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Sodium channel mRNA in the B104 neuroblastoma cell line

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Abstract B104 neuroblastoma cells are excitable, but the ion channels underlying electrogenesis in these cells have not been identified. RT-PCR, restriction enzyme analysis and in situ hybridization were used to study sodium channel mRNAs in B104 cells. High levels of sodium channel α-subunit mRNAs III, NaG and Na6 and β1-subunit mRNA were detected by RT-PCR in B104 cells. Low levels of types I and II α-subunit mRNAs were also present. In situ hybridization with subtype-specific riboprobes detected sodium channel α-subunit mRNAs III, NaG and Na6 and β1-subunit mRNA in B104 cells; analysis of the percentage of B104 cells expressing each α-subunit mRNA subtype suggests that some cells express the mRNAs for several α-subunits.

Key words: In situ hybridization; Neuroblastoma; mRNA; RT-PCR; Sodium channel

1. Introduction

A variety of neuroblastoma cell lines have been developed as model systems to examine mechanisms responsible for the acquisition of properties associated with neurons, including excitability and neurotransmitter synthesis and release. The B104 neuroblastoma cell line, generated from a rat CNS neoplasm, was initially described as exhibiting electrical excitability [1], suggesting the expression of voltage-sensitive sodium channels in these cells. More recently, Baines et al. [2] reported the presence of the mRNA of rat brain sodium channel α-subunit III [3] in B104 cells, but not rat brain types I or II/IIA [4,5]. These results suggested that B104 cells might provide a useful model system, expressing a single isoform of sodium channel α-subunit, in which sodium channel mRNA expression might be correlated with sodium current characteristics in a one-to-one manner.

Subsequent to the study of Baines et al. [2], additional sodium channel α-subunits have been described, including glial sodium channel (NaG) [6], mouse Nav2.3 [7], sodium channel 6 (Na6) [8], human neuroendocrine sodium channel (hNE-Na) [9] (rat PN1 [10,11] and rabbit Schwann cell NaS [12] have been suggested to be species correlates). Thus, it is unresolved whether B104 cells express other sodium channel α-subunits in addition to type III. In the present study, we have used RT-PCR and restriction enzyme mapping, together with in situ hybridization, to further study the expression of sodium channel α-subunit mRNA in B104 cells. In addition, these studies examined whether B104 cells express the mRNA for the β1-subunit of sodium channels (Naβ1). In this paper, we confirm the work of Baines et al. [2] that B104 cells express high levels of sodium channel III mRNA, and extend this work to demonstrate that sodium channel α-subunits NaG and Na6 and Naβ1 are also expressed at high levels in these cells.

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Abbreviations: RT-PCR, reverse transcription polymerase chain reaction.

2. Material and methods

2.1. Culture

Stocks of B104 cells [1] were obtained from D. Schubert (Salk Institute, San Diego) and grown in Dulbecco's modified Eagle's medium containing 20% fetal calf serum and penicillin/streptomycin (500 U/ml) on Corning 75 cm^2 plastic flasks or on 12 mm circular glass coverslips. Cells were maintained at 37°C in a 5% CO_2/95% air atmosphere and fed every fourth day.

2.2. Reverse transcription

Total cellular RNA was isolated by the single step guanidinum isothiocyanate-acid phenol procedure [13]. RNA concentration was determined by optical density measurements at 230, 260 and 280 nm. The quality of the RNA was assessed by electrophoresis in a 1% agarose–2.2 M formaldehyde gel. First strand cDNA was reverse transcribed in a 50 μl final volume using 10 μg total RNA, 1 μM random hexamer (Boehringer Mannheim), 500 units SuperScript II reverse transcriptase (Life Technologies) and 100 units of RNase Inhibitor (Boehringer Mannheim). The buffer consisted of 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT and 5 mM dNTP. The reaction was allowed to proceed at 37°C for 90 min, then terminated by heating to 65°C for 10 min. Controls omitted reverse transcriptase.

2.3. PCR

A sequence encoding a portion of domain 4 (D4) common to α-subunits was selected for amplification. A single consensus sequence was used to synthesize the 5′ primer (5′ CTGCTCTTCCTGGTCATGTTCATC 3′) which corresponds to positions 5277–5300 (αI), 5206–5230 (αII), 5247–5270 (αIII), 4957–4980 (αNa6), 4963–4986 (αhNE-Na), 418–441 (αNaG) and 4407–4430 (αmNav2.3) (Genbank accession numbers: αI: X03638; αII: X03639; αIII: Y00766; αNa6: L39018; αhNE-Na: X82835; αNaG: M965778 and αmNav2.3: L36179). The consensus primer had one mismatch at position 6 (T to C) compared to αI, II and III, and two mismatches at position 3 (G to C) and 4 (C to A) compared to αNaG and αmNaV2.3. Due to codon degeneracy, we could not design a single 3′ primer that matches all six sequences, so we synthesized two 3′ primers (#1: 5′ GGAAGGATATGATGATGTAGCTGACAAA 3′ and #2: 5′ CAACCATGATATGAGGATGTAACTGACA 3′) to maximize the hybridization potential to the corresponding template. The two primers have the same length and predicted thermal profile, and therefore are expected to anneal to their respective templates with comparable efficiency. The 3′ primers are complementary to sequences 5547–5574 (αI), 5476–5503 (αII), 5517–5544 (αIII), 5224–5251 (αNa6), 5233–5260 (αhNE-Na); 690–717 (αNaG) and 4679–4706 (αmNav2.3). The consensus primer has two mismatches at position 8 (G to T) and 23 (C to G) compared to αNa6 and three mismatches at positions 17 (A to G), 20 (A to G) and 23 (A to G) compared to αhNE-Na. The nucleotide mismatches described above are not expected to affect the efficiency of amplification under the PCR conditions used in this study.

The 5′ and 3′ oligonucleotide primers for the rat sodium channel

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β1-subunit correspond to nucleotides 298–320 (5′ GAGACCGAGGCAGTGTATGGGATG 3′) and 872–890 (5′ GCCCAGAGCCAGCGCTATT 3′) of the published sequence [14].

Rat β-actin sequences were amplified from the cDNA pool, using commercial primers (Clontech) to determine the presence of contaminating genomic DNA in the RNA preparations. PCR from a cDNA template results in a 764 bp fragment while a genomic template results in a 1440 bp fragment. A single band of the predicted 764 bp was detected in control PCR reactions which indicates the absence of appreciable genomic DNA contamination of RNA samples (data not shown).

Amplification was performed in 50 μl volume using 2 μl of the first strand cDNA reaction, 1 μM of each primer and 1.75 units of Expand Long Template DNA polymerase enzyme mixture (Boehringer Mannheim). Control PCR reactions in which the template was substituted by water or an aliquot from a control reverse transcription reaction lacking reverse transcriptase produced no amplification products (Figs. 1 and 2 and data not shown). The Expand Long Template enzyme mixture increased the PCR yield without an increase in non-specific amplification [15,16]. The PCR reaction buffer consisted of 50 mM Tris-HCl (pH 9.2), 16 mM $(NH_4)_2SO_4$, 2.25 mM $MgCl_2$, 2% (v/v) DMSO and 0.1% Tween 20. Amplification was carried out in two stages using a programmable thermal cycler (PTC-100, MJ Research, Cambridge, MA). First, a denaturation step at 94°C for 4 min, an annealing step at 58°C for 2 min, and an elongation step at 72°C for 90 s. Second, a denaturation step at 94°C for 1 min, an annealing step at 58°C for 1 min, and an elongation step at 72°C for 90 s. The second stage was repeated 33 times for a total of 35 cycles with the elongation step in the last cycle extended to 10 min.

2.4. Restriction enzyme analysis

Restriction enzymes (New England Biolabs and Boehringer Mannheim) were used in the manufacturer's suggested buffers. Typically, 1/20 of the PCR reaction is digested for 1 h at the recommended temperature and the products resolved by electrophoresis in a 2% agarose gel (Boehringer Mannheim). Fragment sizes were determined by comparison to a 100 bp ladder molecular weight marker (Pharmacia, Inc.). DNA is visualized by ethidium bromide fluorescence.

2.5. In situ hybridization

2.5.1. Probes. The B104 cells were examined for sodium channel α- and β1-subunit mRNA expression utilizing isoform-specific riboprobes as previously described [17–19].

2.5.2. α-Subunit plasmids. The RNA probes derived from 3′ noncoding regions recognize nucleotide sequences 7385–7820, 6807–7302, 6325–6822, 871–1308 and 6461–6761 (GenBank numbering) for sodium channel types I, II, III, NaG and Na6, respectively. RNA blot hybridization analyses have shown that the major hybridizing bands with these riboprobes are 9.0, 9.5, 9.0 and 7.5 kb for NCI, NCII, NCIII and NaG, respectively [17,18,20], consistent with previous descriptions [3,4,6].

2.5.3. β1-Subunit plasmid. The RNA probe derived from the coding sequence of Naβ1 cDNA recognizes the nucleotide sequence 457–790 (GenBank numbering) [21]. RNA blot hybridization analysis shows a major hybridization at 1.5 kb, consistent with previous descriptions [14].

2.5.4. Riboprobes. Digoxigenin-labelled single-strand RNA probes are transcribed using DIG RNA labelling procedure (Boehringer Mannheim). Reaction products were fractionated by gel filtration (Sephadex G-50), and probe integrity and concentrations were determined by RNA electrophoresis, blotting and probing with anti-digoxigenin antibodies.

2.5.5. Cell in situ hybridization. The protocol of Black et al. [18] is used for detection of sodium channel mRNAs in the B104 cells. Control experiments include omitting probe from the hybridization solution, substitution of sense for antisense probes and pretreatment of the coverslips with RNase A prior to hybridization. Negligible to very low specific hybridization signal was detected in these control experiments.

3. Results

3.1. Sodium channel α-subunit mRNAs expressed in brain and B104 cell line

The amplified sequences of the α-subunits encode a stretch of amino acids delineated by the conserved transmembrane segments IVS5 and IVS6. This sequence is highly conserved among the different α-subunits investigated in this study. Individual subunits were differentiated by restriction enzyme polymorphism (Table 1). Fig. 1 shows the results of restriction enzyme analysis of the PCR products using forebrain (lanes 2–10) and B104 (lanes 12–20) cDNA templates. The length of the expected PCR product for the various α-subunits is 294 bp (αNa6), 297 bp (αI, II, III and αhNE-Na) and 299 bp (αNaG and αmNav2.3). The PCR product from brain and B104 templates comigrate with the 300 bp size standard, in close agreement with the predicted length of the amplified fragment.

Despite a small number of mismatches (see section 2.3, the consensus primers are predicted to anneal with comparable efficiency to individual templates in the cDNA pool. Except for rare messages, the representation of any one subunit in the PCR product would be expected to roughly parallel its relative abundance in the RNA pool. Lanes 4–10 show that subunits αII and αNa6 represent the most abundant messages in the forebrain. As noted in Table 1, the enzyme *Dsa*I cleaves both αI and αIII; therefore the signal in lane 6 should be interpreted as a combination of αI (whose contribution could be estimated from lane 4) and αIII. The relative abundance of these subunits is in agreement with published results [8,22]. Also in agreement with published reports, no αhNE-Na, αNaG and αmNav2.3 products were detected in the brain PCR pool [6,7,9,20].

Lanes 14–20 show the results of restriction enzyme analysis for B104 cells. αNaG is the predominant subunit in the PCR pool (lane 19). The restriction enzyme *Ple*I cuts both αNaG and αmNav2.3 PCR products but *Psp*1406I cuts only αmNav2.3 (Table 1). Lane 20 shows that *Psp*1406I did not cleave the input PCR product; therefore we conclude that all of the cleaved DNA in lane 19 must have come from αNaG

Table 1
Predicted subunit-specific enzyme digestion and products

	αI, 297 bp	αII, 297 bp	αIII, 297 bp	αVI, 294 bp	αhNE-NA, 297 bp	αNaG, 299 bp	αmNav2.3, 299 bp	Product size (bp)
*Hinc*II	+	−	−	−	−	−	−	213, 84
*Ava*II	−	+	−	−	−	−	−	183, 114
*Dsa*I	+	−	+	−	−	−	−	254, 43
*Pst*I	−	−	−	+	−	−	−	195, 99
*Bfa*I	−	−	−	−	+	−	−	162, 108
*Ple*I	−	−	−	−	−	+	+	216, 83
*Psp*1406I	−	−	−	−	−	−	+	138, 161

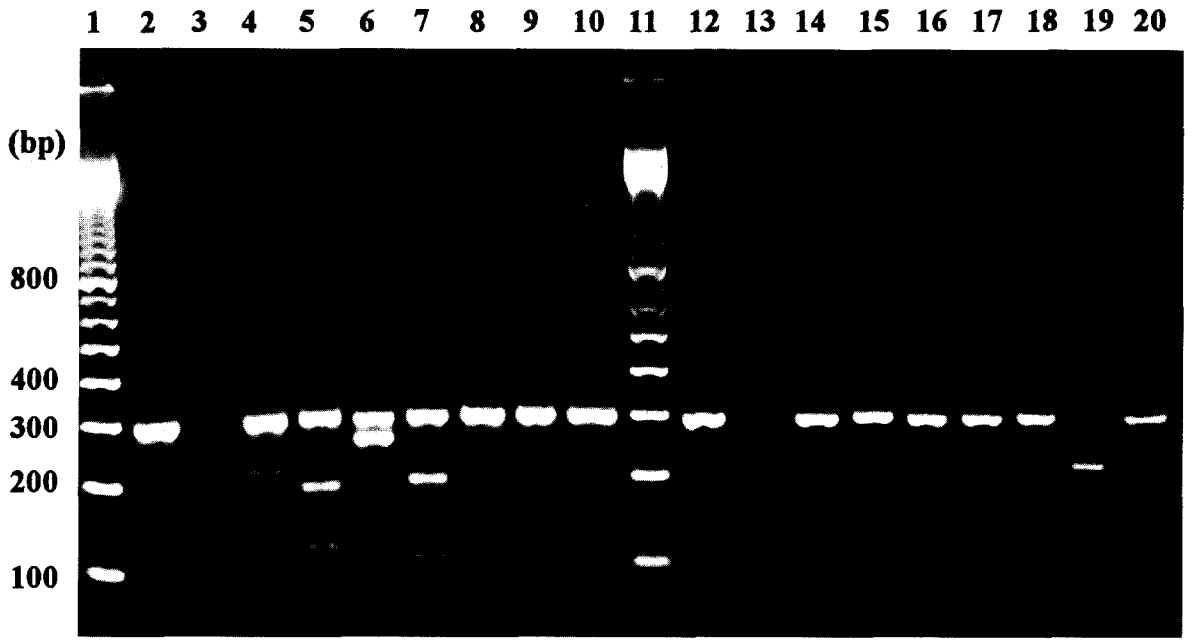

Fig. 1. RT-PCR amplification of sodium channel α-subunits from forebrain and B104 cell line. PCR products and their restriction enzyme analysis: forebrain (lanes 2–10) and B104 cell line (12–20). Lanes 1 and 11 contain the 100 bp ladder size standard (Pharmacia, Inc.). Lanes 2 and 12 contain undigested PCR products. The upstream and two downstream primers were added in a molar ratio of 1:0.5:0.5 to ensure equal access to the respective templates in the cDNA pool. Lanes 3 and 13 contain product of the no template PCR control reaction. Lanes 4–10 and 14–20 contain restriction enzyme products of forebrain and B104 PCR products, respectively. Lanes 4 and 14: *Hin*cII digest (αI-specific); lanes 5 and 15: *Ava*II digest (αII-specific); lanes 6 and 16: *Dsa*I digest (αI- and αIII-specific); 7 and 17: *Pst* I digest (αNa6-specific); lanes 8 and 18: *Bfa* I digest (αhNE-Na-specific); lanes 9 and 19: *Ple* I digest (αNaG- and αmNav2.3-specific); lanes 10 and 20: *Psp*1406 I digest (αmNav2.3-specific). The restriction enzyme products are in agreement with the predicted sizes. Due to low level of representation in the PCR pool, αI and αII restriction products from B104 cell line are not visible in this photographic reproduction of the original gel. Also, the small fragment produced by *Dsa*I restriction of the αI and αIII of the B104 PCR product is not visible but its presence is inferred from the presence of the longer product (compare lanes 6 and 16 for the location of the small fragment).

PCR product. Lane 17 shows that αNa6 is present but in lower amounts compared to αNaG. Lane 16 shows αIII representation that is comparable to that of αNa6. As discussed above, the signal in this lane could, in principle, be an overestimate due to αI contribution. Subunits αI and αII are present at levels barely detectable on the gel (lanes 14 and 15) but the signal was lost during photographic documentation. Therefore, the contribution of αI in lane 16 is minimal. Subunits αhNE-Na (lane 18) and αmNav2.3 (lane 20) were not detected by this assay.

3.2. β1-Subunit

Fig. 2 shows the amplification products of the β1-subunit of the sodium channel from forebrain and B104 cells. The PCR products (lane 2, forebrain; and lane 3, B104 cells) comigrated with the 600 bp size standard in close agreement with the predicted size of 592 bp. Digestion of these PCR products with three restriction enzymes produced a similar pattern in forebrain and B104 samples (compare lanes 5–7, B104 cell line; and 10–12, forebrain). Moreover, the 3′ primer used in this amplification straddles an intron/exon junction [21,23,24]. Taken together, these results strongly suggest that these two PCR products are highly related and were amplified from similar mRNAs that correspond to the β1-subunit.

In situ hybridization. B104 cells were hybridized with riboprobes specific for the mRNAs for sodium channel α-subunits I, II/IIA, III, NaG and Na6 and sodium channel β1-subunit (Naβ1). Sodium channel mRNA I and II were not detectable

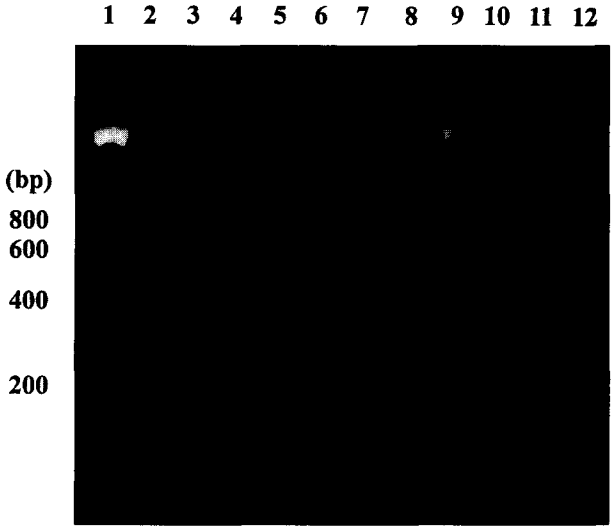

Fig. 2. RT-PCR amplification of sodium channel β1-subunit from forebrain and B104 cell line. PCR products and their restriction enzyme digestion profile from forebrain (lanes 2 and 10–12) and B104 cells (lanes 3 and 5–7). Lane 1 and 9 contain the 100 bp ladder size standard (Pharmacia, Inc.). Lane 4 contains product of the no template PCR control reaction. Lane 8 is empty. Lanes 2 and 3 contain undigested PCR products from forebrain and B104, respectively. *Pvu*II digest produces the expected 434 and 158 bp products (lanes 5 and 10); *Sty*I digest produces the expected 378 and 214 bp products (lanes 6 and 11); and *Hin*cII produces the expected 430 and 162 bp products (lanes 7 and 12).

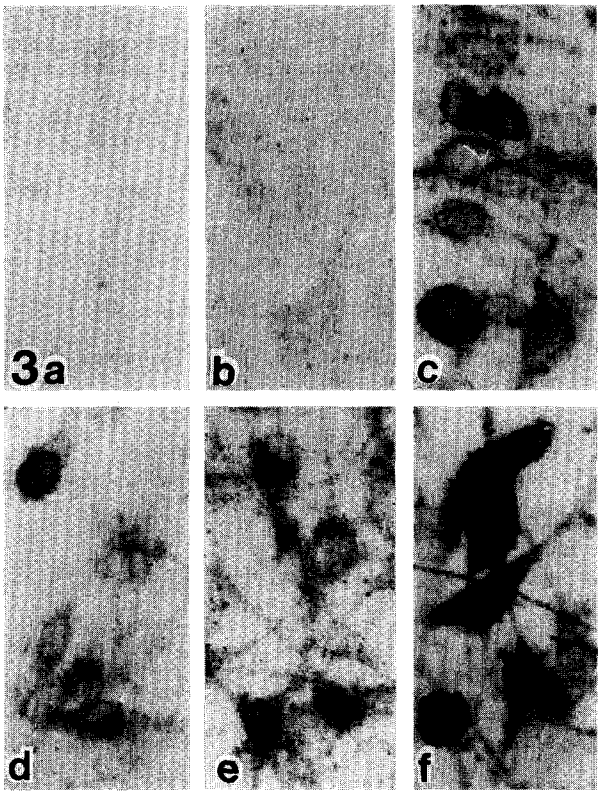

Fig. 3. B104 cells were hybridized with riboprobes specific for sodium channel α-subunit mRNAs I (a), II (b), III (c), NaG (d) and Na6 (e) and β1-subunit mRNA (f). Sodium channel mRNA I and II are not detectable in B104 cells. In contrast, mRNA III, NaG, Na6 and β1 are present in B104 cells. ×500.

(Fig. 3a,b), while hybridization signal was clearly present with riboprobes directed against mRNAs III, NaG and Na6 (Fig. 3c,d,e). 78% (n = 626), 71% (n = 463) and 82% (n = 707) of B104 cells displayed at least some hybridization signal for α-subunit mRNA III, NaG and Na6, respectively. Hybridization signal for Naβ1 mRNA was present at high levels (greater than that for any α-subunits) in many B104 cells (Fig. 3f), although there was a gradient of hybridization signal. 78% (n = 608) of B104 cells were observed to express β1 mRNA, while 22% of the cells did not express detectable levels of this subunit mRNA.

4. Discussion

The results presented here demonstrate that B104 cells express multiple sodium channel α-subunit mRNAs. The mRNA for type III α-subunits is clearly present, as previously described by Baines et al. [2]. We also detected the mRNAs for sodium channels Na6, which has recently been cloned and sequenced [8], and for NaG [6]. We were able to detect the mRNA for types III, NaG and Na6 in 72%, 71% and 82% of B104 cells, respectively. Since the mean sodium channel density ($< 3/\mu m^2$) in B104 cells (Gu, Rizzo and Waxman, unpublished data) is relatively low, it is possible that some cells expressed sodium channel mRNAs at low levels that were below the threshold for detection in our experiments, so that these percentages may represent underestimates. The mRNA for sodium channel β1-subunit is also present in at least 78% of B104 cells, with in situ hybridization signals that are higher than those for any of the individual α-subunits. This may reflect a 1 : 1 stoichiometry between β1-subunits and the multiple α-subunits in B104 cells

Although B104 neuroblastoma cells were originally reported to express only type III sodium channel α-subunit mRNA, and not types I and II [2], our gels detected low levels of type I and II mRNA (too low to be reproduced in photographs, but clearly present), as well as high levels of type III, in B104 cells. Our controls in which the cDNA template was replaced with water did not show any amplification products. Careful examination of the data of Baines et al. [2] (see their Fig. 4) reveals, in fact, light bands consistent with the presence of sodium channel type I and II mRNA in Southern blots of B104 cells. It is also possible that hNE-Na is present in B104 cells, but is not detected by our analysis due to sequence divergence between the rat and human cognates.

It is not clear, at this time, which α-subunit mRNAs are translated in B104 cells. NaG, Na6 and type III mRNA appear to be the predominant α-subunit species, accounting for most of the signal detected in RT-PCR studies. Our results suggest that at least some B104 cells may co-express the mRNAs for two or more α-subunits. It is not clear, however, whether all the sodium channel mRNAs in B104 cells are effectively translated. In this regard, there is evidence for the expression, in some neurons, of transcripts for GABA receptors [25] and NMDA receptors [26] that are not translated.

A notable finding in this study was that B104 cells express the mRNA for NaG. NaG is a partial clone derived from a rat cortical astrocyte library [6]. NaG mRNA is not detected within neurons in the adult CNS, but is present at high levels in DRG neurons [20]. The expression, in B104 cells, of an mRNA for a sodium channel α-subunit that is not expressed in CNS neurons may be due to a loss of normal regulatory control of channel expression in these cells, which were derived from a CNS neoplasm. In this respect, B104 cells may provide a useful model for the study of the regulation of sodium channel expression.

The expression of sodium channel mRNAs in B104 cells (this paper) and the demonstration of electrical excitability in these cells [1] does not necessarily imply a neuronal origin for the B104 cell line. The mRNAs for all the sodium channel α-subunits that are present in B104 cells have been demonstrated in glial cells [6,8,18,27], and mRNA for the β1-subunit is also present in glia [21]. Sodium current densities in spinal cord astrocytes in vitro can exceed 200 pA/pF, implying a channel density of $> 5/\mu m^2$ in some cells [28] and, if spinal cord astrocytes in vitro are held at negative potentials to remove resting inactivation, action potentials can be elicited [29]. The expression of S-100 antigen in B104 cells [1] may reflect origin from a stem cell which can express both neuronal and glial characteristics.

Although our studies confirm the original observation [2] of type III sodium channel mRNA expression in B104 cells, they also demonstrate the presence of other α-subunit mRNAs in these cells. B104 cells thus does not represent a neuronal cell line characterized by expression of a single sodium channel α-subunit. Analysis of the molecular basis for sodium current expression in these cells may be facilitated by single-cell PCR or in situ PCR.

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