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The neuropeptide cerebellin is a marker for two similar neuronal circuits in rat brain

(cartwheel neurons/cochlear nuclei/immunocytochemistry/radioimmunoassay/Purkinje cells)

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ABSTRACT **We report here that the neuropeptide cerebellin, a known marker of cerebellar Purkinje cells, has only one substantial extracerebellar location, the dorsal cochlear nucleus (DCoN). By reverse-phase high-performance liquid chromatography and radioimmunoassay, cerebellum and DCoN in rat were found to contain similar concentrations of this hexadecapeptide. Immunocytochemistry with our rabbit antiserum C_1, raised against synthetic cerebellin, revealed that cerebellin-like immunoreactivity in the cerebellum is localized exclusively to Purkinje cells, while in the DCoN, it is found primarily in cartwheel cells and in the basal dendrites of pyramidal neurons. Some displaced Purkinje cells were also stained. Although cerebellum and DCoN receive their inputs from different brain regions, their organizations show several similarities, with many neuronal cell types present in one having their presumed homologue in the other. Cerebellin is present in Purkinje and cartwheel cells throughout the cerebellum and the DCoN without site predilection. This should be viewed together with recent data indicating that these two classes of neurons share a similar fate in several murine mutations ("nervous," "lurcher," "Purkinje cell degeneration," and "staggerer"). This situation leads us to conclude that cerebellin represents a marker for a small number of spatially related neuronal cell classes, and that it may play a role in an as yet unknown aspect of a circuit function worthy of further exploration.**

The apparent analogy between the structure and microcircuitry of layers 1 and 2 of the dorsal cochlear nucleus (DCoN) (1, 2) and cerebellar cortex (3–6) has been known for some time. The cochlear nuclei contain a system of mossy fibers synaptically connected to granule cells and associated interneurons (Golgi cells) resembling those in the cerebellar granular layer (7). Axons of the cochlear granule cells make up a system of parallel fibers in layer 1 (molecular layer) of the DCoN that synapse with neurons arborizing in this region (8), including the spiny cartwheel cells (9), the nearly aspiny stellate cells (10), and the apical spiny arbor of pyramidal cells (11). Whereas analogy of pyramidal neurons to mammalian cerebellar neurons, if any, remains to be ascertained, cartwheel and stellate neurons bear several distinctive cellular and synaptological similarities to cerebellar Purkinje cells and stellate cells, respectively (9, 10). This analogy even goes beyond the wiring strategy in that some of the neuronal cell types show biochemical similarities, perhaps emphasizing common functions. For instance, Golgi, Purkinje, and stellate cells in the cerebellum (3, 6, 12–14), and Golgi, cartwheel, and stellate neurons in the DCoN are GABAergic (15), and granule cells are believed to use an excitatory amino acid neurotransmitter (16) in both the cerebellum and cochlear nuclei.

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To extend the study of these two structures, we have examined and compared the expression and the localization of the recently discovered neuropeptide cerebellin (17) in the DCoN and the cerebellum by radioimmunoassay (RIA) combined with reverse-phase high-performance liquid chromatography (HPLC) and immunocytochemistry. Cerebellin has been demonstrated previously to be a marker of cerebellar Purkinje cells (17, 18), although its mode of distribution throughout the cerebellum was not explored.

MATERIALS AND METHODS

Biochemistry. One hundred Wistar rats (30–35 days old) were subdivided into four groups of 25 each and decapitated, and the brain was quickly removed. The cerebellum, the lower portion of the medulla oblongata, and the spinal cord were isolated, quickly frozen in liquid nitrogen, and pooled separately. After careful removal of the flocculus and the choroid plexus from the remaining brain stem, the DCoN was dissected free bilaterally from the inferior cerebellar peduncle and severed at its medial and lateral ends with iris scissors and then frozen in liquid nitrogen and pooled. The isolated DCoNs were contaminated by portions of the ventral cochlear nucleus (VCoN). In addition, a number of other brain regions and non-nervous tissues were removed and frozen. Guanidine hydrochloride extracts from the indicated neural structures were desalted on disposable C18 reverse-phase cartridges as described (17). The material eluting between 5% and 60% acetonitrile was collected and dried on a Speed Vac (Savant). After resuspension in 0.1% trifluoroacetic acid, the material was subjected to reverse-phase HPLC on a Vydac C18 column using a linear gradient of acetonitrile (5–60%) over 1 hr. Fractions were collected every 50 sec and dried as described above. These fractions, resuspended in RIA buffer, were assayed for cerebellin immunoreactivity by a specific RIA (18) utilizing an antiserum raised against synthetic cerebellin coupled to thyroglobulin. The details of this assay will appear elsewhere; the antibodies used are those described (18).

Immunocytochemistry. *Light microscopy.* Under sodium pentobarbital anesthesia, 12 Wistar rats (30–180 days old) were perfused through the ascending aorta with a saline solution followed by a fixative containing 4% (vol/vol) commercial formaldehyde, 0.5% zinc dichromate, and 0.9% sodium chloride. After 60 min, the brain was dissected out and placed for 48 hr in chilled 30% (wt/vol) sucrose in saline. Serial frozen sections were cut at 25 μm in the sagittal plane. Floating sections were incubated in 2% gelatin for 1 hr at 20°C and then in rabbit serum C_1 raised against synthetic cerebellin (18) (or in normal rabbit serum for control) diluted 1:1250, for 48–72 hr at 5°C over a shaker. Subsequently, the sections were incubated with standard reagents for the single cycle or

Abbreviations: DCoN, dorsal cochlear nucleus; VCoN, ventral cochlear nucleus; CLI, cerebellin-like immunoreactivity.

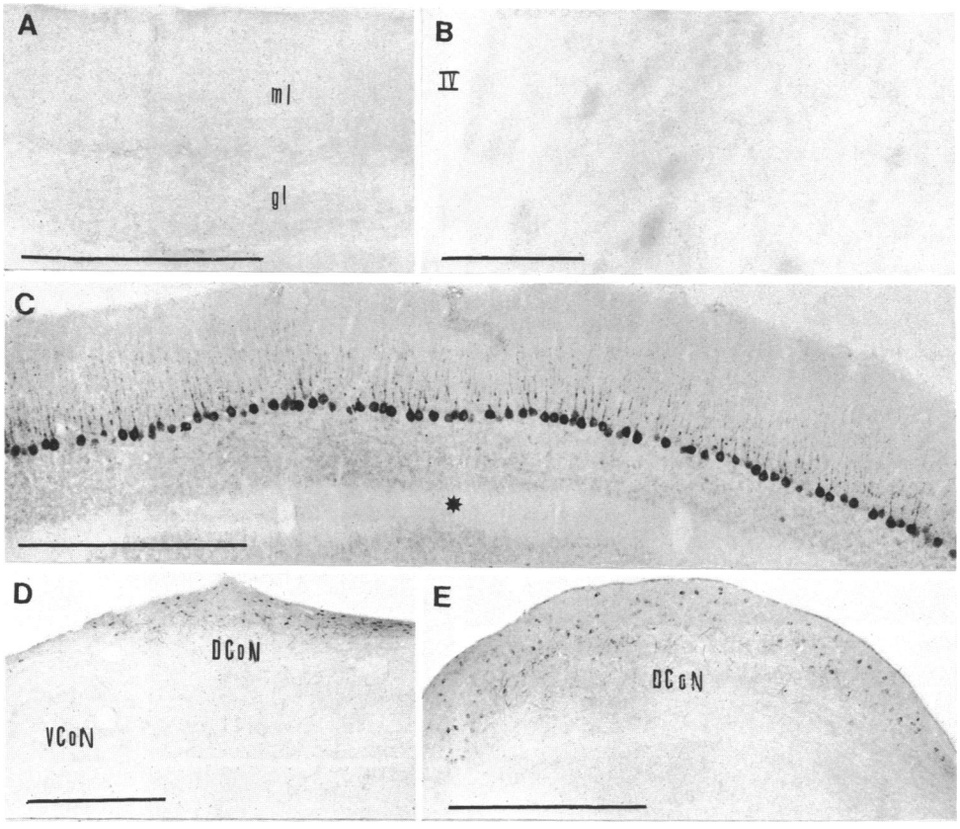

FIG. 1. (*A*) Light micrograph of part of a cerebellar folium from a section incubated with normal rabbit serum as control. Specific immunoprecipitate is absent. ml and gl, molecular and granule layers, respectively. (*B–E*) Light micrographs from sections immunoreacted with antiserum C_1. (*B*) The cerebellar nucleus interpositus anterior contains only unspecific stain. IV, ventricle. (*C*) Immunostaining is present in all Purkinje cells of an anterior lobule folium from midline vermis (asterisk) to intermediate cortex. (*D* and *E*) Rostral and caudal coronal section of the cochlear nuclei show positive cells at all levels of DCoN, while VCoN is unstained. (Bars: *A*, 0.25 mm; *B*, 0.1 mm; *C*, 0.25 mm.)

double cycle (19) avidin-biotin-peroxidase complex (ABC) (20) or peroxidase-antiperoxidase (21) procedure with diaminobenzidine as the chromogen. The diluent for all reagents was 0.5 M Tris buffer (pH 7.6). Control sections were free of specific immunoreaction product (Fig. 1*A*).

Electron microscopy. Four Wistar rats (60 days old) were perfused at room temperature with 100 ml of a Ca^{2+}-free oxygenated Ringer's solution (pH 7.1), followed by 200 ml of a fixative containing 4% freshly depolymerized paraformaldehyde and 0.125% glutaraldehyde in 0.12 M sodium phosphate buffer (pH 7.3), and then by 400 ml of a similar fixative, with glutaraldehyde omitted. Slices were cut on a Vibratome at 30 μm and immunoreacted, as specified above, with the ABC procedure. After rinsing, the immunoreacted slices were postfixed with phosphate-buffered OsO_4 and with aqueous uranyl acetate, dehydrated in ethanol-propylene oxide, and embedded in an Epon-TAAB resin mixture. Ultrathin sections were stained for 2 min with lead citrate and photographed on a Zeiss EM-10 electron microscope operated at 80 kV.

RESULTS

Both cerebellum and DCoN contain cerebellin immunoreactivity that elutes from the HPLC column at the position of authentic cerebellin. High levels of cerebellin are present in the two structures (Table 1); and the respective concentrations are comparable, especially if one takes into consideration that the DCoN sample is contaminated with portions of VCoN, which contains almost no immunoreactive structures (Fig. 2*F*). In contrast, lower medulla and spinal cord, two brain regions adjacent to the DCoN and cerebellum, contain only trace levels of cerebellin immunoreactivity (Table 1). Many other brain regions and non-neural tissues have been examined for cerebellin content by HPLC (17), RIA (Table 1), and immunocytochemistry (data not presented), and have been shown either to be devoid of the peptide or to contain only low levels. Thus, only cerebellum and DCoN contain substantial concentrations of cerebellin, supporting the notion that their analogy might extend to the level of cell-class-

Table 1. Cerebellin immunoreactivity

Tissue	HPLC purified, fmol/mg	Total homogenate, fmol/mg
Cerebellum	740 ± 58	650
Dorsal cochlear nucleus	354 ± 14	ND
Lower medulla	32	ND
Spinal cord	20	ND
Cerebral cortex	10	14
Olfactory bulb	42	28
Hippocampus	12	ND
Pons	18	10
Colliculus	15	ND
Thalamus + hypothalamus	27	21
Liver	<5	ND
Kidney	<5	ND
Adrenals	<5	ND
Olfactory epithelium	<5	ND
Retina	<5	ND
Ileum	<5	ND
Pancreas	<5	ND
Heart	<5	ND
Spleen	<5	ND

Cerebellin content of different brain regions and non-nervous tissues determined by RIA. The CLI in whole tissue homogenates in RIA buffer (total homogenate) or that present in the fraction eluting from HPLC at the retention time of cerebellin (HPLC purified) was determined by RIA. Values are the means of two determinations for all brain regions and non-nervous tissues except for cerebellum and DCoN, which are the mean and SEM of four separate experiments. Cerebellin values are expressed in fmol per mg (wet weight) of tissue. No other significant peaks of cerebellin immunoreactivity were observed to elute from the Vydac column. ND, not determined.

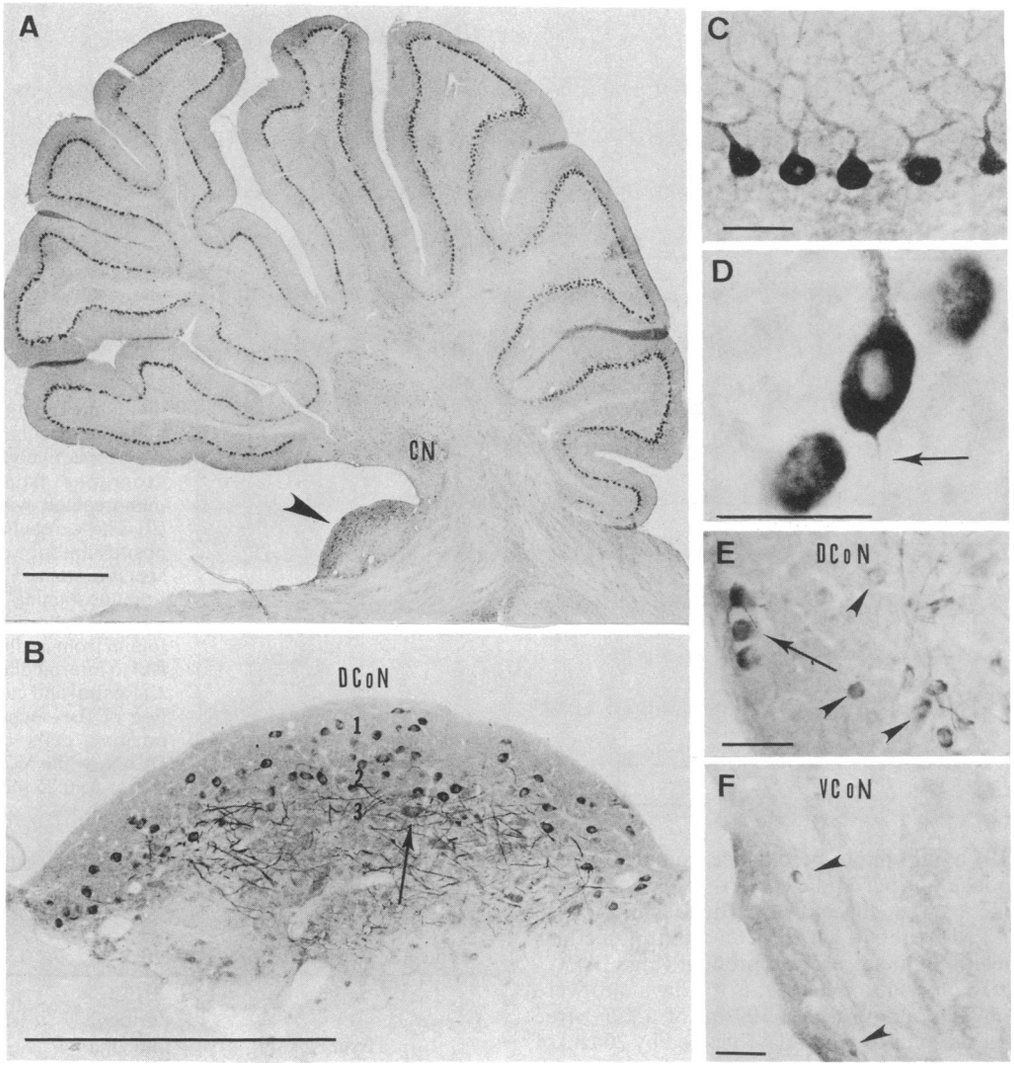

FIG. 2. Light photomicrographs showing cerebellum (*A–C*) and the DCoN (*D–F*) in parasagittal sections. (*A*) Cerebellar hemisphere with part of the underlying DCoN (arrowhead). CN, cerebellar nuclei. (*B*) Immunostaining in DCoN. The cell bodies of cartwheel neurons in the superficial layers (layers 1 and 2) of the DCoN and the plexus in the deeper region (layer 3) predominate. The plexus is most dense in the upper portion of the deep region, which may correspond to layer 3 of the feline nuclei, a zone that contains the basal dendritic arbors of the bipolar pyramidal neurons (2, 11), one of which is indicated by an arrow. (*C*) Immunoreaction product is present in Purkinje cell body and main dendrites. (*D*) In the axon, immunostaining is restricted to the initial axon segment (arrowhead). (*E*) Three subependymal displaced Purkinje cells in DCoN. Smaller cell bodies of several cartwheel neurons (arrowheads) are also shown. (*F*) Portion of the VCoN in which immunostaining is restricted to rare cartwheel cell bodies (arrowheads) displaced in the superficial granular layer. (Bars: *A* and *B*, 0.5 mm; *C–F*, 50 μm.)

specific chemical markers. It was, therefore, compelling to compare the immunocytochemical localization of cerebellin in the two structures.

Three antisera (C_1, R_2, and R_5) were raised in rabbits against synthetic cerebellin and tested in RIA and immunocytochemistry as reported elsewhere (22). By immunocytochemical screening, it was found that the antisera stained Purkinje cells in a differential manner in both adult and developing rats. Cerebellin-like immunoreactivity (CLI) to antisera C_1 and R_5 was present mainly in the dendritic spines. This suggested that the antisera recognize a cerebellin precursor or the peptide itself, respectively (22).

None of the antisera stained the myelinated portions of the Purkinje cell axon and their terminal boutons in adult animals, although it was reported that another rabbit antiserum occasionally revealed portions of the Purkinje axon in the granular layer of immature rats (18). For the present investigation, we selected antiserum C_1 because it reveals neuronal cell bodies most clearly. CLI C_1 is present in the cerebellum and the cochlear nuclei in rats of all ages examined. No other structure in the cerebellar nuclei (Fig. 1*B*), the brain stem, or the spinal cord (not illustrated) contains distinct CLI C_1. Immunostaining in the cerebellum is localized to the cell bodies and main stem dendrites of Purkinje cells (Figs. 1*C* and 2 *A* and *C*) and ceases abruptly at the end of the initial axon segment (Fig. 2*D*) (see also ref. 18). Thus, only the cell compartments that contain a high density of polyribosomes are revealed. Serial sections of the cerebellum and the brain stem cut in the coronal (Fig. 1*C*) and sagittal (Fig. 2*A*) planes demonstrate that all, or nearly all, Purkinje cells are labeled, without any trace of the sagittal banding patterns seen with other Purkinje cell markers (23–31).

In the laminated DCoN, CLI is present throughout the rostrocaudal (Fig. 1 *D* and *E*) and mediolateral (Fig. 2*B*) extent of the nucleus. CLI is localized to four elements: numerous, heavily stained, medium size (11–15 μm) cells located in layers 1 and 2; occasional, heavily stained, large (20–25 μm) and round subependymal cells; scattered, faintly stained, large (20–30 μm) and elongated cells located in layer 2 and in the underlying deep region; a heavily stained fibrous

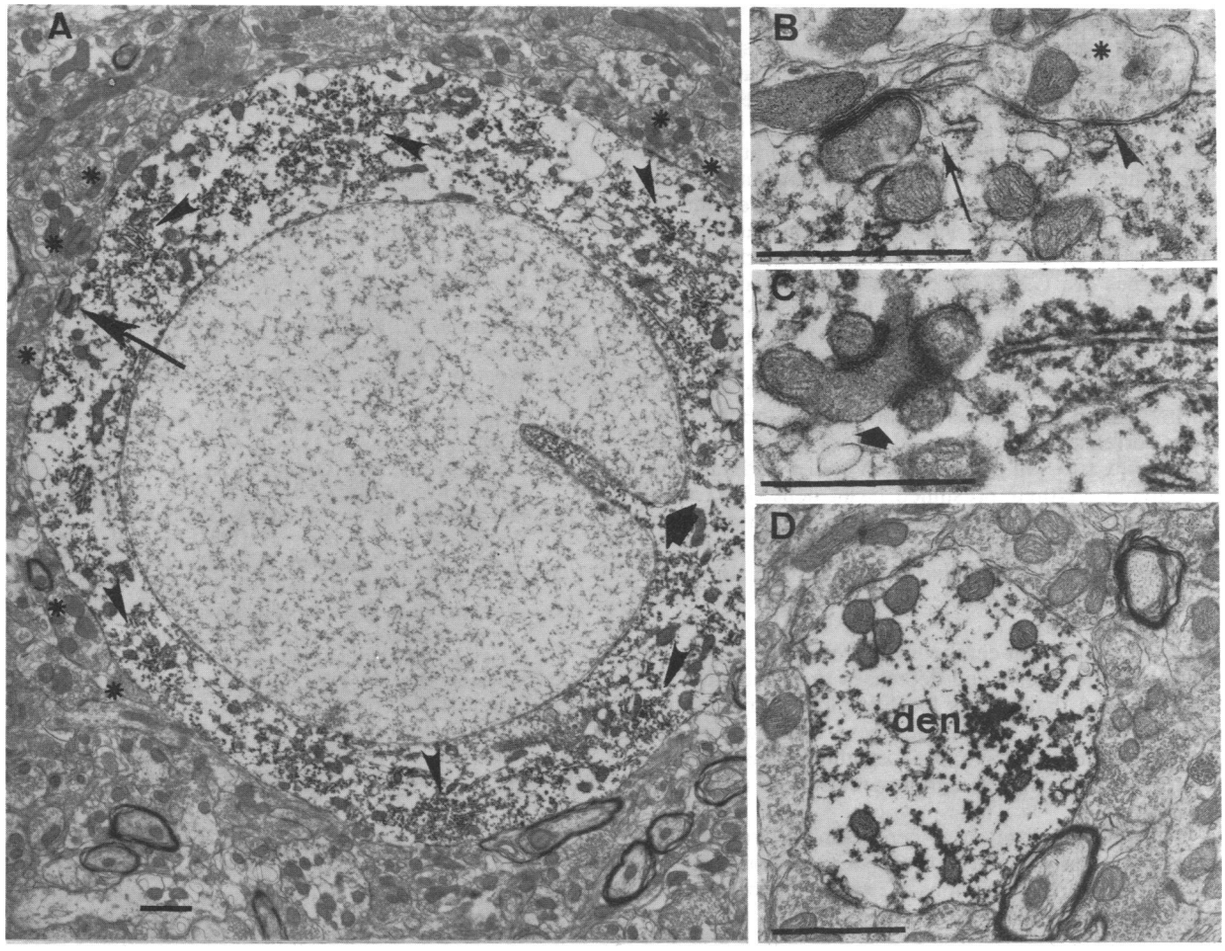

FIG. 3. Electron micrographs showing CLI in DCoN. (*A*) CLI-positive cartwheel cell body. Nuclear infolding (thick arrow), small Nissl bodies (arrowheads), a complex of two serially arranged mitochondria and subsurface cisterns (arrow), and boutons (asterisks) forming axosomatic synapses are indicated. (*B*) Subsurface cistern with associated mitochondrion (arrow) and symmetric synapse (arrowhead) with a bouton (asterisk) containing pleomorphic synaptic vesicles in a CLI-positive cartwheel cell body. (*C*) Association of four mitochondria (thick arrow) with flattened cisterns of the deep endoplasmic reticulum in a CLI-positive cartwheel cell body. (*D*) CLI-positive dendrite (den) surrounded by a nearly complete ring of synaptic boutons in the deep region of the DCoN. (Bars = 1 μm.)

plexus that is oriented predominantly at right angles to the long axis of the DCoN (i.e., in the "trans-strial plane;" ref. 11), extends from layer 2 to the deep region and appears most dense immediately beneath layer 2. The densely labeled medium size neurons resemble in size, shape, frequency, and mode of distribution the cartwheel neurons previously identified with combined Golgi impregnation electron microscopy. Their definitive identification as cartwheel neurons was provided by immunoelectron microscopy, for these CLI-positive cell bodies show a whole set of characteristic features; namely, they contain a vesicular, indented nucleus and small Nissl bodies (Fig. 3*A*); emit stout dendritic trunks (diameter, 2–3 μm) from large, tapering bases (9); display single and multiple assemblies of mitochondria associated either with subsurface cisterns (Fig. 3 *A* and *B*) or with flattened cisterns of the deep endoplasmic reticulum (Fig. 3*C*); and form axosomatic synapses that belong exclusively to the symmetric (Gray type 2) category (Fig. 3 *A* and *B*). Of 18 cartwheel neurons in seven ultrathin sections cut near the surface of immunoreacted slices, where sufficient penetration of immunoreagents is obtained, all were distinctly immunolabeled. The rare large subependymal neurons (Fig. 2*E*) are interpreted as displaced Purkinje cells since they are also densely stained by antisera to a cGMP-dependent protein kinase (32) and glutamate decarboxylase (15). The faintly stained large neurons, barely distinguishable from background, are identical in shape, size, and distribution to the bipolar pyrimidal neurons (2, 11). The densely stained trans-strial plexus appears, in the electron microscope, to consist of dendrites (Fig. 3*D*) provided with few spines. Although the origin of these dendrites remains to be firmly demonstrated, the distribution, orientation, and density of the plexus suggest that it represents the basal dendritic arbors, and also perhaps the primary trunks of some apical arbors, of pyramidal neurons (11). The VCoN is free of CLI (Fig. 1*D*), with the exception of a few cartwheel neurons (Fig. 2*F*) scattered in the granule cell domains (33).

DISCUSSION

The diversity of neuronal phenotypes in the central nervous system has led to a quest for molecules that are expressed with some specificity with respect to cell classes, circuits, and systems. Neurotransmitters and also their biosynthetic enzymes have proven useful markers for specific neurons, as they actually represent part of the functional basis of a given pathway and/or neuronal circuit. However, as useful as such a class of marker molecules is, they are usually distributed in several brain regions (34) and show stereotyped intracellular localizations. Thus, of special use may be a second type of molecule that exhibits a restricted distribution in brain and subserves other special cellular functions.

The present study establishes that while the hexadecapeptide cerebellin is absent in all non-nervous tissues examined, it can be detected by RIA in various brain regions, albeit at relatively low levels when compared to cerebellum or DCoN. Furthermore, all the immunoreactivity present in total homogenates of cerebellum (and other brain regions) appears to be attributable to authentic cerebellin peptide. However, it is only possible to demonstrate cerebellin immunostaining in cerebellar Purkinje neurons and DCoN cartwheel neurons, where it is a distinctive marker. Since both cell types are GABAergic, this finding also provides another example of a peptide that helps to distinguish a functional subset of neurons using the same transmitter substance (35). In addition, the data give further support to the notion of a functional similarity between these two types of neurons and of the overall circuit analogy between the cerebellar cortex and the superficial DCoN layers. The weak localization of CLI C_1 in pyramidal cell bodies and the dense immunostaining of their presumed basal dendrites require additional study. These dendrites are the main target of the dorsal branches of cochlear nerve fibers, but they also make contact with numerous other axons (2). Their immunostaining invites speculation of a role for cerebellin in relation to specific afferent inputs.

It has been shown that cerebellin expression peaks during cerebellar morphogenesis and decreases to a plateau in the adult (17, 18) and that CLI is localized to dendritic spines when antiserum R_2 is used (22). These data suggest that cerebellin is important for synapse formation and is related to postsynaptic functions, although its precise role remains to be established.

The restricted localization of cerebellin to two adjacent laminated gray matters of the brain stem and the specific labeling of two entire populations of nerve cells in these two regions represent unique findings. Thus, Purkinje cells and cartwheel cells share several morphological (9) and neurochemical phenotypes, including expression of glutamate decarboxylase (15), cerebellin, and the recently discovered neuropeptide PEP-19 (unpublished observations), and also have similar fates in several murine neurodevelopmental mutants, including "nervous," "staggerer," "lurcher," and "Purkinje cell degeneration" (36). Therefore, the two cell types may be derived from the same precursors in the region of the rhombic lip. However, there are several reasons to believe that cartwheel cells are not simply Purkinje cells located in the cochlear nuclei. For instance, cartwheel neurons do not have monoplanar dendritic fields; they lack climbing fibers and pinceau formations, and are smaller than Purkinje cells (9). Moreover, in the rat DCoN, we saw occasional, larger, cerebellin-positive neurons, which may truly represent displaced Purkinje cells (32). Further exploration of the morphological and neurochemical phenotype of cartwheel and Purkinje cells, therefore, will be relevant to the general question of regulation of gene products in spatially related neuronal families inserted into smaller, although not identical, circuits and microenvironments.

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