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Immunofluorescent Localization of Three Na,K-ATPase Isozymes in the Rat Central Nervous System: Both Neurons and Glia Can Express More Than One Na,K-ATPase

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In the CNS, there are multiple isozymes of the sodium and potassium ion-stimulated adenosine triphosphatase (Na,K-ATPase) that have differences in affinity for Na^+, ATP, and cardiac glycosides. Three forms of the catalytic subunit (designated $\alpha1$, $\alpha2$, and $\alpha3$) are known to be derived from different genes, but little is known of the cellular distributions of the proteins or their physiological roles. Isozyme-specific monoclonal antibodies permitted the immunofluorescent localization of the 3 Na,K-ATPases in the rat CNS, and markedly different patterns of staining were seen. All 3 isozymes were detected, singly or in combination, in 1 or more neuronal structures, while both $\alpha1$ and $\alpha2$ were detected in glia. Many different neuroanatomic structures or cell types stained for more than 1 isozyme. Even when a structure or region stained for more than 1 isozyme, the pattern of staining was frequently dissimilar, suggesting complex differences in gene expression and cellular localization.

The sodium and potassium ion-stimulated adenosine triphosphatase (Na,K-ATPase) is a membrane-embedded enzyme responsible for the active transport of Na^+ and K^+ ions in most animal cells. The enzyme is present in high concentrations in neurons, where it maintains the gradients of Na^+ and K^+ necessary for nerve impulse conduction, and in glial cells, where ion gradients are used for K^+ buffering and neurotransmitter uptake (reviewed in Stahl, 1986). In addition, the Na,K-ATPase has the potential to play a role in neuronal signaling: it may modulate synaptic transmission by hyperpolarizing nerve membranes, and it may indirectly regulate the Na^+-dependent carriers for neurotransmitters.

Early work in this laboratory established the presence of 2 distinct isozymes of the Na,K-ATPase in the CNS, which were designated "α" and "$\alpha(+)$" (Sweadner, 1979). The isozymes demonstrated a number of differences, the most remarkable of which was the greater sensitivity of $\alpha(+)$ to cardiac glycoside inhibition as compared to that of α in the rat (Sweadner, 1979). The separated α and $\alpha(+)$ forms had differences in affinity for ATP and Na^+ (Sweadner, 1985) and were shown to have different sensitivities to regulation by insulin in adipocytes (Lytton et al., 1985) through an effect on apparent affinity for Na^+ (Lytton, 1985).

The isolation and characterization of nucleic acid sequences subsequently established that there are actually at least 3 isozymes of the Na,K-ATPase, which have been designated $\alpha1$, $\alpha2$, and $\alpha3$ (Shull et al., 1986; reviewed in Sweadner, 1989). Immunoblot and biochemical analyses suggest that "α" and $\alpha1$ are the same isozyme (Felsenfeld and Sweadner, 1988), and that the previously designated "$\alpha(+)$" actually comprises 2 distinct isozymes, $\alpha2$ and $\alpha3$ (Schneider et al., 1988; Urayama and Sweadner, 1988; Hsu and Guidotti, 1989; Urayama et al., 1989).

Messenger RNA for all 3 isozymes has been detected in the CNS (reviewed in Sweadner, 1989). It is therefore of great interest to determine how the isozymes are distributed between different cells and between different parts of cells in the CNS. Monoclonal antibodies have been developed that specifically recognize each of these 3 Na,K-ATPase isozymes (Felsenfeld and Sweadner, 1988; Urayama and Sweadner, 1988; Urayama et al., 1989). Initial immunofluorescent experiments in the kidney and retina indicated that all 3 monoclonal antibodies could be utilized to localize the Na,K-ATPase isozymes (Felsenfeld and Sweadner, 1988; McGrail and Sweadner, 1989). It was anticipated that these antibodies would allow determination of the distribution of the isozymes of the Na,K-ATPase in the CNS with greater specificity and resolution than was previously possible.

Materials and Methods

Antibodies. IgG_1 monoclonal antibodies specific for the $\alpha1$ (McK1) and $\alpha2$ (McB2) isozymes were used as hybridoma culture supernatants with appropriate dilutions as detailed below. The monoclonal antibody specific for the $\alpha3$ isozyme (McBX3), an IgM antibody of lower affinity than those specific for $\alpha1$ and $\alpha2$, was concentrated 7-fold by low-ionic-strength dialysis prior to its use in immunostaining experiments. The specificity of these antibodies has been determined by fine-specificity mapping and protein fragmentation and has been described in previous publications (Felsenfeld and Sweadner, 1988; Urayama et al., 1989). A rabbit polyclonal anti-glial fibrillary acidic protein (anti-GFAP) antiserum (Dako Corporation, Carpinteria, CA) was used in double-label experiments, as well as a monoclonal antibody against a glial filament designated GFII, the generous gift of C. J. Barnstable, Yale University.

The monoclonal antibody specific for $\alpha3$ (McBX3) has been observed to cross-react weakly with $\alpha1$ in immunoblots of detergent-denatured enzyme (Urayama et al., 1989). To determine whether it is sufficiently specific for $\alpha3$ to be used as an immunofluorescence probe in fixed tissue, it was tested for its ability to stain a tissue known to be particularly

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rich in $\alpha1$, the kidney. Whereas the $\alpha1$-specific antibody (McK1) brilliantly stained appropriate kidney structures as previously reported (Felsenfeld and Sweadner, 1988), neither the $\alpha2$ nor the $\alpha3$ antibodies stained anything above control levels (data not shown). We presume that any cross-reactivity to fixed $\alpha1$ is of sufficiently low affinity that it is below the level of detection.

Polyclonal antibodies K2 and Ax2 were made in rabbits injected with purified kidney and axolemma Na,K-ATPases, respectively (Sweadner and Gilkeson, 1985). Kidney Na,K-ATPase is almost exclusively $\alpha1$, while axolemma Na,K-ATPase contains both $\alpha2$ and $\alpha3$ (Urayama and Sweadner, 1988; Hsu and Guidotti, 1989). Both of the antisera, however, contain cross-reactive as well as isoform-specific antibodies, and therefore it was necessary to preabsorb them prior to use for immunofluorescence. K2 was preabsorbed with axolemma membranes, and Ax2 was preabsorbed with kidney membranes, as previously described (McGrail and Sweadner, 1986). Both antisera also contain some antibodies directed against the Na,K-ATPase β subunit. While we expect that much of these would have been removed by the preabsorption, this could not be quantitatively verified and may have reduced the isoform specificity.

Tissue preparation. For immunofluorescence with monoclonal antibodies, adult Wistar rats were anesthetized and then perfused with 2.0 ml of heparinized saline solution followed by 300 ml of periodate-lysine-paraformaldehyde fixative over 20 min (McLean and Nakane, 1974). Following perfusion, the brain, spinal cord, and kidneys were harvested and placed in 25% sucrose with 0.1 M phosphate buffer (pH, 7.4) for 18–48 hr prior to sectioning. Cryostat sections (8–20 μM) were cut at −20°C and affixed to gelatin-coated slides for staining. For immunofluorescence with polyclonal antibodies, procedures were similar except that fixation was with 2–4% paraformaldehyde in 0.12 M phosphate buffer.

Immunofluorescence. Dilutions of all antibodies used in these experiments were in a solution of 0.3% Triton X-100 and 4% goat serum in 0.1 M phosphate buffer (pH, 7.2). The same solution was also used for the washes. Antibodies specific for the $\alpha1$ and $\alpha2$ isozymes were used at 1:5 dilutions, while the antibody specific for the $\alpha3$ isozyme was used at a 1:7.5 dilution. Anti-GFAP antiserum was used at a 1:200 dilution and was incubated simultaneously with the anti-Na,K-ATPase antibodies in the double-labeling experiments. The slides were incubated with these primary antibodies for 2 hr at room temperature followed by 3 10-min washes. The secondary antibody, goat anti-mouse (IgG, IgM, IgA; rhodamine conjugated) was then applied to the slides at a 1:75 dilution for 1 hr. In double-labeling experiments for Na,K-ATPase and GFAP, an additional second antibody, goat anti-rabbit IgG (fluorescein conjugated) was also applied to the slides. The fluorescent secondary antibodies were obtained from Cappel/Cooper Biomedical (Malvern, PA). The slides were then washed 3 times for 10 min and mounted in glycerol containing 15 mM NaCl and 1 mM Na_2PO_4 (pH, 8.0). Slides were examined and photographed with a Zeiss IM-35 fluorescence microscope.

Control slides were concurrently incubated with other monoclonal antibodies that recognized non-CNS antigens, or with secondary antibody alone. No spurious labeling was seen in any of the regions examined that would have interfered with the interpretation of Na,K-ATPase isoform-specific staining (data not shown). Faint, diffuse background fluorescence was barely at the limit of photographic reproduction with the exposure conditions used. In most regions, the distinctive differences in staining patterns provides further assurance that the staining cannot be an artifact due to the secondary antibody.

Results

Monoclonal and polyclonal antibodies compared: cerebellar cortex

The distribution of the Na,K-ATPase in the cerebellar cortex was assessed with monoclonal antibodies specific for the α isoforms and polyclonal antisera that contain isoform-specific antibodies.

The granule cell layer of the cerebellar cortex stained brightly with all 3 monoclonal antibodies, but with different cellular localizations. The $\alpha1$ antibody (Fig. 1*A*) ring stained the densely packed granule cells (single arrow). The granule cells do not have axonal boutons terminating on their somata, indicating that the ring staining observed is likely to be localized either to the membranes of the cell bodies or to closely apposed astrocytic processes. The $\alpha1$ antibody also deeply stained a structure that appears to be the cerebellar glomerulus (double arrow), which is a synaptic complex formed of a mossy fiber rosette, the dendritic terminals of many granule cells, the terminals of Golgi cell axons, and Golgi cell dendrites. The $\alpha2$ (Fig. 1*B*) and $\alpha3$ (Fig. 1*C*) antibodies outlined, but did not ring stain, the cell bodies of granule cells. Both antibodies appeared to be staining neuronal or glial processes within the granular layer. Although the pattern of staining in this area was different for $\alpha2$ and $\alpha3$, it was not possible to determine the particular cell processes labeled by each antibody. The large Purkinje cells of the cerebellar cortex stained intensely for $\alpha3$ (Fig. 1*C*). Bright staining for $\alpha3$ was also seen in the pericellular basket cell arborizations adjacent to the preaxonal region of the Purkinje cell (white arrow). The $\alpha1$ (Fig. 1*A*) and $\alpha2$ (Fig. 1*B*) antibodies did not label Purkinje cell somata and showed only the outlines of their cell bodies, suggesting this cell's exclusive expression of the $\alpha3$ isozyme. The molecular layer exhibited moderate staining with each of the anti-Na,K-ATPase antibodies. Staining for $\alpha1$ and $\alpha2$ was diffuse, and dark circular profiles, some of which may be Purkinje cell dendrites or stellate and basket cell bodies, were unstained. Staining for $\alpha3$ in the molecular layer showed faint vertical striations (Fig. 1*C*, single arrow), and in addition, most of the dark circular profiles were ring stained (double arrow).

The relatively diffuse nature of the staining for the $\alpha2$ isoform of the Na,K-ATPase in the granule cell layer suggests the staining of glia or of neuronal processes (Fig. 1*B*). When the cerebellar cortex was stained with anti-GFAP antibody, which labels the astrocyte cytoskeleton, the pattern of staining was different from that seen with any of the anti-Na,K-ATPase antibodies. The lack of coincident staining was confirmed in double-labeling experiments with each of the anti-Na,K-ATPase antibodies (shown only for the $\alpha2$ isozyme; Fig. 1*B,B'*).

Staining of the cerebellar cortex with polyclonal antibodies is shown in Figure 2. Both of the antisera used (K1 and Ax2) were raised against native Na,K-ATPase preparations; by Western blots, they appeared to be predominantly specific either for $\alpha1$ ("α") or for $\alpha3$ ("$\alpha(+)$") (Sweadner and Gilkeson, 1985). Whether either antiserum recognizes $\alpha2$ in fixed tissue is uncertain; Ax2 reacts weakly with $\alpha2$ on Western blots (not shown). Cross-reactive antibodies in each antiserum were reduced by preabsorption before use for immunocytochemistry. Antiserum K2 (Fig. 2*A,C*) recognizes $\alpha1$ (after preabsorption with axolemma membranes to remove antibodies cross-reactive with $\alpha2$, $\alpha3$, and β subunits), and antiserum Ax2 (Fig. 2*B,D*) recognizes $\alpha3$ (after preabsorption with kidney membranes to remove antibodies cross-reactive with $\alpha1$ and β; McGrail and Sweadner, 1989). Figure 2, *A–D*, shows the molecular layer, Purkinje cells, and granular layer of the cerebellar cortex. In *A* and *C*, the K2 antiserum stained in a pattern essentially indistinguishable from that of McK1: Purkinje cells were unstained; granule cells were ring stained (Fig. 2*C*, single arrow) and the glomeruli were stained brightly (double arrow). Fine processes extending vertically into the molecular layer were prominently stained (Fig. 2*A*, arrowhead), portions of which were also stained for $\alpha1$ in Figure 1*A*. The dendrites of the Purkinje cells were noticeably unstained and showed as negative outlines of their branching trees. The Ax2 antiserum (Fig. 2*B*) was never seen to outline these dendrites, even in comparably angled sections, leading to the suggestion that the dendrites themselves might be stained by this

antibody. In Figure 2*B* and *D*, the Ax2 antiserum ring stained the Purkinje cells and the basket cell arborization at their base (Fig. 2*D*, white arrow), and also stained processes coursing through the granule cell layer. The stain by Ax2 most closely resembled that seen with McBX3, the α3-specific monoclonal antibody, though it was not identical. The more visible staining of what appear to be axonal processes in the granule cell layer by Ax2 might be ascribed to the better fixation conditions tolerated by the polyclonal antibodies, or simply to a more favorable section angle.

Neurons in gray matter structures

Cortex

The somatosensory cortex stained with all 3 monoclonal antibodies (Fig. 3), though the localization of staining was different in each case. All 3 antibodies stained the neuropil. Staining for α1 was seen in vertically oriented fibers in the cortex (Fig. 3*A*, arrow), which were most prominent in layers II–V, but also extended into the deep white matter and corpus callosum. The α1 (Fig. 3*A*) and α2 (Fig. 3*B*) antibodies outlined, but did not stain, pyramidal cell bodies. The α3 antibody, in contrast, appeared to label the membranes of these large cortical neurons (Fig. 3*C*). The somata of these cells thus appear to express predominantly, or exclusively, the α3 isozyme. It is impossible to definitively establish at the light microscope level, however, whether the staining that appears to be localized to neuronal somata is not actually localized to axonal boutons that terminate on these somata.

Hippocampus

Using monoclonal antibodies, large pyramidal cell bodies demonstrated intense staining for α3 (Fig. 4*C*) in all regions of the hippocampus, indicating expression of high levels of this isozyme. Staining for α2 (Fig. 4*B*) also surrounded the pyramidal cells, but ring-staining of the somata was less prominent. Pyramidal cells showed no continuous labeling of the cell body for α1 (Fig. 4*A*). The pyramidal cells did show intense punctate labeling for α1 in the pyramidal cell layer (white arrow), however, which could be due either to synaptic endings or to accumulations of intracellular Na,K-ATPase destined for transport to the pyramidal cell dendrites. The stratum oriens stained brightly and diffusely for α2 and α3, while α1 staining did not exceed control levels. The stratum radiatum stained for all 3 isozymes, but with different intensities and patterns of localization. Staining for α1 was localized to ribbons that extended through the entire layer and appeared to be the dendrites of the pyramidal cells (Fig. 4*A*, asterisk). These structures were seen as negative outlines when the hippocampal sections were stained for the α2 and α3 isozymes, which appeared to be localized to structures between the pyramidal cell dendrites.

Ventral horn of the spinal cord

In Figure 5, the α-motoneurons (asterisk), gray matter, and white matter of the ventral horn of the spinal cord are shown. Most of the α1 labeling was localized to glial processes (Fig. 5*A*), which

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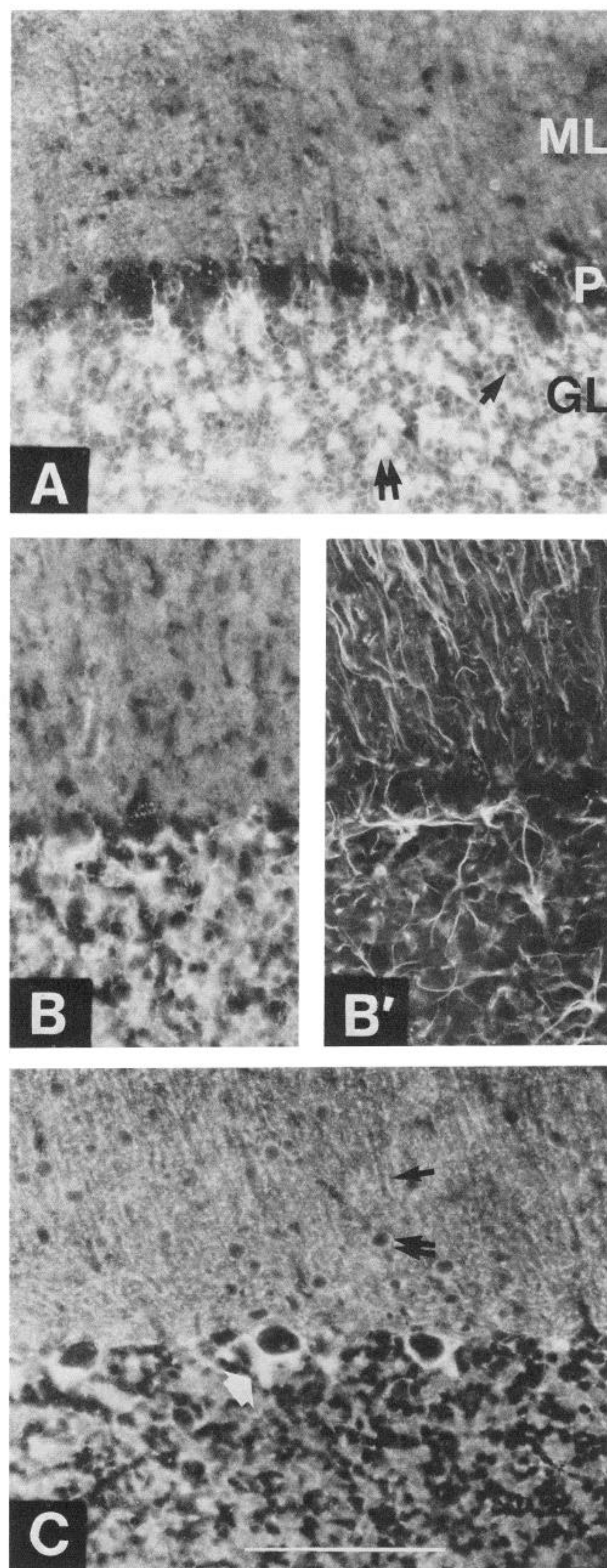
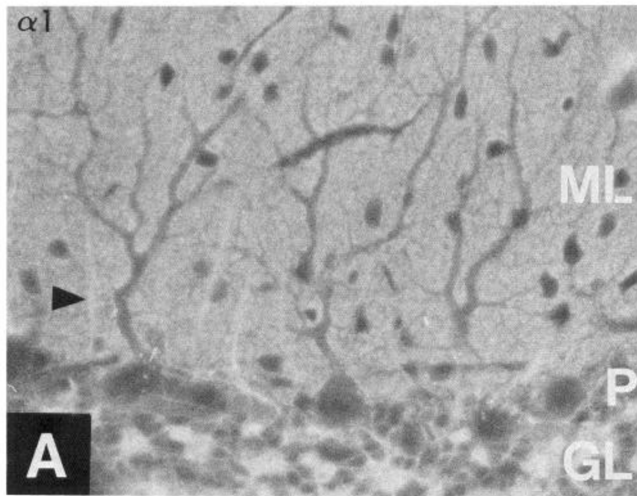
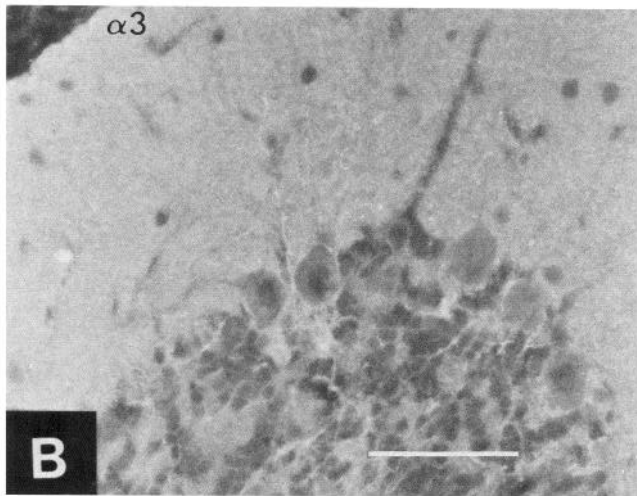
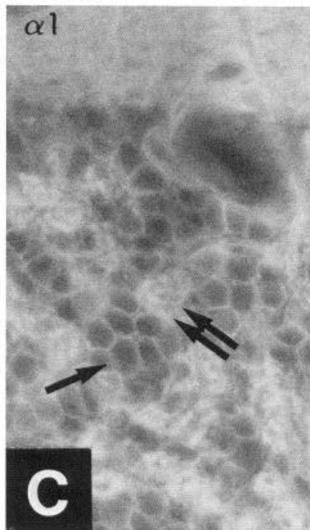


Figure 1. Cerebellar cortex stained with monoclonal antibodies against Na,K-ATPase. Cryostat sections, 6–10 μm thick, were stained with McK1 against α1 (*A*), double labeled with McB2 against α2 (*B*) and an antiserum against GFAP (*B'*), and stained with McB-X3 against α3 (*C*). *ML,* molecular layer; *P,* Purkinje cell layer; *GL,* granule cell layer. In *A,* the *single arrow* points to a granule cell, while the *double arrow* points to a glomerulus. In *C,* the *single arrow* points to vertical striations stained for α3, while the *double arrow* points to a ring-stained profile that is likely to be either a stellate cell body or a Purkinje cell dendrite. The *white arrow* points to a Purkinje cell and surrounding basket cell processes. Scale bar, 100 μm.







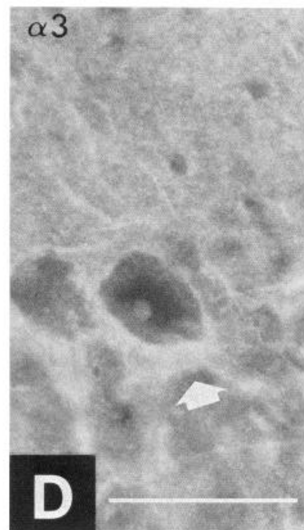
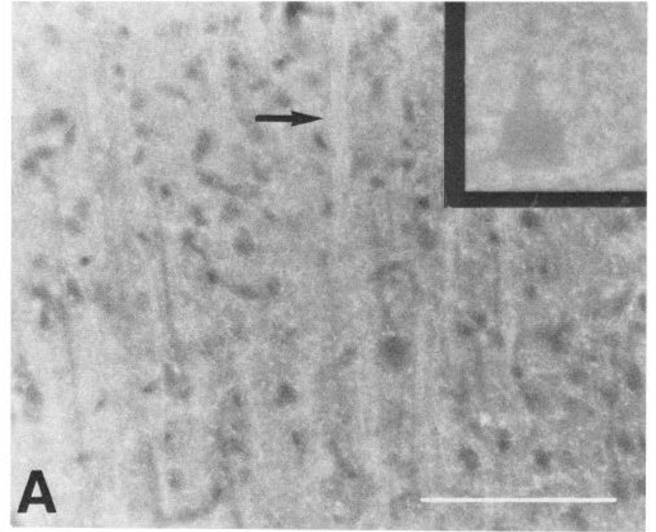
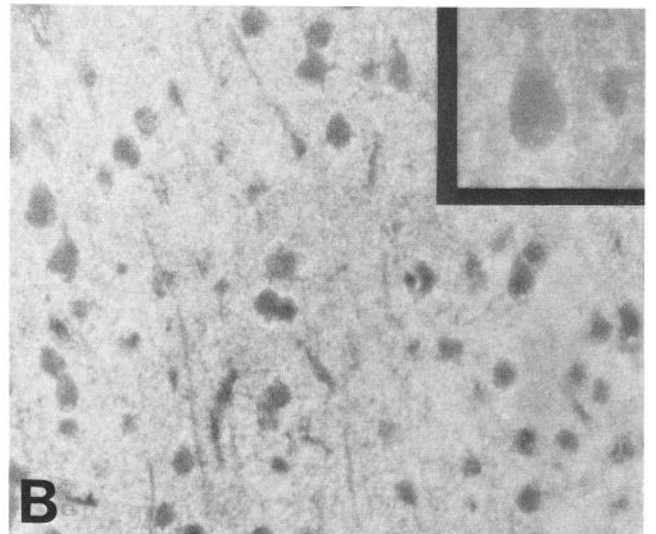


Figure 2. Cerebellar cortex stained with polyclonal anti-Na,K-ATPase antisera. Cryostat sections were stained with K2 antiserum (*A, C*), which stains $\alpha 1$, or Ax2 antiserum (*B, D*), which stains $\alpha 3$. *ML,* molecular layer; *P,* Purkinje cell layer; *GL,* granule cell layer. The *arrowhead* in *A* marks a stained vertical process in the molecular layer. The *single arrow* in *C* marks a granule cell, while the *double arrows* mark a glomerulus. The *white arrow* in *D* marks the basket cell arborization at the base of a Purkinje cell. Scale bars: 100 μm, *A* and *B*; 50 μm, *C* and *D*.





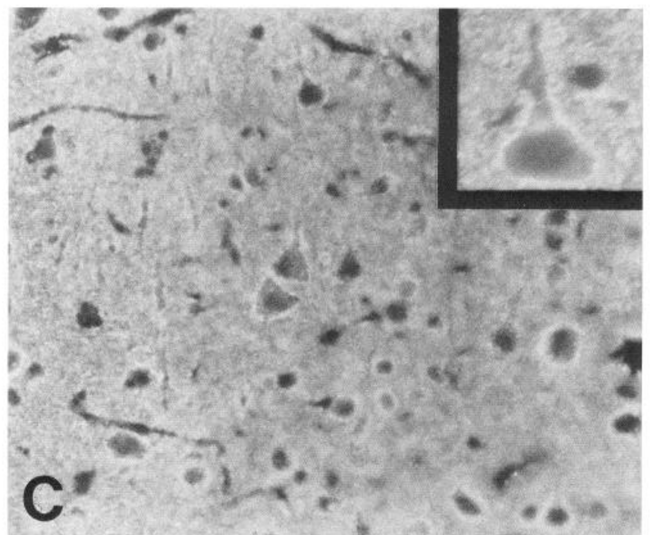


Figure 3. Somatosensory cortex stained with monoclonal antibodies against Na,K-ATPase. Cryostat sections were stained for $\alpha 1$ (*A*), $\alpha 2$ (*B*), or $\alpha 3$ (*C*). *Insets* are high-magnification views of neocortical pyramidal cells. Large-diameter, vertically oriented bundles of fibers stained exclusively for $\alpha 1$ (*A, arrow*). Scale bar, 100 μm.

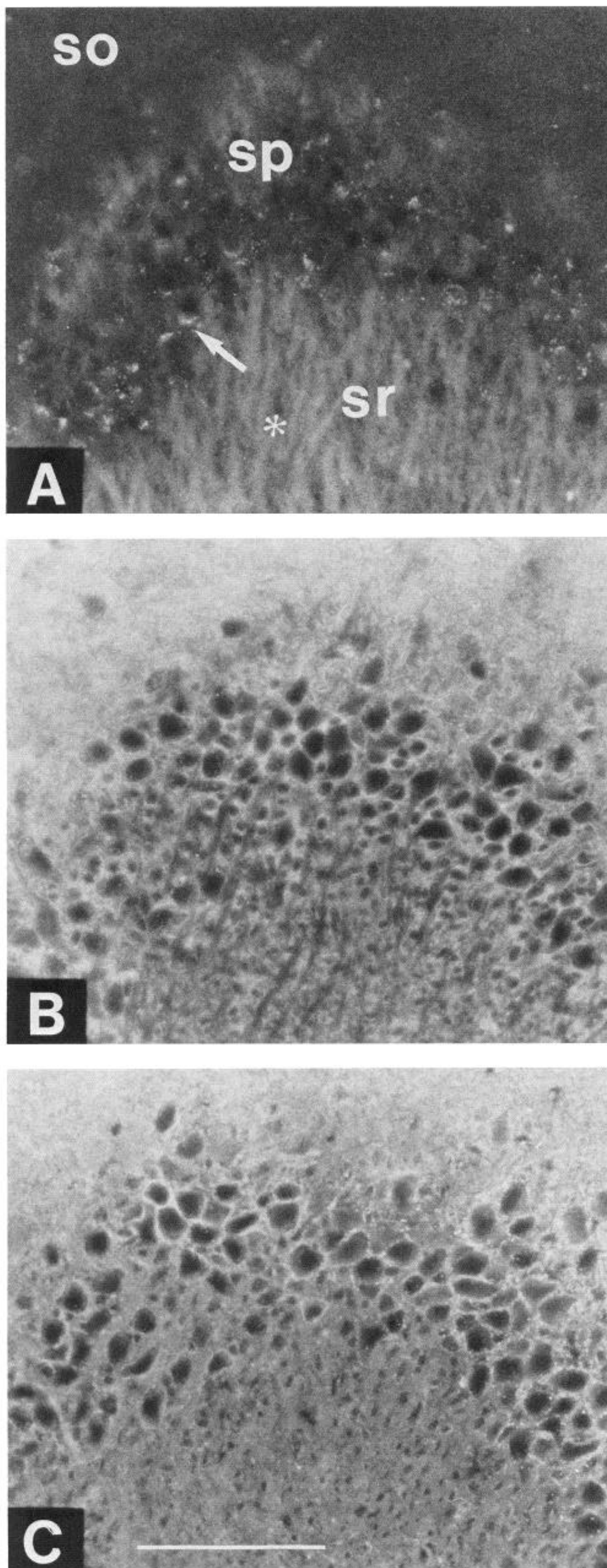


Figure 4. Hippocampus stained with monoclonal antibodies against Na,K-ATPase. Cryostat sections were stained for $\alpha 1$ (*A*); $\alpha 2$ (*B*); or $\alpha 3$ (*C*). *so,* stratum oriens; *sp,* stratum pyramidale, or pyramidal cell layer; *sr,* stratum radiatum. The dendrites of the pyramidal cells are marked with the *asterisk* in *A*; they are more visible as *negative outlines* in *B* and as *unstained profiles* cut obliquely in *C*. Punctate loci of stain for $\alpha 1$ around, or at the base of, the pyramidal cells are marked with the *arrow* in *A*. Scale bar, 100 μm.

also labeled for GFAP in double-labeling experiments (Fig. 5*A'*). The $\alpha 1$ antibody (Fig. 5*A*) did not label α-motoneurons and stained the gray matter at markedly lower levels than the other antibodies. Monoclonal antibody staining for $\alpha 2$ (Fig. 5*B*) and $\alpha 3$ (Fig. 5*C*) was present in the gray matter neuropil and ring stained the cell bodies of the large α-motoneurons. The gray matter in the dorsal horn was also labeled for $\alpha 2$ and $\alpha 3$ (not shown). Some of the $\alpha 2$ staining appeared to localize to GFAP-positive glial cell processes in the spinal cord gray matter in double-labeling experiments (Fig. 5*B'*), while the $\alpha 3$ staining in this region was localized strictly to α-motoneurons and the surrounding neuropil. These findings indicate that $\alpha 2$ and $\alpha 3$ are the predominant isozymes expressed by the neurons of the spinal cord gray matter, while astrocytes in this region appear to express the $\alpha 1$ and $\alpha 2$ isozymes.

White matter structures

Spinal cord

The lateral white matter of the spinal cord was selected for study because of the large-diameter axons present in this region. Cross-sections were made, in which axons should show as circular profiles. Staining for $\alpha 1$ (Fig. 6*A*) in the lateral white matter was only faint to moderate in intensity, while staining for $\alpha 2$ (Fig. 6*B*) was brighter. In the case of both $\alpha 1$ and $\alpha 2$, however, most of the staining was confined to GFAP-positive glial cell processes (Fig. 6*A'*,*B'*). Both large- and small-diameter axons cut in cross-section stained brightly with the $\alpha 3$ antibody (Fig. 6*C*), while no labeling of axons was seen with the $\alpha 1$ and $\alpha 2$ antibodies. The staining of both large- and small-diameter axons for the $\alpha 3$ isozyme was heterogeneous in its intensity. Myelin did not appear to be stained.

Corpus callosum

The corpus callosum stained brightly with both the $\alpha 1$ (Fig. 7*A*) and $\alpha 3$ (Fig. 7*C*) monoclonal antibodies, but the isozymes appeared to have different distributions. Tissue preservation and sectioning limited the resolution, but $\alpha 1$ staining was more diffuse, while $\alpha 3$ staining was more punctate. Staining for $\alpha 2$ was less intense (Fig. 7*B*) and was localized to glial processes and the membranes of small cell bodies that had multiple processes emanating from their circumference. These processes, and the small cell bodies, were stained by anti-GFAP antisera in double-labeling experiments, indicating that they are astrocytes (Fig. 7*B'*).

Pons

The pons is a complex brain-stem structure with many well-defined nuclei and tracts that we have not yet examined in detail. Figure 8 shows a selected region, rostral to the cerebellar peduncles, where white matter tracts in the dorsal and ventral tegmenta showed strikingly different isoform distributions. The $\alpha 1$ isoform (Fig. 8*A*) appeared to be localized in axons in the dorsal tegmentum, but staining for $\alpha 1$ was conspicuously absent in the ventral tegmentum, even at the ventral margin, which is shown in Figure 8. The $\alpha 2$ isoform (Fig. 8*B*) had different distributions in dorsal and ventral tegmenta, but in both cases, the distributions appeared more glial than neuronal. The $\alpha 3$ isoform

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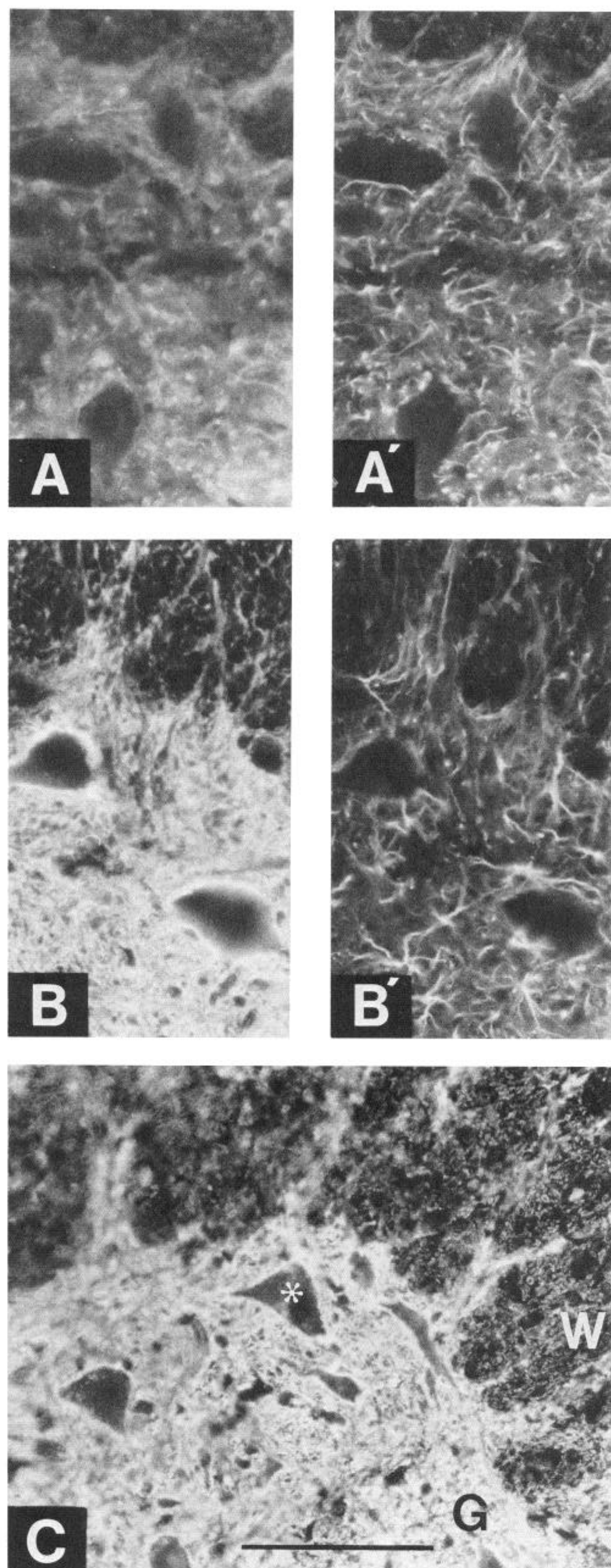

Figure 5. Ventral horn of spinal cord stained with monoclonal antibodies against Na,K-ATPase. Cryostat sections were double labeled for $\alpha1$ (*A*) and GFAP (*A′*), double labeled for $\alpha2$ (*B*) and GFAP (*B′*), or labeled for $\alpha3$ (*C*). In *C*, the *asterisk* marks an α-motoneuron, *W* marks white matter, and *G* marks gray matter. Scale bar, 100 μm.

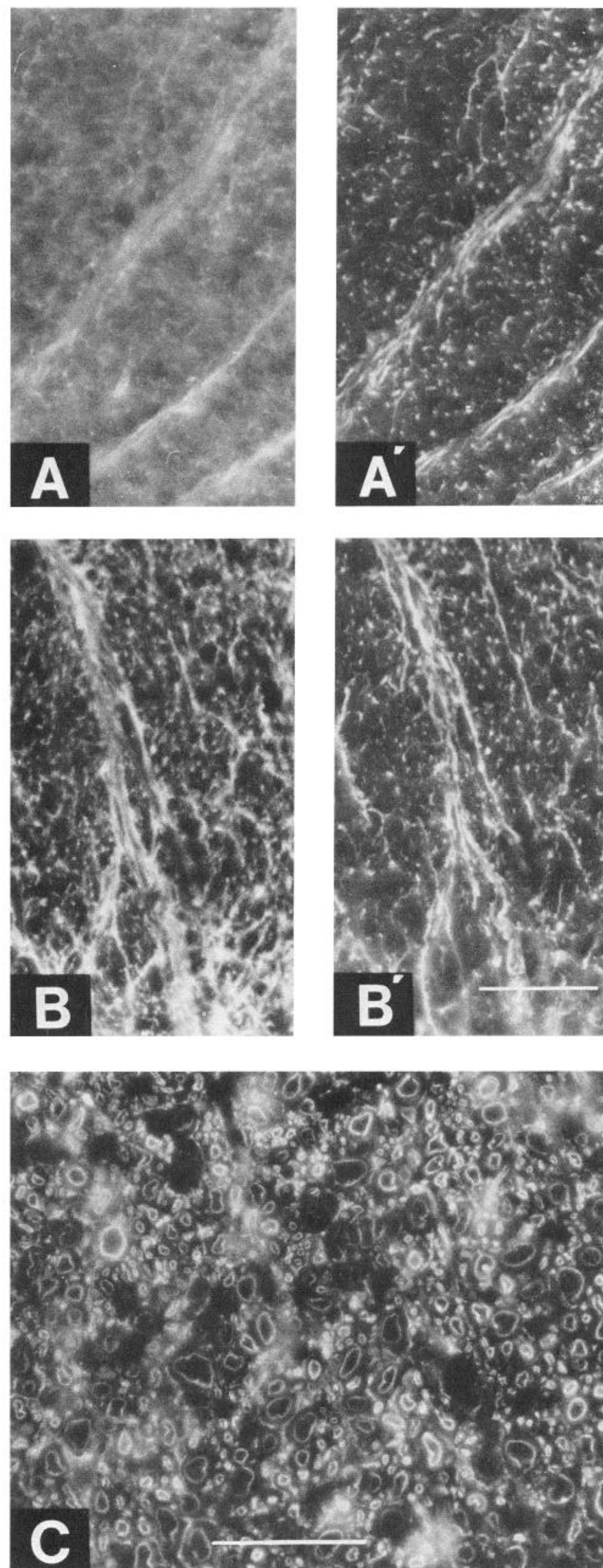

Figure 6. Lateral white matter of spinal cord stained with monoclonal antibodies against Na,K-ATPase. Cryostat cross-sections were double labeled for $\alpha1$ (*A*) and GFAP (*A′*), double labeled for $\alpha2$ (*B*) and GFAP (*B′*), or labeled for $\alpha3$ (*C*). It should be noted that all sections were cut at the same angle, making it very clear that the cellular distribution of the $\alpha3$ isoform was different from the other 2 isoforms. Scale bars, 50 μm.

(Fig. 8*C*) appeared to be in axons in both cases, but the axons in the ventral tegmentum stained much more brightly than those in the dorsal tegmentum, the inverse of the staining intensity seen with antibody to $\alpha 1$. Although we have not unambiguously identified the tracts shown, the figure makes a compelling case that isoform expression is cell specific, even within an ostensibly stereotypical structure such as the myelinated axon.

Cerebellar white matter

Cerebellar white matter was examined with polyclonal antibodies, where better tissue fixation made it possible to see more structural detail. The K2 antiserum against $\alpha 1$ did not stain axons in the white matter, but instead stained isolated cells with the morphology of fibrous astrocytes (Fig. 9*A*, arrow). Double-label experiments with a monoclonal antibody against GFII (a gift of C. J. Barnstable) confirmed that these are glia (Fig. 9*B*). Stained cerebellar granule cells and glomeruli can also be seen in Figure 9*A*, at the edge of the white matter. The axons stained by the Ax2 antiserum against $\alpha 3$ in Figure 9*C* originated in the Purkinje or granule cell layers and extended into the cerebellar white matter, which was brightly stained.

Discussion

Na,K-ATPase isoform expression differs markedly in different cells and tissues. Analyses of both mRNA expression (reviewed in Sweadner, 1989) and protein expression (Hsu and Guidotti, 1989; Shyjan and Levenson, 1989; Urayama et al., 1989) have indicated that most tissues express $\alpha 1$, while some (notably adipocytes and skeletal muscle) express $\alpha 1$ and $\alpha 2$. Substantial levels of $\alpha 3$ have been reported reproducibly so far only in the nervous system and heart, and recently in the pineal (Shyjan et al., 1990). *In situ* hybridization with antisense probes derived from the 3 α-subunit genes can (at high stringency) distinguish among the different isozymes and can provide valuable information about cellular specificity. *In situ* hybridization looks only at mRNA content and not at the expression and cellular localization of the protein, however, which is an especially important limitation in neurons where mRNA remains in the cell body, but the protein is transported over considerable distances. Detection of the individual proteins is essential. The results presented here demonstrate that the 3 isozymes of the Na,K-ATPase can be localized to different structures within the rat CNS and, in many cases, can be provisionally assigned to individual cell types.

The monoclonal antibodies used here are noteworthy in that they recognize both native and SDS-denatured enzyme (Felsenfeld and Sweadner, 1988; Urayama et al., 1989). This made it possible to verify their subunit- and isoform-specificity explicitly with the use of immunoblot techniques and specific protein fragmentation. With any monoclonal antibody, the possibility exists that a closely related determinant on a different protein might be recognized. Preabsorption does not control for this problem, because reactivity to other proteins would necessarily also be removed. The best control for specificity in this case is replication of the observations with other antibodies. We have attempted here to compare the labeling obtained with isoform-specific monoclonal antibodies with that obtained with isoform-specific polyclonal antibodies, with generally similar results.

The monoclonal antibodies have allowed localization of the Na,K-ATPase isozymes with a specificity and resolution not previously possible. In a previous study of the retina and optic nerve, we found that each isozyme had a distinct pattern of

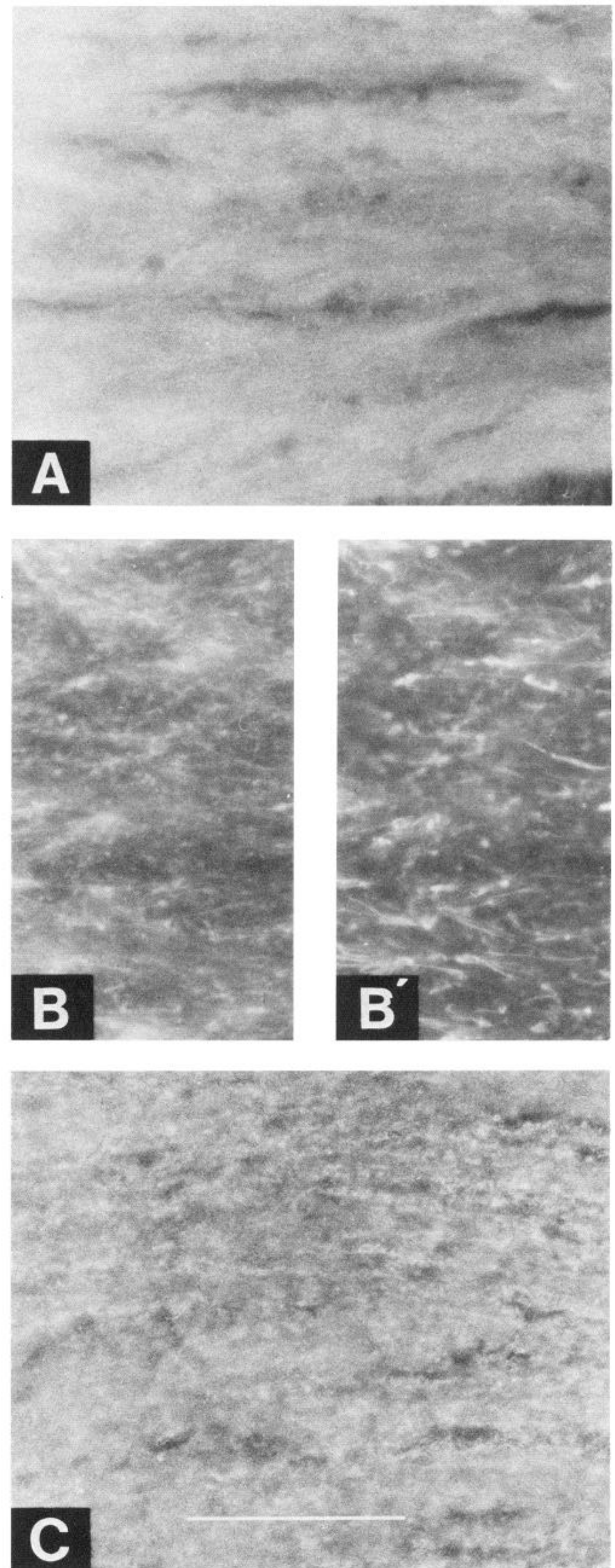

Figure 7. Corpus callosum stained with monoclonal antibodies against Na,K-ATPase. Longitudinal cryostat sections were stained for $\alpha 1$ (*A*), double labeled for $\alpha 2$ (*B*) and GFAP (*B′*), or stained for $\alpha 3$ (*C*). Scale bar, 50 μm.

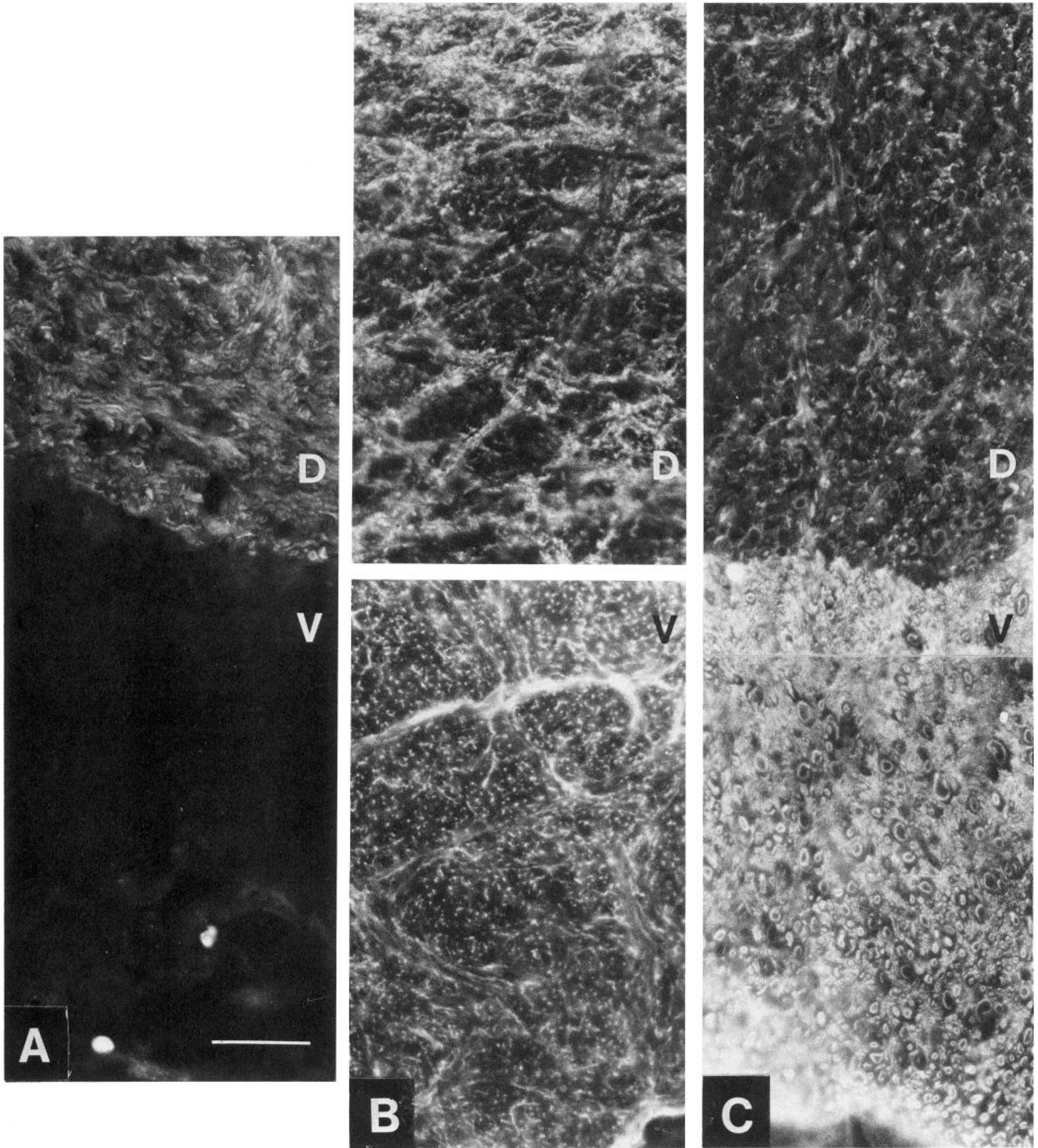

Figure 8. Pons stained with monoclonal antibodies against Na,K-ATPase. Cross-sections of white matter tracts in the pons were stained for $\alpha 1$ (*A*), $\alpha 2$ (*B*), and $\alpha 3$ (*C*). In *A* and *C*, the photographs included the margin between dorsal (*D*) and ventral (*V*) tegmentum; in *B*, 2 separate negatives were used, but were photographed from the same tissue preparation. Scale bar, 50 μm.

localization (McGrail and Sweadner, 1989). Photoreceptors and bipolar cells expressed predominantly $\alpha 3$, horizontal cells expressed high levels of $\alpha 1$ and $\alpha 3$, and the Müller glia expressed $\alpha 1$ and $\alpha 2$. In the retina, $\alpha 2$ expression was relatively low, but was seen particularly in a subclass of amacrine cells, in layers within the inner plexiform layer, and in association with blood vessels. In the optic nerve, $\alpha 3$ was expressed continuously in axons, not confined exclusively to the nodes of Ranvier. GFAP-positive astrocytes stained for both $\alpha 1$ and $\alpha 2$.

Two general conclusions resulted that refuted some old, overly simplified hypotheses: First, different kinds of neurons are capable of expressing any of the Na,K-ATPase isozymes, singly

or in combination, laying to rest the idea that neurons have one isozyme, and non-neuronal cells, another. Second, α2 was found in 2 types of glia: Müller cells and GFAP-positive astrocytes, contradicting our earlier conclusion that glia contained only α1 (Sweadner, 1979; Specht and Sweadner, 1984; McGrail and Sweadner, 1986). We now believe that the antiserum (Ax2) used in the earlier immunofluorescence study (McGrail and Sweadner, 1986) had good reactivity with α3, but poor reactivity with α2.

Localization of the Na,K-ATPase in neurons

The most noteworthy observation in the cerebral hemispheres and cerebellum was consistent, intense staining for the α3 isozyme of the Na,K-ATPase in the cell bodies of large neurons, including neocortical pyramidal cells and cerebellar Purkinje cells, which exclusively expressed α3, and in the pyramidal cells of the hippocampus, which expressed α2 and α3. None of these cells appeared to express significant levels of the α1 isozyme in their perikarya, though α1 staining was seen in dendrites in the hippocampal stratum radiatum. The large neurons all have extensive dendritic arborizations and large-diameter myelinated axons with distant projections. The α3 isozyme may possess determinants that facilitate its intracellular transport over extended distances. It is among the most rapidly transported proteins in optic nerve: Axonal transport of "α(+)" was reported in Specht and Sweadner (1984); the determination that α3, rather than α2, is the isoform in optic nerve fibers was reported in McGrail and Sweadner (1989).

A recent study by Schneider et al. (1988) used an antisense probe specific for α3 to detect the mRNA in the CNS by *in situ* hybridization. Most notably, they saw a selective localization of α3 mRNA in large-diameter cell bodies in the neocortex, hippocampus, and cerebellar cortex, which is in accordance with our results. Hieber et al. (1989) compared the *in situ* hybridization of a probe for α mRNA and immunolocalization of the polypeptide in the mouse cerebellum, retina, and kidney. The antisense probe was derived from the α1 sequence but cross-hybridized demonstrably with α2 and α3 mRNA under the stringency conditions used for hybridization. Heavy labeling was seen of the Purkinje cell bodies, but the result does not necessarily conflict with our finding because of the possibility that abundant α3 mRNA may have been detected by the probe. Filuk et al. (1989) reported *in situ* hybridization of the pyramidal cells of the hippocampus with probes for all 3 mRNA's; in this case, the probes were oligonucleotides designed to minimize the possibility of cross-hybridization. Brines et al. (1988) reported α1 mRNA hybridization in the pyramidal cell layer in the same region.

The demonstration of Na,K-ATPase localization (α3 isozyme) to the membranes of neuronal somata in the somatosensory cortex is in agreement with an immunocytochemical study in the knifefish brain, in which Na,K-ATPase staining was demonstrated in neuronal perikarya (Wood et al., 1977). The vertically oriented processes in the somatosensory cortex, which labeled exclusively for the α1 isozyme and are presumed to be axons or dendrites, appear similar to structures described by Stahl and Broderson (1976) using K^+-stimulated *p*-nitrophenylphosphatase (K^+-*p*NPPase) histochemistry. The patterns of staining seen with all 3 monoclonal antibodies in the cerebellum and hippocampus, taken together as composites, closely resemble those reported by Siegel et al. (1984) and Hieber et al. (1989) in the mouse cerebellum and by Sheedlo et al. (1989) in the rat

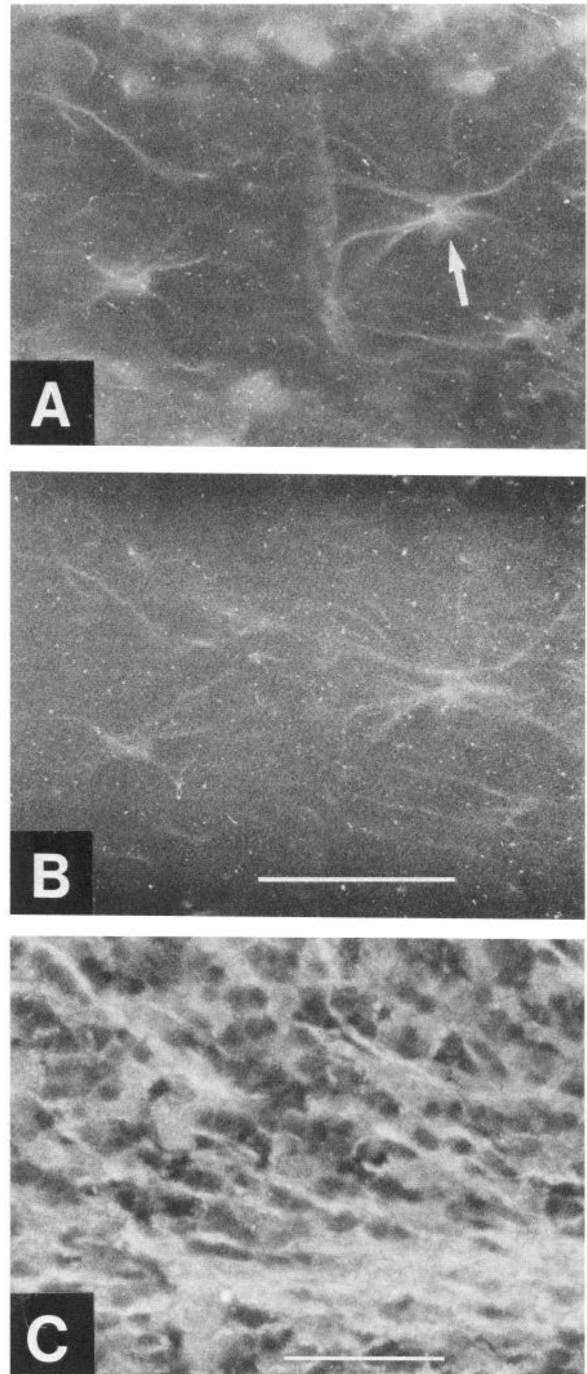

Figure 9. White matter of cerebellum stained with polyclonal antisera against Na,K-ATPase. Longitudinal cryostat sections of cerebellar white matter were double labeled with K2 antiserum against α1 (*A*) and a monoclonal antibody against GFII (a gift of C. J. Barnstable; *B*), or they were stained with Ax2 antiserum against α3 (*C*). The white matter in *C* is only the bright band of stain at the *lower right*; the rest of the picture consists of granule cell layer. Scale bar, 50 μm.

hippocampus, using anti-Na,K-ATPase antisera that did not distinguish among the isozymes.

Localization of the Na,K-ATPase in white matter and myelinated axons

In the spinal cord, the highest levels of the Na,K-ATPase were in the gray matter, with a marked predominance of the α2 and

$\alpha 3$ isozymes. Overall staining in the white matter was less than in the gray, though it was striking in its localization to axons, which stained specifically for $\alpha 3$. Many of these axons undoubtedly originated from neocortical pyramidal cell bodies, which also expressed this isozyme. Some axons did not label with any of the antibodies. It is likely that these axons expressed one of the isozymes of the Na,K-ATPase, but only focally or at levels not detectable by this technique. Our findings in the spinal cord are consistent with previous studies that demonstrated high concentrations of the Na,K-ATPase in the central gray matter, with relatively lower levels in the white matter (Guth and Albers, 1974; Ariyasu et al., 1985; Sheedlo and Siegel, 1987). One of these papers (Ariyasu et al., 1985) reported localization of the Na,K-ATPase to the membranes of spinal cord α-motoneurons as was seen for the $\alpha 2$ and $\alpha 3$ isozymes in this study. In the white matter, Guth and Albers (1974) detected faint K^+-*p*NPPase reactivity in the "axis cylinders" of the ascending and descending pathways. This faint staining resembles the bright staining observed here in the white matter for the $\alpha 3$ isozyme and attributed to axons.

In the corpus callosum, there was markedly brighter staining for $\alpha 1$ than in other myelinated tracts studied. Limitations in the resolution of light microscopy make it impossible to assign this stain to a particular cell type, and the antibody used (McK1) is relatively intolerant of harsher fixation conditions. The abundance of $\alpha 1$ stain there remains puzzling, but may reflect real differences in the isoform composition of the cells present.

Some prior investigators have concluded that the Na,K-ATPase is localized selectively to the nodes of Ranvier in axons from both the CNS and the PNS (Wood et al., 1977; Schwartz et al., 1981; Ariyasu et al., 1985). However, the bright staining of almost all axons in spinal cord cross-sections for the $\alpha 3$ isozyme implies that the Na,K-ATPase must be present in the internodal as well as the nodal regions of myelinated axons. We saw similar labeling for $\alpha 3$ in the optic nerve (McGrail and Sweadner, 1989) and in the lateral white matter of the spinal cord when stained with the Ax2 antiserum (data not shown). Internodal localization of the Na,K-ATPase isozyme has also been reported in other studies (Vorbrodt et al., 1982; Fambrough and Bayne, 1983). The discrepancies may be secondary to the varying sensitivities of the techniques employed. Our data do not rule out a quantitative concentration of Na,K-ATPase at the node, nor does it distinguish between axon plasma membrane and subaxolemmal membrane compartments.

Localization of the Na,K-ATPase in glial cells

It is notable that the $\alpha 3$ isoform has not yet been detected in glia. Processes in the spinal cord gray and white matter that were GFAP-positive stained for $\alpha 1$ and $\alpha 2$, indicating that astrocytes in this region expressed both isozymes, though it could not be determined if they were expressed in the same cell. In the corpus callosum, we were able to detect the presence of only the $\alpha 2$ isozyme in astrocytic processes, while those in the somatosensory cortex were not clearly labeled by any of the antibodies. The bright staining of axons in the corpus callosum for $\alpha 1$, and of the neuropil in the somatosensory cortex for all 3 isozymes, could have masked any astrocytic staining that was present, however. We did not see convincing colocalization of Na,K-ATPase and GFAP staining in any of the layers of the cerebellar cortex with light microscopy, but this may be because the velous morphology of the astrocytes puts the cell membrane at a greater physical distance from the cytoskeletal core than it is in the fibrous astrocytes of white matter. Ariyasu et al. (1985) reported immunostaining for the Na,K-ATPase in velous astrocytic processes located in the granular layer of the cerebellum using electron microscopy, but relatively poor preservation of the granule cell cytoplasm and plasma membrane made the assignment of stain there open to question.

New implications for Na,K-ATPase heterogeneity

The existence of subtly different isoforms of important enzymes has become an important problem in understanding neuronal function. In most cases, the isoforms are discovered as a consequence of molecular biology, and their physiological roles are unknown. A critical step in bridging the gap between knowing a sequence and understanding a protein's role is to be able to identify the protein and know where it is expressed. We have recently observed a major discrepancy between the distribution of the 3 Na,K-ATPase isoforms and their manifestation in a histochemical assay (K^+-*p*NPPase) that should, in principle, detect all of them: histochemical staining for $\alpha 3$ is not observed, implying that this isoform is inactive in some conditions *in situ* (Sweadner et al., 1990). It is hoped that such observations will lead to the discovery of regulatory mechanisms affecting ion transport in excitable cells.

In addition, there is another source of structural heterogeneity in the Na,K-ATPase. The α subunit of the Na,K-ATPase has always been found to be associated with a smaller glycoprotein subunit called β. This subunit is unusual for a transport protein in that it spans the membrane only once and has the majority of its mass exposed to the extracellular surface. Other members of the transport ATPase gene family can function without a β subunit. Recent evidence shows that there are at least 2 different β-subunit genes (Martin-Vasallo et al., 1989; Gloor et al., 1990). Remarkably, the second β-subunit gene was first isolated not as a subunit of the Na,K-ATPase, but as an adhesion protein involved in neuron–glial recognition, called adhesion molecule on glia (AMOG; Antonicek et al., 1987; Antonicek and Schachner, 1988; Pagliusi et al., 1989). In Gloor et al. (1990), it was shown that the AMOG ($\beta 2$) subunit was specifically associated with the $\alpha 2$ Na,K-ATPase isoform. Antibodies specific for each of the β-subunit types are not yet available, but should someday extend our understanding of the cellular distribution and roles of the Na,K-ATPase isoforms.

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