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SHORT COMMUNICATION
NR2 to NR3B subunit switchover of NMDA receptors in early postnatal motoneurons

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Abstract

The NR3B NMDA receptor subunit is selective to somatic motoneurons in the adult nervous system. Here we report its developmental expression in the mouse brain and spinal cord by *in situ* hybridization. NR3B mRNA was detected in few neural regions during embryonic and neonatal periods. It first appeared in motoneurons at postnatal day (P)10–P14, and attained the maximal level at P21 and adult stage. This developmental profile was reciprocal with that of NR2 subunits, of which NR2A mRNA was most predominant in embryonic and neonatal motoneurons and downregulated by P14. Interestingly, mRNA of the NR1 subunit, which is required for functional NMDA receptors, displayed a 'V'-shaped change, decreasing with the early postnatal decline of NR2 mRNAs and increasing with the subsequent appearance of NR3B mRNA. Therefore, the major regulatory subunit of NMDA receptors is likely to switch from NR2 to NR3B in somatic motoneurons during the early postnatal period.

Introduction

The *N*-methyl-D-aspartate (NMDA)-type glutamate receptor channel is involved in activity-dependent changes of synaptic strength, which underlie synapse development, synaptic plasticity and learning and memory (Mayer & Westbrook, 1987; Bliss & Collingridge, 1993). Conventional NMDA receptors are thought to be composed of the obligatory subunit NR1 (GluRζ1) and any of the four regulatory subunits NR2A–D (GluRε1–4; Seeburg, 1993; Nakanishi & Masu, 1994; Mori & Mishina, 1995). Later, the NR3A subunit (originally termed χ1 or NMDAR-L) was cloned and characterized as a novel regulatory subunit that functionally suppresses NR1/NR2 channels in a dominant negative manner (Ciabarra *et al*., 1995; Sucher *et al*., 1995). Subsequently, the NR3B subunit, the second member of the NR3 subunit subfamily, has also been cloned, and shown to suppress NMDA receptor function (Andersson *et al*., 2001; Nishi *et al*., 2001; Matsuda *et al*., 2002). In contrast to higher expression and early onset of NR3A mRNA in various regions of embryonic and neonatal brains (Ciabarra *et al*., 1995; Sucher *et al*., 1995), NR3B mRNA expression is restricted to somatic motoneurons in the adult brain and spinal cord (Nishi *et al*., 2001; Chatterton *et al*., 2002; Matsuda *et al*., 2003). However, the entire ontogenic profile of NR3B expression has not been described.

Previously, we have revealed gene expression profiles of NMDA receptor subunits that are unique and common to brainstem and spinal motor nuclei (Watanabe *et al*., 1994; Oshima *et al*., 2002). In motor nuclei, NR1 and NR2A mRNAs are expressed predominantly during the embryonic period: the former continues to be highly expressed until the adult stage, whereas the latter is downregulated substantially by the end of the second postnatal week. NR2B and NR2D mRNAs are expressed in embryonic motor nuclei, but are downregulated thereafter. NR2C mRNA is below the detection threshold throughout development. This early embryonic onset and postnatal downregulation of NR2A mRNA in motor nuclei differ markedly from its postnatal onset and the following upregulation in most other neural regions (Watanabe *et al*., 1992). To obtain a thorough view of developmental changes of NMDA receptor subunits in motoneurons, we examined NR3B mRNA in the developing brain and spinal cord by *in situ* hybridization. Here we show that, reciprocal with postnatal downregulation of NR2 subunits, NR3B mRNA appears during the second postnatal week and is upregulated toward the adult stage.

Materials and methods

To detect mRNAs for NMDA receptor subunits, antisense oligonucleotide probes were synthesized as follows: oligoNR3a-1 and oligoNR3a-2 for NR3A subunit cDNA (complementary to nucleotide residues 1666–1710 and 1857–1901, respectively, GenBank accession No. L34938), and oligoNR3b-1, oligoNR3b-3, and oligoNR3b-4 for NR3B cDNA (1544–1588, 1742–1786, and 1920–64, respectively, accession No.AF396649). Oligoζ1Ex11 for NR1 cDNA and oligoε1L for NR2A cDNA were reported previously (Fukaya *et al*., 2003). Under deep pentobarbital anaesthesia, the brain and spinal cord were obtained from C57BL/6 J mice at embryonic day (E)15 and E18 and postnatal day (P)1, P7, P10, P14, P21 and adult (4 months). The day after overnight mating was counted as E0. Frozen sections (20 μm thickness) were mounted on glass slides precoated with 3-aminopropyltriethoxysilane. Probe labelling and hybridization were performed

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as described previously (Fukaya *et al*., 1999) with minor modifications. Sections were treated at room temperature with the following incubation steps: fixation with 4% paraformaldehyde−0.1 M sodium phosphate buffer (pH 7.2) for 10 min, 2 mg/mL glycine−phosphate-buffered saline (pH 7.2) for 10 min, acetylation with 0.25% acetic anhydride in 0.1 M triethanolamine-HCl (pH 8.0) for 10 min, and prehybridization for 1 h in a buffer containing 50% formamide, 50 mM Tris-HCl (pH 7.5), 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, 0.6 M NaCl, 0.25% sodium dodecyl sulphate, 200 μg/mL tRNA, 1 mM EDTA and 10% dextran sulphate. Hybridization was performed at 42 °C for 10 h in the prehybridization buffer supplemented with 10 000 c.p.m./μL of ^{33}P-labelled oligonucleotide. Slides were washed twice at 55 °C for 40 min in 0.1 × standard saline citrate (SSC) containing 0.1% sarcosyl. Sections were exposed either to Biomax (Kodak, Rochester, NY) or to Nuclear Track emulsion (NTB-2, Kodak) for 4 weeks. Emulsion-dipped sections were Nissl-stained with methyl green pyronine solution. For semiquantification of mRNA levels, the number of silver grains on motoneurons and the area of motoneuron somata were measured using IPLab software (Nippon Roper, Tokyo, Japan), and the grain density was expressed as the number per 1000 μm^2 motoneuron somal area.

Results

As reported previously (Nishi *et al*., 2001; Chatterton *et al*., 2002; Matsuda *et al*., 2003), motoneuron-specific expression of NR3B mRNA was confirmed by the present *in situ* hybridization. To increase the sensitivity of detection, we used a mixture of three probes. Each of three nonoverlapping oligonucleotide probes for NR3B mRNA yielded identical patterns of labelling (data not shown), and the labelling was abolished almost completely by adding unlabelled oligonucleotide in excess (Fig. 1A, inset), thus verifying the probes' specificity. No NR3B mRNA was detected in the telencephalon and cerebellum. NR3B mRNA was detected in the adult mouse brainstem and spinal cord, including trigeminal motor nucleus, facial nucleus, hypoglossal nucleus, and spinal ventral horn (Fig. 1A, B, H and O). By Nissl staining, we confirmed that these signals originated from cells with large nucleus and soma, i.e. motoneurons (Fig. 1I and P).

We next followed NR3B mRNA expression in the developing mouse brain and spinal cord from E15 through to the adult stage. No significant signals were detected from E15 to P7 in any regions of the brain (parasagittal section, not shown) and spinal cord (Fig. 2), including the trigeminal motor (data not shown), facial (Fig. 1C−E), and hypoglossal (Fig. 1J−L) nuclei. Signals for NR3B mRNA appeared in all of these brainstem motor nuclei at P14 and were maintained until the adult stage (Fig. 1F−H and M−O). Similarly, in the lumbar cord, NR3B mRNA appeared first at P10, and continued to the adult stage (Fig. 2A). This developmental profile was further ascertained by counting the density of silver grains over spinal motoneurons, which were identified in the ventral horn by cell body size (> 20 μm) and dark Nissl staining (Fig. 2B and C). From E15 to P7, the grain density of NR3B mRNA on motoneurons was nearly at the background level, which was determined as the density in the white matter; then, it gradually increased from P10 until the adult stage.

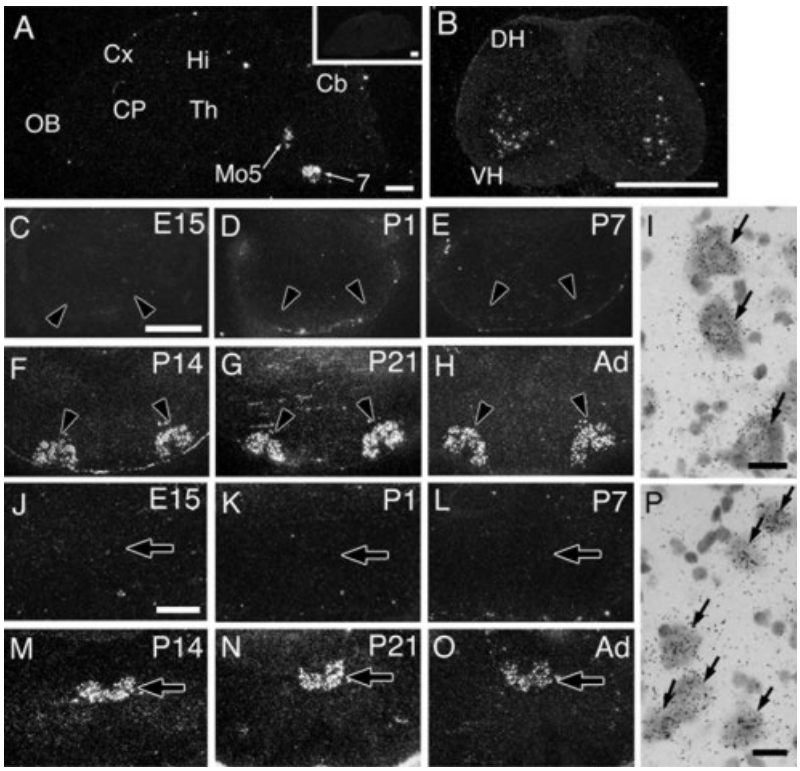

FIG. 1. Distribution of NR3B mRNA in the mouse brain and spinal cord. (A) An X-ray film macroautoradiograph of a parasagittal brain section. Inset indicates the negative control section, in which an excess amount of unlabelled probes was added to the hybridization solution. (B) A dark-field micrograph of a cross-section through the lumbar cord. (C−H and J−O) Dark-field micrographs of coronal brainstem sections through the facial (C−H, arrowheads) and hypoglossal (J−O, arrows) nuclei at E15, P1, P7, P14, P21 and adult. (I and P) Bright-field micrographs showing NR3B mRNA expression on adult motoneurons of the facial (I) and hypoglossal (P) nuclei. Arrows indicate motoneuron somata expressing NR3B mRNA. 7, facial nucleus; Cb, cerebellum; CP, caudate putamen; Cx, cerebral cortex; DH, dorsal horn; Hi, hippocampus; Mo5, trigeminal motor nucleus; OB, olfactory bulb; VH, ventral horn. Scale bars, 1 mm (A and B); 500 μm (C); 250 μm (J); 20 μm (I and P).

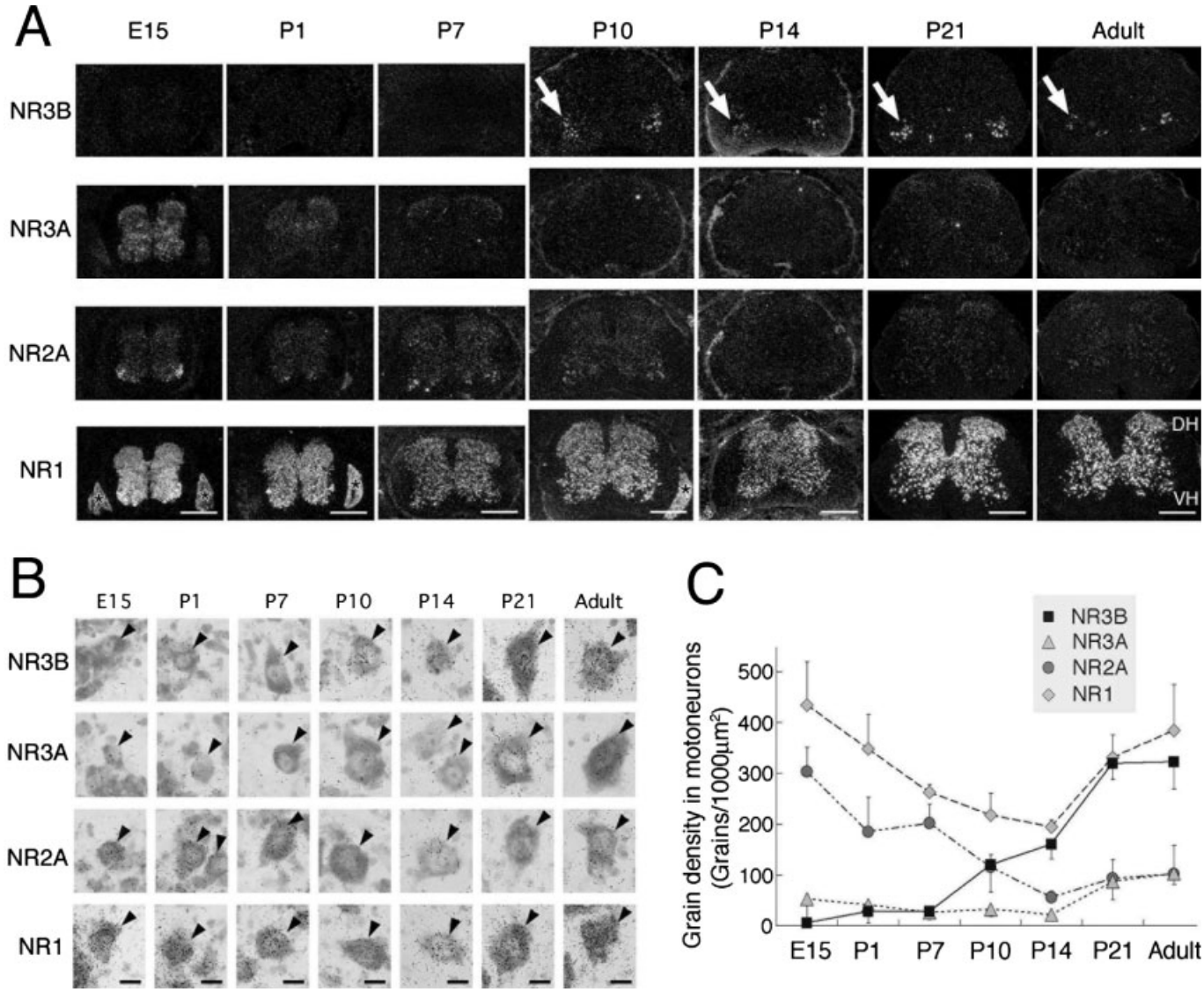

FIG. 2. Developmental expression levels of NR3B, NR3A, NR2A, and NR1 mRNAs in the mouse lumbar cord during development. (A) Dark-field micrographs of spinal cord cross sections. Arrows indicate NR3B mRNA expression in the ventral horn. Asterisks indicate dorsal root ganglia. (B) Bright-field micrographs showing silver grains on Nissl-stained motoneurons (arrowheads). (C) The grain density per 1000 μm^2 motoneuron cell bodies. Error bars represent SD; $n = 6$ or 7 motoneurons at each stage. Scale bars, 500 μm (A); 20 μm (B).

Using the adjacent spinal cord sections, we compared the expression and level of NR3B mRNA with those of other NMDA receptor subunits. Of four NR2 subunits, we examined NR2A mRNA, because it is the major NR2 subunit expressed in spinal ventral horn at each developmental stage whereas other NR2 subunits are either not expressed (NR2C) or limited to very early stages of development (~P1 for NR2B and NR2D; Watanabe *et al*., 1994). NR3A mRNA was detected throughout the spinal cord at E15 and downregulated thereafter (Fig. 2A), consistent with previously reported expression patterns in the brain (Ciabarra *et al*., 1995; Sucher *et al*., 1995). The density of NR3A mRNA on motoneurons was very low throughout development, with slightly higher levels at P21 and adult stage (Fig. 2B and C). NR2A mRNA levels were high in the ventral horn from E15 to P10, and decreased thereafter. The grain density for NR2A mRNA on motoneurons was highest at E15, and gradually decreased before stabilizing at the basal level at P14. Signals for NR1 mRNA were clearly detected throughout the spinal grey matter at all development stages examined. The grain density of NR1 mRNA decreased concomitantly with the decline of NR2A mRNA level. Interestingly, after reaching a minimum at P14, NR1 mRNA increased with NR3B mRNA expression, resulting in a 'V'-shaped profile of expression across development.

Discussion

In the present study, we examined NR3B mRNA expression in the developing brain and spinal cord. Here we found its unique temporal regulation common to somatic motoneurons: NR3B mRNA appears at P10–P14 and increases toward P21 and the adult stage. As NR2A mRNA predominates until P10 or P14 in motoneurons of the spinal ventral horn (Fig. 2) and hypoglossal nucleus (Oshima *et al*., 2002), this developmental profile for NR3B mRNA is thus reciprocal with that for NR2A mRNA. Therefore, the postnatal onset of NR3B mRNA in place of NR2A mRNA indicates that the major regulatory subunit

of NMDA receptor complex switches from NR2A to NR3B subunit during the early postnatal period. Low expression signals for NR2B and NR2D mRNAs in embryonic motoneurons (Oshima *et al*., 2002) and for NR2A and NR3A mRNAs in adult motoneurons (Fig. 2) imply their additional inclusion, leading to functional diversity of NMDA receptors (Palecek *et al*., 1999). Furthermore, the 'V'-shaped change of obligatory NR1 subunit expression might also reflect the developmental switchover of regulatory NMDA receptor subunits in motoneurons. Retrospective analyses of our previous data of NR1 expression profile in cervical cord and hypoglossal nucleus also indicate a similar pattern of regulation (Watanabe *et al*., 1994; Oshima *et al*., 2002).

When coexpressed with NR1 and NR2 subunits, both NR3A and NR3B subunits work as dominant negative subunits to suppress receptor function (Ciabarra *et al*., 1995; Sucher *et al*., 1995; Nishi *et al*., 2001; Matsuda *et al*., 2002). In fact, NR3A knockout mice exhibit augmented NMDA receptor-mediated currents and increased density of dendritic spines (Das *et al*., 1998). Thus, the delayed onset of NR3B subunit may diminish NMDA receptor currents and Ca^{2+} influx in adult motoneurons. In support of this idea, whereas motoneurons in young rodents (< P15) show clear NMDA receptor-mediated responses (Palecek *et al*., 1999; Arvanian *et al*., 2004), adult motoneurons do not show NMDA receptor-mediated currents in response to afferent fibre stimulation or bath-applied NMDA in both control and Mg^{2+}-free media, and are resistant to NMDA receptor-mediated excitotoxicity (Hori *et al*., 2001, 2002a,b). Therefore one possible role of NR3B subunit is to reduce the NMDA receptor-mediated response in juvenile and adult motoneurons. The reduction in early postnatal motoneurons might also be facilitated by the increase of Mg^{2+} sensitivity of NMDA receptors as a result of a decline of NR2D expression (Arvanian *et al*., 2004).

By contrast, a recent study showed that when NR3 subunits are coexpressed with NR1 subunit but without NR2 subunits in a heterologous system, they can constitute excitatory receptor channels gated by glycine but not by glutamate or NMDA (Chatterton *et al*., 2002). This receptor is less permeable to Ca^{2+} and is insensitive to conventional antagonists for both NMDA receptors (AP5 or MK801) and inhibitory glycine receptors (Chatterton *et al*., 2002). If NR3B/NR1 channels act as excitatory glycine receptors, the postnatal expression of the NR3B subunit, and concomitant decrease in NR2 subunits observed in this study, will result in completely different types of NMDA receptors during development in motoneurons: an excitatory glutamate receptor NR1/NR2 in developing motoneurons and an excitatory glycine receptor NR1/NR3B in mature motoneurons. Although no study has provided evidence that the motoneurons contain such receptors, the spinal cord is rich in glycinergic neurons, which provides conventional inhibitory glycinergic synapses and, possibly, novel excitatory glycinergic synapses to motoneurons with NR1/NR3B. Therefore, it will be intriguing to search for biochemical evidence of receptors comprised solely of NR1 and NR3B subunits and also for synaptic localization of NR3B in relation to afferent sources and neurotransmitter species used at such synapses.

Our result is contradictory to a recent report by Qu *et al*. (2004), which reported an early postnatal death of their NR3B-null animals. The animals manifest a progressive paresis of the extremities, particularly of the hind limbs, by P3 and die at approximately P5, probably because of the inability to eat and drink, and therefore eventual dehydration. Histological examination revealed a loss of motor neurons in the spinal cord, including those in the lumbar region but not in any other regions. They thus concluded the demise of motoneurons to be the most likely direct cause of death in NR3B-null mice. Although we did not see any significant expression of NR3B until P10 in all somatic motoneurons examined including those in the lumbar region (Fig. 2), it remains possible that the amount of NR3B subunit required for motoneuron survival is below our detection threshold. Alternatively, it is also possible to assume that the timing of NR3B gene expression is somewhat different between mouse stains or by genetic backgrounds. Furthermore, the chromosomal region involving NR3B is rich in genes and therefore, specificity of gene manipulation must be carefully examined (Niemann *et al*., unpublished data).

In conclusion, our study demonstrates the developmental profile of NR3B, which is critical for understanding the role of NMDA receptor function in motoneurons. Further studies are required to address the exact role of NR3B in this class of neuron, either as dominant negative subunits or excitatory glycine receptors.

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Abbreviations

E, embryonic day; NMDA, N-methyl-D-aspartate; P, postnatal day.

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