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A study of branching in the projection from the inferior olive to the x and lateral c_1 zones of the cat cerebellum using a combined electrophysiological and retrograde fluorescent double-labelling technique

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Summary. The pattern of transverse branching in the olivocerebellar projection to the x zone in the vermis and the lateral c_1 zone in the paravermis of the cat anterior lobe was studied using a combined electrophysiological and retrograde double-labelling tracer technique. Fluorochrome-tagged latex microspheres were well suited for this purpose. The results show that the region of olive that supplies climbing fibres to the two zones forms a continuous, rostrocaudally directed column about 2.25 mm in length, in a caudo-lateral to rostromedial part of the medial accessory olive (MAO), on average between A–P levels 12.50–10.50. This column may be divided into caudal and rostral halves that project respectively to the x and lateral c_1 zones in the apical folia of lobules V/VIa. Partial overlap between these two territories occurs in an intermediate region (A–P levels 12.00–11.00) in middle MAO where olive cells that supply climbing fibres to either x or lateral c_1 are intermingled with a smaller population of cells whose axons branch to provide climbing fibres to both zones. Quantitative analysis showed that, when different tracers were injected into each zone in the same animal, double-labelled cells represented only 5–7% of either single-labelled cell population within this area of overlap. It is concluded that, although some transverse branching is present within the olivocerebellar projection to the x and lateral c_1 zones in the apical folia of lobule V, such branching is not extensive.

Key words: Inferior olive – Cerebellum – Climbing fibres – Branching – Fluorescent tracers

Introduction

It is now well-established that the cerebellar cortex in the cat is divisible into a number of narrow, longitudinal zones each of which receives its climbing fibre afferents from cells in a restricted portion of the inferior olivary complex (for a review see Brodal and Kawamura 1980). Overall, eight zones have been identified on either side of the midline in the anterior lobe of the cerebellum: from medial to lateral these have been designated the a, x and b zones in the vermis, the c_1, c_2 and c_3 zones in the paravermis and the d_1 and d_2 zones further laterally (e.g. Groenewegen and Voogd 1977; Groenewegen et al. 1979). Further studies have shown that among these zones the c_1 and c_3 zones are each subdivisible into a medial and a lateral half (Ekerot and Larson 1982; Campbell and Armstrong 1985; Trott 1989).

Anatomical studies have demonstrated that olivocerebellar axons branch in the rostrocaudal plane (e.g. in the cat: Brodal et al. 1980; Rosina and Provini 1983, 1987 and in the rat: Wiklund et al. 1984; Wharton and Payne 1985; Payne et al. 1985; Hrycyshyn et al. 1989). Additional anatomical studies in the rabbit have shown that some branching also occurs in the mediolateral plane (Takeda and Maekawa 1984). However, none of these studies investigated the pattern of branching in the olivocerebellar projection to individual cortical zones. Anatomical studies at a higher level of resolution are required since physiological investigations have shown that olivocerebellar axons frequently branch in the rostrocaudal plane so that an individual olivary neurone provides climbing fibres to several Purkinje cells located at different points along the length of a single cortical zone. This was initially established by using electrical stimuli to excite in one branch of the parent axon an action potential that propagated antidromically back to the branch point and then orthodromically along another branch to evoke a complex spike in the Purkinje cell innervated by that climbing fibre (Armstrong et al. 1973). Similarly, the axon-reflex technique has been used to show that some olivocerebellar axons also branch in the mediolateral plane so as to provide climbing fibres to two cortical zones which are thereby 'linked' into pairs. Such linking has been found between the x zone and the lateral

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half of the c_1 zone (the latter being therefore sometimes termed the cx zone; Campbell and Armstrong 1985), between the medial halves of the c_1 and c_3 zones and between the d_2 and the lateral half of the c_3 zone (Ekerot and Larson 1982). The existence of such mediolateral branching implies that two zones or sub-zones linked as a pair will contain at least some Purkinje cells that receive their climbing fibre input from the same olivary neurone. This may have important functional consequences in view of the evidence that the zones may be important operational units in the coordinative role of the cerebellum in motor control (Oscarsson 1979). In this connection, cross-linkages between the x and lateral c_1 zones are of particular interest because these two zones are situated respectively in the vermal and the paravermal parts of the cortex. The vermis is thought to be concerned principally with equilibrium and the regulation of body posture while the paravermis is concerned more with the regulation of movements of individual limbs (Chambers and Sprague 1955). Branching in the olivocerebellar pathways to the x and lateral c_1 zones could therefore assist in coordinating the activity of two cerebellar regions with different functional responsibilities.

A previous retrograde transport study in the cat, which used wheatgerm-agglutinin horseradish peroxidase (WGA–HRP) as tracer (Campbell and Armstrong 1985), has shown that olive cells projecting to the x zone are located in the mid-portion of the medial accessory olive while cells projecting to the lateral c_1 zone are located in a region of the medial accessory olive that is centred slightly further rostrally, but which appears to overlap partially with the region projecting to the x zone. It might be predicted that cells projecting to both zones would lie in the region of overlap. In view of the difficulty of restricting WGA–HRP injection sites to a single cortical zone or sub-zone it is desirable that these results are replicated using an alternative tracer. Moreover, the precise location of cells whose axons branch to innervate both zones has not yet been directly determined nor have the relative numbers of cells projecting to both zones as compared with those projecting to only one zone in the pair.

The aim of the present series of experiments was to investigate in detail the olivocerebellar projection to those parts of the x and lateral c_1 zones in the apical folia of lobules V and VIa. A combination of electrophysiological and fluorescent retrograde tracing techniques has been devised suitable to address the following questions:

1. To what extent are cells projecting to the x zone intermingled with or spatially separate from those that project to the lateral c_1 zone?
2. What is the location of olive cells that project to both zones?
3. How numerous are those olive cells that innervate both zones in relation to cells which project to only one member of the pair?

Material and methods

The surgical procedures and electrophysiological recording techniques have been described in detail in previous papers (see for example Trott and Armstrong 1987a, b; Trott et al. 1990) and are therefore considered only briefly below. The experiments were performed on a total of 8 purpose bred cats. At an initial aseptic operation general anaesthesia was induced with sodium pentobarbitone (Sagatal, 40 mg/kg, ip., BDH) and maintenance doses of the anaesthetic were then administered as required via an intravenous saline drip. An antibiotic (Propen, 0.5 ml, i.m., Glaxovet) and atropine sulphate (0.5 ml, s.c., BK) were also routinely administered. A small craniotomy was performed to expose the dorsal surface of the cerebellum in the region of the vermal and paravermal cortex of lobules V and VI (nomenclature after Larsell 1953).

Electrophysiology

Percutaneous stimulation of the left or right forepaws, at a rate of 1 stimulus per 2 secs, was used to set up volleys in the spino-olivocerebellar paths (SOCPs) and this activity was recorded as extracellular climbing fibre field potentials on the cortical surface using a tungsten-in-glass microelectrode. In some experiments the climbing fibre response was facilitated by presenting a pair of stimuli (1 ms separation) at the same rate as above. As the electrode was moved over the surface of a folium from medial to lateral, the latency and pattern of peripheral convergence of the evoked climbing fibre field potential changed in accordance with the known characteristics of the different cortical zones.

The x and lateral c_1 zones were identified by 1) the presence of relatively short latency (range 13.5–18 ms, $n=8$ cats) climbing fibre field potentials evoked in response to stimulation of the ipsilateral (but not the contralateral) forepaw, 2) by their respective positions in the vermal and medial paravermal parts of the cortex and 3) by the different climbing fibre response characteristics of the neighbouring zones. Note, however, that in the present study it was not possible to distinguish electrophysiologically between the lateral and medial parts of the c_1 zone because both receive inputs via common SOCPs. Therefore, in each case in which the lateral c_1 zone was studied, the zone was judged to be located in the lateral 'geographical' half of the overall c_1 zone.

The x zone was found on occasion to be flanked medially by a cortical zone exhibiting long latency responses evoked by percutaneous stimulation of the ipsilateral hindlimb and trunk, a pattern of response consistent with the known characteristics of the a zone. In addition, it was flanked laterally by a zone exhibiting long latency responses evoked by stimulation of both forelimbs as expected for the medial part of the b zone. In comparison, the c_1 zone was found routinely to be flanked medially by a cortical zone exhibiting long latency responses following stimulation of either hindlimb, consistent with the electrophysiological profile of the lateral b zone, whereas recordings made lateral to the c_1 zone typically consisted of long latency responses which could be evoked by stimulation of all four limbs, properties consistent with those of the c_2 zone (for further details see Trott and Armstrong 1987a, b).

Injections

In each experiment the electrophysiological recording (see above) was used to guide the placement of a series of injections of fluorescent tracer material, either into the centre of the x zone or into the lateral-most part of the c_1 zone. In some experiments injections were made into both zones. The tracers used in each experiment are detailed in Table 1 which shows that, with the exception of two experiments (FC2 and FC5), the injected material consisted of latex microspheres or 'beads' (Lumafluor Inc.) which were tagged with either rhodamine (red beads) or coumarin (green beads). These beads, which were obtained as a suspension in water and used in their undiluted form, have the advantage over 'conventional' fluorescent tracers that they can produce at the site of injection a well-defined and highly restricted region of retrograde uptake (Katz et al. 1984; Katz and Iarovici 1990). The electrophysiologically

Table 1. Summary of injections made in each case. Part **A** lists methodological details of tracers used and lobules included within the 'effective' injection site. Part **B** lists the mediolateral width of the effective injection site at its widest point together with the width of the zone, defined electrophysiologically, at the same rostrocaudal level. For cases in which injections were made into the lateral c_1 zone, this latter figure denotes the mediolateral width of the entire c_1 zone (see Methods for details). Note that the value quoted for the effective injection site in Case FC7 is likely to be an overestimate because the green beads were not sonicated prior to injection (see Methods). Note also that, due to the distribution of blood vessels on the cerebellar surface, it was not possible to estimate the electrophysiological width of the c_1 zone in case FC5. Cases in which injections were made into both the x and lateral c_1 zones are indicated by asterisks

Case	Zone	A			B	
		Tracer	Total vol. inj. (nl)	Lobule	Maximum inj. site (mm)	Width of zone (mm)
FC3	x	red beads	300	Vb	1.4	1.3
FC4	x	red beads	300	Vb	2.2	0.5
FC5*	x	4% Fluoro-Gold	160	Va, Vb, Vc	0.9	0.2
FC7	x	green beads	400	VIa	1.3	0.9
FC10*	x	red beads	250	Va, Vb, Vc	1.2	0.7
FC11*	x	red beads	400	Va, Vb, Vc	0.6	0.3
FC2	lat c_1	3% Fast Blue	50	Vb	0.8	1.3
FC5*	lat c_1	red beads	500	Va, Vb, Vc	1.0	–
FC9	lat c_1	red beads	400	Vc	0.8	1.0
FC10*	lat c_1	green beads	400	Va, Vb, Vc	1.7	2.1
FC11*	lat c_1	green beads	450	Va, Vb, Vc	1.6	1.9

defined zones studied in the present investigation are particularly narrow. For example, consistent with previous reports (Ekerot and Larson 1979; Campbell and Armstrong 1985; Trott and Armstrong 1987b), in most animals the maximum width of the x zone was found on average to be only 0.5–0.9 mm (and at most 1.3 mm). The beads were therefore well suited for the present study which required the injections of tracer material to be restricted as much as possible to within the boundaries of a chosen cortical zone.

A glass micropipette (tip diameter 50–70 μm) attached to a 1 μl Hamilton syringe (Field Instruments) was used to deliver the tracers. The details of concentrations of Fast Blue and Fluoro-Gold together with the total volumes of tracer material injected in each experiment are given in Table 1, part A. Briefly, when either type of beads or Fluoro-Gold was used, 3–6 injections (total volume 160–500 nl) were made into a number of adjacent cerebellar folia in lobules Va–c and/or lobule VIa, about 500 μm below the pial surface, to make a rostrocaudally oriented strip of injected material within the chosen zone (see for example, photomicrograph Fig. 4A). In Case FC2, when the tracer used was Fast Blue, the injection consisted of a single delivery of 50 nl. Each injection was made over a 5 minute period. The exposed surface of the cerebellum was then covered in gelfoam (Sterispon, Allen and Hanburys), the craniotomy was sealed with dental acrylic cement and the wound was closed in layers. In later experiments (FC10 and FC11) retrograde transport of the green beads was greatly improved by their being sonicated for about 5 min immediately prior to use (Cornwall and Phillipson 1988).

Survival time and perfusion

Each animal was allowed to recover and analgesia was maintained for a further 24 h postoperatively with buprenorphine (Temgesic, 10 μg/kg, i.m., Reckitt and Coleman). In the earlier experiments (FC2, FC3 and FC4) the animal was maintained for a survival period of 12–16 days while, in all subsequent experiments, a survival period of 5–8 days was found to be sufficiently long to allow for uptake and retrograde transport of the injected material. In each case the animal was then reanaesthetised deeply with barbiturate and perfuse-fixed by transcardial perfusion. In all cases the cardiovascular system was initially flushed with 2 l of heparinised saline. In cases FC4 and FC5 this was followed by 2 l of 4% formalin while the remainder of cases were perfused with 2 l of 4% paraformaldehyde (total perfusion time with fix was about 1 h). The fixative was routinely followed by 2 l of a rinse solution of 10% sucrose in phosphate buffer and the cerebellum and underlying brainstem were removed and stored overnight at 4° C in the rinse solution.

Histology and microscopy

The brainstem was cut into 30 μm transverse sections on a freezing microtome and three separate series of sections were collected. The cerebellum was embedded in gelatin, cut sagittally at 100 μm and two separate series of sections were collected. Both brainstem and cerebellar sections were mounted onto slides via a 1% gelatin/0.1% chrome alum solution, allowed to air dry and stored in the dark at 4° C. The slides were not cleared or coverslipped (even with fluorescent mountants) as both treatments were found to cause potentially serious fading of the fluorescent material.

The sections were scrutinised with a Leitz Diaplan microscope fitted with a 50 W Mercury UV light source (Ploemopak) and high numerical aperture (NA) objectives (PL Fluotar, including × 10/0.3 NA; × 25/0.6 NA; × 40/0.7 NA). Rhodamine fluorescence was viewed with an 'N2' filter block (Dichroic mirror 580 nm, BP 530–560 nm, LP 580 nm) while the coumarin fluorescence was viewed with a 'G' filter block (Dichroic mirror 510 nm, BP 350–460 nm, LP 520 nm). Fast blue and Fluoro-Gold fluorescence were viewed with an 'A' filter block (Dichroic mirror 400 nm, BP 340–380 nm, LP 430 nm).

Analysis of data

Labelling was translated to standard outlines of the inferior olive drawn in transverse section at approximately 250 μm intervals (e.g. Fig. 3). For ease of comparison the labelling in MAO was also depicted on standard two dimensional maps of this olivary subdivision drawn approximately in the horizontal plane (after Brodal 1940, see for example Fig. 1). These maps were prepared by averaging outlines of the olive obtained from 4 cats. In three cases (FC5, FC10 and FC11) injections of different tracer materials were made

into both the x and the lateral c_1 zones. In two of these (FC10 and FC11) a count was made of the number of single- and double-labelled cells within that part of the olive which contained either cells which were double-labelled with both tracers or both types of single-labelled cells intermingled with each other. (In case FC10 the relevant olivary region was a segment of MAO between A–P levels 11.00–11.50. For Case FC11 the corresponding levels within MAO between which cells were counted were 11.25–12.00). The count was restricted to either two (FC10) or one (FC11) of the three prepared series of olive sections and included only those labelled cells in which the nucleus (which did not contain labelled material) was visible in the plane of section.

Interpretation of the injection sites

For each type of tracer (Fast Blue, Fluoro-Gold and fluorochrome-tagged beads) the 'effective' injection site was considered to be the necrotic centre and the surrounding, intensely bright region of fluorescence (see photomicrograph Fig. 4A and Discussion for further details). In cases where multiple injections were made a rostrocaudal strip of labelling, which extended over several folia, was clearly visible on the cerebellar surface. Discontinuities within the cortical strip were however seen in sagittal sections of the cerebellum which showed that the injected material was largely restricted to the apical folia of the injected lobules and never reached the base of the deeper cortical fissures (see for example Fig. 4A).

The folia within lobules V and VI which were included within the 'effective' injection site for each case are listed in Table 1, part A. The mediolateral width of the effective injection site in each folium was also measured histologically and compared with the electrophysiologically defined width of the injected zone in the corresponding folium. Table 1, part B includes a comparison between these two dimensions for the folium where the injection site in each case was widest mediolaterally. Note that in all cases when injections were made into both zones (indicated by asterisks in Table 1) the two strips of injection sites were separated by a substantial area of cortex which was free of injected material.

Results

The data are first considered in terms of those cases in which retrograde transport occurred from injections of tracer centred either in the x zone or in the lateral part of the c_1 zone. These results enable the olivary territory projecting to each of the two zones to be assessed. Further consideration is then given to those cases in which the two zones were injected with different tracers. Data from these latter cases enable the location of olivary cells projecting to both the x and lateral c_1 zones to be compared with the locations of cells projecting to only one of the two zones.

Injections of a single tracer centred in the x zone

In a total of six cats (FC3, FC4, FC5, FC7, FC10, and FC11) the x zone was located electrophysiologically (see Methods) on one side of the cerebellar cortex on the surface of 1–3 adjacent folia between lobules Va and VIa. Hydraulic injections of a single tracer substance were made into the tips of each folium at the centre of the x zone (see Table 1, part A). The resultant retrograde labelling in the contralateral inferior olive was remarkably consistent and only varied to an extent that depended on the size of the injections.

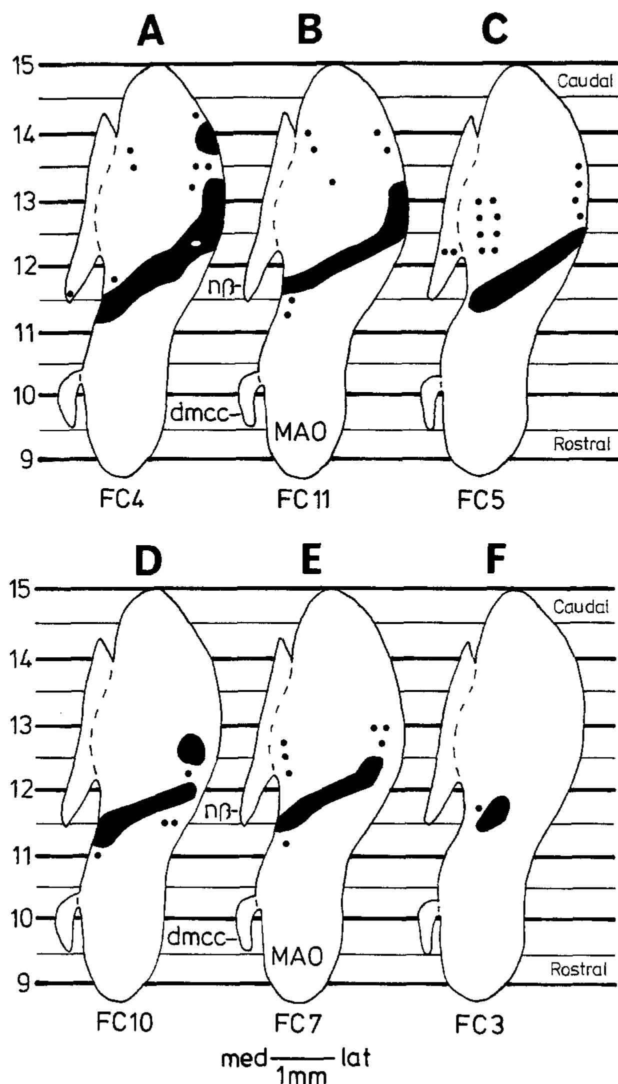

Fig. 1A–F. Distribution of retrograde labelling within medial accessory olive (MAO) in 6 cases in which an injection was made into the x zone. Labelling for each case is depicted on a 2-dimensional representation of MAO which approximates to a horizontal section through the nucleus (reconstructed after Brodal 1940). Note that in this and subsequent Figures, regions of olive where numerous labelled cells were encountered are shown as filled areas whereas regions where labelling was scattered (and perhaps only one or two weakly labelled cells were found) are indicated by dots. nß, nucleus ß; dmcc, dorsomedial cell column

Figure 1 illustrates, for each case, the retrograde labelling in MAO depicted on standard maps of this division of the inferior olive.

It was found that in several cases additional labelling was also present in the caudal part of the dorsal accessory olive, consistent with injectate having spread laterally to encroach on the b zone which is well known to receive its climbing fibres from this olivary region (cf. Groenewegen and Voogd 1977). However, this labelling is not considered further.

The individual cases are ranked within the Figure according to the amount of labelling present within MAO. It can be seen that for 5 of the 6 Cases (Fig. 1 A–E), labelled cells occupied a tightly packed diagonal

band, oriented caudo-laterally to rostro-medially, within the middle portion of MAO. In good agreement with the findings of Campbell and Armstrong (1985), such labelling in MAO was on average found to be present between levels 12.50 and 11.25 (labelling more caudal than this will be considered further below). This represents a total length in the olive of about 1.5 mm which contrasts with a mediolateral width at about the midpoint of the column (level 12.00) of about 0.5 mm i.e. a ratio between the rostrocaudal and mediolateral dimensions of 3:1 respectively. Note also that the band of labelled olive cells transfers from a lateral to a medial position within MAO over only a short length of the olive, between levels 12.25 and 11.50.

Comparison between Fig. 1 and Table 1, Part B shows that in those cases where the mediolateral width of the injection site at its widest point significantly exceeded the width of the x zone, estimated electrophysiologically within the same folium (e.g. Cases FC4, FC5, FC11 and FC10), some additional labelling was present more caudally within MAO. This labelling, which was generally fairly scattered, is consistent with the effective injection site having spread medially in the cortex to encroach on the a zone (cf. for example Groenewegen and Voogd 1977; Campbell and Armstrong 1985). By contrast, the labelling in Case FC3 (Fig. 1F) only occupies a small part of the territory within MAO which appears to project to the x zone. This is consistent with the finding (see Table 1, Part B) that in this case the mediolateral width of the injection site at its widest point only just exceeded the electrophysiological width of the zone in the same folium. Within other folia it is likely therefore that the injection site failed to occupy the entire mediolateral width of the zone and the resulting olivary labelling is therefore likely to underestimate the olivary territory projecting to the x zone.

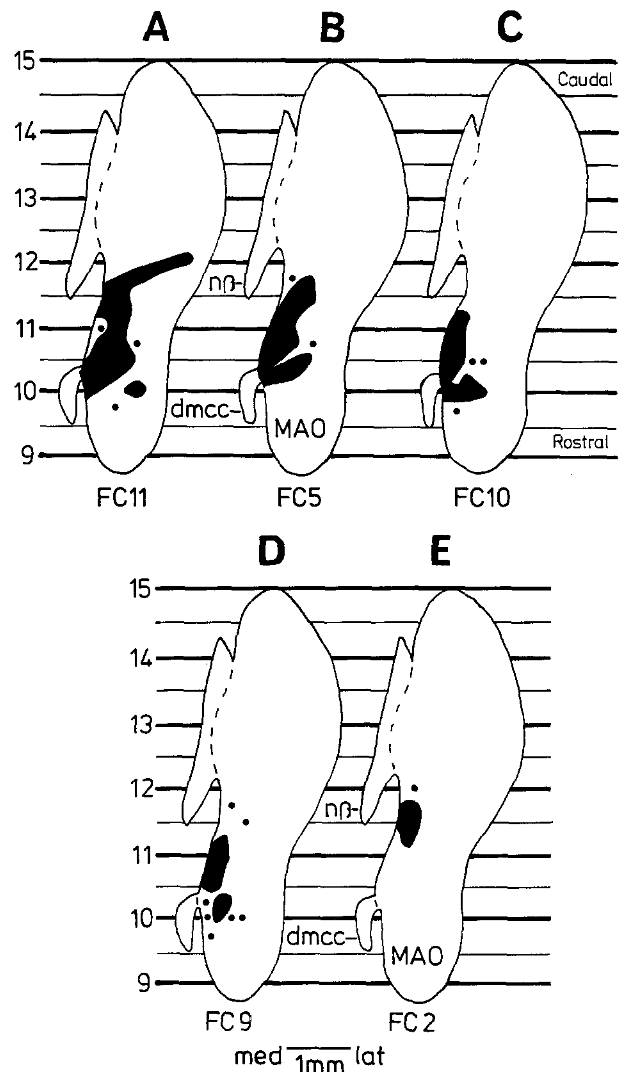

Fig. 2A–E. Distribution of retrograde labelling within medial accessory olive (MAO) in 5 cases in which an injection was made into the lateral part of the c_1 zone. Conventions and abbreviations as in Fig. 1

Injections of a single tracer in lateral c_1

Five cases resulted in retrograde transport from injection sites within the lateral-most part of the c_1 zone (cases FC2, FC5, FC9, FC10 and FC11). Figure 2 illustrates, for all 5 cases, the retrograde labelling in MAO.

In several of these cases additional labelling was also found in the rostral part of the dorsal accessory olive (DAO), consistent with spread of injectate medially within the cerebellar cortex to encroach on the medial part of the c_1 zone (see e.g. Campbell and Armstrong 1985; Trott and Apps 1991). However, as above, this labelling is not considered further. (Note that labelling was never found in caudal DAO suggesting that injected material never spread sufficiently far medially to encroach into the b zone, see for example Groenewegen and Voogd 1977).

The cases in Fig. 2 have been ranked, as in Fig. 1, according to a subjective assessment of the amount of labelling in MAO. It should be noted that it was not possible for these cases to establish the width of each injection site relative to the corresponding width of the lateral c_1 zone since the boundary between the medial c_1 and lateral c_1 zones cannot be determined electrophysiologically (see Methods for further details). It is evident from Fig. 2A–E that in each case labelling is centred more rostrally than in those cases in which injections were made into the x zone (compare for example Figs. 1A and 2A) and in each case the labelling is also biassed towards medial MAO. Taken together, the data suggest that the region of MAO which contains labelled cells forms a continuous, rostrocaudally oriented column, about 2.00 mm in length, on average between about levels 11.75 and 10.00. This compares to a mediolateral width of about 0.5 mm, ie. a ratio between the rostrocaudal and mediolateral dimensions of about 4:1. However, in view of the fact that injections within lateral c_1 were deliberately placed rather close to the c_1/c_2 boundary (see Methods) it is highly likely that part of this labelling, for example the labelling more rostral and lateral in MAO, (Fig. 2A to D) arose from spread of injected material to encroach on the c_2 zone. This point is discussed later.

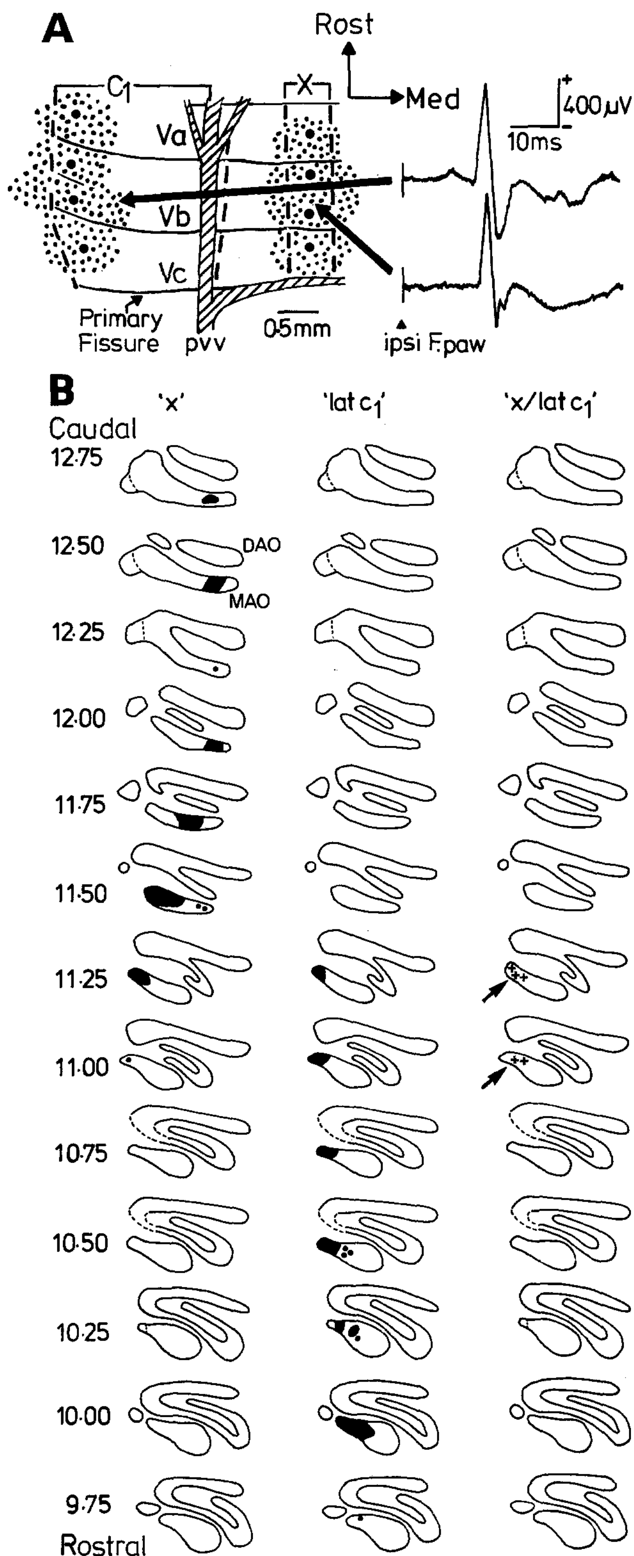

Fig. 3A–B. Case FC10 in which injections of different tracers were made into the x and lateral c_1 zones. Panel **A** depicts a semi-diagrammatic surface view of the left anterior lobe to show part of lobule V. Dashed lines represent approximate locations of medial and lateral boundaries of the c_1 and x zones (estimated electrophysiologically) in each of Va, Vb and Vc. Large filled circles represent sites of injections of either red beads (x zone, cf. Fig. 4A) or green beads (lateral c_1 zone); mediolateral spread of the corresponding effective injection sites is shown by stippling. Pvv, paravermal vein. Records to the right depict climbing fibre field potentials evoked, at sites indicated by arrows, by percutaneous stimulation of the ipsilateral forepaw (each trace average of 4 sweeps). Panel **B** depicts distribution of cells single-labelled with red beads following x zone injections *(left hand column)*, cells single-labelled with green beads following lateral c_1 zone injections *(middle column)* and cells double-labelled with both tracers *(right hand column)*. Note that the distribution of single-labelled cells *(left hand and middle columns)* is depicted according to the same format as for Fig. 1, whereas crosses in the right hand column represent locations of individual double-labelled cells

Again these results are in good agreement with previous WGA–HRP retrograde studies and confirm that the region of olive that supplies the lateral c_1 zone forms a column of cells in rostro-medial MAO (Campbell and Armstrong 1985; Trott and Apps 1991).

Double tracer injections in both x and lateral c_1

Of those cases included in the previous sections, three resulted in retrograde transport of tracer from injections centred in the x zone as well as from injections of a different tracer made into the lateral part of the c_1 zone (FC5, FC10 and FC11). In all these cases no overlap occurred between the injection sites of the two types of tracer.

Figures 3 and 4 depict in detail the results obtained in Case FC10 and panel A of Fig. 3 is a semi-diagrammatic representation of the region of cortex in which the x and lateral c_1 zones were electrophysiologically identified. Examples of climbing fibre field potentials from each zone are shown to the right (for further details see Methods). In this case, red beads were injected into the centre of the x zone in a number of adjacent folia while green beads were injected into the lateral half of the c_1 zone, again in a number of adjacent folia (see Table 1, Part A for further details). In Fig. 3B each series of standard transverse outlines of MAO depicts, from left to right, the distribution of olive cells that were single-labelled with red beads following x zone injections; the distribution of cells that were single-labelled with green beads following lateral c_1 zone injections and the location of cells that were double-labelled with both red and green beads (and which therefore provide climbing fibre terminals to both zones).

As previously described for this case (cf. Figs 1D and 2C), the two single-labelled populations of cells (Fig. 3B, left-hand and middle rows) form two rostrocaudally directed columns in MAO. However overlap between these two columns is present at levels 11.25 and 11.00. Cells double-labelled with both red and green beads are plotted on the right hand series of outlines of Fig. 3B and, as might be expected, such cells are located in the region of overlap in middle MAO (see arrows). One of these double-labelled cells is indicated by arrows in the photomicrographs of Fig. 4B and C.

In Fig. 5 the middle region of MAO has been expanded to show for each double-tracer experiment the location of overlap between the olivary territories that supply the x zone and the lateral c_1 zone. The overlap consistent-

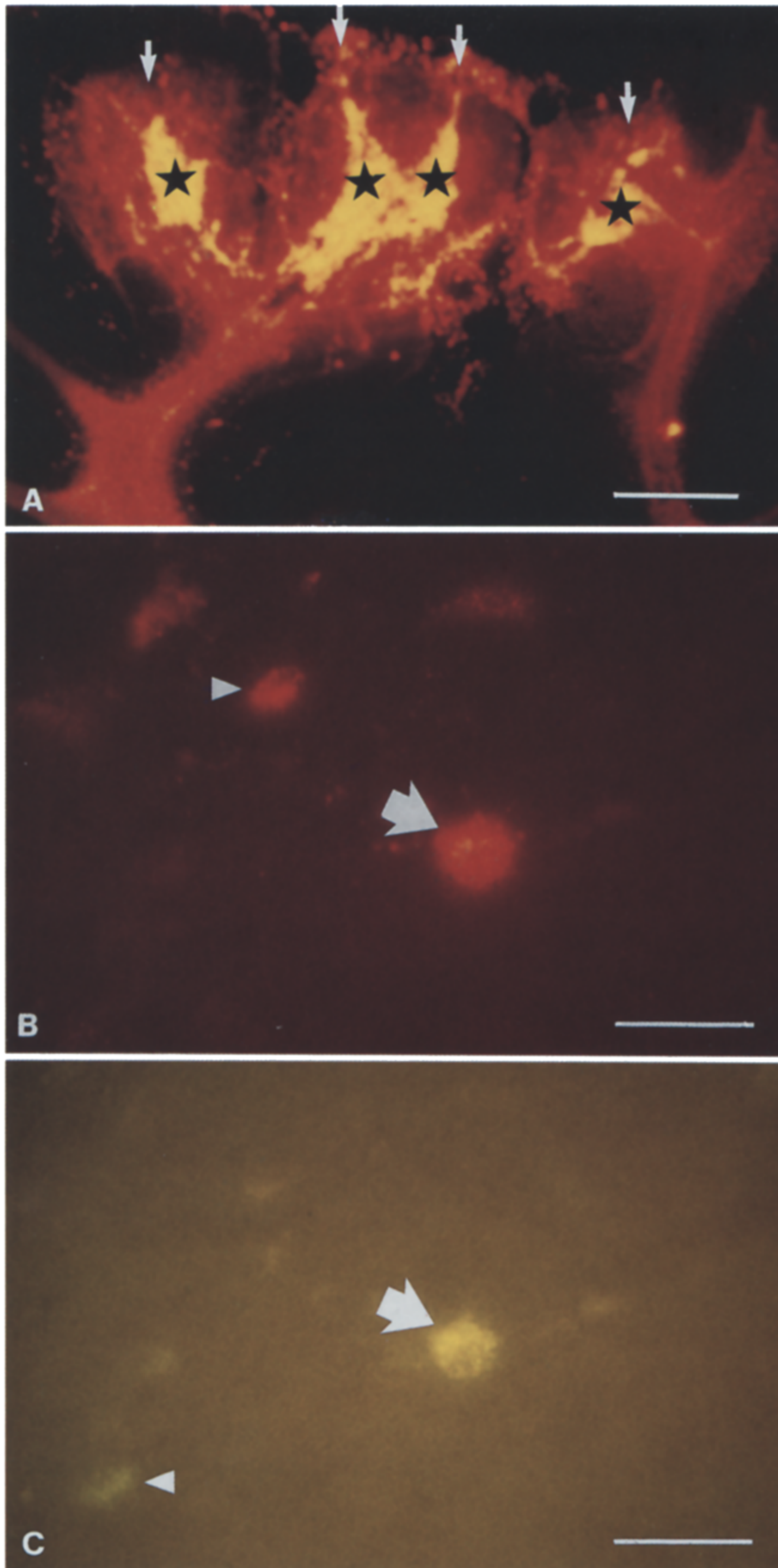

Fig. 4A–C. Photomicrographs from Case FC10 (cf. Fig. 3). **A** Low power sagittal section through the cerebellum viewed for rhodamine fluorescence to illustrate typical injection sites of fluorochrome-tagged beads. In this example 4 injections of red beads were made into the centre of the x zone in the tips of a number of rostrocaudally adjacent apical folia of lobule Va,b and c (*rostral to right*, cf. Fig. 3A). The approximate points of entry of the pipette into the cerebellum are indicated by *arrows*. Note the corresponding regions of intense fluorescence *(marked with stars)* as compared to the surrounding areas of much weaker and more diffuse fluorescence (see Discussion for further details). **B** High power transverse section through middle MAO (about level 11.25, cf. Figs 3B and Fig. 5) viewed for rhodamine *(red)* fluorescence. **C** High power view as depicted in **B** but viewed for coumarin *(green)* fluorescence. In **B, C** an *arrow* indicates a cell double-labelled with *red* and *green beads*. Note the granular appearance of the labelling. In **B** an *arrow head* indicates a cell single-labelled with *red beads* and in **C** an *arrow head* indicates a different cell single-labelled with *green beads*. See text for further details. Calibration bars: **A,** 0.5 mm; **B** and **C,** 50 μm

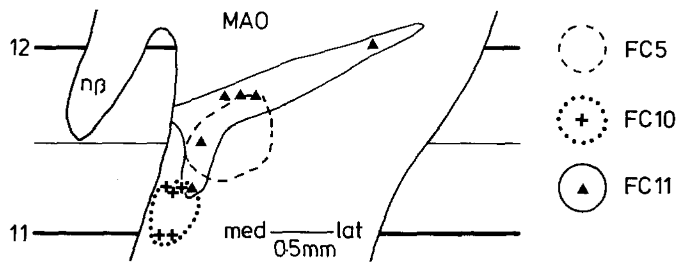

Fig. 5. Results obtained from all 3 cases (FC5, FC10 and FC11) in which injections of different tracers were made into the x zone and the lateral c_1 zone. Figure depicts expanded view of middle region of medial accessory olive (MAO) to show, for each case, the area of overlap between the olivary territories projecting to the two zones. Symbols represent the locations of double-labelled cells in FC10 *(crosses)* and FC11 *(triangles)*

ly occurred between levels 12.00 and 11.00 and was restricted in each case to only a small part of the total area in MAO where labelled cells were found (cf. Figs 1B–D and 2A–C). The positions of the double-labelled cells (see below for further details) are indicated on the Figure by crosses (Case FC10) and triangles (Case FC11). Note that these cells were found scattered throughout the region of overlap.

Quantitative estimate of the numbers of single-labelled as compared to double-labelled cells

In two of the three double-tracer experiments (FC10 and FC11) it was possible to make a quantitative estimate of the number of double-labelled cells as compared to the number of cells labelled with one or other single tracer (see Methods and Table 2 for details). In the third double-tracer experiment (FC5) the area of overlap was similar to that found in the two other cases (see Fig. 5) and it is probable that a few double-labelled cells were present in this region. However, one of the tracers (Fluoro-Gold) produced only weak retrograde labelling which made it difficult to be certain whether or not a particular cell was double-labelled, therefore the results were not considered suitable for quantitative analysis.

In case FC10 labelled cells were counted in two of the three series of sections by independent observers (marked a and b in Table 2). Because the two counts were found to correspond closely, only one out of three sections was counted in the additional experiment (case FC11). Table 2 shows for the two cases, from left to right: the number of cells single-labelled following transport from injections centred in the x zone or lateral c_1 zone; the number of cells double-labelled (and whose axons therefore branch to innervate both zones); and finally the number of double-labelled cells expressed as a percentage of the two different single-labelled cell populations. Note, however, that these values should be considered with some caution since they do not take into account variations in the size and location of the injections in the two cases and any consequent differences in the extent to which the injection sites involved the x or lateral c_1 zones (particularly in terms of involvement in the injections of apical versus deep folia, see Discussion). Nonetheless the percentile values are consistently low and therefore presumably reflect a genuine low incidence of transverse axonal branching between the two zones.

Table 2. Comparison between numbers of single- and double-labelled cells present within the middle region of MAO in Cases FC10 and FC11. In Case FC10 counts were obtained from 2 of the 3 series of olive sections, in Case FC11 only one series of sections was counted. Note that the count was restricted to that area of MAO which contained either double-labelled cells or both types of single-labelled cells intermingled (see Methods for details)

Expt	Single labelled cells (x)	Single labelled cells (lat c_1)	Double labelled cells (x/lat c_1)	Double labelled as % of x	Double labelled as % of lat c_1
FC10	a 77	81	5	6.5%	6.2%
	b 78	70	4	5.1%	5.7%
FC11	84	84	6	7.1%	7.1%

Discussion

Interpretation of injection sites

In the present study a number of different fluorescent retrograde tracers were used but in each case the size of the 'effective' injection site could be estimated according to a general principle. For each tracer, the region of retrograde uptake was taken as the central 'core' of the injection site, where occasional pipette damage and necrosis were evident, together with a surrounding region where the fluorescence was intense (equivalent to 'zones 1 and 2' described by Schmued and Fallon 1986). In all cases the intensely fluorescent area was in turn surrounded by an area of weaker, more diffuse fluorescence (see for example the photomicrograph of Fig. 4A). This outer region was not included in the 'effective' injection site (see below).

This interpretation was based on the following factors: 1) The effective injection site was considered to have exceeded the size of the central core because in all cases this region was restricted to within the boundaries of a given zone yet on a number of occasions the distribution of retrograde olivary labelling was compatible with there having been uptake of injected material by more than one cortical zone. The effective injection site for each type of tracer must therefore include (at least part of) the surrounding region of fluorescence. 2) The outer area of weaker fluorescence was considered not to contribute to the effective injection site because in some cases the core and intensely fluorescent region were *confined within* a single physiological zone but the weakly fluorescent outer region *spread beyond* the boundaries of that zone. In such cases the retrograde labelling was always restricted to only that part of the olive associated with the cortical zone at the centre of the injection site. For instance, injections where the core and intensely fluorescent areas were confined to within the boundaries of the physiologically defined x zone consistently resulted in

retrograde labelling confined to middle MAO in the contralateral olive. In such cases, the weakly fluorescent outer area occasionally spread beyond the x zone, for example laterally into the neighbouring b zone, but no corresponding retrograde labelling was found in the associated region of the olive, in this case the caudal half of the dorsal accessory olive (cf. Groenewegen and Voogd 1977).

It is likely that this method somewhat overestimates the size of the injection sites because of the assumption that the effective site includes all of the area of intense fluorescence. In fact, the various tracers differed mainly in the extent to which the intense fluorescent region was found to spread. Since this region was smallest with injections of the fluorochrome-tagged beads the estimate of injection site size in these cases is likely to be correspondingly more accurate.

Olivary territories projecting to x and lateral c_1

The present study confirms and extends previous reports (e.g. Campbell and Armstrong 1985; Trott and Apps 1991) on the olivary projection to the x and lateral c_1 zones. The region of olive supplying the x zone was found to be located in a band extending across the middle of MAO in a caudo-lateral to rostro-medial direction between about levels 12.50 and 11.25. In comparison, the region of olive containing labelled cells following injections made in the lateral c_1 zone was located more rostrally in a medial part of MAO between about levels 11.75 and 10.00. These caudal and rostral limits are based on a synthesis of the data and must be considered with some caution because it is possible that part of the most caudal labelling in MAO arising from x zone injections resulted from spread of the injection site to the a zone (cf. Groenewegen and Voogd 1977; Brodal and Walberg 1977). Similarly, some of the most rostral labelling in MAO arising from c_1 zone injections could have resulted from spread of the injection site to the c_2 zone (cf. Brodal and Walberg 1977; Groenewegen et al. 1979), particularly as injections made in the c_1 zone were deliberately placed close to the c_1/c_2 boundary to minimise the possibility of these injection sites overlapping with those centred in the x zone.

In a recent WGA–HRP retrograde transport study (Trott and Apps 1991) a comparison was made between the olivary labelling arising from injections that were centred in the c_2 zone (but encroached on lateral c_1) and the labelling that resulted from injections that were centred in lateral c_1 (but encroached on c_2). This revealed the 'core' lateral c_1 territory to be situated medially within MAO between levels 12.00 and 10.50, suggesting that the most rostral labelling within MAO obtained in the present results, did indeed arise as a consequence of the inclusion of the c_2 zone within the effective injection site.

Region of overlap

Collectively, this and previous studies (see above) demonstrate that the olivary territory that supplies either x and/or lateral c_1 in the apical folia of lobules V/VIa forms a continuous, rostrocaudally oriented column, across the middle of MAO between about levels 12.50 and 10.50. Such a columnar organisation would seem to be a common feature in at least part of the olivocerebellar projection in two species: the rat (Buisseret-Delmas 1988; Apps 1990) and the cat (Trott and Apps 1991). The results indicate, in agreement with those of Campbell and Armstrong (1985), that a middle portion of this olivary column, between levels 12.00 and 11.00, supplies both x and lateral c_1. The region caudal to this would seem to be an olivary territory that supplies only the x zone while the region rostral to this would seem to supply only the lateral c_1 zone. On this basis the olivocerebellar projection can be considered to be organised such that three different topographical areas within middle MAO project to the x and lateral c_1 zones in apical folia of lobules V/VIa (cf. the nucleotopic organisation suggested by Payne et al. 1985, their Fig. 1f).

The results in the present double-labelling study demonstrate that in the central overlap area located in middle MAO, there are some olive cells whose axons branch to provide climbing fibres to both the x and lateral c_1 zones. However, within this area of overlap, double-labelled cells represent only 5–7% of either single-labelled cell population. Moreover, it should be emphasised that these percentile values are based on the number of 'single x' and 'single lateral c_1' cells assessed only from that region of middle MAO where overlap of the two olive territories occurred. Since the count excluded those additional caudal and rostral areas which were found to project only to the x or lateral c_1 zones respectively the results imply that considerably fewer than 5–7% of the olive cells projecting to one of the two zones also project to the other zone of the pair, at least in the apical folia of lobule V.

Given the good agreement found in case FC10 between the number of labelled cells counted in two out of the total of three series of sections and since fragments were not included in those counts (see Methods), it seems reasonable to estimate the total number of labelled cells in this case by multiplying the value observed in any one series of sections by a factor of three. When this calculation includes those additional areas described above then the corrected value for the *total* number of cells supplying the x zone within apical folia of lobule Va,b and c in case FC10 is estimated to be 389 (single-labelled) + 15 (double-labelled) = 404 cells and the *total* number of cells supplying the corresponding part of the lateral c_1 zone is estimated to be 441 (single) + 15 (double) = 456 cells i.e. the large majority (almost 97%) of labelled cells in the central area of MAO would seem to provide a climbing fibre to only one of the two zones and these are intermingled with a much smaller population of cells whose axons branch to innervate both zones.

Quantitative analysis compared to previous studies

The results of this numerical analysis would appear to be at odds with the results of Ekerot and Larson (1982) who

concluded from their study of axon-reflexes elicited between the two zones, that "...it is not unlikely that the x zone is exclusively innervated by climbing fibres belonging to the x–c_1 group." It should be emphasised that what is at issue is the *lack* of double-labelled cells observed in the present results (as compared to the positive identification of climbing fibre axon-reflexes) and it is therefore important to consider any factors in the present study which may have resulted in the identification of a spuriously low number of double-labelled cells.

It may be significant, for example, that only a relatively small part of the overall territory within the x and lateral c_1 zones was investigated in the present study: in both cases in which double-labelled cells were counted, injections were restricted to lobule V whereas Voogd (1983) estimates that the x zone spans from lobule IV to lobule VI inclusive. In the axon-reflex study of Ekerot and Larson (1982), the area of cortex explored included both lobules V and VI but excluded lobule IV. Furthermore, it is possible that a majority of the olive cells that branch to innervate the two zones project to deeper regions of the cortex that were unlikely to be included in the injection sites in the present study. It is noteworthy in this respect that in the study by Ekerot and Larson (1982), the majority of Purkinje cells in the x zone found to have climbing fibres that also had branches innervating the lateral c_1 zone were located in two clusters in deeper folia, (cf. their Fig. 2C and F).

It is also possible that uptake of both types of beads by a given cell may have resulted in the two tracers 'interfering' with each other's retrograde transport: such an effect could produce a spuriously low number of observed double-labelled cells. This option seems however to be most unlikely, in light of experiments, currently in progress, in which the beads have been used to investigate rostrocaudal branching within the olivocerebellar projection. Preliminary experiments have revealed a high proportion of double-labelled cells, suggesting that considerable numbers of olivary cells have axons which branch in the rostrocaudal plane (a finding consistent with previous anatomical and physiological studies in the cat e.g. Armstrong et al. 1973; Brodal et al. 1980; Rosina and Provini 1983, 1987) and that the method employed in the present study is capable of revealing double-labelled cells in large numbers.

A third explanation for the discrepancy between the numerical results of the present study and those of Ekerot and Larson (1982) is that the two techniques used to investigate axonal branching may differ in their sensitivity. For example, whereas electrical stimulation of a single climbing fibre terminal is likely to be an adequate stimulus for exciting an axon-reflex in another branch of the same olivary stem axon, retrograde fluorescent labelling of an olive cell at levels above threshold for detection might require tracer uptake by more than one climbing fibre terminal originating from that cell. Such a requirement could give rise to a spuriously low number of observed double-labelled cells, particularly if large numbers of olive cells provide only a single climbing fibre terminal to either one (or both) of the x and lateral c_1 zones in the region of cortex investigated.

It is interesting to note, in this respect, that in the 2 experiments illustrated in their Fig. 2, Ekerot and Larson (1982) categorised 15 Purkinje cells in the x zone as receiving climbing fibre input from an axon that also branched to innervate lateral c_1. However, only 3 of these cells were innervated by a climbing fibre which could be shown to provide more than one termination point in the lateral c_1 zone. Furthermore, 2 of these cells were innervated by a climbing fibre which, despite providing at least 2 branches to the lateral c_1 zone, did not appear to provide any additional branches elsewhere within the x zone i.e. transverse branching within the olivocerebellar projection to the x and lateral c_1 zones may be asymmetrical in distribution, with most of the probable ten axon collaterals derived from each olivary stem axon (see Mlonyeni 1973) terminating within one of these zones as compared with only one or two branches terminating in the other zone. This would imply that, even if large numbers of olive cells give rise to transverse branches, such branching is much less frequent than that found in the rostrocaudal axis. This suggestion must, of course, be treated with caution since Ekerot and Larson (1982) were unable to explore inaccessible cortical regions, for example at the depths of the primary fissure, but the existence of such an asymmetrical branching pattern in the olivocerebellar projection to the two zones could help to reconcile the numerical discrepancy between the electrophysiological and neuroanatomical studies. It would, furthermore, be reminiscent of the asymmetry described on a lobular basis by Rosina and Provini (1987), for rostrocaudal branching within the olivocerebellar projection.

It is also noteworthy in this connection that a comparison between the size of climbing fibre field potentials generated as a result of axon-reflexes evoked in the transverse plane between the lateral c_1 and x zones and those evoked in the transverse plane between the medial halves of the c_1 and c_3 zones reveals a marked difference (Ekerot and Larson 1982, their Figs. 2 and 3 respectively). The axon-reflexes between the medial c_1 and c_3 zones generated climbing fibre potentials about 300 μV in amplitude (see their Fig. 3G and H), consistent with values obtained for branching in the rostrocaudal plane (cf. Armstrong et al. 1973). In contrast, Ekerot and Larson (1982) recorded axon-reflexes between the lateral c_1 and x zones that generated climbing fibre potentials only about 80 μV in amplitude (see their Fig. 2G and H). This would suggest that in the latter situation a much smaller number of climbing fibres were contributing to the potentials, again making it likely that transverse axonal branching between these two zones is not extensive. Comparisons such as these again suggest that the electrophysiological data is not inconsistent with the conclusion drawn in the present study, namely that there is a low numerical incidence of transverse branching in the olivocerebellar projection to the x and lateral c_1 zones, at least in those parts of the zones so far studied.

Although the various factors outlined above may, at least in part, account for the differences between this study and that of Ekerot and Larson (1982) it is nevertheless important to emphasise that the present results, in

agreement with Campbell and Armstrong (1985), showed that the olivary territory that projects to the lateral c_1 zone was invariably centred rostral to that region of MAO projecting to the x zone. Moreover, injections into lobule V resulted in a pattern of olivary labelling indistinguishable from the retrograde labelling following injections into lobule VIa i.e. regardless of which lobule was injected with tracer material the results consistently showed that only a partial overlap was evident between the x and lateral c_1 territories. Such a nucleotopic organisation indicates that at least some olive cells provide climbing fibres that terminate in only one of the pair of zones in apical folia of lobules V and VIa. It would seem therefore most unlikely that all olive cells innervating the region of cortex studied have axons that branch to terminate in both zones.

Afferents to the middle part of MAO

The most direct route that is likely to generate the climbing fibre fields recorded in the c_1 and x zones following forelimb stimulation is via the dorsal funiculus-SOCP, a path that is known to relay in the cuneate nucleus of the dorsal column nuclei (Oscarsson 1969; Ekerot and Larson 1979; Andersson and Eriksson 1981). A number of anatomical studies have demonstrated a direct projection from the cuneate nucleus to the contralateral MAO (e.g. Boesten and Voogd 1975; Berkley and Worden 1978; Berkley and Hand 1978; Gerrits et al. 1985). In particular, Gerrits et al. (1985), in a detailed autoradiographic study, demonstrated a direct cuneo-olivary projection that was found to terminate in two discrete regions in rostro-medial and caudo-lateral parts of MAO (see their Fig. 7, Case H9823). Interestingly, the more rostral area of termination corresponds closely with the intermediate region of middle MAO (levels 12.00–11.00) where overlap was found, in the present study, between cells that project to either lateral c_1 or x and where cells are present whose axons branch to innervate both zones. In contrast, inspection of their Fig. 7 suggests that the caudal area of MAO, shown in the present study to project only to the x zone, lacks any direct cuneo-olivary projection. Similarly, the more rostral area in MAO, shown in the present study to project only to the lateral c_1 zone, receives, at best, only a modest cuneo-olivary input. The absence of labelling may of course have resulted from incomplete staining of the cuneate nucleus with tritiated leucine but in light of the present findings it is tempting to conclude that the result reflects a genuine difference between the three olivary regions.

It is also of interest in this respect that Courville et al. (1983), in an autoradiographic study of afferent pathways projecting to the olive, describe a projection from the contralateral spinal trigeminal nucleus which appears to be restricted, within MAO, to the region of overlap between olivary territories projecting to both the x and the lateral c_1 zones. The fact that this pathway does not appear to terminate within the regions of MAO found to project to only one or the other zone reinforces the idea that functional differences may exist between the three olivary regions.

The present study has demonstrated that certain *efferent* pathways from MAO contain partial overlap within their organisation, in that different cerebellar cortical zones receive input from partially overlapping regions of the olive. A recent anatomical study (Bull et al. 1990) has suggested that certain *afferent* pathways terminating within MAO may also contain an element of partial overlap in their organisation. Thus, Bull et al. (1990) described partially convergent inputs to the inferior olive from the pretectum and dorsal column nuclei, such that an area within caudal MAO which received input from both sources was flanked by regions which received input only from the pretectum or the dorsal column nuclei (see for example their Fig. 5A and C). It is therefore possible that such partial overlap may be an organisational feature common to a number of afferent and efferent pathways innervating the olive, enabling certain information to be relayed to more than one cortical zone while other information is targeted to individual zones.

Other anatomical studies have demonstrated that the lateral c_1 and x zones have different corticonuclear targets. Both retrograde and orthograde pathway tracing studies have shown that the x zone projects to the junctional region between nucleus fastigius and nucleus interpositus posterior (Voogd 1983; Trott and Armstrong 1987b) while the lateral c_1 zone was found to project to nucleus interpositus anterior (Trott and Armstrong 1987a). In conclusion it would seem that there is a growing body of evidence to suggest that the lateral c_1 and x zones, although sharing some common anatomical features, are sufficiently different to make it unlikely that they are functional homologues.

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