BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **251,** 394–398 (1998)
ARTICLE NO. RC989480

Differential Expression of Rat Brain Synaptic Proteins in Development and Aging

Shun Shimohama,*[,1] Sadaki Fujimoto,† Yasuo Sumida,† Kimio Akagawa,‡ Tomoaki Shirao,§ Yasuji Matsuoka,‖ and Takashi Taniguchi‖

**Department of Neurology, Faculty of Medicine, Kyoto University, Sakyoku, Kyoto 606, Japan; †Department of Environmental Biochemistry and ‖Department of Neurobiology, Kyoto Pharmaceutical University, Yamashinaku, Kyoto 607, Japan; ‡Department of Physiology, Kyorin University School of Medicine, Mitakashi, Tokyo 181, Japan; and §Behavior Research Institute, Gunma University School of Medicine, Maebashishi, Gunma 371, Japan*

Received September 9, 1998

We have previously reported the differential involvement of synaptic proteins in Alzheimer's disease (AD). As AD is an aging-associated disease, in the present study we examined the developmental and aging-related changes in synaptic proteins such as synaptophysin, synaptobrevin, synaptotagmin, synaptosomal-associated protein 25 (SNAP-25), syntaxin 1/HPC-1 and drebrin in the rat brain. Immunoblot analyses of brain extracts from embryonic day 19 (E19) to postnatal 96-week-old rats indicated that the protein level of synaptophysin and synaptobrevin increased after birth, being highest at 24 weeks, and then decreased with aging. Synaptotagmin was detected at E19, with levels increasing after birth to 96 weeks. SNAP-25 levels were highest at 4 weeks, and then decreased with aging. Syntaxin 1/HPC-1 levels were high at E19 and 1 week, decreasing rapidly from 2 weeks onwards, and drebrin levels were highest at E19 and 1 week, and decreased during aging. The present results suggest that the expression of each synaptic protein is differentially regulated in development and aging.

Alzheimer's disease (AD) is a neurodegenerative disease characterized by the progressive deterioration of cognitive function and memory (1). Recent studies have shown that synaptic loss in the cortex and hippocampus is the major reason for cognitive decline in AD (2–4). In a previous study we found that synaptophysin and synaptobrevin, localized mainly in transmitter-containing synaptic vesicles (5–7), are more vulnerable than synaptosomal-associated protein 25 (SNAP-25) and syntaxin 1/HPC-1, found in presynaptic plasma membranes (8–10), in autopsy brains from patients with AD (11).

As AD is an aging-associated disease, it is important to consider such alterations in the context of normal developmental and aging-associated changes in synaptic proteins, but these are little understood. In the present study we examined the developmental and aging-related changes in synaptic protein levels in the hippocampus from the embryonic (E19) to postnatal 96-week-old Wistar rats, and found a unique and differential expression of each synaptic protein during development and aging.

MATERIALS AND METHODS

Materials. The characteristics and specificity of the polyclonal antibodies against syntaxin 1/HPC-1 and drebrin have been described previously (9, 12). Other specific antibodies were obtained commercially: anti-synaptobrevin and anti-SNAP-25 antibodies from Chemicon International Inc. (Temecula, CA); anti-synaptotagmin antibody from Transduction Laboratories (Lexington, KY); anti-synaptophysin and anti-neurofilament 68 antibodies from Sigma Chemicals (St Louis, MO); and anti-glial fibrillary acidic protein (GFAP) antibody from Dako Japan (Tokyo). An enhanced chemiluminescent detection system (ECL kit; Amersham, Buckinghamshire, UK), and a Vectastain ABC Elite kit (Vector, Burlingame, CA) were used. Other chemicals were of reagent grade and were obtained commercially.

Brain samples. Wistar rats pregnant for 19 days (E19) were anesthetized under ether inhalation, and rat fetuses were removed from the uteri by Caesarean section. Brains were taken from E19 fetuses as well as from 1-, 2-, 4-, and 8-week-old male Wistar rats purchased from Japan SLC (Kyoto). 24-, 36-, 48-, 72-, and 96-week-old male Wistar rats were bred in our laboratory. The animals were treated in accordance with the guidelines published in the *NIH Guide for the Care and Use of Laboratory Animals.*

[1] Address correspondence to Shun Shimohama, M.D., Ph.D., Department of Neurology, Faculty of Medicine, Kyoto University, 54 Shogoinkawaharacho, Sakyoku, Kyoto 606-8507, Japan. Fax: 81-75-751-9541. E-mail: i53367@sakura.kudpc.kyoto-u.ac.jp.

Abbreviations used: AD, Alzheimer's disease; SNAP-25, synaptosomal associated protein 25; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; BSA, bovine serum albumin; HRP, horseradish peroxidase; GFAP, glial fibrillary acidic protein.

0006-291X/98 $25.00

Preparation of brain extracts. Brain tissue samples from the hippocampus were homogenized with a Teflon-glass homogenizer in 4 volumes of 10 mM Hepes buffer (pH 7.0) containing 0.32 M sucrose, 0.05 % NaN_3, 100 μM orthovanadate, 0.1 mM phenylmethylsulfonyl fluoride, 0.5 mM diisopropylfluorophosphate, 1 mM dithiothreitol, 10 μg/mL aprotinin, 5 μg/mL pepstatin A, 5 μg/mL leupeptin, 5 mM benzamidine, and 4 mM ethylene glycol tetraacetic acid. The homogenate was centrifuged at 105,000 $\times$ g for 60 min and the supernatant thus obtained was used as the cytosolic fraction. The pellet was washed twice, suspended in homogenization buffer, and used as the particulate fraction. Protein concentrations were measured by the method of Bradford (13).

Immunoblotting assay. The cytosolic and particulate fractions in Laemmli sample buffer were subjected to 4–20% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), blotted onto Immobilon (Millipore, Bedford, MA), blocked with phosphate buffered saline (PBS) containing 0.1% Tween 20 (TPBS) for 1 hr, and incubated with anti-synaptophysin (1:2000), anti-synaptobrevin (1:2000), anti-synaptotagmin (1:1000), anti-SNAP-25 (1:3200), anti-syntaxin 1/HPC-1 (1:50000), anti-drebrin (1:2000), anti-neurofilament 68 (1:400), or anti-GFAP (1:3000) antibodies in PBS containing 3% bovine serum albumin (BSA) for 18 hr at 4°C. Blots then were washed with TPBS, incubated with horseradish peroxidase (HRP)-linked secondary antibody (1:1000) against rabbit (for the anti-syntaxin 1/HPC-1 and the anti-GFAP antibodies) or mouse (for all other primary antibodies) immunoglobulin. Subsequently, HRP-labeled antibodies present in the fractions were detected by the enhanced chemiluminescence detection system. Protein bands reacting with antibodies could be detected on radiographic films (X-Omat JB-1; Kodak, Rochester, NY) 5 to 60 sec after exposure. The integrated optical density for the 38 kDa protein band recognized by the anti-synaptophysin antibody, for the 18 kDa protein band recognized by the anti-synaptobrevin antibody, for the 65 kDa protein band recognized by the anti-synaptotagmin antibody, for the 25 kDa protein band recognized by the anti-SNAP-25 antibody, for the 35 kDa protein band recognized by the anti-syntaxin 1/HPC-1 antibody, for the 100 kDa protein band recognized by the anti-drebrin antibody, for the 68 kDa protein band recognized by the anti-neurofilament 68 antibody, and for the 50 kDa protein band recognized by the anti-GFAP antibody, was measured by a scanning densitometer (Arcus II, Agfa, Germany) and taken to indicate the relative quantity of the respective protein. To test if synaptophysin, synaptobrevin, synaptotagmin, SNAP-25, syntaxin 1/HPC-1, drebrin, neurofilament 68 and GFAP immunostaining on the blots was linear within the protein range examined, their immunoreactivity in samples containing 5-50 μg of protein of sample was measured and plotted versus protein. All of these graphs were linear for protein values between 5 and 50 μg (r= 0.90 to 0.99, respectively) (data not shown). Prestained SDS-PAGE standards (Bio-Rad, Richmond, CA) were used as molecular weight markers. The apparent molecular weight of phosphorylase B, BSA, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and lysozyme was 112, 84, 53.2, 34.9, 28.7 and 20.5 kDa respectively, according to the manufacturer's instructions. Results are given as mean $\pm$ standard error of the mean (SEM).

RESULTS

Immunoblots for Synaptophysin, Synaptobrevin, Synaptotagmin, SNAP-25, Syntaxin 1/HPC-1, and Drebrin in the Cytosolic and Particulate Fractions of Rat Brain

On the immunoblot, the anti-synaptophysin, -synaptobrevin, -synaptotagmin, -SNAP-25, and -syntaxin 1/HPC-1 antibodies chiefly detected their respective antigens in the particulate but not the cytosolic fraction of rat brains, whereas the anti-drebrin antibody mainly detected its antigen in the cytosolic fraction. Neurofilament 68 and GFAP were detected in both the cytosolic and particulate fractions, but more abundantly in the particulate fraction (data not shown).

Developmental and Aging Changes of Synaptic Protein Levels in the Rat Hippocampus

Synaptophysin. Synaptophysin was minimally detected at E19 and 1 week. Its protein level increased after birth, being highest at 24 weeks of age, and then gradually decreased with development and aging (Fig. 1 and Fig. 2A).

Synaptobrevin. Synaptobrevin was minimally detected at E19, and its protein level increased after birth, being highest at 24 weeks of age, and then gradually decreased with development and aging (Fig. 1 and Fig. 2B).

Synaptotagmin. Synaptotagmin was slightly expressed at E19 and its protein level increased with development and aging after birth until 96 weeks of age (Fig. 1 and Fig. 2C).

SNAP-25. SNAP-25 was apparently not expressed until 2 weeks after birth and its protein level was highest at 4 weeks of age, and then gradually decreased with development and aging (Fig. 1 and Fig. 2D).

Syntaxin 1/HPC-1. Syntaxin 1/HPC-1 was highly expressed at E19 and its protein level was highest at 1 week of age, decreased rapidly at 2 weeks, remaining constant until 96 weeks (Fig. 1 and Fig. 2E).

Drebrin. The protein level of drebrin was highest at E19 and 1 week, and then gradually decreased with development and aging (Fig. 1 and Fig. 2F).

Developmental Changes in Neurofilament 68 and GFAP Levels in the Rat Hippocampus

Neurofilament 68 and GFAP were expressed in both the cytosolic and particulate fractions, but more abundantly in the particulate fraction. They were both minimally detected at E19, and their level increased with development, being highest at 48 weeks, and then gradually decreased with aging (Fig. 3).

DISCUSSION

The present study revealed a differential and unique expression of each synaptic protein in the brain during development and aging.

Synaptophysin and synaptobrevin are the major polypeptide components of small presynaptic transmitter-containing vesicles in neurons and of similar vesicles in neuroendocrine cells (5–7). Because the for-

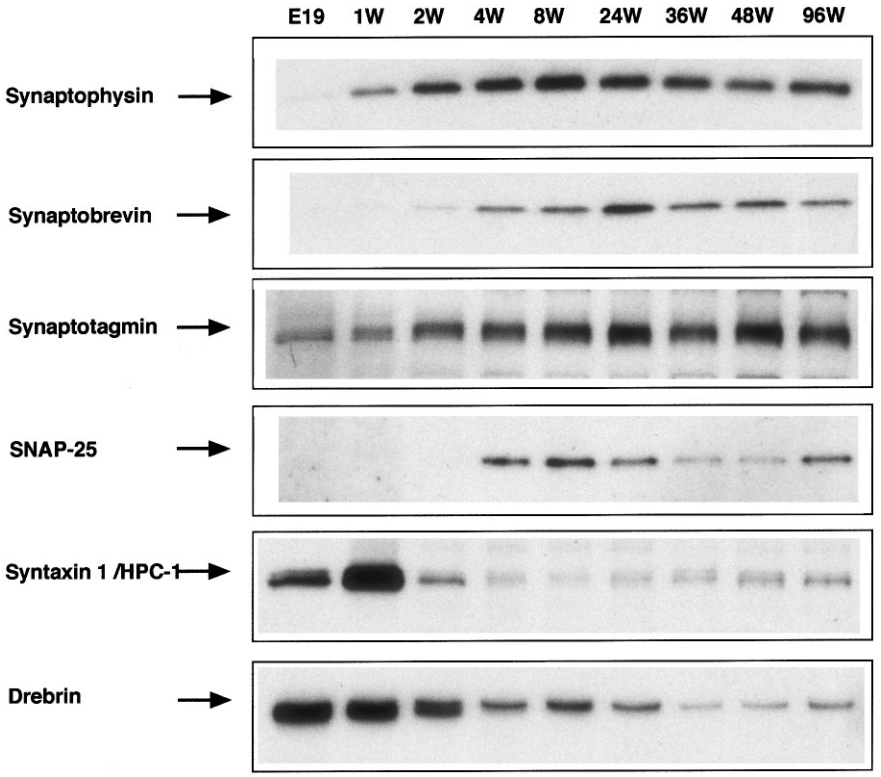

FIG. 1. A typical demonstration of immunodetection of synaptophysin, synaptobrevin, synaptotagmin, SNAP-25, syntaxin 1/HPC-1 and drebrin in development and aging. Particulate (synaptophysin, synaptobrevin, synaptotagmin, SNAP-25, and syntaxin 1/HPC 1) and cytosolic fractions (drebrin) from the hippocampus of Wistar rat brains were loaded (10 μg protein/lane), and immunoblot assays were performed using antibodies against these proteins. E19; embryonic day 19, W; postnatal week

mation of a mature nerve terminal requires the accumulation of large quantities of synaptic vesicles, the expression of synaptic vesicle proteins would be expected to correlate with synaptogenesis. The present study indicated that synaptophysin and synaptobrevin were detected minimally at E19, and that their level increased gradually after birth peaking at 24 weeks, and decreased gradually with aging in a similar fashion, although higher levels of synaptobrevin than synaptophysin were detected at E19 and 1 week. This is consistent with the result that in primary cultures of mouse neurons cultivated for 3 to 4 days, synaptobrevin appears earlier than synaptophysin on small synaptic vesicles and in synaptic contacts (14).

Synaptotagmin is another as an integral membrane protein associated with synaptic vesicles (15). In contrast to synaptophysin and synaptobrevin, synaptotagmin was detected minimally at E19, and then increased gradually until 96 weeks. The difference between synaptotagmin and synaptophysin or synaptobrevin might be in part due to the involvement of synaptotagmin in the exocytosis of both small synaptic vesicles and large dense-core catecholaminergic vesicles (15), although a further role of synaptotagmin might be involved. The expression of synaptotagmin was higher than that of synaptophysin and synaptobrevin at early stages of development, which might reflect the involvement of synaptotagmin in neuronal differentiation (16).

SNAP-25 and syntaxin 1/HPC-1 are plasma membrane proteins at presynaptic sites (8–10). SNAP-25, syntaxin 1/HPC-1 and synaptobrevin have recently been recognized as the three SNARE proteins (soluble NSF attachment protein receptor; NSF, N-ethylmaleimide-sensitive fusion protein) that form the core complex involved in synaptic vesicle docking and subsequent fusion with target membranes (17–19). However, the present study indicated a differential expression of each of the SNARE complex proteins during development and aging. SNAP-25 was barely detected until 2 weeks, and its level was highest at 4 weeks, and then gradually decreased until 96 weeks. In contrast, syntaxin 1/HPC-1 was highly expressed at E19 with a peak protein level at 1 week, which abruptly decreased after 2 weeks. Axonal syntaxin 1/HPC-1 physiologically suppresses the excess axon-collateral sprouting, and downregulation of syntaxin 1/HPC-1 expression at 2 weeks may underlie the control of collateral sprouting and synapse formation during development and memory processing (20). The expression pattern of SNAP-25 is essentially consistent with the report that both SNAP-25 protein and mRNA were present at low

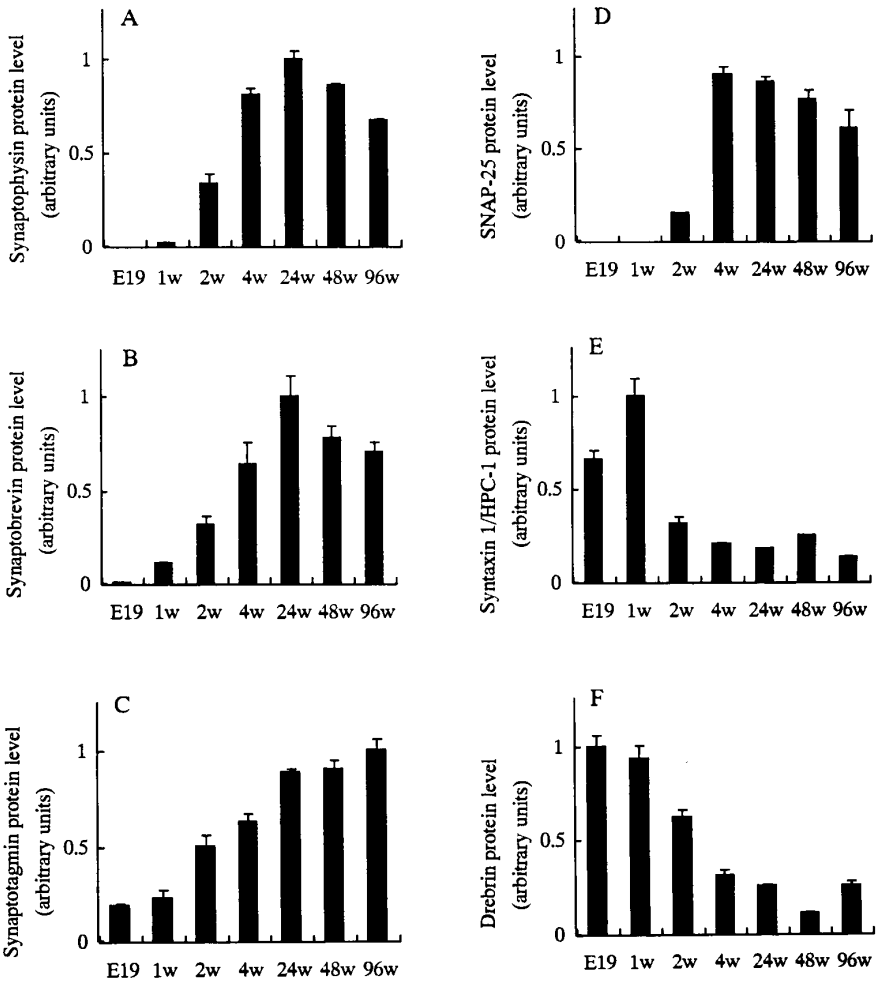

FIG. 2. Developmental and aging changes in levels of synaptophysin, synaptobrevin, synaptotagmin, SNAP-25, syntaxin 1/HPC-1 and drebrin in the rat hippocampus. A, Synaptophysin protein level. B, Synaptobrevin protein level. C, Synaptotagmin protein level. D, SNAP-25 protein level. E, Syntaxin 1/HPC-1 protein level. F, Drebrin protein level. The method of immunochemical detection is described in Materials and Methods. Bars indicate standard error of mean (SEM) (n=6).

levels in embryonic day 15 rat brain, that levels of both increased during postnatal maturation and that a 25-kDa peptide was the major isoform in brain, which increased steadily from embryonic day 15 into adulthood. A second 27-kDa immunoreactive form was present in the brain only during early development (21), although our antibody did not detect a 27-kDa protein band. This data suggests that SNAP-25 may play a role in the establishment and stabilization of specific presynaptic terminals in the brain.

Drebrin, an actin-binding protein, is a developmentally regulated protein which was first isolated from the brains of 10-day chicken embryos. Immunocytochemistry and in situ hybridization analysis of the embryonic cerebellum indicated that drebrin mRNA is first transcribed in postmitotic neurons and that there seems to be a relationship between cell migration and the expression of drebrin (12). The present study also clearly indicated that the drebrin levels were highest at E19, decreasing gradually after birth, suggesting a role for drebrin in cell migration.

In addition, immunoblot analysis showed that neurofilament 68 and GFAP immunoreactivity increased during the postnatal development of the rat brain peaking at 48 weeks in a very similar manner, suggesting intimate cross-talk between the neurons and glia. Moreover, their expression pattern is parallel to that of synaptophysin and synaptobrevin, although the peak levels of neurofilament 68 and GFAP were later than those of synaptophysin and synaptobrevin.

The careful application of stereological techniques to several species, including humans, has led to the conclusion that age-related decline in neuron number through neuronal cell death is not significantly involved in normal aging, at least with respect to the neocortex and hippocampus. In contrast, anatomically, there have been changes in dendritic arbor, spines, and synapse morphology that could have an impact on the

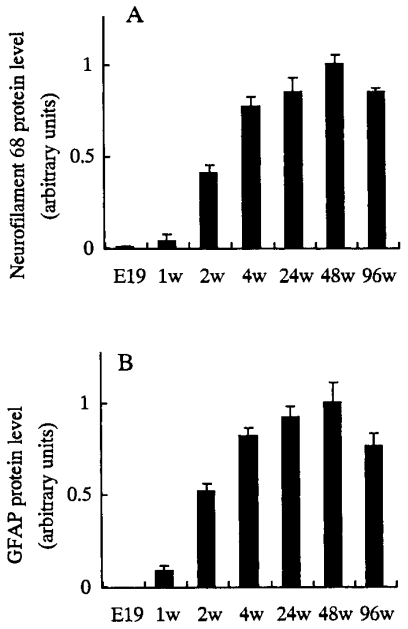

FIG. 3. Developmental and aging changes in neurofilament 68 and GFAP protein levels in the rat hippocampus. A, Neurofilament 68 protein level. B, GFAP protein level. The method of immunochemical detection is described in Materials and Methods. Bars indicate standard error of mean (SEM) (n=6).

function of hippocampal circuits but would not be reflected as neuronal loss (22). The present study first clarified differential aging-related changes in several synaptic proteins. The synaptic density in the hippocampus, as determined by synaptophysin, synaptobrevin and SNAP-25 immunoreactivity, decreased after 24 weeks, until at 96 weeks of age, which correspons to 70–80 years of age in humans, their density decreased to 60–70% of that at 24 weeks. This remarkable reduction might result in a decrease in neurotransmitter release, followed by a decline in cognitive performance during aging. It is of interest that this decrease can be prevented by rearing under an enriched environment (23, 24), suggesting the importance of environmental factors in cognitive function through the maintenance of synaptic proteins.

The present study was intended to consider the alteration of synaptic proteins in AD in the context of their normal developmental and aging-associated changes. In aged rat brains, the expression of SNAP-25 and synaptotagmin proteins were high along with that of synaptophysin and synaptobrevin. In AD, however, synaptophysin and synaptobrevin were more vulnerable than SNAP-25 and synaptotagmin (11). These results suggest that the neurodegenerative events underlying AD are distinct from the events that mediate age-related synapse impairment, and that age-related changes and AD are not part of a continuum.

ACKNOWLEDGMENTS

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan, and grants from the Ministry of Welfare of Japan, the Mitsui Life Social Welfare Foundation, and the Smoking Research Foundation. We also thank Dr. T. Tsuji for technical assistance.

REFERENCES

1. Katzman, R. (1986) *N. Engl. J. Med.* **314,** 964–973.
2. Masliah, E., Terry, R. D., DeTeresa, R. M., and Hansen, L. A. (1989) *Neurosci. Lett.* **103,** 234–239.
3. Masliah, E., Terry, R. D., Alford, M., DeTeresa, R., and Hansen, L. A. (1991) *Am. J. Pathol.* **138,** 235–246.
4. Terry, R. D., Masliah, E., Salmon, D. P., Butters, N., DeTeresa, R., Hill, R., Hansen, L. A., and Katzman, R. (1991) *Ann. Neurol.* **30,** 572–580.
5. Wiedenmann, B., and Franke, W. W. (1985) *Cell* **41,** 1017–1028.
6. Navone, F., Jahn, R., Di-Gioia, G., Strukenbrok, H., Greengard, P., and De-Camilli, P. (1986) *J. Cell Biol.* **103,** 2511–2527.
7. Baumert, M., Maycox, P. R., Novone, F., De-Camilli, P., and Jahn, R. (1989) *EMBO J.* **8,** 379–384.
8. Oyler, G. A., Higgins, G. A., Hart, R. A., Battenberg, E., Billingsley, M., Bloom, F. E., and Wilson, M. C. (1989) *J. Cell Biol.* **109,** 3039–3052.
9. Barnstable, C. J., Hofstein, R., and Akagawa, K. (1985) *Brain Res.* **352,** 286–290.
10. Yoshida, A., Oho, C., Omori, A., Kuwahara, R., Ito, T., and Takahashi, M. (1992) *J. Biol. Chem.* **267,** 24925–24928.
11. Shimohama, S., Kamiya, S., Taniguchi, T., Akagawa, K., and Kimura, J. (1997) *Biochem. Biophys. Res. Commun.* **236,** 239–242.
12. Imamura, K., Shirao, T., Mori, K., and Obata, K. (1992) *Neurosci. Res.* **13,** 33–41.
13. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254.
14. Ahnert-Hilger, G., Kutay, U., Chahoud, I., Rapoport, T., and Wiedenmann, B. (1996) *Eur. J. Cell. Biol.* **70,** 1–11.
15. Perin, M. S., Brose, N., Jahn, R., and Sudhof, T. C. (1991) *J. Biol. Chem.* **266,** 623–629.
16. Berton, F., Iborra, C., Boudier, J. A., Seagar, M. J., and Marqueze, B. (1997) *J. Neurosci.* **17,** 1206–1216.
17. Osen-Sand, A., Staple, J. K., Naldi, E., Schiavo, G., Rossetto, O., Petitpierre, S., Malgaroli, A., Montecucco, C., and Catsicas, S. (1996) *J. Comp. Neurol.* **367,** 222–234.
18. Boschert, U., O,Shaughnessy, C., Dickinson, R., Tessari, M., Bendotti, C., Catsicas, S., and Pich, E. M. (1996) *J. Comp. Neurol.* **367,** 177–193.
19. Jagadish, M. N., Fernandez, C. S., Hewish, D. R., Macaulay, S. L., Gough, K. H., Grusovin, J., Verkuylen, A., Cosgrove, L., Alafaci, A., Frenkel, M. J., and Ward, C. W. (1996) *Biochem J.* **317,** 945–954.
20. Yamaguchi, K., Nakayama, T., Fujiwara, T., and Akagawa, K. (1996) *Brain Res.* **740,** 185–192.
21. Oyler, G. A., Polli, J. W., Wilson, M. C., and Billingsley, M. L. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 5247–5251.
22. Morrison, J. H., and Hof, P. R. (1997) *Science* **278,** 412–419.
23. Saitoh, S., Kobayashi, S., Ohashi, Y., Igarashi, M., Komiya, Y., and Ando, S. (1994) *J. Neurosci. Res.* **39,** 57–62.
24. Kempermann, G., Kuhn, H. G., and Gage, F. H. (1997) *Nature* **386,** 493–495.