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Organization of Pontocerebellar Projections to Identified Climbing Fiber Zones in the Rat

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ABSTRACT

The organization of pontocerebellar projections to the paravermis and hemisphere of the posterior cerebellum of the rat was studied in relation to the organization of climbing fibers. Small injections of cholera toxin subunit B were placed in the cerebellar cortex at locations predetermined by evoked climbing fiber potentials from selected body parts or based on coordinates. The injection site was characterized with respect to the zebrin pattern and by the distribution of retrogradely labeled neurons in the inferior olive. The following zones were studied: hindlimb-related zones C1 and C2 of lobule VIII; forelimb-related zones C1, C2, and D0/D1 of the paramedian lobule; and face-related zones A2 of the paramedian lobule and C2 and D0 of crus 2B. The results show that the distribution of pontine neurons is closely related to the climbing fiber somatotopy. Injections centered on face-related zones result in distribution of pontine neurons within the pontine core region. Forelimb regions surround this core, whereas hindlimb regions are mostly supplied by caudal pontine regions and by a single patch of more rostrally located neurons. This distribution fits well with published data on the somatotopy of the corticopontine projection from the rat primary somatosensory cortex. However, apart from differences in the participation of ipsilaterally projecting cells, the distribution of pontine neurons does not change significantly when the injection covers different zones of the same lobule such as C1 and C2 of lobule VIII; C1, C2, and D0/D1 of the paramedian lobule; A2 of the paramedian lobule; and C2 and D0 of crus 2B. J. Comp. Neurol. 496:513–528, 2006.

Indexing terms: mossy fibers; inferior olive; modules; pontine nuclei; somatotopy

The cerebro-cerebellar connection represents one of the most powerful pathways in the mammalian brain. The basilar pontine nuclei (Pn) essentially form the intermediary in this pathway (Brodal and Bjaalie, 1992). They receive their major input from the cerebral cortex and project as mossy fibers to the cerebellar cortex. One of the most interesting questions in the organization of this massive corridor relates to the how and why the somatotopic pattern of cerebrum (e.g., Leergaard et al., 2000c) is transposed to a fractured cerebellar map (Welker, 1987), which supposedly results from combining peripheral and cerebro-cerebellar tactile receptive fields (Morissette and Bower, 1996). Also, as yet, it is far from clear how the organization of the other main afferent system of the cerebellum, i.e., the climbing fibers, relates to the cerebro-ponto-cerebellar system. Some of these questions will be addressed in the present study performed in the Wistar rat, as they were in a very recent study by Odeh et al. (2005).

In the rat, the ipsilaterally organized corticopontine projection has been shown to originate from layer V neurons located throughout most of the cerebral cortex (Legg et al., 1989). However, clear regional differences have been described, and the densest projections seem to originate from the sensorimotor and visual cortices (for review, see Ruigrok, 2004). Although initially a clear somatotopic pattern could not be detected in the corticopontine projections that arise from the somatosensory cortex (SI),

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recent studies by Leergaard and colleagues (2000c) have shown that the SI map is converted to concentric shells in the Pn. A description of the translation from the concentric somatosensory pontine maps onto the somatotopic cerebellar maps described for peripherally induced climbing fiber and mossy fiber activations (Atkins and Apps, 1997; Brown and Bower, 2001) will obviously help us to understand the anatomical substrate of the fractured cerebellar somatotopy (Bower, 1996) and will provide significant clues to general cerebellar functioning. Indeed, a recent study by Odeh et al. (2005) showed that the organization of the pontocerebellar projection not only is in line with previously described aspects of the corticopontine projection from the somatosensory cortex (Leergaard et al., 2000b) but also adheres to the somatotopic and zonal pattern of the climbing fiber system.

In this respect it is important to note that recent studies by Cicirata and his group (Serapide et al., 1994, 2001, 2002b) also have shown that small injections with anterogradely transported tracers result in multiple longitudinally oriented strip-like patterns of mossy fibers. In the anterior cerebellum, a spatial correspondence of climbing fiber zones and subjacently positioned mossy fiber rosettes both labeled from the posterior cerebellum has been suggested by Voogd et al. (2003). Similar observations have been made in the vestibulocerebellum (Ruigrok, 2003).

The zonal pattern of the organization of the climbing fibers adheres well to the organization of the Purkinje cell projections to the cerebellar nuclei (Voogd and Glickstein, 1998; Voogd, 2004; Pijpers et al., 2005). For the cat, these zonal relations can be subdivided into a microzonal level, which may constitute the entities of cerebellar functioning (for review, see Apps and Garwicz, 2005) Somatotopic relations have also been documented for the rat concerning the connections between the dorsal accessory olive (DAO) and the C1 zone of the cerebellar cortex (Atkins and Apps, 1997; Pardoe and Apps, 2002; Voogd et al., 2003; Odeh et al., 2005), i.e., a strip of ipsilateral hindlimb responsive climbing fibers terminates within the C1 zone of the copula pyramidis (COP; lobule VIII), whereas the ipsilateral forelimb is represented by climbing fibers in both the C1 and C3 zones of the paramedian lobule (PMD; lobule VII); face-receptive fields are found within the A2 zone of the PMD. However, receptive fields of the anatomically defined C2 zone encompass both sides of the body (Atkins and Apps, 1997).

In the present study we have examined the relation between several identified climbing fiber zones in the paravermal and hemispheral cortex of lobules VII and VIII and the organization of the pontine origin of the mossy fiber projections to these zones. Small injections with a retrograde tracer covering both the molecular and granular layer resulted in retrogradely labeled neurons in the Pn as well as in the olivary subnuclei. The latter allowed identification of the zonal location of the injection and thus allowed direct study of the relation between the (pontine) mossy fiber projection and the climbing fiber zones. In line with results presented by Odeh et al. (2005), we show that the pontine origin of pontocerebellar projections is related to the somatotopy of climbing fiber zones as well as to the organization of the somatosensory corticopontine projections. However, apart from differences in the amount of ipsilaterally located pontine neurons, a clear distinction in distribution of pontine cells that could be related to different climbing fiber zones within the same lobule was not revealed in the present study.

Photomicrographs were prepared from selected sections by using a Leica DMR microscope equipped with a digital camera (Leica DC-300). Photo panels were constructed in CorelDraw 11.0, after some correction for brightness and contrast in Corel Photopaint 11.0.

Abbreviations

β	beta subnucleus
BSA	bovine serum albumin
COP	copula pyramidis
CTb	cholera toxin B subunit
DAB	3,3′-diaminobenzidine tetrahydrochloride
DAO	dorsal accessory olive
DC	dorsal cap
dfDAO	dorsal fold of the DAO
dlPO	dorsal leaf of the PO
DM	dorsomedial group
DMCC	dorsomedial cell column
lfp	longitudinal fascicle of pons
iMAO	intermediate part of the MAO
rMAO	rostral part of the MAO
PB	phosphate buffer
PBS	phosphate-buffered saline
PMD	paramedian lobule
Pn	pontine nuclei
PO	principal olive
Py	pyramidal tract
rMAO	rostral medial accessory olive
SI	primary somatosensory cortex
TBS	Tris-buffered saline
vfDAO	ventral fold of the DAO
vlPO	ventral leaf of the PO

MATERIALS AND METHODS

For this study experiments performed in 10 male Wistar rats were selected out of a batch of 16 cases with cholera toxin B subunit (CTb) injections in the posterior cerebellum. All procedures adhered to the NIH *Guide for the Care and Use of Laboratory Animals* according to the principles expressed in the declaration of Helsinki and were approved by a national committee overseeing animal welfare.

Animals were anesthetized with a ketamine/xylazine mixture (100 mg/kg + 3 mg/kg) or with pentobarbital (40 mg/kg) administered intraperitoneally (i.p.). Surgical levels of anesthesia were monitored by the absence of rhythmic whisker movements and pinch withdrawal reflex. When necessary, supplementary doses were administered to maintain surgical levels of anesthesia. During surgery, body temperature was monitored and kept within physiological limits. After inducing anesthesia, the animals were placed in a stereotactic head holder, and the posterior cerebellum was accessed as described earlier (Ruigrok et al., 1995; Voogd et al., 2003). Subsequently, a single iontophoretic injection was made with CTb in selected places of the right hemisphere of lobule VIII (copula pyramidis: COP), lobule VII (paramedian lobule: PMD), or crus 2B. In most cases the actual place of injection was determined by prior mapping of field potentials resulting from stimulation of the ipsilateral hindlimb, forelimb, or contralateral or ipsilateral face by percutaneous stimulation with pairs of fine needles (Table 1).

The stimulation and recording paradigm has been described by Atkins and Apps (1997; also see Voogd et al., 2003). In these cases, pentobarbital was used as the an-

TABLE 1. List of Experiments and Distribution and Number of Labeled Cells in Inferior Olive and Basal Pontine Nuclei[1]

Exp. no.	Lobule	Evoked potentials	Olivary region with labeling	Corresponding cortical zone	Labeled olivary cells (no.)	Labeled pontine cells (no.)
GR03	COP	Not tested	vfDAO	C1	167	790
981	COP	Not tested	rMAO	C2	24	276
A40	PMD	Contralateral face	MAO b/c + DM	A2 + ?	27	488
AP03	PMD	Ipsilateral forelimb	vfDAO	C1	105	631
979	PMD	Not tested	vfDAO	C1	89	709
AP09	PMD	Ipsilateral and contralateral forelimb	rMAO	C2	40	612
978	PMD	Not tested	vIPO/DM	D0/D1	17	459
AP02	PMD	Ipsilateral forelimb	vIPO/DM	D0/D1	59	1093
A41	Crus 2B	Ipsilateral face	rMAO	C2	29	389
A39	Crus 2B	Ipsilateral face	DM	D0	33	146

[1]For abbreviations, see list.

esthetic of choice. Depending on the site of maximum response, or based on coordinates, CTb (#104, lot #10426A, low salt: List Biological Laboratories (Campbell, CA), 1% w/v in 0.2 M phosphate buffer, pH 7.4) was injected at the apex of the lobule by using a glass pipette (with filament, tip 10–15 μm; Ruigrok et al., 1995; Voogd et al., 2003) and applying 7 seconds on, 7 seconds off pulses of 4 nA anodal current for 5–10 minutes. After injection, muscles and skin were sutured, and the animal was allowed to recover. Postoperative analgesia was provided by a single dose of subcutaneously administered buprenorphine (0.05 mg/kg). All animals recovered uneventfully and were checked daily, but additional analgesic therapy was not considered necessary.

After a survival time of 7 days, rats were given a lethal dose of sodium pentobarbital (240 mg/kg), and the rats were perfusion-fixed by cannulation of the ascending aorta and by subsequent infusion of 500 ml of saline, 1,000 ml of fixative, made up of 4% freshly prepared paraformaldehyde and 0.1% glutaraldehyde in 0.05 M phosphate buffer (PB) containing 4% sucrose. After perfusion, the brains were extracted, blocked, and postfixed in the same fixative for 3–5 hours. They were rinsed and stored overnight in 0.05 M PB containing 10% sucrose and embedded in 11% gelatin containing 10% sucrose. The gelatin block was hardened for 3 hours in a 4% formaldehyde solution (containing 30% sucrose) and again stored overnight in 0.05 M PB containing 30% sucrose.

Histology

Histological procedures have been detailed elsewhere (Ruigrok et al., 1995; Voogd et al., 2003). Briefly, sections were cut at 40 μm on a freezing microtome and serially collected in glass vials containing 0.05 M PB. CTb immunohistochemistry was carried out on free-floating sections within selected glass vials by using subsequent incubations in a high-titer polyclonal anti-choleragenoid raised in goat (goat anti-CTb: lot #703: List Biological Laboratories, 1:15,000 in PBS and containing 0.4% Triton X-100 for 48–72 hours at 4°C), biotinylated donkey anti-goat antibody (List Biological Laboratories, 1:2,000 for 1½ hours at room temperature), avidin-biotin complex (ABC Elite [Vector, Burlingame, CA] for 1½ hours at room temperature), and, finally, 3,3′-diaminobenzidine tetrahydrochloride (0.025% DAB and 0.005% H_2O_2 in PB at room temperature). This procedure resulted in dense staining of CTb present at the injection site and within neuronal profiles throughout brainstem and cerebellar regions that have been known to be related to the injection site (Luppi et al., 1990; Voogd et al., 2003; Ruigrok, 2004; Voogd, 2004).

Some vials were selected for double staining with zebrin II immunohistochemistry. In these vials, cobalt (0.01%) and nickel (0.01%) ions supplemented the DAB incubation bath, resulting in a black deposit of reaction product, and sections were subsequently incubated with the mouse monoclonal anti-zebrin II, which was produced by immunization with a crude cerebellar homogenate of the weakly electric fish *Apteronotus* and recognizes a single polypeptide antigen with a molecular weight of approximately 36 kD (Brochu et al., 1990). This antibody was kindly provided by Dr. R. Hawkes [Calgary, Canada]. Briefly, sections were incubated, free floating, for 48–72 hours in anti-zebrin II, 1:150 in Tris-buffered saline containing 0.4% Triton X-100 (TBS) and 2% normal horse serum at 4°C. After thorough rinsing, sections were incubated for 2 hours in rabbit anti-mouse antibody conjugated to horseradish peroxidase (HRP; p260 Dako [Carpinteria, CA], 1:200 in TBS and 2% normal horse serum). Subsequently, sections were thoroughly rinsed in PB, incubated in a second DAB staining (without heavy metal ions) for 15–20 minutes, and rinsed in 0.05 M PB, resulting in brown staining of zebrin-positive Purkinje cells in a pattern that was identical to that described in detail earlier (Voogd et al., 2003; Voogd and Ruigrok, 2004; Pijpers et al., 2005). No cellular zebrin II staining was found in the brainstem, and no zebrin staining of Purkinje cells resulted when the primary antiserum was omitted. All sections were mounted on slides in a chromic alum solution, air-dried, and counterstained with thionin. Subsequently, slides were dehydrated in graded alcohol and xylene and coverslipped with Permount.

Analysis

Sections were analyzed with an Olympus microscope equipped with a Lucivid miniature monitor and with Neurolucida software (Microbrightfield, Williston, VT). Brainstem and nuclear contours were plotted at low magnification (2.5× objective), and retrogradely labeled cells were subsequently indicated at high-power magnification (10–20× objective). Maps of the labeling in the inferior olive as well as of the Pn were based on sequential series of one of four sections (i.e., every 160 μm), resulting in a series of 12 coronal plots throughout the Pn. The width and height of the sections were adjusted with respect to a standard series of sections, allowing comparison of the results between animals (Brevik et al., 2001). Subsequently, a 160 × 160-μm grid overlay was used to count the number of

labeled neurons within every grid square and served as a measure of the density of labeled neurons. Using standard Matlab routines and interpolation (The Mathworks, Natick, MA), this density was visualized in color-coded density plots. In addition, by rearranging the grids, the same data could also be used to obtain horizontal and sagittal planes. By summation of grids, summed plots of the coronal, dorsal, and sagittal views were obtained. The 12 coronal plots also served as the basis of the three-dimensional plots of the Pn. Section by section alignment was performed by using the Neurolucida software. The midline ventral surface of the pons was used as a reference, and new sections were shifted and rotated to obtain a best fit of contours. Three-point smoothing of contours was performed before final three-dimensional rendering by Neurolucida.

The photomicrographs of Figure 2 were made with a Leica DMR microscope equipped with a digital camera (Leica DC-300). Photo panels were constructed in CorelDraw 11.0, after some correction for brightness and contrast in Corel Photopaint 11.0.

RESULTS

A key objective of the present study was to relate the pontocerebellar projection to the organization of climbing fiber input to the cerebellar cortex. Because the relation between the zebrin pattern and the organization of the olivary projections to the cerebellar cortex has recently been established (Sugihara and Shinoda, 2004; Voogd and Ruigrok, 2004; Pijpers et al., 2005), an accurate classification of injection sites as based on their position relative to the zebrin pattern (see below), as well as with respect to the resultant retrograde labeling in the contralateral inferior olive, was possible. Within the posterior part of the paravermal, or intermediate, cerebellar cortex, three parasagittally oriented climbing fiber zones have been identified (Buisseret-Delmas and Angaut, 1993; Voogd et al., 2003; Sugihara and Shinoda, 2004). Most medially is the C1 zone, which receives its climbing fiber input from the ventral fold of the dorsal accessory olive (vfDAO). Directly lateral to the C1 zone is the C2 zone, which is innervated by climbing fibers derived from the rostral part of the medial accessory olive (rMAO); the C3 zone laterally borders C2 and is again supplied by the vfDAO. Lateral to the C zones are the hemispheral D zones, and they receive their climbing fiber input from the principal olive (PO). Medial to the C zones, a vermal B zone is found in lobule VIII, whereas a vermal A2 zone is noted in lobule VII (Atkins and Apps, 1997; Sugihara and Shinoda, 2004; Voogd and Ruigrok, 2004).

Identification of the C zones as well as of the A2 zone was possible prior to injection by determining the location of the receptive fields of these climbing fiber zones (Atkins and Apps, 1997; Voogd et al., 2003). However, although most of the injections (n = 6) were based on the latency and laterality of the evoked climbing fiber responses from different body parts, in all cases the ultimate classification of the injection site was based on the resulting labeling in the inferior olivary complex (Table 1).

Classification of injection sites

Figure 1 presents the location of 10 injections that were analyzed in detail together with their resultant labeling within the contralateral inferior olivary complex. The injections are shown relative to their position with respect to the zebrin pattern banding as examined in the double-labeled sections. Terminology of zebrin banding is adapted from Voogd and Ruigrok (2004; also see Sugihara and Shinoda, 2004; Pijpers et al., 2005). Three of these cases were centered on the C1 zone. In case GR03, the injection was placed in the copula pyramidis (COP), where it covered part of the zebrin-positive patch "e" (Fig. 2A). It resulted in retrogradely labeled neurons that were located in the caudolateral aspect of the vfDAO (Fig. 2B). In the COP, climbing fiber-evoked potentials in C1, usually referred to as c1 (Voogd et al., 2003), can be triggered by percutaneous stimulation of the ipsilateral hindlimb and tail (Atkins and Apps, 1997). In the paramedian lobule (PMD), two injections (cases AP03 and 979, respectively) involved the ventral and dorsal aspects of C1. Both injections were centered on the zebrin-negative zone directly medial to P5+ and resulted in labeling of olivary neurons in the rostromedial aspects of the vfDAO. In case AP03, evoked climbing fiber potentials could be triggered by percutaneous stimulation of the ipsilateral forelimb, and they are typical of the C1 zone in the PMD (Atkins and Apps, 1997). Medial to the C1 injections, the A2 zone was targeted in case A40. As described by Atkins and Apps (1997), contralaterally evoked responses from the face could be recorded at this site (not shown), and the subsequent CTb injection involved both the P4+ and P4− zebrin bands. The resultant olivary labeling was noted in the b/c groups of the caudal MAO but also included neurons in the caudal part of the DM group of the PO.

Three injections were centered on the C2 zone. In case 981, the injection was positioned within the COP and was found within the medial part of the lateralmost zebrin-positive zone (P5/6/7+). Olivary labeling was restricted to the lateral aspect of the rostral part of the medial accessory olive (rMAO), thus confirming the C2 location of the injection site (Pijpers et al., 2005). In addition, in case AP09, the injection site was located within the P5+ band of PMD, and labeled neurons were found within the rMAO at a more medial position compared with case 981. Finally, the injection in case A41 involved the P5+ band in crus 2B and resulted in labeling of neurons within the dorsal aspect of the rMAO. Receptive fields of c2 are typically found bilaterally on the hindlimbs for the COP and on both forelimbs for the PMD (Atkins and Apps, 1997), as was confirmed for case AP09, but was not tested in case 981 (Table 1). For case A41, ipsilateral face responses could be recorded prior to injection; however, the contralateral face was not tested (inset in Fig. 1A).

Three injections resulted in olivary labeling that was exclusively or mostly confined to the principal olive (PO). Labeling was specifically found in its ventral leaf and within the dorsomedial group (DM), which forms a dorsomedial extension of the ventral leaf of the PO (vlPO). The injections of cases 978 and AP02 were both located in the ventral part of the PMD and covered to varying extents zebrin zones P5− and P6/7+. In case 978, the injection was placed somewhat lateral and ventral to case AP02. In addition to the PO labeling, this resulted in several labeled neurons within the rMAO. Because no involvement of C1 or C3 was apparent due to the lack of olivary labeling in the vfDAO, it is likely that the rMAO labeling resulted from a small lateral extension of the main C2 zone (present within the P5+ zebrin band) within the medial aspect of the P6/7+ zone (cf. Sugihara and Shi-

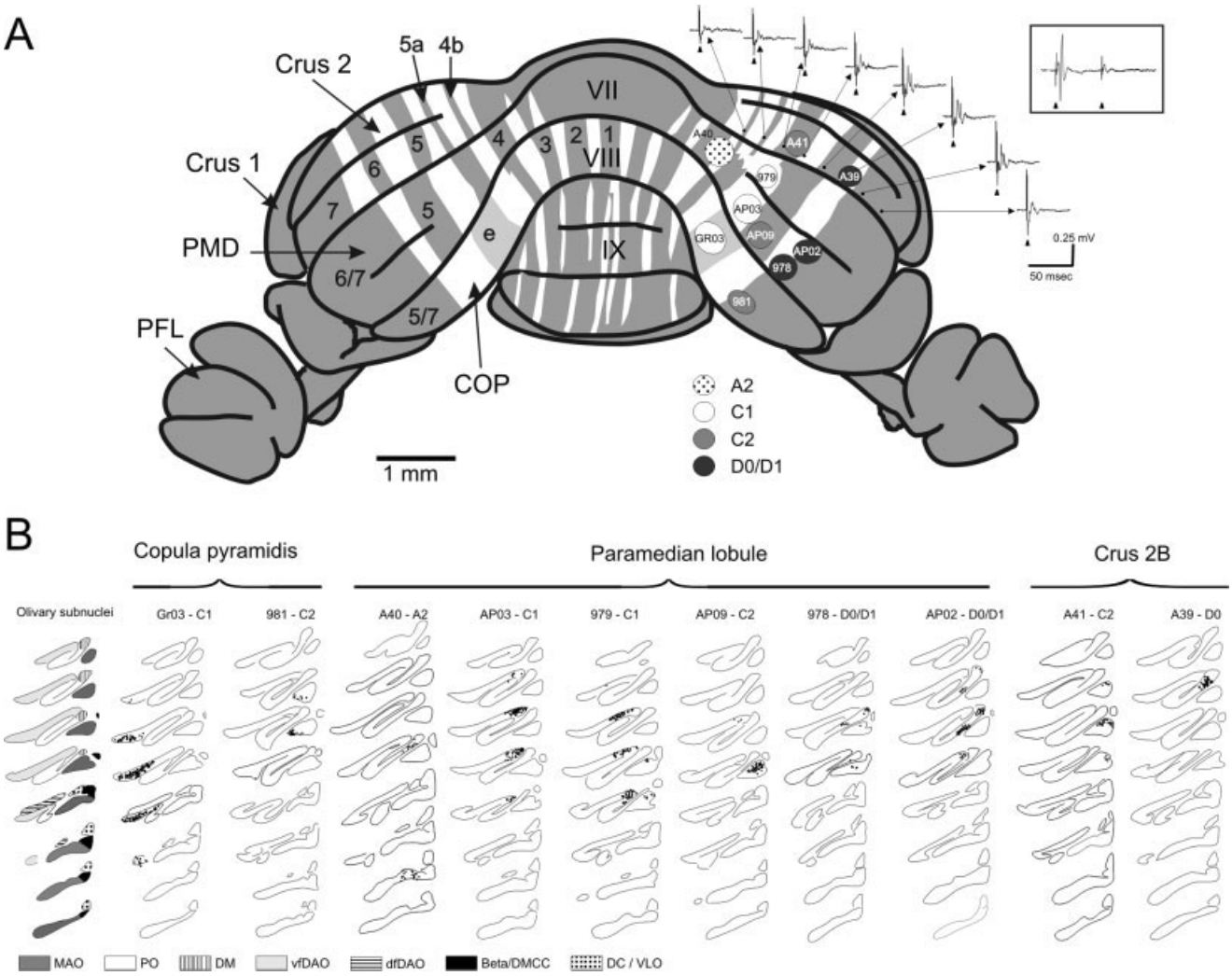

Fig. 1. **A:** Diagram depicting the site and size of the CTb injections described in this study. Approximate positions of the injections are indicated on a reconstruction of the posterior view of a sectioned rat cerebellum stained for zebrin (Voogd and Ruigrok, 2004). Injections are gray-scale coded to indicate their zonal position as based on the retrograde labeling of neurons in the inferior olive. For case A39, averaged evoked potentials (10 sweeps) triggered from electrical stimulation of the ipsilateral whiskerpad and upper lip region at 300 μA are shown as recorded from the indicated positions on folium B of crus 2. Arrowheads indicate time of stimulation and the ensuing sharp positive deflections are suggested to be related to climbing fiber activation of Purkinje cells. Latency of the peak response at the place where the injection was made measured 6.6 msec. Inset shows paired pulse stimulation (interval of 50 msec) in another experiment (A40). Note that the second pulse failed to trigger an evoked response, which is highly indicative that the evoked potentials indeed reflect climbing fiber activation (Atkins and Apps, 1997). At the indicated positions no responses could be evoked from ipsi- or contralateral hindlimb or forelimb stimulation. **B:** Plots of retrograde labeling in the inferior olive at 160-μm intervals. Each dot indicates a single-labeled olivary neuron. Cases are grouped according to their lobular location and distribution of most of the retrogradely labeled cells in the inferior olivary complex. A key to the different subdivisions of the inferior olive is shown on the left. For abbreviations, see list. Scale bar = 1 mm.

noda, 2004). PO labeling in case AP02 was rather similar to that obtained in case 978, but here additional labeling was not observed in the rMAO but rather in the vfDAO. This identified the injection site as centered on the D1 zone (vlPO) and involving the D0 zone (DM group) laterally and the zebrin-negative C3 zone (vfDAO) medially.

The third injection involving the D zones (case A39) was placed in crus 2B, in a region in which stimulation of the ipsilateral face (whiskerpad and upper lip region) resulted in short latency-evoked climbing fiber potentials (Fig. 1A). In this case, the resulting olivary labeling was confined to the DM group, establishing the D0 location of the injection site (Buisseret-Delmas and Angaut, 1993; Voogd et al., 2003; Sugihara and Shinoda, 2004; Pijpers et al., 2005).

Pontine labeling

Figure 2 shows the results of the CTb injection placed in the medial aspect of the COP (case GR03). As indicated above, this case resulted in labeled olivary neurons that were confined to the caudolateral aspect of the vfDAO, thereby demonstrating the C1 locus of the injection site (Fig. 2A,B; also see Fig. 1). In this particular case, retro-

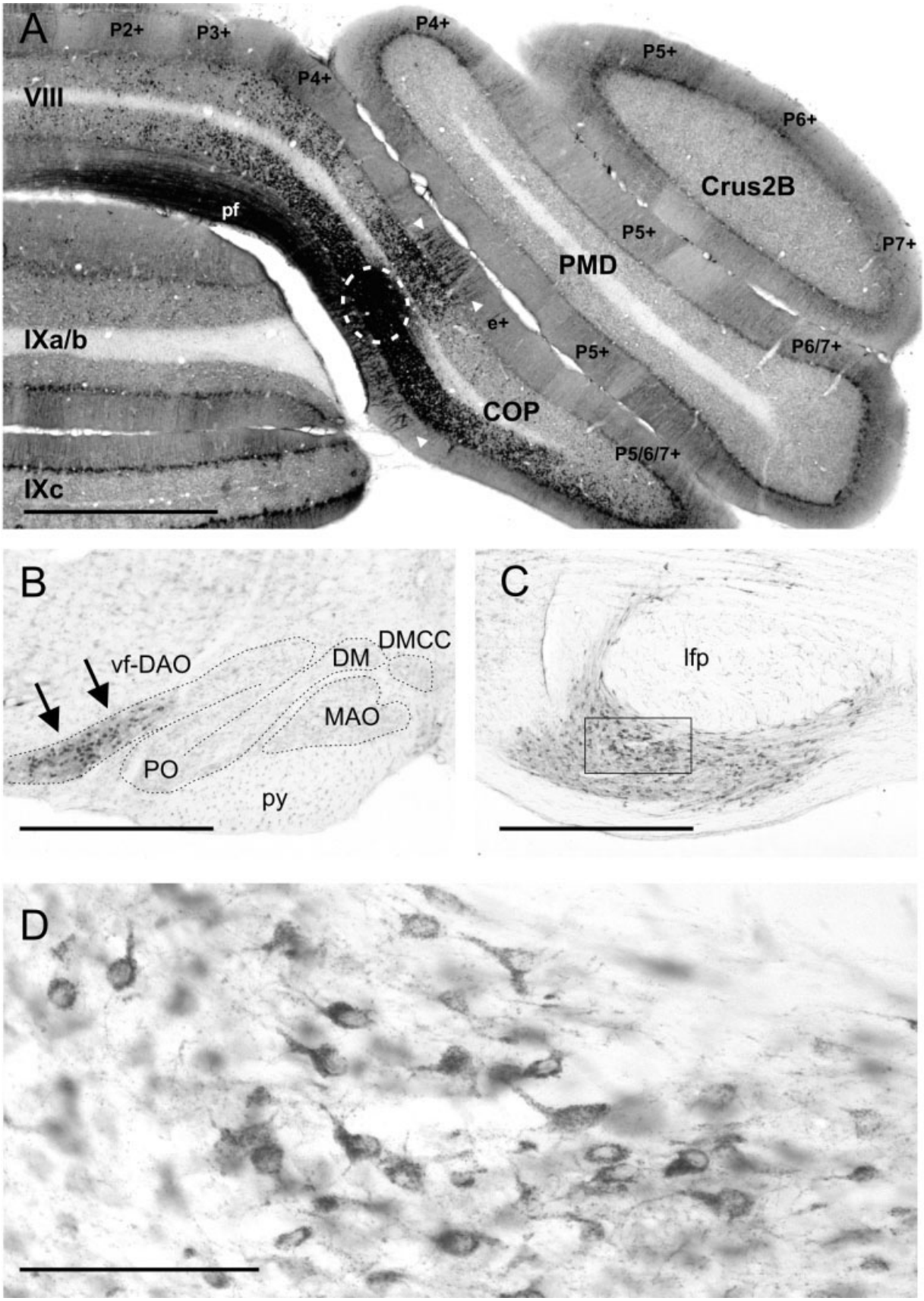

Fig. 2. Microphotographs showing results from case GR03. **A:** Transverse section of the posterior cerebellum incubated for zebrin showing the CTb injection centered at the COP (white hatched circle). Zebrin-positive bands are indicated according to Voogd and Ruigrok (2004). Note that collaterals of labeled mossy fibers are visible throughout the granular layer of lobule VIII, as are labeled parallel fibers (pf) and climbing fibers in the molecular layer (arrowheads). **B:** Retrogradely labeled olivary neurons (arrows) are confined to the lateral aspect of the contralateral vfDAO. **C:** retrogradely labeled neurons in the contralateral Pn. **D:** Higher magnification of labeled neurons shown in the boxed area of C. All sections were counterstained with thionin. For abbreviations, see list. Scale bar = 1 mm in A–C; 100 μm in D.

gradely labeled cells in the Pn were mostly, but not exclusively, located in their caudal aspect at the contralateral side (Fig. 2C,D). An additional cluster of labeled neurons, dissociated from the main body of labeling, was found at the rostral aspect of the Pn. Three-dimensional plots, based on a one of four series of sections and showing both dorsal and caudal views, provide additional information on the overall distribution of labeled neurons throughout the Pn. These plots were constructed for all 10 cases, thus allowing direct comparison of the Pn labeling resulting from the different zonal injections (Fig. 3). From these plots it can be appreciated that the injection in the C2 region of the COP (case 981) resulted in a rather similar general distribution of pontine labeling, as in case GR03 (Fig. 3, lower panels).

The middle row of panels in Figure 3 shows three-dimensional reconstructions of three cases with injections in the C1, C2, and D0/D1 zones of the PMD, respectively. In these cases, labeling was found at distinctively more central regions compared with the COP injections (cases GR03 and 981), although, for injections of the C1 and C2 zones (cases 979 and AP09), the focus point of the labeling could still be found in the caudal half of the contralateral Pn. Projections to the D0/D1 regions of the PMD originated from a medioventral sheath of cells at the center of the pons. Finally, three-dimensional reconstructions of the A2 zone injection (case A40) in the PMD and the C2 and D0 injections of crus 2B, which all receive face-triggered climbing fiber-evoked potentials, show that in all these cases a fairly circumscribed patch of neurons was found at the center of the Pn. Within this center area, no clear caudal or rostral bias of distribution was noted, which is in contrast to all formerly described cases. The latter three cases, however, did show differences with respect to the amount of labeled neurons within the ipsilateral Pn (see below).

To facilitate further a comparison of differences in the distributions and densities of labeled neurons in the various cases, the results were quantified and represented in color-coded density distributions (Fig. 4). Note that the resulting interpolated diagrams of Pn labeling, such as those shown in Figure 4C, provide both a quick and visually attractive way to evaluate the plot of the same section shown in Figure 4A. By rearranging the grids of consecutive transverse sections (one of four series of 40-μm sections), horizontally as well as sagittally oriented sectional views of similarly color-coded diagrams could be constructed (Fig. 5A). In this way, the distribution of labeled cells can be readily appraised in all three dimensions, and the most optimal view for comparing the resulting distribution of labeled neurons between cases can be chosen (Fig. 5B). For the present analysis, both sliced (Fig. 6A) as well as summed horizontal views (Fig. 6B) were constructed for all cases.

Figure 6A provides sliced diagrams arranged into horizontal sections at different dorsoventral levels of eight representative cases (cf. middle column of Fig. 5A). These diagrams clearly show that the labeling resulting from COP injections (cases GR03 and 981) was focused not only in the caudal part of the Pn but also at their ventralmost levels. In contrast, the patch of labeling in the rostral pons in both cases was noted at distinctively more dorsal levels. The highest densities of labeled neurons after PMD injections into C1/C2 cases were found not only more rostrally but also more dorsally compared with cases GR03 and 981. The injection that aimed for the forelimb c3 zone but resulted in coverage of mostly the D0/D1 zones (case AP02) also showed a pontine distribution quite similar to that of the C1 and C2 related zones and related region. Finally, the injections in face-related regions of both the PMD and crus 2B (cases A40, A41, and A39) appear to be centered in the rostrocaudal as well as the dorsoventral axes.

From these horizontal views, it can be appreciated that in several cases a considerable amount of labeled neurons was located within the ipsilateral Pn (Fig. 6). A further quantification of the distribution of contra- and ipsilateral labeling is shown in Table 1. Note that the case involving the contralateral face regions (A2 of the PMD) resulted in the highest percentage of ipsilaterally labeled neurons, followed by both cases that were centered on the C1 zone of the PMD. This contrasts in particular with the C1 injection in the COP (GR03) and the three hemispheral injections that all resulted in less than 5% of ipsilaterally located pontine neurons.

Figure 6B is a summary of the results, with figurines of the summed dorsal views of the pontine labeling shown on the approximate locations of their respective injection sites on the posterior cerebellum and with respect to their zonal positions. In this view, it can be appreciated that the distribution of pontine cells resulting from injections that were made in the same lobule (e.g., the COP) were rather similar, whereas the results from injections made in the same zone (e.g., the C2) but in different lobules were not. Also note that the distribution of neurons in the contralateral pons after injection into the A2 zone of the PMD was quite confined and was rather similar to that resulting from the C2 and D0 cases of crus 2B. In fact, the most striking difference between these injections related to the amount of ipsilaterally labeled neurons that rapidly decreased after more laterally placed injections. A similar tendency was observed in the other injections of the PMD but was not evident in the two COP cases (Table 1). The injection into the D0 zone of crus 2 resulted in the most confined distribution of labeled neurons that were all located in the center of the contralateral Pn.

DISCUSSION

Pontocerebellar projection and its relation with the corticopontine projection

The cerebrocerebellar connection by way of the Pn is likely to be the largest extracerebral connection in most mammalian species. However, only recently has detailed insight into the fine anatomical organization of the first leg, i.e., the cerebropontine projection from the primary sensory cortex (SI), been obtained by using small electrophysiologically guided cortical injections of multiple anterogradely transported tracers and 3-dimensional reconstruction techniques (Leergaard et al., 2000a,b). In these and earlier studies that investigated the ontogeny of the cerebropontine projections (Leergaard et al., 1995), it was shown that the spatial relations existing within the SI essentially are maintained in a more clustered form within the Pn. However, here, the rather clearly recognizable cortical somatosensory map of the rat is transposed in sphere-like concentric rings with the head in the center and surrounded by the forelimbs, body, and, finally, the hindlimbs and tail (Leergaard et al., 2000b).

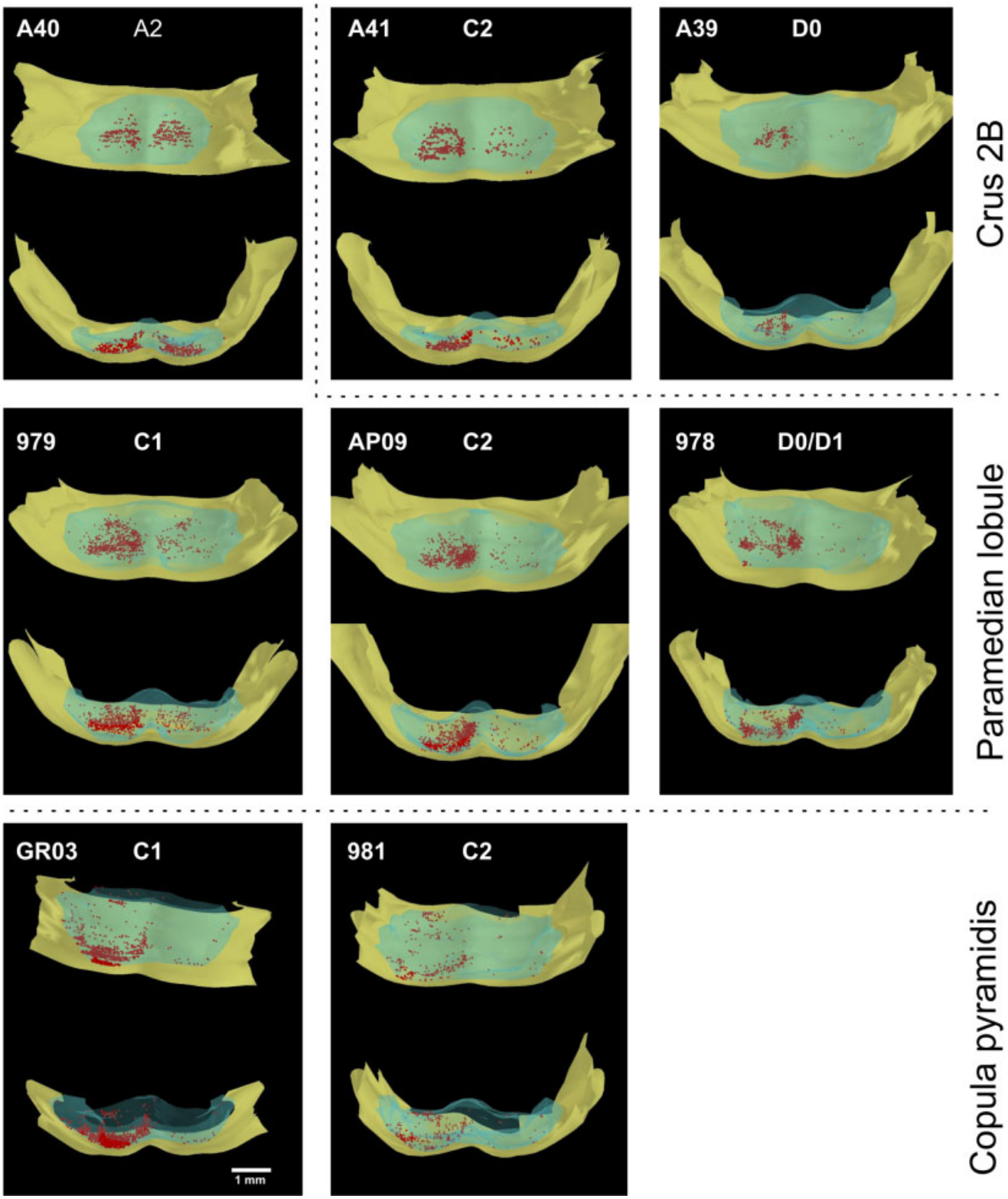

Fig. 3. Three-dimensional reconstructions of eight cases showing the distribution of retrogradely labeled neurons in the basal pons. Cases are arranged according to position of the injection site. In the top row, the two rightmost panels reflect the three-dimensional reconstructions of cases A41 and A39, in which the injection was placed in C2 and D0 of crus 2B, respectively. Top row, left-hand panel shows pontine labeling in case A40 with an injection involving the A2 zone of PMD. Middle row shows other PMD injections: from left to right, cases 979, AP09, and 978 with an injection in C1, C2, and D0/D1, respectively. Refer to Figure 1 for location of injections and determination of climbing fiber zone. Bottom row shows the pontine reconstructions of cases GR03 and 981 with injections in C1 and C2 of the COP, respectively. Both a dorsal (top) and a caudal (bottom) three-dimensional view are shown of the Pn (blue), the pial surface (yellow), and all labeled neurons (red dots) as plotted using a one of four series of consecutive sections. Scale bar = 1 mm in bottom left panel (applies to all).

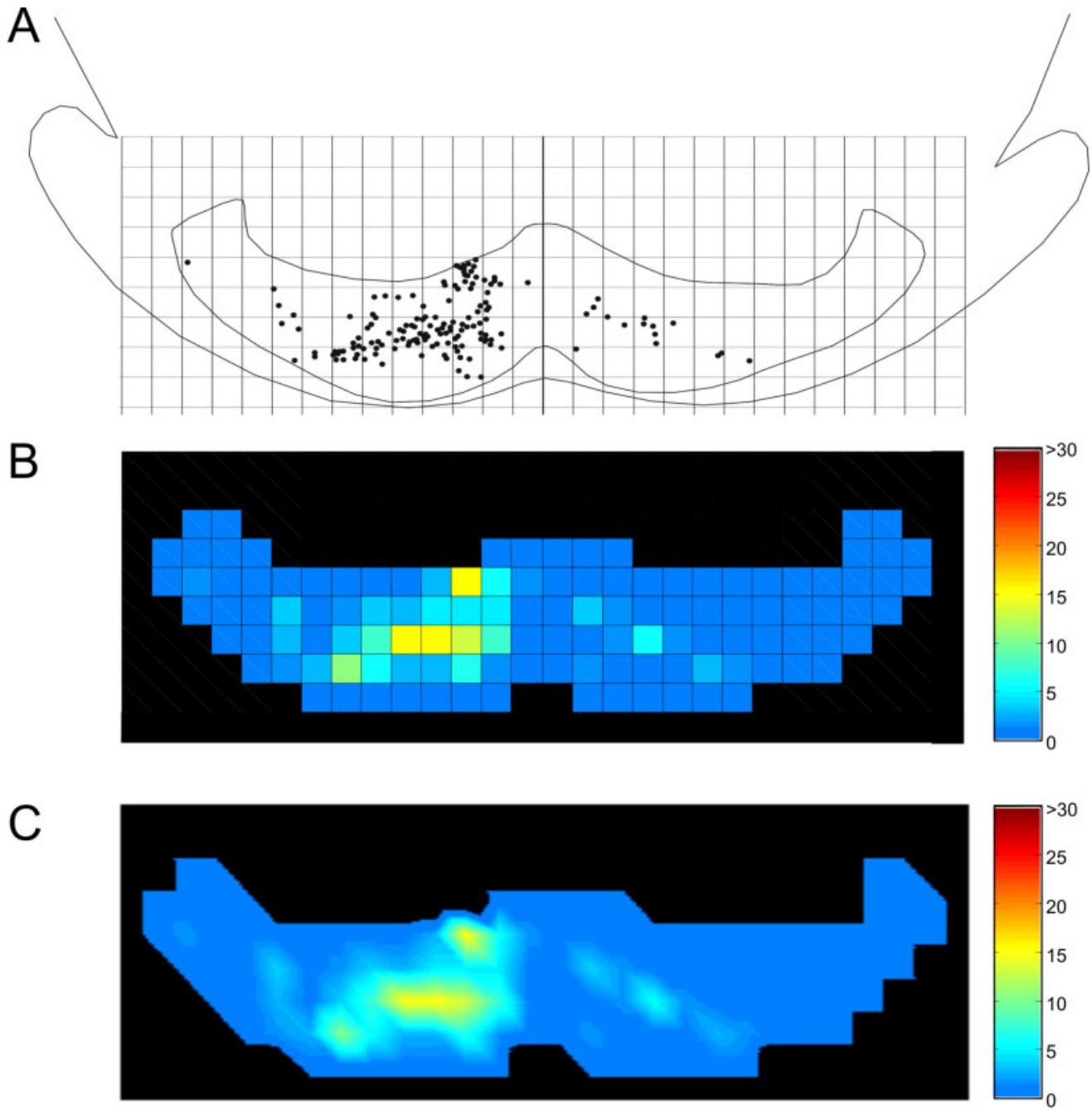

Fig. 4. Conversion of plotted reconstruction of individual sections into color-coded density plots in order to obtain an objective measure of the distribution of pontine labeling (case 979). **A:** Plot of a pontine section indicating borders of the nucleus and position of labeled neurons (using Neurolucida software). The plot is overlaid with a 160 × 160-μm grid, and the number of dots is counted within every grid square. **B,C:** The ensuing matrix is visualized by using Matlab software (B) using interpolation (C). Color coding reflects the number of labeled cells within a grid square (i.e., density of labeled neurons).

Here we show that the topography of corticopontine projections is generally maintained in the pontocerebellar projection (Fig. 7). Indeed, our injections in lobule VIII, which is considered to be involved in control of the hindlimb (for review, see Manni and Petrosini, 2004), mostly labeled pontine neurons in a location that was very similar to the corticopontine projections from the hindlimb region of the SI (Leergaard et al., 2000b). Likewise, the origin of the pontine projections to the PMD, controlling the forelimb, overlaps with cerebral projections from the forelimb SI. Finally, the injections into crus 2B, as well as the medialmost injection in the PMD, all in a face-responsive area, only resulted in labeled pontine neurons that took up rather central positions in the contralateral

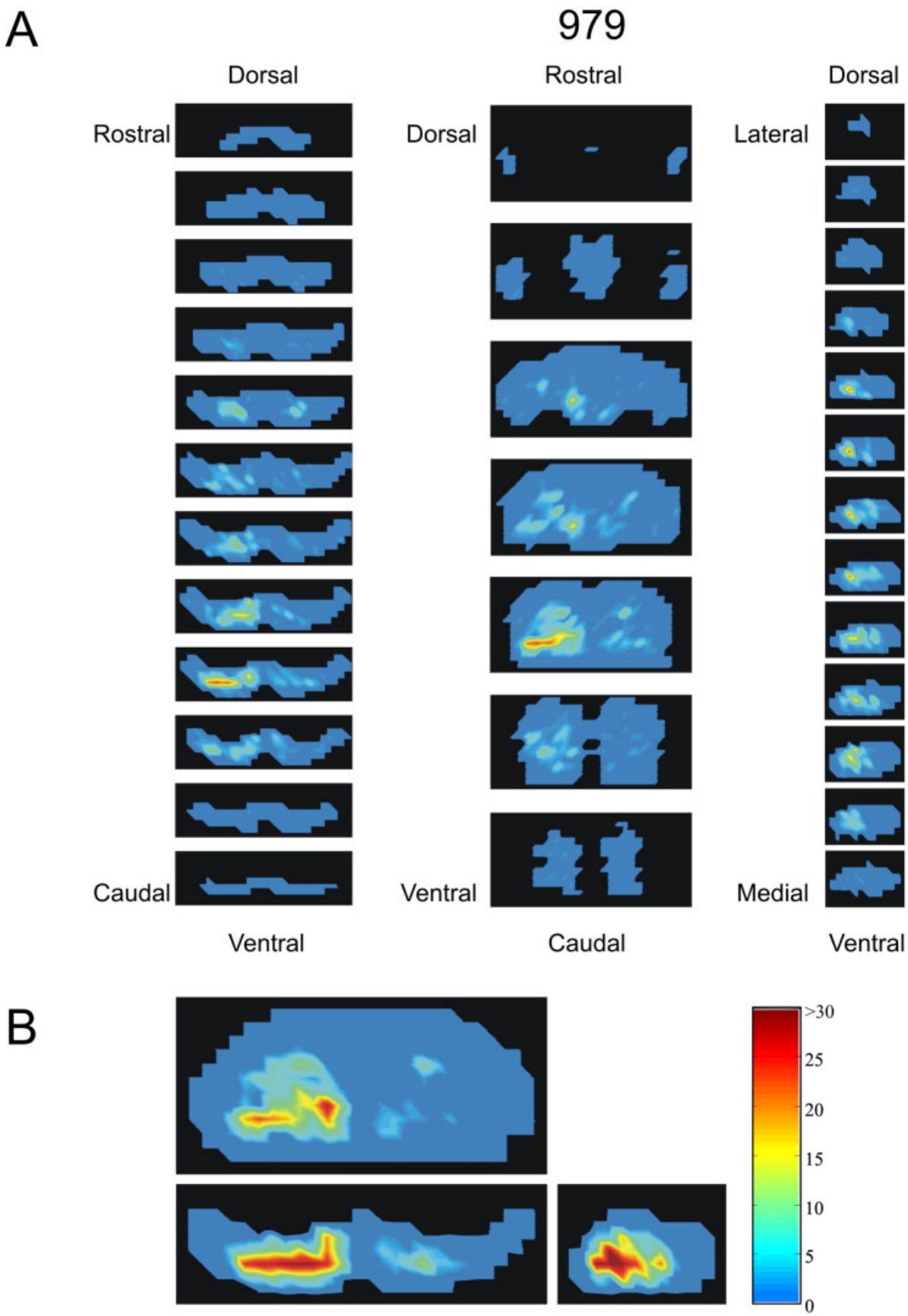

Fig. 5. Color-coded density profiles of the Pn of case 979 as constructed from a series of one out four transverse sections (of 40 μm each). **A:** Original conversions of transverse series are shown in the left column. By rearranging the 160 × 160-μm, grids it was possible to obtain horizontal (middle column) and sagittal (right column) cross-sectional views of the Pn and distribution of labeled cells. **B:** Summing all grids into either the transverse, horizontal, or sagittal direction results in color-coded views of the density profiles in rostrocaudal (top), dorsoventral (bottom left), and lateral-medial (bottom right) views, respectively. Together they provide a three-dimensional indication of the distribution of labeled neurons. Note that the dorsoventral summed view seems to provide the most useful information on the distribution of labeled neurons. Also note that the sagittal sections in A and sagittal summation in B only involve the contralateral side of the Pn.

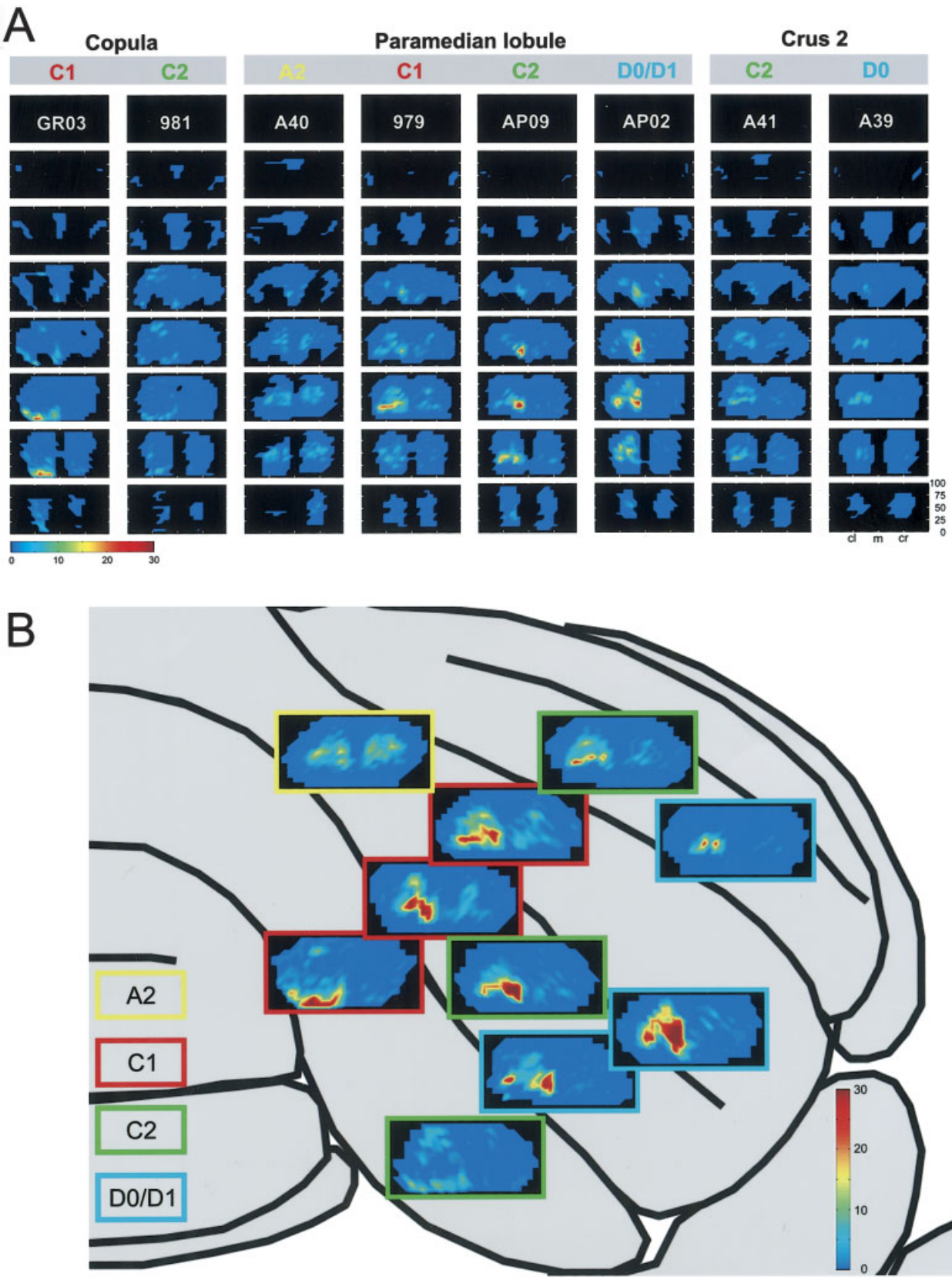

Fig. 6. **A:** Color-coded density profiles of the distribution of retrogradely labeled neurons in the basal pons viewed with horizontal sections taken at 160-μm intervals in eight cases with injections into identified zonal regions of the posterior cerebellum. Note the similarity in distribution patterns resulting from injections into the COP (hindlimb: cases GR03 and 981), zones C and D zones of the PMD (forelimb: cases 979, AP09, and AP03), and zones A2 of the PMD and C2 and D0 of crus 2B (face: cases A40, A41, and A39), respectively. However, also note the differences in the contribution of ipsilaterally labeled neurons (cf. Table 1). Conventions for representing the figurines are similar to those of Figure 5. In addition, tic marks have been introduced to facilitate comparison between cases and indicate center left (cl), midline (m), and center right (cr) on the horizontal axis and caudorostral extent of the Pn in percentage on the vertical axis. **B:** Color-coded density profiles of the summed diagrams depicting the dorsoventral distribution of retrogradely labeled neurons in 10 cases with CTb injections in the posterior part of the intermediate cerebellum. Diagrams are placed at the approximate position of the injection site on the surface of the posterior cerebellum (also see Fig. 1), and the zonal identification is indicated by yellow (A2), red (C1), green (C2), or blue (mostly D0/D1) borders.

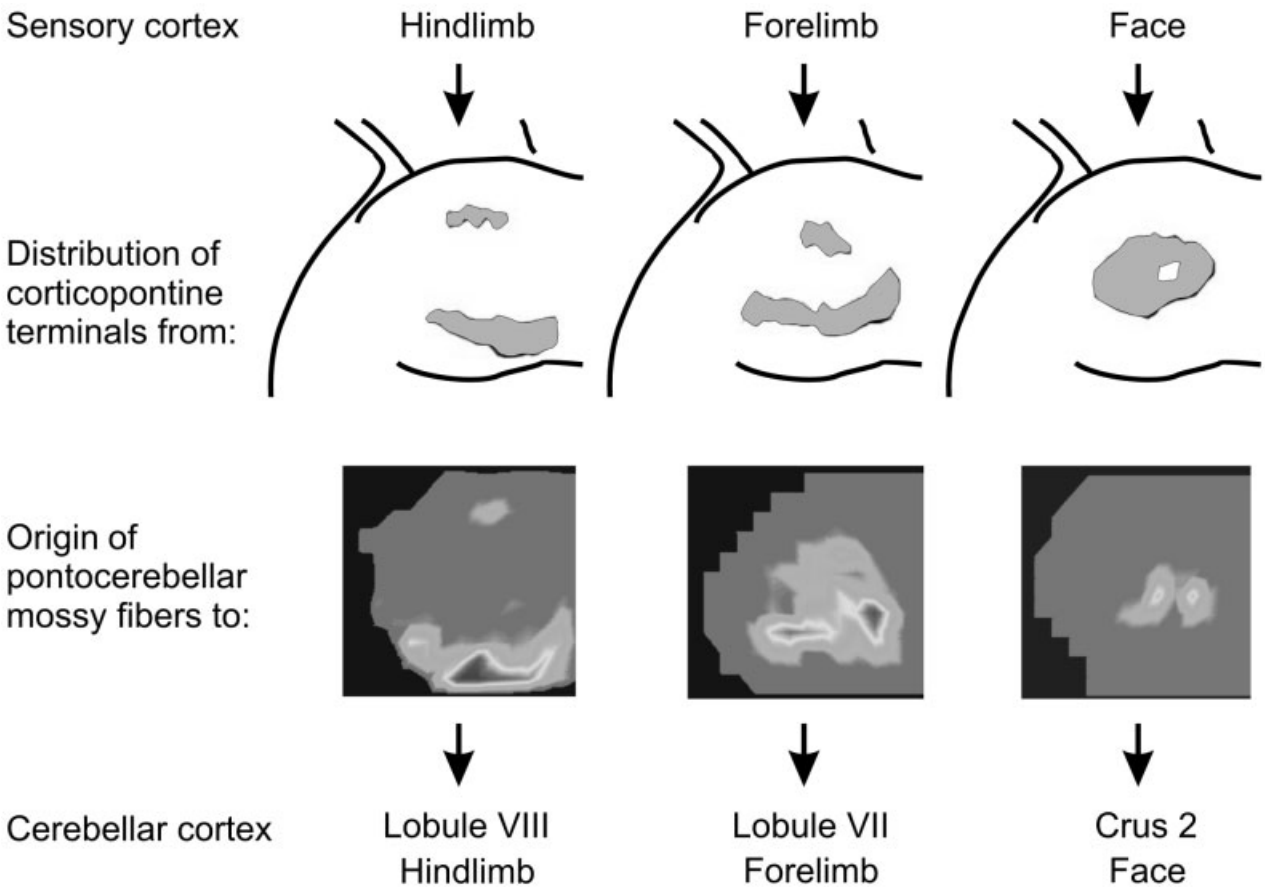

Fig. 7. Similarity in the organization of corticopontine projection from the primary somatosensory cortex and pontocerebellar projection to regions with climbing fiber receptive fields from either the hindlimb (ipsi- or bilateral), forelimb (ipsi- or bilateral), or face (ipsilateral). Distribution of corticopontine terminals is redrawn from Leergaard et al. (2000b). Distribution of pontocerebellar neurons is based on average density (in percentages of total number present in the contralateral Pn) calculated for cases GR03 and 981 (hindlimb), AP03, 979, AP09, and AP02 (forelimb), and AP40, AP41, and A39 (face).

Pn and hence at a position where terminals from the face region of the somatosensory cortex are likely to terminate. Similar conclusions were reached in a recent study by Odeh and collaborators (2005), who studied the distribution of retrogradely labeled neurons in the Pn after injections of fluorescent beads in the C1 and A2 zones of the PMD and the C1 zone of the COP. However, it should be noted that the C1 zone has climbing fiber receptive fields located on body parts ipsilateral to the cerebellar zone, whereas the A2 zone is mostly activated from contralateral face regions (Atkins and Apps, 1997). Nevertheless, as in our material, the pontine distribution of retrogradely labeled cells after an injection in A2 seems to overlap with the distribution after our crus 2B injection in C2 or D0. This suggests that all face-related regions of the cerebellar cortex may receive their pontine "face" information from essentially the same region but receive their climbing fiber input from rather different parts of the inferior olivary complex.

The observed coincidence between patterns of corticopontine terminations from the SI and distribution of pontocerebellar neurons to these regions suggests that at least aspects of the organization of corticopontine projection patterns are maintained in the organization of pontocerebellar connections. This seems at odds with the general notion that the mossy fiber projections in general and the pontocerebellar projection in particular are characterized by a rather divergent and patchy organization that ultimately results in the transformation of a single cortical somatosensory map into multiple noncontinuous or fractured somatosensory regions on the cerebellar cortical surface (Brodal, 1982; Welker, 1987; Bower, 1996; Voogd et al., 1996; Wu et al., 1999). The regions investigated in the present paper are selected and classified with respect to the origin of their climbing fiber input from either hindlimb, forelimb, or face rather than with the fine, and fractured, topographic relations that have been described for mossy fiber inputs to these regions (Bower et al., 1981; Welker, 1987; Manni and Petrosini, 2004). Nevertheless, in agreement with Odeh et al. (2005), we are able to make statements on the correlation between the organization of pontocerebellar and olivocerebellar systems.

Pontocerebellar projection and climbing fiber zones

By relating retrograde labeling within the Pn and the inferior olivary complex after individual injections into the cerebellar cortex, it was possible to verify the relation

between the cortico-ponto-cerebellar input and the climbing fiber receptive fields in the COP, PMD, and crus 2B. For the rat, climbing fiber receptive fields in the COP and PMD were first described by Atkins and Apps (1997) and were later confirmed by others (Teune et al., 1998; Pardoe and Apps, 2002; Voogd et al., 2003). In these studies, the C1 zone of lobule VIII was shown to respond to short-latency stimulation of the ipsilateral hindlimb (i.e., physiological c1 zone) and to receive its climbing fibers from the lateral part of the dorsal accessory olive (DAO). The C2 zone of lobule VIII, by definition, receives its climbing fibers from the rostral part of the medial accessory olive (rMAO) and can be activated from both hindlimbs at a longer latency. In between, a small CX zone or lateral c1 zone is located, which is characterized by short-latency hindlimb receptive fields corresponding with climbing fibers that are derived from the intermediate part of the medial accessory olive (iMAO: Atkins and Apps, 1997; Voogd et al., 2003). This latter zone was not specifically studied in the present study.

Although both COP injections were not placed using prior determination of the receptive fields, by relating the injection site to the cortical pattern of zebrin II (Sugihara and Shinoda, 2004) and, more importantly, to the resultant labeling in the inferior olive, it was inferred that our two COP injections typically were centered on the C1 (GR03) and C2 (981) zones. However, no obvious differences were noted in the overall distribution of labeled neurons within the contralateral Pn, although the more or less separated rostral patch of neurons seems to take up a somewhat larger area in case 981 (C2) compared with case GR03 (C1). The observation that the percentage of ipsilaterally labeled neurons was more than twice as high in case 981 (C2) compared with GR03 (C1: Table 1) may correspond with the notion that the climbing fiber receptive fields of C2 involve both hindlimbs, rather than solely the ipsilateral hindlimb in C1 (Atkins and Apps, 1997).

Within the PMD, six injections were described in the present study that, based on both its location with respect to the zebrin pattern as well as their distribution of retrogradely labeled cells within the inferior olivary complex, were judged to be located in either the A2, C1, C2, or D0/D1 zones. Furthermore, four of these injections had been placed based on the size and distribution of evoked potentials triggered from electrical stimulation of the ipsi- or contralateral forelimbs or contralateral face. Even though the zonal location of these injections was rather different, the distribution of pontine cells appeared to be remarkably similar, with the exception of the A2 injection (case A40). Peak density in all other cases was noted in a focused region, approximately 500–600 μm rostral to the caudal pontine border. This distribution is similar to that observed by Odeh et al. (2005) after selective injections of the C1 zone in the PMD. Because these authors also noted a systematic difference in pontine labeling between A2 and C1 zone injections of the PMD, they concluded that a precise cerebro-ponto-cerebellar topography exists that can be defined by climbing fiber somatotopy.

The present study is in general agreement with this conclusion, provided that the term climbing fiber somatotopy specifically refers to the general part of the body from which climbing fiber responses may be elicited rather than to a more detailed relation with a zonal topography as based on differences between ipsi- and bilateral representation of body parts and related to origin of the climbing fibers within the olivary complex (i.e., C1 or C2 of either the COP or PMD). In line with this notion was the observation that although all three cases with injections of the face-responsive regions in the PMD and crus 2B were localized, based on their resultant olivary labeling, within different climbing fiber zones (i.e., A2 of the PMD and C2 and D0 of crus 2B, respectively), most labeling was confined to a central core of the contralateral Pn in all three cases. Hence, the general distribution of pontine neurons that send mossy fiber rosettes to areas subjacent to the characterized climbing fiber zones depends more on the body region from which climbing fiber evoked potentials can be generated (i.e., face, forelimb, hindlimb) than on the zonal location of these regions as based on the olivary origin of these climbing fibers (i.e., A2, C1, C2, or D0/D1 zone).

Although the general pontine distribution was rather similar, conspicuous differences between injections covering different zones were also noted. They concern the percentages of ipsilaterally labeled Pn cells. Quite contrary to the injections in the C1 zone of the COP, PMD injections of C1 resulted in rather high densities of ipsilaterally located neurons, whereas the highest ipsilateral percentage was noted after the A2 injection (Table 1). These numbers were quite similar to the observations of Odeh et al. (2005). However, although with respect to the predisposition of ipsilaterally located neurons, C1 of the COP and PMD were markedly different, this was not the case after injection into C2 of the PMD (case AP09) and COP (981). C2 of crus 2B resulted in labeling of twice as many neurons in the ipsilateral Pn as the other C2 cases (Table 1).

Finally, injections involving D zones resulted in very few ipsilaterally localized pontine neurons. Indeed, the ipsilateral face-related region of D0 in case A39 clearly contrasts the contralateral face-related region of A2 in case A40. In this respect it is interesting to note that the micromapping studies of Welker and his group (1987) show that in the medial regions of crus 2 and PMD (both probably covered by the A2 zone: Voogd and Ruigrok, 2004), patches of granule cells are found that respond to either contralateral or bilateral regions of the face. If, indeed, a close correspondence is present between the organization of the climbing fiber system and that of the mossy fibers (Brown and Bower, 2001; Ruigrok, 2003; Voogd et al., 2003; Apps and Garwicz, 2005), a more refined mapping of climbing fiber receptive fields in these regions likewise might reveal a different location for contralaterally and bilaterally located receptive fields. This could be related to the fact that within this region several narrow zebrin zones are located (i.e., P4+, P4−, P4a+, P4a−, etc.), which have been shown to receive their climbing fibers from separate olivary subnuclei (Sugihara and Shinoda, 2004). Indeed, our injection A40 covered both the P4+ region as well as the adjacent P4− zone and resulted in retrograde labeling in the caudal DM group (which was shown to project selectively to the P4+ band) and the b/c group of the caudal MAO (projecting to several narrow zones lateral to P4+). Because bilateral projections from the trigeminal nucleus to the b/c groups of the MAO in particular have been well documented in rabbit (Van Ham and Yeo, 1992), the P4− region could very well be involved in processing information from both sides of the face, whereas other, adjacent, zones might be more selectively

involved in processing either ipsi- or contralateral face information.

In conclusion, the location of the pontine neurons that project to the granular layer subjacent to either the A2, C1, C2 or D0/D1 climbing fiber zones of the posterior cerebellum is not simply based on this zonal identification but correlates quite well with the general part of the body that can activate these climbing fibers. Zone-dependent differences that were noted mostly concerned the laterality of retrogradely labeled pontine neurons, which, taken together, suggests a highly structured organization of pontocerebellar projections (Mihailoff et al., 1981; Serapide et al., 2002b).

Modular organization of climbing and mossy fiber connections

One of the main aims of this study was to see whether the cerebellar cortical regions that receive a characteristic climbing fiber input also differ with respect to the origin of their mossy fiber inputs from the basal Pn. This possibility was suggested by the work of Serapide and collaborators (1994, 2002a), who showed in the rat that small injections with anterograde tracers in the Pn or the reticular tegmental nucleus of the pons resulted in a number of longitudinal zones of mossy fibers. Indeed, Voogd and colleagues (2003) have noted that collaterals of climbing fibers, labeled from localized CTb injections in the caudal cerebellum and arranged in longitudinal patterns, were always accompanied by mossy fiber rosettes labeled from the same injection. However, the origin of the participating mossy fibers was not specified in that study. Congruence of peripherally triggered mossy fibers and climbing fibers has been shown by comparing the receptive fields of peripheral mossy and climbing fibers responses in a selected region of crus 2 (Brown and Bower, 2001) and by relating this to the zonal distribution of the zebrin pattern of Purkinje cells (Hallem et al., 1999). Cerebrally evoked somatosensory mossy fiber responses have been shown to be in register with the peripherally evoked patterning (Bower et al., 1981).

So, if one accepts a close correlation between the receptive fields of climbing and mossy fibers, and most people agree that the climbing fiber organization follows zonal, or at least elongated, patterns (Sugihara and Shinoda, 2004; Voogd and Ruigrok, 2004; Apps and Garwicz, 2005; Pijpers et al., 2005), it follows that the mossy fiber organization is also likely to adhere to these patterns in general. However, our results in this retrograde study do not seem to endorse this conclusion. Apart from the differences in the contribution of ipsilaterally projecting pontine neurons, no conspicuous differences could be noted between the distribution of pontine neurons that supply mossy fibers to C1 or C2 zones of the COP and PML. This could imply that within the pontine regions that supply afferents to either the COP or PML, differences in distribution may exist but that they escape the present level of analysis. Indeed, Eisenman (1981a,b; see also Mihailoff et al., 1981) has noted small regional differences of pontine projections to medial, intermediate, and lateral parts of lobule VIII, but it did not appear to be possible to make positive correlations with the olivocerebellar patterning. A similar conclusion has been drawn for pontine projections to zonal regions of the PMD in the cat (Hoddevik and Walberg, 1979) and for projections of the reticular tegmental nucleus of the pons to the PMD in the rabbit (Grottel et al., 1989).

Recently, Herrero et al. (2002), in studying collateralization of pontine and olivary projections to c1 zones of both the simple lobule and PMD in the rat, noted that there was no correlation in the incidence of double-labeled neurons between both cell groups, although one was noted between olivary labeling and labeling in the lateral reticular nucleus. They concluded that the mossy fiber projections from the pons are not zonally organized. Finally, Morissette and Bower (1996) provided evidence that the spatial distribution of cortico-ponto-cerebellar-mediated peripheral responses measured within crus 2B have a wider spatial distribution compared with the direct trigeminocerebellar responses. Therefore, it is quite possible that the zonal distribution of mossy fiber collaterals and their relation to climbing fiber collaterals (Voogd et al., 2003; Pijpers et al., 2005) is primarily based on collateralization of mossy fibers from nonpontine regions such as the spinal cord and lateral reticular formation (King et al., 1998; Herrero et al., 2002). However, in that case the zonal patterning observed after making small injections into the pons as well as the zonal differences in ipsilateral pontine labeling cannot be easily explained (Serapide et al., 2001; Voogd et al., 2003). Hence, it is also possible that subtle differences in location of the retrogradely labeled Pn neurons escape the level of the currently used analysis. Indeed, the considerable differences noted in the percentage of ipsilaterally located neurons suggest that zone-related differences in Pn location are indeed possible. Double or triple retrograde tracer studies employing injections into the same cerebellar lobule that characterized climbing fiber zone (or mossy fiber patch) may help to establish such patterning.

Functional considerations

Cerebellar modules and their underlying micromodules are thought to act as the functional units of the cerebellum (Apps and Garwicz, 2005). The organization of the climbing fiber input system and the corticonuclear output seems to be perfectly related (Pijpers et al., 2005). However, a climbing fiber-related, modular organization of the mossy fiber system is much less obvious. Only recently have studies in cat and rat suggested that different parts of the mossy fiber input system (e.g., lateral reticular nucleus and Pn) are connected in a distinctive way to the cerebellar modular system (King et al., 1998; Herrero et al., 2002). The present study shows that the organization of pontine projections to the posterior parts of the intermediate cerebellum of the rat can be characterized by patterns of body representations of the overlying climbing fibers and seems to be less obviously related to the olivary origin of the climbing fiber zones. Nevertheless, more subtle zonal differences may still be present, as was indicated by zonal differences in ipsilateral projections.

Highly structured patterning of mossy fibers within the cerebellar cortex will have to be taken into account in models of cerebellar functioning, i.e., a potential narrow spatial correlation between the functional identity of mossy and climbing fibers suggests that when both systems are (nearly) simultaneously active, local interactions with other constituents of the cerebellar cortex, i.e., granule cells, interneurons, and of course the Purkinje cells, are likely to be of a quite different nature compared with regions receiving mossy fiber collaterals without related

climbing fibers (Jorntell and Ekerot, 2003; Apps and Garwicz, 2005). These findings suggest that our knowledge of the spatial and temporal interrelations between the cerebellar afferents and the cortical network (Simpson et al., 2005) has only just begun to deepen.

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