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Alternative RNA Splicing of the NMDA Receptor NR1 mRNA in the Neurons of the Teleost Electrosensory System

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The sequence for cDNA encoding the NMDA receptor subunit 1 (aptNR1) of the weakly electric fish *Apteronotus leptorhynchus* has been determined. The deduced amino acid sequence is ~88% identical to other vertebrate NR1 proteins, with sequence homology extending to the alternatively spliced cassettes N1 and C1. The fish and mammalian N1 and C1 splice cassettes are identical at 20 of 21 and 30 of 37 amino acid positions, respectively. We did not detect a C2 splice cassette in aptNR1 mRNA, but we did find two novel C-terminal alternative splice cassettes labeled C1′ and C1″.

The relative levels of NR1 transcripts containing the N1 and C1 splice cassettes were determined by using RNase protection and *in situ* hybridization analysis. N1-containing mRNAs are more abundant in caudal brain regions, similar to the patterns reported for mammalian brain. In contrast, the relative levels of transcripts containing the C1 splice cassette are much lower in fish than in mammals, averaging only 9% for the whole brain. The levels of C1 splicing increased in more rostral brain regions. *In situ* hybridizations with N1- and C1-specific probes demonstrated that N1 cassette splicing occurs in most neurons but that C1 splicing is heterogeneous and is restricted to a subset of neuronal types in the electrosensory system.

Key words: NMDA receptor; electrosensory system; NR1 subunit; RNA splicing; evolution; fish neurons

Glutamate is the principal excitatory transmitter in the vertebrate brain. The receptors for glutamate are classified broadly into families of ionotropic and metabotropic receptors, the ionotropic receptors subclassified as either AMPA/kainate or NMDA types. The NMDA types are distributed throughout the CNS and display a unique combination of voltage sensitivity and calcium ion permeability that is important for neuronal differentiation, synaptic plasticity, and neurotoxicity.

In mammals the NMDA receptor is an oligomeric protein complex containing the obligatory NR1 subunit and one or more of the four NR2 subunits (Hollmann and Heinemann, 1994). Gene expression from the single NR1 gene generates nine different NR1 receptor subunit proteins via the use of alternative RNA splice cassettes (Zukin and Bennett, 1995). In contrast, each of the NR2 subunits NR2A/2B/2C/2D is encoded by a different gene. (Ikeda et al., 1992; Kutsuwada et al., 1992; Meguro et al., 1992; Monyer et al., 1992; Ishii et al., 1993). The voltage dependencies and kinetics of receptor responses depend on which of the NR1 splice variants and NR2 subunits are assembled into the receptor complex. Mapping of the NMDA receptor subunits in rat brain has revealed a complex mosaic of the NR1 splice variants and NR2 subunits that is believed to tailor synaptic responses to the requirements of different neural networks (Ishii et al., 1993; Buller et al., 1994; Laurie and Seeburg, 1994; Monyer et al., 1994; Petralia et al., 1994; Standaert et al., 1994, 1996; Landwehrmeyer et al., 1995; Laurie et al., 1995; Dunah et al., 1996; Johnson et al., 1996; Paupard et al., 1997).

NR1 subunits have been characterized by molecular cloning from rat (Moriyoshi et al., 1991), mouse (Yamazaki et al., 1992), human (Planells-Cases et al., 1993), duck (Kurosawa et al., 1994), and *Xenopus* (Soloviev et al., 1996). The rat NR1 gene contains 22 exons, four of which (exons 3, 5, 21, and 22) undergo alternative RNA splicing to generate nine splice variants (Anantharam et al., 1992; Sugihara et al., 1992; Durand et al., 1993; Hollmann et al., 1993). Splice insertion of the cassette N1 (exon 5) in the N-terminal region of NR1 yields NMDA receptors with reduced agonist affinity, increased current amplitudes, decreased sensitivity to proton inhibition, altered responses to activated PKC, and a failure to respond to Zn^{2+} and polyamines (Durand et al., 1993; Hollmann et al., 1993; Zhang et al., 1994; Zheng et al., 1994; Paoletti et al., 1995; Traynelis et al., 1995). The C1 cassette (exon 21) contains major regulatory sites phosphorylated by protein kinases A and C (Tingley et al., 1993, 1997; Leonard and Hell, 1997) and the high-affinity calmodulin binding site (Ehlers et al., 1996). Phosphorylation of the receptor potentiates the NMDA currents, possibly via an inhibition of calmodulin binding to the C1 segment (Tingley et al., 1993, 1997; Tong et al., 1995; Ehlers et al., 1996; Hisatsune et al., 1997). C1 also may play a role in the subcellular targeting of NMDA receptors (Ehlers et al., 1995). Thus alternative splicing at the C-terminal segment of subunit NR1 can affect both subcellular localization and modulation of the NMDA receptor.

We are studying the molecular mechanisms that are critical for the extraction of specific sensory features by the neurons of the electrosensory system of the electric fish *Apteronotus leptorhynchus*. NMDA receptor-mediated neurotransmission is central to this process, as it is for the mammalian sensory systems (Bastian,

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1993, 1995; Daw et al., 1993; Collingridge and Bliss, 1995). A critical question concerns the extent to which the features of NMDA receptor structure and regulation have been conserved since the divergence (≈400 million years ago) of teleosts such as *Apteronotus* from the vertebrate lineage leading to mammals. In a previous study we have shown that an mRNA encoding the NR1 subunit is distributed broadly in the *Apteronotus* CNS and is expressed by many neurons of the electrosensory system (Bottai et al., 1997). We now have determined the full structure of the fish NR1 protein, including the sequences of the alternatively spliced cassettes. The patterns of alternative RNA splicing of aptNR1 in the fish CNS provide evidence for neuron-specific control of NR1 splice cassette selection and for evolutionary conservation of the regulation of this process.

MATERIALS AND METHODS

RNA isolation

Tissue samples were dissected from *A. leptorhynchus* liver, brain, and subregions of the brain, including electrosensory lateral line lobe (ELL), cerebellum, forebrain, brainstem, midbrain (optic tectum plus torus semicircularis), and hypothalamus. Tissues were frozen in liquid nitrogen immediately after dissection, and RNA was prepared by extraction with TRIzol reagent (Life Technologies, Gaithersburg, MD), as described by Chomczynski and Sacchi (1987). The RNA concentration was estimated from UV absorbance at 260 and 280 nm.

Isolation of cDNAs

A. leptorhynchus brain cDNA libraries were constructed in λZap II and HybriZap (Stratagene, La Jolla, CA). These libraries were probed with the rat NMDAR1 cDNA nucleotides (nt) −269 to 3500 (Moriyoshi et al., 1991) and the 768 bp partial aptNR1 cDNA previously reported [nt 158–915 (Bottai et al., 1997)]. Hybridization-positive phages were purified, and the cDNA inserts were analyzed by DNA sequence analysis.

The central region of the AptNR1 cDNA (nt 1576–2543) was recovered by PCR amplification from cDNA prepared from *A. leptorhynchus* brain mRNA. The cDNA was prepared with the reagents supplied in the Superscript Preamplification System (Life Technologies). Fish brain mRNA (1 μg) was annealed with 150 ng of random hexamers in an 11 μl reaction. After being heated at 70°C for 10 min, the reaction was chilled in ice for 1 min and then diluted to a final volume of 20 μl containing (in mM) 20 Tris-HCl, pH 8.4, 50 KCl, 2.5 $MgCl_2$, and 2.5 dNTPs plus 0.01 M dithiothreitol (DTT). After 5 min at 25°C, 200 U of Superscript II RT were added, and the solution was incubated for 10 min at 25°C and for 50 min at 42°C. The enzyme was heat-inactivated at 70°C for 15 min, the reaction was chilled on ice, and 2 U of RNase H were added and then incubated at 37°C for 20 min. The PCR step was performed in (in mM) 20 Tris-HCl, pH 8.4, 50 KCl, and 1.5 $MgCl_2$ plus 200 μM dNTPs, a 5 μM concentration of the two primers Rob 1 and 2, and 1.25 U of Tsg DNA polymerase (Sangon, Canada). The cycle conditions included one cycle at 94°C/2 min and 30 cycles at 94°C/1 min, 56°C/1.5 min, and 72°C/2.5 min, with a final extension at 72°C/10 min. The products were analyzed by agarose gel electrophoresis, gel-purified, and subcloned in pGemT vector (Promega, Madison, WI).

The 5′ terminus of the cDNA was recovered by using the 5′ race system for rapid amplification of cDNA ends (RACE), version 2.0 (Life Technologies). Primer Dano 26 (2.5 pmol) was mixed with 1 μg of brain mRNA in a 14 μl vol of H_2O. The mixture was denatured for 10 min at 70°C and chilled in ice. cDNA synthesis was performed in 20 μl of (in mM) 20 Tris-HCl, pH 8.4, 50 KCl, 2.5 $MgCl_2$, and 0.4 each dNTPs plus 0.01 M DTT, and 200 U Superscript II RT at 42°C for 50 min. The enzyme was heat-inactivated at 70°C for 15 min and chilled in ice; then RNase H (2 U) was added and incubated at 37°C for 30 min. The cDNA was purified through a Glass Max DNA isolation spin cartridge and recovered in 50 μl of H_2O.

The 5′ tailing reaction was performed by incubation (37°C/10 min) of 10 μl of the purified cDNA product in PCR buffer [(in mM) 10 Tris-HCl, pH 8.4, 25 KCl, and 1.5 $MgCl_2$] containing 20 μM dCTP and 1 μl of terminal transferase in a final volume of 25 μl. The reaction mixture was heat-inactivated at 65°C for 10 min and chilled on ice. The 5′ RACE product was amplified from the tailed cDNA, using primers Dano 27 and Abridge Anchor Primer. The reaction contained (in mM) 20 Tris-HCl, pH 8.4, 50 KCl, and 1.5 $MgCl_2$ plus 200 μM each dNTPs, 400 nM primers, 1/10 dilution of the cDNA, and 2.5 U of Tsg polymerase. The cycle conditions included one cycle at 94°C/2 min, 35 cycles at 94°C/45 sec, 55°C/1 min, and 72°C/1.5 min, and the last cycle at 94°C/45 sec, 55°C/1 min, and 72°C/7 min. The single product was purified by agarose gel electrophoresis and cloned into vector pGemT (Promega).

Isolation of alternatively spliced cDNAs

N1 cassette. Brain RNA was treated with DNase I (50 μg/ml) in 2 mM $MgCl_2$ for 30 min at 4°C. The reaction was stopped with 5 mM EDTA and then heat-inactivated at 65°C for 10 min. cDNA synthesis (0.2 μg of RNA) was performed in 50 mM KCl, 10 mM Tris-HCl, pH 9.0, 1% Triton X-100, 0.01 M DTT, 200 μM dNTPs, 0.16 μM primer Dano 9, 25 U of RNasin, and 200 U of Superscript II RT at 42°C for 1 hr. After heat inactivation (95°C/5 min), the cDNA was mixed in 50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1% Triton X-100, 2 mM $MgCl_2$, 200 μM dNTPs, 0.2 μM primers Dano 8 and Dano 9, and 2.5 U of Taq DNA polymerase (Promega). The cycle conditions were one cycle at 94°C/1 min, 40 cycles of 92°C/1 min, 55°C/1.25 min and 72°C/2.5 min, with the last cycle of 92°C/1 min, 55°C/1.25 min, and 72°C/7 min. The products were analyzed by agarose gel electrophoresis, gel-purified (Micropure separators, Amicon, Beverly, MA), and subcloned in pGemT.

C-terminal segments. cDNAs for the C-terminal RNA splice isoforms were isolated by PCR amplification, using primers on the 5′ (Dano 20) and 3′ (Dano 21, 22) sides of the junction region. The cDNA synthesis and PCR amplifications were performed as described above for the isolation of the central region. Dano 22 is located in the C1 cassette. The program PCR Primer (DNASTAR, Madison, WI) was used to design PCR primers.

RNase protection assay

Probes for RNase protection were prepared from cDNA segments inserted into the vector pGemT. The cDNA segments included (1) a 446 bp *Bam*HI–*Bam*HI fragment (363–808) product containing the N1 cassette; (2) a 328 bp fragment (2765–3092) (primers 23 and 21) containing the C1 cassette; and (3) a 185 bp fragment (2667–2851) (primers 25 and 24) containing C1′–C1″–C1 cassettes. The ^{32}P-labeled RNA probes were prepared by transcription with SP6 or T7 RNA polymerase (Promega), using the conditions recommended by the manufacturer. The RNA probes were purified by electrophoresis as described (Bottai et al., 1997). The hybridization solution contained 40 mM PIPES, pH 6.4, 0.4 M NaCl, 1 mM EDTA, and 80% formamide. For each reaction 5 μg of total RNA was combined with 10^6 cpm of labeled probe and hybridized overnight at 55°C. Nonhybridized RNA was digested with RNase A (40 μg/ml) and RNase T1 (2 μg/ml) for 1 hr at 37°C. Samples were treated further with SDS (0.04%) and proteinase K (90 μg/ml) for 30 min at 37°C. The protected RNA/RNA duplexes were denatured and separated by electrophoresis in a 5% polyacrylamide/8 M urea gel. After drying, the gels were exposed to x-ray films (BioMax MR, Kodak, Rochester, NY) for 35–60 hr with an intensifier screen at −80°C.

Oligonucleotide primers

The RT-PCR N1 cassette included Dano 9, tgtacacagcggcagcatcctctt; Dano 8, ctcacgcccaccccagtctccta; Dano 10, gggagaccaagaataaaaa; and Dano 11, tgccttgggtcctcgcttg. The RT-PCR for 3′end splice isoforms included Dano 20, gctacaagcgccacaaagac; Dano 21, agagaagttcccgctggtagtt; Dano 22, ggccaccagcatcaagag; Dano 23, agagcagacccagaacc; Dano 24, ggatgacctacgtctcttg; and Dano 25, acaataaggaaagtgctgggagt. The Race PCR included Dano 26, acgtgcgcctggtgagc; Dano 27, cggtacgtaggaaggaaaggtggat; and Abridge Anchor, ggccacgcgtcgactagtacgggiigggiigggiig. The RT-PCR for the central domain included Rob1, ggagccgttcgtgtatgtga; and Rob2, gctggcctgtgatgtgtgtg.

Northern blot

RNA (20 μg) from brain and liver was subjected to electrophoresis in a formaldehyde agarose gel (1%). The RNA was blotted to nylon membrane (Hybond N^+; Amersham Life Science, Arlington Heights, IL) and hybridized to 1.3 kb of fish NR1 cDNA at 42°C in 50% formamide, 40 mM phosphate buffer, pH 6.8, 5× SSC, 1% SDS, 1% glycine, 5× Denhardt's, 100 μg/ml herring sperm DNA, and 100 μg/ml tRNA. The membrane was washed twice at room temperature for 5 min with 2× SSC/0.1% SDS, twice at room temperature with 0.2× SSC/0.1% SDS for 5 min, and 15 min at 42°C with 0.2× SSC/0.1% SDS. The membrane was

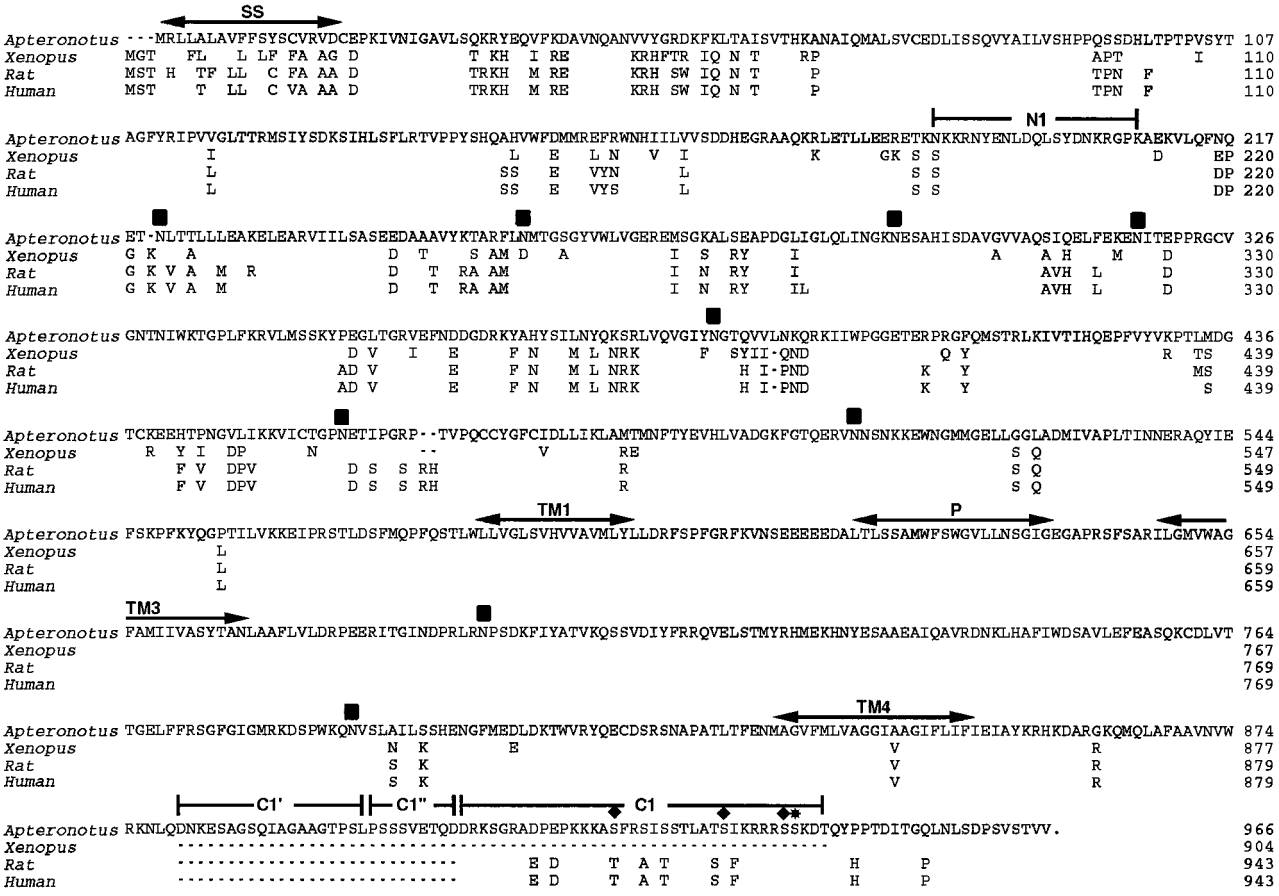

Figure 1. Sequence comparison of the NR1 proteins. The *Apteronotus* NR1 protein sequence is shown and compared with NR1 sequences of *Xenopus* (Soloviev et al., 1996), rat (Anantharam et al., 1992), and human (Foldes et al., 1993; Karp et al., 1993; Planells-Cases et al., 1993). Residues that are identical to the aptNR1 sequence are omitted in the other sequences. Positions of deletions are indicated by *dashes*. The amino acid positions are indicated on the *right*. *TM1*, *TM3*, and *TM4* indicate the putative transmembrane segments, and *P* indicates the proposed pore segment. Glycosylation (*filled squares*), protein kinase C (*filled diamonds*), and protein kinase A (*filled star*) are indicated *above* the aptNR1 sequence. *N1*, *C1*, *C1′*, and *C1″* indicate the positions of the alternatively spliced cassettes.

exposed overnight to x-ray film (BioMax MR, Kodak) with an intensifier screen at −80°C.

In situ *hybridization*

In situ hybridization was performed by using a modified version of the protocol of Simmons et al. (1989) as described previously (Bottai et al., 1997). For the N1 cassette an RNA probe was transcribed from a 77 bp (nt 579–656) segment of NR1 cDNA that was amplified by PCR with the primers Dano 10 and 11. The probe for the C1 cassette experiment was transcribed from an 87-bp-long NR1 cDNA fragment (nt 2764–2851; primers Dano 23 and 24). All glassware was baked for 4 hr at 250°C; distilled water for all solutions was treated with 0.1% diethylpyrocarbonate (DEPC) and then autoclaved.

After hybridization the slides were air-dried and apposed to x-ray film (Kodak BioMax MR film); after 2–5 d the film was developed, and the slides were dipped in emulsion (1:1 dilution of NTB2 gel in 600 mM ammonium acetate) and exposed for 15 d. The slides were developed and lightly counterstained with neutral red. Approximately one-half of the slides routinely were coverslipped with Permount; the other one-half were coverslipped with glycerol in PBS to permit the use of differential interference contrast (DIC) microscopy, which facilitated the identification of specific cell types.

Slide autoradiographs were photographed on an Olympus microscope, and the film was scanned (1500 dpi; Polaroid SprintScan). Montages were made with Photoshop 4 and Illustrator 7. Cell groups were identified with reference to an atlas of the brain of *A. leptorhynchus* (Maler et al., 1991). The only image processing used was to adjust the output range in Photoshop to match the input range. Quantitative analysis was done on the emulsion-coated material. Individual cells were selected under DIC; grain counts and cell areas were measured, and grain densities were computed with National Institutes of Health Image software. Background grain densities (measured in the sense controls) were subtracted from all measurements. The results were analyzed by ANOVA, using Statistica on a Macintosh computer.

RESULTS

Sequence conservation of NR1 proteins expressed by fish and mammals

The NR1 cDNAs were identified from *Apteronotus* cDNA libraries by hybridization with rat NR1 probes. Initially, two cDNAs were recovered, encoding 1.2 kb of sequence at the 5′ region of the mRNA and 1.3 kb of 3′ sequence that included the C-terminal region of the mRNA and 1175 nt of 3′ nontranslated mRNA sequence. The remainder of the cDNA was obtained by a combination of PCR approaches. The central segment (nt 1576–2543) was isolated by RT-PCR, using primers from the termini of the previously cloned cDNAs. The 5′ terminal sequence was obtained by using the RACE PCR approach (Frohman et al., 1988) to obtain a product of 550 bp that extended the cDNA sequence in the 5′ direction, as described in Materials and Methods. The complete nucleotide sequence of the aptNR1 cDNA has been deposited in GenBank (accession number AF060557).

Figure 1 presents the sequence of aptNR1, the predicted protein product encoded in the sequence of the *Apteronotus* NR1

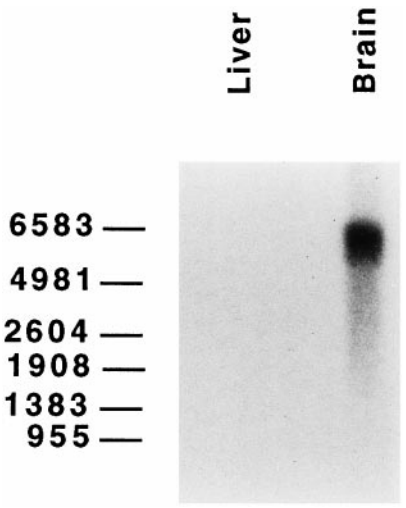

Figure 2. Analysis of aptNR1 mRNAs. RNA samples (20 μg) from liver and brain were fractionated by formaldehyde agarose gel electrophoresis, and the Northern blot was probed with a 1.3 kb fragment of the aptNR1 cDNA. RNA size markers are indicated on the *left*.

cDNA. The first methionine codon in this sequence is preceded by 29 nt of 5′ nontranslated sequence and is designated as the presumptive initiation codon. The nucleotide sequence context of this methionine codon matches the Kozak consensus sequence at 6 of 10 positions (agCACaAUGc), including the critical adenosine residue at position −3 (Kozak, 1991). This methionine corresponds to the third amino acid of the predicted *Xenopus*, rat, and human sequences (Moriyoshi et al., 1991; Foldes et al., 1993; Karp et al., 1993; Zimmer et al., 1995; Soloviev et al., 1996), indicating that the fish NMDAR1 protein is slightly shorter at the N terminus than the previously described NR1 sequences.

Figure 1 illustrates the high level of sequence conservation between aptNR1 and its higher vertebrate homologs. The aptNR1 amino acid sequence, excluding the alternatively spliced exons, is identical at 87.6, 88.3, and 88.7% positions when compared with *Xenopus*, rat, and human sequences, respectively. Sequence homologies are most pronounced in the carboxyl half of the protein, where only eight amino acid positions are divergent (homology >98%) in the segment stretching between aptNR1 amino acid 486 and the site of C-terminal alternative splicing. This highly conserved segment includes the three proposed transmembrane segments, the pore segment and the ligand-binding domains S1 (defined as the 150 amino acids N-terminal to TM1) and S2 (defined as the extracellular loop between TM3 and TM4) (O'Hara et al., 1993; Kuryatov et al., 1994; Stern-Bach et al., 1994). The very high sequence conservation of the segments S1 and S2 in contrast to the much lower sequence conservation at the N terminus (amino acids 1–485) suggests that strong functional constraints have acted to maintain the sequence and structure of these segments.

Figure 2 illustrates a Northern blot analysis of the aptNR1 mRNA. In this experiment the probe included nucleotides corresponding to the 3′ nontranslated sequence and a short segment (200 nt) of the C-terminal coding sequence, which should hybridize to all forms of the alternatively spliced aptNR1 mRNA described below. The brain mRNAs that hybridize to this probe appear as a broad band of ~6000–7000 nt in length and are absent in the hybridization to liver mRNA. The broad nature of the signal in brain RNA suggests that there is considerable heterogeneity in the lengths of the aptNR1 mRNAs, possibly resulting from a combination of alternative RNA splicing and the use of alternative polyadenylation signals. Alternative RNA splicing within the coding segments of the mRNAs is described below.

N1 alternative RNA splicing in the electric fish CNS

Mammalian NR1 genes exhibit two alternatively spliced N-terminal splice isoforms that differ in the presence or absence of the short 21 amino acid N1 sequence (Anantharam et al., 1992; Durand et al., 1992; Nakanishi et al., 1992). To determine whether N1 alternative RNA splicing is conserved in *Apteronotus*, we identified cDNAs for both alternatively spliced transcripts for aptNR1, using a PCR approach. Primers flanking the presumed splice junction were used to amplify cDNAs prepared from *Apteronotus* brain RNA; two PCR products were obtained. DNA sequence analysis confirmed that these represented the fish homologs of the $N1^-$ and $N1^+$ variants observed for mammalian NR1 subunits. Analysis of the intensity of these two PCR products after electrophoresis indicated that the $N1^-$ form was 5–10 times more abundant than the $N1^+$ form. As shown in Figure 1, 20 of the 21 amino acids of the aptNR1 N1 sequence are identical to those found in *Xenopus*, rat, and human NMDAR1, which identifies the N1 insert sequence as one of the most highly conserved segments within the N-terminal region of the protein.

To provide a more reliable estimate for the relative amounts of $N1^-$ and $N1^+$ mRNAs than the estimate obtained from the PCR assay, we analyzed selected brain regions, using an RNase protection assay that differentiated between the two splice isoforms. The RNA probe was complementary to 446 nt of aptNR1 mRNA that included the 63 nt N1 sequence (Fig. 3*A*). The mRNAs containing the $N1^+$ insert were predicted to protect the full 446 nt of this probe, whereas $N1^-$ mRNAs should protect two smaller fragments of 156 and 227 nt. Figure 3*B* illustrates the results obtained when this assay was applied to RNAs prepared from different brain regions. Quantitation of the relative amounts for $N1^-$ and $N1^+$ are given in Table 1. With total brain RNA, strong signals were detected for both the $N1^+$ and $N1^-$ products, with the $N1^-$ form approximately five times more abundant than the $N1^+$ form. In control assays, no signal was detected in the liver RNA sample.

Although the $N1^-$ form is predominant in all regions of the brain, the relative amount of the $N1^+$ form varies between regions such that the relative amounts of $N1^+$ transcript increase in more caudal regions of the brain, from a level of 18% in forebrain to 43% in brainstem. The hypothalamus expresses a low level of the $N1^+$ mRNA. In the electrosensory relay nucleus (ELL), where we have shown previously that pyramidal cells express high levels of aptNR1 mRNA (Bottai et al., 1997), the $N1^+$ insert is present in approximately one-fourth of these transcripts. This result indicates that the first steps of electrosensory processing involve a significant fraction of the NMDA receptors with properties that depend on the presence of the N1 peptide insert.

The cerebellum of *Apteronotus* contains a only a small amount of aptNR1 mRNA, <1% of the amount in forebrain (Bottai et al., 1997). To facilitate the analysis of the N1 splicing in cerebellum, we increased the amount of input RNA fourfold for the RNase protection experiment (Fig. 3*B*). The results show that, although the NR1 gene is expressed at only low levels in cerebellum, 50% of the NR1 mRNAs contain the N1 insert, a ratio that is higher than in any other brain area. In addition, a novel fragment of ~200 nt is detected in the cerebellar RNA. This fragment also is

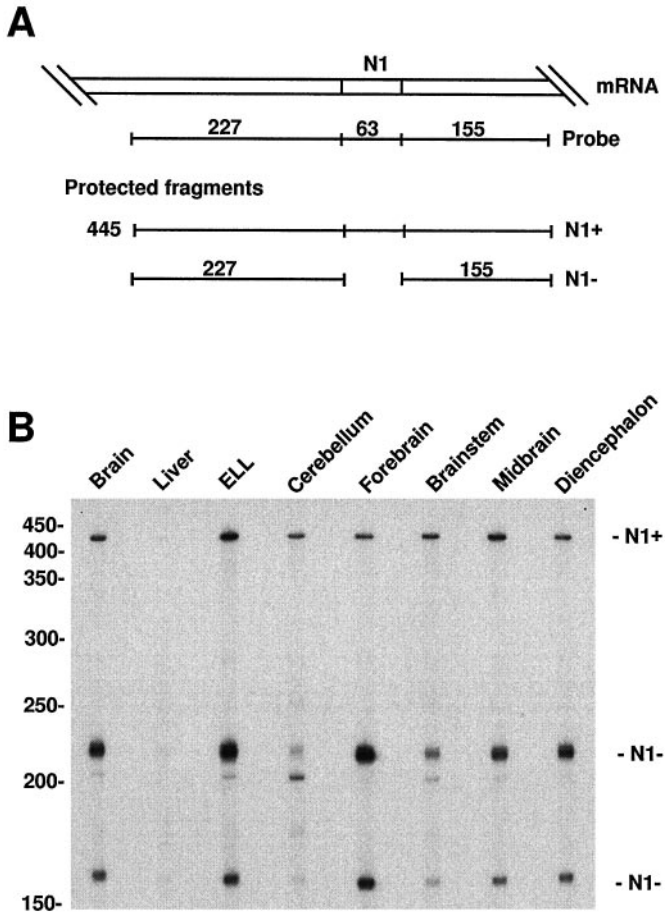

Figure 3. Estimation of the relative amounts of $N1^+$ and $N1^-$ NR1 mRNA in various regions of the *Apteronotus* CNS. *A*, Schematic representation of the RNase protection assay. AptNR1 mRNA is shown at the *top* as a *double line*. The structures of the probe and products are shown *below* as *single lines*. The length of each fragment is given in nucleotides. *B*, The products of RNase protection analysis with RNA isolated from different regions of the *Apteronotus* brain were analyzed by electrophoresis on a 5% polyacrylamide gel. RNA (5 μg) was used in each assay, except for ELL and cerebellum, which used 20 μg each. DNA size markers are indicated on the *left*. The splice isoforms to which the bands correspond are indicated on the *right*.

detected at lower levels in the ELL, brainstem, and midbrain samples. The absence of this band in both the liver and forebrain assays indicates that it is not an artifact of the protection assay. The identify of this fragment is unknown, but it may represent a novel splice isoform of aptNR1 expressed in cerebellum and brainstem neurons.

Novel isoforms of aptNR1 generated by alternative RNA splicing within the C-terminal region

Mammalian NR1 transcripts are subject to two separate alternative RNA splicing events within the region encoding the C terminus of the receptor. Consequently, mammalian NR1 mRNAs either lack or include two short segments, one encoding 37 amino acids (segment C1) that occurs close to the COOH terminus, and the second encoding 38 amino acids (segment C2) that lies at the COOH terminus. The presence or absence of segment C2 determines which of two alternative sequences make up the C terminus of the protein.

In our initial screen of the fish cDNA library, a total of four

Table 1. Relative levels of the alternatively spliced NR1 mRNAs in different brain areas

Tissue	$N1^+$ (%)	$C1^+$ (%)
Brain	17	9
ELL	24	12
Cerebellum	50	5
Forebrain	8	13
Brainstem	43	6
Midbrain	31	10
Hypothalamus	15	24

The levels of the $N1^+$ and $C1^+$ mRNAs are expressed as a percentage of the total NR1 mRNA from the relative densities illustrated in Figures 3*B* (N1) and 5*B* (C1). The relative amounts of radioactivity in each band were estimated by densitometry of the autoradiographic films, using a SciScan 500 densitometer (United States Biochemical, Cleveland, OH). The signals were adjusted to account for the different lengths and numbers of labeled cytidine residues in each transcript to obtain the values reported.

independent cDNAs containing sequences for the C-terminal region of aptNR1 were obtained, all lacking both the C1 and C2 segments. This result suggested that the C1 and C2 alternatively spliced forms are present at low levels, if at all, in the *Apteronotus* brain. To determine whether even rare mRNAs containing C1 and C2 are present in the population of aptNR1 mRNAs, we undertook a PCR approach by using primers flanking the putative C1 and C2 insertion sites (see Materials and Methods). The DNA sequences of the products recovered from this experiment revealed the presence of three alternative splice segments, which we have labeled C1, C1′, and C1″ in Figure 1. The cassette labeled C1 encodes a 37 amino acid peptide with high sequence homology to the C1 cassette described for mammalian NR1 mRNAs. The two other cassettes encode novel sequences not found in the mammalian homologs. The most abundant product from the PCR amplification lacked all three of these inserts. We did not find evidence for additional splice isoforms encoding the C2 cassette found in mammalian NR1 proteins.

The PCR screen described above uncovered two novel segments that have not been reported in mammalian NR1 genes. To provide additional evidence for the presence of these segments in fish brain RNA, we performed an RNase protection assay with an antisense RNA probe complementary to the mRNA encoding C1′–C1″–C1, as outlined in Figure 4*A*. In agreement with the PCR analysis discussed above, the sizes of the protected fragments confirm the presence of four splice isoforms: C1, C1′–C1″, C1″–C1, and C1′–C1″–C1 (Fig. 4*B*). This result, and the failure to detect a segment homologous to C2 in the PCR experiment, indicates considerable evolutionary variability in C-terminal RNA splice cassettes of NR1.

The C1 splice insert contains recognition sites for both protein kinases A and C and therefore may be an important modulation site for the NMDA receptor. To determine whether the presence of the C1 splice cassette is regulated in fish neurons, we used RNase protection analysis to determine the distribution of the C1 cassette in different brain subregions. The probe extended from the beginning of the C1 segment through the C-terminal region and into the 3′ nontranslated sequence of the aptNR1 mRNA. Protection of this probe by $C1^+$ and $C1^-$ mRNAs yields fragments of 325 and 230 nt, respectively. The results of the RNase protection assays are shown in Figure 5. As predicted from the PCR analysis, the amount of $C1^+$ mRNA was generally much lower than that of the $C1^-$ isoform, with levels of 9% estimated for whole brain RNA (Table 1). In general, the inclusion of $C1^+$

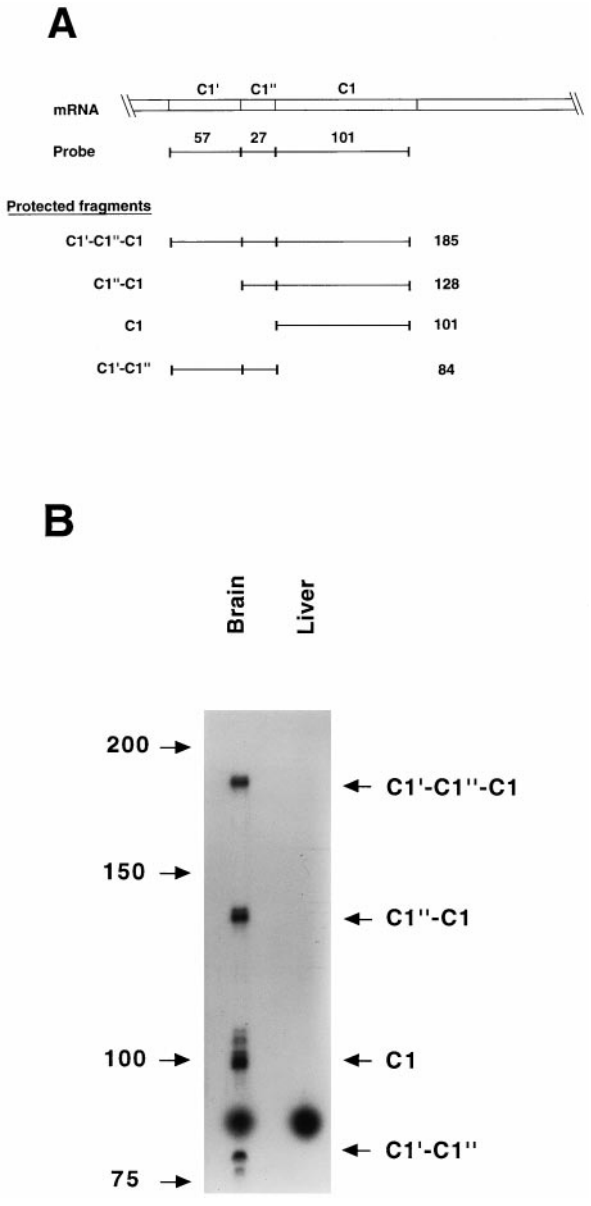

Figure 4. RNase protection analysis confirms the presence of novel NR1 splice isoforms containing cassettes C′ and C″. *A*, Schematic representation of the RNase protection assay. AptNR1 mRNA is shown at the *top* as a *double line*. The structures of the probe and products are shown *below* as *single lines*. The length of each fragment is given in nucleotides. *B*, The products of RNase protection analysis with RNA (20 μg) isolated from *Apteronotus* brain and liver were analyzed by electrophoresis on a 5% polyacrylamide gel. DNA size markers are indicated on the *left*. The splice isoforms to which the bands correspond are indicated on the *right*.

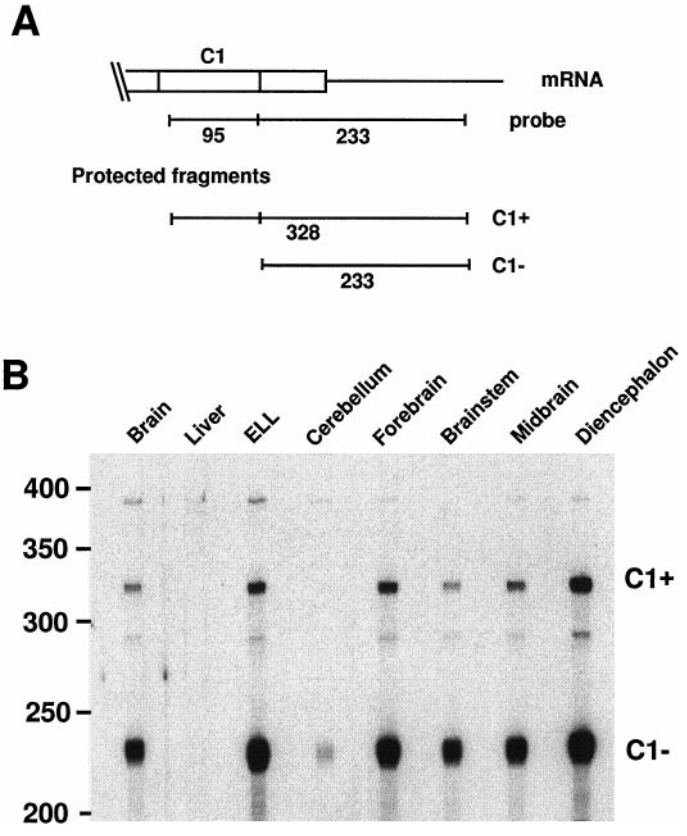

Figure 5. Estimation of the relative amounts of $C1^+$ and $C1^-$ NR1 mRNA in various regions of the *Apteronotus* CNS. *A*, Schematic representation of the RNase protection assay. AptNR1 mRNA is shown at the *top* as a *double line*. The structures of the probe and products are shown *below* as *single lines*. The length of each fragment is given in nucleotides. *B*, The products of RNase protection analysis with RNA isolated from different regions of the *Apteronotus* brain were analyzed by electrophoresis on a 5% polyacrylamide gel. RNA (5 μg) was analyzed in each assay, except for ELL, which used 20 μg. DNA size markers are indicated on the *left*. The splice isoforms to which the bands correspond are indicated on the *right*.

cassette varied in an opposite manner to that observed for the $N1^+$ cassette, $C1^+$ being higher in forebrain and lower in more caudal areas. However, because the assays used do not measure directly the presence or absence of both exons in an individual mRNA, we have not been able to determine directly whether there is a coordinate regulation of the two alternatively spliced exons. Hypothalamus recorded the highest ratio of $C1^+$-containing NR1 transcripts.

In the studies described above, we were unable to detect the presence of the C2 splicing cassette that is a prevalent isoform of mammalian NMDAR1 mRNAs. As an approach to isolation of the fish C2 sequence, an RT-PCR amplification was performed to identify fragments spanning the C1 cassette and extending into the 3′ untranslated sequence of aptNR1. In this amplification the presence of a sequence homologous to C2 is predicted to produce a product larger than the product obtained from the $C1^+$ splice variant described above. Analysis of the products of this amplification by using Southern blot hybridization with a probe that hybridized with all of the splice variants showed only a single product, corresponding to the $C1^+C2^-$ isoform (data not shown). In summary, these results have failed to find evidence for the existence of the C2 splice cassette in fish NR1 mRNA.

Cell-specific splicing of the NR1 transcript in neurons of the electrosensory system

To determine which specific neuronal populations in the electrosensory system express the alternatively spliced NR1 mRNAs, we performed *in situ* hybridizations on coronal sections of the *Apteronotus* brain with probes specific for N1 and C1. Labeling with these probes was distributed specifically over gray matter regions, with no label detected in fiber tracts. Control probes representing sense strands of both N1 and C1 produced only a very low and homogenous distribution of grains (data not shown).

The electroreceptive inputs from skin receptors project to the

ELL nucleus of the medulla, which is the primary processing station for electrosensory information. Tuberous electroreceptors encode the amplitude of the electric organ discharge (EOD) through excitatory glutamatergic synapses to the large ELL pyramidal cells and smaller ELL granule cell interneurons. Additional excitatory glutamatergic synaptic inputs to both of these cell types arise from feedback pathways that originate higher in the CNS and terminate on the prominent apical dendrites of pyramidal cells (Bastian, 1993; Wang and Maler, 1994; Berman et al., 1997). The ELL pyramidal cells express NR1 mRNA to a very high level (Bottai et al., 1997) and are labeled strongly with both N1 and C1 probes (Fig. 6*I*, Table 2), indicating that both splice cassettes contribute to NMDA receptor populations in these cells. The major class of inhibitory interneuron in the ELL is the type 1 granule cells, which provide inhibitory inputs to the pyramidal cells that contribute to temporal filtering in this system. These granule cell interneurons express moderate levels of NR1 mRNA that is labeled with both the pan NR1 probe and the N1-specific probe but shows no detectable label with the C1 probe (Fig. 6*I*). This result indicates that pyramidal cell projection neurons contain NMDA receptors that are regulated via both the N1 and C1 regulatory domains, whereas the granule cell interneuron NMDA receptors specifically lack the C1 form.

Pyramidal cells of the ELL transmit electrosensory signals via projections to two secondary processing nuclei, the midbrain torus semicircularis and the hindbrain nucleus praeminentialis. The N1 probe labeled neurons to moderate levels in both of these nuclei, as did the pan NR1 probe. In contrast, the C1 probe demonstrated a only very weak labeling in both nuclei (Fig. 6*II*). The relatively low level of C1 splicing in these areas suggests that C1-mediated regulation of NMDA receptors in these secondary nuclei may be less important than it is in the primary processing areas of the ELL.

Electrosensory and visual signals are combined and compared in the optic tectum of the electrosensitive teleost brain. We previously detected only low levels of NMDA receptor expression in most tectal neurons, except for the pyramidal cells of the stratum fibrosum et griseum superficiale. These cells have dendrites that receive signals from both electrosensory and visual systems and are thought to be important for the integration of information from both systems. Although these pyramidal cells are intensely labeled by the pan NR1 probe, they are labeled only very weakly by either the N1 or C1 probes (Table 2). Although the N1 probe weakly labeled most pyramidal cells, the C1 probe appeared to label only a subset (approximately one-half) of these cells (the mean density given in Table 2 includes only labeled cells).

Electrosensory information also is projected to forebrain, although the pathways of these projections are not well defined. The RNase protection assay indicated that the N1 splice is expressed at low levels in forebrain; consistent with this, *in situ* hybridization also revealed a homogenous low level of expression of the N1 splice that paralleled the expression of NR1 pan (data not shown). The RNase protection experiment indicated that cells in the forebrain contain low levels of $C1^+$ mRNA (comparable to ELL; see Table 1). Labeling with the C1 probe in forebrain was heterogeneous and confined mainly to two forebrain regions: ventral intermediate region and the dorsal posterior forebrain. Both regions may be involved in electrocommunication behavior (Wong, 1997). The significance of this localized expression of the C1 splice is unclear, because specific roles for different forebrain areas in processing of electrosensory signals have not been defined.

The control of the electric organ discharge is influenced by electrosensory information via a neuronal network that extends from the nucleus electrosensorius to the thalamic prepacemaker nucleus and finally to the pacemaker nucleus (containing pacemaker and relay cells), which directly control spinal electromotor neurons. Neurons in all three of these nuclei contain low-to-moderate levels of NR1 mRNA detected with the pan NR1 probe and uniformly low levels of N1 insert detected with the N1 probe. A much more heterogeneous pattern was observed by using the C1 probe. Neurons of the nucleus electrosensorius and the prepacemaker nucleus showed no label with the C1 probe. However, in the final stage of the control pathway, the medullar pacemaker nucleus, relay cells were strongly labeled with C1, whereas adjacent pacemaker cells were not labeled (Fig. 6*III*). These results indicate that only NMDA receptors in the relay cell, the final cell of this motor control pathway, contain the C1 regulatory segment.

DISCUSSION

Evolution of the teleost NR1 sequence

The fish NR1 sequence reported here is the first complete teleost NMDA receptor subunit sequence to be established and represents the most evolutionarily distant NR1 sequence to date. Phylogenetic comparison to human, rodent, avian, and *Xenopus* sequences confirms the relatively distant evolutionary origin of the fish sequence (Fig. 7), which is clearly evident in the amino acid sequence comparisons shown in Figure 1. When compared with the mammalian sequences, the fish NR1 displays twice the number of amino acid nonidentities than does the *Xenopus* NR1 (Soloviev et al., 1996). A striking aspect of the evolution of the NR1 gene is that, except for the C-terminal splice cassettes, sequence variation is restricted mostly to the N-terminal 470 amino acid residues. The sequence of the C-terminal half of the protein, including the proposed transmembrane, ion pore, and glycine-binding segments, is conserved very highly between the fish and mammalian sequences. Thus the ligand-binding properties and pharmacology of aptNR1 should match closely those of mammalian NR1, because all of the residues known to participate in binding glycine (Laube et al., 1993; Kuryatov et al., 1994; Wafford et al., 1995; Hirai et al., 1996) are conserved in the fish sequence. On the other hand, the high sequence divergence of the N-terminal segment suggests that it may form a surface domain of the receptor, in which more variation of sequence is tolerated (Wilson et al., 1977). One segment within the divergent N-terminal region that does not diverge is the 21 amino acid alternative RNA splice cassette N1, in which 20 amino acids are identical in all four species (see Fig. 1). This result and the well conserved regulation of N1 splicing discussed below strongly suggest that the controlled expression of NR1 subunits containing N1 is an important feature of NMDA receptor function.

In the proposed topological models of the NR1 subunit, the segment C0 is the intracellular 30 amino acid peptide immediately C-terminal to the final transmembrane segment TM4, where it contributes to the regulation of NMDA receptor activities via interactions with both calmodulin and α-actinin (Ehlers et al., 1996; Wyszynski et al., 1997). The C0 sequence has been highly conserved in the fish receptor, with only a single amino acid substitution, suggesting that C0-mediated regulation of NMDA receptors is also important in fish neurons.

The 37 residue alternatively spliced segment C1 is also a feature of the fish NR1 gene. The amino acid sequence of the fish

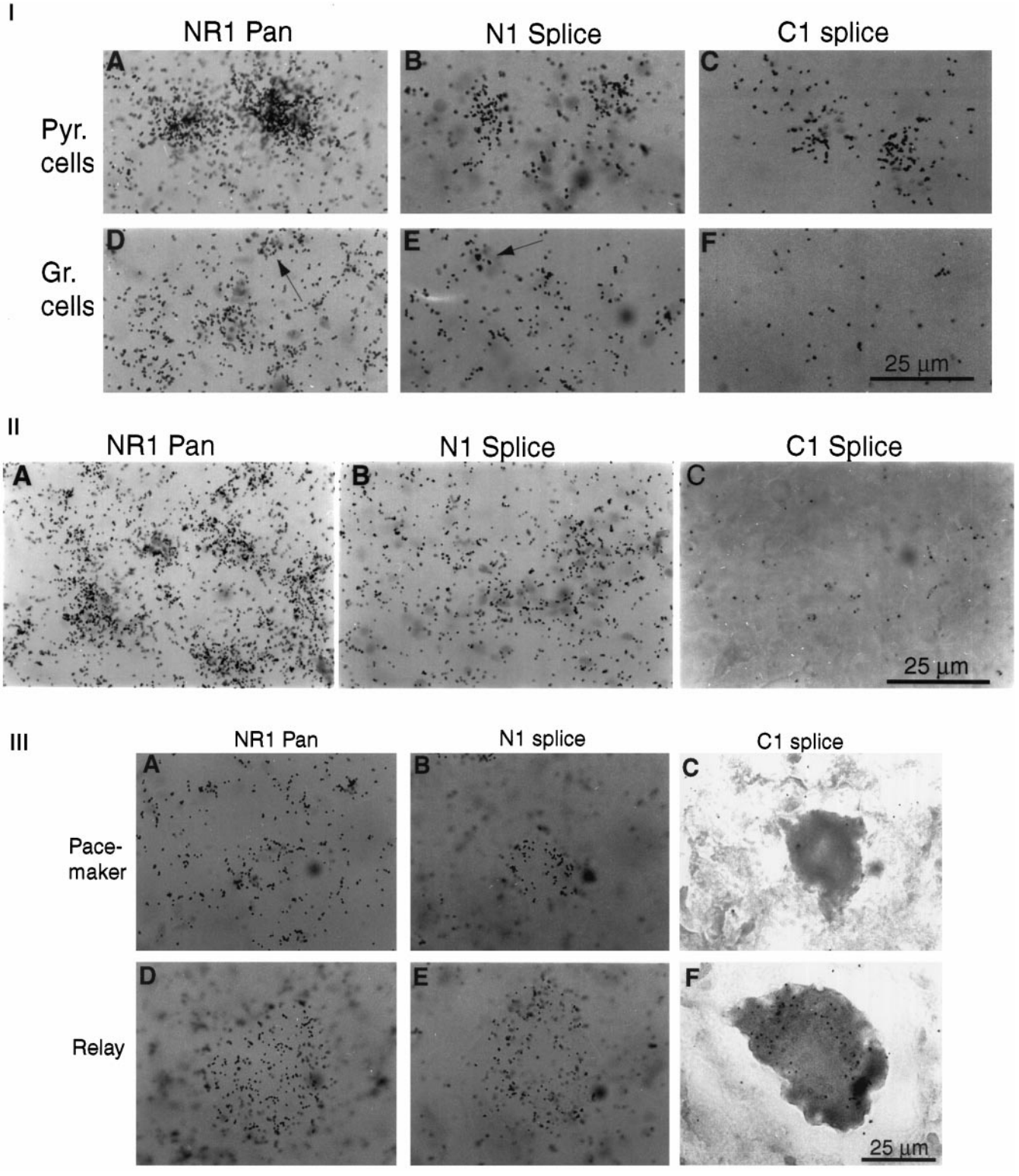

Figure 6. Localization of aptNR1 alternatively spliced transcripts in neurons of the electrosensory system. *I*, *In situ* hybridization of NR1 splice variants in the electrosensory lateral line lobe. *A, D*, Both pyramidal cells (*Pyr. cells*) and granular interneurons (*Gr. cells*) of the ELL are labeled with the NR1 pan probe. *B, E*, Pyramidal cells and interneurons also express the N1 splice cassette. *C, F*, The C1 splice cassette is expressed in pyramidal cells but appears to be absent from ELL interneurons. *II*, *In situ* hybridization of NR1 splice variants in layers 7–8A of the torus semicircularis dorsalis. *A*, The pan probe reveals strong labeling in cells of this region; as previously reported (Bottai et al., 1997), this region of the torus has the highest density of NR1 expression. *B*, The N1 probe also appears to label most cells, although at a much lower level. *C*, There does not appear to be any specific hybridization with the C1 probe to neurons in the torus. *III*, *In situ* hybridization of NR1 splice variants in the pacemaker nucleus. Pacemaker and relay cells were identified on the basis of somatic diameter in cases in which they were sectioned through their nuclei (relay cells are far larger). *A, D*, Both pacemaker and relay cells are labeled with the NR1 pan probe. *B, E*, Both pacemaker and relay cells also strongly express the N1 splice cassette. *C, F*, The C1 splice cassette appears to be absent from pacemaker cells but is strongly expressed in relay cells.

Table 2. Corrected grain densities from *in situ* hybridization studies with probes specific for the N1 and C1 splice isoforms

	N1 splice			C1 splice		
Brain region	Density (grains/100 μm^2)	SD	*n*	Density (grains/100 μm^2)	SD	*n*
DC	11.2	3.2	17	7.8	3.2	20
DLd	12.3	3.4	24	ND	—	—
TeO	7.6	2.5	22	8.4	3.5	31
nM Crest cells	17.4	4.0	26	14.5	5.4	15
ELL pyramidal cells	25.0	6.9	26	19.1	4.9	59
ELL granule cells	20.5	4.7	51	ND	—	—

Statistics were done by ANOVA with Tukey's unequal *n post hoc* test.

N1 splice: The grain density in pyramidal cells is significantly greater than in granule cells ($p < 0.01$) and all other cell types ($p < 0.0001$). Granule cells and nM crest cells have significantly higher grain densities than the forebrain or tectal pyramidal cells ($p < 0.0005$ for granule cells; $p < 0.001$ for crest cells). Grain density in forebrain cells (DC, DLd) are greater than in tectal cells ($p < 0.01$ for DLd; $p < 0.05$ for DC). Note that, whereas tectal cells had far higher levels using the panNR1 probe (Bottai et al., 1997) than forebrain cells, they are significantly lower with the N1 probe. For most other cell types examined, however, the levels of N1 and panNR1 appear to be correlated.

C1 splice: Label was not detected in dorsolateral forebrain or in ELL granule cells. Pyramidal cells have significantly greater grain densities than crest cells ($p < 0.05$) and all other cell types ($p < 0.00001$). Crest cells have higher grain densities than forebrain (DC) or tectal cells ($p < 0.001$). Tectal pyramidal cells have a heterogeneous distribution of grains, with some cells having no detectable label; the unlabeled cells were not included in the statistical analysis, which, therefore, overestimates the grain density of this cell type.

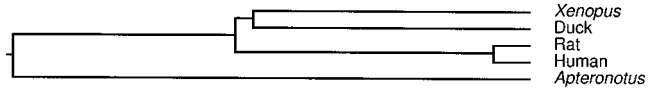

Figure 7. Phylogenetic tree comparing the ancestral relationships among the NR1 protein sequences from *Apteronotus*, *Xenopus* (Soloviev et al., 1996), duck (Kurosawa et al., 1994), rat (Moriyoshi et al., 1991), and human (Planells-Cases et al., 1993). The length of each branch is proportional to evolutionary distance. The sequences were aligned by using the multiple sequence alignment program Clustal V in the DNASTAR sequence analysis package.

C1 cassette contains seven amino acid substitutions as compared with the mammalian sequences, but only the serine at position 926 of the fish sequence fails to maintain the charge and hydrophilic character of the mammalian homologs. The mammalian C1 segment has been shown to contain sites phosphorylated by protein kinase A (PKA) and protein kinase C (PKC) (Tingley et al., 1993, 1997; Hisatsune et al., 1997; Leonard and Hell, 1997). The three PKC and single PKA phosphorylation sites demonstrated in the rat NR1 C1 segment by Tingley et al. (1997) are conserved in the fish sequence (see Fig. 1). The positions of key positive residues and hydroxyl acceptors have been maintained, with only the substitution of serine for threonine at position 923 directly affecting the target sites. Phosphorylation of C1 inhibits the interaction with calmodulin and subsequent calmodulin inhibition of receptor activity (Ehlers et al., 1996; Hisatsune et al., 1997). The calmodulin interaction sites (Ehlers et al., 1996) on C0 (amino acids 854–879) and C1 (amino acids 919–942) are well conserved in the fish sequence, with only a single substitution in the C0 site and five amino acid substitutions, four of which are highly conservative, in the C1 site. This high level of sequence conservation within the putative regulatory domains of the fish C0 and C1 segments indicates that many, if not all, of the regulatory mechanisms associated with the mammalian C1 segment were features of NMDA receptors in primitive vertebrate organisms existing before the divergence of fish and tetrapod lineages.

In heterologous cells expressing NR1, PKC activators increase both NMDA responses and phosphorylation of the NR1 subunit (Durand et al., 1992, 1993; Tingley et al., 1993, 1997; Sigel et al., 1994; Zheng et al., 1997). However, direct involvement of the C1 segment in this process has been questioned because homomeric NR1 receptors containing C1 are less responsive than C1$^-$ receptors to PKC activators (Durand et al., 1993). The presence of C1-containing NR1 subunits in pyramidal cells, but not granule cells, of the ELL (see Fig. 6*I*), where excitatory synaptic responses are analyzed readily (Berman et al., 1997), provides an opportunity to examine these effects of the C1 segment on the regulation of NMDA receptors located at functional synapses.

A study of *Xenopus* NR1 failed to find evidence for either C1 or C2 segments in frog brain RNA (Soloviev et al., 1996). The finding of C1 in the fish NR1 suggests either that this segment is present at such low abundance in the frog that it was not detected or that the C1 sequence was present in the ancestral jawed vertebrate but has been lost in the lineage leading to modern frogs.

The most striking evolutionary divergence within the fish NR1 sequence is the presence of the novel segments C1′ and C1″. These segments were recovered by using an RT-PCR experiment designed to probe for sequences of relatively low abundance in fish brain NR1 mRNA. The presence of these two splice cassettes in a variety of splice isoforms was indicated by DNA sequence analysis of the PCR products and confirmed by an RNase protection analysis (see Fig. 4). The location of C1′ and C1″ between C0 and C1 suggests a possible role in the regulation of the calmodulin and α-actinin interactions that occur at adjacent sites, possibly via phosphorylation of the multiple serine residues in C1′ and C1″. The presence of these novel sequences within the C-terminal domain of NR1 appears to be an evolutionary development of the teleost lineage that has not been observed in either frog or mammalian NR1 transcripts.

We failed to detect the presence of the C2 splice cassette in fish NR1 transcripts, using a sensitive PCR and Southern blot procedure. C2 also was not detected in frog brain RNA (Soloviev et al., 1996), suggesting that the alternative splice junction that generates C2 in the mammalian NR1 gene arose after the divergence of the frog and mammalian lineages. The most significant characteristic of the C2 splice isoform is that it lacks the canonical amino acid sequence S/TXV for recognition by the PSD class of synaptic localization proteins (Sheng and Kim, 1996), whereas the C2′ isoforms (including aptNR1) terminate with TVV (Kornau et al., 1995). The absence of the C2 splice isoform means that all NMDA receptors in fish contain NR1 isoforms that participate directly in the PSD interaction.

Regulation of alternative RNA splicing of NR1 in neurons of the electrosensory system

Alternative RNA splicing of mammalian NR1 transcripts regulates the presence of the 21 amino acid N1 peptide encoded by exon 5 (Anantharam et al., 1992; Nakanishi et al., 1992; Hollmann et al., 1993). This short peptide contains a high percentage of charged residues and is located adjacent to the ligand-binding domain of the receptor. The presence of the N1 results in NMDA receptors that express higher current amplitudes (Hollmann et al., 1993; Zheng et al., 1994), lower agonist affinities (Nakanishi et al., 1992; Hollmann et al., 1993), resistance to inhibition by extracellular protons (Traynelis et al., 1995), and lack of potentiation by magnesium (Paoletti et al., 1995). These properties suggest that N1-containing receptors should produce larger EPSCs at physiological pH and undergo more rapid deactivation because of lower agonist affinity.

The regulation of N1 splicing in the teleost brain has the same general pattern as that of mammalian NR1. The total amount of N1 in fish brain is estimated to be 17% (see Table 1), which compares very well with the estimates of 20% for rat brain (Nakanishi et al., 1992). Furthermore, the ratio of N1 is higher in more caudal areas of both fish and rat brains, with the highest ratios in cerebellum for both species. This high degree of evolutionary conservation for splice regulation further supports a fundamental role for N1 in NMDA receptor function.

In the fish brain *in situ* hybridization study, the pattern of neurons labeled with N1 generally matched the pattern with the use of the pan NR1 probe (Bottai et al., 1997) except that the N1 probe produced lower grain densities. Almost all cells that express NR1 contain at least a low level $N1^+$ mRNA. An exception to this general pattern are the pyramidal cells of the optic tectum. These cells labeled heavily with the pan NR1 probe to levels significantly above those of forebrain cells but were labeled only slightly above background with the N1-specific probe (data not shown).

We also have examined the patterns of alternative RNA splicing for the C-terminal segment C1 in neurons of the fish CNS. The level of C1-containing transcripts measured by RNase protection is low in the fish brain (9%) and is distributed in a nonuniform manner that is generally higher in more rostral regions (see Table 1). This pattern also has been observed in rat brain, where C1 transcripts were highest in forebrain, hippocampus, caudate putamen, and olfactory bulb (Laurie et al., 1995). Apparently, the presence of higher levels of C1 alternative splicing in these rostral areas has been a feature of the very early vertebrate nervous system.

In situ hybridization that used the C1-specific probe showed a heterogeneous labeling pattern in many regions of the fish brain. In neurons of the electrosensory system, two clear examples of cell-specific regulation were found. In the ELL, expression of the C1 sequence was restricted to pyramidal cells and was not detectable in adjacent granule cells (see Fig. 6*I*, Table 2). Pyramidal cells are the major integrative elements for processing of electrosensory spike trains in the ELL, and the presence of the C1 sequence may provide a mechanism to regulate excitatory currents during signal integration and processing. There is strong binding of both forskolin and phorbol ester associated with the feedback input to pyramidal cells (Maler and Wang, 1997), which is involved in adaptive regulation of electrosensory processing (Bastian, 1995, 1996a,b; Maler and Wang, 1997), suggesting that modulation of NMDA receptor currents by PKA and/or PKC may be involved in this process. Granule cells, which provide inhibitory relays to the pyramidal cells, may not require similar regulation of excitatory inputs. Thus in the ELL, neuron-specific regulation of alternative RNA splicing controls the availability of C1-mediated regulatory mechanisms in two of the major cell types in this neuronal network.

The pacemaker nucleus is the second example of cell-specific regulation of the C1 splice insert. In this nucleus only the relay cells express the C1 isoform of NR1, whereas pacemaker cells express the $C1^-$ form. Pacemaker and relay cells receive glutamatergic input from three different sources, and these inputs subserve distinct electrocommunicatory behavior (Heiligenberg et al., 1996). Inputs to both cell types involve NMDA receptors that mediate slow increases in EOD frequency, so it will be interesting to determine whether the presence of the C1 isoform in relay cells confers different kinetic and/or regulatory properties to the relay cell input.

The sequence and regulation of the N1 and C1 splice cassettes are remarkably well conserved in the fish NR1 subunit gene. Further, in the electrosensory system the splicing of the C1 cassette and, therefore, C1-mediated modulation of NMDA receptor currents are restricted to synapses on the pyramidal cells in the ELL. The well characterized anatomy and physiology of the early steps in signal processing in the ELL now provide an opportunity to investigate the role of C1 modulation in this process.

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