*Proc. Natl. Acad. Sci. USA*
Vol. 88, pp. 2850–2853, April 1991
Neurobiology

# Nuclear and axonal localization of $Ca^{2+}$/calmodulin-dependent protein kinase type Gr in rat cerebellar cortex

**(cerebellum/parallel fibers/climbing fibers/cerebellar granule cell/synapsin)**

KARL F. JENSEN*†, CAROL-ANN OHMSTEDE‡, ROBIN S. FISHER†§, AND NAJI SAHYOUN‡

*Neurotoxicology Division, Environmental Protection Agency, Research Triangle Park, NC 27711; ‡Cell Biology Division, Wellcome Research Laboratories, Research Triangle Park, NC 27709; and §Departments of Anatomy & Cell Biology, Psychiatric & Biobehavioral Sciences, and †Mental Retardation Research Center, University of California School of Medicine, Los Angeles, CA 90024

*Communicated by Gertrude B. Elion, January 3, 1991*

ABSTRACT **The granule cell-enriched $Ca^{2+}$/calmodulin-dependent protein kinase (CaM kinase-Gr) is a recently discovered neuron-specific enzyme. The kinase avidly phosphorylates synapsin I and contains a polyglutamate sequence, which suggests an association with chromatin as well. A possible role in synapsin I phosphorylation and in nuclear $Ca^{2+}$ signaling was supported by immunochemical and ultrastructural examination of CaM kinase-Gr distribution. CaM kinase-Gr immunoreactivity was present in the molecular and granule cell layers of the rat cerebellum. This pattern corresponded to the occurrence of the enzyme in the granule cell axons and nuclei, respectively. Immunoblots confirmed these findings. Thus, CaM kinase-Gr may mediate and coordinate $Ca^{2+}$-signaling within different subcellular compartments.**

$Ca^{2+}$/calmodulin-dependent protein kinases (CaM kinases) have been implicated in neuronal communication by regulating neurotransmitter biosynthesis, neurotransmitter release, alterations in cytoskeletal components, and possible regulation of gene expression (1–5). A brain-specific CaM kinase has recently been isolated and purified and part of its encoding nucleotide sequence has been cloned (6). This kinase shows certain catalytic and regulatory similarities to CaM kinase II but exhibits a number of unique characteristics, including amino acid sequence, subunit organization, subcellular distribution, and immunohistochemical localization. Because this kinase protein is enriched in the granule cells of the cerebellar cortex, it has been called the granule cell-enriched CaM kinase (CaM kinase-Gr). The enzyme has been purified to homogeneity and consists of two polypeptides with apparent $M_r$ values of 65,000 and 67,000 (6). Partial cDNA sequence data indicate that mouse and human brains contain homologues to the rat enzyme that, nevertheless, exhibit considerable divergence in their nucleotide sequence (7, 8).

The potential physiological roles of CaM kinase-Gr depend on its substrate specificity and its subcellular availability. This kinase phosphorylates synapsin I on the head and tail domains (6) and may thereby promote neurotransmitter release by analogy to the action of CaM kinase II (9, 10). Moreover, CaM kinase-Gr contains a polyglutamate-rich sequence (6), which characterizes several chromatin-associated proteins (11). Thus, CaM kinase-Gr may regulate different neuronal reactions in different subcellular compartments. The objective of this study was to determine the subcellular compartments in which CaM kinase-Gr occurs.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

## MATERIALS AND METHODS

**Primary Antiserum.** Rabbit antibodies were raised against a β-galactosidase fusion product of CaM kinase-Gr expressed in and purified from *Escherichia coli*. The antiserum was affinity-purified by adsorption to mammalian CaM kinase-Gr isolated from rat cerebellum (6). The resulting monospecific antibody preparation was employed throughout this study.

**Immunoblots.** Punches from the molecular layer and granule cell layer of cerebellar vermis of adult rats (0.1 mg of each tissue) were suspended in SDS-containing sample buffer and were heated for 5 min at 90°C. Equivalent aliquots of these as well as of a cerebellar cytosol extract were applied to duplicate SDS/10% polyacrylamide gels (12) and were then electroblotted onto nitrocellulose (13) or stained with 0.1% Coomassie blue. In addition, rat cerebellar tissue was homogenized in 2 M sucrose and centrifuged at 100,000 × *g*, and the nuclear pellet was washed twice in 0.32 M sucrose; the postnuclear supernatant was dialyzed against 10 mM Tris·HCl, pH 7.5/1 mM EDTA/1 mM dithiothreitol. Similar aliquots from these fractions were also analyzed by immunoblotting and by Coomassie staining. Antibody incubations were performed with a 1:20 dilution of the affinity-purified antibody followed by a 1:2000 dilution of alkaline phosphatase-conjugated secondary antibody. We had previously demonstrated that the monospecific antibody reacted solely with $M_r$ 65,000 and 67,000 subunits of CaM kinase-Gr in crude soluble extracts from rat forebrain or cerebellum (6). The immunoreactivity of CaM kinase-Gr in all cerebellar fractions tested was abrogated by omitting the monospecific antibody or by neutralizing it with purified antigen (Fig. 1 *B* and *C*).

**Immunohistochemistry.** Adult Long–Evans or Wistar rats were sacrificed by an overdose of Nembutal (100 mg/kg) and perfused through the heart with saline followed by aldehyde fixative (4% paraformaldehyde/0.1% gluteraldehyde/0.1% acrolein in 0.1 M phosphate buffer, pH 7.4) or only with 10% glycerol. Three rats were prepared for correlative light and electron microscopy, 12 rats were for light microscopy of fixed tissue, and 3 rats were for light microscopy of unfixed-glycerol perfused tissue. Brains from fixative-perfused animals were removed and postfixed for 12 hr. The cerebellum was then sectioned sagittally through the vermis at a thickness of 60 μm. Sections were incubated for 72 hr in the affinity-purified primary antibody, in dilutions ranging from 1:200 to 1:2, as was determined optimal for the particular antibody preparation. A fluorescein-labeled secondary antibody (1:200) or a peroxidase-antiperoxidase method (1:200) was used to detect the antigen-bound primary antibody. The peroxidase label was visualized with diaminobenzidine enhanced with cobalt chloride and nickel ammonium sulfate.

Abbreviations: CaM kinase II, $Ca^{2+}$/calmodulin-dependent protein kinase II; CaM kinase-Gr, granule cell-enriched CaM kinase.

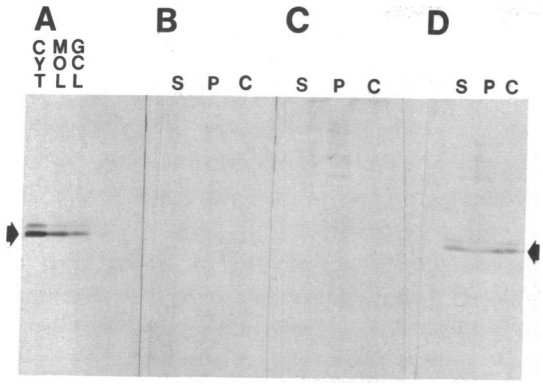



FIG. 1. Immunoblot analysis of CaM kinase-Gr immunoreactivity in cerebellar extracts. (*A*) Immunoblot demonstrating the presence of CaM kinase-Gr ($M_r$ 65,000 and 67,000, arrow) in punches from the molecular layer (MOL) and granule cell layer (GCL) as well as the cytosolic fraction (CYT) from whole cerebellum. (*B–D*) Immunoblot of CaM kinase-Gr in the nuclear pellet (P), postnuclear supernatant (S), and cytosol (C) fractions from cerebellum. The immunoreactive polypeptides (*D*, arrow) were not observed in control experiments in which the antibody was omitted (*B*) or preadsorbed with antigen (*C*). Similar amounts of protein were applied to the SDS/polyacrylamide gels as determined by Coomassie staining.

Following light microscopic observations, selected regions of the cerebellar cortex were osmicated and prepared for transmission electron microscopy (14). Nonspecific immunoreactivity was shown to be absent by primary antibody deletion and by neutralizing the primary antibody with fusion protein prior to incubation with tissue sections (Fig. 2*C*).

## RESULTS

**Laminar Distribution of CaM Kinase-Gr in the Cerebellar Cortex.** Immunoblots indicated that CaM kinase-Gr is abundant in the granule cell layer and molecular layer (Fig. 1*A*). Immunohistochemistry confirmed this laminar distribution of CaM kinase-Gr and further revealed that the Purkinje cell and medullary layers were devoid of immunoreactivity (Fig. 2*A*). Most immunoreactive cellular profiles were located in the granule cell layer (Fig. 2*B*). All of the immunoreactive cellular profiles within the granule cell layer were likely to be granule cells based on their uniformly small diameter (5–7 μm). These granule cells exhibited varying degrees of staining density. A greater degree of immunoreactivity was apparent in the neuropil of the molecular layer than in the neuropil of the granule cell layer. The perikarya of Purkinje cells did not display any evidence of immunostaining.

**Presence of CaM Kinase-Gr in Axons and Nuclei.** Electron microscopy disclosed that immunoreactive cellular profiles in the granule cell layer had the limited cytoplasm and chromatin arrangement characteristic of granule neurons (Fig. 3). Immunoreactivity within granule cells was most abundant in nuclei where it was largely associated with regions of dispersed chromatin. The nuclear localization of CaM kinase-Gr was corroborated directly by immunoblot experiments that revealed that CaM kinase-Gr was the only specifically immunoreactive species in a purified nuclear preparation (Fig. 1 *B–D*).

Immunoreactivity was also present, though sparse, in the cytoplasm of the granule neurons (Fig. 3). The degree of immunoreactivity varied widely even in adjacent cells within the same section; however, no evidence of immunoreactivity was found in Purkinje or Golgi II neurons.

In the molecular layer, immunoreactivity was apparent in cross-sectional profiles of thin-caliber unmyelinated axons (Fig. 4*B*) and elongated profiles of larger caliber axons. The morphology of the thin-caliber axonal profiles was characteristic of the parallel fibers (15), whereas the larger caliber axonal profiles (Fig. 4*A*), particularly those apposed to primary Purkinje cell dendrites, were likely to be climbing fibers originating from the inferior olive (15).

**Relative Abundance of CaM Kinase-Gr in the Molecular and Granule Cell Layers.** Since the association of protein kinases with the nucleus may alter their sensitivity to immunohistochemical detection (16) we attempted to determine whether the pattern of CaM kinase-Gr immunohistochemical labeling was influenced by aldehyde fixation. This was achieved by comparing the distribution of labeling in fixed and unfixed sections using immunofluorescent procedures. In fixed sections, a greater intensity of immunofluoresence was apparent in the granule cell layer (Fig. 5*A*). Yet, in sections of unfixed tissue, greater immunofluoresence was observed in the molecular layer (Fig. 5*B*). Diminished staining intensity in the granule cell layer could be attributed to the shorter incubation times required by the use of fresh tissue. Thus, it appeared that soluble CaM kinase-Gr from the molecular layer might be more sensitive to denaturation by aldehyde fixation than nuclear CaM kinase-Gr, whose immunoreactivity may be

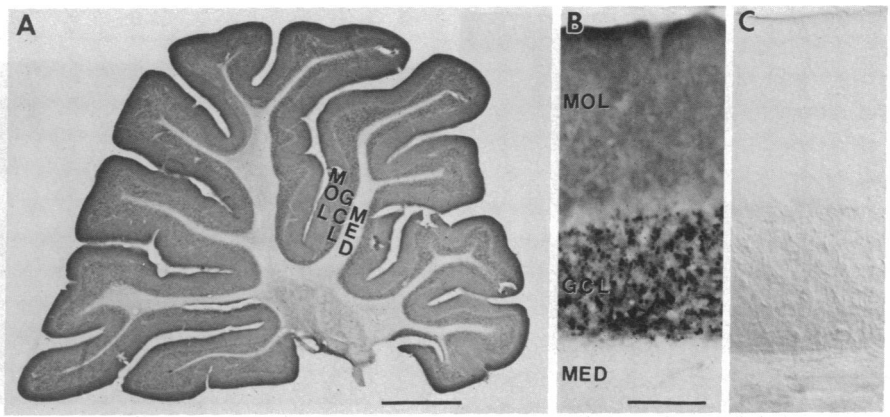



FIG. 2. Laminar distribution of CaM kinase-Gr in the cerebellar cortex. (*A*) Low-power photomicrograph of CaM kinase-Gr immunoreactivity in a sagittal section through the vermis of the rat cerebellum. The most intense immunoreactivity occurs in neurons of the granule cell layer (GCL), with less intense immunoreactivity in the molecular layer (MOL). Minimal immunoreactivity is apparent in the medullary layer (MED). (Bar = 1 mm.) (*B*) Higher-power photomicrograph of CaM kinase-Gr immunoreactivity in a sagittal section of the cerebellar vermis; granule cells exhibit varying degrees of immunoreactivity. Staining was absent from Purkinje cells located at the interface of the molecular layer (MOL) and granule cell layer (GCL). MED, medullary layer. (Bar = 0.1 mm.) (*C*) Labeling in all layers of the cerebellum was absent from sections incubated with primary antisera preadsorbed with purified antigen. (Scale same as in *B*.)

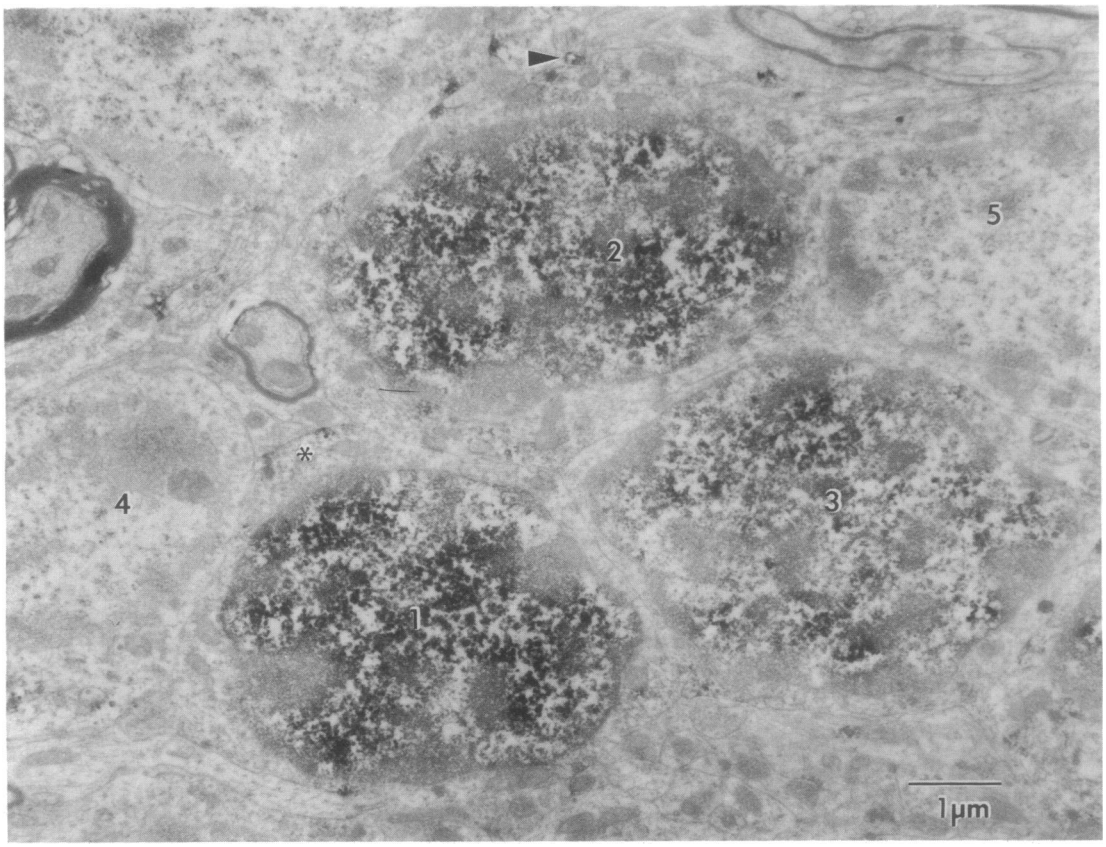
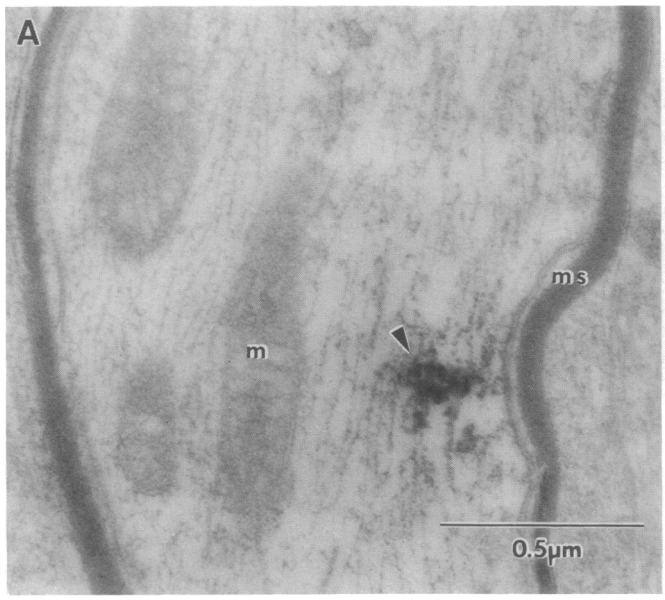



FIG. 3. Localization of CaM kinase-Gr in nuclei of granule cells. Electron micrograph of the granule cell layer indicating the range of label density in the nuclei, in descending order from cell 1 (heavy label) to cell 5 (no label). Sparse immunoreactivity, indicated by asterisk, can be seen in the cytoplasm of cell 1.

protected by its association with chromatin. Comparison of immunoblots and immunohistochemistry should be made with caution due to conformational differences in the enzyme following SDS denaturation and *in situ* aldehyde fixation. Nonetheless, the possibility that the diminished labeling within the molecular layer reflects a particular sensitivity to *in situ* aldehyde fixation is supported by the relative abundance of CaM kinase-Gr in the cerebellar molecular layer and nuclear extracts demonstrated by immunoblot. Thus, the combined examination of fixed and unfixed tissue, as well as the subcellular distribution of immunoreactive enzyme, indicate that CaM kinase-Gr is abundant in multiple neuronal compartments.

## DISCUSSION

The present immunochemical and immunohistochemical observations indicate that CaM kinase-Gr is located in the nucleus as well as processes of granule cells. The density of label within granule cell nuclei is one of the most striking features of the pattern of immunoreactivity. The association of CaM kinase-Gr with dispersed chromatin of granule cell



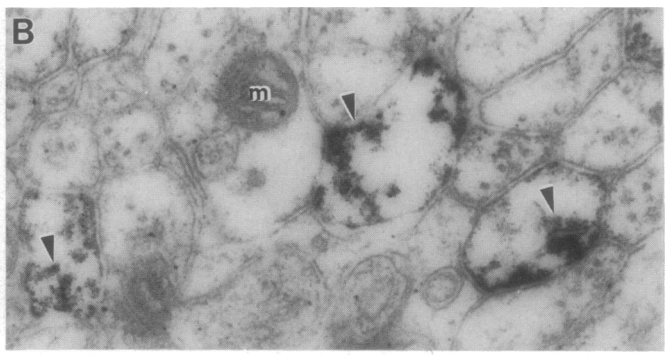




FIG. 4. Localization of CaM kinase-Gr immunoreactivity in axons of rat cerebellar cortex. (*A*) Longitudinal profile of a thick-caliber myelinated climbing fiber passing through the granule cell layer (m, mitochondrion; ms, myelin sheath). Arrowheads indicate dense immunoreactivity. (*B*) Cross-sectional profiles of thin-caliber, unmyelinated parallel fibers in the molecular layer. (Scale same as in *A*.)

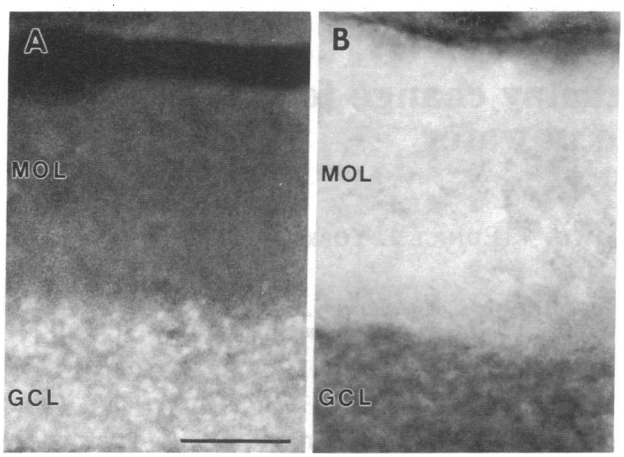



FIG. 5. Relative CaM kinase-Gr immunoreactivity in the molecular and granule cell layers. (*A*) Intense immunofluoresence of the granule cell layer (GCL) is apparent in aldehyde-fixed tissue, whereas only slight immunofluoresence is apparent in the molecular layer (MOL) following a 72-hr incubation with primary antibody. (Bar = 0.1 mm.) (*B*) Intense immunofluoresence is apparent in the molecular layer of unfixed tissue, whereas only slight immunofluoresence is apparent in the granule cell layer following a primary incubation of 2 hr. The lack of immunofluoresence in Purkinje cells is similar in fixed and unfixed tissue. (Scale same as in *A*.)

nuclei implies that this enzyme could be involved with regions of active gene transcription. The association of CaM kinase-Gr with chromatin may be specified by the regions of the molecule containing glutamate-rich amino acid sequences (11) and suggests that CaM kinase-Gr may serve as a target for nuclear $Ca^{2+}$ signaling. Such a role is compatible with observations that neuronal activity as well as increased intracellular $Ca^{2+}$ levels can modulate neuronal gene expression (17) and that CaM kinases phosphorylate chromatin polypeptides (18, 19).

The association of CaM kinase-Gr with parallel fibers within the molecular layer corresponds to the previous demonstration of synapsin I immunoreactivity in the same region (20–24). This codistribution of CaM kinase-Gr and synapsin I in the parallel fibers is significant in light of the previous observation that CaM kinase-Gr phosphorylates the tail domain of synapsin I (6). Thus, by analogy with the action of CaM kinase II, it is likely that CaM kinase-Gr plays a significant role in neurotransmitter release.

The relative contribution of CaM kinase-Gr and CaM kinase II to the regulation of neurotransmitter release in the cerebellum has not been evaluated. However, whereas CaM kinase-Gr is enriched in the molecular layer of the cerebellum, CaM kinase II is less abundant in the cerebellum than in the forebrain (25–27). Moreover, in contrast to CaM kinase-Gr, CaM kinase II has lower levels in the molecular layer than in either the granule or Purkinje cell layer (28). Thus, distinct synaptic systems may differentially rely on the two kinases for the regulation of neurotransmitter release. The simultaneous presence of two or more CaM kinases with similar substrate specificities in the same neuronal system provides a complex and diversified $Ca^{2+}$-signaling pathway.

The heterogeneity of CaM kinase-Gr immunoreactivity within granule cell nuclei appears to be paralleled by a heterogeneity of immunoreactivity within the granule cell axons. Likewise, climbing fibers (Fig. 4) and their cells of origin within the inferior olivary complex contain CaM kinase-Gr (6), indicating that several neuronal subpopulations may express CaM kinase-Gr in their cell bodies as well as their processes. The localization of CaM kinase-Gr in the nuclei and processes of particular populations of neurons may provide them with an integrated and coordinated response to activity-induced changes in $Ca^{2+}$ levels.

The excellent technical assistance of J. Asai and J. K. Olin is greatly appreciated. An abstract of this work was presented at the 1989 Annual Meeting of the American Society for Cell Biology. This research was supported in part by Public Health Service Grants HD05958 and NS24596 to R.S.F. Portions of this work were conducted by K.F.J. while he was a visiting scientist at the Mental Retardation Research Center, University of California, Los Angeles, through an Interagency Personnel Agreement with the Environmental Protection Agency.

1. Nestler, E. J. & Greengard, P. (1983) *Nature (London)* **305,** 583–588.
2. Nairn, A. C., Hemmings, H. C. & Greengard, P. (1985) *Annu. Rev. Biochem.* **54,** 931–976.
3. Browning, M. D., Huganir, R. & Greengard, P. (1985) *J. Neurochem.* **45,** 11–23.
4. Schulman, H. (1988) *Adv. Second Messenger Phosphoprotein Res.* **22,** 39–112.
5. Colbran, R. J., Schworer, C. M., Hashimoto, Y., Fong, Y. -L., Rich, D. P., Smith, M. K. & Soderling, T. R. (1989) *Biochem. J.* **258,** 313–325.
6. Ohmstede, C.-A., Jensen, K. F. & Sahyoun, N. (1989) *J. Biol. Chem.* **264,** 5866–5875.
7. Ohmstede, C.-A. & Sahyoun, N. (1990) *FASAB J.* **4,** 2078 (abstr.).
8. Sikela, J. M., Law, M. L., Kao, F. T., Hartz, J. H., Wei, Q. & Ahan, W. E. (1989) *Genomics* **4,** 21–27.
9. Llinas, R., McGuinness, T. L., Leonard, C. S., Sugimori, M. & Greengard, P. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 3035–3039.
10. Nichols, R. A., Sihra, T. S., Czernik, A. J., Nairn, A. C. & Greengard, P. (1990) *Nature (London)* **343,** 647–651.
11. Earnshaw, W. C. (1987) *J. Cell. Biol.* **105,** 1479–1482.
12. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
13. Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 4350–4354.
14. Fisher, R. S., Hull, C. D., Buchwald, N. A., Adinolfi, A. M. & Levine, M. S. (1987) *Dev. Brain Res.* **33,** 215–234.
15. Palay, S. L. & Chan-Palay, V. (1974) *Cerebellar Cortex: Cytology and Organization* (Springer, New York).
16. Sikorska, M., Whitfield, J. F. & Walker, P. R. (1988) *J. Biol. Chem.* **263,** 3005–3011.
17. Morgan, J. T. & Curran, T. (1986) *Nature (London)* **322,** 552–555.
18. Sahyoun, N., LeVine, H., III, Bronson, D. & Cuatrecasas, P. (1984) *J. Biol. Chem.* **259,** 9341–9344.
19. Sahyoun, N., LeVine, H., III, & Cuatrecasas, P. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 4311–4315.
20. Bloom, F. E., Udea, T., Battenburg, E. & Greengard, P. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 5982–5986.
21. Haas, C. A. & DeGennaro, L. J. (1988) *J. Cell Biol.* **106,** 195–203.
22. De Camilli, P., Cameron, P. R. & Greengard, P. (1983) *J. Cell Biol.* **96,** 1337–1354.
23. De Camilli, P. & Greengard, P. (1986) *Biochem. Pharmacol.* **35,** 4349–4357.
24. Mason, C. A. (1986) *Neuroscience* **19,** 1319–1333.
25. McGuinness, T. L., Lai, Y. & Greengard, P. (1985) *J. Biol. Chem.* **260,** 1696–1704.
26. Miller, S. G. & Kennedy, M. B. (1985) *J. Biol. Chem.* **260,** 9039–9046.
27. Ouiment, C. C., McGuinness, T. L. & Greengard, P. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 5604–5608.
28. Fukunaga, K., Goto, S. & Miyamato, E. (1988) *J. Neurochem.* **51,** 1070–1077.