Motoneuron-Specific Expression of NR3B, a Novel NMDA-Type Glutamate Receptor Subunit That Works in a Dominant-Negative Manner

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We have identified a novel glutamate receptor subunit on the human and mouse genome. Cloning of the mouse cDNA revealed a protein consisting of 1003 amino acids encoded by at least nine exons. This protein showed the highest similarity (51%) to the NR3A subunit of the NMDA receptor and therefore was termed NR3B. NR3B has a structure typical of glutamate receptor family members with a signal peptide and four membrane-associated regions. Amino acids forming a ligand-binding pocket are conserved. When coexpressed with NR1 and NR2A in heterologous cells, NR3B suppressed glutamate-induced current similarly to NR3A. Thus members of the NR3 class of NMDA receptors act as dominant-negative subunits in the NMDA receptor complex. NR3B shows very restricted expression in somatic motoneurons of the brainstem and spinal cord. Its expression in other types of motoneurons, including autonomic motoneurons in Onuf's nucleus and oculomotor neurons, is significantly weaker. Our results indicate that NR3B is important as a regulatory subunit that controls NMDA receptor transmission in motoneurons. It may be involved in the pathogenesis of neurodegenerative diseases involving motoneurons as well.

Key words: NMDA-type glutamate receptor; genomic sequence; cDNA cloning; motoneurons; in situ *hybridization; amyotrophic lateral sclerosis; Onuf's nucleus*

Glutamate is the major excitatory neurotransmitter in the mammalian CNS. Its action is mediated by two distinct classes of glutamate receptors, ionotropic and metabotropic glutamate receptors (Seeburg, 1993; Nakanishi et al., 1996; Dingledine et al., 1999). The ionotropic glutamate receptor is a ligand-gated cation channel that passes cation with glutamate binding. It consists of several different subclasses including NMDA, AMPA, kainate, and δ, which, in turn, are hetero-oligomers of several subunits (Seeburg, 1993; Hollmann and Heinemann, 1994; Dingledine et al., 1999). The NMDA receptor is composed of hetero-oligomers of NR1, NR2A–NR2D, and NR3A subunits (also called χ-1 or NMDA receptor-like subunits). NR1 is a key subunit that confers essential functions of the NMDA receptor and is expressed ubiquitously in the CNS. In contrast, other subunits show more limited expression and confer a functional diversity. For example, during development of the visual cortex, NR2B is expressed predominantly in the early stages. After functional maturation of the cortex, predominant expression switches to NR2A (Quinlan et al., 1999). Concomitantly, the decay time course of the NMDA receptor-mediated synaptic current becomes faster (Carmignoto

and Vicini, 1992; Philpot et al., 2001). Dark rearing of animals, which prolongs the critical period of the visual cortex, delays this conversion (Quinlan et al., 1999).

In contrast to NR2 subunits, less is known about the NR3 class of subunits. To date, the NR3A subunit has been the only reported member of this class (Ciabarra et al., 1995; Sucher et al., 1995). NR3A is expressed ubiquitously during development and its expression level reaches a maximum at approximately the first postnatal week. Thereafter, the level gradually decreases, and in adult animals, NR3A is confined to limited nuclei in the thalamus, amygdala, and nucleus of the lateral olfactory tract (Ciabarra et al., 1995; Sucher et al., 1995). The NR3A subunit binds to NR1 and NR2 and acts in a dominant-negative manner against the NMDA receptor to reduce whole-cell current as well as single-channel conductance (Ciabarra et al., 1995; Sucher et al., 1995; Das et al., 1998; Perez-Otano et al., 2001). Consistent with this role for NR3A, mice lacking NR3A have a larger NMDA receptor-mediated current and an increased dendritic spine density in cerebrocortical neurons (Das et al., 1998), suggesting that the NR3A

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subunit is important for the development and plasticity of the CNS through a modulation of NMDA receptor function. Therefore, understanding the NR3 class of NMDA receptor subunits is important for understanding the physiology of the CNS.

In this report, we describe a novel NR3 subunit termed NR3B and characterize the structure, expression pattern, and electrophysiological properties of mouse NR3B.

MATERIALS AND METHODS

Identification of a novel glutamate receptor. We performed a basic local alignment search tool (BLAST) search on the published human genomic sequence using the amino acid sequence of the mouse $\delta 2$ subunit as a query sequence. This search detected a stretch of genomic sequence with significant homology to members of the glutamate receptor family on a contig from human chromosome 19p13.3 (GenBank accession number AC004528). We then used this sequence to search the unfinished high throughput genomic sequences database (including phases 0, 1, and 2) and identified the mouse homolog (accession numbers AC087114 and AC073805) on chromosome 10. A search of the expressed sequence tag (EST) database with these sequences identified the following ESTs: accession number AL040053 (human); accession numbers AW045848, BE864387, AW048083, and BE955769 (mouse); and accession numbers AW525909, BE108608, and BE112464 (rat). AW045848 and BE864387 are the same clone sequenced from opposite ends. We used this clone, which corresponds to the extracellular domain of the putative receptor, for screening of a mouse spinal cord cDNA library and *in situ* hybridization (Clontech, Palo Alto, CA).

Reverse transcriptase-PCR and in situ *hybridization.* We performed reverse transcriptase (RT)-PCR with primers spanning predicted exons 1 (CCTCTATAACCTTTCCCGAGG) and 2 (CTAGAGCAATGTCCTCCCAGG) of mouse NR3B. As a positive control for RT-PCR, we used a primer set for NR1 (5′ primer, GATCCTCGAGCCATGGAGATCGCCTACAAGCGACAC; 3′ primer, GATCGGGATCCGCATGCTCAGCTCTCCCTATGACGGG). For *in situ* hybridizations on brain and spinal cord sections from male mice (C57BL/6, 6 weeks of age), we used either [33]P- or digoxigenin-labeled RNA probes (Simmons et al., 1989; Lu et al., 2001). Serotonin was immunohistochemically detected with anti-serotonin antibody (Incstar, Stillwater, MN).

Electrophysiology in human embryonic kidney 293. To express the receptors in human embryonic kidney (HEK)293 cells, we transfected the cells with 1 μg of each plasmid unless otherwise stated (Shi et al., 1999). We tagged the NR1 and NR2A with green fluorescent protein (GFP) on the extracellular domain to facilitate the identification of transfected cells. Such constructs have been shown to preserve the properties of the glutamate receptor (Shi et al., 1999). NMDA receptor-mediated current was recorded in the presence of 10 μM glycine. AMPA receptor current was recorded as described previously (Shi et al., 1999). Pipette solutions contained (in mM): cesium methanesulfonate 110, CsCl 30, NaCl 4, HEPES 10, EGTA 5, and CaCl$_2$ 0.5, adjusted to a pH of 7.3 with CsOH. A 1 mM concentration of glutamate (for NMDA receptor) or kainate (for AMPA receptor) was pressure-applied (1.0 psi) through a puffer pipette positioned ~10 μm to the cells. We used a program based on Igor to acquire data on a Macintosh computer.

RESULTS

Identification and cloning of a new mouse glutamate receptor

A BLAST search of the published human and mouse genomic sequences identified a unique sequence with significant homology to the glutamate receptor family (Fig. 1A). We examined transcript levels as well as the tissue distribution of mRNA by RT-PCR of mouse RNA using a primer set spanning the predicted exons 1 and 2 (Fig. 2A). It revealed a band of the expected size for spliced product in brainstem and spinal cord. We also detected a fainter band in the cerebellum. Using other primer sets covering exons 2 and 3, we detected a similar pattern of expression (data not shown). More rostral structures did not show any significant expression of this transcript, whereas RT-PCR for the NR1 subunit performed in parallel showed positive bands in all brain
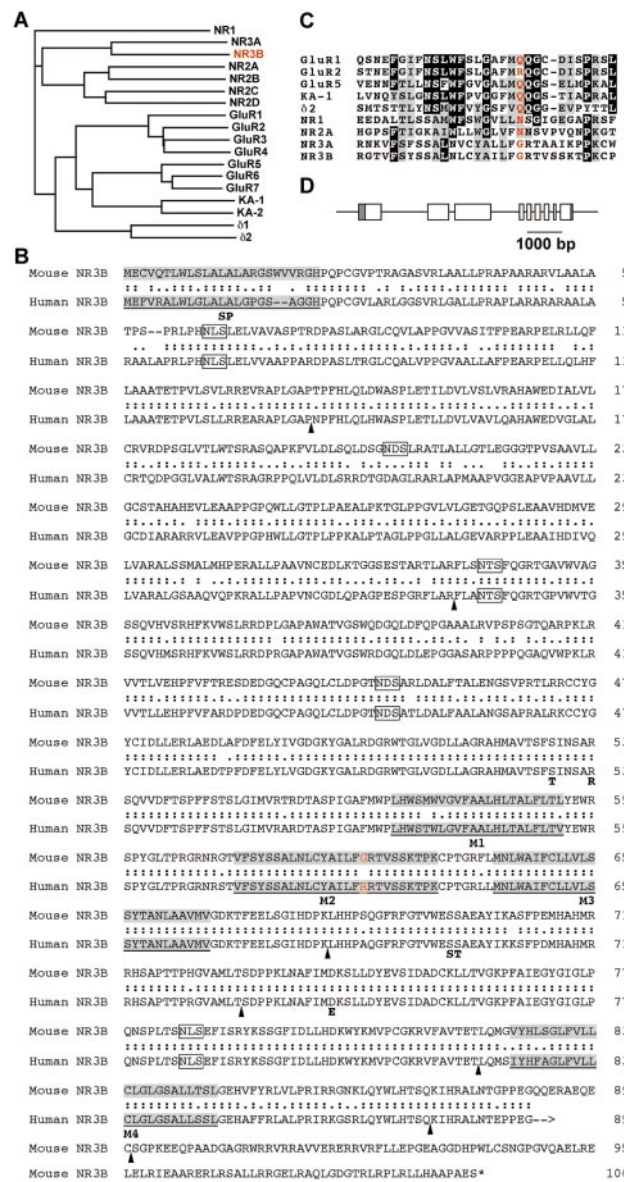

Figure 1. Structure of the glutamate receptor subtype NR3B. *A*, Phylogenic tree of the members of the glutamate receptor family based on the neighbor-joining method. The *horizontal* length of the branch indicates the distance between proteins. *B*, Mouse and human NR3B sequence. The human sequence is assembled from the genomic sequence and is not complete at the C terminus. Identical amino acids between two species are indicated by ":" and homologous ones are indicated by ".". *SP* and *M1–M4* indicate the predicted signal peptide and membrane-associated region, respectively. Putative glycosylation sites are *boxed*. *Upward arrowheads* indicate exon boundaries. Amino acids implicated in ligand binding of GluR2 from a crystallographic study are shown below the mouse sequence at the corresponding positions. *C*, A comparison of the M2 domain that forms a channel pore. The critical amino acid at the Q/R/N site, which controls ion permeability and rectification, is glycine followed by an arginine in both NR3B and NR3A (shown in *red*). In human NR3B, the Q/R/N site is an arginine (*B*). *D*, Genomic structure of mouse NR3B. *Open* and *shaded boxes* are coding and noncoding regions, respectively. The 5′ noncoding region may extend further upstream.

regions. We did not detect the band when we omitted reverse transcriptase from the reaction (Fig. 2A, −*RT*), ruling out the possibility of amplification from contaminated genomic DNA. An additional BLAST search identified EST clones from humans, mice, and rats. When the mouse EST clone AW045848 was used
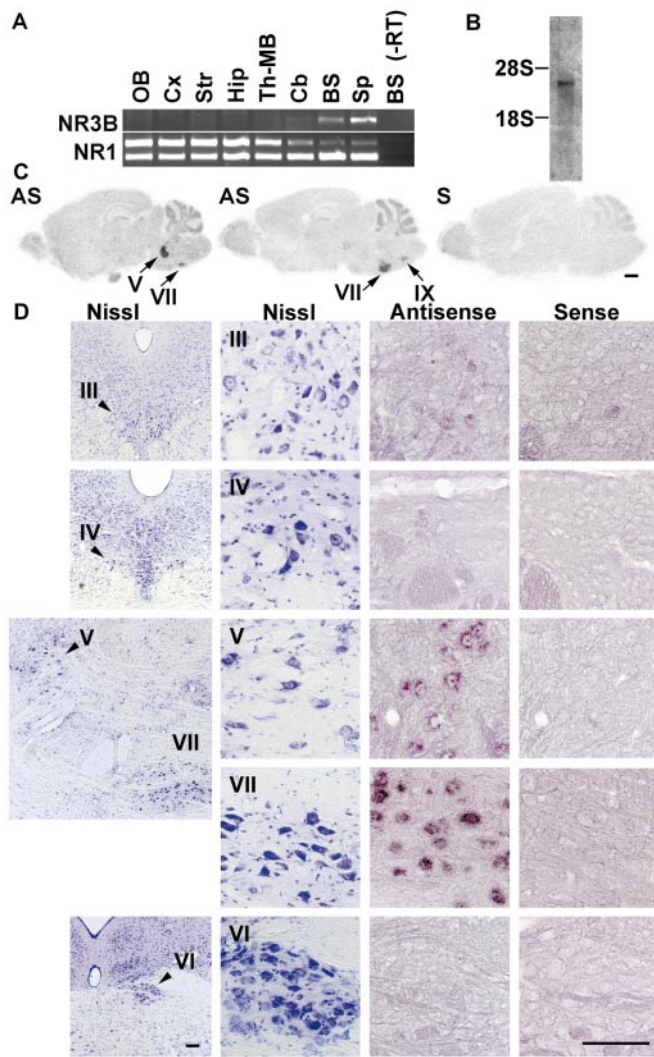
Figure 2. Tissue distribution of mouse NR3. *A,* NR3B transcripts detected by RT-PCR with primers that span exons 1 and 2. The PCR product of the expected length was detected in the brainstem (*BS*) and spinal cord (*Sp*) and to a much lesser extent in the cerebellum (*Cb*). When reverse transcriptase was omitted (−*RT*), the product was not detected. As a positive control for RT-PCR, the NR1 was amplified in parallel, producing two bands that correspond to different slice variants. *OB,* Olfactory bulb; *Cx,* cerebral cortex; *Str,* striatum; *Hip,* hippocampus; *Th-MB,* thalamus to midbrain region. *B,* Northern blot analysis of mouse spinal cord polyA $^+$ RNA detecting a single band at ∼3.5 kb. *C, In situ* hybridization of sagittal sections of mouse brain with ^{33}P-labeled antisense (*AS*) and sense (*S*) probes. The antisense probe detected a discrete signal in trigeminal motor (*V*), facial (*VII*), and ambiguus (*IX*) nuclei. The cerebellum also had a faint signal. *D,* Higher magnification of cranial nerve nuclei. A restricted expression of NR3B was detected in motoneurons that control somatic movement but not in those controlling ocular movement. *Left,* Low- and high-magnification images of Nissl-stained sections of oculomotor (*III*), trochlear (*IV*), trigeminal motor (*V*), facial (*VII*), and abducens (*VI*) nuclei are shown. *Right, In situ* hybridization using antisense and sense probes labeled with digoxigenin. A strong signal was observed in trigeminal motor and facial nuclei but not in those innervating extraocular muscles. Scale bars: *C,* 1 mm; *D,* 100 μm.

to probe polyA $^+$ RNA from mouse spinal cord, it hybridized to a band at ∼3.5 kb (Fig. 2*B*). We used this clone to screen a mouse spinal cord cDNA library. The screening of ∼10^6 clones yielded three positive clones, one of which contained full-length cDNA (GenBank accession number AF396649).

Structure of the NR3B gene, cDNA, and protein product

Sequence analysis of the clone revealed a cDNA of 3283 bp encoding a protein of 1003 amino acids (Fig. 1*B*). The cDNA is encoded by at least nine exons in the mouse genome spanning ∼6.5 kb (Fig. 1*D*). A TATA box is found in the genomic sequence at −339 from the start codon ATG, whereas the cDNA starts at −185, suggesting that a 5′ untranslated sequence exists beyond our cDNA. All nucleotides in the genomic sequence and the cDNA are identical, indicating that RNA editing does not occur in this gene. The predicted protein has a significant similarity to NR3A (51%); therefore, we named it NR3B (Fig. 1*A*). A glutamate receptor member named χ-2 has been reported in abstract form, but because of a lack of detailed information, the identity of NR3B with χ-2 is obscure (Sevarino et al., 1996). The human sequence was assembled from the genomic sequence deposited in GenBank (Fig. 1*B*) (third-party annotation section of DNA Data Bank of Japan/European Molecular Biology Laboratory/GenBank accession number Bk 000070). The C terminus of the human clone loses homology after Gly890, which is followed by a possible splice donor site (GG<u>G</u> <u>T</u>), indicating that there may be one or more additional exons in the human sequence. All of the other exon–intron boundaries are preserved between the two species and conform to a consensus splice donor–acceptor site (GT-AG). The homology between the human and mouse sequence is 81.3%.

NR3B has a transmembrane topology that is typical of glutamate receptors with an N-terminal signal peptide and four membrane-associated regions. Five (in mice) or four (in humans) consensus sequences for N-glycosylation sites are found on the N-terminal domain, and one (for both species) is found at the loop between the third and fourth membrane-associated regions. The intracellular C terminus of mouse NR3B has three threonines and five serines, which may serve as regulatory phosphorylation sites. The C terminus ends with Ala–Glu–Ser, which does not conform to the consensus (PSD-05, Dlg, Z0-1) domain protein-binding site sequence typical of other glutamate receptor family members (Songyang et al., 1997; Sheng and Sala, 2001).

The amino acid residues forming the glutamate-binding pocket have been elucidated by structural studies of the crystallized ligand-binding domain of glutamate receptor 2 (GluR2) (Armstrong and Gouaux, 2000). Importantly, the 2-carboxyl group of glutamate binds to the guanidium group of Arg485 and the amino H group of Thr480; the 2-amino group interacts with the carboxyl group of Glu705 in GluR2. These residues can be mapped precisely on the sequence of NR3B with Thr480 of GluR2 corresponding to Ser533 of NR3B, Arg485 to Arg538, and Glu705 to Asp745. Other amino acids forming the putative ligand-binding pocket are also well conserved (Fig. 1*B*), indicating that NR3B itself is likely to bind to glutamate.

Expression of NR3B is limited to motoneurons

The RT-PCR revealed that NR3B is expressed selectively in a limited number of brain regions. *In situ* hybridization of a sagittal section of a mouse brain consistently detected signal in limited regions in the brainstem (Fig. 2*C*). A counterstaining of the sections (data not shown) showed that these are the trigeminal motor (*V*), facial (*VII*), and ambiguus (*IX*) nuclei. A higher magnification of these structures revealed hybridization signals in motoneurons with large cell bodies (Fig. 2*D*). In contrast, the signal was significantly weaker in motoneurons in the nuclei
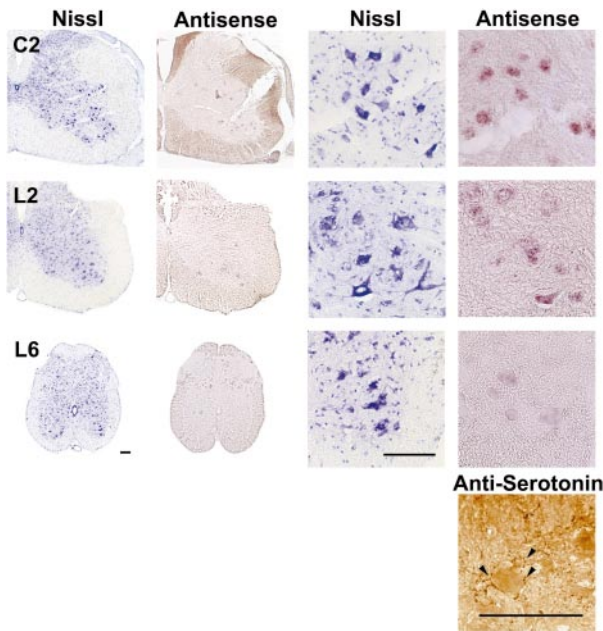
Figure 3. Expression of NR3B in spinal cord. *Left*, Low-magnification images of sections stained with Nissl and adjacent sections hybridized with antisense probe. *Right*, Higher magnification of the same sections. Somatic motoneurons are strongly labeled at the *C2* and *L2* levels. In contrast, the expression of NR3B was significantly weaker in the motoneurons at the *L6* level. There was a plexus of serotonin immunoreactive fibers (*arrowheads*) surrounding these cells, which is one of the characteristics of motoneurons in Onuf's nucleus. Scale bars, 100 μm.

controlling the external ocular muscles (Fig. 2*D*, *III*, oculomotor; *IV*, trochlear; and *VI*, abducens).

In the spinal cord, the signal was also detected in the motoneurons in the anterior horn (Fig. 3). However, at the L6 level, the signal intensity of NR3B in motoneurons was significantly weaker than at higher levels, although the presence of motoneurons at this level was confirmed by Nissl staining. These motoneurons were classified as those in Onuf's nucleus, which controls external anal and urethral sphincters, based on plexus formation of serotonin immunoreactive fibers on the cell body (Fig. 3, *bottom*) (Micevych et al., 1986) and a comparison with the cytoarchitecture of the mouse spinal cord (Sidman et al., 1971) as well as with previous studies on a rat counterpart (Schroder, 1980; McKenna and Nadelhaft, 1986). However, because of a lack of systematic analyses of Onuf's nucleus in the mouse, this assignment is tentative and awaits more extensive study (for example, a retrograde labeling) to be conclusive.

NR3B acts as a dominant-negative subunit
Finally, we tested the electrophysiological properties of NR3B by expressing NR3B in HEK293 cells. A conventional NMDA receptor channel is formed by a heteromeric combination of NR1, which is a key subunit for functionality, and at least one NR2 subunit, which is modulatory (Dingledine et al., 1999). NR3B cannot substitute for either of these subunits, because expression of NR3B alone ($n = 6$) or coexpression with NR1 ($n = 6$) or NR2A ($n = 5$) did not result in electrophysiologically functional channels (data not shown), whereas coexpression of NR1 and NR2A gave a robust current (Fig. 4). However, coexpression of all three subunits markedly depressed the whole-cell current compared with the combination of NR1 and NR2A (Fig. 4*A,C*). Increasing the amount of NR3B versus the other subunits had a stronger sup-
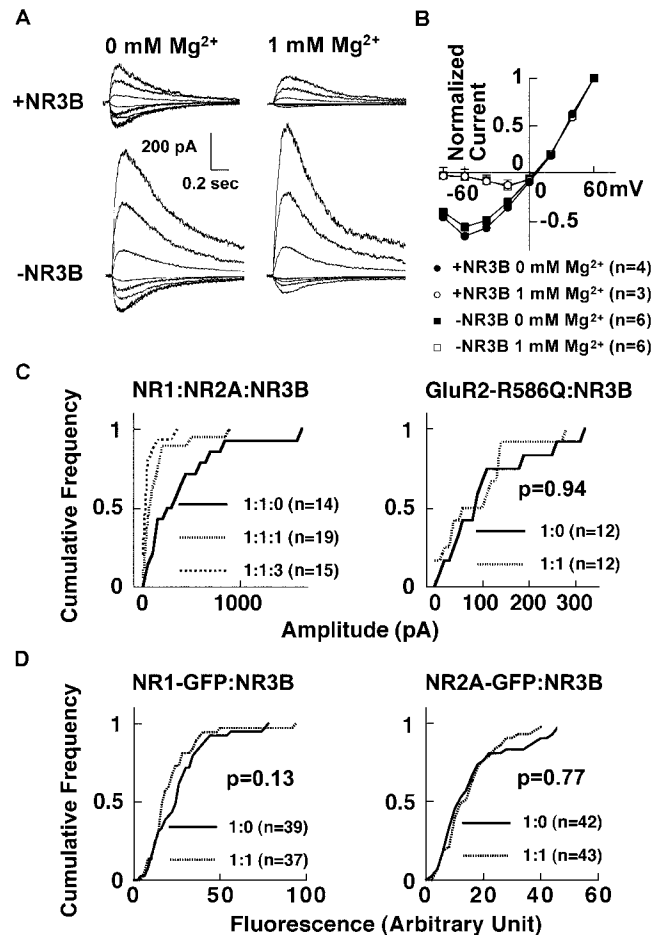

Figure 4. Glutamate-induced whole-cell current recorded from HEK293 cells expressing NR3B. NR3B was coexpressed with the NR1 and NR2A subunits. *A*, Sample traces of glutamate-induced current recorded at −80 to +60 mV (20 mV step) in the presence or absence of 1 mM Mg^{2+}. *B*, The remaining current in NR3B-expressing cells exhibits a Mg^{2+} block indistinguishable from cells not expressing NR3B. The slight block at −60 and −80 mV may be attributable to residual Mg^{2+}. *C*, NR3B acts as a dominant-negative subunit and suppresses glutamate-induced whole-cell current in cells coexpressing NR1 and NR2A. The distribution of averaged responses obtained at +60 mV in Mg^{2+}-free solution is shown in a cumulative plot. Increasing the amount of NR3B plasmid (0, 1, and 3 μg) versus other subunits (each 1 μg) caused a concomitant decrease in current amplitude. Statistical significances were as follows: 1:1:0 vs 1:1:1, $p < 0.05$; 1:1:0 vs 1:1:3, $p < 0.01$; and 1:1:1 vs 1:1:3, $p < 0.01$. AMPA receptor-mediated current was unchanged by coexpression with NR3B. GluR2-(R586Q)-GFP was used because we know empirically that this construct gives a reliable response. *D*, Coexpression with NR3B did not change the expression levels of NR1 or NR2A. NR1 and NR2A tagged with GFP were individually expressed with NR3B and their expression levels were measured by the fluorescence intensity. The statistical significance in this figure was assessed by a Kolmogorov–Smirnov test.

pressive effect (Fig. 4*C*). This is not attributable to a nonspecific reduction of the protein expression level, because a direct quantification of expression levels of NR1 and NR2A tagged with GFP using fluorescence as a measure did not show any significant difference in the presence or absence of NR3B (Fig. 4*D*). The effect of NR3B is specific to the NMDA receptor, because the AMPA receptor-mediated current was not changed by the presence of NR3B (Fig. 4*C*). The sensitivity of the remaining current to Mg^{2+} block was not affected by the presence of NR3B subunit (Fig. 4*B*).

DISCUSSION

We have identified a novel glutamate subunit, NR3B. The most intriguing feature of this receptor is its expression pattern. It is almost exclusively expressed in somatic motoneurons in cranial nerve nuclei (including trigeminal motor and facial nuclei) and in the spinal anterior horn, which control somatic movement. In addition to these somatic motoneurons, there are other classes of motoneurons. The extraocular muscles are controlled by motoneurons originating from the oculomotor, trochlear, and abducens nuclei. Also, external urethral and anal sphincter muscles are controlled by a distinct group of motoneurons present in Onuf's nucleus in lower spinal cord (Mannen et al., 1982; Anneser et al., 1999). These different classes of motoneurons are affected differently under pathological conditions. As a typical example, in amyotrophic lateral sclerosis (ALS), a selective loss of somatic motoneurons is observed but no loss of those autonomic motoneurons and oculomotor neurons is apparent (Mannen et al., 1982; Anneser et al., 1999; Nimchinsky et al., 2000). Given the importance of glutamate neurotoxicity in various neurodegenerative diseases (Choi, 1992), we tested the expression of NR3B in those motoneurons. Strikingly, less expression was clearly observed in these structures compared with the somatic motoneurons (Figs. 2 and 3). This selective expression of NR3B in somatic motoneurons versus other types of neurons may explain a selective loss of somatic motoneurons in ALS. The low but not absent level of expression is consistent with the fact that incontinence and opthalmoplegia still appear at the terminal stage after prolonged respiratory support (Palmowski et al., 1995).

In the glutamate receptor family, the second membrane-associated domain forms a channel pore. The critical site for determining ion channel permeability and the selectivity of glutamate receptors is called the Q/R/N site (Seeburg, 1993; Hollmann and Heinemann, 1994; Dingledine et al., 1999). Non-NMDA receptors have either a glutamine or an arginine, and the presence of arginine confers a linear IV relationship and the monovalent cation selectivity of this class of channel. In NR1 and NR2 subtypes of NMDA receptor, this site is asparagine and it is critical for the Mg^{2+} block at negative potential and the Ca^{2+} permeability of this channel. The corresponding residue of NR3B is glycine followed by an arginine, which may also be important for determining the channel properties. This feature is shared with NR3A (Fig. 1C). Previous reports indicate that coexpression of NR3A with NR1 and NR2 also decreases whole-cell current (Ciabarra et al., 1995; Sucher et al., 1995) as well as single-channel conductance (Das et al., 1998; Standley et al., 2000). A similar mechanism may explain an observed decrease in the whole-cell current in NR3B-expressing cells. In fact, in an outside-out patch obtained from motoneurons, one study detected a decreased conductance channel (Paleček et al., 1999).

Alternatively, this decrease in whole-cell current could be explained by a decrease in the surface delivery of functional receptors. For example, NR1A has an endoplasmic reticulum retention signal at the intracellular C terminus (Standley et al., 2000; Scott et al., 2001). Also, the AMPA receptor subtype GluR1 has a delivery signal to the synaptic surface, which retains GluR1 in the absence of Ca^{2+}/calmodulin-dependent protein kinase II activity (Hayashi et al., 2000; Shi et al., 2001). A similar mechanism may be involved in the regulation of the NMDA receptor by the NR3B subunit.

Because of this dominant-negative role of the NR3B subunit,

downregulation or dysfunction of NR3B would lead to enhanced NMDA receptor activity, which may increase the vulnerability of neurons to excitotoxicity. Considering the relatively specific expression of NR3B in somatic motoneurons, reduction of NR3B (for example, by a genetic mutation) may specifically affect somatic motoneurons. Alternatively, an autoimmune antibody against NR3B could have a similar impact on somatic motoneurons, as has been shown for other classes of receptors and channels (Appel et al., 1993; Rogers et al., 1994; Smitt et al., 2000). Such mechanisms may explain the selective loss of somatic motoneurons in ALS. Further characterization of NR3B is indispensable for a precise understanding of the physiology and pathophysiology of motoneurons.

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