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Characterization in Cultured Cerebellar Granule Cells and in the Developing Rat Brain of mRNA Variants for the NMDA Receptor 2C Subunit

Amina Rafiki, Anne Bernard, Igor Medina, Henri Gozlan, and Michel Khrestchatisky

INMED-INSERM Unité 29, Marseille, France

Abstract: *N*-Methyl-D-aspartate (NMDA) receptors are heteromeric structures resulting from the association of at least two distantly related subunit types, NR1 and one of the four NR2 subunits (NR2A–NR2D). When associated with NR1, the NR2 subunits impose specific properties to the reconstituted NMDA receptors. Although the NR1 mRNAs are expressed in the majority of central neurons, the NR2 subunits display distinct patterns of expression in the developing and adult rat brain. The NR2C subunit is barely expressed in the rat forebrain, whereas its expression increases substantially in the granule cells in the course of cerebellar development. We have identified novel NR2C splice variants in cultured cerebellar granule cells as well as in the developing cerebellum. When compared with the prototypic NR2C mRNA, these variants carry one (NR2Cb) or two (NR2Cd) insertions or a deletion (NR2Cc) and encode putative NR2C polypeptides that terminate between the third and fourth membrane segments or between the first and second membrane segments. RT-PCR analysis and in situ hybridization show that expression of the splice variants is developmentally regulated, both in the cerebellum and in the hippocampus. Electrophysiological recordings and microfluorimetry emissions in transfected human embryonic kidney 293 cells indicate that the NR2Cb variant, when expressed in combination with NR1, does not contribute to the formation of functional receptor channels. The significance of theses findings is discussed. **Key Words:** Cerebellum—Hippocampus—RT-PCR—In situ hybridization—HEK 293 cells—Transfections—Splicing—Introns.
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The *N*-methyl-D-aspartate (NMDA) receptor is a subtype of the glutamate ionotropic receptors implicated in brain development (reviewed by Feldmeyer and Cull-Candy, 1996), in long-term potentiation (Collingridge and Bliss, 1995), and in several neurological disorders (reviewed by Mody and MacDonald, 1995). NMDA receptor channels are highly permeable to Ca^{2+} and are unique among glutamate receptors in that they are blocked by Mg^{2+} in a voltage-dependent manner (reviewed by Ozawa et al., 1998).

Three gene families encoding NMDA receptor subunits have been characterized: NR1, NR2, and NR3A (also termed NR-L or χ^{-1}) (Moriyoshi et al., 1991; Monyer et al., 1992; Ciabarra et al., 1995; Sucher et al., 1995). Eight splice variants have been reported for NR1 (reviewed by Hollmann and Heinemann, 1994), whereas the NR2A–NR2D subunits are encoded by different genes (Monyer et al., 1992; Ishii et al., 1993).

When combined with the NR1 subunits, members of the NR2 subunit family form channels with different biophysical and pharmacological properties (reviewed by Hollmann and Heinemann, 1994; Sucher et al., 1996). When expressed in vitro, NMDA receptor channels containing the NR2C subunit are less sensitive to glutamate, to NMDA receptor agonists, to Mg^{2+}, and to MK-801 blockade, and they have a lower conductance than other subunit assemblies (Kutsuwada et al., 1992; Ishii et al., 1993; Laurie and Seeburg, 1994). When compared with other heteromeric receptor complexes, those containing the NR2C subunit show no significant inactivation by intracellular Ca^{2+} and have a lower affinity to the zinc voltage-independent inhibition (Krupp et al., 1996; Gray et al., 1997).

In situ hybridization and immunohistochemical studies demonstrate differential distributions of the NR1 and NR2 subunit families in the CNS. The NR2A subunits are detected in almost all brain structures, NR2B subunits are essentially expressed in the forebrain, and NR2D subunits are found essentially in the midbrain and in the brainstem (Brose et al., 1993; Akazawa et al., 1994; Monyer et al., 1994; Petralia et al., 1994*a*,*b*; Laurie et al., 1995; Fritschy et al., 1998). In the cerebellum NMDA receptors are involved in the development and maturation of neuronal networks (Rabacchi et al., 1992; Komuro and Rakic, 1993). Although the NR2B subunits are expressed transiently in the developing cerebellum,

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Address correspondence and reprint requests to Dr. M. Khrestchatisky at INMED-INSERM Unité 29, Route de Luminy, B.P. 13, 13273, Marseille Cedex 09, France.

Abbreviations used: HEK, human embryonic kidney; NMDA, *N*-methyl-D-aspartate; OD, optical density; P, postnatal day; tNR, tobacco nitrate reductase.

TABLE 1. *Oligonucleotide sequence and nucleotide position of primers used in this study*[a]

Primer	Oligonucleotide sequence (5′–3′)	Nucleotide position
C1	GTGTGGGTGATGATGTTCGTGATG	2,224–2,247
C2	CAACCAGAACCTCACCAAGGGCAA	2,307–2,324
C3	GACCATGTGGGTGTGCATGTCAC	2,651–2,673
TM3	GCGGCCTTCATGATCCAGGAGCAA	2,503–2,526
C4	GAGCAAGCCACTCCTACCCTCTTCGTCCAGCTCCC	2,851–2,886
C5	GCAGGACCCTCCAGGTGACATGG	1–25
C6	CCACACGGACTTGCCAATGGTGAA	2,351–2,373
C7	CGGATGTTCCTCTCTGTGCTTCTT	2,557–2,639
C9	TCAGAGCCTGGTAGGAACTTCGTCACCGGCCAGCCCACCC	2,366–2,406
C10	GTCTTGAGGCCGCTGAAACTTCTTGTCACTAAGGC	2,545–2,580
C11	GAAAGATGGTCCACCAGGTTC	2,331–2,352
C12	ACGGATGTTCCTCTCTGTGCTTCTTGTCACTAAGGC	2,545–2,640

[a] The sequence is deposited under EMBL accession no. RN08259.

they are replaced during ontogeny by the NR2C subunit. In the developing brain NR2C expression increases during the first week after birth, mainly in the cerebellar granule cells, and peaks in the adult (Pollard et al., 1993; Akazawa et al., 1994; Monyer et al., 1994). Low levels of NR2C mRNA are also expressed in the developing hippocampus (Pollard et al., 1993). Expression of the NR2C subunit appears to be regulated in some neurological disorders (Marianowski et al., 1995; Kadotani et al., 1998) and in hippocampal slice models of anoxia-aglycemia (Small et al., 1997). Combined targeted disruption of the NR2A and NR2C genes results in a motor discoordination abnormality in the deficient mice (Kadotani et al., 1996). Although splice variants have been described for the NR1 subunit, of the NR2 subunits, only the human NR2C mRNA has been shown to undergo alternative splicing in its coding region leading to the hNR2C1–4 subunits (Daggett et al., 1998).

In the present work, we describe novel splice variants of the rat NR2C subunit mRNAs that were identified in cultured cerebellar neurons and in the developing rat cerebellum. These splice variants carry one or two insertions or a deletion, and they encode truncated forms of the NR2C subunit. We studied their regional and cellular distribution during rat brain ontogeny, and we assessed whether one of these splice variants formed functional NMDA receptor channels in transfected human embryonic kidney (HEK) 293 cells.

MATERIALS AND METHODS

RT-PCR and Southern blot analysis

Total RNA and cDNA were prepared from the cerebellum and hippocampus of developing and adult Wistar rats ranging from birth [postnatal day (P) 0] to adult and from cultured cerebellar granule neurons (prepared from P7–P8 rat pups) as described by Ferhat et al. (1993). PCR procedures were performed in the following conditions: 50-μl reactions; 30 (cerebellum and cultured cerebellar cells) to 32 (hippocampus) cycles; 30 s of denaturation at 94°C, 30 s of annealing at 56–65°C (depending on the primer pairs), and 30 s of elongation at 72°C. To assess quantitative differences by RT-PCR, total RNA (1 μg) from the different tissues was spiked with 40 pg of in vitro-transcribed tobacco nitrate reductase (tNR) RNA. Following cDNA synthesis and PCR, the tNR PCR products were quantified on Southern blots and used as standards as described by Ferhat et al. (1993). In each set of experiments, PCR controls included RT-PCR with no RNA input, mock cDNA synthesis (no RT), and PCR procedures in the absence of cDNA input.

Following gel analysis, specificity of the PCR products was further checked by Southern blot hybridization with labeled nested oligonucleotides (Table 1) as described previously (Ferhat et al., 1993). Membranes were washed and exposed to Amersham MP films at −80°C for 2–12 h. Multiple exposures were performed to ensure linearity of the autoradiographic responses, and the least exposed autoradiograms were used for quantifications. The relative quantities of mRNA transcripts were determined by comparing the ratios of the hybridization signals for specific PCR products to the coamplified tNR PCR product. At least two independent PCR procedures were performed for each experiment and highly similar results were obtained when optical density (OD) values of the bands were assessed.

cDNA cloning and plasmid constructs

The cDNAs encoding the NR1, NR2A, and NR2C subunits used in this study were spliced out from their native pBluescript plasmids (Stratagene, San Diego, CA, U.S.A.) and subcloned into the eukaryotic expression vector pcDNA I/Amp (Invitrogen, Carlsbad, CA, U.S.A.). Constructs were verified by sequencing. The cDNAs corresponding to the various NR2C variants were generated by RT-PCR from total RNA extracted from cerebellum or cerebellar cultures, using the primers described in Table 1. All inserts were subcloned into PCRII vector (Invitrogen) and were sequenced on both strands. The full-length NR2Cb cDNA was constructed by replacing a *Sca*I–*Xho* cassette (1,370 bp) digested from the native NR2C insert cloned into pcDNA I/Amp by a *Sca*I–*Xho* cassette (770 bp) digested from the NR2Cb PCRII construct. This construct was fully sequenced.

In situ hybridization

Sagittal brain sections (15 μm) of rats at different postnatal ages were prepared as described by Rafiki et al. (1998). Antisense oligonucleotide probes (Table 1) were labeled, and in situ hybridizations were performed as described by Rafiki et al. (1998). High-stringency wash was in 0.5 × saline–sodium citrate (SSC) for 30 min at 55°C. Slices were dehydrated,

air-dried, and exposed to X-Omat film (Kodak) for 6–12 days. Some sections were dipped in nuclear emulsion (NTB-2 from Kodak), exposed at 4°C for 20 days, and counterstained with cresyl violet. The specificity of the hybridization reaction was determined for each probe by competition experiments in which excess (200-fold) unlabeled probe was added to the hybridization mixture. The resulting autoradiograms were blank.

Cell transfections and electrophysiological recordings

HEK 293 cells were cultured on 10-mm-diameter glass culture wells ($1–2 \times 10^5$ cells per well) and were transfected as described previously (Medina et al., 1995; Rafiki et al., 1997) with green fluorescent protein, NR1–NR2A, NR1–NR2C, or NR1–NR2Cb plasmids. Electrophysiological recordings were performed 24–48 h later from isolated green fluorescent protein-positive cells using patch-clamp in the whole-cell configuration. Currents were recorded using an EPC-9 amplifier (Heka Electronik, Lambrecht, Germany). Data were filtered at 2 kHz and digitized at 20 kHz using pClamp version 6 software (Axon Instrument, Foster City, CA, U.S.A.). The same software was used for data analysis. The internal pipette solution contained 140 m*M* CsCl, 1.1 m*M* EGTA, 0.06 m*M* $CaCl_2$, 2.0 m*M* $MgCl_2$, and 10 m*M* potassium HEPES (pH 7.2). The resistance of the recording borosilicate glass pipettes was 2–4 Mohms, and series resistance was in the range of 10–30 Mohms. The external solution contained 140 m*M* NaCl, 2.0 m*M* $CaCl_2$, 2.0 m*M* $MgCl_2$, and 10 m*M* potassium HEPES (pH 7.4). Glutamate (100 m*M*) and glycine (1 m*M*) were dissolved in the external solution and applied through the micropipette using a picospritzer as described previously (Medina et al., 1995).

Intracellular calcium measurements were performed using indo-1 (Molecular Probes, Leiden, The Netherlands) during glutamate and glycine application and were monitored using a PhoCal system (Applied Imaging, Cambridge, U.K.). The intracellular calcium concentration was calculated using the PhoCal program according to the equation of Grynkiewicz et al. (1985). R_{max} and R_{min} were estimated during application of ionomycin with 5 m*M* Ca^{2+} or 5 m*M* BAPTA, respectively.

RESULTS

Cloning and characterization of NR2C splice variants

cDNAs were prepared from total RNA extracted from cerebellar granule cells in culture and from adult rat cerebellum. RT-PCR was performed using primers C1 and C3 (Fig. 1A). Of the three PCR products obtained from the granule cells, two hybridized to the nested probe TM3 following Southern transfer. It is likely that the PCR product that is not detected by probe TM3 (asterisk on Fig. 1B) carries a deletion of the region hybridizing with this probe. One of the two PCR products detected by probe TM3 was of the expected size (427 bp), whereas the other was larger (768 bp). OD values of this PCR product on autoradiograms were 30–50% lower than ODs of the 427-bp band (depending on the developmental stage of the cells) (Fig. 1B). Similar PCR products were obtained from adult rat cerebellum in addition to a smaller PCR product (368 bp; lower arrow, Fig. 1B). Ratios between the 427- and 768-bp bands differ substantially from those in the cerebellar cultures as the 427-bp product represents typically 90% of the PCR products. The two hybridizing PCR products indicated by arrows (Fig. 1B) were gel-isolated, cloned, and sequenced. The 768-bp PCR corresponds to an NR2C variant containing a 341-bp insertion (Ins I) encoding a premature stop codon (Fig. 1C). The 368-bp PCR product corresponds to another NR2C variant with a 59-bp deletion (Δ I) that alters the open reading frame and introduces a premature stop codon (Fig. 1D). The insertion and the deletion, if they are not associated with other insertions or deletions upstream, could lead to truncated NR2C polypeptides that we named NR2Cb and NR2Cc, schematized in Fig. 1E. We next assessed whether in cultured granule cells Ins I was associated with other insertions or deletions in the upstream coding region of the NR2C mRNA. An NR2Cb plasmid encoding the NR2Cb open reading frame was constructed by inserting (via an *Sca*I restriction site) the C1–C3 PCR product into the NR2C coding region cloned into a pcDNA I plasmid (Fig. 2A). RT-PCR was performed from RNA extracted from cultured cerebellar cells with primers C5 and C4 (primer C4 was selected within Ins I), which span the entire coding region of NR2Cb, whereas PCR with the same primers was performed from the NR2Cb plasmid (Fig. 2B). Comparison of the digestion profiles of the RT-PCR and PCR products indicates that in the cultured granule neurons there are no insertions or deletions upstream of the NR2Cb insertion (Fig. 2C).

Several minor DNA products were detected by the TM3 probe on Southern blots with RT-PCR products from rat cerebellum (Fig. 1B). We used PCR primers C2 and C4 (Fig. 3A) to assess whether in developing cerebellum Ins I was associated with other deletions and insertions in the region spanning primers C1 and C3. Two RT-PCR products were obtained from developing cerebellum: the expected DNA fragment (456 bp) and a larger one (587 bp) with a 131-bp insertion (Ins II) whose levels of amplification were higher than those of the expected product. As expected from the RT-PCR results with primers C5–C4 (Fig. 2B), only one DNA fragment was obtained from the cultured granule cells (Fig. 3B). Cloning and sequencing of the 587-bp product show that Ins II is located between the regions encoding the hydrophobic domains I and II and that its sequence contains a stop codon (Fig. 3C). Apparently, Ins I is not associated with Δ I. Hence, the splicing events leading to the NR2Cb and NR2Cc mRNAs are mutually exclusive. The hypothetical polypeptide translated from the mRNA containing Ins II is named NR2Cd and is schematized in Fig. 3D.

The Ins II sequence has no homology with database sequences, whereas a portion of the Ins I sequence (nucleotides 29–95) is 76% homologous to the minus strand of a segment of the human and mouse fibroblast growth factor FGF8 mRNA sequences. Comparison with the exon–intron boundaries of the murine NR2C gene (Suchanek et al., 1995) strongly suggests that Ins I and Ins II correspond to unspliced introns (Fig. 3E). Comparisons with splice variants of the human NR2C mRNA [hNR2C-2, -3, and -4 (Daggett et al., 1998)] show that

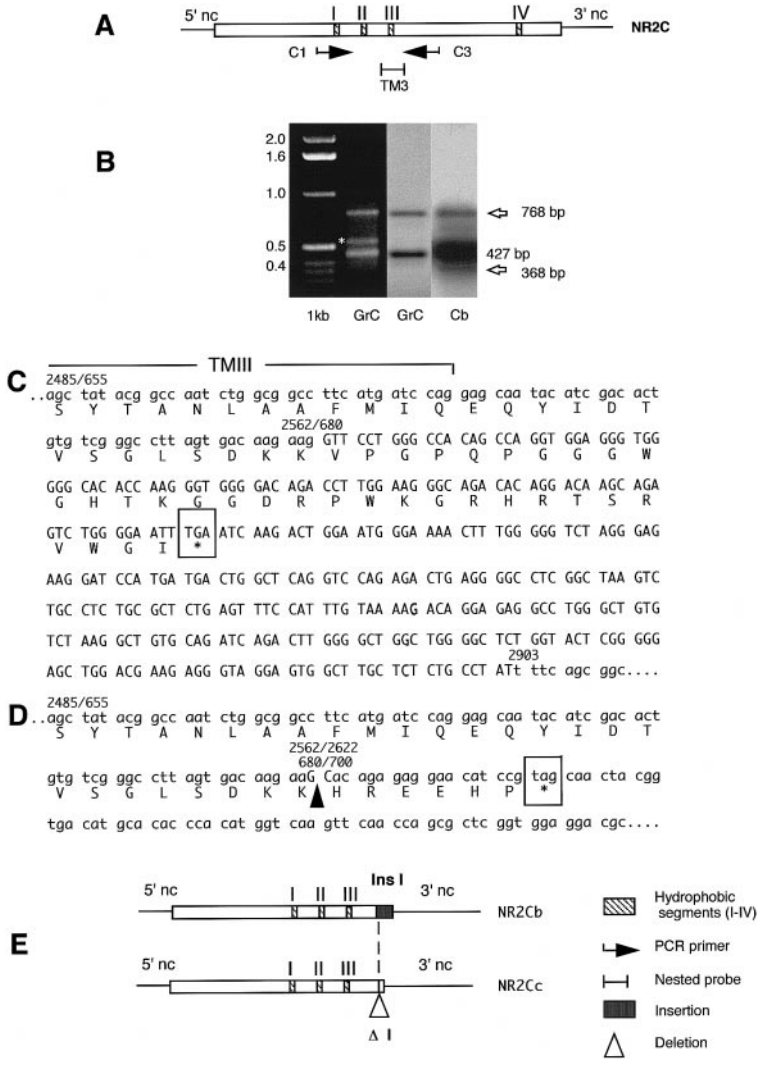

FIG. 1. Characterization of the NR2Cb and NR2Cc splice variants. **A:** Schematic representation of the position of PCR primers C1 and C3 and nested probe TM3 relative to the NR2C subunit. Coding regions are boxed; thin lines represent 5′ and 3′ untranslated regions. **B:** Ethidium bromide-stained agarose gel (two left lanes) with the PCR-amplified products from cultured cerebellar granule cells (GrC). 1 kb, DNA ladder from Life Technologies. The two right lanes are autoradiograms of a Southern blot of the GrC and adult cerebellar tissue (Cb) RT-PCR products hybridized with probe TM3. Open arrows indicate the bands that were gel-isolated and cloned, and the asterisk the PCR product undetected by probe TM3. **C** and **D:** Sequences of the RT-PCR products that were isolated. Numbers refer to nucleotide position in the reference rat NR2C sequence (see Table 1). In C, the Ins I sequence is in capital letters. In D, the site of the Δ I deletion is indicated by an arrow. Stop codons are boxed. **E:** Schematic diagram of the putative NR2C protein variants encoded by the NR2Cb and NR2Cc splice variants. Coding regions are boxed; thin lines represent 5′ and 3′ untranslated regions.

the five-amino acid deletion of the hNR2C-2 variant occurs at the same site identified for Ins II in the rat mRNA (K604 in the rat NR2C protein). In contrast, there is no correspondence between the sites for rat Ins I and Δ I (K680 in the rat NR2C protein) and the sites for the human eight-amino acid insertion (hNR2C-3) and 17-amino acid deletion (hNR2C-4) (G721 in the human NR2C protein sequence) (Fig. 3F).

Developmental expression of the NR2C splice variants

We used the C1–C3 primer pairs (Fig. 4A) to study the developmental expression of the NR2C splice variants in cultured cerebellar neurons (Fig. 4B) and in developing cerebellum and hippocampus (P0 to adult) (Fig. 4C). Following RT-PCR and Southern transfer, membranes were hybridized with the TM3 oligonucleotide probe. In cultured cerebellar cells, the level of the NR2Cb variant increases with the number of days in vitro relative to the expected NR2C variant. In developing cerebellum, short exposure times of the autoradiograms allow only detection of the expected NR2C splice variant. At longer exposure times, the other splice variants are detected. The NR2Cb variant is transiently expressed at P0 and is next detectable at P10. At P4 and P7, the NR2Cd variant is expressed at higher levels than NR2Cb. The NR2Cc variant with the 59-bp deletion is also expressed starting at P0. Another unidentified RT-PCR product appears at P10 (star on Fig. 4C). In the hippocampus, the expected NR2C mRNA is detected by RT-PCR at all stages of development. The NR2Cd splice variant is detected but only at P0, even following long exposure times of the autoradiograms.

We next used the C2–C4 and C1–C6 primer pairs (Fig. 4E and G) to study in more detail the developmental expression of the NR2Cb and NR2Cd splice variants in developing cerebellum and hippocampus. The TM3 and C11 oligonucleotide probes were used to detect the C2–C4 and C1–C6 PCR products, respectively. The results obtained confirm those of the previous experiments. In the cerebellum, the NR2Cd variant is expressed at low

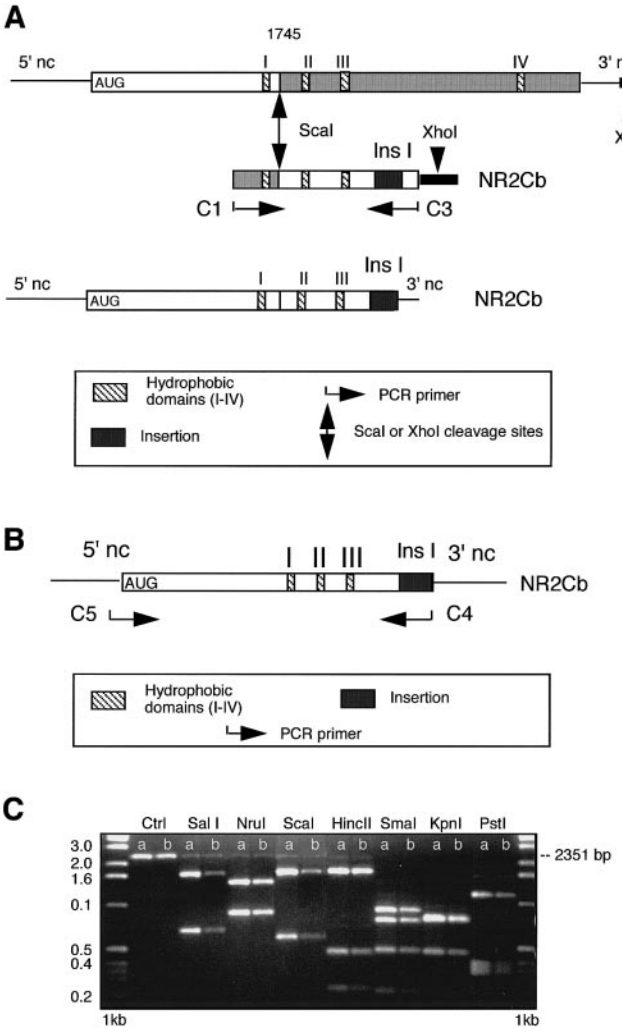

FIG. 2. A: NR2Cb cDNA construct. The C1–C3 PCR product and a plasmid containing the full-length NR2C cDNA were digested by *Sca*I and *Xho*I. The *Sca*I–*Xho* cassette from NR2Cb was ligated into the NR2C plasmid. **B:** Schematic representation of the position of PCR primers C5 and C4 (selected in Ins I) relative to the NR2C subunit. Coding regions are boxed; thin lines represent 5′ and 3′ untranslated regions. **C:** The restriction patterns of the PCR products obtained from the cloned NR2Cb plasmid (A) and from cerebellar granule cell cDNA are identical on an ethidium bromide-stained gel. Ctrl, control.

levels at P0 relative to NR2Cb and NR2C. Then NR2Cd is expressed at higher levels at P4 and P7, at low levels at P10, and again transiently at P14 (Fig. 4F and H). At these stages, the NR2Cd variant represents ~10% of the native NR2C PCR product (Fig. 4H). Later, the NR2Cb variant predominates (Fig. 4F). In the hippocampus, we detect only the NR2Cd splice variant at P0 (Fig. 4F and H) and in proportions that are important relative to the expected NR2C variant. Low levels of NR2Cb are detected until P21 (Fig. 4F). The NR2Cb and NR2Cd splice variants are absent from the adult hippocampus. Finally, we used primers C1–C7 to amplify selectively the NR2Cc splice variants with the 59-bp deletion (Fig. 4I). These were only found in the cerebellum and are expressed at P14 and later (Fig. 4J).

Expression of the NR2C splice variants in brain sections as revealed by in situ hybridization

Oligonucleotide probes were designed to recognize the NR2C, NR2Cb, NR2Cc, and NR2Cd mRNA splice variants (Fig. 5A). Plasmids containing cDNA inserts corresponding to each NR2C splice variant were digested, and inserts were gel-separated (Fig. 5B). Identical Southern blots were hybridized sequentially with the different oligonucleotide probes. Probe C10 detects the fully spliced NR2C mRNAs as it spans domains adjacent to Ins I and probe C12 spans domains adjacent to Δ I. A pan probe (C11) detects all four inserts, whereas probes C4 and C9 were selected to hybridize in Ins I and Ins II domains, respectively. Southern blots indicate that at high stringency all probes are specific. For in situ hybridization, we also used as control an oligonucleotide probe that was used by various authors (Monyer et al., 1992; Pollard et al., 1993) to detect the NR2C subunit mRNA. Each one of these probes was used for in situ hybridization.

The intensity of the hybridization signals differs for a given probe in the course of development, indicating that alternative splicing of the NR2C variants is developmentally regulated and among the different probes in agreement with our RT-PCR results. Splice variants can be detected early in postnatal development in different brain structures. By P14, the pattern of labeling becomes adult-

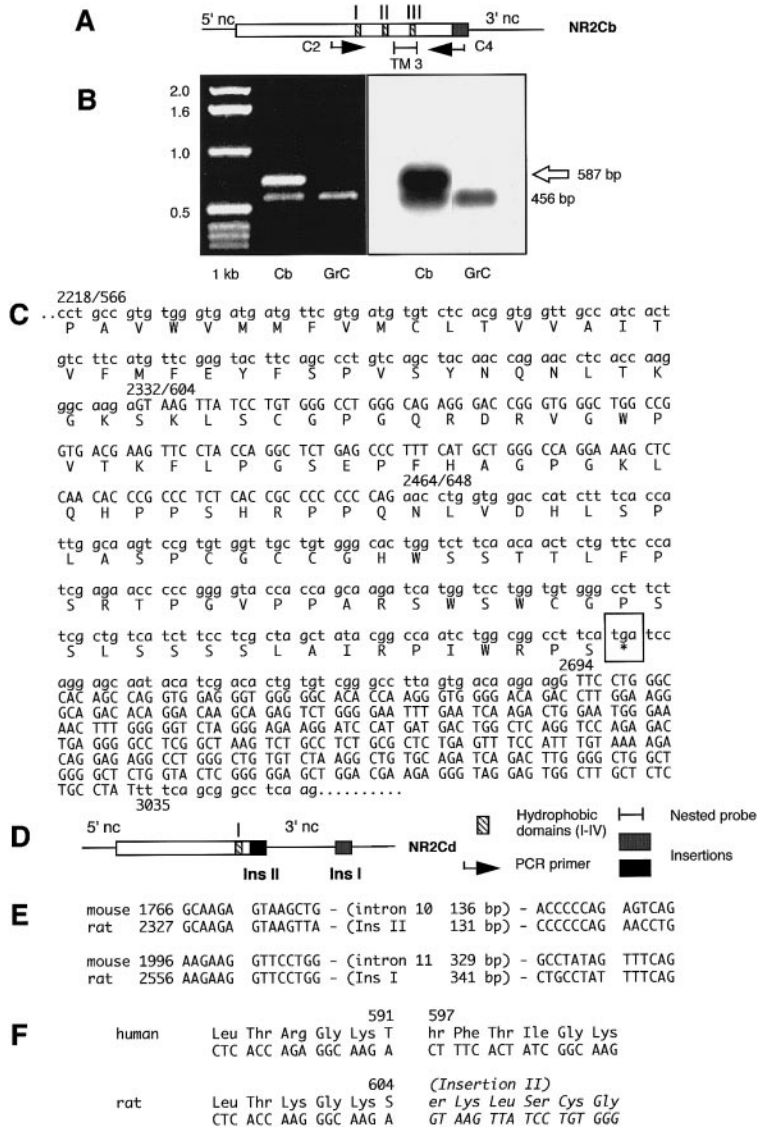

FIG. 3. Characterization of the NR2Cd splice variant. **A:** Schematic representation of the position of PCR primers C2 and C4 (selected in Ins I) and nested probe TM3 relative to the NR2C subunit. The coding region is boxed; thin lines represent 5′ and 3′ untranslated regions. nc, noncoding. **B:** Ethidium bromide-stained agarose gel (three left lanes) with the PCR-amplified products from cerebellum (Cb) and from cultured cerebellar granule cells (GrC). The two right lanes are autoradiograms of a Southern blot of the Cb and GrC RT-PCR products hybridized with probe TM3. The open arrow indicates the band that was gel-isolated and cloned. **C:** Sequence of the cloned RT-PCR product. Numbers refer to nucleotide and amino acid positions in the rat NR2C sequence of reference (see Table 1). The Ins II sequence is in capital letters. The stop codon is boxed. **D:** Schematic diagram of the putative NR2C protein encoded by the NR2Cd splice variant. **E:** Comparison of the sequences adjacent to introns 10 and 11 in the mouse NR2C gene (Suchanek et al., 1995) and to rat Ins I and Ins II. Numbers indicate nucleotide positions in the mouse and rat NR2C mRNA sequences. **F:** Comparison of the sequence adjacent to the five-amino acid deletion of the human NR2C-2 variant (Daggett et al., 1998) and adjacent to rat Ins II (italics). Numbers indicate amino acid residue positions in the human and rat NR2C protein sequences.

like and essentially restricted to the cerebellum. Labelings obtained with probes C10 (Fig. 5C) and NR2C (data not shown) are identical throughout development, with strong labeling in the cerebellum and weak labeling in hippocampus. Probe C4 (Fig. 5C) should detect both the NR2Cb (Ins I only) and NR2Cd (Ins I and Ins II) variants. We find increased labeling during cerebellar development with maximal signal in the adult cerebellum. Probe C4 also labels the hippocampus with higher labeling in the early stages of postnatal development (P0 and P4). Probe C9 (Fig. 5C) detects Ins II and labels the cerebellum most prominently during the first 2 weeks of development, although labeling is substantially lower than that obtained with other probes. The hippocampus is slightly labeled during the first postnatal days. Probe C12 labels the developing cerebellum starting at P14 (Fig. 5C). Emulsion-dipped slices (data not shown) indicate that in the adult brain all the NR2C variants are expressed in the granule cell layer of the cerebellum and are absent from the Purkinje cells. In the hippocampus, silver grains are observed over the dentate gyrus and to a lower extent in the CA1 region.

Transfection of NR1 and NR2C splice variants in HEK 293 cells and recordings

HEK 293 cells were cotransfected with plasmid constructs expressing NR1–NR2A, NR1–NR2C, or NR1–NR2Cb combinations, and we assessed recordings and microfluorimetry emissions from isolated cells and from groups of cells (Fig. 6). In HEK 293 cells expressing NR1–NR2A (used for comparison) and NR1–NR2C assemblies, glutamate induced typical inward currents whose amplitudes averaged 500 and 100 pA with a τ_{off} of 200 and 300 ms, respectively (Fig. 6A). In cells transfected with NR1–NR2Cb

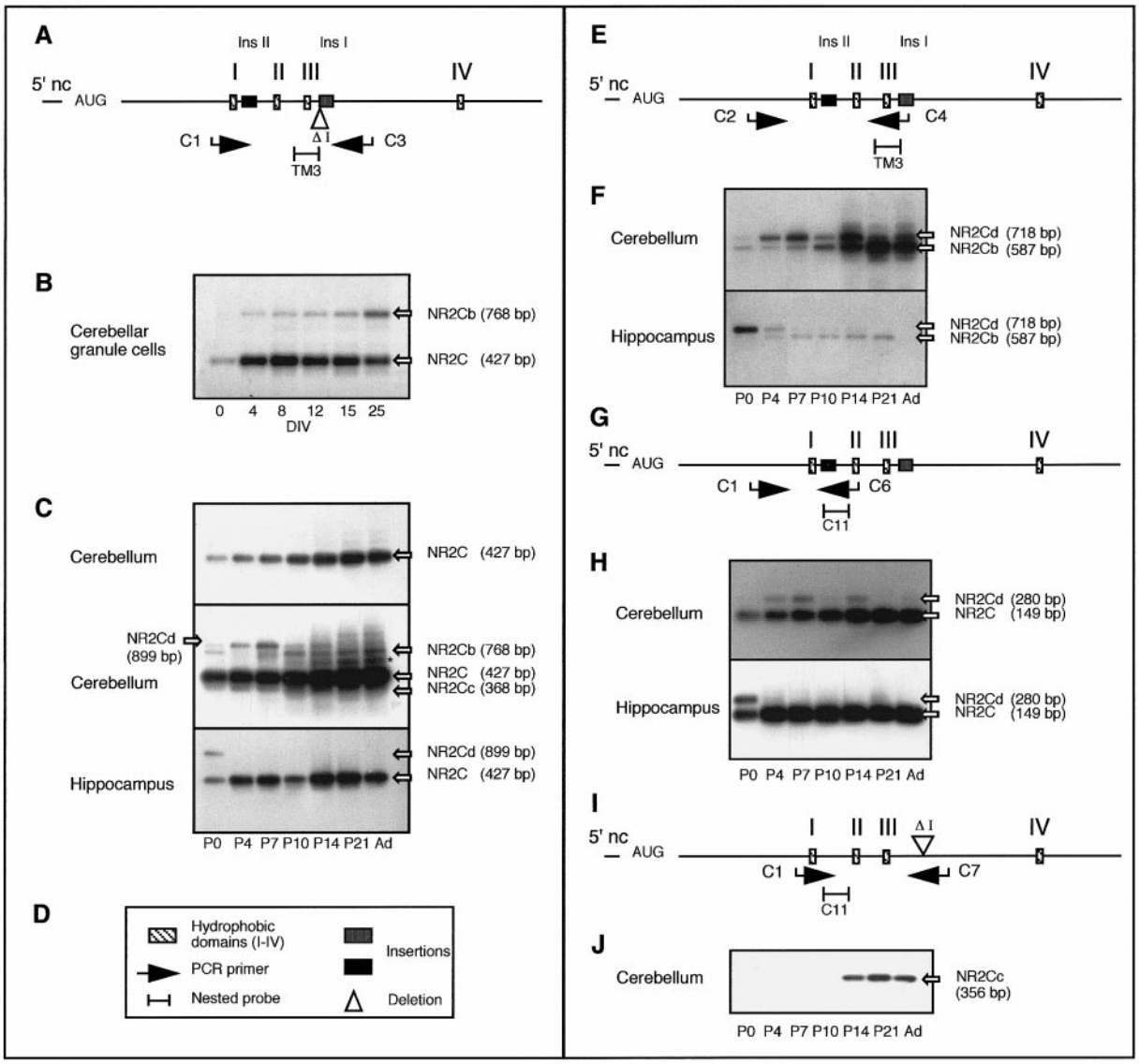

FIG. 4. Developmental expression of the NR2C splice variants in cultured cerebellar granule cells and in developing cerebellum and hippocampus. **A, E, G,** and **I:** Schematic representation of the position of PCR primers C1–C3, C2–C4 (C4 is selected in Ins I), C1–C6, and C1–C7 (C7 spans, and hence is specific for, deletion Δ I) and nested probes TM3 and C11 relative to Ins I, Ins II, and Δ I. nc, noncoding. **B:** Autoradiogram of a Southern blot with the RT-PCR products from cerebellar granule cells grown from 0 (disrupted tissue, before plating) to 25 days in vitro, hybridized with probe TM3. **C, F, H,** and **J:** Autoradiograms of Southern blots with the RT-PCR products from developing rat cerebellum and hippocampus, from P0 to adult, hybridized with probes TM3 and C11. In C, the same membrane was exposed for 2 h (**upper panel**) and 6 h (**lower panel**); with the C1–C3 primer pair, we do not detect the NR2Cb or NR2Cc PCR products in the hippocampus. **D:** Legend. In F, with primer C4 specifically selected in Ins I, low levels of NR2Cb were detected in hippocampal tissue from P0 (at this stage, Ins I is associated with Ins II) to P21.

cDNAs, we were not able to record any glutamate-induced responses (n = 150, 11 independent transfections). To confirm that the NR1–NR2Cb assemblies did not form functional channels, we assessed microfluorimetry emission from transfected cells loaded with indo-1 acetoxymethyl ester following glutamate stimulation. Significant increases were obtained in the NR1–NR2C-transfected cells, whereas no differences were observed in those transfected with the NR1–NR2Cb constructs (Fig. 6B).

DISCUSSION

Ins I and Ins II correspond to unspliced introns characterized in the mouse NR2C gene

We describe here the isolation, developmental expression, and distribution of rat NR2C mRNA variants that we have named NR2Cb, NR2Cd, and NR2Cc. The first two contain, respectively, one and two insertions, whereas the latter carries a deletion when compared with the prototypic NR2C sequence. In all three cases, the

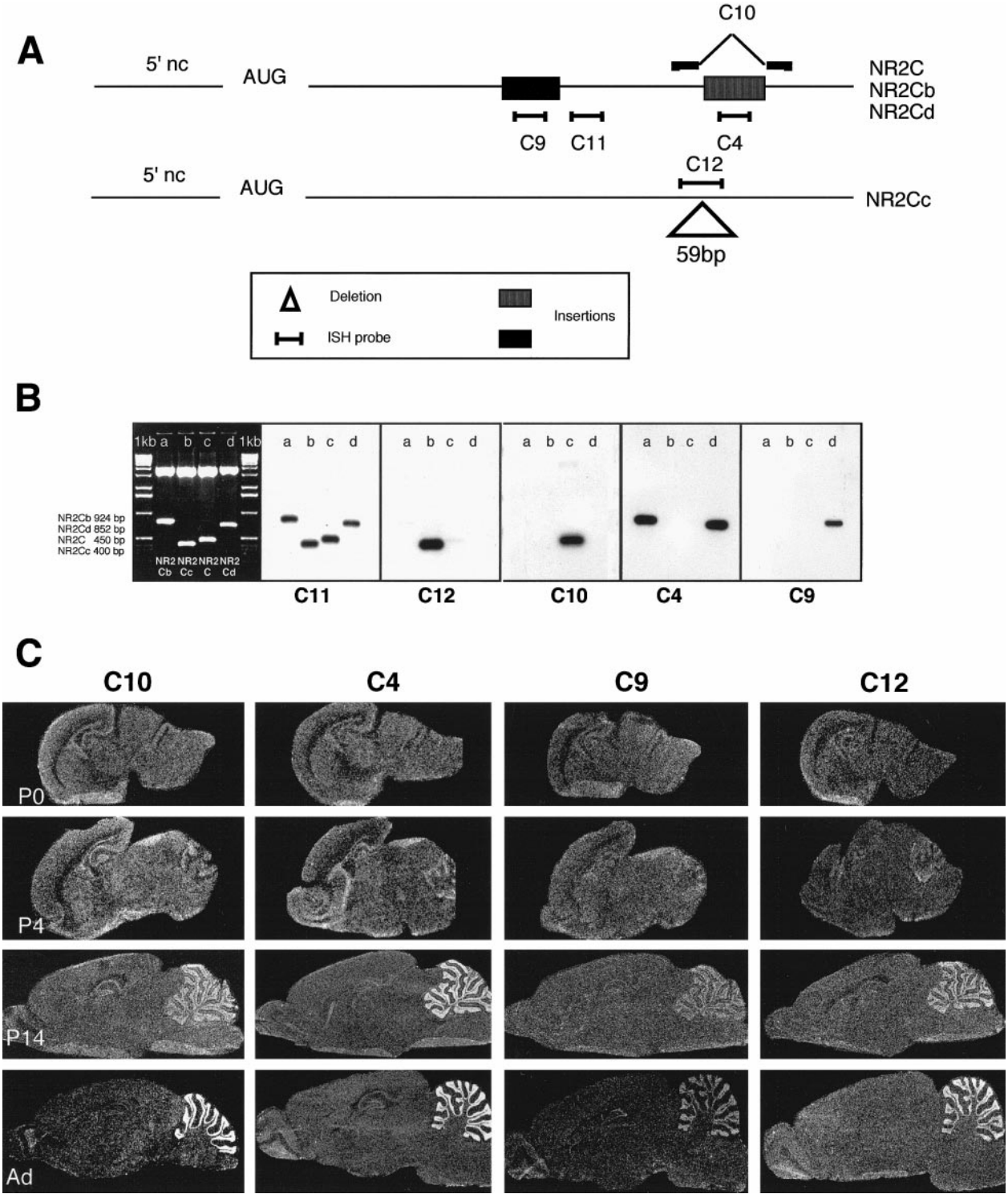

FIG. 5. Expression of the NR2C splice variants in the developing rat brain. **A:** Schematic representation of the position of probes C4, C9, C10, C11, and C12, relative to Ins I, Ins II (boxed), and Δ I. nc, noncoding. **B:** Ethidium bromide-stained agarose gel (left panel) with cloned cDNA fragments corresponding to NR2Cb, NR2Cd, NR2Cc, and the native NR2C. Numbers on the left indicate the sizes of the cDNAs following restriction enzyme digestion of the plasmids. The five right panels show autoradiograms of Southern blots of gels identical to the ethidium bromide-stained gel, probed with C11, C12, C10, C4, and C9, respectively, showing specificity of these probes. **C:** Dark-field photomicrographs obtained from representative autoradiograms from sagittal brain sections hybridized with the C10, C4, C9, and C12 oligoprobes at different stages of development [P0, P4, P14, and adult (Ad)].

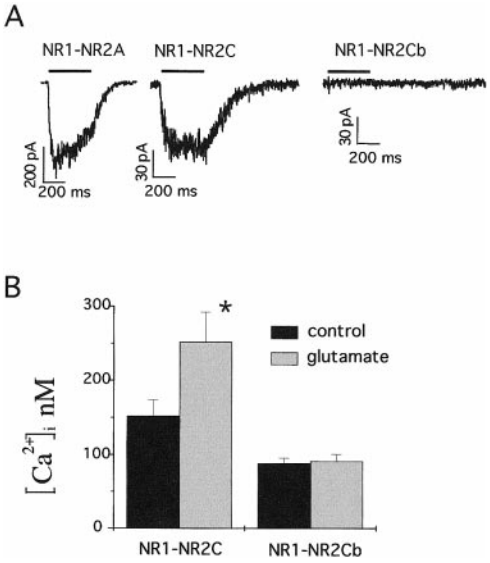

FIG. 6. Functional analysis of the NR1/NR2Cb combination in transfected HEK 293 cells. **A:** Typical recordings from isolated cells transfected with NR1/NR2A, NR1/NR2C, and NR1/NR2Cb plasmids. No current was observed during glutamate application to cells transfected with the NR1/NR2Cb combination. **B:** Microfluorimetry emission from transfected cells loaded with indo-1 acetoxymethyl ester following glutamate stimulation. No changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) were observed in HEK cells transfected with the NR1/NR2Cb plasmids. Data are mean ± SEM (bars) values of measurements from at least 50 cells in four independent transfections. $^*p < 0.05$ versus control.

open reading frames are prematurely interrupted, and the mRNAs thus encode putative truncated NR2C polypeptides. The C-terminal end of two of these polypeptides is located between the third and fourth hydrophobic domain; they are thus lacking the large C-terminal intracellular domain. The C-terminus of the NR2Cd polypeptide is located downstream of the first hydrophobic domain. Sequence analysis of the localization of the inserts in NR2Cb and NR2Cd strongly suggests that they correspond to unspliced introns. Indeed, inserts II and I are very similar in length to introns 10 and 11 described in the mouse NR2C gene, and sequences adjacent to the insertions are nearly identical to the exon–intron boundaries of this gene for introns 10 and 11 (Suchanek et al., 1995). Alternative NR2C splice products have been previously isolated in mouse and rat brain that differ in the 5′ untranslated region and thus do not affect the NR2C coding region (Suchanek et al., 1995). Three NR2C splice variants (hNR2C-2, -3, and -4) have also been isolated from human brain that contain, respectively, a five-amino acid deletion, an eight-amino acid insertion, and a 17-amino acid deletion when compared with the prototypic hNR2C-1 (Daggett et al., 1998). The five-amino acid deletion of the hNR2C-2 variant occurs at the very same splice acceptor site we identified for Ins II in the rat mRNA, hence just upstream from hydrophobic domain II (K604 in the rat NR2C protein), whereas there is no correspondence between the sites for rat Ins I and Δ I (K680 in the rat NR2C protein) and the sites for the human eight-amino acid insertion and 17-amino acid deletion (G721 in the human NR2C protein sequence). In the case of the human splice variants, neither the eight-amino acid insertion nor the five- and 17-amino acid deletions alter the open reading frames: thus, the hNR2C splice variants encode nontruncated NR2C polypeptides. Our results thus demonstrate that some of the splice acceptor sites are conserved among the rat, mouse, and human but that at some of these sites, there is a fair degree of variability in splicing.

Expression of the rat NR2C splice variants is regionally and developmentally regulated

Both RT-PCR and in situ hybridization confirm the presence of NR2C mRNAs in the developing cerebellum and hippocampus, in agreement with previous reports (Pollard et al., 1993; Monyer et al., 1994). Overall, the RT-PCR-based patterns of expression of the NR2C splice variants we characterized were confirmed by in situ hybridization, most notably in the cerebellum, where high levels of NR2C mRNA expression are observed. In the hippocampus, confirmation of the RT-PCR results with in situ data is more difficult because NR2C mRNAs are expressed at lower levels than in the cerebellum and increased exposure times generate background. Our RT-PCR-based study of the expression of the various NR2C splice variants indicates that culture conditions alter the splicing pattern of the NR2C transcript when compared with developing brain in vivo and that splicing of the NR2C mRNA is developmentally and regionally regulated. For instance, we show that Ins I is present in a significant proportion of the NR2C mRNAs expressed in aging cerebellar cultures, where this insertion does not seem to be associated with Ins II or with Δ I. In the hippocampus, NR2Cd is expressed at relatively high levels when compared with the prototypic NR2C, but only at P0, and the NR2Cb splice variant is barely detectable in the following stages of development. In cerebellar tissue, and at all stages of ontogeny, the splice variants that differ from the prototypic NR2C represent only a small proportion of the NR2C mRNAs. The most complex pattern is observed in the developing cerebellum, where several RT-PCR schemes confirm an alternative expression of NR2Cb and NR2Cd with a higher expression of NR2Cd over NR2Cb between P4 and P7. These observations could result from the heterogeneity in the degree of maturation of the cerebellar granule neurons, which differ significantly in their differentiation in the external and internal granule layers. It is, however, difficult to associate incomplete splicing of the primary transcripts with neuronal differentiation as RT-PCR results differ substantially between cerebellum and hippocampus. Indeed, the increasing number of PCR products (some of which we characterized) that are detected by the NR2C-specific oligonucleotide probe (TM3) on Southern blots at P21 and in the adult cerebellum, combined with the results obtained with the cerebellar cultures, suggests that incomplete splicing of introns in-

creases with neuronal development and differentiation. However, the RT-PCR results obtained from the developing hippocampus suggest the opposite. In addition, in our in situ hybridization study, we were not able to assign specific NR2C splice variants to the external versus internal granule cell layers of the developing cerebellum, suggesting that there is no strict correlation between splicing efficiency and granule cell maturation.

The rat NR2Cb splice variant does not display agonist responses in transfected cells

Overall, it appears that there is complex alternative splicing of the NR2C mRNA in various species and that the NR2C mRNA splice variants, including those that have the potential to encode truncated NR2C polypeptides, are accumulated in neurons and thus readily detectable, not only by RT-PCR but also by in situ hybridization. Our data indicate that the expression of the intron-containing splice variants is developmentally regulated and, at least in the cultured cerebellar cells and in the early stages of hippocampal development, can represent a significant proportion of the NR2C mRNAs. Our observations are surprising as it has been shown that nonsense mRNAs are highly unstable in various species, including the mouse and human. As a result of RNA surveillance (Pulak and Anderson, 1993), nonsense mRNAs are degraded by a pathway called nonsense-mediated mRNA decay (Leeds et al., 1992). To date, we have no evidence of the expression of truncated NR2C polypeptides in cerebellar neurons or evidence of whether such truncated polypeptides would be inserted in neuronal membranes and contribute to the formation of NMDA receptor complexes. Antibodies directed against the specific domains of the truncated NR2C protein variants should help address these issues. Indeed, even if presently available polyclonal antibodies directed against the N-terminal region of the rat NR2C subunit detect polypeptides of smaller size than NR2C on western blots, we cannot exclude at this point that these are merely breakdown products of the native NR2C protein (or of NR2A, considering that the NR2C antibodies also recognize other NR2 protein variants). In any case, the apparent stability of the rat NR2C mRNA splice variants and the developmental regulation of their expression raise the question of a potential role for truncated NR2C protein isoforms. HEK 293 cells cotransfected with NR1 and NR2Cb did not display glutamate-induced responses or an elevation of intracellular free calcium level, suggesting that NR2C subunits that lack the fourth membrane segment and the entire intracellular C-terminal domain cannot contribute to the formation of functional NMDA receptor–channels. Sprengel et al. (1998) have shown that C-terminal truncation of NR2 subunits downstream of the fourth membrane segment does not interfere with the formation of gateable receptor channels and with the induction of Ca^{2+} transients. It thus appears that in NR2 subunits the fourth membrane segment is critical for the formation of gateable receptor channels.

In conclusion, it remains to be proven that truncated NR2C subunits are indeed expressed in neurons and, if such is the case, the functional significance of truncated subunits will need to be assessed. One possibility is that nonfunctional truncated subunits, following their incorporation into receptor–channel complexes, could contribute to their downshifting.

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