

Brain and heart sodium channel subtype mRNA expression in rat cerebral cortex

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ABSTRACT The expression of mRNAs coding for the α subunit of rat brain and rat heart sodium channels has been studied in adult and neonatal rat cerebral cortex using the reverse transcription–polymerase chain reaction (RT–PCR). Rat brain sodium channel subtype I, II, IIA, and III sequences were simultaneously amplified in the same PCR using a single oligonucleotide primer pair matched to all four subtype sequences. Identification of each subtype-specific product was inferred from the appearance of unique fragments when the product was digested with specific restriction enzymes. By using this RT–PCR method, products arising from mRNAs for all four brain sodium channel subtypes were identified in RNA extracted from adult rat cerebral cortex. The predominant component was type IIA with lesser levels of types I, II, and III. In contrast, the type II and IIA sequences were the predominant RT–PCR products in neonatal rat cortex, with slightly lower levels of type III and undetectable levels of type I. Thus, from neonate to adult, type II mRNA levels decrease relative to type IIA levels. Using a similar approach, we detected mRNA coding for the rat heart sodium channel in neonatal and adult rat cerebral cortex and in adult rat heart. These results reveal that mRNAs coding for the heart sodium channel and all four previously sequenced rat brain sodium channel subtypes are expressed in cerebral cortex and that type II and IIA channels may be differentially regulated during development.

Voltage-dependent sodium channels are responsible for action-potential generation and propagation in many excitable cells, including cardiac and skeletal muscle and neurons (1). Although virtually all sodium channels appear to have very similar functional properties, minor differences are observed among sodium channels from various cells in the same organism and, in some cases, within the same cell. One notable functional property that differs considerably among tissues is the sensitivity to the guanidinium toxins tetrodotoxin (TTX) and saxitoxin (STX). Sodium channels in central nervous system neurons and skeletal muscle are normally blocked by nanomolar concentrations of TTX and STX, whereas channels in cardiac cells and denervated skeletal muscle are relatively TTX/STX-resistant, requiring 0.1–10 μ M toxin for block (2). Some cell types, including developing skeletal muscle myocytes (3–5), certain peripheral (dorsal root ganglion) neurons (6, 7), and some brain glial cells (astrocytes) (8–10), express both high-TTX-affinity and low-TTX-affinity sodium channels. The physiological consequences of these differences are not known.

Biochemical and molecular biological studies have provided considerable information about the molecular structures of voltage-gated ion channels. All sodium channels thus far characterized contain a large (260 kDa) α subunit and, in some cases, one or two smaller (30–40 kDa) β subunits (11–13). Hydropathy analysis of the primary amino acid

sequence suggests that the α subunit consists of four homologous domains each containing six transmembrane segments (14). Molecular cloning studies have also revealed that there are four sodium-channel α -subunit mRNAs in rat brain, designated types I, II, and III (15, 16) and IIA (17). Types II and IIA differ in only six amino acids and 36 (out of >6000) nucleotides, and most of these differences are clustered in a “hypervariable” region in the first putative homologous domain. Rat skeletal muscle expresses a different sodium channel subtype with a high TTX affinity that is homologous to, but distinct from, those in rat brain (18). Expression of all brain and intact skeletal muscle sodium channel transcripts in *Xenopus* oocytes has revealed voltage-dependent inward sodium currents that were blocked by nanomolar TTX and STX (17–20). Recently, another sodium channel subtype was identified in neonatal rat heart (21), and subsequently, a channel with a virtually identical sequence was detected in denervated skeletal muscle (22). That this sequence codes for the sodium channels with low TTX sensitivity characteristic of these tissues was demonstrated recently when channels expressed from the cloned heart and denervated skeletal muscle genes were found to be at least two orders of magnitude less sensitive to TTX and STX than neuronal channels (23, 24). The basis of this differential toxin sensitivity appears to be that the heart and denervated skeletal muscle sequences have a positively charged arginine residue at the putative TTX binding site (25), which would be expected to lower the toxin binding affinity.

Our interest is in understanding the physiological significance of the differential expression of TTX/STX-sensitive and -insensitive sodium channels in the central nervous system. To this end, we have developed a reverse transcription–polymerase chain reaction (RT–PCR) based method for detecting expression of mRNAs for five of the known sodium channel isoforms in a single sample. In this report we describe the method and its application to detecting sodium channel subtype mRNA expression in rat cerebral cortex and heart. Our results demonstrate that the heart subtype mRNA is expressed in cerebral cortex and that expression of type II and IIA sodium channels, which are >99% homologous in nucleotide sequence, may be differentially regulated during brain development.

MATERIALS AND METHODS

RNA Preparation. Total RNA was prepared from adult (30-day-old female) and neonatal (P1) rat cerebral cortex and adult rat heart using either a modification of the method of Cathala *et al.* (26) or the method of Chomczynski and Sacchi (27). No differences were observed in the results obtained

Abbreviations: RT, reverse transcription; TTX, tetrodotoxin; STX, saxitoxin.

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using RNA prepared by the two methods. All experiments reported in this paper were repeated using at least two RNA preparations.

RT. The first-strand cDNA was synthesized using Moloney murine leukemia virus (M-MLV) reverse transcriptase [BRL Technical Bulletin 8025-1 (28–30)]. Total RNA (1 μ g) was incubated (final volume, 20 μ l) at 37°C for 60 min with 10 units of cloned M-MLV reverse transcriptase (BRL)/50 mM Tris-HCl, pH 8.3/75 mM KCl/3 mM MgCl₂/10 mM dithiothreitol/(dT)_{12–18} (25 μ g/ml; Pharmacia) or random hexamer primers (25 μ g/ml; BRL)/bovine serum albumin (0.1 mg/ml; Boehringer Mannheim)/0.5 mM dATP/0.5 mM dCTP/0.5 mM dGTP/0.5 mM dTTP (BRL). For each RT, a blank was prepared using all the reagents except the RNA sample; an equivalent volume of water was substituted for the latter. This RT blank was used to prepare the PCR blank (see below).

PCR. Oligonucleotide primers for the PCR were synthesized in the Biopolymer Laboratory of the Department of Microbiology and Immunology, University of Maryland School of Medicine, by the phosphoramidite method on an Applied Biosystems model 380B DNA synthesizer. The oligonucleotides, in water, were used without further purification. Two pairs of primers were used in these studies. The first pair, designated primer pair B, bracketed a 626-base-pair (bp) segment near the 5' end of the four rat brain channel sequences. Primer pair B was perfectly matched to all four rat brain sodium channel sequences. Primer pair H bracketed a 390-bp segment in the intracellular loop between the first and second domains in the heart channel sequence. The sequences of the primers are shown in Table 1.

PCR was carried out according to GeneAmp DNA amplification reagent kit instructions (Perkin-Elmer/Cetus) with minor modifications (29, 30). The PCR mixture (50 μ l) contained 10 mM Tris-HCl (pH 8.3), 2.5 mM MgCl₂, 50 mM KCl, 0.001% gelatin, 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP, 0.2 mM dTTP (Perkin-Elmer/Cetus or BRL), 1 unit of cloned *Thermus aquaticus* DNA polymerase (AmpliTaq, Perkin-Elmer/Cetus), 0.01% bovine serum albumin, 50 pmol of each primer in water, and 1 μ l of RT sample. The components were concentrated in the bottom of the tube by centrifugation and overlaid with 50 μ l of light mineral oil (Sigma). PCR incubations were carried out in a programmable thermal controller (MJ Research, Cambridge, MA). During each PCR cycle, the samples were heated to 94°C to denature template complexes (120 sec initially and 30 sec during all subsequent cycles), cooled to 60°C to anneal template and primers (30 sec), and heated to 72°C for optimal extension (90 sec). Amplification was carried out for 35 cycles (primer pair B) or 30 cycles (primer pair H). The final 72°C incubation step was extended for an additional 7 min, as recommended in the GeneAmp kit instructions. The samples were then rapidly cooled to 2°C and kept on ice or frozen until analyzed. Each time a PCR was carried out, a blank was prepared using all reagents, including primers, and 1 μ l of the matching RT blank. In all experiments, the RT-PCR reagent blank lane was devoid of DNA bands (see Fig. 3A). In control experiments, a 1:10 dilution of the tissue RNA resulted in a substantial reduction in the amount of PCR product; more-

over, the relative amounts of sodium channel isoform sequences were unchanged by this dilution.

Gel Electrophoresis. A sample of the PCR mixture (8 μ l) was added to 2 μ l of 5 \times loading buffer [30% (wt/vol) Ficoll/0.2 M EDTA/0.25% bromophenol blue/0.25% xylene cyanole FF] and size-fractionated by electrophoresis in a 0.75-mm-thick nondenaturing 8% polyacrylamide gel (7 \times 8 cm, SE250 apparatus, Hoefer) using a constant 60-V field. Electrophoresis was stopped when the bromophenol blue dye front had migrated at least 6 cm (\approx 90 min). Standard size markers (in 10 μ l) were ϕ X174 replicative form DNA *Hae*III fragments (0.37 μ g per lane; 1353, 1078, 872, 603, 310, 271/281, 234, 194, 118, and 72 bp; BRL) or pGEM markers (0.5 μ g per lane; 2645, 1605, 1198, 676, 517, 460, 396, 350, 222, 179, 126, 75, 65, 51, and 36 bp; Promega). Gels were stained for 30 min in ethidium bromide (0.5 μ g/ml), destained in water for 15 min, and examined on a 312-nm ultraviolet transilluminator. Gels were photographed using an orange filter and Polaroid 665 positive/negative film.

Restriction Enzyme Analysis. Restriction enzymes and matched 10 \times buffers were obtained from Amersham or BRL. A PCR sample (5–7 μ l) was incubated for 1 h at 37°C with enzyme as described by the manufacturer's instructions (final volume, 10 μ l). The digest, along with an untreated sample and size standards, was then precipitated with ethanol, fractionated by electrophoresis, stained, and examined, as described above.

RESULTS

Rat Brain Sodium Channel Subtypes in Cerebral Cortex. When cDNA derived from adult (P30) or neonatal (P1) rat cerebral cortex RNA was amplified using primer pair B, a single major band was observed at the size (626 bp) of the product expected from each of the four rat brain sodium channel subtypes using these primers (Figs. 1 and 2, lane 2). All bands >100 bp represent amplification of cDNA derived from rat brain RNA because no PCR product was observed in the blanks from which RNA had been omitted in the RT step; for example, see Fig. 3A. This also rules out contamination of reagents by spurious RNA or cDNA. In addition, no PCR product was observed when reverse transcriptase was omitted from the RT step, demonstrating that the product is not the result of amplification of genomic DNA (data not shown). Since the length of the PCR product for each of the four brain subtypes is identical, it is not possible to tell, from size alone, which isoforms are represented in the 626-bp band. However, because each subtype sequence has a unique restriction enzyme site located between the primers, the presence of each subtype can be detected by the appearance of the appropriate restriction enzyme fragments. This strategy is illustrated in Table 2, which shows the predicted fragments for each channel subtype.

The PCR products from neonatal and adult rat cerebral cortex were incubated with each restriction enzyme, ethanol-precipitated, subjected to polyacrylamide gel electrophoresis, and stained with ethidium bromide. When cDNA prepared from adult rat cortex RNA was amplified using primer pair B, evidence for the presence of all four sodium channel

Table 1. Sodium-channel α -subunit sequences for primer hybridization

	3'-AAGTACCCGTTGGACTCCTTAT-5'
Primer Pair B: 5'-CATTCTCCAGAGATGGTGTC	TTCATGGGCAACCTGAGGAATA-3'
	3'-AGTCGAGGGCCAATGCAAGA-5'
Primer Pair H: 5'-CCGTAGCTCTCTGGAGATGT	TCAGCTCCGGTTACGTTCTT-3'

Lower line shows the coding sequences bracketing the amplified region for each primer pair; the primer sequences are in boldface type. Primer pair B corresponds to bases 201–221 and 804–826 of type I and III α subunits and 204–224 and 807–829 of types II and IIA α subunits. Primer pair H corresponds to bases 1374–1393 and 1744–1763 of the heart and denervated skeletal muscle α subunit.

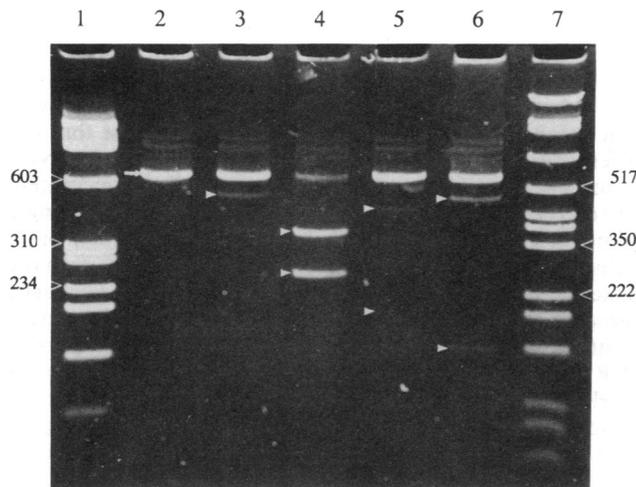


FIG. 1. Ethidium bromide-stained polyacrylamide gel of RT-PCR product and restriction endonuclease fragments using adult rat cerebral cortex RNA and primer pair B. Lanes: 1, ϕ X174 standards; 2, PCR product (expected size using primer pair B, 626 bp) prior to incubation with restriction enzymes; 3, PCR product incubated with *Msp* I (cuts type I sodium channel sequence); 4, PCR product incubated with *Bam*HI (cuts type IIA); 5, PCR product incubated with *Sfu* I (cuts type II); 6, PCR product incubated with *Dra* I (cuts type III); 7, pGEM standards. The arrow (lane 2) indicates the uncut 626-bp PCR product. The arrowheads (lanes 3–6) indicate the restriction endonuclease fragments for the respective channel subtypes (see Table 2).

subtypes was obtained (Fig. 1). The highest level of restriction enzyme products appeared with *Bam*HI, indicating that type IIA mRNA was probably present in the highest amounts. Fragments from types I and III were observed at intermediate levels, and fragments from type II PCR product were barely detectable.

With the PCR product from neonatal rat cortex (Fig. 2), the predicted restriction enzyme fragments were observed with *Bam*HI, *Sfu* I, and *Dra* I, indicating the presence of mRNAs for rat brain type IIA, II, and III sodium channels in the original RNA, respectively. The predominant restriction fragments were from types II and IIA channels, suggesting approximately equal levels of the corresponding mRNAs in neonatal brain; there was no evidence of an effect of *Msp* I, indicating very low or negligible levels of type I sodium channel mRNA in neonatal cortex. Levels of the type III PCR product were nearly as high as those of types II and IIA in neonatal brain.

Rat Heart Sodium Channel Subtype in Cerebral Cortex. A second primer pair (H; cf. Table 1) was used to amplify cDNA specific for the sodium channel isoform identified in rat heart (21) and denervated rat skeletal muscle (22). The primers bracketed a 390-bp region in the putative intracellular loop between homologous domains I and II. As shown in Fig. 3A, amplification of cDNA reverse transcribed from adult rat heart RNA (lane 2) or adult cortex RNA (lane 3) using primer

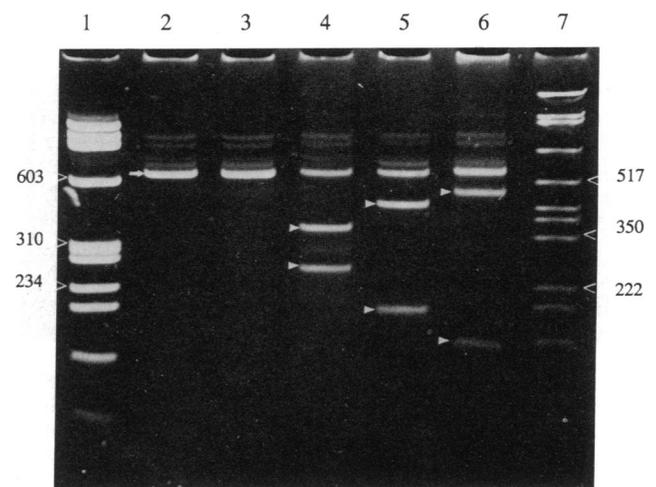


FIG. 2. Ethidium bromide-stained polyacrylamide gel of RT-PCR product and restriction endonuclease fragments using neonatal rat cerebral cortex RNA and primer pair B. Lanes: 1, ϕ X174 standards; 2, PCR product (expected size using primer pair B, 626 bp) prior to incubation with restriction enzymes; 3, PCR product incubated with *Msp* I (cuts type I sodium channel sequence); 4, PCR product incubated with *Bam*HI (cuts type IIA); 5, PCR product incubated with *Sfu* I (cuts type II); 6, PCR product incubated with *Dra* I (cuts type III); 7, pGEM standards. The arrow (lane 2) indicates the uncut 626-bp PCR product. The arrowheads (lanes 3–6) indicate the restriction endonuclease fragments for the respective channel subtypes (see Table 2).

pair H produced a major product at 390 bp. No bands were observed using a blank sample (lane 4) from which the cellular RNA had been omitted at the RT step. To confirm the identity of this band the PCR product was incubated with restriction endonucleases specific for sites in the amplified sequence between the primers. Thus, the 390-bp PCR product was expected to be cut into two fragments by *Alu* I (AG/CT; 220 and 148 bp; predicted 16- and 6-bp fragments are not visible on the gels). The 390-bp PCR product and the same *Alu* I restriction enzyme products were observed using RNA from rat heart (Fig. 3B) and from adult (Fig. 3C) and neonatal (Fig. 3D) rat cerebral cortex, indicating the presence of mRNA coding for heart-type sodium channel α subunit in both of these tissues. The 390-bp band from adult and neonatal cortex and heart was also digested by *Rsa* I (GT/AC) into 318- and 72-bp fragments as expected (data not shown). Although we observed low levels of the heart-type 390-bp PCR product in brain, we do not believe that this is due to contamination of brain samples by heart RNA. The experiments have been repeated at least four times for both adult and neonatal cortex with identical results; moreover, all PCR blanks, which were processed in parallel with the brain samples, were devoid of bands (see Fig. 3A).

Although the heart channel-specific primers might be expected to hybridize to brain-type channel sequences (there were four mismatches with brain sequences in each primer),

Table 2. Expected restriction endonuclease fragment sizes for primer pair B

Rat brain sodium channel subtype	Size, bp			
	<i>Msp</i> I	<i>Bam</i> HI	<i>Sfu</i> I	<i>Dra</i> I
I	100 + 526	NC	NC	NC
IIA	NC	361 + 265	NC	NC
II	NC	NC	445 + 181	NC
III	NC	NC	NC	498 + 128

The 626-bp product expected for rat brain sodium channel subtypes I, IIA, II, and III amplified with primer pair B was analyzed by cleavage with *Msp* I (C/CGG), *Bam*HI (G/GATCC), *Sfu* I (TT/CGAA), or *Dra* I (TTT/AAA). The sizes of the expected fragments for each subtype are shown. NC indicates restriction enzyme would not be expected to cut the 626-bp segment from the indicated subtype.

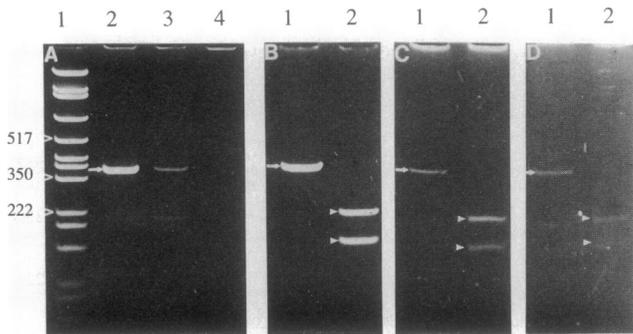


FIG. 3. Ethidium bromide-stained polyacrylamide gel of RT-PCR product and restriction endonuclease fragments using primer pair H. (A) cDNA from rat heart (lane 2) or adult rat cerebral cortex (lane 3) was used as template; lane 1 is pGEM standards and lane 4 is the blank (RNA omitted from RT step). (B) Rat heart cDNA was used as template. (C) Adult rat cerebral cortex cDNA was used as template. (D) cDNA from neonatal rat cerebral cortex was used as template. (B–D) Lanes: 1, uncut PCR product using primer pair H showing expected 390-bp product (arrow); 2, PCR product was cut with *Alu* I (expected 220- and 148-bp products identified by arrowheads; 16- and 6-bp products not visible on these gels).

a product of 512 bp would be predicted for the brain sequences. No product of that size was observed under the PCR conditions used. When primer pair B was used to amplify cDNAs from rat heart, evidence for types I, II, and III and minimal levels of IIA was obtained (data not shown).

DISCUSSION

In this study, we have used the RT-PCR to amplify segments of mRNA coding for previously reported rat brain and rat heart sodium channels. A single pair of primers, which were perfectly matched to identical sites in type I, IIA, II, and III rat brain sequences, amplified all four subtypes in rat brain. Identification of individual subtypes in each sample was made by virtue of unique restriction endonuclease sites in each subtype sequence. The results indicate that all four rat brain subtypes are represented in rat cerebral cortex, although some appear to be present at higher levels than others and the relative levels of expression change during development. By using a similar strategy, mRNA coding for the previously reported rat heart sodium channel sequence (21), which is virtually identical to that reported for the denervated rat skeletal muscle sodium channel (22), was also detected in both neonatal and adult rat cerebral cortex.

In this strategy, each sodium channel subtype identification is made at two levels. (i) The PCR product must be of the appropriate size as defined by each primer pair. (ii) Since the primer sites were chosen so that the resulting product for each subtype would contain a unique restriction endonuclease site (see Table 2), the appropriate restriction enzyme fragments must be generated. Thus, for example, when primer pair B is used, the 361- and 265-bp *Bam*HI restriction enzyme products arising from the 626-bp PCR product cannot be due to type I, II, or III sequences because they do not contain a *Bam*HI site in their respective sequences bounded by the primers.

Only 5–10% of the entire (≈ 6000 bp) sodium-channel α -subunit coding sequence was amplified in each PCR, and thus our conclusions are rigorously confined to the presence of the amplified segments and not necessarily to the whole sequence. Thus, we cannot rule out that we may be detecting a sodium channel sequence that differs from types I, II, IIA, and III in a region outside the one we amplified; however, no additional rat brain sodium channel sequences have been reported to our knowledge.

A potential source of error in this approach is that differences in the amount of PCR product may not accurately reflect differences in mRNA levels. In general, it is considered to be somewhat difficult to derive absolute quantitative conclusions from PCR (31). However, we suggest that the differences in brain sodium channel subtype product levels within each sample reported here are likely to accurately reflect relative differences in mRNA levels because the single primer pair used is perfectly matched to all four known brain sodium channel sequences, all amplified sequences are in the same region of the α subunit and show minimal sequence variation, all products are of the same size, and sequences coding for all four subtypes are amplified in the same RT-PCR reaction sequence. Thus, we are not comparing product levels produced by different primer pairs carried out in separate incubations, and we would not expect any significant differences in amplification efficiencies among the different subtypes.

Neurons in the central nervous system generally display voltage-gated sodium currents that are blocked by nanomolar concentrations of STX and TTX and thus would be expected to reflect the presence of the rat brain type I, II, IIA, and/or III subtypes. The presence of the heart/denervated skeletal muscle isoform in brain is not entirely unexpected, however. Recent studies have shown that cultured astrocytes (glial cells) from neonatal rat cerebral cortex express not only neuron-like sodium channels with high STX affinity but also a second population of channels that require micromolar concentrations for block (8–10). Thus, expression of heart-type sodium channel mRNA, which is presumed to code for channels with low STX affinity, in rat cerebral cortex may be due to the presence of cortical astrocytes, whereas the brain subtypes may be present in both astrocytes and neurons (32). In preliminary experiments using primer pair H, we have found that primary cultures of rat cortical astrocytes express quite high levels of heart-type sodium-channel α -subunit mRNA (unpublished data). Two other cell types present in brain, oligodendrocytes and fibroblasts, have not been shown to express voltage-dependent sodium channels (33, 34).

Types I, II, and III sodium channel subtypes were identified in rat brain by Noda *et al.* (15). Type IIA, reported in rat brain by Auld *et al.* (17), differs from type II in only six amino acids and 36 bp out of ≈ 6000 bp, and most of these differences are located in a "hypervariable" region in the first homologous domain. The hypervariable region is within the segment amplified in our experiments. One of the six amino acid differences, which is not in the region we amplified, has been reported to be due to a cloning artifact (35). We detected both II and IIA sequences in rat cerebral cortex (Figs. 1 and 2); however, we found that the amount of type IIA PCR product was much higher than that of type II in adult (P30) cortex. In contrast, we found that type II sodium channel PCR product levels were about equal to those of type IIA in neonatal rat cortex. Although we cannot determine absolute RNA levels, it is clear that the amount of type II mRNA decreases dramatically relative to type IIA mRNA during development and that type IIA is the predominant mRNA expressed in adult rat cortex. Recently, Ahmed *et al.* (36), using PCR and RNase protection assays directed toward the 5' untranslated region of the α -subunit mRNA, reported the presence of both type II and IIA sequences in adult rat brain. Our results are in qualitative agreement with this finding; however, whereas we find that type IIA mRNA is predominant in adult (P30) rat cortex, Ahmed *et al.* (36) reported that the levels of type II message were 10–20 times higher than that of type IIA in adult (P14) whole rat brain. Possible explanations for this apparent discrepancy are that type II channels are highly concentrated in subcortical brain structures that were not included in our samples of cerebral cortex (cortex represents $\approx 40\%$ of rat forebrain) or that mRNA levels from the

noncoding regions may not be indicative of mRNA levels coding for the hypervariable coding region, perhaps due to alternative splicing mechanisms.

Our results are generally consistent with the two previous studies on the regional and developmental expression of sodium channel subtypes. Rat brain type I sodium channel expression has been reported to be low at all ages and to increase from neonate to adult (37, 38). Similarly, we found that type I expression was undetectable in the neonate and increased to relatively low levels in adult cortex. Our finding that relative type III mRNA expression was higher in neonatal than in adult cortex is consistent with the results of Beckh *et al.* (38) who reported that type III mRNA expression (in both cortex and whole brain) is highest around birth and then declines. In that study, type II sodium channel mRNA expression in cerebral cortex was relatively constant throughout development; however, no attempt was made to distinguish between types II and IIA levels. Our results indicate that, from neonate to adult, relative levels of type II sodium channel mRNA decrease as compared to type IIA levels, suggesting that the expression of these very similar sodium channel molecules is differentially regulated throughout development.

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