Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 7145–7148, October 1985
Neurobiology

Cerebellin: A quantifiable marker for Purkinje cell maturation

(cerebellum/development/immunocytochemical localization/peptide analysis)

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Communicated by Allan H. Conney, June 26, 1985

ABSTRACT **The cerebellum-specific hexadecapeptide cerebellin has been localized by immunocytochemical means to the perikarya and dendrites of cerebellar Purkinje cells. Biochemical analysis using ion-pairing HPLC shows cerebellin to first appear 5 days after birth, whereafter levels rise to a maximum at 25 days postpartum, and then decline to stable adult values. This same pattern of development occurs with a lag of approximately 5 days for the major metabolite of cerebellin, des-Ser[1]-cerebellin. The immunocytochemical picture of cerebellin in developing Purkinje cells mirrors the biochemical data. These results show that cerebellins represent unique quantifiable markers for the investigation of Purkinje cell maturation and lend support to the feasibility of using unique endogenous peptides to chart neurodevelopment.**

We have proposed that by systematically identifying naturally occurring unique peptides of neural structures these could be used as quantifiable molecular markers with which to investigate neurodevelopment (1). In a prototype study two cerebellum-specific peptides were isolated and sequenced and shown to be a hexadecamer termed cerebellin (NH_2-Ser-Gly-Ser-Ala-Lys-Val-Ala-Phe-Ser-Ala-Ile-Arg-Ser-Thr-Asn-His-OH) and an apparent metabolite des-Ser[1]-cerebellin (1). It thus remained to establish the utility of the cerebellins as molecular markers for neurodevelopment and to determine their cellular location. Since both cerebellins have been synthesized (1), we have been able to develop antibodies to the peptides that can be used for their localization at the level of the light microscope. Furthermore, as sensitive biochemical analysis of the peptides is possible (1), immunocytochemical evidence could be corroborated and quantitated in adult and developing cerebellum. In this study the immunocytochemical localization of cerebellin in Purkinje cells is reported as well as the characteristics of cerebellin expression during ontogeny. The demonstration of these parameters lends support to the feasibility of using native endogenous peptides to map development in the brain.

MATERIALS AND METHODS

Preparation of Immunogen. Cerebellin immunogen was synthesized by incubating together 2 mg of cerebellin and 30 mg of bovine thyroglobulin (Sigma) in 2.8 ml of 50 mM sodium phosphate (pH 7.8) containing a final concentration of 0.5% glutaraldehyde for 2 hr at room temperature. The complex was dialyzed against incubation buffer, and then 1-mg aliquots were used to immunize rabbits. Details of the immunization procedures are as previously reported (2).

Affinity Purification of Rabbit Antiserum to Cerebellin. One milliliter of rabbit antiserum (titered by using a cerebellin radioimmunoassay; unpublished data) was passed over a 1-ml protein A-Sepharose column (Pharmacia) under the conditions recommended by the suppliers. The IgG fraction was neutralized with 0.2 vol of 1 M sodium phosphate (pH 7.6) and passed over a 1-ml cerebellin-agarose affinity column at a flow rate of 10 ml/hr. After loading, the column was washed with 20 ml of 0.1 M sodium phosphate buffer (pH 7.2) and then back eluted with 8 ml of 50 mM glycine·HCl (pH 2.5). The eluted IgG was immediately neutralized with 2 ml of 1 M sodium phosphate (pH 7.6) containing 5 mg of goat IgG (Sigma). Only IgG eluted at this last step had binding activity in the cerebellin radioimmunoassay (unpublished data). The cerebellin affinity column was prepared by dissolving 1 mg of synthetic cerebellin in 1 ml of dimethyl sulfoxide and adding this to a 1-ml pellet of Affi-Gel 10 (Bio-Rad) that had been washed in 20 vol of dimethyl sulfoxide. Coupling proceeded for 18 hr at 21°C, whereafter 10 mg of alanine was added for 1 hr to block remaining esters in the gel. The gel was then washed four times with 30 ml of 0.1 M sodium phosphate (pH 7.2) and packed into a column and equilibrated with buffer.

Immunocytochemistry. Rats were anesthetized with Nembutal and perfused via the aorta with 300 ml of 0.1 M sodium phosphate containing 4% paraformaldehyde and 1% glutaraldehyde. After dissection, the brain was postfixed for 18 hr in 0.1 M sodium carbonate containing 4% paraformaldehyde. The tissue was then immersed in 0.1 M sodium phosphate containing 4% sucrose for 2 days, whereafter it was cryoprotected with 30% sucrose. The cerebellum was quick-frozen by submersion in a −76°C Freon bath and then was sectioned (15 μm) on a cryostat by standard techniques. For immunocytochemistry, all buffers were composed of 75 mM sodium phosphate, pH 7.2/75 mM sodium chloride. When used to dilute antibodies, the buffer also contained 0.2% Nonidet P-40 (Sigma). Sections were first blocked for 20 min with 10% goat serum before incubation for 2 hr with affinity-purified anti-cerebellin antibody (diluted to 300 ng/ml and containing 0.5 mg of goat IgG per ml as carrier). Detection of antibody was accomplished with the Vectastain ABC Kit (Vector Laboratories, Burlingame, CA) using the protocol provided by the supplier.

Analytical Procedures. The isolation procedures for cerebellin and des-Ser[1]-cerebellin and their quantitation by ion-pairing HPLC were as previously described. Synthetic standards of both peptides were used to calibrate the HPLC.

RESULTS

Immunocytochemical analysis of the mature rat cerebellum revealed specific cerebellin immunoreactivity to be restricted to the perikarya and dendrites of Purkinje cells (Figs. 1 and 2). Occasionally staining of Purkinje cell axons was observed, primarily in neonatal rats (Fig. 2), although we were unable to detect a positive reaction in the cerebellar nuclei (data not shown). In all experiments reported here, prior incubation of the affinity-purified antiserum with excess synthetic cerebellin resulted in a loss of staining of Purkinje cells (data

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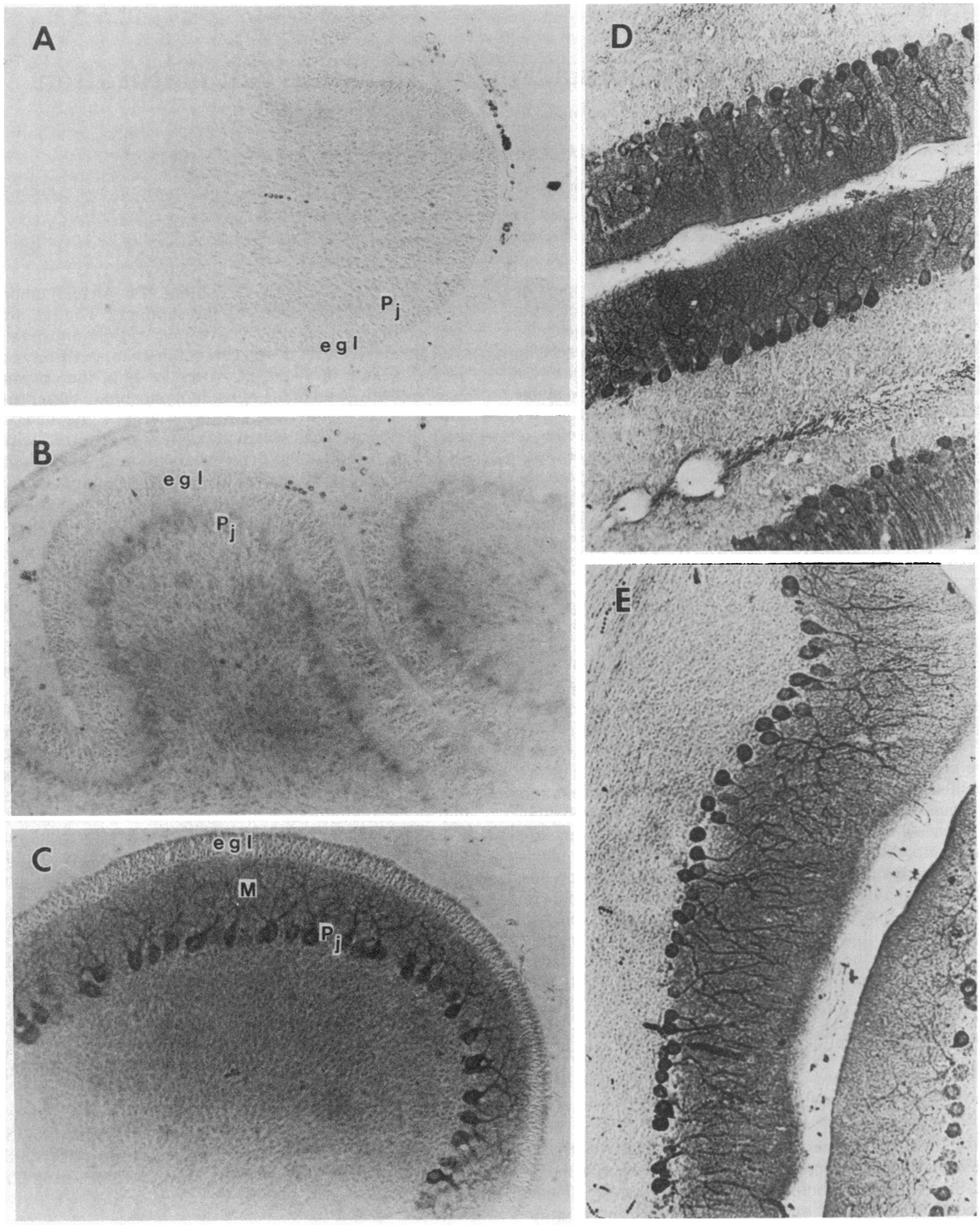

FIG. 1. An immunocytochemical analysis of the distribution of cerebellin immunoreactivity in the developing rat cerebellum at 3 days (*A*), 6 days (*B*), 8 days (*C*), 18 days (*D*), and 60 days (*E*) postpartum. In *A–C*, egl is the external granular layer, Pj is the Purkinje cell layer, and M marks the molecular layer. Details of the preparation of affinity-purified antiserum and immunocytochemical procedures are as described previously. (×200.)

not shown). During the postpartum development of the cerebellum, weak cerebellin immunoreactivity first appeared in the soma of immature Purkinje cells at about day 5 (Fig. 1). At this point the Purkinje cells are just aligning as a monolayer in the Purkinje cell layer and have not established significant dendritic arbors, but there is a large external granular layer (3–5). By day 8 cerebellin staining was pronounced in both the soma and developing dendrites of Purkinje cells as cerebellar development proceeds with the initial formation of the internal granular layer (Figs. 1 and 3; refs. 3–5). By day 18 the external granular layer had largely disappeared, and the intensity of cerebellin staining was

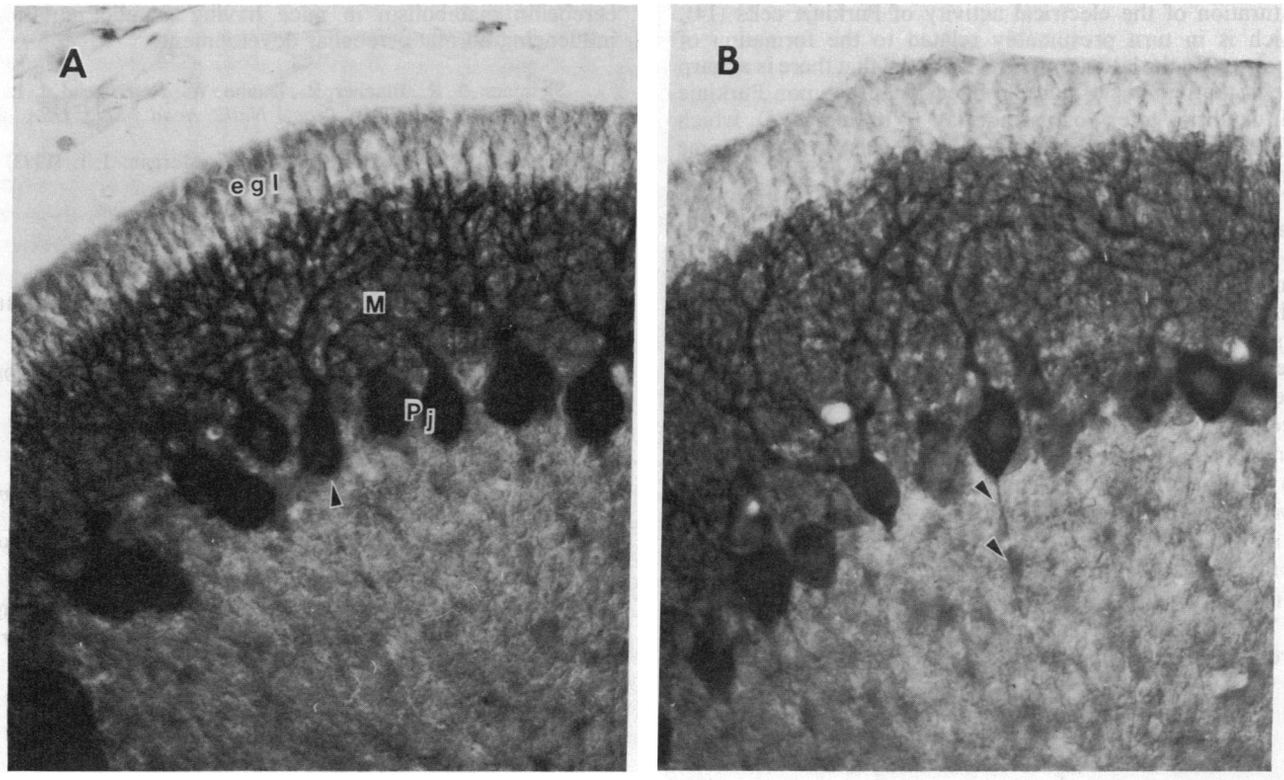

FIG. 2. Localization of cerebellin immunoreactivity in 8-day postpartum Purkinje cells. (*A*) Position of the external granular layer (egl), the early molecular layer (M), and the Purkinje cell layer (Pj). The arrow shows the position of a Purkinje cell perikaryon. (*B*) Another field in which Purkinje cell axons may be visualized (arrows) passing through the developing internal granular layer. (×700.)

indistinguishable from the adult (Fig. 1). Immunoreactivity was still localized to the soma and dendrites of the Purkinje cells (Fig. 1).

In a parallel study, rats of various ages were sacrificed and the cerebellin content of their cerebella was determined by HPLC (Fig. 3). The analysis revealed that cerebellin was first detectable at day 5 after birth. Its concentration then increased rapidly to a maximum at day 25 postpartum and then declined to a stable adult value by about day 60 (Fig. 3). Levels of des-Ser^1-cerebellin paralleled the rise of cerebellin but with a lag of about 5 days (Fig. 3). Furthermore, whereas cerebellin was the major form of the peptide in neonates, des-Ser^1-cerebellin was the more abundant species in the adult (Fig. 3). These data could be explained if there were a precursor–product relationship between cerebellin and des-Ser^1-cerebellin. In addition, they support and add quantitative evidence to the immunocytochemical findings documented above. Thus, the initial rise in cerebellin levels are attributable both to an increase in the concentration of the peptides in individual Purkinje cells (as evidenced by denser staining) and an increase in unit cell volume due to dendritic arborization. The reason for the decline in cerebellin levels after day 25, presumably reflecting alterations in biosynthetic rates or degradation and export, is unclear.

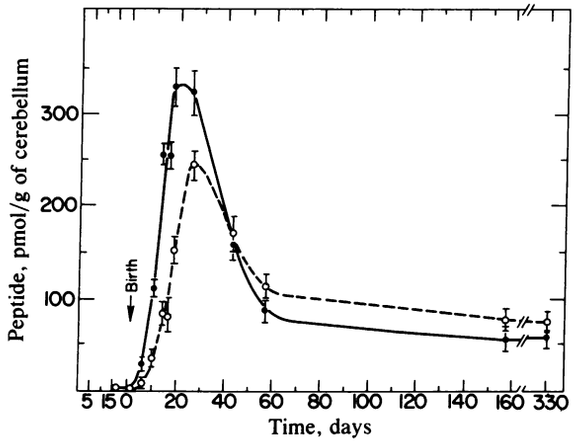

FIG. 3. Chemical determination of cerebellin and des-Ser^1-cerebellin levels in the developing rat cerebellum. Peptides were isolated from cerebella, and cerebellin concentrations were determined by ion-pairing HPLC with sodium dodecyl sulfate as pairing reagent as described (1). Synthetic cerebellin (1) was used to calibrate the HPLC system. Data are represented as the mean cerebellin content (pmol/g of cerebellum) ± 1 SEM. ●, Cerebellin levels; ○, des-Ser^1-cerebellin levels.

DISCUSSION

From the perspective of developmental neurobiology, it is of interest to relate cerebellin metabolism to the cellular events occurring within the cerebellum during equivalent time periods. In general, the absolute levels of cerebellin closely parallel the formation of the internal granular layer up to day 25 after birth (Figs. 1 and 3; refs. 3–7). It might be inferred, therefore, that granule cells play some explicit role in regulating cerebellin biosynthesis in Purkinje cells. One such action could be the initial triggering of cerebellin expression on postpartum days 4–5 (Figs. 1 and 3), perhaps by the formation of the subproliferative zone or by migration itself (3–5). However, the appearance of cerebellin also coincides with the entry of climbing fibers into the cerebellum (postpartum days 3–5 in rat; refs. 8 and 9) and their formation of cerebellar nests around immature Purkinje cells (8, 10, 11). Since climbing fibers establish somatic synapses onto Purkinje cells during this period (8, 11–13) they might also supply a trigger for cerebellin expression. The rapid rise in cerebellin levels between days 8 and 25 (Fig. 3) parallels the

maturation of the electrical activity of Purkinje cells (14), which is in turn presumably related to the formation of synapses. In the latter context it is known that there is a sharp rise in the number of parallel fiber synapses upon Purkinje cell dendrites after postpartum day 14 in the rat (4), which might account for the rise in cerebellin levels during this same time period (Fig. 3).

The decline in cerebellin levels after day 25 may have no real significance and merely be a reflection of an equilibration of the many influences impinging upon the developing Purkinje cell. However, in the mouse a morphological correlate does exist that could explain this phenomenon. It might first be added that the mouse cerebellum contains the identical molecular forms of cerebellin and has qualitatively the same developmental characteristics as rat, including a decline in peptide levels approximately 25–30 days after birth (unpublished data). In the mouse it is known that, after day 20 postpartum, there ensues a significant loss of dendritic spines on Purkinje cells (15, 16). If a loss of dendritic spines is responsible for the reduction of cerebellin levels, then the control of cerebellin metabolism in mature animals may be a function of granule cell–Purkinje cell interaction.

The various propositions elaborated above may be addressed either by lesioning experiments or by investigating cerebellin metabolism in mice having genetic mutations influencing normal cerebellar development.

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