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Identification of a long variant of mRNA encoding the NR3 subunit of the NMDA receptor: its regional distribution and developmental expression in the rat brain

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Abstract A longer variant of rat mRNA encoding the NR3 subunit of the NMDA receptor has been identified. It contains a 60-bp insertion at the nucleotide position 3007 in the intracellular domain of the C-terminal of the previously cloned variant. Therefore, the NR3 mRNA exists in at least two variants – with the insert (NR3-long; NR3-l) and without the insert (NR3-short; NR3-s). The NR3-l variant is expressed throughout the adult rat brain. Moreover, this variant predominates in the occipital and entorhinal cortices, thalamus and cerebellum. Analysis of NR3-l development indicates that it is regulated in a region-specific manner.

Key words: NMDA receptor; NR3 subunit; Variant; Cloning; Phosphorylation; Distribution; Development

1. Introduction

The NMDA receptors constitute a major class of the excitatory amino acid-gated ion channels. Molecular cloning efforts have identified six rat NMDA receptor subunits: NR1, NR2A, 2B, 2C and 2D (NR2A–D) and NR3 (the latter also known as NR3A, NR-like and χ-1) [1–9]. Heterologous co-expression studies demonstrated that NR1 is an essential channel-forming NMDA receptor subunit. In contrast, the NR2A–D subunits do not by themselves produce functional channels but potentiate NR1 activity and confer functional variability to the NMDA receptors [1–4,10]. NR3 is also a regulatory subunit. Its presence decreases the channel currents in NR1/NR2 heteromeric receptors [7–9]. The existence of multiple NMDA receptors composed of different subunit combinations may underlie a great functional diversity characteristic of the native NMDA receptors.

The diversity of the NMDA receptors could be further enhanced by the alternative splicing of pre-mRNAs of receptor subunits. Until now, only the NR1 variants have been identified [11–14]. The eight known variants of this subunit display a high degree of functional heterogeneity. The homomeric NMDA receptors assembled from these NR1 splice variants differ in agonist affinity, non-competitive antagonist affinity, current amplitude, pH sensitivity, Zn^{2+} modulation, polyamine potentiation, and regulation by protein kinase C (PKC) [15–19].

In this paper, we report that mRNA encoding the NR3 subunit also exists as at least two forms. The new variant, which we named NR3-long (NR3-l), differs from the previously described form by a 60-bp insertion within the intracellular domain of the C-terminal. The earlier cloned form of NR3 will now be referred to as NR3-short (NR3-s). We also examined the regional and developmental expressions of the new NR3 variant.

2. Materials and methods

2.1. RNA extraction and cDNA synthesis

Total RNA was extracted from the rat brain tissue using RNeasy columns (QIAGEN, Chatsworth, VA, USA). Poly(A)$^+$ RNA was prepared employing a Poly(A)-pure mRNA Isolation Kit (Ambion, Austin, TX, USA). First strand complementary DNA (cDNA) synthesis was primed with random hexamers using First-strand beads (Pharmacia, Piscataway, NJ, USA).

2.2. cDNA cloning

Single-strand cDNA, reversely transcribed from rat telencephalon poly(A)$^+$ RNA, was used in the PCR reactions with two pairs of primers: (i) ACATAGTGCACAGACTGCTGTT (forward) and TCGTTGGTTGTCATGACTCA (reverse); (ii) CTAGACGCCTTCATCATGGAC (forward) and CTCTGTCTTCCTGCTTACAGC (reverse). The first pair was designed based on the unique sequence of NR3, which spans the intracellular C-terminal region (Fig. 1A). The second pair amplifies the portion encompassing transmembrane regions III–IV (TMs III–IV) extracellular loop and the C-terminal (Fig. 1A). The above-mentioned PCR reactions were conducted on a DeltaCycler System (ERICOMP, San Diego, CA, USA) using PCR beads (Pharmacia, Piscataway, NJ, USA). Thirty-two cycles of amplification were performed with denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min. The final cycle was followed by a 10-min extension step at 72°C. The concentration of both sets of primers was 0.25 μM and the reaction volume was 50 μl. The amplified cDNA fragments were identified by gel electrophoresis, followed by a confirmation of their specificity by digestion with restriction enzymes (*Pst*I and *Alu*I). The fragments were then subcloned into pCR2.1 vector (Invitrogen, Carlsbad, CA, USA) and sequenced on an Automatic ABI 373 DNA sequencer (Perkin-Elmer, Foster, CA, USA). Sequence analysis was performed with BLASTN program (NCBI, Bethesda, MD, USA).

2.3. Northern blot

To prepare the ^{32}P-labeled antisense RNA probe, the newly-discovered 60-base insertion into NR3 cDNA was subcloned into pGEM-TEasy vector (Promega, Madison, WI, USA). The resulting plasmid was linearized by restriction digestion with *Nco*I, and antisense RNA was transcribed with SP6 RNA polymerase (Promega, Madison, WI, USA) in the presence of α-^{32}P-UTP (New Life Science Product, Boston, MA, USA).

For analysis, 5 μg of poly(A)$^+$ RNA prepared from diencephalon/telencephalon and cerebellum was separated on 1% agarose-formaldehyde gel and transferred to positively charged nylon membrane (Ambion, Austin, TX, USA). Membrane was hybridized overnight at 68°C with the ^{32}P-labeled antisense RNA probe and then washed in 2×

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SSC and 0.5% SDS solution three times at room temperature. The final wash was in 0.1× SSC and 0.5% SDS solution for 60 min at 68°C. To visualize the radiolabeling, the membranes were exposed to Kodak XAR film.

2.4. Analysis of regional distribution and development

The relative levels of NR3 variants were compared in multiple brain regions of adult rats. The levels of these variants were also examined in total rat brain tissue at embryonic days E15, E17 and E19, and in the combined rat telencephalon and diencephalon (without the olfactory bulb), cerebellum, and olfactory bulb at postnatal days P0, P3, P5, P7, P10, P14, P20, P25 and P60 (the latter age represented the mature animal). At least three rats were examined for each brain region and developmental age.

The identical amounts (10 ng) of cDNAs reversely transcribed from samples of total RNAs isolated from the above-mentioned tissues were used as templates in quantitative PCR assays. The primers GAAGAAAAGCAGCCACGTTCC (forward) and TCGTTGGTTGTCATGACTCA (reverse) were used to amplify 180-bp (long form) and 120-bp (short form) cDNA fragments. For all samples, 28 cycles of amplification were performed with the cycle parameters described above for the PCR procedure used in the cDNA cloning.

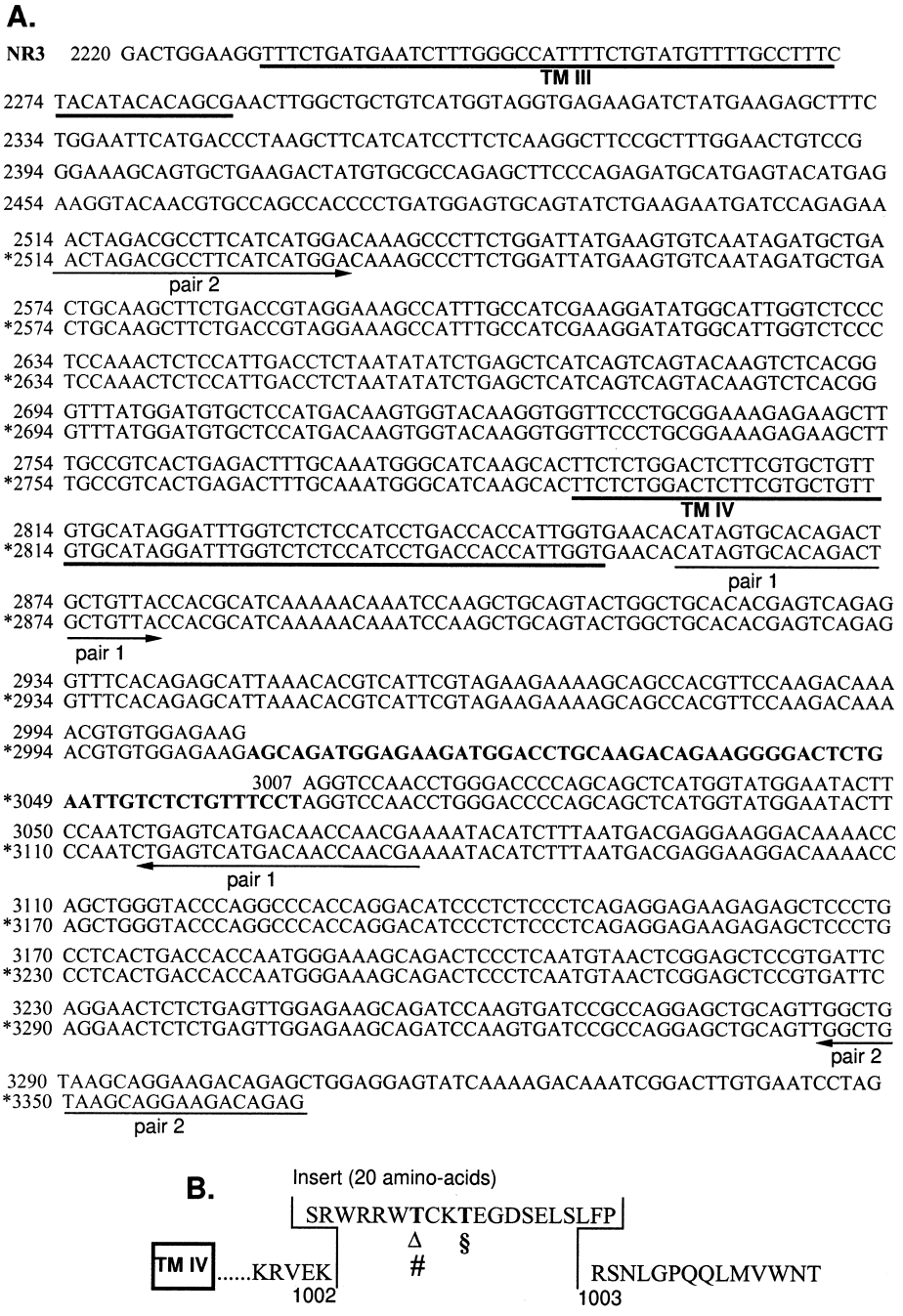

Fig. 1. A: Alignment of the previously reported sequence of the TM III C-terminal of the NR3 cDNA [7,8] and the large RT-PCR product obtained in the present study (marked by asterisk). The 60-base insertion between nucleotides 3006 and 3007 is in bold. The TM III and TM IV are underlined. Primers used for RT-PCR cloning are indicated with arrows. B: Deduced amino acid sequence of a portion of the C-terminal of the long NR3 variant. The upper portion depicts the 20 amino acid insertion between amino acids 1002 and 1003. The potential phosphorylation sites for PKC (△), PKA (#) and CaMK-II (§) are indicated. The numbering of the nucleotide and amino acid sequences is according to Ciabarra et al. [7]. The GenBank accession number of the new nucleic acid sequence is AF061945.

The concentration of primers was 0.4 μM, and the reaction volume was 50 μl. Our preliminary studies established that, under these conditions, the PCR products of serial dilutions of NR3 short and long form-containing plasmids and the corresponding PCR products of dilutions of cDNA reversely transcribed from the total brain RNA, produced parallel linear dilution curves. Furthermore, comparison of the above-mentioned dilution curves for PCR products of plasmids containing long form and short form of NR3 demonstrated similar efficiencies of amplification for fragments of both forms. Finally, our preliminary studies showed that PCR conditions employed in this study amplify fragments of both short and long NR3 forms below saturation levels for all tissues examined (data not shown). This demonstrates that our RT-PCR protocols were appropriate for quantitative analysis of the levels of the NR3 mRNAs [20]. Upon completion of amplification, 15 μl of each amplified sample was run in parallel with a 50-bp DNA ladder (Gibco, Gaithersburg, MD, USA) on a 2% ethidium bromide-stained agarose gel.

The regional levels of NR3 variants in adult rat were estimated visually using the above-mentioned gels containing PCR products of these variants. For developmental studies the quantification of the RT-PCR products obtained at different ages was performed with the Universal Software for Electrophoresis and TLC (Advanced American Biotechnology, Fullerton, CA, USA) using digitized images of the ethidium bromide-stained gels. Series of known amounts of PCR products placed in the same gels served as standards. The final results of quantitative RT-PCR were expressed as moles of amplified transcripts per ng of cDNA reversely transcribed from total tissue RNA.

2.5. Southern blot

PCR reaction products obtained from adult rat brain regions listed above were electrophoresed on 2% agarose gels and blotted onto Hybond-N membrane (Amersham, Buckinghamshire, UK). The membrane was hybridized overnight at 68°C with the α-^{32}P-labeled antisense RNA probe complementary to the 60-base insert of the long form, or hybridized at 42°C with an oligonucleotide probe common to both long and short forms. The common probe was complementary to nucleotides 3016–3075 of the original clone and it was labeled at its 3′-end with α-^{32}P-dTTP. The membrane was washed as described above for the Northern blot, except that the last wash of the membranes labeled with the oligonucleotide probe was conducted at 42°C. The radiolabeled membranes were exposed to Kodak XAR film.

3. Results and discussion

3.1. Isolation and characterization of NR3 variants

RT-PCR of the telencephalic poly(A)$^+$ RNA using primers flanking the unique C-terminal region of NR3 (pair 1, Fig. 1A), generated two products. The smaller product contained 213 nucleotides, constituting the 2857–3075 region of the previously described NR3 form. The sequence of the larger product was identical to the smaller one, except for a 60-bp insertion at position 3007. To confirm that the novel insert is associated with a variant of the NR3 subunit mRNA, an additional RT-PCR was performed using the second pair of primers surrounding the fragment of NR3 from TMs III–IV extracellular loop to the C-terminal (pair 2, Fig. 1A). Here also two products were amplified: an 800-bp fragment which was identical to the previously cloned NR3, and an 860-bp fragment which had the same nucleotide sequence as the shorter fragment except for the 60-bp insertion (Fig. 1A). Finally, to demonstrate that the insert was not an RT-PCR artifact and that mRNA containing such an insert exists in vivo, we performed Northern blot analysis of poly(A)$^+$ RNA from diencephalon/telencephalon and cerebellum with the probe specific for the insert. The Northern blot identified an approximately 8-kb transcript in both brain regions (Fig. 2). The size of the detected transcript was close to that which would be expected for the NR3 mRNA [7,8].

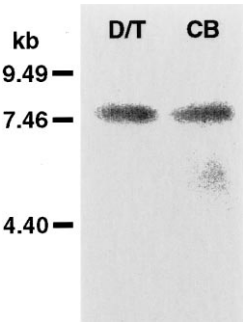

Fig. 2. Northern blot of the NR3-l variant from diencephalon/telencephalon (D/T) and cerebellum (CB). Poly(A)$^+$ RNA was loaded on 1% agarose-formaldehyde gel and then transferred to nylon membrane. The blots were visualized by hybridization with insert-specific ^{32}P-antisense RNA probe followed by exposure to Kodak XAR film.

These findings clearly demonstrate the existence of a previously unidentified longer form of mRNA encoding the NR3 subunit of the NMDA receptor and, therefore, this subunit may exist in at least two variants. We propose to designate the newly discovered long form NR3-l, and the previously cloned short form NR3-s. Based on the fact that NR3-l and NR3-s are identical, with the exception of a 60-bp insert, and the observation that alternative splicing is fairly common in ionotropic glutamate receptors [21], it is very likely that these two variants are generated by alternative splicing of a single gene transcript.

The deduced amino acid sequence of the insert of NR3-l shows that it consists of 20 amino acid residues at the position of amino acid 1003 within the intracellular domain of the C-terminal (Fig. 1B). A structural analysis of these 20 amino acid residues revealed two potential sites for phosphorylation. One of these sites may be phosphorylated by PKC and cAMP-dependent protein kinase A, while the other site may be phosphorylayed by calmodulin-dependent protein kinase II (Fig. 1B). This finding may be significant since a large body of evidence implicates phosphorylation of subunit proteins in regulating major properties of NMDA receptors, such as potentiation and subcellular distribution [22–25]. The presence of the potential phosphorylation sites within the insert also parallels the situation observed in the NR1 subunit where PKC-induced phosphorylation is mainly restricted to four serines located on the C1 splice cassette with the alternative splicings determining the presence or absence of these phosphorylation sites in the resulting form [26]. This is postulated to be one of the major mechanisms regulating functional activity of the NR1 subunits [26]. We hypothesize that the same mechanism operates in determining the sensitivity to phosphorylation, and thus the functional properties, of the NR3 subunits.

3.2. Regional expression of NR3-l and NR3-s variants

RT-PCR was used to examine the regional expression of the NR3-l and NR3-s variants in the olfactory bulb, frontal, occipital, entorhinal and pyriform cortices, hippocampus, striatum, thalamus, cerebellum and spinal cord of adult rat. Two fragments with the sizes expected to be produced by the NR3-l (180 bp) and NR3-s (120 bp) mRNAs were amplified from all tissue samples (Fig. 3A). To confirm that these fragments represent the transcripts of the NR3 forms, Southern blots of these products were performed with the insert-specific

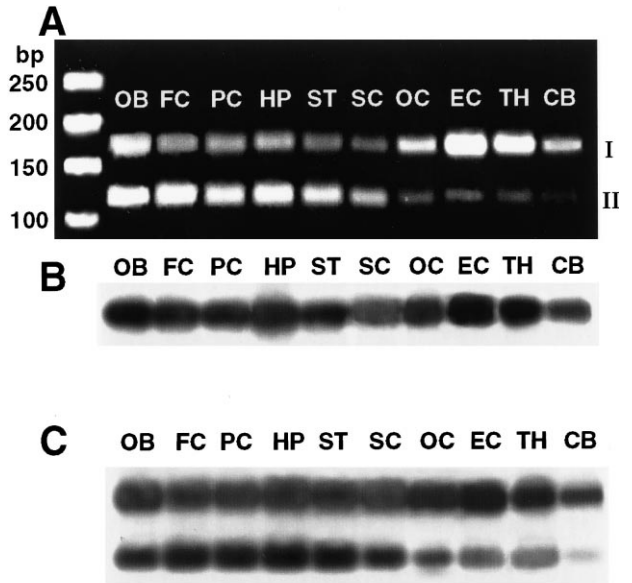

Fig. 3. Distribution of the transcripts of the NR3-l and NR3-s variants in different regions of the central nervous system. 10-ng samples of cDNA reversely transcribed from total RNA from nine regions of the rat brain were amplified, and 15 μl of each amplified sample eletrophoresed on 2% agarose gels as described in Section 2. The agarose gels were blotted to a nylon membrane and the specificity of the amplified fragments was checked by hybridization with an insert specific antisense ^{32}P-probe and a ^{32}P-probe common for both long and short NR3 variants. A: The agarose gel electrophoresis of RT-PCR products stained with ethidium bromide and illuminated with UV light. Note that two fragments with the size expected to be produced by PCR of the NR3-l (180 bp; I) and NR3-s (120 bp; II) mRNAs were amplified from all tissue samples. This gel demonstrates that the olfactory bulb (OB) has relatively similar levels of both NR3 variants. NR3-s predominates in the frontal cortex (FC), pyriform cortex (PC), hippocampus (HP), striatum (ST) and spinal cord (SC). NR3-l predominates in the occipital cortex (OC), entorhinal cortex (EC), thalamus (TH) and cerebellum (CB). B: Hybridization with the insert-specific probe. Note that this probe hybridized only to the large fragment. C: Hybridization with the probe common for both long and short NR3 variants. Note that both long and short fragments were hybridized to this probe.

probe and the probe to a sequence common for both variants. As predicted, the insert-specific probe hybridized only to the larger fragment while the common probe hybridized to both PCR products (Fig. 3B, C).

While both long and short forms of the NR3 mRNA were present in all the brain regions studied, their relative levels differ from region to region (Fig. 3A). The visual examination of the ethidium bromide gels containing RT-PCR products showed that the olfactory bulb was characterized by relatively close levels of NR3-l and NR3-s variants (Fig. 3A). The short form clearly predominated in the frontal and pyriform cortices as well as in the hippocampus and spinal cord (Fig. 3A). In contrast, the long form was predominant in the occipital and entorhinal cortices, thalamus and cerebellum. Moreover, the NR3-s variant was barely detectable in the latter region (Fig. 3A). This indicates that, similar to the NR1 splicing variants, expression of forms of the NR3 mRNA is regulated in a region-specific manner. The regional variations in the relative expression of the long and short NR3 forms may provide for more functionally distinct NMDA receptors in different parts of the brain.

3.3. Developmental expression of NR3 variants

The prenatal expression of the NR3 variants was examined in the whole rat brain. Both forms were detectable as early as E15 with NR3-s being the predominant form (Fig. 4, inset).

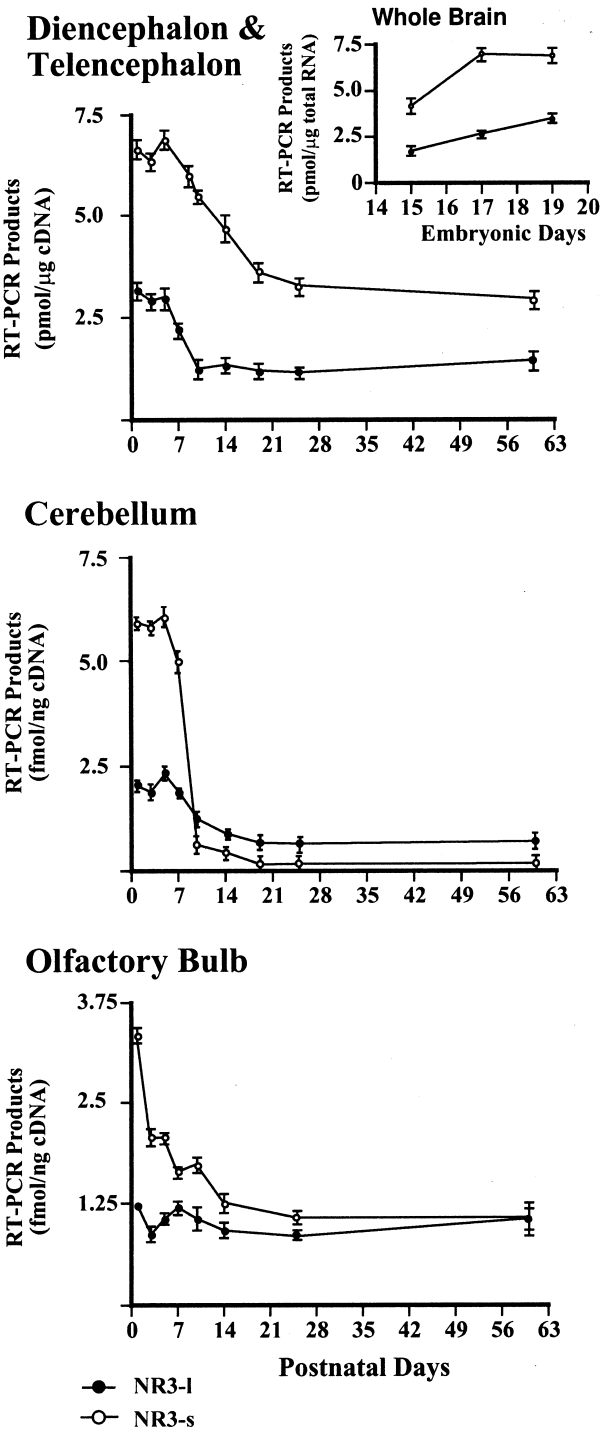

Fig. 4. Developmental changes in the expression of the NR3-s and NR3-l mRNAs. The transcript levels of the variants were determined by quantitative RT-PCR. The prenatal levels were measured in the whole brain (insert). The postnatal levels were examined in the combined diencephalon and telencephalon (without the olfactory bulb), cerebellum and olfactory bulb. The results were expressed as pmol of RT-PCR product per ng of cDNA reversely transcribed from the total tissue RNA. Each point is an average of three reactions ± S.E.M.

By E17, the levels of both forms increased by approximately 50%. At E19, the levels of the NR3-l variant were increased further by an additional 25%. In contrast, the NR3-s variant remained at the levels seen at E17 (Fig. 4, inset). Therefore, while the expression of the NR3-l form in the brain increases steadily throughout the third trimester, the expression of the NR3-s form remains virtually unchanged during the second half of this trimester.

The postnatal development of the NR3 mRNAs was studied in the combined telencephalon and diencephalon (without the olfactory bulb), cerebellum and olfactory bulb (Fig. 4). In the diencephalon/telencephalon of newborn rats, the expression of both NR3 variants was nearly twice as high as in adult animals, with the NR3-s clearly being the predominant form (the levels of NR3-l were only 50% of the levels of NR3-s). The levels of both variants remained steady throughout the first postnatal week and then began to decline to the adult levels, which were reached for the NR3-l form by P14 and for the NR3-s form by P25. NR3-s continued to be the predominant variant in the diencephalon/telencephalon of adult animals (Fig. 4). In the cerebellum of newborn rats, the levels of the NR3 mRNAs also were much higher than in adult animals (more than eight times higher for NR3-s and nearly twice as high for NR3-l) with NR3-s being the predominant variant (the levels of NR3-l were only 35% of the levels of NR3-s). After P7, the expressions of both NR3 variants declined sharply reaching close to the adult levels by P10. The decline was much more significant for NR3-s then for NR3-l, and the latter becomes the predominant form in the cerebellum of adult animals (Fig. 4). In the olfactory bulb of newborn animals, the expression of NR3-s was almost twice as high as that of NR3-l. In addition, the levels of NR3-s were much higher at birth than in adults. The expression of this variant declined steadily within the first two postnatal weeks reaching adult levels at P14. In contrast, the levels of the NR3-l variant remained virtually unchanged from birth to adulthood. This resulted in similar levels of expression of both NR3 variants in the adult olfactory bulb (Fig. 4).

Our observations confirm the previous developmental studies of the NR3-s mRNA which showed that the highest levels of its expression occur within the late prenatal and early postnatal periods, indicating that the NR3-s subunit may be important for the developmental activities of the NMDA receptors [7,8]. Our investigations also demonstrate that NR3-s is the predominant form during these developmental periods, with NR3-l being expressed at much lower levels, which points to NR3-s as the primary NR3 variant involved in regulation of brain development. NR3-l, however, may also be involved in some developmental actions of the NMDA receptor. This is suggested by its overexpression in the diencephalic/telencephalic and cerebellar tissues of newborn animals. It may be significant that, in contrast to other brain regions examined, the olfactory bulb does not display a significant postnatal decline in the NR3-l expression. This may be related to the fact that the olfactory bulb retains many properties of the immature nervous system and undergoes continued reorganization in adult individuals [27].

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