medially, i.e. in the dorsolateral part of the medial nucleus (Mdlp), distribute divergent axon collaterals to the diencephalon and to the medulla oblongata, probably primarily its medial reticular formation. However, only few of the neurons, which distribute descending collaterals to the spinal cord or the medulla oblongata, distribute ascending collaterals to the superior colliculus.

D. After injections in the medulla oblongata a population of small sized single labeled neurons was encountered especially in the lateral and interpositus nuclei. On the basis of other findings in rat they were assumed to represent cerebello-olivary neurons.

Key words: Cerebellar nuclei – Axon collaterals – Cerebellar efferents – Double labeling – Fluorescent tracers

Introduction

The efferent projections of the deep cerebellar nuclei have been extensively investigated anatomically. In different mammalian species a similar pattern of organization has been described (Cajal 1972; Faull 1978; Faull and Carman 1978; Flood and Jansen 1966; Jansen and Jansen 1955; Martin et al. 1974; Rand 1954; Voogd 1964). The brachium conjunctivum represents the principal efferent pathway, which takes origin from the lateral (dentate) and interpositus nuclei and decussates outside the cerebellum, i.e. in the caudal midbrain. The ascending limb of the brachium conjunctivum courses through the contralateral red nucleus and adjacent midbrain tegmentum. In the mesencephalon the fibers of the brachium conjunctivum terminate in the red nucleus and in other midbrain structures, such as the deep layers of the superior colliculus (Castro 1978; Caughell and Flumerfelt 1977; Chan Palay 1977; Cohen et al. 1958; Faull and Carman 1978; Rand 1954; Voogd 1964). The brachium conjunctivum continues from the mesencephalon into the diencephalon to terminate in the subthalamic area and in the thalamus: primarily in the ventral nuclear complex and intralaminar nuclei (Castro 1978; Chan Palay 1977; Cohen et al. 1958; Faull and Carman 1978; Haroian et al. 1981; Hendry et al. 1979; Rand 1954; Stanton 1980; Sugimoto et al. 1981). Immedi-

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ately rostral to the decussation, the brachium conjunctivum gives off a descending limb, which proceeds through the medial pontine and medullary reticular formation and terminates in the pons, medulla oblongata and presumably also in the spinal cord (Achenbach and Goodman 1968; Asanuma et al. 1980; Cajal 1972; Carpenter and Stevens 1957; Castro 1978; Faull 1978; Martin et al. 1974; Rand 1954; Voogd 1964). In the medulla oblongata some fibers terminate in the medial reticular formation but the inferior olive represents the main termination area (Dom et al. 1973; Graybiel et al. 1973; Kalil 1979; Martin et al. 1976; Tolbert et al. 1976b). A pathway descending ipsilaterally from the brachium conjunctivum to the lateral medullary reticular formation has also been described in rodents (Achenbach and Goodman 1968; Cajal 1972; Castro 1978; Chan Palay 1977; Faull 1978). The bulk of the efferent fibers of the medial (fastigial) cerebellar nucleus are incorporated in the uncinate tract and decussate mainly within the cerebellum (Batton et al. 1977; Carpenter 1959; Flood and Jansen 1966; Haroian et al. 1981; Jansen and Jansen 1955; Thomas et al. 1956; Voogd 1964). The ascending fastigial fibers (ascending limb of the uncinate tract) course in the mesencephalon dorsal to the brachium conjunctivum and terminate in the superior colliculus and ventral thalamus (Angaut and Bowsher 1970; Batton et al. 1977; Haroian et al. 1981; Kievit and Kuypers 1972; Sugimoto et al. 1981; Voogd 1964). The descending fibers (descending limb of the uncinate tract) are distributed to the caudal brainstem, e.g. to the vestibular complex and medial reticular formation, and to the spinal cord (Achenbach and Goodman 1968; Batton et al. 1977; Cohen et al. 1958; Thomas et al. 1956; Voogd 1964; Walberg et al. 1962; Ware and Mufson 1979). In addition some uncrossed fastigial fibers are distributed to the medulla oblongata (Batton et al. 1977; Voogd 1964). These fibers terminate primarily in the vestibular complex (Batton et al. 1977).

Cajal (1972) reported that the fibers of the crossed descending limb of the brachium conjunctivum mainly represent collaterals of its ascending fibers. Recent studies in cat, employing retrograde horseradish peroxidase (HRP) transport and electrophysiological techniques, reported that cerebellofugal axons also give off collaterals descending to pons or inferior olive (Ban and Ohno 1977; Gould and Graybiel 1976; McCrea et al. 1978; Tolbert et al. 1976a, 1978).

The recently introduced double retrograde fluorescent tracer technique has been used to ascertain anatomically in cat the existence of axonal branching in the cerebellar efferent fibers (Bharos et al. 1981). In the present study this technique has been used in rat in order to elucidate:

a) whether single cerebellar neurons, which give rise to fibers to the diencephalon also distribute collaterals to the tectum;

b) whether single cerebellar neurons, which give rise to fibers ascending to mesencephalon and diencephalon also give rise to descending collaterals to medulla oblongata and spinal cord.

Two types of fluorescent retrograde tracer combinations have been used:

a) tracers which fluoresce in different colours at the same excitation wavelength and label different structures in the cell (Kuypers et al. 1980) and

b) tracers which fluoresce in different colours at different excitation wavelengths (Kuypers et al. 1977; van der Kooy et al. 1978). However, as a first step the distribution of the cells of origin of the various cerebellar efferents in the rat was studied by means of the retrograde HRP technique.

Material and Methods

A. *HRP Injections*

Nine rats were injected unilaterally with HRP. The injections were made with a Hamilton microsyringe under Nembutal anaesthesia. A 30% HRP (Miles) aqueous solution was injected stereotaxically in the ventral thalamus (0.4 µl) in 2 cases; in the midbrain tegmentum including the red nucleus (0.3 µl) in 1 case and in the superior colliculus (0.3 µl) in 2 cases. Further, in 2 cases HRP (0.2 µl) was injected in the medulla oblongata from dorsally under direct vision, and in 2 cases multiple HRP injections (0.8 µl in total) were made in the white and grey matter of the upper cervical segments of the spinal cord. The latter injections were made from the dorsal side through a laminectomy.

Fig. 1. Semidiagrammatic representation of the distributions of retrogradely labeled neurons in the cerebellar nuclei after TB injections in diencephalon, midbrain tegmentum and superior colliculus respectively

List of Abbreviations. Dlh dorsolateral hump; *Dmc* dorsomedial crest; *I* interpositus nucleus; *IC* internal capsule; *IO* inferior olive; *Is* interpositus nucleus, small-celled part; *L* lateral nucleus; *LD* laterodorsal nucleus; *Ll* lateral nucleus, large-celled part; *Ls* lateral nucleus, small-celled part; *M* medial nucleus; *Mcm* medial nucleus, caudomedial part; *MD* mediodorsal nucleus; *Mdlp* medial nucleus, dorsolateral protuberance; *Mm* medial nucleus, middle part; *R* red nucleus; *Rgc* reticular formation, gigantocellular part; *Rpc* reticular formation, parvocellular part; *SC* superior colliculus; *VL* ventrolateral nucleus; *VM* ventromedial nucleus; *Vsp* nucleus of the spinal tract of the trigeminal nerve

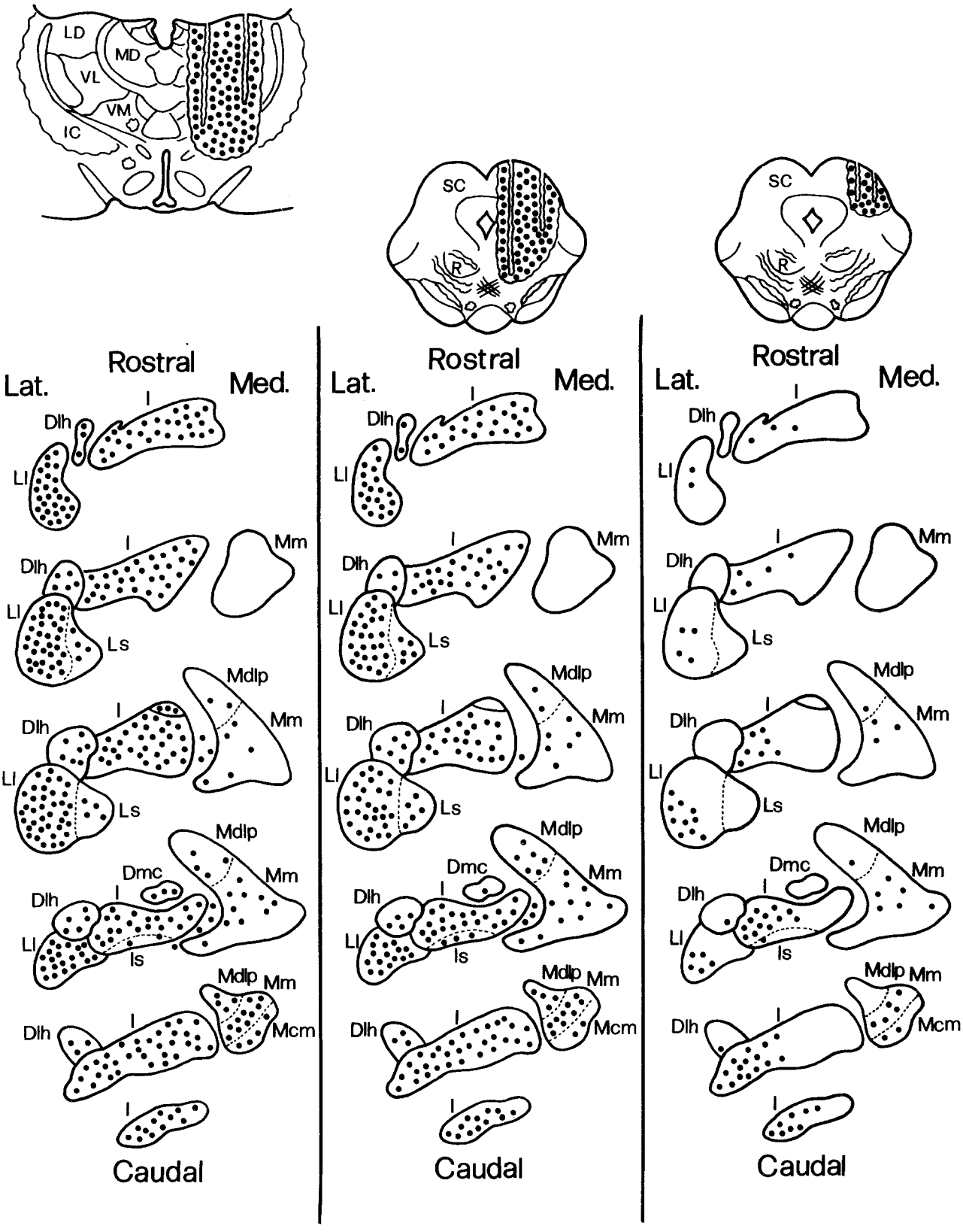


Fig. 1

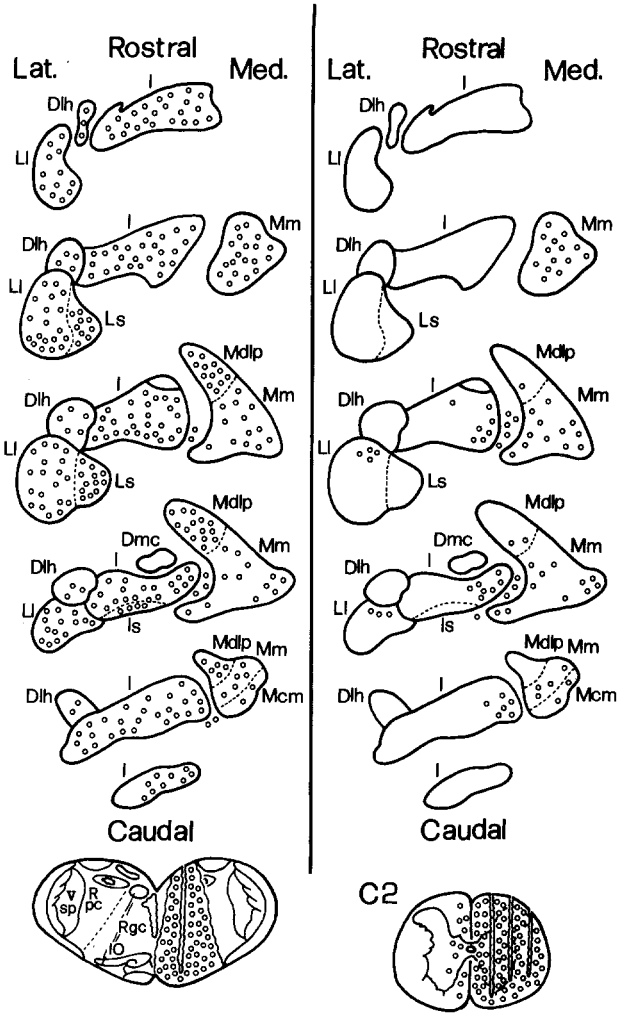


Fig. 2. Semidiagrammatic representation of the distributions of retrogradely labeled neurons in the cerebellar nuclei after NY injections in medulla oblongata and cervical cord respectively. (Abbreviations: see Fig. 1)

After 24–36 h survival the animals were deeply anesthetized and perfused transcardially with 6% dextran, followed by a solution of 0.5% paraformaldehyde and 2.5% glutaraldehyde in cacodylate buffer (pH 7.2). The injection sites and the cerebella were soaked overnight in 30% buffered sucrose (pH 7.2). They were then cut transversally in 40 µm thick frozen sections, which were incubated according to Malmgren and Olsson (1978), counterstained with cresyl violet and studied under darkfield and brightfield illumination. The extent of the injection areas and the distribution of the labeled neurons in the cerebellar nuclei were charted with the aid of an X–Y plotter. The atlas by Korneliussen (1968) was used as a reference for the morphology of the cerebellar nuclei in the rat.

B. *Injections with Fluorescent Tracers*

The following fluorescent retrograde tracers were used:

a) "True Blue" (TB), "Granular Blue" (GB) (Bentivoglio et al. 1979a), mixture of DAPI (Serva) and Primuline (Eastman) DAPI-Pr) (Kuypers et al. 1977); they produce a blue fluorescent labeling of the cytoplasm when illuminated with light of 360 nm wavelength and DAPI produces also blue labeling of the nucleus;

b) Bisbenzimide (Bb; Hoechst 33258) (Kuypers et al. 1979) and "Nuclear Yellow" (NY; Hoechst S7691217) (Bentivoglio et al. 1980b) which produce a green or yellow fluorescent labeling when illuminated with light of 360 nm or 390 nm wavelengths. After short survival times the Bb and NY labeling is largely restricted to the neuronal nucleus (Bentivoglio et al. 1980a);

c) Evans Blue (EB) (Kuypers et al. 1977) which produces a red fluorescent labeling of the cytoplasm and the nucleus when illuminated with light of 550 nm wavelength.

All the substances were injected in aqueous solutions or suspensions (for concentrations and volumes see Tables 1, 2). The total injected volumes were divided over several injections of 0.1–0.2 µl each. The injections were made in the same way as the HRP injections; each tracer was injected with a different syringe needle and glass barrel. As indicated in Tables 1 and 2, TB was injected in combination with Bb or NY (Kuypers et al. 1980); EB was injected in combination with DAPI-Pr (van der Kooy et al. 1978) or GB (Catsman-Berrevoets et al. 1980; Rosina et al. 1980).

The findings obtained by means of the fluorescent tracers, to be described, are based on observations in a total of 40 rats. They are divided in three groups. In each group several different tracer combinations were used. In group A (Table 1) two different tracers were injected in two termination areas of ascending fibers, i.e. in the ventral thalamus and in the superior colliculus respectively. In groups B (Table 1) and C (Table 2) two different tracers were injected in the trajectories and in some of the termination areas of the ascending and of the descending cerebellar efferent fiber bundles. In all cases the two injections were made on the same side.

Survival times are indicated in Tables 1 and 2. When Bb or NY was injected, the survival time was restricted to a few hours in order to prevent diffusion of the tracers out of retrogradely labeled cell bodies which is revealed by fluorescent labeling of adjacent glial nuclei (Bentivoglio et al. 1980a). Therefore, in these cases TB was injected first and Bb or NY were injected later, some hours before perfusion of the animal (Kuypers et al. 1980). After the appropriate survival times the animals were anesthetized and perfused transcardially with 0.9% saline followed by 10% buffered formalin (pH 7.2). In general the injection sites and the cerebella after being dissected were soaked in 30% cacodylate buffered (pH 7.2) sucrose solution for 6–12 h. The tissue was then cut transversely in 30 µm thick frozen sections, which were immediately mounted from distilled water and air dried. In some cases the formalin perfusion was followed by perfusion with 8% buffered sucrose, after which the cerebella and injection sites were immediately frozen and cut; each section was floated in distilled water and immediately mounted (Bentivoglio et al. 1980a). The sections were always airdried and were not coverslipped (Bentivoglio et al. 1980a). They were stored at 4° C (Kuypers et al. 1980).

The material was studied with a Leitz Ploemopak fluorescence microscope, equipped with Leitz filter-mirror systems N2, D and A providing excitation light of 550 nm, 390 nm and 360 nm

Fig. 3. Semidiagrammatic representation of the distribution of single and double retrogradely labeled cells after NY injection in the diencephalon and TB injection in the superior colliculus *(central column)*. The *left column* portrays the distribution of neurons labeled from the diencephalic injection and the *right column* the distribution of neurons labeled from the tectal injections in the same case: note that the vast majority of neurons labeled from the tectum are also labeled from the diencephalon. (Abbreviations: see Fig. 1)

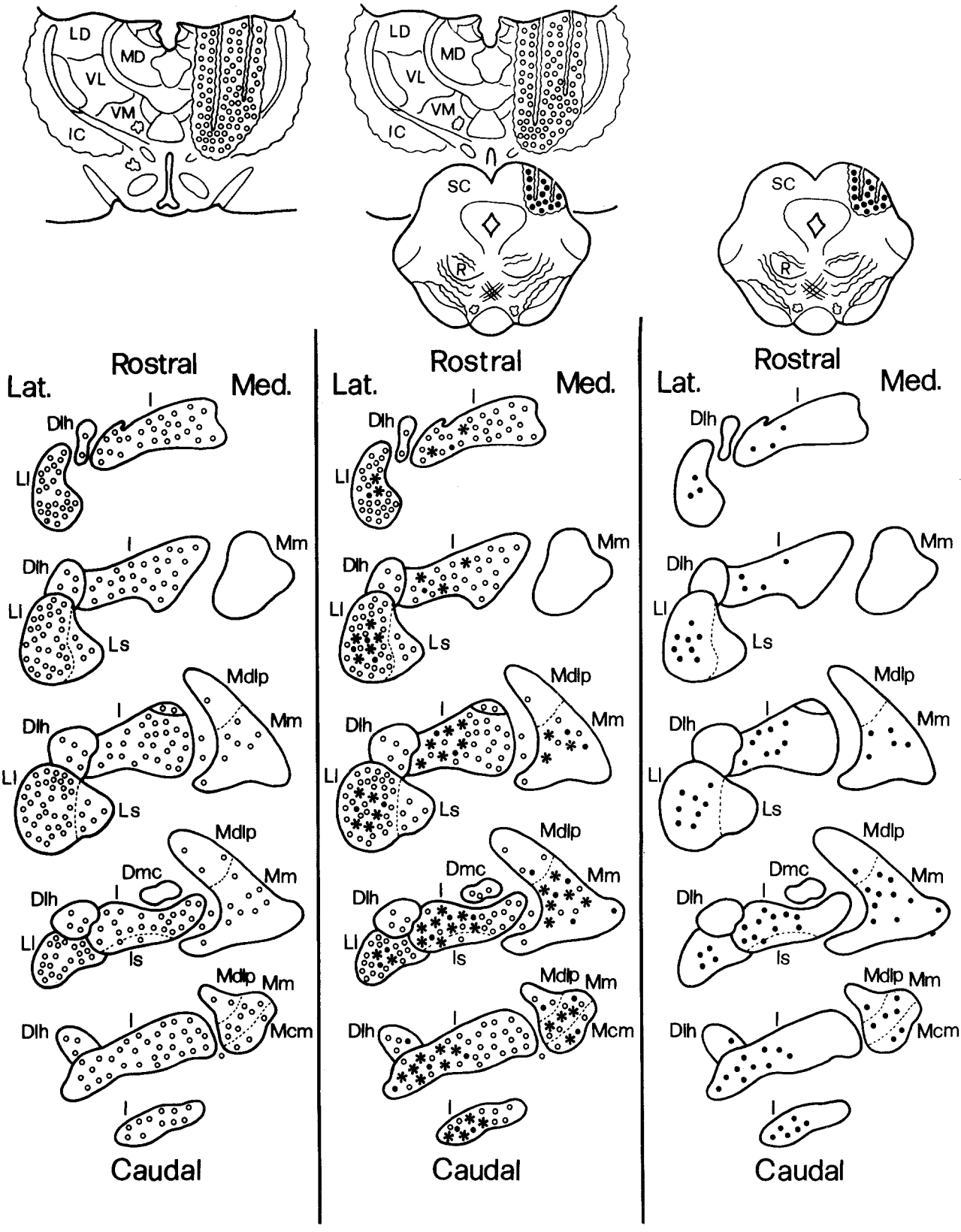

LD
MD
VL
VM
IC
SC
R
Rostral
Lat.
Med.
Caudal
Dlh
Ll
Mm
Ls
Mdlp
Dmc
Mcm
True Blue
Nuclear Yellow
double labeled

Fig. 3

wavelengths respectively. After injections of TB in combination with Bb or NY, the sections were studied with filter-mirror system A. After injections of EB in combination with DAPI-Pr or GB they were studied with filter-mirror systems N2 and A, continuously switching from one filter-mirror system to the other during the observation.

The extent of the injection areas and the distribution of labeled cells in the cerebellar nuclei were charted with an X–Y plotter attached to the microscope stage by means of transducers. The charts were compared with Nissl stained standard series of the rat cerebellum. In addition, some sections containing the retrogradely labeled neurons were counterstained with cresyl violet after plotting.

Results

Part 1. Distribution of the Neurons Retrogradely Labeled from the Different Injection Areas

In order to facilitate the description of the results obtained in double labeling experiments, first the rat's cerebellar nuclei and the distribution of neurons retrogradely labeled from each of the injected area will be described.

The Cerebellar Nuclei in Rat. According to Korneliussen (1968) three main nuclei can be identified in the rat's cerebellum, i.e. the lateral, interpositus and medial nuclei. In addition, two other cell groups can be recognized: the dorsolateral hump (Dlh) and the dorsomedial crest (Dmc) (Figs. 1–3, 5, 7).

The lateral nucleus extends farther laterally and ventrally than the other nuclei. It consists of a large-celled part (Ll) located laterally and an adjoining small-celled part (Ls) located medially and ventrally. The Dlh, which mainly contains large cells, is located dorsal to the border between the lateral and interpositus nuclei, and is present along their entire rostro-caudal extent. The interpositus nucleus is located between the lateral and medial nuclei and extends further caudally than the other two. In transverse sections, no clear border could be distinguished between the anterior and posterior parts of the interpositus nucleus. Therefore, the two subnuclei will not be dealt with separately. The interpositus nucleus contains large, medium and small sized cells. A region with predominantly small cells (Is) occupies the most ventral part of the nucleus, mainly at caudal levels. The Dmc is located dorsal to the medial part of the interpositus nucleus, at the level of the rostro-caudal middle third of the cerebellar nuclei. The medial border of the interpositus nucleus is separated from the lateral border of the medial nucleus by streams of fibers. However, at caudal levels the ventral portions of the two nuclei come into close apposition.

The medial nucleus can be divided into three subdivisions (see also Beitz and Chan Palay 1979a, b). The medial portion (Mm) is present throughout the rostro-caudal extent of the nucleus, and is characterized by a predominance of medium sized cells. In the caudal two-thirds of the medial nucleus the dorsolateral protuberance (Mdlp) occurs, which contains predominantly large neurons and extends more dorsally than the other cerebellar nuclei. This protuberance is particularly prominent in the rostro-caudal middle third of the nucleus, which at these levels displays a triangular shape. The caudomedial part (Mcm) can only be distinguished at the caudal pole of the nucleus and mainly contains small neurons.

Distribution of Retrogradely Labeled Neurons in the Cerebellar Nuclei. The distributions of the labeled neurons obtained by means of the various tracers, including HRP, were very similar. However, in all cases injected with fluorescent tracers a larger number of retrogradely labeled neurons was observed than in the cases injected with HRP, although the areas containing the HRP reaction products at the injection sites were generally of the same dimensions as the fluorescent injection areas. The retrogradely labeled neurons were most numerous in the double labeling experiments using TB and NY (see further Part 2). The description of the distribution of neurons labeled from the various injection sites will therefore be based on results obtained with these two tracers. The HRP experiments were especially used to verify the location of labeled cells in counterstained sections.

A. Injections in the Diencephalon. Injections in the diencephalon were made with each of the tracers (see Tables 1 and 2). The following description is based on the injections with TB followed by 5 days survival (group Cl, Table 2, Fig. 1).

The TB diencephalic injection area consisted of two concentric fluorescent zones (Huisman et al. 1981). They involved the thalamic ventromedial and ventrolateral nuclei, the intralaminar nuclei, the laterodorsal and lateroposterior nuclei, as well as the mediodorsal nucleus, and extended ventrally into the zona incerta and dorsal hypothalamus.

Many blue fluorescent TB labeled neurons, which were predominantly large to medium in size, were present in the contralateral cerebellar nuclei. The cytoplasm of cell body and dendrites as well as the nucleoli were intensely blue fluorescent (see Fig. 6A). Labeled neurons were distributed throughout the rostro-caudal extent of the lateral and interpositus nuclei and in the caudal part of the medial nucleus, especially in Mm-labeled neurons were also present in Dlh and Dmc and a few were scattered

Table 1

Group	Number of animals	%	Tracer	µl	Injection site	%	Tracer	µl	Injection site	Survival time	Survival time
	2	1%	NY	0.4	Ventr. thal.	2%	TB	0.6	Sup. coll.	NY: 13, 15 h	TB: 8,5 days
	3	1%	Bb	0.4	Ventr. thal	2%	TB	0.7	Sup. coll.	Bb: 6, 12, 15 h	TB: 4 days
A	1	10%	EB	0.3	Ventr. thal.	10%	DAPI-			EB: 3 days	DAPI-Pr:
						2.5%	Pr	0.7	Sup. coll.		3 days
	1	10%	DAPI-	0.6	Ventr. thal.	10%	EB	0.3	Sup. coll.	DAPI-Pr: 3 days	EB: 3 days
		2.5%	Pr								
	1	2%	GB	0.6	Ventr. thal	10%	EB	0.3	Sup. coll.	GB: 3 days	EB: 3 days
B1	3	2%	TB	0.6	Ventr. thal. +	1%	NY	1.0	Spin. C2	TB: 5 days	NY: 17, 18 h
				0.6	Sup. coll.						
B2	2	2%	TB	0.8	Midbrain tegmentum	1%	NY	1.2	Spin. C2	TB: 5 days	NY: 19 h
B3	5	2%	TB	0.8	Ventr. thal.	1%	NY	1.0	Spin. C2	TB: 5 days	NY: 17–19 h
	1	10%	EB	0.5	Ventr. thal.	5%	GB	1.0	Spin. C2	EB: 4 days	GB: 4 days
B4	2	2%	TB	0.6	Sup. coll.	1%	NY	1.0	Spin. C2	TB: 5 days	NY: 17, 19 h
	1	5%	TB	0.6	Sup. coll.	1%	Bb	1.0	Spin. C2	TB: 5 days	Bb: 11 h

Table 2

Group	Number of animals	%	Tracer	µl	Injection site	%	Tracer	µl	Injection site	Survival time	Survival time
	4	2%	TB	0.8	Ventr. thal.	1%	NY	0.5	Inf. olive, med. Ret. form.	TB: 4–5 days	NY: 14–15 h
	1	2%	TB	0.7	Ventr. thal.	1%	Bb	0.4	Inf. olive, med. Ret. form	TB: 6 days	Bb: 8 h
	1	2%	TB	0.9	Ventr. thal.	1%	NY	0.3	mainly Inf. olive[a]	TB: 6 days	NY: 15 h
	1	2%	TB	0.8	Ventr. thal.	1%	Bb	0.4	mainly Inf. olive[a]	TB: 5 days	Bb: 11 h
C1	1	1%	Bb	0.4	Ventr. thal.	2%	TB	0.4	mainly Inf. olive[a]	Bb: 11 h	TB: 3 days
	1	1%	Bb	0.4	Ventr. thal.	2%	TB	0.4	Inf. olive, med. Ret. form.	Bb: 14 h	TB: 4 days
	1	10%	EB	0.5	Ventr. thal.	2%	GB	0.5	Inf. olive, med. Ret. form.	EB: 2 days	GB: 2 days
	2	2%	TB	0.8	Midbrain tegmentum	1%	NY	0.6	Inf. olive, med. Ret. form.	TB: 4–6 days	NY: 14–16 h
C2	1	5%	TB	0.8	Midbrain tegmentum	1%	Bb	0.4	Inf. olive, med. Ret. form.	TB: 6 days	Bb: 10 h
	1	10%	EB	0.6	Midbrain tegmentum	2%	GB	0.4	Inf. olive, med. Ret. form.	EB: 3 days	GB: 3 days
	3	2%	TB	0.5	Sup. coll	1%	NY	0.6	Inf. olive, med. Ret. form.	TB: 4 days	NY: 14–15 h
C3	1	10%	EB	0.4	Sup. coll.	2%	GB	0.5	Inf. olive, med. Ret. form.	EB: 3 days	GB: 3 days

[a] In these cases there was only little involvement of the medial reticular formation in the injection area

among the fiber bundles between the interpositus and medial nuclei. No labeled cells were seen in the rostral part of the medial nucleus.

Some labeled cells were scattered throughout the ipsilateral cerebellar nuclei in the same areas as on the contralateral side; they were particularly numerous in the most caudal part of the medial nucleus.

After the longest TB survival times (6–8 days) silver fluorescent granules occured in the blue TB fluorescent cytoplasm (cf. Bentivoglio et al. 1979a). When the formalin perfusion had been followed by perfusion with 8% sucrose, the TB labeling of neurons mainly consisted of blue granules in the cytoplasm, resembling the GB labeling.

In 2 rats with NY injections in the diencephalon and in 3 rats with Bb injections (group A, Table 1) the distributions of the NY and Bb labeled neurons were roughly the same as that of the TB labeled ones. The NY labeled neurons after 13 and 15 h survival time were surrounded by only a few dull fluorescent glial nuclei, indicating only a minimal diffusion of the tracers out of labeled neurons. Therefore they could with confidence be considered as genuinely retrogradely labeled (Bentivoglio et al. 1980a; Kuypers et al. 1980). In the cases with Bb injections followed by 6 h survival, a few dull Bb fluorescent neuronal nuclei were present. After 12 h they were more numerous and more intensely fluorescent and were surrounded by dull fluorescent glial nuclei, which were more numerous after 15 h.

B. Injections in the Mesencephalic Tegmentum. TB and EB were injected in the mesencephalic tegmentum (group B2, Table 1; group C2, Table 2). The injection area as well as the distribution of retrogradely labeled cerebellar neurons after TB injection is shown in Fig. 1. The injection area involved red nucleus, periaqueductal central grey, mesencephalic reticular formation and superior colliculus. It therefore involved both the trajectory of fibers of the ascending limb of the brachium conjunctivum, which were interrupted by the needle penetrations, as well as their termination areas in e.g. red nucleus and tectum (see Faull and Carman 1978).

The distribution of the TB labeled neurons in the contralateral cerebellar nuclei was roughly the same as after injections in the thalamus. However, the neurons in the lateral and interpositus nuclei were in general less fluorescent than in the previous cases, indicating that retrograde transport from broken fibers gives a less pronounced retrograde labeling than retrograde transport from the termination area. A few labeled cells were also seen ipsilaterally in the same regions as on the contralateral side, without the prevalence in the caudal part of the medial nucleus which was observed in the cases injected in the diencephalon.

C. Injections in the Superior Colliculus. TB, EB and DAPI-Pr were injected (groups A and B4, Table 1; group C3, Table 2). The present description is based on the TB injection shown in Fig. 1. The fluorescent injection area was confined to the lateral two-thirds of the superior colliculus, including its deep layers.

In the contralateral cerebellar nuclei, TB labeled neurons were scattered in the central and ventral portions of Ll, in the lateral part of the interpositus nucleus, especially its caudal half, and in the caudal part of the medial nucleus, mainly in Mm. A very few TB labeled neurons were seen in the caudal part of Dlh, but none were present in Dmc and in the rostral pole of the medial nucleus (Mm). Very few labeled neurons (from 1 to 3 in some sections) were seen in the ipsilateral cerebellar nuclei in the same regions as contralaterally.

D. Injections in the Medulla Oblongata. NY, Bb and GB were injected in the medulla oblongata (group C, Table 2). The case shown in Fig. 2 portrays the distribution of the NY labeled neurons after a NY injection in the medulla oblongata followed by 14 h survival. The injection area consisted of three concentric zones (Bharos et al. 1981) which involved the medial gigantocellular reticular formation and the inferior olive (Fig. 2). The first and second zones around the needle track displayed yellow tissue fluorescence with numerous fluorescent glial nuclei. The third zone, displayed an accumulation of fluorescent glial nuclei but no tissue fluorescence. In the medullary injections, the needle penetration may have also interrupted some cerebello-spinal fibers, which are located close to the inferior olive (Achenbach and Goodman 1968; Asanuma et al. 1980; Asanuma et al. 1980; Batton et al. 1977; Faull 1978; Ware and Mufson 1979).

Many retrogradely labeled neurons were seen throughout the rostro-caudal extent of the contralateral lateral, interpositus and medial nuclei. In most of the neurons only the nucleus was NY labeled, which showed a brilliant yellow fluorescence with a brilliant ring around the nucleolus (see Fig. 6C). In a few NY labeled neurons some yellow fluorescent fine grains were present in the cytoplasm. No fluorescent glial nuclei were seen around the NY labeled neurons, indicating that after 14 h survival the tracer had not diffused out of the cell. The vast majority of the NY labeled neurons in the lateral and interpositus nuclei were small. They were very numerous in Ls and Is, but were also distributed over the other regions. In addition, some medium and large sized NY labeled neurons were observed in Ll and in the interpositus nucleus. Large and small sized labeled neurons were also seen in Dlh, where they were less numerous than in the lateral and interpositus nuclei. No labeled cells were seen in Dmc, but some large and medium sized NY labeled cells were scattered among the fibers between the interpositus and medial nuclei. In the medial nucleus the NY labeled neurons were very numerous in the rostral part of Mm; proceeding caudally their number decreased. The majority of the NY labeled neurons in Mm were medium sized. Some small labeled neurons were also observed in Mm, mainly located in its ventral part. Moreover, strikingly enough, in these cases the Mdlp

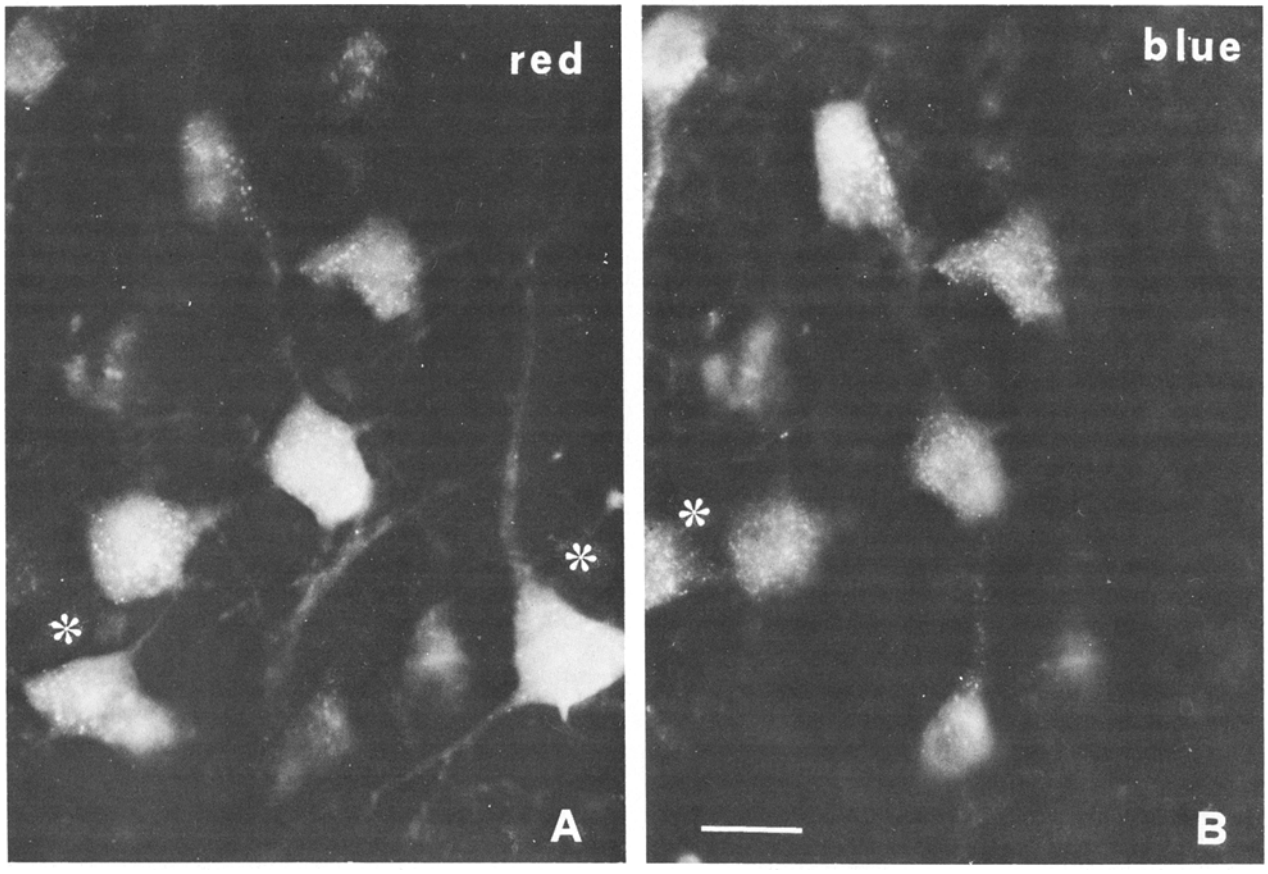


Fig. 4A, B. Photomicrographs of fluorescent retrogradely labeled neurons in the lateral part of the interpositus nucleus after Evans Blue injections in the diencephalon and Granular Blue injections in the superior colliculus. **A** red fluorescent Evans Blue labeled neurons illuminated with 550 nm light excitation wavelength; **B** blue fluorescent Granular Blue labeled neurons in the same field, illuminated with 360 nm excitation wavelength. Note the occurrence of several double labeled both red and blue fluorescent neurons. White asterisks indicate single Evans Blue labeled *(on the left)* and single Granular Blue labeled *(on the right)* neurons. Scale bar: 20 μm

was packed with large NY labeled neurons throughout its rostro-caudal extent. A few NY labeled medium or large sized neurons were seen throughout the rostro-caudal extent of the ipsilateral medial nucleus (1–2 cells per section) as well as in the ipsilateral Dlh. The same distribution as described above was observed in most of the other cases with injections in the medulla oblongata.

In three cases injected with NY, Bb and TB respectively (group C1, Table 2) the medullary injection area was largely confined to the inferior olive with little involvement of the medial reticular formation. In these cases some differences from the preceding cases were observed. The lateral and interpositus nuclei and Dlh contained almost exclusively small sized labeled neurons and only a few medium and large sized labeled neurons were seen, especially in the most lateral part of the interpositus nucleus. In these cases, as in the preceding ones, some large and medium sized labeled cells were seen in the medial part of the caudal half of the interpositus nucleus as well as scattered among the fibers between the interpositus and medial nuclei. In the medial nucleus the most striking feature, in contrast with the preceding cases, was the fact that only a few NY, Bb or TB labeled neurons were present in Mdlp. Moreover, in Mm the medium sized labeled cells were less numerous than in the preceding cases, whereas small sized labeled neurons were still present in the ventral part of the nucleus.

E. Injections in the C2–C3 Spinal Segments. After a large unilateral NY injection in the cervical cord, which slightly extended to the other side, followed by 17 h survival (group B1, Table 1; Fig. 2) no NY labeled neurons were seen in the rostral third of the contralateral lateral and interpositus nuclei and in Dlh. However, NY labeled neurons did occur laterally: in the dorsal part of the middle third of Ll at the border between Ll and Dlh (2 to 6 neurons per section) and medially: in the medial extremity of the caudal half of the interpositus nucleus, in the medial

nucleus and scattered among the fiber bundles between these two nuclei (Fig. 2). In the medial nucleus NY labeled neurons were present in its caudal half but several were also present in the rostral half of Mm; only few NY labeled neurons were present in Mdlp.

Part 2. Single and Double Labeling of Neurons After Injections of Two Tracers in Different Termination Areas

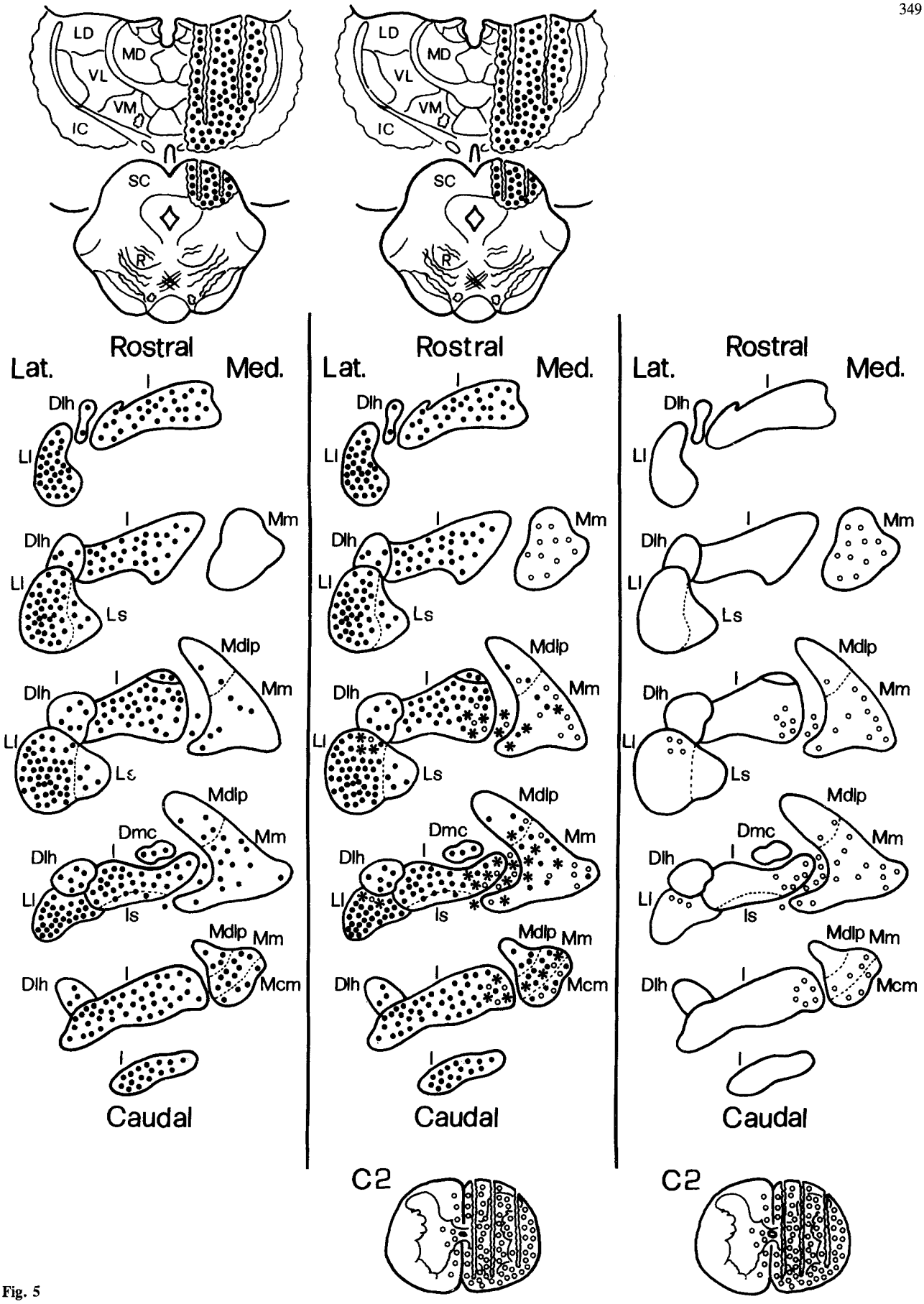
Group A. Injections of One Tracer in Diencephalon and of the Other in Superior Colliclulus. In the cases with NY injections in diencephalon and TB injections in superior colliculus followed by 8 days TB survival and 15 h NY survival (Table 1, Fig. 3) the neurons in the contralateral cerebellar nuclei which were NY labeled from the diencephalon were distributed as described above. In addition, several of the many NY labeled neurons were NY-TB double labeled, showing a blue fluorescent TB labeled cytoplasm and a brilliantly yellow NY labeled nucleus (see Fig. 6B). They were located, intermixed with a few single TB labeled ones, in: the central part of Ll, the lateral part of the interpositus nucleus and the caudal two-thirds of the Mm portion of the medial nucleus. The same results were obtained in the other cases, including those injected with other tracers (i.e. Bb and TB, EB and DAPI-Pr, GB and EB, Table 1). However, in the cases injected with a red fluorescent and a blue fluorescent tracer (EB and DAPI-Pr; EB and GB) double labeled neurons were red fluorescent and blue fluorescent when illuminated with light of 550 nm and 360 nm wavelength respectively (Fig. 4). These double labeled neurons were in general less brilliantly fluorescent in either colour than the single labeled ones (cf. van der Kooy et al. 1978).

In all the cases at least two-thirds of the neurons labeled from the tectum were also labeled from the diencephalon. This indicates that the fibers from the deep cerebellar nuclei to the tectum consist for a major part of collaterals of cerebello-diencephalic fibers.

Group B. Injections of One Tracer in the Diencephalon and Mesencephalon and of the Other in Cervical Cord. In the cases injected with TB in both diencephalon and superior colliculus followed by 5 days TB survival and injected with NY in C2–C3 spinal segments followed by 17 h NY survival time (group B1, Table 1, Fig. 5) the following findings were obtained. In keeping with the preceding description, in the rostral third of the cerebellar nuclei TB labeled neurons were located in the lateral and interpositus nuclei, while NY labeled ones were located in the medial nucleus. However, at more caudal levels, especially in the rostro-caudal middle third of the cerebellar nuclei, the two populations of TB and NY labeled neurons overlapped in several regions and in these regions many double labeled neurons were present, i.e. laterally: in the dorsal part of Ll, close to the border with Dlh, and medially: in the medial parts of the interpositus nucleus, between this nucleus and the medial nucleus, as well as in the lateral portion of the medial nucleus, mainly in Mm. These double labeled neurons (Fig. 6B) were particularly numerous in the medial tip of the interpositus nucleus. A similar distribution pattern was observed in the other cases in which either diencephalic or mesencephalic tegmentum injections were combined with cervical injections (groups B1, B2, B3, Table 1).

Thus, in the cerebellar nuclei two concentrations of branching neurons projecting to the spinal cord appear to exist, which are located medially and laterally. These branching neurons project rostrally either to the diencephalon or to the superior colliculus or to both. In the cases of group B4, however, in which TB injections in the superior colliculus were combined with NY injections in the C2–C3, relatively few double labeled neurons were observed. Thus in Ll single TB labeled neurons and a few more dorsally located single NY labeled ones were present. In the interpositus nucleus single TB labeled neurons were located laterally and single NY labeled ones occured medially. Finally in the medial nucleus single NY labeled neurons were present both rostrally and caudally, while single TB labeled ones were present caudally. In addition, a limited number of double labeled neurons was present in the caudal two-thirds of the medial nucleus, mainly in Mm. These findings indicate that the majority of the branching neurons in the cerebellar nuclei, which project to the spinal cord, also project rostrally to the diencephalon, while

Fig. 5. Semidiagrammatic representation of the distribution of single and double retrogradely labeled cells after TB injections in diencephalon and tectum and NY injections in the cervical cord *(central column)*. The left column portrays the distribution of neurons labeled from the diencephalic and tectal injections and the right column the distribution of neurons labeled from the cervical injection in the same case. (Abbreviations: see Fig. 1)


LD
MD
VL
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IC
SC
R
Rostral
Lat.
Med.
Dlh
Ll
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Ls
Mdlp
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Is
Mcm
Caudal
C2
:: True Blue
:: Nuclear Yellow
** double labeled

Fig. 5

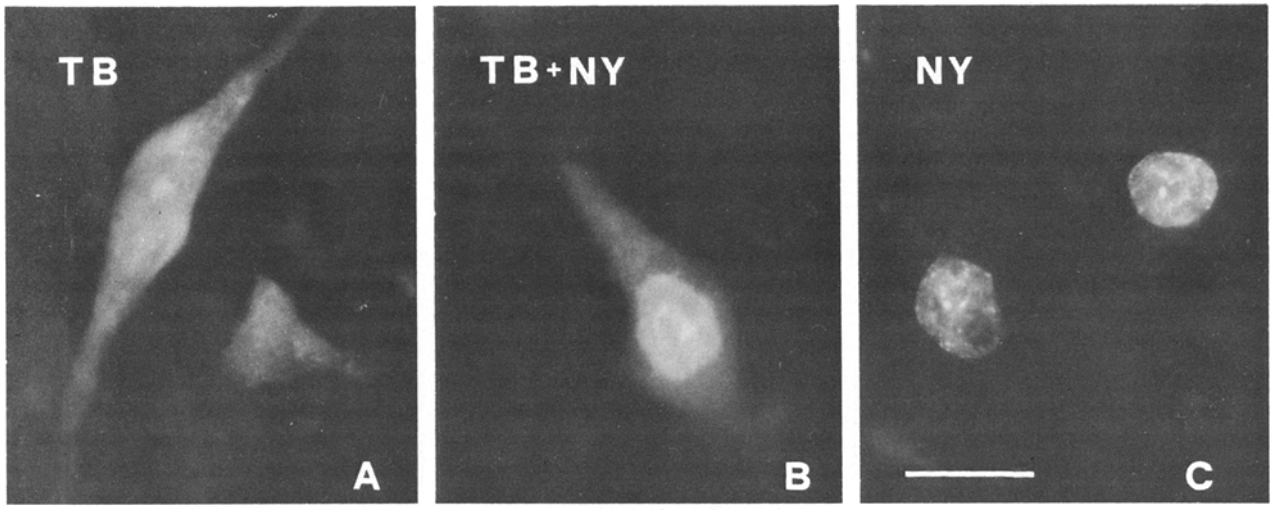

Fig. 6A–C. Photomicrographs of fluorescent retrogradely labeled neurons illuminated with 360 nm light excitation wavelength in the medial part of the interpositus nucleus after *TB* injections in diencephalon and tectum and *NY* injections in cervical cord. **A** *TB* single labeled neurons, with blue fluorescence of the cytoplasm and nucleolus; **B** *TB-NY* double labeled neuron displaying the features of labeling of both tracers; **C** *NY* single labeled neurons with yellow fluorescence of the nucleus including a brilliant ring around the nucleolus. Scale bar: 20 μm

only few of them project to the superior colliculus. The latter are located in the caudal part of the medial nucleus.

Group C. Injections of One Tracer in Diencephalon and Mesencephalon and the Other in Medulla Oblongata. In some rats injections were made in medulla oblongata instead of in the spinal cord. In the cases with TB injections in diencephalon and NY injections medially in medulla oblongata involving inferior olive and medullary medial reticular formation (group C1, Table 2, Fig. 7) the following findings were obtained. As observed in the preceding cases, single TB labeled neurons were present throughout the lateral and interpositus nuclei and in the caudal portion of the medial nucleus. These neurons were intermixed with single NY labeled ones, many of which were rather small. Further, many single NY labeled neurons were present in the rostral part of the medial nucleus. Double labeled neurons were also present, which in general were large to medium in size. Some of these double labeled neurons were distributed in the same way as after injections in diencephalon and tectum combined with injections in the spinal cord (group B1, Table 2). Thus, they were located laterally: in the dorsal part of the caudal third of Ll and medially: in the medial part of the caudal two-thirds of the interpositus nucleus and the lateral parts of the caudal two-thirds of the medial nucleus (Mm) as well as in the area between these two nuclei. In view of this distribution, the NY labeling of these double labeled neurons may have resulted from the interruption of cerebello-spinal fibers, which are located close to the inferior olive. However, in the cases of this group with medullary injections, in contrast to those with spinal cord injections, double NY-TB labeled neurons were also present in other areas i. e. medially: in the Mdlp portion of the medial nucleus and laterally: in the rostal two-thirds of the Dlh as well as in the most lateral part of the rostral two-thirds of the interpositus nucleus at the border with the Dlh. Therefore, in addition to the branching neurons in the medial and lateral portion of the cerebellar nuclei, which project to the spinal cord, other branching neurons appear to exist, which project to medulla oblongata.

Finally, in the three cases in which the medullary

Fig. 7. Semidiagramatic representation of the distributions of single and double retrogradely labeled neurons after TB injection in diencephalon and NY injection in medulla oblongata *(left column)*, and after TB injection in superior colliculus and NY injection in medulla oblongata *(central column)*. The *right column* portrays the distribution of labeled cells after TB injections in diencephalon and tectum and NY injection in the cervical cord in the case represented also in fig. 5. Notice that medullary injection typically results in labeling of neurons in Mdlp. Note also that the distribution of double labeled neurons medially and laterally in the cerebellar nuclei is quite similar after medullary and spinal injections (cf. *left* and *right columns*) but that after medullary injections the laterally located double labeled neurons are relatively more numerous. (Abbreviations: see Fig. 1)

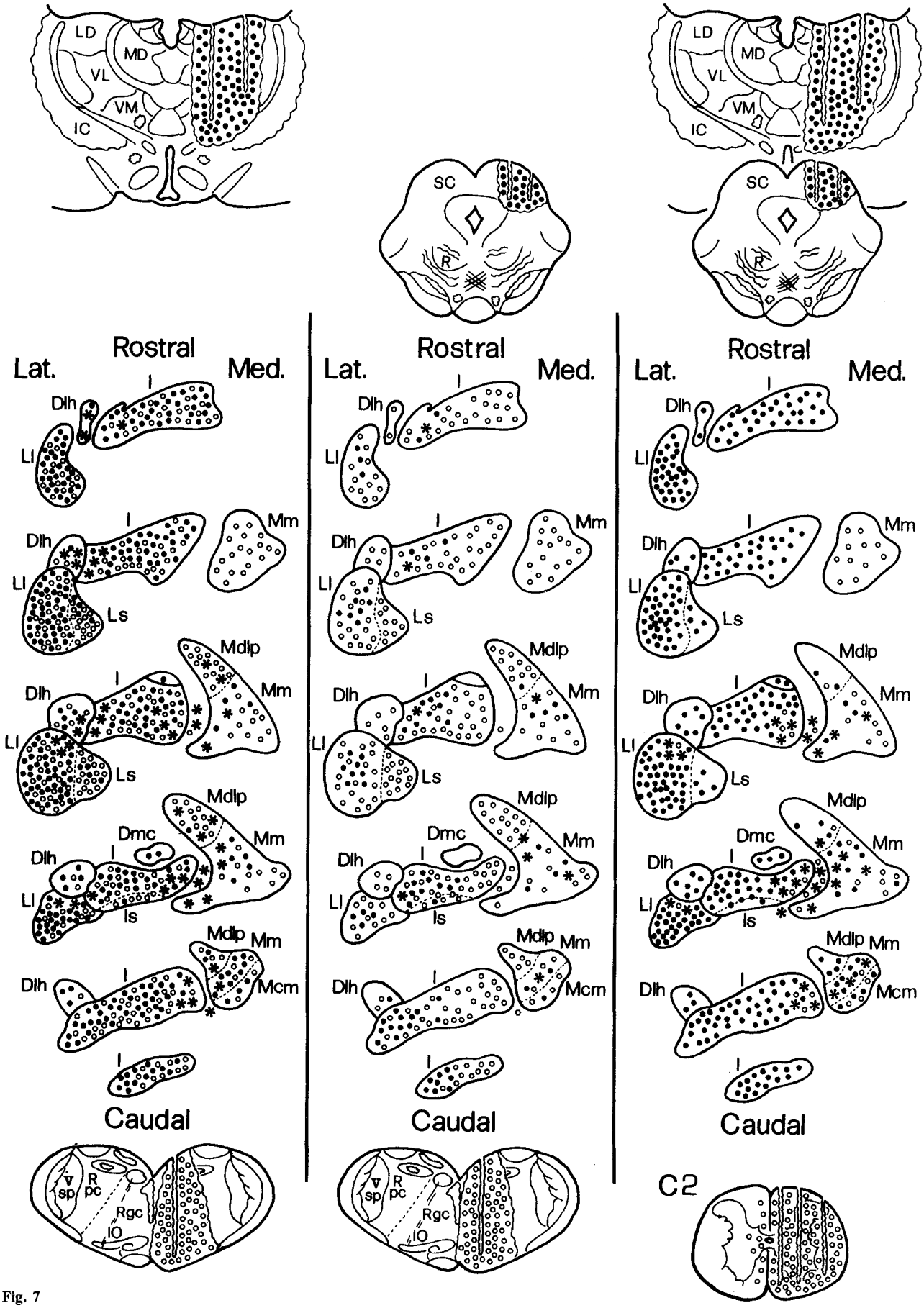
LD
MD
VL
VM
IC
SC
R
Rostral
Lat.
Med.
Dlh
LI
Ls
Mm
Mdlp
Dmc
Is
Mcm
Caudal
v
sp
R
pc
Rgc
IO
C2
True Blue
Nuclear Yellow
double labeled

Fig. 7

injection areas mainly involved the inferior olive and largely spared the medial reticular formation (group C1), mainly small sized labeled neurons in the lateral and interpositus nuclei were single labeled as observed also in the preceding cases. Moreover, as in the preceding cases some double labeled neurons were present, the distribution of which suggests that the labeling from the medullary injections may have resulted from interruption of cerebello-spinal fibers. However, some differences with the preceding cases were also noted, such that an extremely limited number of double labeled neurons occurred laterally: in the Dlh, the lateral part of the interpositus nucleus at the border with the Dlh and medially: in the Mdlp. These findings suggest that the bulk of the branching neurons in these two areas which project to the medulla oblongata distribute their descending collaterals mainly to the medial reticular formation.

In the four cases (group C2) with injections in the midbrain tegmentum (interrupting cerebellar fibers to red nucleus, mesencephalic tegmentum, tectum and diencephalon) combined with injections in the medulla oblongata, the double labeled neurons were distributed in much the same way as after diencephalic injections combined with medullary ones (group C1). However, the double labeled neurons in the Mdlp were slightly more numerous than after the diencephalic injections (group C1). On the other hand in the four cases with TB injections in the tectum and NY injections in the medulla oblongata (group C3, Table 2; Fig. 7) only few double labeled neurons were present, as after combined injections in tectum and spinal cord (group B4, Table 1). These few double labeled neurons were located in the lateral parts of the rostral two-thirds of the interpositus nucleus and in the caudal half of the medial nucleus. From these findings it was inferred that the majority of the branching neurons which project to the medulla oblongata distribute their ascending axon collaterals to diencephalon but not to the superior colliculus.

Discussion

The results of the present study confirm earlier findings and provide new data on the anatomical organization of the cerebellar efferents in rat. Thus, the present findings indicate that the vast majority of the neurons in the deep cerebellar nuclei distribute fibers to the contralateral diencephalon, probably mainly to the thalamus. These cerebello-thalamic neurons are mainly medium to large in size. They occur throughout the rostro-caudal extent of the interpositus and lateral nuclei and in the caudal part of the medial nucleus. This is in keeping with earlier retrograde degeneration (Jansen and Jansen 1955) and retrograde transport findings (Nakano et al. 1980; Tolbert et al. 1978) in cat.

Anterograde degeneration findings in rat (Faull and Carman 1978) emphasized that ascending fibers of the brachium conjunctivum, which probably included the adjoining ascending limb of the hook bundle (Batton et al. 1977; Voogd 1964), are also distributed to the contralateral tectum. The present retrograde neuronal labeling findings (Part 1) indicate that in rat this projection is mainly derived from neurons in the central and ventral part of the lateral nucleus, in the lateral part of the interpositus nucleus and in the caudal part of the medial nucleus. The double labeling results obtained in the group A experiments (Part 2) further demonstrate that the fibers to the contralateral tectum mainly consist of collaterals of neurons projecting to the contralateral diencephalon. In view of earlier electrophysiological (Deniau et al. 1978) and anatomical (Bentivoglio et al. 1979b; Steindler and Deniau 1980) findings, there thus appears to exist a parallel between the cerebellar and nigral projections to the tectum and the thalamus, because also many of the fibers from substantia nigra pars reticulata to the tectum represent collaterals of fibers to the thalamus.

Anterograde degeneration findings (Achenbach and Goodman 1968; Faull 1978) showed that in rat fibers from the deep cerebellar nuclei are also distributed to the spinal cord and the medulla oblongata. The present retrograde neuronal labeling findings (Part 1) indicate that in rat the cerebello-spinal neurons are situated medially: in the intermediate and caudal parts of the medial nucleus (Mm) and in the adjoining medial parts of the interpositus nucleus, in agreement with retrograde labeling findings in cat (Bharos et al. 1981; Matsushita and Hosoya 1978). Moreover, according to the present findings, in rat neurons in the rostral part of the medial nucleus also contribute to this projection as it was also reported in cat (Fukushima et al. 1977). Further, as in cat (Bharos et al. 1981) some cerebello-spinal neurons were found laterally: in the dorsal part of the caudal half of the Ll portion of the lateral nucleus.

The double labeling results in the group B experiments (Part 2) indicate that the cerebellar projections from the medially and laterally located neurons to the spinal cord in part consist of collaterals of neurons distributing ascending fibers to the mesencephalon and diencephalon. The double labeling findings in the cases with injections in the tectum and the spinal cord further showed that only few of these branching neurons which project to the spinal cord also distribute collaterals to the tectum.

The present retrograde labeling findings (Part 1) indicate that cells of various sizes throughout the deep cerebellar nuclei distribute fibers to the medulla oblongata. In the majority of the present cases the large extent of the medullary injection area made it difficult to differentiate between neurons projecting to the medullary medial reticular formation (Achenbach and Goodman 1968) and those projecting to the inferior olive (Chan Palay 1977). According to previous retrograde HRP transport findings in rat (Brown et al. 1977) the cerebello-olivary neurons in general are small and are located in the lateral and interpositus nuclei, in particular in their ventral parts. Similar results were obtained in cat (Buisseret Delmas and Batini 1978; Mc Crea et al. 1978; Tolbert et al. 1976b, 1978). The many small neurons, which were labeled from the medulla oblongata and which were especially numerous in the ventral part of the nuclei, therefore may represent cerebello-olivary neurons. However, a recent HRP study in cat (Dietrichs and Walberg 1981) described that many medium sized neurons project to the inferior olive. Therefore, it cannot be excluded in the present study that some medium sized cerebellar neurons labeled from the medulla oblongata also represent cerebello-olivary neurons. A few small neurons labeled from the medulla oblongata were found in the ventral part of the medial nucleus. These findings may suggest that in rat the cerebello-olivary pathway takes also origin from the medial nucleus, in agreement with anterograde degeneration findings in rat (Achenbach and Goodman 1968) and anterograde as well as retrograde transport findings in cat (Bharos et al. 1981; Dietrichs and Walberg 1981; Sugimoto et al. 1980).

In three cases (group C1; Part 2) the medullary injections largely spared the medial reticular formation. In these cases the labeled medium and large neurons in the lateral and interpositus nuclei and especially in the dorsolateral protuberance of the medial nucleus (Mdlp) were less numerous than in the other cases with medullary injections. These neurons therefore probably project mainly to the medullary medial reticular formation.

The double labeling results of the group C experiments (Part 2) produced further information concerning the branching neurons projecting to the medulla oblongata. First, the vast majority of the small neurons in the lateral and interpositus nuclei, which are assumed to be cerebello-olivary neurons, were single labeled. The majority of the cerebello-olivary neurons therefore probably do not distribute axon collaterals to mesencephalon, diencephalon or spinal cord. Further, after medullary injections combined with mesencephalic tegmentum and diencephalic injections double labeled medium to large sized neurons were present in the same areas as after the spinal injections (see above). These neurons may have represented the branching neurons, which project to the spinal cord and in these cases were retrogradely labeled due to interruption of their fibers in the medulla oblongata. However, after medullary injections double labeled large to medium sized neurons were also present laterally: in the Dlh, in the lateral part of the interpositus nucleus, and medially: in the dorsolateral protuberance of the medial nucleus (Mdlp). These double labeled neurons were not observed after spinal injections. They therefore must represent branching neurons which project to the medulla oblongata. In the three cases of group C1 in which the medullary injections largely spared the medial reticular formation, much fewer double labeled neurons were present in these two areas. This makes it likely that these double labeled neurons in the medial and lateral parts of the cerebellar nuclei are branching neurons the descend-

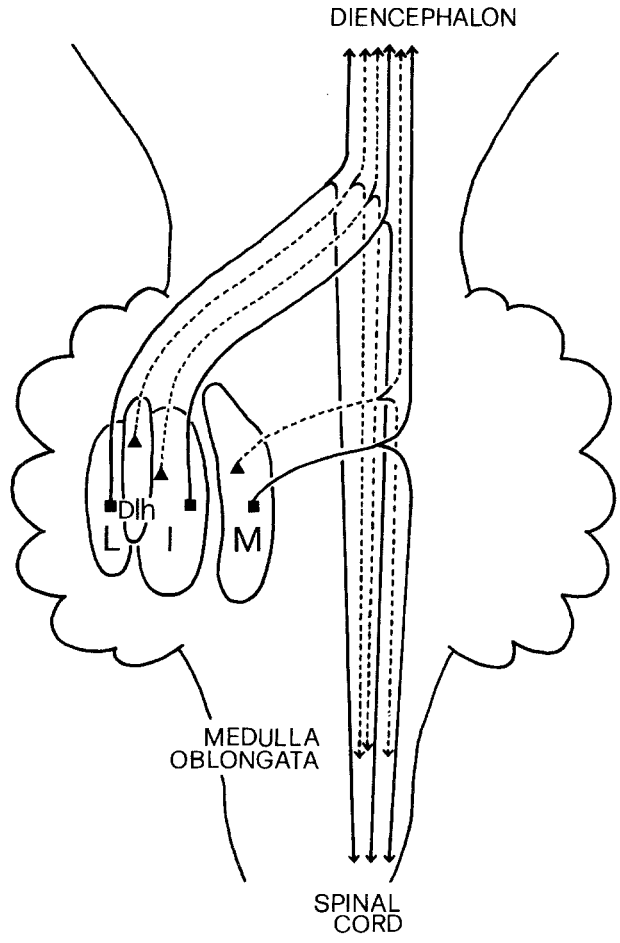

Fig. 8. Diagram of the distribution of divergent axon collaterals from medially and laterally located cerebellar neurons to the diencephalon, medulla oblongata and spinal cord

ing collaterals of which project to medullary structures outside the inferior olive, i.e. the medullary medial reticular formation. Finally, a comparison of the findings after injections in the medulla oblongata combined with injections in the diencephalon and in the tectum respectively indicate that only few of the branching neurons projecting to the medulla oblongata distribute collaterals to the tectum and that the majority projects to the diencephalon.

The present double labeling findings in rat (Part 2), described diagrammatically in Fig. 8, are in keeping with those in cat (Bharos et al. 1981). In cat also a laterally and medially located group of branching neurons was encountered, the descending collaterals of which are distributed either to the spinal cord or to the medulla oblongata. In cat as in rat they were found laterally: at the border between the lateral and interpositus nuclei and medially: in the medial nucleus and in the adjoining part of the interpositus nucleus. On the other hand, the present conclusions regarding the branching neurons may be at variance with electrophysiological findings (Ban and Ohno 1977; McCrea et al. 1978; Tolbert et al. 1978) which indicated that some cerebello-olivary neurons are branching neurons with ascending collaterals to thalamus while in the present study the vast majority of the small neurons which were assumed to be the cerebello-olivary ones, were found to be single labeled. However, further anatomical double labeling studies are necessary to clarify this point since in the present experiments the neuronal population projecting to the medullary reticular formation and to the inferior olive could not be differentiated properly.

The descending collaterals from the two populations of branching neurons i.e. in the lateral and medial parts of the cerebellar nuclear complex, probably reach their destination along two different routes. Thus, the laterally located neurons in all likelihood contribute fibers to the crossed descending limb of the brachium conjunctivum (Chan Palay 1977; Cohen et al. 1958; Faull 1978; Graybiel et al. 1973; Thomas et al. 1956; Voogd 1964). According to Cajal (1972) this bundle, which he regarded as a cerebello-olivo-spinal pathway, consists largely of contralaterally descending collaterals of ascending fibers of the brachium conjunctivum. This is in keeping with the double labeling of some of the laterally located neurons. On the other hand, the contralaterally descending collaterals of the medially located branching neurons in all likelihood reach their destination by way of the descending limb of the crossed hook bundle (uncinate tract) (Batton et al. 1977; Cohen et al. 1958; Thomas et al. 1956; Voogd 1964).

It has been seldom reported that cerebellar neurons projecting to the medullary medial reticular formation and the spinal cord are located not only medially in the cerebellar nuclei: in the medial nucleus and in the medial part of the interpositus nucleus, but also laterally: in the lateral nucleus, the Dlh and the adjoining part of the interpositus nucleus. The dual location of these neurons was therefore somewhat surprising. However, in retrospect this dual location seems to be in keeping with the fact that in cat (cf. Sugimoto et al. 1981) and in monkey (cf. Batton et al. 1977; Chan Palay 1977; Kievit 1979; Kievit and Kuypers 1972; Thach and Jones 1979) the projections both from the medial and lateral parts of the cerebellar nuclei (i.e. from the interpositus and medial nuclei and from the dentate nucleus) to the thalamus in part overlap in the medial portion of the ventrolateral nuclear complex. This would imply that the cerebello-thalamic connections in part are established by two sets of overlapping fiber projections, each of which gives rise to some descending collaterals to medulla oblongata and spinal cord.

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