—Minireviews—

A Neuron-Specific Isoform of Brain Ankyrin, 440 kD Ankyrin$_B$, As a Useful Tool in Neurobiology and Neurotoxicology

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Two isoforms of brain ankyrin, 440 kD and 220 kD ankyrin$_B$, are generated from the same gene by alternative splicing of pre-mRNA. The larger isoform shares the same NH_2-terminal and COOH-terminal domains as the smaller isoform and contains, in addition, a unique inserted domain of about 220 kD in size. Both isoforms were expressed in primary neurons in a manner similar to that *in vivo*; the larger isoform appeared first when axogenesis is actively conducted and the smaller isoform appeared later. 440 kD ankyrin$_B$ was localized in the axons of neurons both *in vivo* and *in vitro*, while the 220 kD isoform was rather localized in the cell bodies and dendrites of neurons. The expression of 440 kD ankyrin$_B$ is intimately associated not only with neurite outgrowth but also with neurite retraction in neuronal cells, and is regulated at the mRNA level. Therefore, 440 kD ankyrin$_B$ is a specific and useful marker for neuritogenesis and is also a useful tool for investigating the effects of neurotoxic substances like methylmercury on the developing nervous system.

Keywords —— brain ankyrin, neuron, neuritogenesis, neurotoxicity

Ankyrins are a family of spectrin-binding proteins that link the spectrin/actin network to cytoplasmic domains of integral membrane proteins, which include ion channels and cell adhesion molecules.[1,2] Three different ankyrins are currently known to be expressed in brain tissue: ankyrin$_R$, which is also expressed in erythrocytes; ankyrin$_B$, the major ankyrin in the brain; and ankyrin$_G$, which is localized to axonal initial segments and the nodes of Ranvier of myelinated axons.[3] Ankyrin$_B$ includes two isoforms of 220 kD and 440 kD which are generated from a single gene by alternative splicing of pre-mRNA.[4] 220 kD ankyrin$_B$ is the major ankyrin isoform in adult rat brain, while 440 kD ankyrin$_B$, in contrast, is maximally expressed in the developing neonatal rat brain.[5] Immunocytochemical staining of developing rat cerebellum and optic nerve implies that 440 kD ankyrin$_B$ is localized to unmyelinated axons and premyelinated axons.[5,6]

It has also been shown that 440 kD ankyrin$_B$ is up-regulated concomitantly with the neurite outgrowth in human neuroblastoma NB-1 cells, whose neurite extension can be promoted by external stimuli such as dibutyryl cAMP.[7] Thus, 440 kD ankyrin$_B$ can be regarded as a neuronal growth-associated protein like GAP (growth-associated protein)-43.[8]

Taken together, these observations strongly suggest that 440 kD ankyrin$_B$ has the potential to be a useful tool in neurobiology and neurotoxicology. Our recent studies using this protein for investigating neuronal development and neuronal degeneration will be presented.

A Neuron-Specific Isoform of Brain Ankyrin, 440 kD Ankyrin$_B$, Is Targeted to the Axons of Rat Cerebellar Neurons[9]

440 kD ankyrin$_B$ has been localized to unmyelinated axons and premyelinated axons.[5,6] Localization of 220 kD ankyrin$_B$, however, has not been clearly demonstrated, because this

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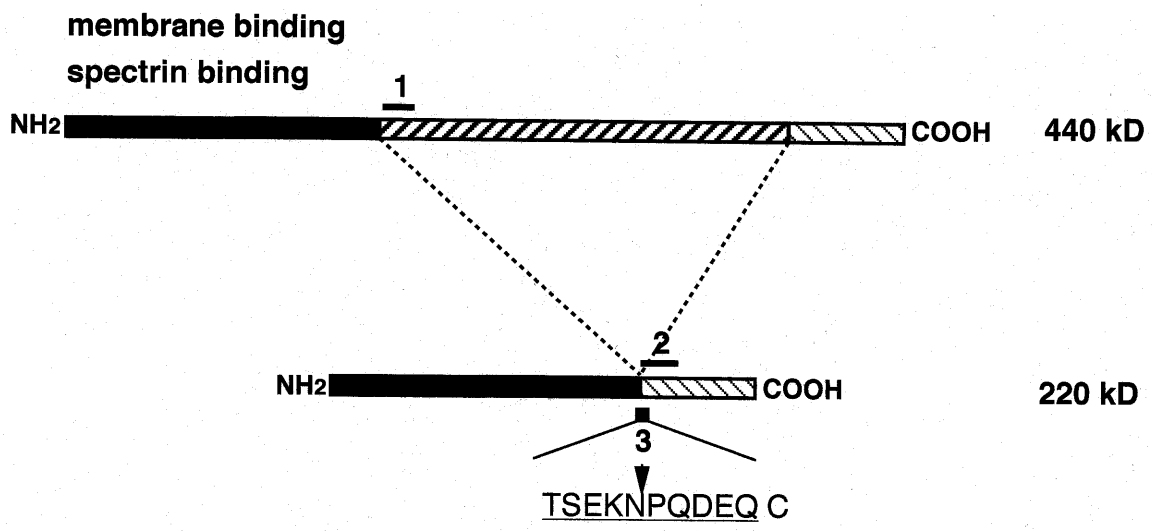

Fig. 1. Schematic Organization of Ankyrin$_B$ Isoforms

Recombinant proteins corresponding to Regions 1 and 2 were used as antigens to prepare polyclonal antibodies 1 and 2, respectively and synthetic peptide corresponding to Region 3 was used to prepare antibody 3.

isoform is totally subsumed within the 440 kD isoform (Fig. 1), which prevents the production of specific antibodies by standard strategies using recombinant proteins as antigens. Therefore, a synthetic peptide corresponding to the splice site (ten amino acids, TSEKNPQDEQ, corresponding to the splice site plus an artificial C at the carboxy terminus for coupling to bovine serum albumin, Fig. 1, Region 3) was designed to prepare a polyclonal antibody which can recognize the 220 kD isoform specifically. Such an antibody (antibody 3), raised against the synthetic peptide specifically recognizes 220 kD ankyrin$_B$ by Western blot analysis of rat cerebellum, while an antibody against Region 1 in Fig. 1 (antibody 1) recognizes 440 kD ankyrin$_B$ specifically and an antibody against Region 2 (antibody 2) recognizes both 440 kD and 220 kD isoforms. Using these antibodies, the expression and localization of the two ankyrin$_B$ isoforms in cerebellar cells was investigated.

In primary cerebellar cells stained by double-label immunofluorescence, 440 kD ankyrin$_B$ is localized to the axons (Fig. 2 A (red)), while MAP2 (microtubule associated protein 2, a neuronal marker) is localized to the dendrites and cell bodies of neurons (Fig. 2 A (green)).[10] Conversely, the staining of 220 kD ankyrin$_B$ is quite similar to that of MAP2 (Fig. 2 B). In addition, 220 kD ankyrin$_B$ is localized to astroglial cells which are not stained with anti-MAP2 antibody (Fig. 2 B, indicated by arrows), but stained with anti-GFAP (glial fibrillary acidic protein, an astroglial marker) antibody (data not shown). This is the first example of alternative splicing in neurons which produces protein isoforms differentially targeted to axons and dendrites/cell bodies.

These results clearly indicate that 440 kD ankyrin$_B$ is a neuron-specific isoform, while 220 kD ankyrin$_B$ is expressed both in neurons and glial cells. In addition, 440 kD ankyrin$_B$ is sorted to the axons, while the 220 kD isoform, which can be regarded as a naturally-occurring deletion mutant of 440 kD ankyrin$_B$ lacking the 220 kD inserted domain, remains both in the dendrites and cell bodies of neuronal cells, even though both ankyrin$_B$ isoforms share the same membrane binding domain (Fig. 1). Taken together, these data suggest that the 220 kD inserted domain is essential for the sorting of 440 kD ankyrin$_B$ to the axons. It is also conceivable that the differential sorting mechanism, but not the membrane binding domain itself, may be responsible for determining the target sites of each ankyrin isoform, the cytoplasmic domains of integral membrane proteins. To date, only a few proteins, such as tau and GAP-43, have been shown to be targeted to growth cones and/or axons, but the sorting mechanism has not been elucidated. Ankyrin$_G$ is targeted to the nodes of Ranvier and the initial segments of axons and has a unique insert which may be responsible for this targeting.[3] Ankyrins may be a useful model for investigating protein sorting in neurons.

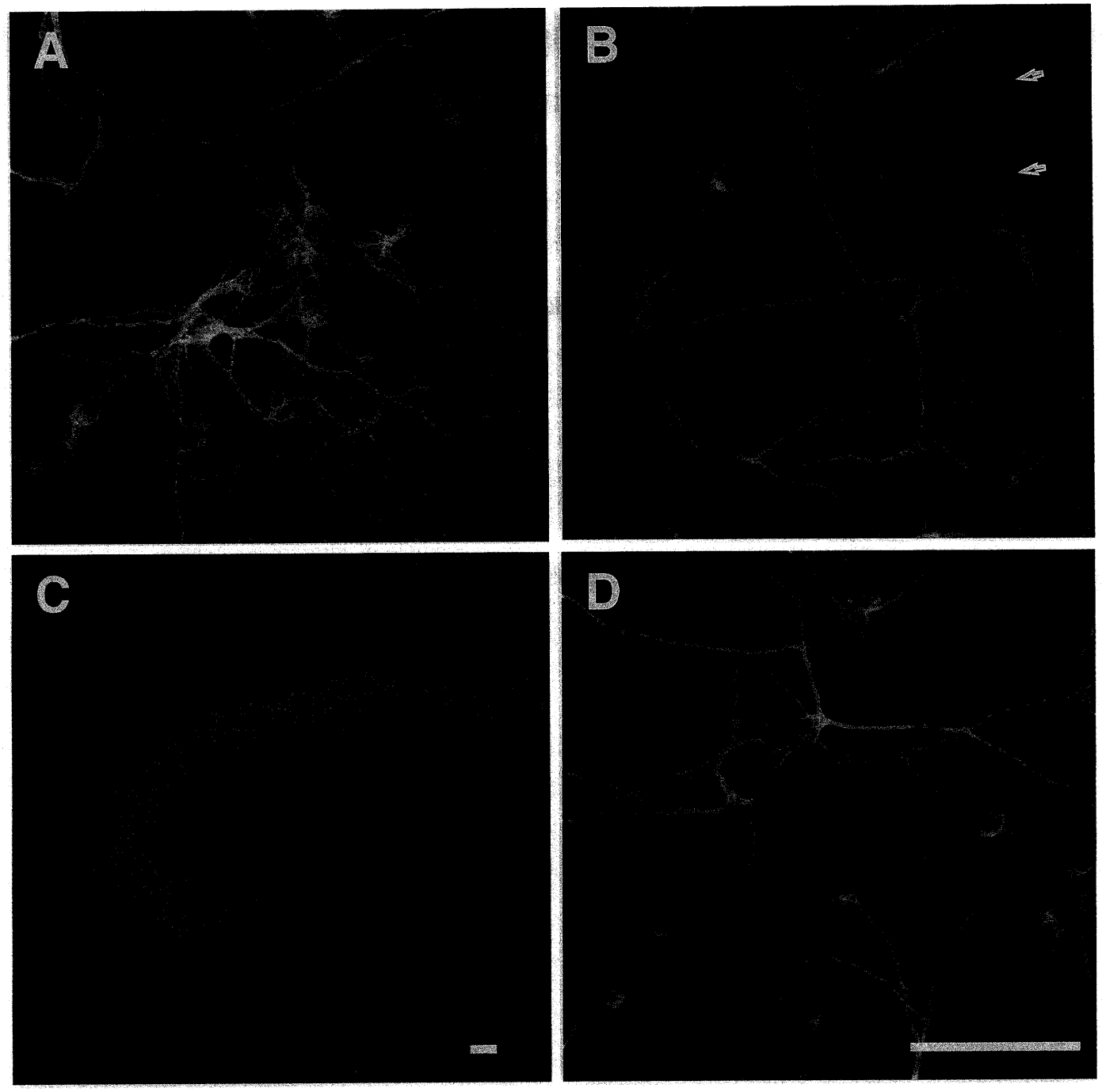

Fig. 2. Immunocytochemical Localization of Ankyrin$_B$ Isoforms and L1 in Primary Cerebellar Cells of Rats and in Prenatal Rat Brain

Cerebellar cells cultured for 9 days were stained with double-label immunofluorescence using antibody 1 (A), antibody 3 (B) or anti-L1 (D) (red) and anti-MAP2 (green), simultaneously. Cryosections of the brains from the E18 rat fetus were stained with double-label immunofluorescence using antibody 1 (red) and anti-MAP2 (green) (C). Scale bar, 50 μm.

Immunocytochemical Localization of Brain Ankyrin Isoforms and Cell Adhesion Molecule L1 in Fetal Rat Brains and Cerebellar Cells in Primary Culture[11]

L1 is a member of the neurofascin/L1/NrCAM family of cell adhesion molecules in the the nervous system[12] and is known to interact with brain ankyrins.[13,14] In sagittal sections of the brains of the E18 rat fetus, the staining of 440 kD ankyrin$_B$ was quite similar to that of L1 and both complementary to that of MAP2 (Fig. 2 C).

In primary cerebellar cells cultured for 9 days *in vitro*, however, the staining of 440 kD ankyrin$_B$ was confined to the axons and was

totally different from that of MAP2, which was localized in the dendrites and cell bodies of neurons (Fig. 2 A). In contrast to 440 kD ankyrin$_B$, the staining of 220 kD ankyrin$_B$ and L1 was similar to that of MAP2 (Fig. 2 B and Fig. 2 D, respectively).

The membrane binding domain of brain ankyrin can bind to the cytoplasmic domains of the neurofascin/L1/NrCAM family of cell adhesion molecules in the nervous system.[13,14] It is possible that the cytoplasmic domains of integral membrane proteins like L1 are involved in the differential localization of the two brain ankyrin isoforms, or *vice versa*. In such cases, a modifying role of the inserted domain of 440 kD ankyrin$_B$ must be essential for the differential sorting, because the two brain ankyrin isoforms share the same membrane binding domain. It is, therefore, likely that ankyrin$_B$ isoforms are not solely responsible for the localization of L1 and additional or independent factor(s) interacting with the brain ankyrin isoforms or L1 may be involved in the localization of L1. Furthermore, in primary cerebellar neurons, their environment is totally different from that *in vivo* and very little cell-cell interaction involving L1 is expected. This may be one of the reasons for the differente localization of L1 in the brain and cultured neurons.

Selective Down-Regulation of 440 kD Ankyrin$_B$ Associated with Neurite Retraction Induced by Methylmercury[15]

440 kD and 220 kD ankyrin$_B$ are both expressed in human neuroblastoma NB-1 cells and the expression of the larger isoform is increased upon induction of neurite outgrowth.[7] Exposure to methylmercury, a potent neurotoxic compound, at a sublethal dose, induced dramatic retraction of neurites in NB-1 cells. Concomitantly, the polypeptide and mRNA of 440 kD ankyrin$_B$ were selectively attenuated in methylmercury treated cells, while those of the 220 kD isoform were unaffected. These results indicate that the expression of 440 kD ankyrin$_B$ is intimately associated not only with neurite outgrowth but also with neurite retraction in neuronal cells, and is regulated at the mRNA level. In addition, methylmercury can specifically induce perturbation of neuritogenesis without affecting cell viability, which may account for its higher toxicity to developing nervous systems.

It is still unclear whether the increased expression of 440 kD ankyrin$_B$ is essential for the outgrowth and maintenance of neurites in neuronal cells. Only specific inhibition of the expression of this isoform will answer this question, and this may be helped by the use of antisense oligonucleotide strategies.

In conclusion, our results show that the expression of 440 kD ankyrin$_B$ is intimately associated not only with neurite outgrowth but also with neurite retraction in neuronal cells, and is regulated at the mRNA level. Therefore, 440 kD ankyrin$_B$ is a specific and useful marker for neuritogenesis and is also a useful tool for investigating the effects of neurotoxic substances like methylmercury on the developing nervous system.

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