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Developmentally regulated alternative RNA splicing of rat brain sodium channel mRNAs

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ABSTRACT

Two rat brain Na channel α-subunit cDNAs, named RII and RIIA, have almost identical coding regions, with a divergence of only 36 nucleotides (0.6%) over a total length of 6015 residues. A cluster of 20 divergent residues occurs within a 90 nucleotide segment of cDNA sequence. We now demonstrate that this 90 nucleotide segment is encoded twice in the RII/RIIA genomic sequence. Furthermore, the mutually exclusive selection of these two exons is developmentally regulated. RII mRNAs are relatively abundant at birth but are gradually replaced by RIIA mRNAs as development proceeds. The two mRNAs also appear to have different regional distributions in the developing rat brain. Strikingly, although 30 amino acids are encoded by each alternative exon, only amino acid position 209 is altered between the two, specifying asparagine in RII and aspartate in RIIA. Alternative RNA splicing may modulate the RII/RIIA sodium channel properties during neuronal development.

INTRODUCTION

Voltage activated K^+, Ca^{++} and Na^+ channels conduct the ionic currents that regulate membrane potential during nerve signaling (1, 2). Biophysical studies have shown that neurons contain channel subtypes which differ in properties such as ion permeability, sensitivity to blocking agents, voltage dependance and kinetics of response. This diversity is most pronounced among the K^+ selective channels where three major groups of voltage-activated channels have been identified; the delayed rectifiers, the transient 'A' types and the inward rectifiers (3). In addition to these voltage-activated types, K^+ channels activated by Ca^{++} ions or by second messengers are found in many neurons. The diversity of K^+ channels is further increased by the expression of many subtypes within each of the major classes. Molecular cloning of K^+ channel cDNAs have shown that this diversity is reflected in the expression of several distinct K^+ channel subunits that are generated by two mechanisms (4). One mechanism is through the use of a number of distinct genes, and the second through the selection of alternative exons within individual genes. For example, in Drosophila the four genes *Shaker, Shab, Shaw* and *Shal* express different subtypes of K^+ channel subunit (5, 6, 7). One of these, *Shaker*, can be expressed as five distinct polypeptides due to alternative usage of exons (5).

Although electrophysiological studies indicate that Na^+ channels possess a more uniform spectrum of properties than K^+ or Ca^{++} channels (2), differences in neuronal Na^+ channels have been shown to exist. This diversity has been indicated by differences in the sensitivity to channel blockers, in the voltage dependencies of activation and in inactivation properties (8, 9, 10, 11). Molecular genetic studies of mammalian Na^+ channels have also provided evidence for multiple subtypes. Four distinct cDNAs encoding Na^+ channel α-subunits have been isolated from rat brain (12, 13, 14). Three of these, RI, RII and RIII, appear to arise from separate genes as they differ by approximately 15% at the amino acid level, with these differences spread throughout the sequences. The amino acid sequence of a fourth rat brain Na^+ channel α-subunit, RIIA, is almost identical to the RII sequence, having only 6 amino acid differences (13). At the nucleotide level, the coding sequence of RIIA differs from RII at 36 positions, including a cluster of 20 that lie within a 90 nucleotide segment of the coding region. Previously, we had suggested that alternative RNA splicing might have resulted in this clustering of nucleotide differences between the RII and RIIA coding regions (13).

We now demonstrate that a short sequence corresponding to the the cluster of nucleotide sequence differences is encoded by two repeat exons in the genomic RII/RIIA sequence, named exon N (neonatal) and exon A (adult). Exon N encodes a sequence corresponding to the RII cDNA, and exon A to the RIIA cDNA for this short region. RNAase protection and PCR experiments indicate that the RII and RIIA mRNA sequences are generated from a single gene by an alternative splicing mechanism. Examination of the abundance of RII and RIIA during the first month after birth indicates that the selection of the two exons is regulated in the brain during development.

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MATERIALS AND METHODS

Isolation of a fragment of rat genomic DNA containing alternatively spliced exons for the sodium channel cDNA

A single DNA fragment of approximately 1800 bp was amplified from rat genomic DNA (Sprague-Dawley) using the 5′-flanking primer VA 20 (5′-agg ggc ttt tgt cta gaa ga-3′), corresponding to residues 529−548 of the published sequence for Na^+ channel α-subunit cDNA (13) and VA 23 (5′-tgt tca ccg tcc tat tga agg c-3′) as the 3′ flanking primer corresponding to residues 916−937 of the Na^+ channel α-subunit cDNA. The amplification was carried out using the polymerase chain reaction employing the following cycles: 30 cycles of 92°C, 45s, 45°C, 45 s, 72°C, 1 min; 1 final cycle of 92°C, 45s, 45°C, 45 s, 72°C, 7 min. The amplified DNA fragment was gel-purified, kinased and ligated into the Sma I site of pGEM 7z. This subclone was denoted as R-18. The complete sequence of the insert was obtained in both orientations by digesting the plasmid R-18 with Xba I and Sph I and employing the Erase-a-base system (Promega) to obtain 5′ and 3′ deletion subclones. Dideoxynucleotide sequencing were carried out using the Sequenase version 2.0 (U.S. Biochemicals, Cleveland, OH).

Peparation of subclones containing specific exons for the sodium channel cDNAs

The plasmid R-18 was digested with BamHI to recover an 1857 bp fragment. The 1857 bp fragment was further digested with HpaII to obtain a BamHI-HpaII 514 bp fragment (containing the exon IIN) and a HpaII-HpaII 314 bp fragment (containing the exon IIA). These fragments were subcloned into AsuII-BamHI and AsuII digested pGEM 7z, and were designated pRSIIN and pRSIIA respectively. The sequence of the inserts were further verified by sequencing in both orientations.

RNAase protection assay

RNAase protections were carried out as described in (15) with modifications. The plasmids pRSIIA and pRSIIN were linearized with BamHI and DraI respectively and by using T7 RNA polymerase (Pharmacia Canada Inc.). Antisense RNA transcripts were synthesized in vitro under conditions suggested by the manufacturer. The reaction was terminated by the addition of 10 units of RNAase-free DNase I and incubation at 37°C for 15 min. The reaction mixture was brought up to 100 ul with core buffer (50 mM Tris-HCl pH 7.4, 4 mM EDTA, 300 mM NaCl, 0.1% SDS) and 20 μg yeast tRNA was added as carrier. The reaction mixture was extracted once with phenol:$CHCl_3$ (1:1 v/v) and the RNA subsequently purified on a NucTrap™ Push Column (Stratagene Cloning Systems) and precipitated with 2.5 volumes of 95% EtOH at −20°C. The precipitate was denatured in 80% formamide, 0.1% w/v bromophenol blue, 0.1 w/v xylene cyanol, 1×TBE (1×TBE used as electrophoresis buffer was 134 mM Tris-borate (pH 8.3), 5 mM EDTA) at 85°C for 5 min and fractionated by 4% urea-PAGE on a 0.75 mm gel. The labelled RNA was eluted at 37°C for 4 hours in 0.5 M NH_4OAc, 1 mM EDTA, 0.1% SDS, 400 μg/ml tRNA and then precipitated with EtOH. The hybridizations of the antisense RNA to RNA preparations from rat brain were carried out overnight at 50°C using $2.5-10\times10^4$ cpm of probe and 30 μg RNA in 30 ul of 80% formamide, 400 mM NaCl, 40 mM PIPES pH 6.8, 1 mM EDTA. Hybridizations to 30 μg yeast tRNA and 30 μg rat liver RNA were carried as negative controls. RNAase digestions were carried out in a total volume of 350 ul of 200 mM NaCl, 100 mM LiCl, 30 mM Tris-HCl pH 7.5, 3 mM EDTA containing 5 μg/ml (2.5 μg/ml for N transcript protections) RNAase A (Boehringer Mannheim) and 100 units/ml RNAase T1(Boehringer Mannheim). Digestions were carried out at 30°C for 30 min., the reaction mixture was then made 0.6% SDS and treated with 50μg of Proteinase K (Sigma Chemical Co) at 37°C for 15 min. 20μg of tRNA was added as carrier and the reaction mixture extracted once with phenol: $CHCl_3$ and precipitated with EtOH. The RNA pellets were recovered by centrifugation, denatured and separated on 8% urea-PAGE. The DNA molecular weight markers were pBR322-HaeIII fragments end-labelled with ^{32}P (15).

PCR amplification from total cellular RNA and hybridization with exon-specific oligonucleotide probes

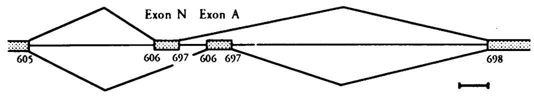
Total RNA was prepared from brains of 0−4 week old Sprague-Dawley rats and from different sections of 0 and 2 week old rat brains using the guanidinium thiocyanate-CsCl method (16). Na^+ channel sequences were amplified from total RNA after first strand cDNA synthesis primed with an RII specific 3′ primer VA23. cDNA from 1 μg of RNA was synthesized in a total volume of 20 μl containing 20 pmoles of VA 23 primer, 40 units of RNAguard (Pharmacia Canada Inc.), 1 mM dNTPs, 400 units M-MLV reverse transcriptase (Gibco-BRL Canada) in 50 mM Tris HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT at 37°C for 1 hour. The cDNA-RNA hybrids were heated to 90°C for 5 min and quickly cooled on ice. Forty pmoles of 5′ primer (VA20) and 20 pmoles of 3′ primer (VA23) were added to a total reaction volume of 100 μl containing amplification buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% w/v gelatin). Prior to amplification, 2.5 units of AmpliTAQ (Cetus-Perkin Elmer) and 100 μl light mineral oil overlay were added. 30 amplification cycles of 92°C, 45s, 45°C, 45 s, 70°C, 1 min and a final cycle of 92°C, 45s, 45°C, 45 s, 70°C, 10 min were employed. 1/10 of each PCR reaction was electrophoresed on a 1% agarose gel and blotted onto Zeta-Probe membrane (Biorad Laboratories) using 0.4 M NaOH as transfer buffer (15). The membranes were baked at 80°C for 30 min. Oligonucleotide probes specific for exons IIA and IIN (VA 25A 5′-gac ctt act gac tat aa-3′ and VA 25N 5′-gac ctt aat gtc ttt at −3′) were labelled to a specific activity of $1-2\times10^8$ cpm/μg using γ-^{32}P-ATP (3000 Ci/mmole, Amersham Canada) and T4 polynucleotide kinase (Boehringer Mannheim). The membranes were prehybridized in 3×SSC, 20 mM sodium phosphate, 10×Denhardt's, 10 mg/ml sonicated salmon sperm DNA , 7% w/v SDS for 1 hour. The membranes were then hybridized using 10^6 cpm/ml in prehybridization buffer at 47°C for 20 hours, and then washed three times for 5 min each in 6×SSC, 0.05% sodium pyrophosphate, 1% SDS prewarmed to 47°C. The final wash was at 50°C for 15 min.

RESULTS

Thirty Amino Acids of the Na^+ Channel α-subunit are Encoded Twice in the RII/RIIA Gene

The coding sequences of the two cDNAs reported for the RII/RIIA Na^+ channel α-subunit diverge significantly in one short segment spanning nucleotides 605 to 690 (12, 13). This restricted localization of 20 nucleotide differences within less than 100 residues suggested an alternative splicing mechanism. Such a mechanism would require the existence of two separate exons within the RII/RIIA gene encoding the peptide sequence for the

A



B

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--- gly phe cys leu glu asp phe thr phe leu arg asp pro trp asn
--- GGC TTT TGT CTA GAA GAT TTC ACA TTT CTA CGG GAT CCC TGG AAT          5' Exon

trp leu asp phe thr val ile thr phe ala
TGG TTG GAT TTC ACA GTC ATT ACT TTT GC gtaagtatttctgtaatact-----
                                     605

<---- 328 nucleotides ---->   ----- actctgatttaatcctacag G
                                                        606

tyr val thr glu phe val asn leu gly asn val ser ala leu arg thr
TAT GTA ACA GAA TTT GTA AAC CTA GGC AAT GTT TCA GCT CTT CGA ACT         Exon N

phe arg val leu arg ala leu lys thr ile ser val ile pro
TTC AGA GTC TTG AGA GCT TTG AAA ACT ATT TCT GTA ATT CCA Ggtaagaag
                                                         697

aagccggtataaggtggtaggctccttatatctccaactgtttcttgtgttctgtcattgtgtttgtgtgtgaactccctattacag A
                                                                                        606

tyr val thr glu phe val asp leu gly asn val ser ala leu arg thr
TAT GTG ACA GAG TTT GTG GAC CTG GGC AAT GTC TCA GCG CTG AGA ACG         Exon A

phe arg val leu arg ala leu lys thr ile ser val ile pro 697
TTC AGA GTT CTC CGA GCA TTG AAA ACA ATA TCA GTC ATT CCA G gtgagagct

aggttaaacac -----      <---- 872 nucleotides ---->

                              gly leu lys thr ile val gly ala
----- ctctctggttttatttgtag GC CTG AAG ACT ATC GTG GGG GCC
                         698
leu ile gln ser val lys lys leu ser asp val met ile leu ---             3' Exon
CTG ATC CAG TCA GTG AAG AAG CTC TCT GAC GTC ATG ATC CTC ---
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Figure 1. The structure of the genomic region encompassing exons N and A. A. The arrangement of exons N and A in the RII/RIIA sodium channel α-subunit gene. The stippled boxes represent the exon sequences. The connecting lines show the mutually exclusive selection of exon N and exon A in the RII and RIIA mRNAs. The numbers below the shaded boxes are nucleotide residue positions in the sodium channel α-subunit cDNA as denoted by Auld et al. (13). The line bar indicates 100 bp. B. The nucleotide sequence of the genomic fragment containing exons N and A in the sodium channel a subunit gene. The sequence in capital letters are exons while lowercase letters indicate intron sequence. The corresponding nucleotide positions from the cDNA sequences are at the beginning and end of each exon. The asterisks indicate nucleotides in exon N that differ from the sequence of exon A. The predicted amino acid residues are noted above the exon sequences, with variant residue 209 highlighted.

region around amino acid residues 202 to 230. To examine this possibility, two oligonucleotide primers flanking the polymorphic sequence were used to amplify the corresponding genomic DNA. These primer sequences (nucleotides 529–548 and 916–937 of the RII sequence numbered according to (13) are identical in both the RII and RIIA cDNAs, but sequence 916–937 diverges at 12/22 and 11/22 positions from the corresponding positions in the two other rat brain Na^+ channel mRNAs, RI and RIII, respectively.

Amplification of genomic DNA with these primers produced a 1800 nucleotide fragment which was sequenced. Figure 1B presents the sequence of the exons found within this fragment. The most striking feature is the occurrence of two exons corresponding to residues 606–697 of the RII/RIIA cDNAs. The sequences of the flanking exons match both the RII and RIIA sequences. The two repeat exons match the sequences of the two cDNAs perfectly, such that the more 5′ exon is identical to the nucleotide sequence 606–697 of Noda et al. (12) and is labelled exon N. The more 3′ exon is identical to the sequence 606–697 of Auld et al. (13) and is labelled exon A. Both exons have consensus sequences for 5′ and 3′ splice junctions, including the

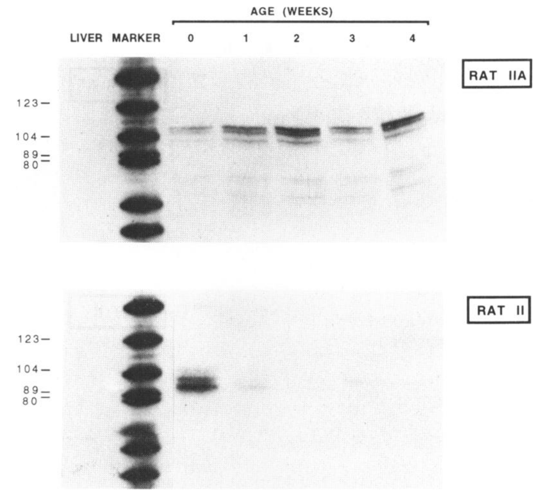

Figure 2. RIIA and RII mRNAs during post-natal development measured by RNAase protection. The first lane represents RNAase protection using liver RNA (30 μg) as a negative control. The sizes of labelled DNA markers are indicated on the left hand side of each panel. Lanes to the right of the marker depict the comparative levels of RIIA and RII mRNAs in the developing rat brain from 0 to 4 weeks as indicated. Gel-purified antisense riboprobes (315 bases for RIIA and 350 bases for RII) were hybridized to RNA (30 μg) prepared from 0 to 4 week post-natal rat brain. The RNA-RNA hybrids were treated with RNAase A and RNAase T1, and the protected fragments were separated on 8% polyacrylamide gels. Exposure was for 4 days, only the RII panel using an intensifying screen.

3′ splice-site CAG, the 5′ splice-site GTPuPu, and a pyrimidine rich sequence 5′ to the 3′ splice-site.

Figure 1A illustrates the proposed intron/exon organization for exons N and A that is indicated by the genomic sequence. The presence of adjacent exons N and A explains the clustered nature of the majority of nucleotide sequence differences between the coding regions of the RII and RIIA cDNAs. The RII mRNA results from splice selection of the N exon, while RIIA results from selection of the A exon. This alternative splicing appears to be mutually exclusive as we have not detected cDNAs with both exons in either cDNA cloning experiments or in PCR studies of mRNA. Indeed, the splice donor at the 3′ end of exon N splices between the first and second position of codon 233, while the splice acceptor at the 5′ end of exon A splices between the second and third position of codon 202. Thus joining of exon N to exon A would result in a frameshift of the channel coding sequence.

Exon N Selection Decreases and Exon A Selection Increases during Post-natal Development

Na^+ channel RII/RIIA α-subunit gene expression is detectable in the brain as early as embryonic day 10, and builds to a peak by post-natal day 7–10 (17, 18). This period of brain development in the rat is characterized by major cellular changes including neuron cell death, synaptogenesis, and myelin formation (19). If the two exons, N and A, impart unique properties to the two types of RII/RIIA channel and/or are expressed in distinct cell types, then the changes that occur during brain maturation might result in different requirements for exons N and A. We have examined this possibility by measuring the levels of exon N and exon A in developing rat brain using a sensitive RNAase protection assay. For this purpose, the two exon sequences were subcloned individually into plasmids with the T7 polymerase

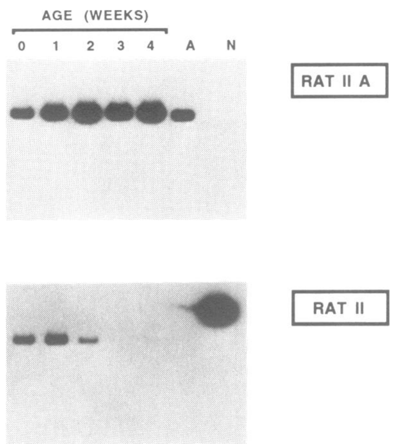

Figure 3. RIIA and RII mRNAs during post-natal brain development measured by PCR amplification of cDNAs. Lanes 0 to 4 in the upper panel depict the levels of exon A in PCR products obtained from 0 to 4 wk post-natal rat brain RNA preparations. The lower panel shows levels of exon N during the same developmental period. Lanes A and N represent controls for exons A and exon N respectively obtained by restriction of the plasmids pRSIIA and pRSIIN to yield fragments containing exon A and exon N respectively. First strand synthesis was carried out using 1 μg of total RNA from 0 to 4 wk post-natal rat brain and primer VA 23, specific for both RIIA and RII mRNAs. Amplification of the first strand cDNA with primers VA 23 and VA 20 yielded a product of approx. 400 bp. Ten percent of each PCR reaction was subjected to electrophoresis and transferred to Zeta-probe membrane. Duplicate blots were hybridized with 32P labelled oligonucleotide probes (25A and 25N) under conditions which selected for either RIIA or RII as described in Methods.

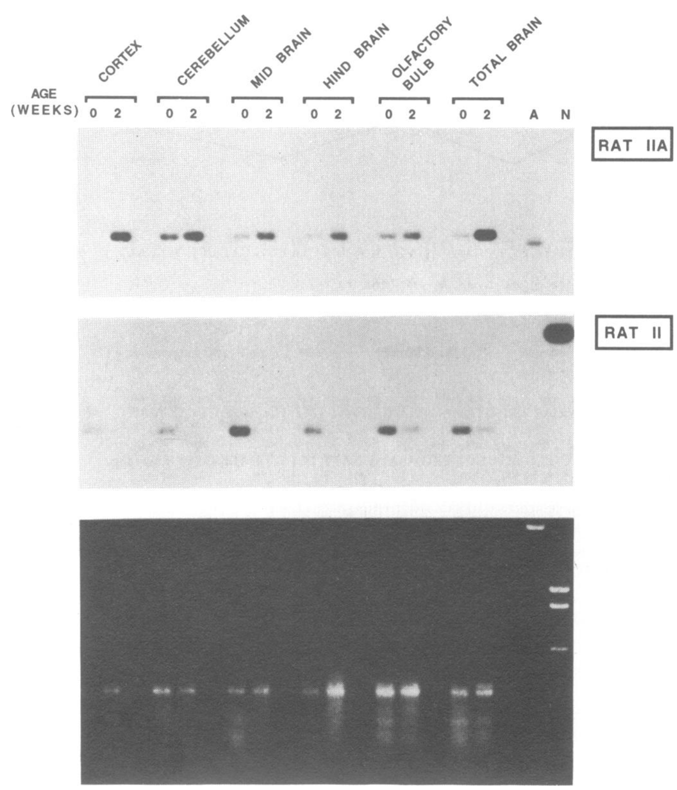

Figure 4. Regional distribution of RIIA and RII mRNAs using PCR amplification. The top panel shows the relative abundance of exon A containing PCR products at 0 and 2 week post-natal development in different parts of the brain as indicated. The middle panel shows the distribution of exon N and the bottom panel represents the ethidium bromide stained gel from which the blots were prepared. Lanes A and N show controls for exon A and exon N respectively (see legend for Figure 3) Total RNA was extracted from different regions of the brain at 0 and 2 weeks post-natal development. First strand synthesis was carried out using 1 μg of RNA and amplified and probed with exon A and exon N specific probes as described in Figure 3.

promoter and antisense transcripts complementary to each exon used to measure mRNA levels after hybridization and RNAase treatment.

Figure 2 shows the dramatic changes in the amounts of each exon measured in the RII/RIIA mRNA as brain development proceeds. In this assay, each mRNA derived from each exon should protect 92 residues of complementary RNA probe. The rat N probe protected the correct size fragment, but surprisingly the strength of the signal decreased markedly after birth, indicating that the RII mRNA is predominantly an early message that disappears soon after birth. On the other hand, the exon A probe protected three fragments of approximately 106, 105 and 100 nucleotides. The absence of protected fragments in rat liver RNA showed that these were brain specific, and we concluded that incomplete digestion of the exon A protected fragment at its extremities resulted in the multiple, closely migrating bands with the A probe (Fig. 2). The RIIA mRNA increased in abundance from birth, reaching a peak by 2–4 weeks. This pattern is similar to the previously reported pattern for the developmental expression of RII/RIIA mRNAs by Northern blot analysis (17, 18).

To confirm and extend these observations, we used a PCR strategy to detect the presence of the two exon sequences. In this procedure the two PCR primers, described above for amplification of the genomic fragment, were used to amplify complementary DNA sequences extending across nucleotides 529 to 937 of the RII/RIIA mRNA. The sequences of these primers are shared by both RII and RIIA mRNAs, but not by other rat α-subunit mRNAs. Amplification of cDNAs prepared from rat brain RNA of different ages produced a predominant fragment of 409 nucleotides in length (Figs. 3 and 4). The relative amounts of N and A exons in the 409 nucleotide product were then determined by hybridization with oligonucleotides specific for each form. Control plasmids (pRSIIA and pRSIIN as described in Materials and Methods) included on each DNA blot demonstrate the specificity of the hybridizations (Figs. 3 and 4). Although the quantitative estimates from PCR amplification are not necessarily linear, we ensured that amplifications were not taking place at saturating levels. We have found that increasing the template RNA from 1 μg to 2, 4, or 8 μg yielded increased signals for both RII and RIIA probes (results not shown). The relative levels and temporal changes in exon A and N observed in the PCR assay were qualitatively similar to the RNAase protection studies shown in Figure 2. Figure 3 shows that the exon N containing RII sequence PCR product was most abundant just after birth and then declined to the point were it was virtually absent at 4 weeks. Exon A containing PCR products gradually increase after birth, with the highest abundance obtained using RNA from the brain of the 4 week old rat.

Given the non-linear characteristics of PCR amplification, the assays in Figure 3 can be viewed as qualitative confirmation of the results presented in Figure 2. This confirmation is important because of the incomplete digestion observed for RNAase protection of the RIIA probe (Fig. 2). The strength of hybridization signals in Figure 3 indicate that mRNAs containing exons N and A are approximately equally abundant at birth, in agreement with the RNAase protection data shown in Figure 2. In Figure 2, the apparent higher strength of signal for RII protected fragments at the 0 week time point is due to the use of an enhancing screen to increase the relatively weak RII signals from the later time points (see legend to Figure 2). Thus both the RNAase protection and PCR assays indicate that the splicing of the two exons is developmentally controlled, with the exon N containing RII mRNA progressively replaced by the exon A containing RIIA mRNA as post-natal brain development proceeds.

RIIA and RII mRNAs Differ in Regional Distribution in the Neonatal Rat Brain

The observed shifts in the levels of RII and RIIA mRNAs in the developing rat brain through alternative splicing suggested that a modulation of exon selection in the RII/RIIA mRNA might be taking place in different regions of the brain. Previous reports have shown that Na^+ channel expression reaches a peak level by post-natal day 7–10 in developing rat brain (17, 18). Certain rostral regions of the brain maintained high levels of the message, while more caudal regions showed a marked decrease as development proceeded. To determine the relative regional distributions of RII and RIIA mRNAs, total RNA was extracted from various regions of the rat brain, viz. the olfactory bulb, cerebral cortex, midbrain, cerebellum and hindbrain, at birth and at 2 weeks post-natal. Using the PCR amplification protocol and hybridization with exon-specific probes as described above, the levels of RII and RIIA mRNAs were determined in various regions of the brain. As depicted in Figure 4, RII mRNAs were localized mostly to the midbrain (composed mainly of the hypothalamus and thalamus) and to the olfactory bulb at birth, but were barely detectable in the cortex, cerebellum and hindbrain at birth. All regions of the brain showed decreases in RII mRNA levels by 2 weeks after birth. In contrast, levels of RIIA mRNAs increased in all regions of the brain, with the greatest increase taking place in the cortex. The semi-quantitative results obtained by these experiments indicate that at birth RII mRNAs are expressed in abundance in the mid-brain, while increases in RIIA mRNAs by 2 weeks after birth take place most noticeably in the cortex and in the cerebellum.

DISCUSSION

RII and RIIA Isoforms

The studies described in this report were initiated when two closely related, but non-identical cDNA sequences were isolated for the RII/RIIA rat brain α-subunit (12, 13). When considering the origin of these two cDNAs, it is helpful to compare the two sequences first within the coding region and secondly within the 5′ and 3′ non-coding regions.

Within the coding regions, the majority of the nucleotide differences between the RII and RIIA cDNAs (20/36) result from the mutually exclusive selection of either exon N or exon A. The origins of the 16 nucleotide differences that lie outside the sequence of exon N/A are known only for a few positions. One of these, at nucleotide position 2580, was shown to result from a cDNA synthesis artifact in the original RIIA clone (20). Two others, at nucleotide positions 564 and 708, are included within the genomic sequence shown in Figure 1. In this sequence, position 564, which specifies Asn in the RII sequence, and asp in the RIIA sequence, is equivalent to the RIIA cDNA sequence, while the silent T/C difference in the third codon position of Thr at position 708 corresponds to the RII cDNA. It is probable that these and most of the remaining 14 nucleotide differences in coding sequence result from natural polymorphisms within the RII/RIIA gene.

The body of the sequence data supports the hypothesis that RII and RIIA transcripts derive from the same gene. The main supporting evidence is that within the two coding sequences the two cDNAs are identical throughout 6015 nucleotides except for 16 positions scattered through the sequence and for 20 positions within the alternatively selected exons A and N. We propose now to restrict the terms RII and RIIA to the two proteins produced from the RII/RIIA gene by the alternative splicing mechanism operating on nucleotides 606 to 697. In this scheme, the RII protein is defined as the protein encoded by the exon N containing transcript, the RIIA protein by the exon A containing transcript. These two forms differ at amino acid position 209 which is asparagine in RII and aspartate in RIIA. The original RIIA cDNA also differed at amino acid positions 189, 538, 579, 1062 and 1356 (13). We interpret these and the 11 silent nucleotide differences outside of exon N/A to the occurrence of nucleotide polymorphisms or cDNA synthesis errors.

The RII and RIIA cDNAs also diverged within both their 5′ and 3′ untranslated sequences. At the 5′ end, the divergence appears to result from a second alternative splicing process or from the use of alternative promoters (21). Our experiments indicate that the selection of one or the other of the two 5′ untranslated sequences is not correlated with the exon N/A splicing choice. Thus, in mature rat brain, the transcript with exon A is predominant, but is associated mainly with the RII 5′ untranslated sequence. Apparently, the 5′ non-coding sequences reported for the original RIIA cDNA do not appear on the majority of these transcripts.

The demonstration that the structure of the major isoform of the α-subunit is altered during brain development raises the question of functional differences in Na^+ channels which result from the expression of either the RII or RIIA α-subunit. Structural models of the α-subunit place the variant residue 209 on the extracellular surface of the membrane adjacent to proposed transmembrane helix S3 within the first homology domain (12, 22). In this position, the presence or absence of a negative charge at 209 might influence interactions of the α-subunit with elements at the surface of neurons. These elements could include other proteins in the membrane of the neuron, proteins on other cells or the extracellular matrix, or non-protein membrane components such as glycolipids. A second possibility is that the negative charge could have a direct influence on the properties of the ion channel itself. An example of this type of effect is the nicotinic acetylcholine receptor, where removal of negative charges adjacent to the M2 transmembrane helix affects channel permeability (23). In a similar way, if Na channel residue 209 is positioned adjacent to the ion pore, the presence of the negative charge encoded by exon A at the extracellular surface might increase channel permeability of RIIA relative to RII.

The expression pattern of the RII α-subunit containing exon N resembles the temporal expression of another rat brain α-subunit gene, RIII (17). The fact that both RII and RIII are expressed early in rat brain development may indicate that that these two isoforms are adapted for functions required in neurons within the nascent CNS.

Alternative selection of exons is a common feature of ion channel gene expression

Alternative RNA splicing can generate sequence diversity in multiple proteins encoded from single genes (24). The mutually exclusive splicing of the N and A exons is similar in many respects to an alternative splicing event reported for the Drosophila *para* Na^+ channel (25). Two short exons of 166 nucleotides are alternatively spliced into the *para* mRNA, and although these two exons differ at 34 nucleotide positions they encode only two amino acid changes. Thus, both RII and *para* undergo a mutually exclusive alternative splicing event that changes only one or two amino acid residues, respectively. Although the functional significance of these changes is not known in either case, mutagenesis studies indicate that even small changes in amino acid sequence can have marked effects on the properties of Na^+ channels (20, 26).

Previously, evidence for apparent alternative splicing of rat brain α-subunit mRNAs had been obtained from the sequence of cDNAs for two α-subunit genes, RI and RIII. In both cases, alternative splicing modifies the amino acid sequences that are proposed to form the cytoplasmic linkers that join two of the four homology regions. The modifications observed are insertions of 11 and 32 amino acids between the first and second homology domains of RI and RIII respectively (12, 27). Similar inserts have not been observed for RII/RIIA cDNAs to date.

Alternative RNA splicing has been described in many ion channel systems, most notably the *Shaker* class of K channel from Drosophila (4). Splicing in this case modifies the inactivation properties of the *Shaker* channel by altering the sequences of the amino and carboxyl regions of the *Shaker* protein. In this case, ample evidence for functional effects of the alternative splicing events has been obtained by expression of the different spliced transcripts in frog oocytes (28, 29, 30, 31). Similar studies are now needed for the two forms of the RII protein.

Mutually exclusive exon switching also occurs in the vertebrate Ca^{++} channels. Studies on the mRNA sequences of the IV homology domain of L-type Ca^{++} channels showed an alternative sequence encoding the S3 segment of this domain, and several other alternative exons following the S3 (32, 33, 34). Although the number of splicing variants is greater than we have observed in the RII Na^+ channel, many of the Ca^{++} channel variations do occur in the amino acids that follow the S3 transmembrane segment. This concentration of sequence variability in several types of channel protein localized to a segment with proposed structural similarities suggests that the region following S3 may have a critical role in molding the properties of these channels for their specific roles in neuronal function.

Mutually Exclusive Splicing of Exons N and A

Two questions arise from the demonstration of alternative exon selection by the RII/RIIA gene: first, what causes the alternative splicing pattern to alter during neuronal development and secondly, what ensures the mutual exclusivity of the splicing event (i.e. exons N and A remain incompatible with one another during the splicing of pre-mRNAs)? The alteration in levels of RII mRNAs containing exon N or exon A is probably trans-directed. Emerging studies of the ribonucleoprotein components of the spliceosome and their function in the splicing event suggest that protein-RNA interactions mediate the regulation of alternative splicing (35, 36). From studies of 3′ site selection of the Drosophila genes P-transposase and *tra*, selection of the appropriate 3′ acceptor site is accomplished by the binding of an inhibitory factor to prespliced transcripts (37, 38, 39). Analogous examples of repressors directing 3′ site selection have been demonstrated for mammalian genes including CGRP and β-tropomyosin (40, 41). Thus a possible explanation for the reduction of exon N selection is that a repressor of splicing is produced in the maturing neurons of the CNS and that it progressively reduces the amount of splicing to the 3′ site on exon N.

The mutual incompatibility of exons N and A, on the other hand, is probably cis-directed. Since no products containing both exon N and exon A were detected by the highly sensitive PCR amplification process, it is probable that the splicing of exon N to exon A does not take place. Thus, although both N and A 3′ splice sites can be selected on different transcripts, they cannot both be selected on the same transcript. The nucleotide sequence of the small intron A-N suggests a mechanism for this restriction of splice site selection. Studies on mammalian branch-point locations have shown that the minimum distance between the branch-point and the 5′ splice site is at least 70–80 nucleotides. Examination of intron A-N indicates two possible branch points at positions 31–37 nt and 60–66 nt downstream of the 5′ splice site of exon N (UCCUUAU and CUGUCAU respectively). The branch-point 31–37 nts downstream of the 5′ splice site of exon N matches the mammalian consensus sequence YNYURAY 5/6, while the sequence at 60–66 downstream matches only 4/6. Therefore, the best consensus branch point is at 31–37, but it is too close to the 5′ splice site to be used efficiently in the formation of the lariat. Thus use of the strong branch-point exclusively over the weaker would select for the more distant upstream 5′ site and exclude the too closely situated A intron 5′ site . This situation, where the branch point is too close to the 5′ splice site of an exon to be used efficiently, has been well documented in mutually exclusive mRNA splicing of α-tropomyosin transcripts (42).

Although originally thought to be relatively uniform in their biophysical properties, it is becoming evident that many subtypes of Na^+ channel exist in mammalian neurons. For the α-subunit, this diversity is generated by expression from different genes, and as suggested in this report, by changes in the selection of alternative exons. In addition, post-translational differences in carbohydrate modification and phosphorylation increase the functional repertoire of the Na^+ channel. Thus the neuron can select channels with properties that will produce Na^+ currents with temporal, regional and spatial properties that are appropriate for the particular electrical activity of each cell. For the RII/RIIA α-subunit, this process must involve the selection of the alternative exons N and A, probably through the action of trans-acting factors that effect 3′ splice site selection.

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REFERENCES

1. Hodgkin, A. and Huxley, A. (1952) *J Physiol.*, **117**, 500–544.
2. Hille, B., *Ionic channels of excitable membranes* (Sinauer Associates, Sunderland, MA, 1984).
3. Rudy, B. (1988) *Neuroscience*, **25**, 729–750.
4. Jan, L. Y. and Jan, N. Y. (1990) *Trends Neurosc.*, **13**, 415–419.
5. Schwarz, T. L., Tempel, B. L., Papazian, D. M., Jan, Y. N. and Jan, L. Y. (1988) *Nature*, **331**, 137–142.
6. Butler, A., Wei, A., Baker, K. and Salkoff, L. (1989) *Science*, **243**, 943–947.
7. Wei, A., Covarrubias, M., A., B., Baker, K., Pak, M. and Salkoff, L. (1990) *Science*, **248**, 599–603.
8. Rogart, R. B. (1986) *Annals New York Acad. Sci.*, **479**, 402–430.
9. Gilly, W. F. and Armstrong, C. M. (1984) *Nature*, **309**, 448–450.
10. Llinas, R. and Sugimori, M. (1980) *J. Physiol.*, **305**, 171–195.
11. Llinas, R. and Sugimori, M. (1980) *J. Physiol.*, **305**, 197–213.
12. Noda, M., Ikeda, T., Kayano, T., Suzuki, T., Takeshima, H., Kurasaki, M., Takahashi, T. and Numa, S. (1986) *Nature*, **320**, 188–192.
13. Auld, V. J., Goldin, A. L., Krafte, D. S., Marshall, J., Dunn, J. M., Catterall, W. A., Lester, H. A., Davidson, N. and Dunn, R. J. (1988) *Neuron*, **1**, 449–461.
14. Kayano, T., Noda, M., Flockerzi, V., Takahashi, H. and Numa, S. (1988) *FEBS. Lett*, **228**, 187–194.
15. Sambrook, J. and Fritsch, E. F., *Molecular cloning: a laboratory manual,* (Cold Spring Harbour Press, Cold Spring Harbour, NY, 1989).
16. Chirgwin, J. M., Pryzybyla, A. E., MacDonald, R. J. and Rutter, W. J. (1979) *Biochemistry*, **18**, 5294–5299.
17. Beckh, S., Noda, M., Lubbert, M. and Numa, S. (1989) *EMBO J.*, **8**, 3611–3616.
18. Scheinman, R. A., Auld, V. J., Goldin, A. L., Davidson, N., Dunn, R. J. and Catterall, W. A. (1989) *J. Biol. Chem.*, **264**, 10660–10666.
19. Jacobson, M., *Developmental Neurobiology* (Holt, Rinehart and Winston, Inc., New York, 1970).
20. Auld, V. J., Goldin, A. L., Krafte, D. S., Catterall, W. A., Lester, H. A., Davidson, N. and Dunn, R. J. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 323–327.
21. Ahmed, C. M. I., ııAuld, V. J., Lester, H. A., Dunn, R. and Davidson, N. (1990) *Nucl. Acids. Res.*, **18**, 5807.
22. Guy, H. R. and Conti, F. (1990) **13**, 201–206.
23. Imoto, K., Busch, C., Sakmann, B., Mishina, M., Konno, T., Nakai, J., Bujo, H., Mori, Y. and Fukuda, K. N., S. (1988) *Nature*, **335**, 645–648.
24. Leff, S. E., Rosenfeld, M. G. and Evans, R. M. (1986) *Ann. Rev. Biochem.*, **55**, 1091–1117.
25. Loughney, K., Kreber, R. and Ganetzky, B. (1989) *Cell*, **58**, 1143–1154.
26. Stuhmer, W., Conti, F., Suzuki, H., Wang, X. D., Noda, M., Kubo, H. and Numa, S. (1989) *Nature*, **339**, 597–603.
27. Joho, R. H., Moorman, J. R., VanDongen, A. M. J., Kirsch, G. E., Silberberg, H., Schuster, G. and Brown, A. M. (1990) *Mol. Brain Res*, **7**, 105–115.
28. Timpe, L., Schwartz, T., Tempel, B., Papazian, D., Jan, Y. and Jan, L. Y. (1988) *Nature*, **331**, 143–145.
29. Timpe, L., Jan, Y. and Jan, L. (1988) *Neuron*, **1**, 659–667.
30. Iverson, L., Tanouye, M., Lester, H., Davidson, N. and Rudy, B. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 5723–5727.
31. Stocker, M., Stuhmer, W., Wittka, R., Wang, X., Muller, R., Ferrus, A. and Pongs, O. (1990) *Proc. Nat. Acad. Sci. U.S.A.*, **87**, 8903–8907.
32. Koch, W. J., Ellinor, P. T. and Schwartz, A. (1990) *J. Biol. Chem*, **265**, 17786–17791.
33. Perez-Reyes, E., Wei, X., Castellano, A. and Birnbaumer, L. (1990) *J. Biol. Chem.*, **265**, 20430–20436.
34. Snutch, T., Tomlinson, W., Leonard, J. and Gilbert, M. (1991) *Neuron*, **7**, 45–57.
35. Padgett, R., Grabowski, P., Konarska, M., Seiler, S. and Sharp, P. (1986) *Ann. Rev. Biochem.*, **55**, 1119–1150.
36. Maniatis, T. (1991) *Science*, **251**, 33–34.
37. Sosnowski, B. A., Belote, J. M. and McKeown, M. M. (1989) *Cell*, **58**, 449–459.
38. Inoue, K., Hoshijima, K., Sakamoto, H. and Shimura, Y. (1990) *Nature*, **344**, 461–463.
39. Siebel, C. and Rio, D. (1990) *Science*, **248**, 1200–1208.
40. Emeson, R. B., Hedjran, F., Yeakley, J. M., Guise, J. W. and Rosenfeld, M. G. (1989) *Nature*, **341**, 76–80.
41. Helfman, D., Roscigno, R., Mulligan, G., Finn, L. and Weber, K. (1990) *Genes Dev.*, **4**, 98–110.
42. Smith, C. W. J. and Nadal-Ginard, B. (1989) *Cell*, **56**, 749–758.