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The β1 subunit mRNA of the rat brain Na^+ channel is expressed in glial cells

(astrocyte/Schwann cell/ion channel)

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ABSTRACT **Although the molecular characteristics of glial Na^+ channels are not well understood, recent studies have shown the presence of mRNA for rat brain Na^+ channel α subunits in astrocytes and Schwann cells. In this study, we asked whether the mRNA for the rat brain Na^+ channel β1 subunit is expressed in glial cells. We performed *in situ* hybridization using a complementary RNA probe for the coding regions of the rat brain Na^+ channel β1 subunit mRNA and detected β1 subunit mRNA in cultured rat optic nerve astrocytes and sciatic nerve Schwann cells. The β1 subunit was amplified by reverse transcription–polymerase chain reaction in rat optic and sciatic nerves, which lack neuronal somata but contain astrocytes and Schwann cells, respectively. Doublet bands of the β1 subunit mRNA were amplified from both optic and sciatic nerves. Through the cloning and sequencing of these bands, we confirmed the amplification of a mRNA highly homologous to the previously cloned rat brain Na^+ channel β1 subunit ($\beta_{1.1}$) and a novel form of the β1 subunit mRNA ($\beta_{1.2}$), which is closely homologous to $\beta_{1.1}$ but contains an additional 86-nucleotide insert in 3′ noncoding regions. Two β1 subunit mRNAs were also amplified from rat brain and skeletal muscle, but not from rat liver or kidney. These results indicate that rat brain Na^+ channel β1 subunit mRNAs are expressed in glial cells as well as in neurons.**

Na^+ channels in excitable cells have been well characterized, and their primary amino acid sequences show that multiple channel subtypes are present in mammals (1–3). The physiological significance of the expression of multiple Na^+ channel subtypes is not yet understood, although it is known that their expression is regionally and developmentally regulated (1–5). Voltage-sensitive Na^+ channels are composed of subunits (α and β), but their compositions vary depending upon the tissue and species (1, 2). For example, Na^+ channels purified from chicken heart and eel electroplax contain only an α subunit (6, 7), whereas rat skeletal muscle and brain contain α and one or two β subunits (8, 9). Thus far, three distinct α subunits (termed subtypes I, II, and III) and one β subunit (β1) of Na^+ channels have been cloned from rat brain (2, 3). Some studies suggest the importance of the Na^+ channel β subunits in normal Na^+ channel function (10–12), but less is known about the biochemistry and molecular biology of the Na^+ channel β subunits than about the α subunit.

The presence of extraneuronal Na^+ channels in rabbit sciatic nerve was suggested on the basis of studies using radiolabeled saxitoxin (13). Following this suggestion, patch–clamp studies showed that cultured Schwann cells can express voltage-sensitive Na^+ currents with biophysical characteristics similar (although not identical) to those present in neuronal cells (14–16). The patch–clamp technique was also used to demonstrate the presence of voltage-sensitive Na^+ currents in astrocytes cultured from various regions of the central nervous system (CNS), including cerebrum (17), hippocampus (18), spinal cord (19, 20), and optic nerve (17, 21–24). Two distinct Na^+ currents have been recorded in astrocytes with different biophysical and pharmacological characteristics (17–21, 24). However, little information is available about the biochemical and molecular characteristics of the channels underlying these distinct Na^+ currents in glial cells.

Astrocytes and Schwann cells express rat brain Na^+ channel α subunits (refs. 25 and 26; Y.O. and S.G.W., unpublished data) and another type of Na^+ channel α subunit that was partially cloned (27). In the present study, we asked whether the rat brain Na^+ channel β1 subunit is expressed in astrocytes and Schwann cells by applying *in situ* hybridization and reverse transcription–polymerase chain reaction (RT-PCR) techniques. We demonstrate here that these glial cells express rat brain Na^+ channel β1 subunits both *in vitro* and *in vivo* and also demonstrate the presence of an isoform* of the rat brain Na^+ channel β1 subunit mRNA, which we refer to as $\beta_{1.2}$ using nomenclature similar to that for K^+ channels (28), together with a mRNA highly homologous to the originally cloned (12) β1 subunit mRNA, $\beta_{1.1}$.

MATERIALS AND METHODS

Cell Cultures. Optic nerve astrocytes and sciatic nerve Schwann cells were prepared from postnatal day 5–7 Sprague–Dawley rats. Tissue was minced and incubated in solution containing 30 units of papain per ml, Earle's salts, 0.5 mM EDTA, and 1.65 mM L-cysteine at 37°C followed by trituration in Dulbecco's modified Eagle's medium containing 10% (vol/vol) fetal bovine serum and penicillin/streptomycin (500 units/ml each) supplemented with trypsin inhibitor and bovine serum albumin (1.5 mg/ml each). Resuspended cells were plated onto 25-cm^2 culture flasks, maintained at 37°C for 3 days *in vitro* in 5% CO_2/95% air with a medium change on the second day. The cells were then dissociated with trypsin/EDTA and replated onto 24-well culture plates containing 12-mm untreated circular glass coverslips and maintained for an additional 3–4 days. After 6–7 days in culture, >95% of the cells from optic nerve were immunostained by anti-GFAP (glial fibrillary acidic protein) antibody (Boehringer Mannheim). Schwann cells were readily differentiated from contaminating fibroblasts by their characteristic bipolar spindle-shape morphology in culture (14, 15).

RT-PCR. Total cellular RNA was prepared, and RT-PCR was performed as described (25). In brief, RNA was extracted from postnatal day 7–14 Sprague–Dawley rat sciatic

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Abbreviation: RT-PCR, reverse transcription–polymerase chain reaction.

*The sequence reported in this paper has been deposited in the GenBank data base (accession no. L34417).

and optic nerves and from adult rat tissues (brain, skeletal muscle, liver, and kidney) with a single-step guanidinium thiocyanate/acid phenol/chloroform method (29). RT-PCR was performed by synthesizing cDNA from the prepared RNA with avian myeloblastosis virus (AMV) reverse transcriptase followed by PCR reaction in 1× PCR buffer (60 mM Tris·HCl, pH 9.5/15 mM $(NH_4)_2SO_4$/2.5 mM $MgCl_2$). cDNA was synthesized from the prepared RNA by either oligo(dT)- or random hexamer-priming methods; both methods yielded essentially the same RT-PCR results. The 5′ and 3′ primers for the rat brain Na^+ channel β1 subunit correspond to nucleotides 299–316 (numbered according to GenBank) and 1255–1272, respectively, to give a predicted PCR product of 974 bp. This amplified region contains most of the coding region and about two-thirds of the 3′ noncoding region. Each PCR cycle consisted of a denaturation step (94°C for 1 min), an annealing step (58°C for 2 min), and an elongation step (72°C for 3 min). DNA was amplified for 30–35 cycles in a programmable thermal controller (PTC-100, MJ Research, Cambridge, MA).

For controls, RNA samples were pretreated with RNase A for 30 min at 37°C and subjected to RT-PCR; in these controls, we did not observe any specific amplifications. We also amplified rat β-actin from the same RNA preparations used to amplify Na^+ channels. The use of β-actin primers (Clontech) gives predictably different sizes of PCR products depending upon whether the DNA is amplified from genomic DNA (1440 bp) or mRNA (764 bp) because of the presence of introns in the amplified regions. We amplified β-actin mRNA from all RNA samples in this study and observed only one size (764 bp) of PCR products amplified from mRNA, demonstrating that there was only a negligible amount of genomic DNA contamination in our RNA samples (data not shown).

Analysis of PCR Products. Besides noting the expected sizes of amplified PCR products, to validate them we performed (*i*) DNA sequencing and (*ii*) restriction mapping analysis with restriction enzymes. PCR products were cloned into either the TA vector (Invitrogen) or pCR-Script vector (Stratagene) according to the manufacturer's suggested protocols and were sequenced in the Keck Biotechnology Laboratory at Yale University. Restriction digestions were performed with restriction enzymes (New England Biolabs or Boehringer Mannheim) in the manufacturer's suggested buffer and temperature for 1 hr. PCR products used in enzyme digestion reactions were purified from the gel by using Gene-Clean II (Bio 101), and the digested PCR products were run on 3% agarose gel.

***In Situ* Hybridization.** Amplified Na^+ channel β1 subunits via RT-PCR were digested with *Alu* I, and a fragment of 334 bp corresponding to nucleotides 457–790 (coding regions of the Na^+ channel β1 subunit) was cloned into pCR-Script SK(+) cloning vector (Stratagene) and confirmed by DNA sequencing. From this cloned plasmid, digoxigenin-labeled sense or antisense single-strand complementary RNA probes were generated by using "Dig RNA labeling kit" (Boehringer Mannheim) after linealization of the plasmids with *Sac* II or *Hin*dIII, respectively. Unincorporated nucleotides were removed with Chroma Spin-100 column (Clontech). Specific staining of cultured glial cells was observed when antisense but not sense complementary RNA probes were used for *in situ* hybridization.

We used the protocol of Black *et al.* (26) for *in situ* hybridization with minor modifications. Cells were washed with phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde in 0.14 M Sorensen's buffer (pH 7.4) for 10 min at room temperature. The cells were then sequentially incubated in (*i*) PBS, three changes for 5 min each; (*ii*) prehybridization solution containing 50% formamide, 5× SSC (1× = 0.15 M NaCl/0.015 M sodium citrate, pH 7), 5× Denhardt's solution (1× = 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin), and 100 μg of salmon sperm DNA (Sigma) per ml for at least 1 hr at 58°C; and (*iii*) hybridization solution containing 50% formamide, 10% dextran sulfate, 5× SSC, 1× Denhardt's solution, 100 μg of salmon sperm per ml, and 0.25–0.5 ng of probe per μl overnight at 58°C. The complementary RNA probes were incubated for 10 min at 68°C before addition into hybridization solution. After hybridization, coverslips were sequentially incubated in (*i*) 4× SSC, two changes for 5 min each; (*ii*) 2× SSC, two changes for 5 min each; (*iii*) 10 mM Tris/500 mM NaCl, pH 7.5, containing RNase A (50 μg/ml; Boehringer Mannheim) for 1 hr at 37°C; (*iv*) 2× SSC, two changes for 5 min each; (*v*) 0.2× SSC, two changes for 20 min each at 58°C; (*vi*) 100 mM Tris/150 mM NaCl, pH 7.5, for 5 min; (*vii*) blocking solution containing 100 mM Tris/150 mM NaCl/2% (vol/vol) normal sheep serum/1% bovine serum albumin for at least 1 hr; (*viii*) anti-digoxigenin antibody conjugated to alkaline phosphatase (1:500–1000 in blocking solution; Boehringer Mannheim) for 2 hr; (*ix*) 100 mM Tris/150 mM NaCl, two changes for 5 min each; (*x*) 100 mM Tris/150 mM NaCl/50 mM $MgCl_2$, pH 9.5, three changes for 5 min each; and (*xi*) color substrate solution containing 420 μg of nitro blue tetrazolium (NBT) and 188 μg of 5-bromo-4-chloro-3-indolyl phosphate (X-phos) per ml in 100 mM Tris/150 mM NaCl/50 mM $MgCl_2$ overnight. The color reaction was stopped by several changes of 10 mM Tris/1 mM EDTA, pH 8.0, and coverslips were mounted on glass slides with aqua-poly/mount (Polysciences). Dark blue-purple precipitate, indicating the presence of the Na^+ channel β1 subunit mRNA, was revealed with bright-field microscopy (Leitz Aristoplan).

Control experiments performed to establish the specificity of the *in situ* hybridization protocol were (*i*) hybridization of cells in the absence of labeled probes and (*ii*) pretreatment of the cells with RNase A. We did not observe any specific labeling in these controls. The specificities of the digoxigenin-labeled probes were also tested on Northern blot analysis (see Fig. 2).

Northern Blot Analysis. Poly(A)$^+$ mRNAs were purified from total cellular RNAs prepared as described above from adult rat total brain and liver using Poly(A) Quick mRNA purification kit (Stratagene). Four micrograms of poly(A)$^+$ mRNA were electrophoresed through 1.2% agarose/2.2 M formaldehyde gel (equivalent loading of the mRNAs was checked by ethidium bromide staining), transferred to nylon membranes (Boehringer Mannheim), UV-cross-linked, probed with the digoxigenin-labeled antisense riboprobe, and detected with a chemiluminescent detection protocol (Boehringer Mannheim).

RESULTS

***In Situ* Hybridization.** Optic nerve astrocytes cultured from postnatal day 5–7 rats exhibit two morphologically distinct cell types (5, 21, 24): flat fibroblast-like astrocytes and process-bearing astrocytes. As shown in Fig. 1, astrocytes were hybridized with a digoxigenin-labeled antisense riboprobe made against the rat brain Na^+ channel β1 subunit mRNA. The hybridization signal, revealed by using a reaction with anti-digoxigenin antibody conjugated with alkaline phosphatase, was detectable in the cell body and processes of stellate astrocytes *in vitro*, indicating the presence of mRNA in both of these regions; similar observations on mRNA localization have been reported in cultured neurons (30). In general, the staining was more intense in the cell body than in the processes. We did not detect expression of the Na^+ channel β1 subunit mRNA in flat astrocytes *in vitro* (data not shown).

Fig. 1 also shows the expression of the Na^+ channel β1 subunit mRNA in cultured Schwann cells but not the sur-

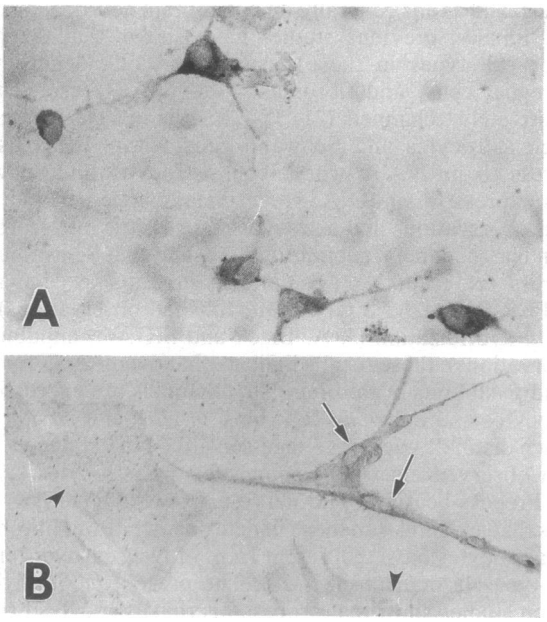

FIG. 1. *In situ* hybridization of the rat brain Na^+ channel $\beta 1$ subunit mRNA in optic nerve astrocytes (*A*) and sciatic nerve Schwann cells (*B*) *in vitro*. Note the staining of optic nerve astrocytes and Schwann cells (arrow) but not the surrounding fibroblasts (arrow heads). (*A*, ×415; *B*, ×225.)

rounding fibroblasts. Both cell bodies and bipolar processes of Schwann cells were stained. The specificity of the probes, demonstrated by the detection of hybridization signal in Schwann cells but not in contaminating fibroblasts, was further demonstrated on Northern hybridization studies. On Northern blot analysis, the antisense riboprobe used in the *in situ* hybridization was hybridized to about 1.5 kb of mRNA transcript in brain poly(A)$^+$ mRNAs but not in liver poly(A)$^+$ mRNAs (Fig. 2), consistent with a previous report (12).

RT-PCR. Fig. 3 shows the amplification of the rat brain Na^+ channel $\beta 1$ subunit mRNA from optic and sciatic nerves. These nerves lack neuronal cell bodies but contain astrocytes and Schwann cells, suggesting that these messages are expressed in glial cells *in vivo*. Treatment of the Na^+ channel $\beta 1$ subunit amplified from sciatic nerve RNAs with *Pvu* II or *Bst*YI predictably generated fragments of the expected sizes [(816 bp + 158 bp) or (631 bp + 216 bp + 127 bp), respectively] (Fig. 3 *Middle* and *Bottom*). Doublet bands of the $\beta 1$ subunit were consistently amplified from rat brain, sciatic nerve, and optic nerve RNAs (Fig. 3 *Top*), and both bands were digested by restriction enzymes in a similar pattern from

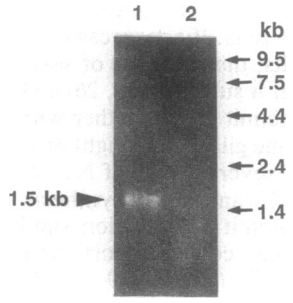

FIG. 2. Northern blot analysis of mRNA from rat brain (lane 1) and liver (lane 2). Four micrograms of poly(A)$^+$ RNA was electrophoresed through a 1.2% agarose/formaldehyde denaturing gel and transferred to Nylon membrane. The RNA was probed with the digoxigenin-labeled antisense riboprobe and detected by the chemiluminescent method.

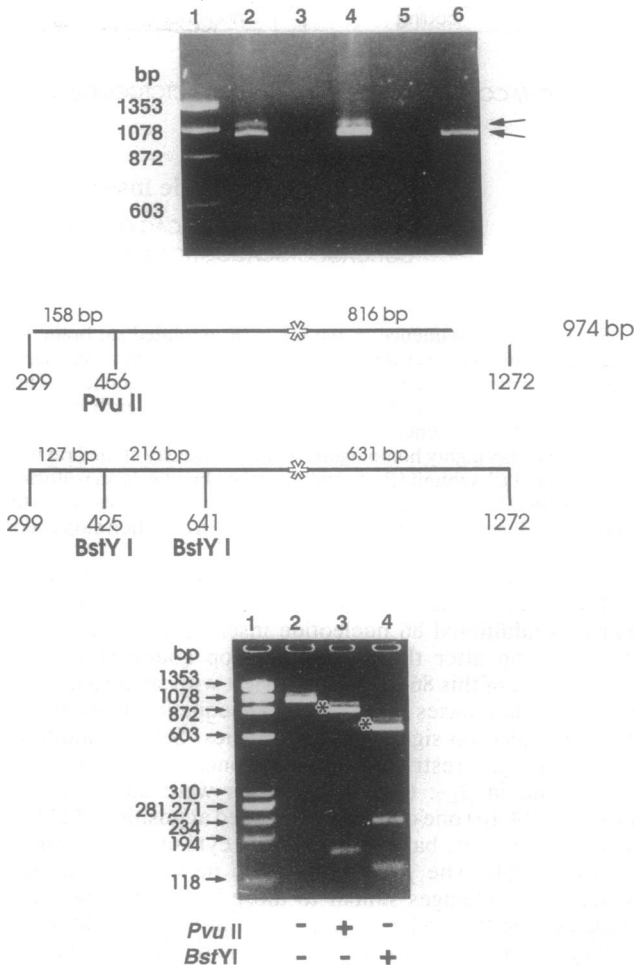

FIG. 3. RT-PCR amplification of the rat brain Na^+ channel $\beta 1$ subunit. (*Top*) The rat brain Na^+ channel $\beta 1$ subunit was amplified from rat brain (lane 2), kidney (lane 3), skeletal muscle (lane 4), liver (lane 5), and optic nerve (lane 6). Lane 1 contains ϕX174/*Hae* III digests. Note the amplification of doublet bands (arrows) of the Na^+ channel $\beta 1$ subunit. Samples were analyzed on a 2% agarose gel. The results show that the rat brain Na^+ channel $\beta 1$ subunit is expressed in both optic and sciatic nerves and in rat skeletal muscle. (*Middle*) Partial restriction map of the amplified PCR products ($\beta_{1.1}$) including *Pvu* II and *Bst*YI. Asterisks indicate the location of the 86-nucleotide insert in $\beta_{1.2}$ observed from DNA sequencing. (*Bottom*) Restriction mapping analysis of the amplified PCR product from sciatic nerve (analyzed on a 3% agarose gel). Lanes: 1, ϕX174/*Hae* III digests; 2, PCR products without enzyme treatment; 3 and 4, PCR products after treatment with *Pvu* II and *Bst*YI, respectively. Note doublet bands (asterisks) of the largest fragment after enzyme digestion, which are consistent with the location of an 86-nucleotide insert in $\beta_{1.2}$ observed from DNA sequencing.

all of these tissues (Fig. 3 *Bottom*), suggesting that alternative splicing may generate different sizes of rat brain Na^+ channel $\beta 1$ subunit mRNAs as demonstrated previously for the Na^+ channel α subunit (31–33). Doublet bands of the $\beta 1$ subunit were also amplified from rat skeletal muscle (Fig. 3 *Top*), indicating that the same Na^+ channel $\beta 1$ subunit is expressed in rat brain and skeletal muscle. The absence of amplification of the $\beta 1$ subunit from liver and kidney suggests that the rat brain Na^+ channel $\beta 1$ subunit is specifically expressed only in excitable tissues.

Three independent clones were isolated and sequenced from each of the doublet bands amplified from sciatic nerves with identical results. Sequencing of the doublet bands showed that the lower band was highly homologous to the previously cloned (12) rat brain Na^+ channel $\beta 1$ subunit ($\beta_{1.1}$)

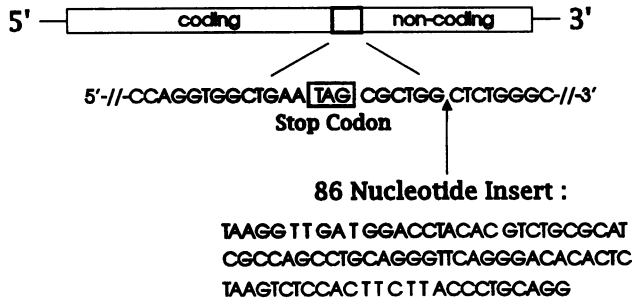

FIG. 4. DNA sequence of the RT-PCR-amplified rat brain Na^+ channel $\beta 1$ subunits. The doublet bands amplified from sciatic nerves were gel-purified, cloned into plasmid vectors, and sequenced. Three different clones were isolated and sequenced from each band; the resulting DNA sequences from these clones were identical. The lower band was highly homologous to the previously cloned rat brain Na^+ channel $\beta 1$ subunit ($\beta_{1.1}$), and the upper band was an isoform of the $\beta 1$ subunit mRNA ($\beta_{1.2}$), which had an additional 86-nucleotide insert in its 3′ noncoding regions 6 bp after the translation stop codon.

and that the upper band was another form of the $\beta 1$ subunit mRNA ($\beta_{1.2}$), which is closely homologous to $\beta_{1.1}$ but contains an additional 86 nucleotide insert in its 3′ noncoding region, 6 bp after the translation stop codon (Fig. 4). A comparison of this 86-nucleotide insert with sequences in the GenBank data bases using the GCG sequence analysis software revealed no significant homologies. Several additional subtle changes, restricted to the 3′ noncoding regions, were also found in $\beta_{1.2}$: (*i*) two cytosines were added at base position 914, (*ii*) one cytosine was added at positions 1220 and 1247, and (*iii*) one base changed from cytosine to thymine at position 1250. The $\beta_{1.1}$ from sciatic nerve also exhibited several base changes similar to those in $\beta_{1.2}$: (*i*) one nucleotide change from adenine to guanine at position 548, which will change the corresponding amino acid from asparagine to serine; (*ii*) one cytosine deletion at position 1187 in the 3′ noncoding region; and (*iii*) all of the changes noted above in $\beta_{1.2}$ except for the 86 nucleotide insert. Interestingly, these base changes observed in sciatic nerve $\beta_{1.1}$ and $\beta_{1.2}$ were concentrated in 3′ noncoding regions.

DISCUSSION

Rat brain Na^+ channels are known to be composed of three subunits: α, of 260 kDa; $\beta 1$, of 36 kDa; and $\beta 2$, of 32 kDa (2, 9). Expression studies have shown that the α subunit alone can encode functional voltage-sensitive Na^+ channels in oocytes and mammalian cell lines (2). However, the kinetic properties observed in these expression systems are slightly different from those in intact neurons in that Na^+ currents are inactivated rather slowly, and Na^+ current voltage-dependence is shifted to more positive membrane potentials. Isom *et al.* (12) recently cloned the rat brain Na^+ channel $\beta 1$ subunit (which we refer to as $\beta_{1.1}$) and coexpressed rat brain α and $\beta 1$ subunits in oocytes to show that these altered kinetics could be corrected and, further, that the level of Na^+ channel expression could be increased. Thus, the $\beta 1$ subunit may play a role in normal Na^+ channel activity in intact neurons.

Four lines of evidence indicate that the Na^+ channel messages amplified from optic and sciatic nerves are likely derived from astrocytes and Schwann cells, respectively. First, the presence of voltage-sensitive Na^+ channels in astrocytes from optic nerve and Schwann cells from sciatic nerve has been well documented electrophysiologically (14, 15, 17, 21, 24). Second, our *in situ* hybridization studies show the expression of the Na^+ channel $\beta 1$ mRNA in cultured astrocytes and Schwann cells. Third, we studied the intact optic and sciatic nerves, which do not contain neuronal cell bodies, and axons generally do not contain neuronal mRNAs (34). Finally, previous studies have shown that the other major cell types in these tissues—i.e., oligodendrocytes, microglial cells, and fibroblasts—do not express voltage-sensitive Na^+ channels (21, 35–37). It is not clear precisely which astrocytes and Schwann cells within these tissues express $\beta 1$ mRNA or whether all astrocytes and Schwann cells express this mRNA. Nevertheless, together with previous observations that astrocytes express the mRNA for rat brain Na^+ channel α subunits (25, 26) and that Schwann cells express rat brain Na^+ channel α subunits as shown by RT-PCR (Y.O. and S.G.W., unpublished observations) and *in situ* hybridization (J. A. Black and S.G.W., unpublished observations), these results indicate that the mRNAs for both α and β subunits of rat brain Na^+ channels are expressed in astrocytes and Schwann cells both *in vitro* and *in situ*.

Two distinct types of voltage-sensitive Na^+ channels have been observed electrophysiologically in astrocytes (17–21, 24). Process-bearing optic nerve astrocytes have Na^+ currents that are similar to those in neurons, while flat fibroblast-like astrocytes have a different form of Na^+ current, termed glial-type Na^+ current (17, 24). The present *in situ* hybridization studies show that process-bearing astrocytes express Na^+ channel $\beta 1$ subunit mRNAs in culture. Cultured Schwann cells, which also exhibit neuronal-type Na^+ currents (15, 38), are shown in this study to express Na^+ channel $\beta 1$ subunit mRNA. Therefore, it might be hypothesized that cells expressing the Na^+ channel $\beta 1$ subunit mRNA have neuronal-type Na^+ currents, while the cells without $\beta 1$ subunit mRNA expression express glial-type Na^+ currents.

The DNA sequence information from sciatic nerve $\beta_{1.1}$ includes several minor nucleotide changes (most of them concentrated in the 3′ noncoding region) that were detected compared to the previously cloned (12) rat brain $\beta_{1.1}$. It might be argued that these nucleotide changes reflect a cloning artifact or errors that might have occurred during DNA amplification by *Taq* polymerase and/or DNA sequencing. However, the 86-nucleotide insert in $\beta_{1.2}$ was consistently detected in RT-PCR amplification from various tissues (Fig. 3 *Top*); moreover, the RT-PCR and restriction mapping analysis (Fig. 3 *Bottom*) are consistent with respect to the location of the 86-nucleotide insert predicted from DNA sequencing (Fig. 4). Because the coding regions are essentially the same in these two isoforms, it seems reasonable to speculate that the differences between $\beta_{1.1}$ and $\beta_{1.2}$ may be involved in the regulation of mRNA processing. It has recently been reported that mRNAs having the same coding sequences with variable 3′ noncoding sequences can have different half-lives (39), suggesting the possibility that $\beta_{1.1}$ and $\beta_{1.2}$ may have different half-lives.

It is not yet clear whether other forms of Na^+ channel α and/or β (e.g., $\beta 2$) subunit mRNAs are expressed in astrocytes or Schwann cells. If coexpression of α and β subunits occurs in glial cells, the presence of several subtypes of rat brain Na^+ channel α subunits (25, 26) and of a putative glial Na^+ channel α subunit (27), together with a $\beta 1$ subunit that is expressed in some glial cells, might provide a substrate for the expression of several types of Na^+ current observed in astrocytes and Schwann cells. Synthesis of complete Na^+ channels, with subunit composition similar to that of Na^+ channels in neurons, could support the expression of Na^+ currents in glial cells (17, 19, 20, 24, 38) similar to those observed in neurons.

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