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The glial voltage-gated sodium channel: Cell- and tissue-specific mRNA expression

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ABSTRACT **Previous electrophysiological and pharmacological studies on central and peripheral glia revealed the presence of voltage-gated Na channels with properties that are similar but not identical to those of neuronal Na channels. Here we report the isolation and characterization of a cDNA encoding the C-terminal portion of a putative glial Na-channel (Na-G) α subunit. The amino acid sequence deduced from this cDNA indicates that the Na-G represents a separate molecular class within the mammalian Na-channel multigene family. By Northern blot, RNase protection, and *in situ* hybridization assays, we demonstrate that, in addition to brain astroglia, the Na-G mRNA is expressed in cultures of Schwann cells derived from dorsal root ganglia or from sciatic nerve. *In vivo*, the Na-G mRNA is detected not only in brain, dorsal root ganglia, and sciatic nerve, but also in tissues outside the nervous system including cardiac and skeletal muscle and lung. Its level varies according to the tissue and is developmentally regulated. The sequence and expression data concur in designating Na-G as an distinct type of Na channel, presumably with low sensitivity to tetrodotoxin.**

Voltage-gated Na channels are essential for voltage-dependent modulation of Na ion permeability, inherent to the generation and propagation of action potentials in nerve and muscle (1). They consist of a large (≈260 kDa) transmembrane glycoprotein—the α subunit (2)—that, in brain and skeletal muscle, is associated with smaller glycosylated β-subunit polypeptides (3, 4). cDNAs encoding the α subunits of four closely related rat brain Na-channel isotypes (I, II, III, and IIa) were cloned and characterized (5–7). Two Na-channel subtypes were identified in skeletal muscle and two were identified in cardiac muscle, of which one is common to both tissues (8–11).

Voltage-gated ionic channels are not restricted to excitable cells (reviewed in refs. 12 and 13) and evidence is available for their presence in both central and peripheral glia in culture (14–18) as well as *in vivo* (19, 20). The fundamental properties of the glial channels are quite similar to those found in central nervous system neurons. They are, however, not identical and differ in channel kinetics (13) and sensitivity to neurotoxins (12, 21). Particularly striking is their low sensitivity to the Na-channel blocker tetrodotoxin (TTX), and to a lesser extent to saxitoxin, observed in cortical astrocytes in culture (12, 15, 17) and Schwann cells *in vivo* (19). Thus, the question that arose was whether such differences have a structural basis or are the consequence of channel environment.

Probes specific for brain Na-channel isotypes were recently shown to hybridize to mRNA from cultured central neurons but not to mRNA from astroglial cells (ref. 22; unpublished data). Moreover, a "common" Na-channel probe (23) that, in brain and muscle, recognizes Na-channel mRNAs of 9 ± 0.5 kilobases (kb) reveals in astroglia an mRNA species of 7.5 kb (22). In the present study, we report the partial sequence of this 7.5-kb glial mRNA, show its homology to known voltage-gated Na channels, and describe its cell and tissue specificity.‡

MATERIALS AND METHODS

Cell Cultures. Astrocyte cultures were prepared from cerebral hemispheres of 1-day-old Wistar rats as described (17). Cells were harvested for RNA isolation at 2–8 weeks as secondary or tertiary cultures; >95% of the cells were defined as type 1 astroglia, according to criteria established by Raff *et al.* (24). The "contamination" by neuronal cells was rigorously excluded on the basis of morphology, lack of tetanus toxin binding, lack of staining with antibodies to neurofilament or γγ-enolase proteins, and by PCR analysis of mRNA (25). Schwann cell cultures were prepared from fetal or newborn rat dorsal root ganglia (DRG) or from newborn rat sciatic nerves as described (26). C6 is a rat brain glioma cell line (27); C6Bu1 is a bromodeoxyuridine-resistant mutant derived from C6 (28); BN1010 is a rat brain neurosecretory tumor cell line (27).

Isolation of Astroglial Na-Channel cDNA and Nucleotide Sequence Determination. A λgt10 cDNA library was constructed by random priming (29) with poly(A)$^+$ RNA from cultured rat astrocytes and a cDNA synthesis kit (Amersham). Screening of 10^5 recombinants at moderate stringency with a "common" Na-channel probe [nucleotides (nt) 4835–5142 from rat brain Na-channel II] (23) led to the isolation of 20 clones. The cDNA inserts were subcloned as overlapping restriction fragments in pSK$^-$ vector (Stratagene) and sequenced by the method of Sanger et al. (45) using Sequenase (United States Biochemical).

Surgical Denervation. Adult rats were anesthetized with ether, and the sciatic nerve was exposed on one side at the hip level and transected. After 4 weeks, the denervated and the control muscles (extensor digitorum longus and tibialis anterior) were dissected separately.

RNA Purification and Analysis. Total RNA was extracted from cultured cells or rat tissues by the method of Chirgwin et al. (46); poly(A)$^+$ RNA was isolated by oligo(dT)-cellulose chromatography. Northern blot analysis of total RNAs was carried out by a standard procedure (30): denaturation for 30 min at 50°C (1 M glyoxal/50% dimethyl sulfoxide/10 mM phosphate buffer), gel electrophoresis in 1% agarose, and transfer onto Hybond N$^+$ filters (Amersham). Hybridization conditions and ^{32}P-labeled single-stranded probe preparations were as described (31). The glial Na-channel (Na-G) probe consisted of a 370-nt fragment complementary to the 3′

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Abbreviations: TTX, tetrodotoxin; DRG, dorsal root ganglia; nt, nucleotide(s); cRNA, complementary RNA.

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‡The sequence reported in this paper has been deposited in the GenBank data base (accession no. M96578).

coding region adjacent to domain IV (G probe in Fig. 1). The amount of RNA per slot was monitored by hybridization of the blots with ^{32}P-labeled oligodeoxynucleotides specific for rRNAs (28S and 18S). Quantification was done by densitometer scanning of autoradiograms. RNase protection assays were conducted as in Sambrook *et al.* (30). The results shown were obtained with a 480-nt complementary RNA (cRNA) probe corresponding to the 3′ portion of the Na-G mRNA, spanning 434 nt in the coding region and 46 nt extending into the noncoding region (G′ probe in Fig. 1).

***In Situ* Hybridization.** Anti-sense Na-G ^{35}S-labeled cRNA (^{35}S-cRNA) probes, positioned identically to those used for RNase protection assays, were hybridized to cultured cells according to the procedure of Sassoon and coworkers (32). In each experiment, a corresponding "sense" RNA probe was included as a negative control. Pretreatment of twin coverslips with RNase A was also performed as a second type of control.

RESULTS

Partial Sequence of the Na-G cDNA. A rat astrocyte cDNA library was constructed and screened with a Na-channel common probe. The clones selected were found to contain a nucleotide sequence with an open reading frame encoding part of a protein remarkably similar in overall structure to the known Na-channel α subunits. This channel was designated Na-G. The deduced amino acid sequence of its C-terminal portion (Fig. 1) discloses a protein organized in hydrophobic (S1–S6) and hydrophilic segments, analogous to that of brain and muscle Na channels. Sequence alignment of the glial polypeptide with the fourth domain and the adjacent cytoplasmic region of rat brain (5) and muscle (8, 9, 11) Na-channel α subunits shows a striking homology in the region spanning S5–S6. Comparison of Na-G with brain channel II shows an overall homology of ≈55% but 91%, 76%, and 64% homology in segments S5, SS1 + SS2, and S6, respectively. Although the amino acid sequence of the S4 segment here is rather dissimilar (36%), it nevertheless shows a characteristic distribution of positively charged (basic) residues at every third position. The number of basic residues is, however, reduced (six compared to eight in channel II) and, in two positions, arginine is replaced by histidine.

Expression of Na-G Transcripts in Cultured Glial Cells. To explore the expression of the Na-G mRNA, specific probes were generated from the 3′-terminal portion of the corresponding cDNA indicated in Fig. 1. As in the case of cross-hybridization with the common Na-channel probe, Northern blot experiments with the Na-G probes revealed a 7.5-kb mRNA species in cultured astrocytes (Fig. 2*A*, lane 1). In addition, a minor band of ≈5 kb hybridized under stringent conditions; its relation to the main species remains to be explored.

With the aim of determining whether some or all of the astroglial cells in culture express the Na-G mRNA, *in situ* hybridization assays were performed with anti-sense ^{35}S-RNA probes. As illustrated in Fig. 2*B*, in confluent cultures, >90% of the cells display clear autoradiographic signals, with most silver grains circumscribed to the cytoplasmic area surrounding the nucleus. Cultured Schwann cells, whether from DRG or from sciatic nerve, express fairly high Na-G mRNA levels, as demonstrated by Northern blot (Fig. 2*A*, lane 2) and *in situ* hybridization assays (Fig. 2*C*). Fibroblasts, identifiable in Fig. 2*C* by their large nuclei, show only

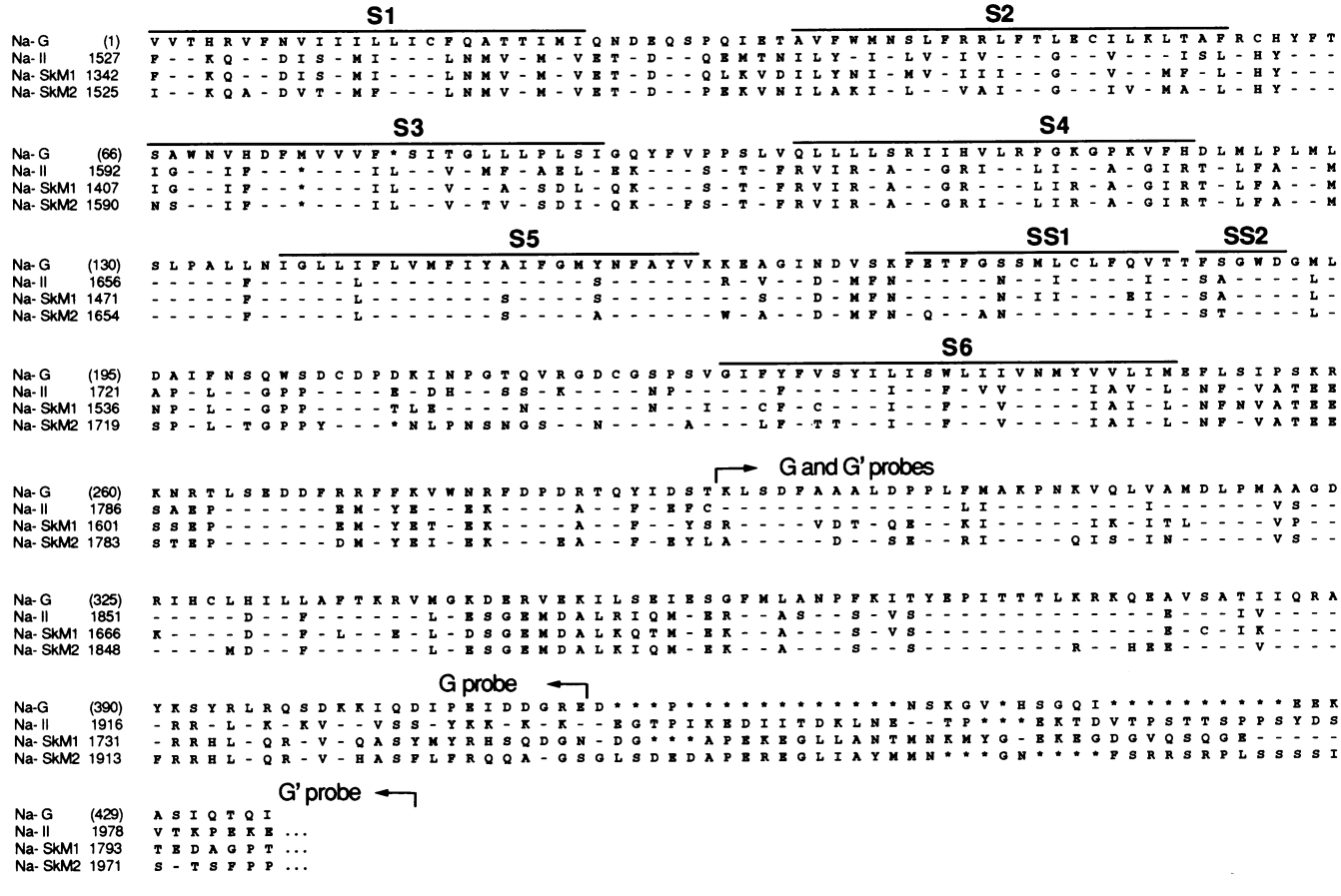

FIG. 1. Amino acid sequence comparison between Na-G, rat brain channel II (Na II), and two rat muscle channels (SKM1 and SKM2). The sequence shown encompasses channel domain IV and the adjacent C-terminal region. The presumptive transmembrane segments (S1–S6) and the position of the Na-G probes used in this study are indicated.

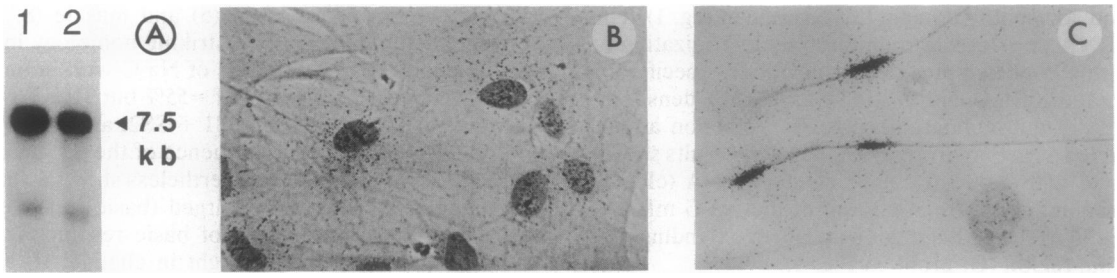

FIG. 2. (*A*) Northern blot hybridization of Na-G probe to cultured astroglial and Schwann cell RNA. Single-stranded [^{32}P]cDNA Na-G probe (probe G in Fig. 1) was hybridized to 5 μg of total RNA from cultured astrocytes (lane 1) or 1 μg of total RNA from sciatic nerve Schwann cell cultures (lane 2). Autoradiographic exposure was overnight. (*B* and *C*) *In situ* hybridization of Na-G probe to cultured glia. Rat cerebral hemisphere astroglia (*B*) and sciatic nerve Schwann cells (*C*) were hybridized with a ^{35}S-Na-G anti-sense RNA transcript (probe G′ in Fig. 1) as described. (×280.)

background levels of labeling and thus apparently lack Na-G mRNA.

Three rat brain tumor cell lines were examined: C6 and C6Bu1 gliomas and a neurosecretory tumor cell line BN1010. Only the latter two express low levels of Na channels, as previously shown by specific neurotoxin binding and ion-flux assays (refs. 27 and 28; unpublished results). RNase protection experiments illustrated in Fig. 3 (lanes 5–7) show that protection of the complete length of the Na-G cRNA fragments is obtained only with the RNAs of the two Na-channel-expressing cell lines.

Na-G Transcripts in the Rat Nervous System. The next step was aimed at determining whether Na-G mRNA is expressed in cerebral tissue *in vivo*. In Northern blot experiments with brain RNA preparations, a 7.5-kb hybridization signal could be detected upon prolonged exposure only. Thus, to increase the sensitivity of mRNA detection and, at the same time, to control the identity of the hybridizing sequence, RNase protection assays were carried out with cRNA probes. Results in Fig. 3 (lanes 1–4) show protection of the complete length of the Na-G cRNA fragments in hybrids with brain, astrocyte, and Schwann cell RNA preparations. Similar results were obtained in another series of RNase protection assays, performed with a different cRNA probe (630 nt from domain II)—namely, the size of the protected fragments was concordant with the size of the Na-G cRNA insert (data not shown). Thus, at least in the two regions examined, no alternative splicing of Na-G mRNA occurs in brain, astroglia, and Schwann cells. Taking into account the differences in the amounts of total RNA per assay and the intensity of the respective autoradiographic signals, it is obvious that the level of Na-G message in brain is quite low.

To explore the regional distribution of Na-G messenger, RNAs from several areas of the central and peripheral nervous systems were separately extracted and subjected to Northern blot analysis. The results in Fig. 4 demonstrate that Na-G mRNA is differentially expressed in the central nervous system: very low levels are present in cerebral hemispheres and cerebellum, moderate levels are found in midbrain and medulla–pons, and higher levels are found in spinal cord. In the latter, the Na-G mRNA is less abundant in the adult than in early postnatal tissue (lanes 10 and 11). Fairly high levels of Na-G transcripts are expressed in peripheral nervous tissue: a progressive accumulation can be observed in developing DRG (lanes 13–15) and significant amounts are found in the adult sciatic nerve (see Fig. 6, lane 4).

Na-G mRNA Outside the Nervous System. Whether the Na-G mRNA is restricted to the nervous system was investigated in Northern blots with total RNA extracts from several peripheral tissues. Na-G message is found in moderate amounts in skeletal muscle, spleen, and intestine (Fig. 5) as well as in uterus, pancreas, and adrenals (data not shown). It is not detected in liver and kidney. Surprisingly high amounts are found in lung and cardiac muscle. In all tissues where the Na-G mRNA was detected, its expression is developmentally regulated. With the exception of skeletal muscle, where the messenger is transiently expressed at an early postnatal stage, it tends to accumulate with age. In lung tissue, adult levels of the message are already present at birth.

Effect of Nerve Transection on Na-G mRNA Level in Muscle and Sciatic Nerve. To explore whether the expression of Na-G mRNA in skeletal muscle is dependent on innervation, RNAs were extracted from denervated (4 weeks) and from contralateral control thigh muscles and analyzed by Northern blotting. From the results shown in Fig. 6, it may be noted

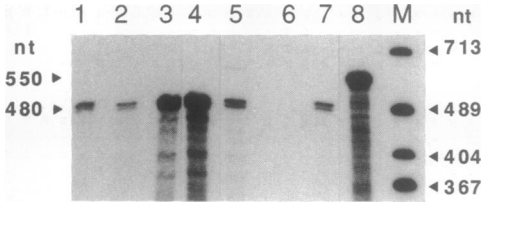

FIG. 3. RNase protection analysis of Na-G transcripts in rat brain tissue and brain-derived cell lines. A [^{32}P]cRNA Na-G probe (550 nt), consisting of 480 nt complementary to Na-G messenger (probe G′ in Fig. 1) and 70 nt complementary to the plasmid vector, was hybridized to total RNAs from the sources indicated and subjected to digestion by RNases A and T1. Lanes: 1, adult brain (100 μg); 2, postnatal day 1 brain (100 μg); 3, astroglial culture (2 μg); 4, embryonic day 16 DRG-derived Schwann cell culture (20 μg); 5, C6Bu1 glioma cells (20 μg); 6, C6 glioma cells (lacking Na channels) (20 μg); 7, BN1010 neurosecretory cells (20 μg); 8, undigested probe; M, molecular size markers. Exposure time, 2 days.

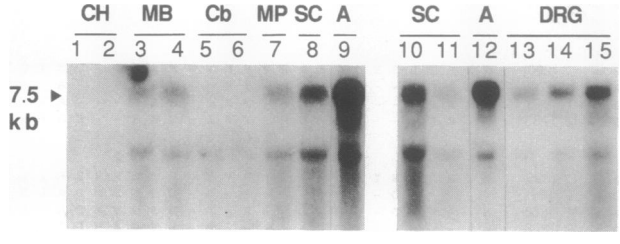

FIG. 4. Northern blot analysis of regional Na-G mRNA distribution in the rat nervous system. Data are from two separate experiments (lanes 1–9 and lanes 10–15) using total RNAs from the following sources: lanes 1 and 2, cerebral hemispheres [postnatal day 1 (PN1) and adult]; 3 and 4, midbrain (PN1 and adult); 5 and 6, cerebellum (PN1 and adult); 7, medulla–pons (adult); 8, spinal cord (adult); 10 and 11, spinal cord (PN1 and adult); 13–15, DRG (embryonic day 17, PN1, and adult); 9 and 12, cultured astrocytes. The amount of RNA deposited per slot was 2 μg in lanes 9 and 12, 5 μg in lanes 13–15, and 20 μg in all other lanes. Hybridization probe was as in Fig. 2*A*. Exposure times, 6 days for lanes 1–9 and 3 days for lanes 10–15.

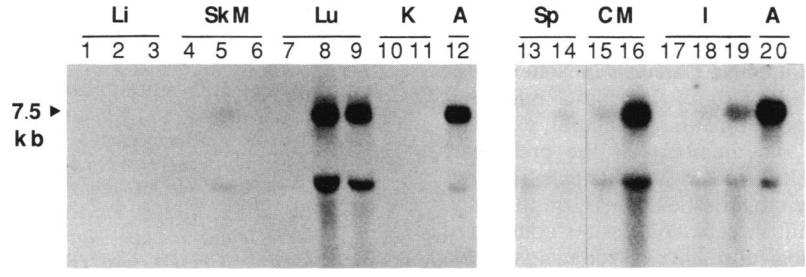

FIG. 5. Northern blot analysis of Na-G transcripts in rat nonnervous tissues. Data are from two separate experiments (lanes 1–12 and lanes 13–20). Total RNAs were from the following sources: lanes 1–3, liver [embryonic day 15 (E15), postnatal day 1 (PN1), and adult]; 4–6, skeletal muscle (E15, PN1, and adult); 7–9, lung (E15, PN1, and adult); 10 and 11, kidney (PN1 and adult); 12 and 20, cultured astrocytes; 13 and 14, spleen (PN1 and adult); 15 and 16, cardiac muscle (PN1 and adult); 17–19, intestine (E15, PN1, and adult). The amount of RNA deposited per slot was 10 μg in lanes 7–9, 2 μg in lanes 12 and 20, and 20 μg in all other lanes. Hybridization probe was as in Fig. 2*A*. Exposure time, 3 days.

that while Na-G mRNA is present in trace amounts in normal adult skeletal muscle, its level is notably increased in denervated muscle. In another set of experiments, the Na-G mRNA content was assayed in the normal and transected sciatic nerve itself. As shown in Fig. 6, an increase in Na-G mRNA level can be observed in extracts from the distal part of the sectioned nerve.

DISCUSSION

Search for the Glial Voltage-Gated Na Channel. A glial cDNA was isolated, found homologous to domain IV of Na channels from rat brain and muscle (5, 8, 9), and designated Na-G. An outstanding degree of homology between Na-G and the other Na channels is found in the sequence spanning segments S5–S6, including the short hydrophobic segments SS1 and SS2, postulated to be at or within the channel pore (33). Two residues in each of the four SS2 segments of brain channel II were recently shown to be important for the channel sensitivity to blocking by TTX and saxitoxin (34); only one is conserved in domain IV of the Na-G protein. This may have a bearing on the lower sensitivity to TTX of Na channels in mouse and rat astroglia (K_i in the micromolar range) (12, 15, 17) as compared to neurons (K_i in the nanomolar range) (1, 21). S4 segments of voltage-gated Na^+, K^+, and Ca^{2+} channels contain a periodic motif of positively charged residues at every third position, presumed to form the voltage sensor (5, 35). S4 in Na-G shows relatively little sequence homology with the corresponding segments of the previously identified Na channels; it nevertheless displays the typical motif of positively charged amino acids.

Although not correlated with excitability, the expression of Na-G mRNA is correlated with the presence of Na channels as shown for cultured astrocytes, Schwann cells, and cell lines C6Bu1 and BN1010. Normal rat fibroblasts and the C6 glioma are negative for both. Only a 7.5-kb mRNA is detected

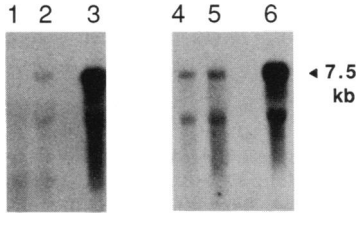

FIG. 6. Effect of denervation on Na-G expression in rat skeletal muscle and sciatic nerve. Data are from two separate Northern blot experiments (lanes 1–3 and lanes 4–6). Total RNAs were from the following sources: lane 1, normal adult skeletal muscle (20 μg); lane 2, denervated (4 weeks) skeletal muscle (20 μg); lanes 3 and 6, cultured astrocytes (5 μg); lane 4, normal sciatic nerve (2 μg), lane 5, distal stump of sectioned sciatic nerve (5 days) (2 μg). Hybridization probe was as in Fig. 2*A*. Exposure time, 4 days.

in astroglia and Schwann cells with either the common or the Na-G-specific probes, indicating strongly that little or none of the other known brain or muscle Na channels (mRNAs 9 ± 0.5 kb) are expressed in these cells. Since voltage-activated Na currents in cultured astrocytes are relatively insensitive to TTX, one may conclude that Na-G encodes a newly discovered TTX-insensitive Na-channel species. The definitive proof of its functional properties is yet to come.

Na-G mRNA Expression in the Central Nervous System and the Peripheral Nervous System. The low levels of Na-G mRNA found in brain contrast with the relatively high levels found in cultured astrocytes. As hypotheses to account for this discrepancy, one may evoke positive regulation of Na-G mRNA expression in culture or, alternatively, selection of an astroglial subpopulation enriched in Na-G but poorly represented in brain. Higher levels of Na-G mRNA are found in spinal cord than in any other region of the central nervous system. This observation is in agreement with previous studies at the protein (36, 37) and mRNA (23) levels, suggesting the presence in rat spinal cord of an unidentified Na-channel type in addition to those examined. Na-G protein is a likely candidate for that channel.

At this stage, it cannot be excluded that both Schwann cells and neurons contribute to the Na-G mRNA content in DRG. It is known that spinal ganglion neurons exhibit two types of Na channels—one is TTX-sensitive and the other is relatively insensitive (38). The former may correspond to neuronal type(s) of Na channel with 9.0-kb mRNAs (39) and the latter may correspond to the Na-G channel with 7.5-kb mRNA.

A notable level of Na-G transcripts was observed in the sciatic nerve. Since little, if any, of the sciatic nerve RNA can originate from the axons, it can be assumed that we are essentially looking at mRNA from Schwann cells. The increase in Na-G level observed in transected sciatic nerve agrees with data on transection-induced modulation of Na-channel content in sciatic nerve (40, 41). The relation of these changes to axon–Schwann cell interactions and/or to the myelination state of the latter remains to be explored.

Na Channels and Na-G mRNA in Peripheral Tissues. Earlier physiological studies and recent molecular studies on cardiac and skeletal muscle described both TTX-sensitive and TTX-resistant Na channels (42, 43). Their relative proportion varies during development and depends on innervation. The pattern of Na-G mRNA expression is similar to that of the TTX-resistant channels: it accumulates during cardiac ontogenesis, is transiently expressed in neonatal skeletal muscle, and is induced in adult muscle by denervation. It could thus correspond to an additional, maybe minor, TTX-resistant Na-channel form in these tissues.

The low levels of Na-G mRNA detected in some of the other peripheral tissues can possibly come from the parasympathetic and the enteric nervous systems. Finally, in the lung, Na-G may be associated with the neural crest-derived

"oat" cells. The precise localization of the Na-G mRNA and/or protein is bound to be most informative.

On the Role of Voltage-Gated Na Channels in Nonexcitable Cells. Data were presented to show that Na-G mRNA is found in a variety of tissues: neural and nonneural, excitable and nonexcitable. While the members of the previously characterized family of voltage-gated Na channels are preferentially expressed in excitable tissues, Na-G, on the other hand, is relatively abundant in cells and tissues considered nonexcitable. The physiological finality of voltage-gated Na channels in nonexcitable tissues remains to be determined. It was hypothesized that Schwann cells may serve as donors of Na channels to peripheral axons (16). A minimal prerequisite for that would be a demonstration that the Na channels in both are structurally the same. The arguments that (*i*) Na-G is apparently the only Na-channel mRNA species expressed in Schwann cells and (*ii*) Na currents in Schwann cells *in vivo* (19) are about 2 orders less sensitive to TTX than nodal Na currents (1) plead against it. More probable seems the implication of the Na-G-type Na channels in some general cellular functions such as the regulation of resting potential and ion homeostasis, possibly via regulation of Na,K-ATPase, whose activity is highly dependent on intracellular Na^+ concentration (44). Finally, by creating local depolarizations, the activation of small numbers of Na channels in nonexcitable cells may lead to activation of other voltage-sensitive ionic channels (e.g., K^+ and Ca^{2+} channels) and may thus be an early step in a cascade of metabolic reactions. The biological significance of the Na-G channel in its various cellular locations represents an interesting challenge for future studies.

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