

Selective Disappearance of an Axonal Protein, 440-kDa Ankyrin_B, Associated With Neuronal Degeneration Induced by Methylmercury

Motoharu Sakaue,¹ Hiromi Takanaga,¹ Tatsumi Adachi,² Shuntaro Hara,¹ and Manabu Kunimoto^{1*}

¹Department of Public Health, School of Pharmaceutical Sciences, Kitasato University, Minato-ku, Tokyo, Japan

²Department of Basic Medical Sciences, National Institute of Minamata Disease, Minamata, Kumamoto, Japan

The 440-kDa isoform of brain ankyrin, 440-kDa ankyrin_B, is a neuron-specific protein and is confined to axons. Cerebellum is one of the areas characteristically altered by methylmercury intoxication both in the adult and during development. When rat cerebellar neurons matured for 7 days *in vitro* were exposed to methylmercury at 0.03 μ M for 48 hr, viability of the cells was unaffected. However, the immunocytochemical staining of 440-kDa ankyrin_B diminished drastically, whereas that of microtubule-associated protein-2, which is localized in dendrites and cell bodies, and of glial fibrillary acidic protein (GFAP), a marker for astroglial cells coexisting in the culture, remained unchanged. To confirm these observations, a simplified dot blot assay was established to determine 440-kDa ankyrin_B and several other marker proteins in cultured cell samples. With this assay, we found that methylmercury at a submicromolar range induced a decrease of 440-kDa ankyrin_B and an increase of GFAP in a dose-dependent manner in cerebellar cells in primary culture. Surprisingly, another axonal protein, tau, remained mostly in its intact molecular sizes even in the presence of 0.3–1.0 μ M methylmercury, though its immunocytochemical localization was substantially altered. These results indicate that selective loss of the axonal protein 440-kDa ankyrin_B is associated with the early stage of degeneration of cerebellar neurons induced by methylmercury. Therefore, 440-kDa ankyrin_B should be useful as a specific and sensitive marker for the neurotoxicity of methylmercury.

© 2003 Wiley-Liss, Inc.

Key words: methylmercury; neurotoxicity; 440-kDa ankyrin_B; tau; GFAP; cerebellar cells; primary culture; rat

Methylmercury has been recognized as an extremely hazardous environmental pollutant. Selective vulnerability of various parts of the nervous system to injury from methylmercury intoxication is a well-established though as yet unexplained phenomenon in man and experimental

animals (Chang, 1977; Clarkson, 1987). One of the areas characteristically altered is the cerebellum, both in the adult and during development. It has been shown that methylmercury can induce apoptotic death of cerebellar granule neuron at lower doses both *in vitro* and *in vivo*. However, the precise mechanism for the induction of apoptotic death by methylmercury has not yet been elucidated. To understand the mechanism of the neuronal degeneration caused not only by methylmercury but also by other neurotoxicants, sensitive detection of early signs of neuronal alteration caused by neurotoxic substances is extremely important, which should also be quite useful as a marker for neurodegenerative diseases. Neuron-specific proteins are promising candidate for these purposes.

We have shown that the 440-kDa isoform of brain ankyrin (ankyrin_B), a member of the family of spectrin-binding proteins that link the spectrin/actin network to the cytoplasmic domain of integral proteins (Bennett and Baines, 2001), is a neuron-specific isoform, whereas 220-kDa ankyrin_B is rather general in neural tissue and is expressed both in neurons and in glial cells (Kunimoto, 1995b). In neuronal cells, 440-kDa ankyrin_B is sorted to the axons, whereas the 220-kDa isoform is abundant in the dendrites and cell bodies. Furthermore, 440-kDa ankyrin_B is concentrated at the tip of growing neurites in primary cerebellar neurons at the very beginning of culture. Expression of polypeptide and mRNA of 440-kDa ankyrin_B is increased upon induction of neurite outgrowth in human neuroblastoma NB-1 cells (Kunimoto, 1995a). The

Contract grant sponsor: Ministry of Education, Science and Culture of Japan.

*Correspondence to: Manabu Kunimoto, PhD, Department of Public Health, School of Pharmaceutical Sciences, Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan.
E-mail: kunimotom@pharm.kitasato-u.ac.jp

Received 11 April 2003; Revised 15 May 2003; Accepted 28 May 2003

expressed 440-kDa ankyrin_B is also concentrated at the tip of growing neurites and is partly colocalized with GAP-43. These results suggest that 440-kDa ankyrin_B plays an important role in axogenesis.

In this study, we investigated the effects of methylmercury on the expression and localization of 440-kDa ankyrin_B in cerebellar neurons of rats to see whether 440-kDa ankyrin_B can be used as a sensitive marker for neuronal degeneration caused by methylmercury. By comparing the responses of 440-kDa ankyrin_B with other neuronal and glial proteins, we have shown that 440-kDa ankyrin_B is one of the useful indicators for the early stage of neuronal degeneration caused by methylmercury.

MATERIALS AND METHODS

Materials

Iodine 125-protein A was from ICN (Costa Mesa, CA). All tissue culture media and supplements were from Gibco BRL (Grand Island, NY). Mouse monoclonal antibodies against microtubule-associated protein-2 (MAP2; clone AP20) and tau (PC1C6; clone tau-1) were from Cosmo Bio Co., Ltd. Glial fibrillary acidic protein (GFAP), mouse monoclonal antibody against GFAP (clone G-A-5), rabbit anti-mouse IgG, goat anti-rabbit IgG (TRITC-labeled), goat anti-mouse IgG (FITC-labeled), normal goat serum, Triton X-100, pepstatin A, leupeptin, and diisopropyl fluorophosphate (DFP) were from Sigma (St. Louis, MO). An antibody specific to 440-kDa ankyrin_B and a recombinant protein corresponding to the sequence specific to this isoform were prepared as described previously (Kunimoto et al., 1991).

Cell Culture

Cerebellar cells were prepared from neonatal Jcl-Wistar rats within 24 hr after birth, plated onto polylysine-coated dishes or glass coverslips, and maintained in serum-free medium as described previously (Kunimoto et al., 1992).

Gel Electrophoresis And Immunoblot Analysis

Brains were dissected from adult rats and homogenized in 9 volume of 0.32 M sucrose containing 1 mM EGTA, pH 7.4, 1 mM NaN₃, 10 µg/ml pepstatin A, leupeptin, and 0.01% DFP. The homogenates were mixed with sodium dodecyl sulfate (SDS) sample buffer and heated at 65°C for 20 min. Samples were electrophoresed on SDS-polyacrylamide gradient gels and were immunoblotted using ¹²⁵I-protein A to visualize antibodies as described by Davis and Bennett (1983) after electrophoretic transfer to a PVDF membrane (Bio-Rad, Hercules, CA). Cultured cells on plastic plates were washed once with Dulbecco's phosphate-buffered saline (PBS), mixed with SDS sample buffer, scraped from the plates, and heated at 65°C for 20 min. Samples were electrophoresed and immunoblotted in the same way.

Dot-Blot Analysis

Samples prepared for SDS-polyacrylamide gel electrophoresis (PAGE) were spotted (2 µl per spot) on nitrocellulose membranes (Bio-Rad) sandwiched with plastic screens of 1-cm meshes. Air-dried membranes were then fixed with 10% acetic

acid containing 20% isopropyl alcohol for 5 min, washed well with distilled water, and immunoblotted as described above. Radioactivities of the spots were analyzed with a radioluminography analyzer (BAS 2000; Fuji Film) and with an autogamma counter (Packard).

Immunocytochemical Procedures

Cells grown on coverglasses coated with polylysine were fixed with 4% formaldehyde for 15 min at room temperature and washed three times with PBS. Fixed cells were permeabilized with 0.5% Triton X-100 in PBS and blocked with 10% normal goat serum and 1% bovine serum albumin in PBS for 30 min at room temperature. The coverglass was then incubated with 4 µg/ml rabbit polyclonal antibodies against brain ankyrin isoforms and mouse monoclonal antibodies against MAP2 or GFAP in the presence of 0.05% Triton X-100 overnight at 4°C and washed five times with PBS containing 0.1% Tween 20. Ig molecules were visualized with TRITC-conjugated goat anti-rabbit IgG and FITC-conjugated goat anti-mouse IgG. Stained samples were observed and photographed under a confocal laser fluorescent microscope system (TCS 4D; Leica).

Other Procedures

The amount of total proteins in the samples was determined with the Bio-Rad Protein Assay according to the manufacturer's instruction.

RESULTS

Effects of Methylmercury on the Cerebellar Neurons in Primary Culture

Cerebellar cells in primary culture were made up of more than 90% neurons and small numbers of astrocytes (Kunimoto, 1995b). After 5–7 days of culture in vitro, neurons extended extensive axons as revealed by the staining with anti-440-kDa ankyrin_B antibody (Fig. 1A), and astrocytes extended their processes as revealed by staining with anti-GFAP antibody (Fig. 2A,C). When the cerebellar cells precultured for 7 days were exposed to methylmercury at 0.03 µM for 48 hr, the 440-kDa ankyrin_B staining was drastically reduced (Fig. 1D). However, the GFAP staining did not show significant alteration with the exposure (Fig. 2D,F). It is noteworthy that the staining of MAP2, a marker protein for neuronal cell bodies and dendrites, was also reduced by the exposure, but to a much lesser extent (Fig. 1B,E).

A Simplified Dot-Blot Assay for 440-kDa Ankyrin_B and GFAP

A simple dot blot assay for 440-kDa ankyrin_B and GFAP was established by a slight modification of the

Fig. 1. Immunocytochemical localization of 440-kDa ankyrin_B and MAP2 in cerebellar cells in primary culture. Cerebellar cells matured for 7 days in vitro were then exposed to 0 (A–C) or 0.03 µM methylmercury (D–F) for 48 hr. Cells were then fixed, permeabilized, and stained with double-label immunofluorescence using anti-440-kDa ankyrin_B (A,D) and anti-MAP2 (B,E) antibodies. Corresponding phase-contrast micrographs are also shown (C,F, respectively). Scale bar = 50 µm.

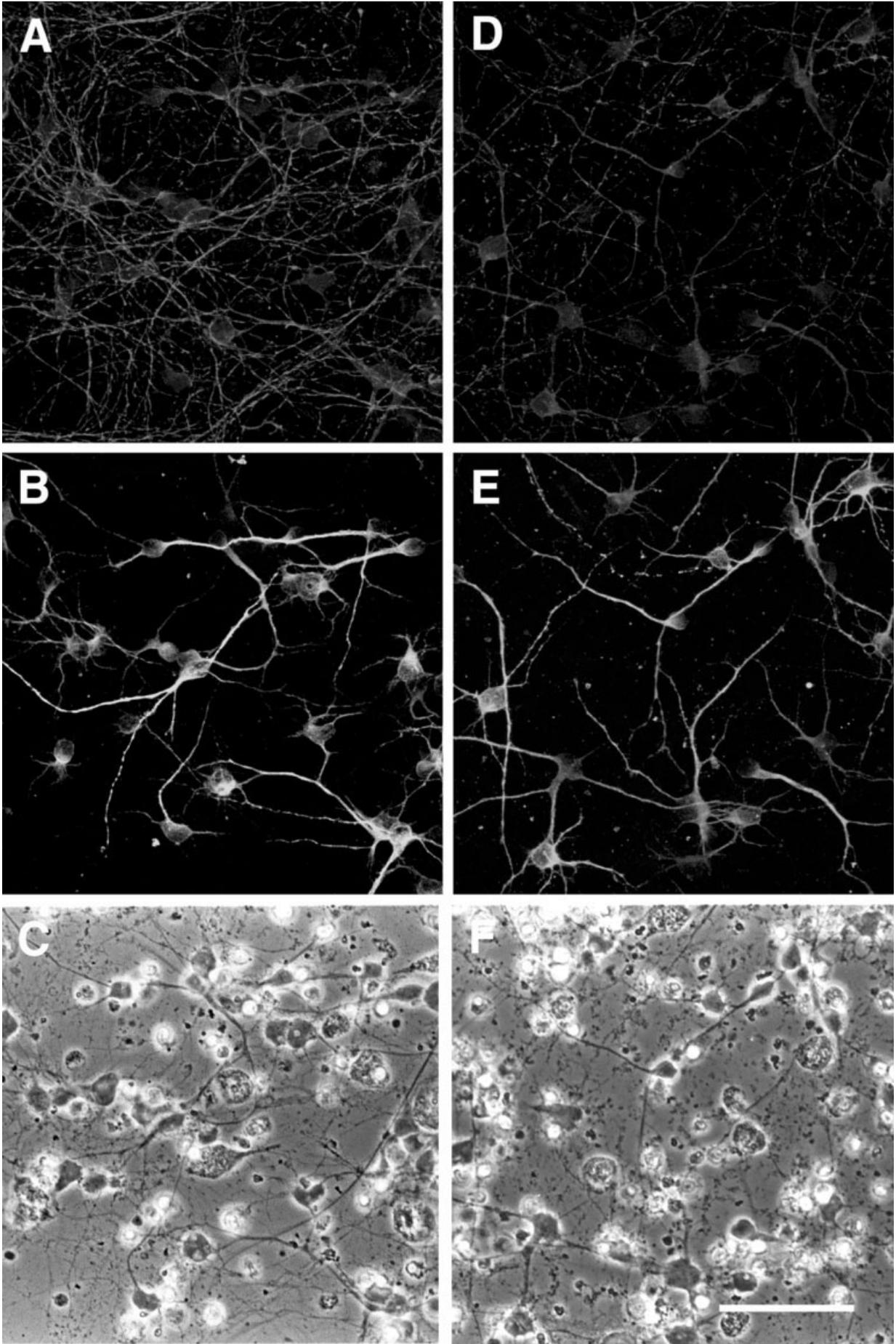


Figure 1.

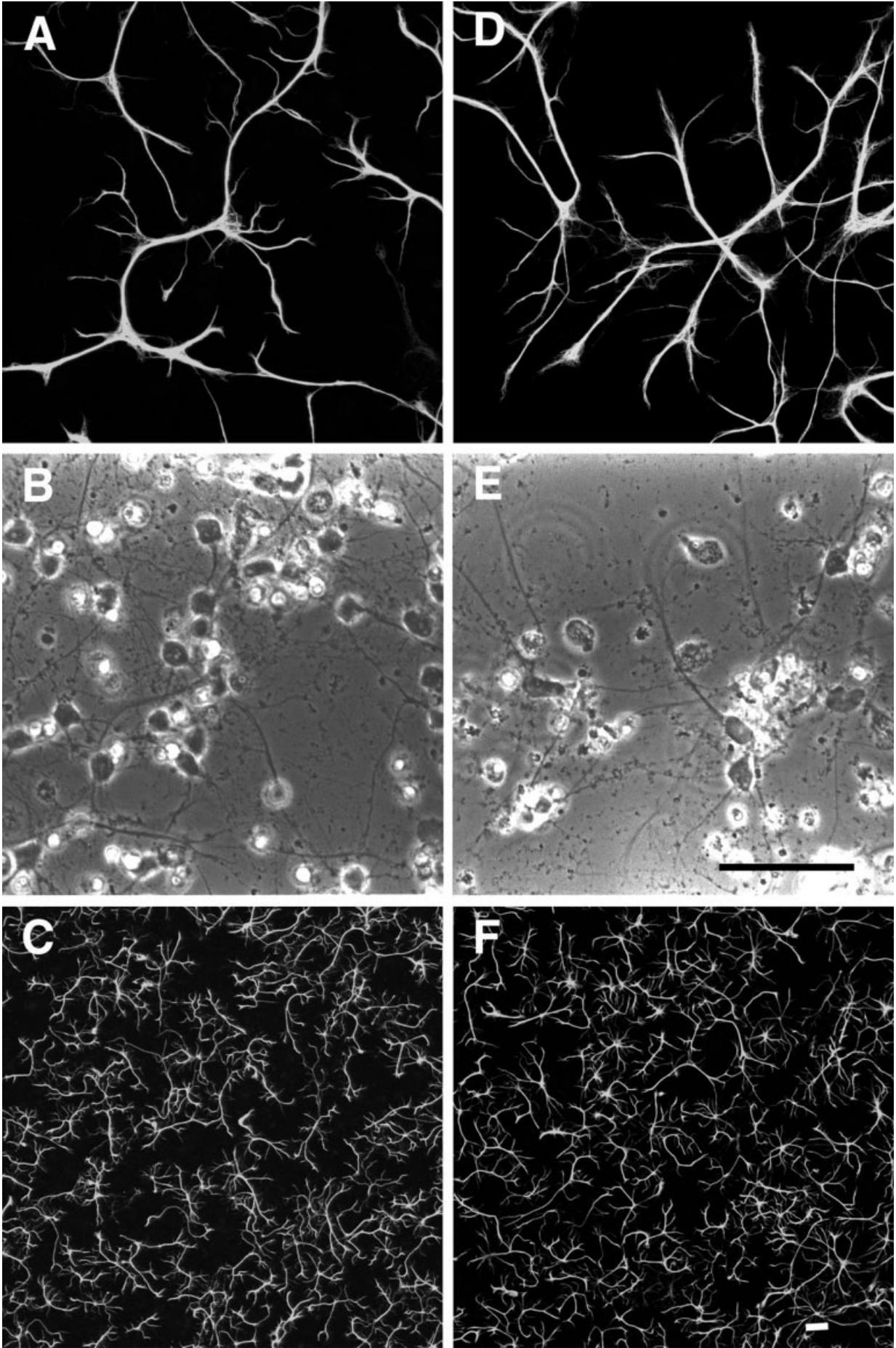


Figure 2.

procedure of O'Callaghan (1991). In this method, samples prepared for SDS-PAGE were directly spotted on nitrocellulose membranes sandwiched with plastic screens of 1-cm mesh, which allows whole proteins in the samples to be retained on the membranes as dots arranged regularly (see Fig. 4). The antibodies used recognized their antigens specifically in the samples from cultured cerebellar cells and brain tissue (data not shown). By using this dot blot assay, standard curves were obtained for GFAP and a recombinant protein corresponding to the sequence specific to 440-kDa ankyrin_B (Fig. 3). The total amount of proteins in the standard sample was adjusted to 2 mg/ml by using bovine serum albumin (BSA). The curves clearly indicate that this dot blot assay provides a wide range of linearity for both proteins. In addition, the radioactivities estimated with BAS 2000 (a radioluminography analyzer) coincide well with those measured with a gamma counter (Fig. 3).

Effects of Methylmercury on the Amount of GFAP and 440-kDa Ankyrin_B in Rat Cerebellar Cells in Primary Culture

To confirm the immunocytochemical observation of cerebellar cells exposed to methylmercury, the amount of each protein expressed in the culture was determined with the dot blot assay (Fig. 4). Although the amount of 440-kDa ankyrin_B decreased depending on the methylmercury concentration, the amount of GFAP increased upon exposure to methylmercury at 0.01–0.3 μ M (Fig. 5). At a higher concentration (1 μ M) of methylmercury, however, the amount of GFAP started decreasing. These results suggest that astrocytes might be activated in the presence of methylmercury at doses at which neurons were degenerating.

The amount of MAP2, a neuronal protein localized in dendrites and cell bodies, also decreased, but at higher methylmercury concentrations. It is noteworthy that the amount of tau, a microtubule-associated protein localized mainly in the axon, remained almost unchanged even at 1 μ M of methylmercury.

Differential Effects of Methylmercury on the Expression and Localization of Axonal Proteins in Rat Cerebellar Neurons

To confirm further the differential effects of methylmercury on the two axonal proteins, 440-kDa ankyrin_B and tau, in cerebellar neurons, localization and intactness of tau were examined by immunofluorescent microscopy and Western blotting analysis and compared with those of

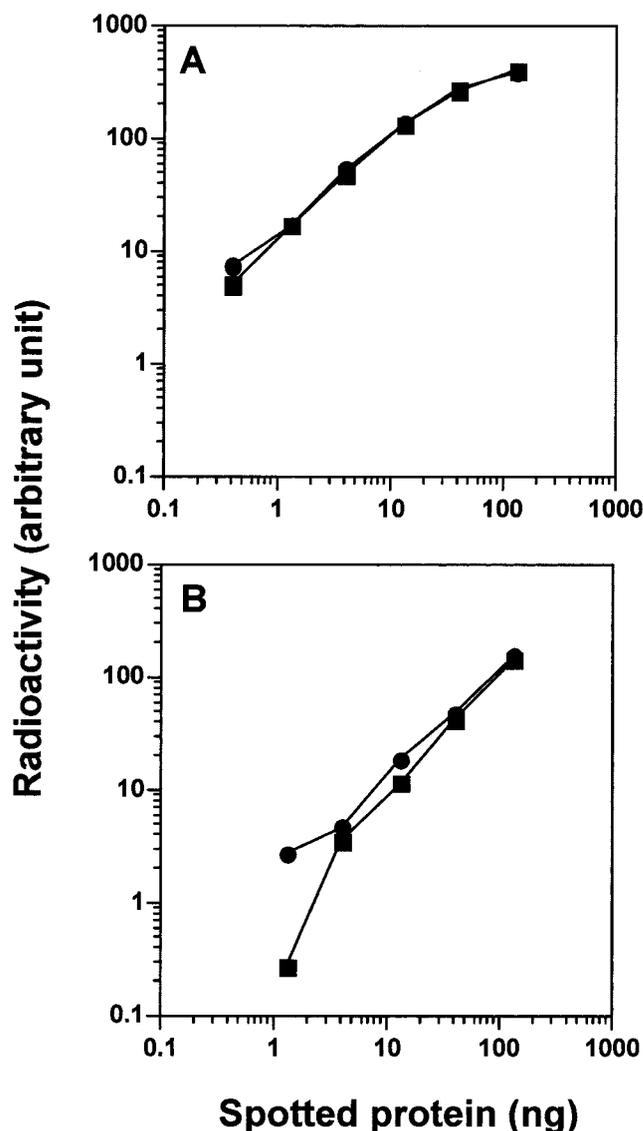


Fig. 3. Dose-response curves of recombinant 440-kDa ankyrin_B fragment (A) and GFAP (B) in the dot blot assay. Radioactivity in each spot was determined with BAS 2000 (squares) and an autogamma counter (circles). Each point represents the mean of triplicate assays.

Fig. 2. Immunocytochemical localization of GFAP in cerebellar cells in primary culture. Cerebellar cells matured for 7 days in vitro were then exposed to 0 (A–C) or 0.03 μ M (D–F) methylmercury for 48 hr. Cells were then fixed, permeabilized, and labeled with indirect immunofluorescence using anti-GFAP antibodies. Phase-contrast micrographs corresponding to A,D are shown (B,E, respectively). Immunofluorescent images with lower magnification (C,F) are also shown. Scale bars = 50 μ m in E (for A,B,D,E); 50 μ m in F (for C,F).

440-kDa ankyrin_B. Although the immunostaining of 440-kDa ankyrin_B decreased upon exposure to methylmercury at 0.1 μ M (Fig. 6A,D), that of tau did not show a significant decrease in its intensity but did so in the pattern of the staining; fibrous staining pattern decreased, whereas dotted or aggregated staining increased (Fig. 6B,E). Tau proteins in the dotted aggregates could be degraded and denatured peptides still holding epitopes. However, Western blot analysis of tau in the cerebellar cell cultures treated with methylmercury revealed that tau proteins were mostly intact, at least based on their molecular sizes (Fig. 7B), whereas the immunoreactivity of 440-kDa ankyrin_B almost completely disappeared at higher methylmercury concentrations (Fig. 7A).

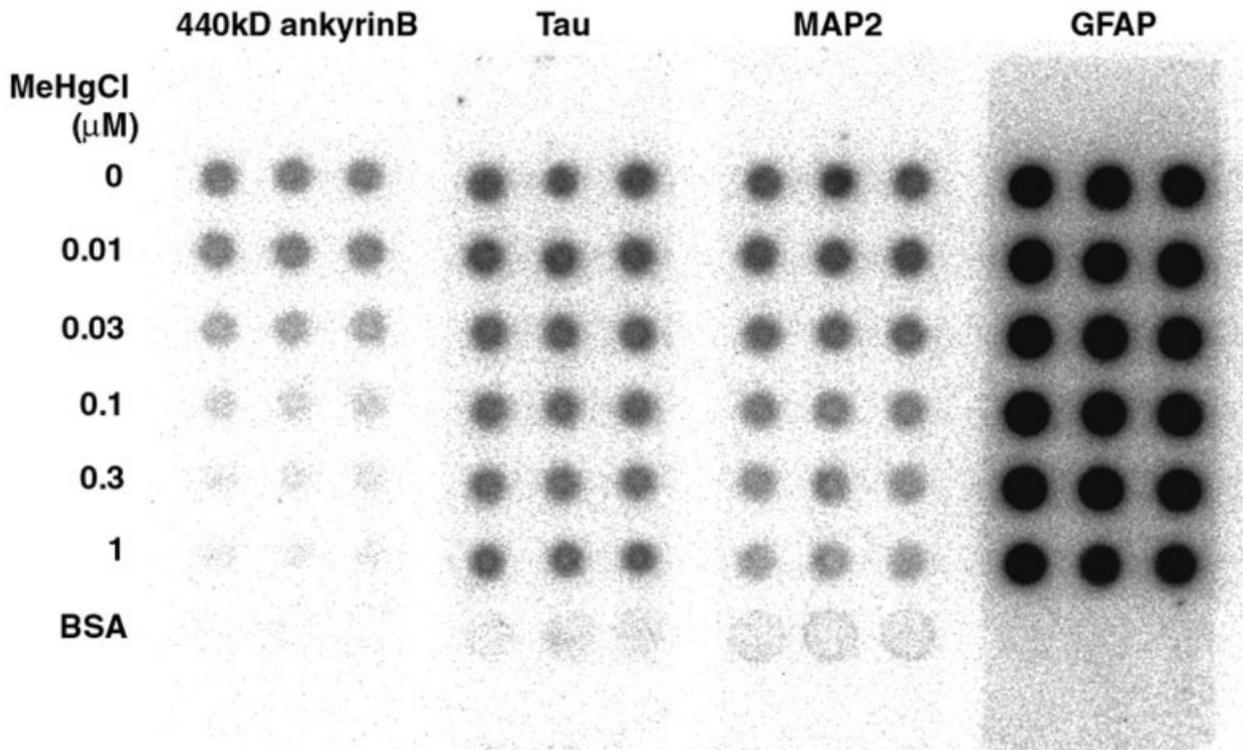


Fig. 4. Dot blot analyses of marker proteins expressed in cerebellar cells in primary culture. Cerebellar cells matured for 7 days in vitro were then exposed to methylmercury at 0–1.0 μM for 48 hr. Total proteins of the cells solubilized with SDS sample buffer were subjected to dot

blot immunoassay using 440-kDa ankyrin_B, tau, MAP2, or GFAP antibodies as described in Materials and Methods. Radiographs obtained with BAS 2000 are shown.

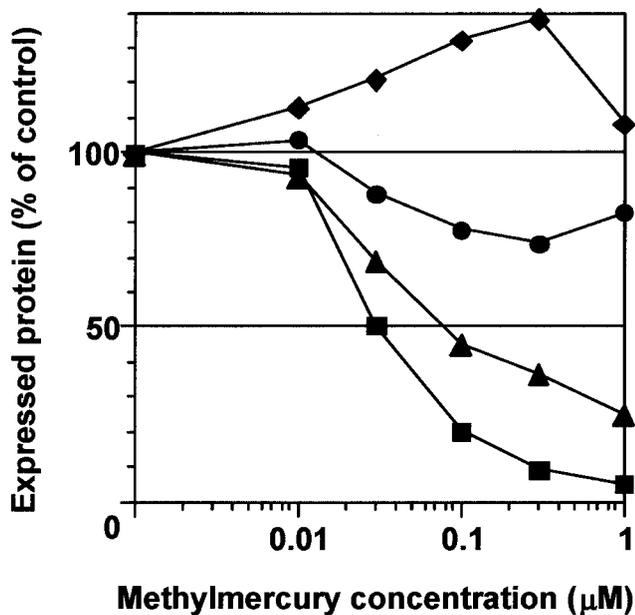


Fig. 5. Changes in the amount of expressed marker proteins in cerebellar cells in primary culture upon exposure to methylmercury. Cerebellar cells matured for 7 days in vitro were then exposed to methylmercury at 0–1.0 μM for 48 hr. Total proteins of the cells solubilized with SDS sample buffer were subjected to the blot immunoassay using 440-kDa ankyrin_B (squares), MAP2 (triangles), tau (circle), or GFAP (lozenges) antibodies. Radioactivity at each spot was determined with BAS 2000 and standardized with the amount of total protein in each sample. Values expressed as the percentage of control represent the mean of triplicate assays.

DISCUSSION

This report describes differential effects of methylmercury at the submicromolar range on the expression of several marker proteins in rat cerebellar cells in primary

culture. Two axonal proteins, 440-kDa ankyrin_B and tau, showed different patterns of disappearance/degradation in cerebellar neurons exposed to methylmercury. Whereas 440-kDa ankyrin_B disappeared with methylmercury treatment at as low as 10–30 nM, most of tau remained apparently intact even in the presence of 1 μM methylmercury. Another neuronal protein, MAP2, which is localized in dendrites and cell bodies of neurons, also disappeared upon exposure to methylmercury, but at higher concentrations. These results clearly indicate that selective loss of the axonal protein 440-kDa ankyrin_B is associated with an early stage of the degeneration of cerebellar neurons induced by methylmercury in vitro. One possible candidate responsible for the degradation of 440-kDa ankyrin_B is calpain, a protease activated by calcium, insofar as ankyrin_B has been shown to be degraded by calpain

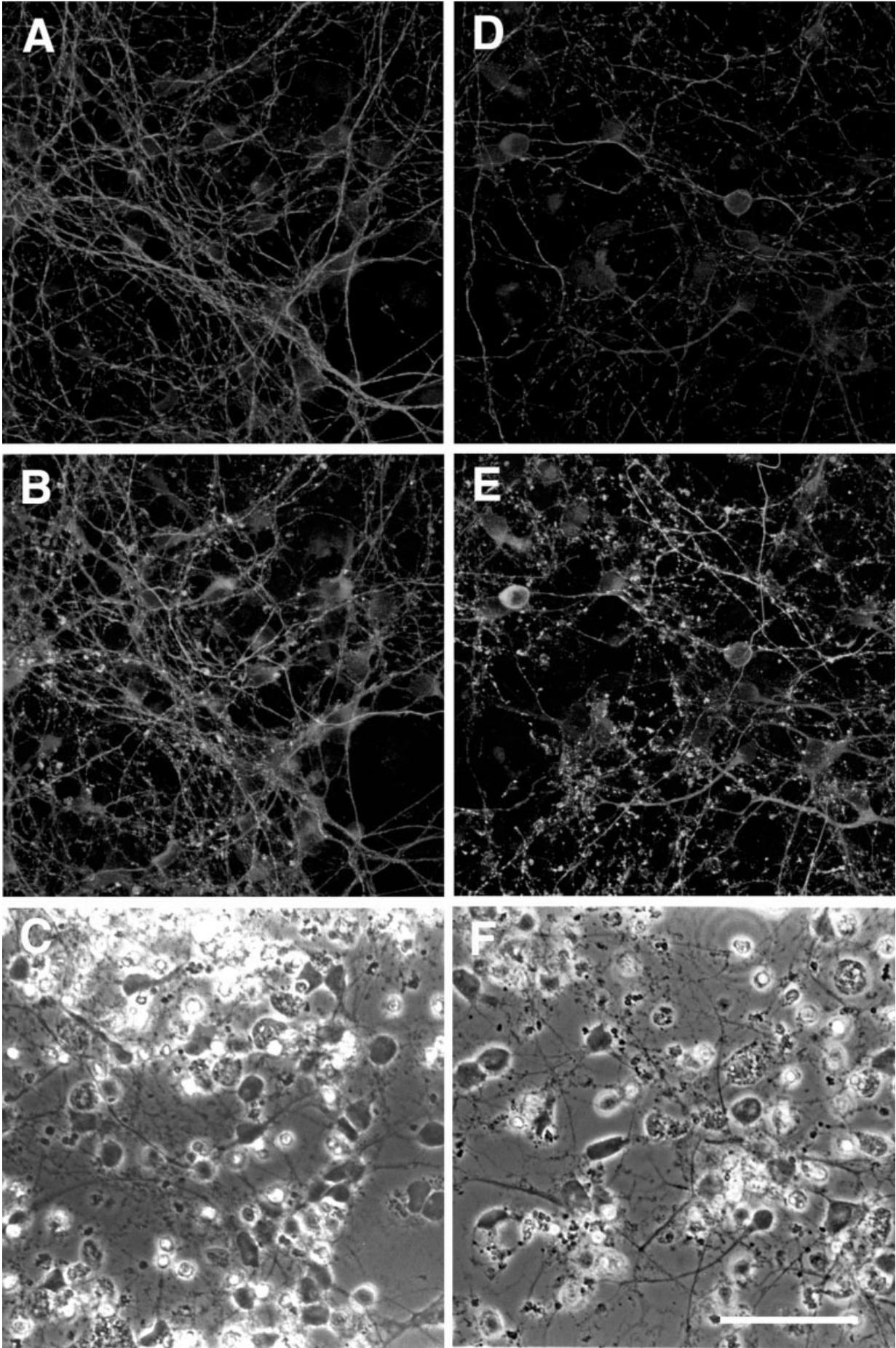


Figure 6. (Legend on following page.)

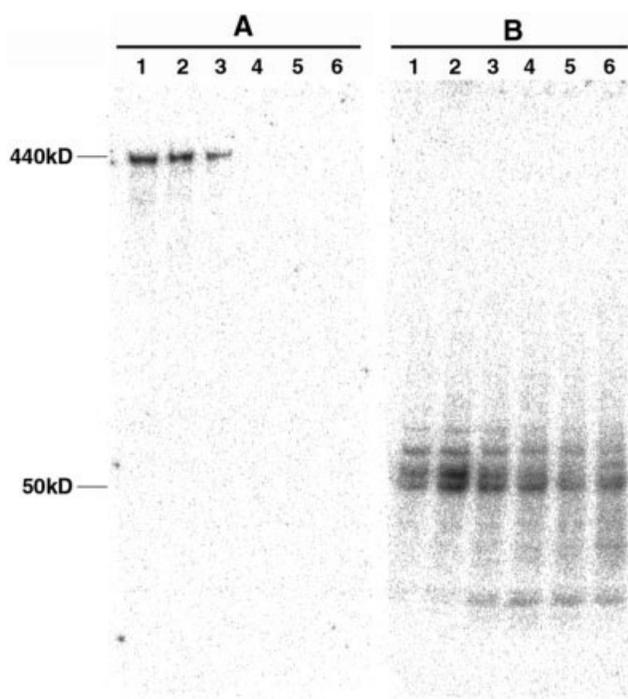


Fig. 7. Western blot analyses of 440-kDa ankyrin_B and tau in cerebellar cells in primary culture. Cerebellar cells matured for 7 days *in vitro* were then exposed to 0, 0.01, 0.03, 0.1, 0.3, or 1 μ M methylmercury (lanes 1–6, respectively) for 48 hr. Total proteins of the cerebellar cells were separated on SDS-polyacrylamide gel, transferred to Immobilon P membrane, and immunoblotted using 440-kDa ankyrin_B (A) or tau (B) antibodies as described in Materials and Methods. Radioluminographs obtained with BAS 2000 are shown.

(Harada et al., 1997), and exposure to methylmercury at 2–5 μ M increases the intracellular calcium level in NG108–15 cells (Atchison and Hare, 1994). It is also likely that 440-kDa ankyrin_B is down-regulated at the transcriptional level, insofar as it has been shown that methylmercury at 1 μ M induced down-regulation of 440-kDa ankyrin_B mRNA in human neuroblastoma NB-1 cells (Kunimoto and Suzuki, 1995). However coexistence of calpain inhibitor (calpain inhibitor II) prevented the methylmercury-induced decrease of 440-kDa ankyrin_B protein in rat cerebellar neurons almost completely, indicating that calpain is mainly involved in the selective disappearance of 440-kDa ankyrin_B (manuscript in preparation).

Fig. 6. (Figure appears on preceding page.) Immunocytochemical localization of 440-kDa ankyrin_B and tau in cerebellar cells in primary culture. Cerebellar cells matured for 7 days *in vitro* were then exposed to 0 (A–C) or 0.1 μ M (D–F) methylmercury for 48 hr. Cells were then fixed, permeabilized, and stained with double-label immunofluorescence using anti-440-kDa ankyrin_B (A,D) and anti-tau antibodies (B,E). Corresponding phase-contrast micrographs are also shown (C,F, respectively). Scale bar = 50 μ m.

The 440-kDa ankyrin_B is localized in unmyelinated axons, such as parallel fibers in cerebellum. Its 220-kDa inserted polypeptide is considered to form a long tail domain, which is essential for its axonal localization and plays an important role in the connection of axonal membranes and cytoskeletons. Therefore, the disappearance of 440-kDa ankyrin_B may result in disorganization and degeneration of axons, which is inconsistent with the finding that degeneration of the synaptic boutons of parallel fibers was observed as an early sign of methylmercury intoxication in rats (Su et al., 1996). In addition, it is well-known that ankyrins, including 440-kDa ankyrin_B, contain a death domain at their C-terminal, but its roles in cell death processes have not yet been clarified (Bennett and Baines, 2001). Although there is no experimental evidence, it is imaginable that the degradation of 440-kDa ankyrin_B may activate the death domain, which results in the induction of apoptotic death of cerebellar neurons.

As for the resistance of tau to proteolytic degradation in methylmercury-treated cerebellar neurons, one possibility is hyperphosphorylation of tau by the exposure to methylmercury, in that hyperphosphorylated tau is resistant to proteolytic cleavage and hence is accumulated in the brain of Alzheimer's disease patients as part of paired helical filaments or neurofibrillary tangles (Litersky et al., 1993; Billingsley and Kincaid, 1997). However, the monoclonal antibody used to detect tau (PC1C6; clone tau-1) in this study has been shown to be specific to dephosphorylated tau (Billingsley and Kincaid, 1997). Therefore, tau proteins that survived methylmercury-induced degeneration of cerebellar neurons are likely not hyperphosphorylated but modified in another way.

The amount of GFAP increased upon exposure to methylmercury at doses (0.01–0.3 μ M) at which neurons were degenerating, indicating that astrocytes were affected by methylmercury in a way different from neurons. Gliosis has been commonly observed in the brains of methylmercury-intoxicated humans and experimental animals (Tsubaki and Takahashi, 1986; Charleston et al., 1994), suggesting that methylmercury may stimulate the proliferation of astrocytes directly or indirectly. However, in our preliminary experiments, the methylmercury exposure did not increase the number of 5-bromo-2'-deoxyuridine-incorporating astrocytes, suggesting that methylmercury may stimulate the expression of GFAP proteins (data not shown).

A new dot blot method developed in this study, the "spot blot immunoassay," does not require any expensive apparatus. This method also can be used according to a nonradioactive protocol (data not shown). Therefore, it provides a simple and quantitative way to determine the amount of proteins with very high molecular weights, such as 440-kDa ankyrin_B, that are hard to electrotransfer to nitrocellulose membranes quantitatively.

In conclusion, 440-kDa ankyrin_B should be useful as a specific and sensitive marker for the early stage of methylmercury intoxication, especially for axonal degeneration. In addition, the neurotoxic action of methylmercury

could be ascribed partially to its selective effects on axon-specific proteins, including 440-kDa ankyrin_B.

ACKNOWLEDGMENTS

The authors thank Drs. James P. O'Callaghan, Vann Bennett, and Tsuguyoshi Suzuki for valuable discussions.

REFERENCES

- Atchison WD, Hare MF. 1994. Mechanisms of methylmercury-induced neurotoxicity. *FASEB J* 8:622–629.
- Bennett V, Baines AJ. 2001. Spectrin and ankyrin-based pathways: meta-zoan inventions for integrating cells into tissues. *Physiol Rev* 81:1353–1392.
- Billingsley ML, Kincaid RL. 1997. Regulated phosphorylation and dephosphorylation of tau protein: effects on microtubule interaction, intracellular trafficking and neurodegeneration. *Biochem J* 323:577–591.
- Chang LW. 1977. Neurotoxic effects of mercury: a review. *Environ Res* 14:329–373.
- Charleston JS, Bolender RP, Mottet NK, Body RL, Vahter ME, Burbacher TM. 1994. Increases in the number of reactive glia in the visual cortex of *Macaca fascicularis* following subclinical long-term methyl mercury exposure. *Toxicol Appl Pharmacol* 129:196–206.
- Clarkson TW. 1987. Metal toxicity in the central nervous system. *Environ Health Perspect* 75:59–64.
- Davis J, Bennett V. 1983. Brain spectrin: isolation of subunits and formation of hybrids with erythrocyte spectrin subunits. *J Biol Chem* 258:7757–7766.
- Harada K, Fukuda S, Kunimoto M, Yoshida K. 1997. Distribution of ankyrin isoforms and their proteolysis after ischemia and reperfusion in rat brain. *J Neurochem* 69:371–376.
- Kunimoto M. 1995a. Possible involvement of 440 kDa isoform of ankyrin_B in neuritogenesis in human neuroblastoma NB-1 cells. *FEBS Lett* 357:217–220.
- Kunimoto M. 1995b. A neuron-specific isoform of brain ankyrin, 440 kDa ankyrin_B is targeted to the axons of rat cerebellar neurons. *J Cell Biol* 131:1821–1829.
- Kunimoto M, Suzuki T. 1995. Selective down-regulation of 440 kDa ankyrin_B associated with neurite retraction. *Neuroreport* 6:2545–2548.
- Kunimoto M, Otto E, Bennett V. 1991. A new 440 kDa isoform is the major ankyrin in neonatal rat brain. *J Cell Biol* 115:1319–1331.
- Kunimoto M, Aoki Y, Shibata K, Miura T. 1992. Differential cytotoxic effects of methylmercury and organ tin compounds on mature and immature neuronal cells and non-neuronal cells in vitro. *Toxicol In Vitro* 4:349–355.
- Litersky JM, Scott CW, Johnson GVM. 1993. Phosphorylation, calpain proteolysis and tubulin binding of recombinant tau isoforms. *Brain Res* 604:32–40.
- O'Callaghan JP. 1991. Assessment of neurotoxicity: use of glial fibrillary acidic protein as a biomarker. *Biomed Environ Sci* 4:192–276.
- Su M, Kakita A, Yamada M, Takahashi H, Ikuta F. 1996. Degeneration of the synaptic boutons of parallel fibers in rats treated with methylmercury: chronological observations. *Neuropathology* 16:172–177.
- Tsubaki T, Takahashi H. 1986. Recent advances in Minamata disease studies. Tokyo: Kodansha Ltd. p 24–39.