Viral Delivery of NR2D Subunits Reduces Mg$^{2+}$ Block of NMDA Receptor and Restores NT-3-Induced Potentiation of AMPA-Kainate Responses in Maturing Rat Motoneurons

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INTRODUCTION

Considerable development of motoneurons and their synaptic inputs occurs during the first 2 postnatal weeks as adult motor patterns are established (Jamon and Clarac 1998; Lanuza et al. 2002; Walton et al. 1992). Plasticity of synaptic inputs to spinal motoneurons during this perinatal period plays an important role because activity affects motoneuron development (Hockfield and Kalb 1993; Kalb and Hockfield 1994), dendrite growth (Kalb 1994), and maturation of motor behavior (Vinay et al. 2000). Motoneurons in neonatal rat spinal cord can be activated monosynaptically by two fiber systems: a peripheral segmental input via dorsal root (DR) afferents and a central input via fibers in the ventrolateral funiculus (VLF) (Fulton and Walton 1986; Pinco and Lev-Tov 1993, 1994). VLF and DR inputs form during late embryonic stages, and their monosynaptic responses are mediated via glutamate receptors in postnatal rats (Pinco and Lev-Tov 1993, 1994).

A prior study in the in vitro spinal cord from postnatal pups ±1 wk of age showed that brief administration of neurotrophin-3 (NT-3) could induce an immediate and long-lasting facilitation of the monosynaptic response in motoneurons to DR stimulation (Arvanian et al. 2000). However, VLF inputs to the same motoneurons were not affected by NT-3. Furthermore, NT-3 affected neither DR nor VLF responses in motoneurons recorded from spinal cords of postnatal week 2 pups. These studies also revealed that N-methyl-D-aspartate receptor (NMDAR) function in motoneurons, which normally becomes subject to Mg$^{2+}$ block at resting membrane potential after postnatal week 1, is a requirement for the observed neurotrophin effects (Arvanian and Mendell 2001a,b; Arvanov et al. 2000; Mendell and Arvanian 2002).

Susceptibility of the NMDAR to Mg$^{2+}$ block is an important determinant of NMDAR function at resting membrane potential (Nowak et al. 1984). In previous work we showed that abolition of this block, by removing extracellular Mg$^{2+}$, reversed the loss of NMDAR-mediated transmission in the second postnatal week (Arvanian and Mendell 2001b). In the work reported here, mRNA analysis showed that NR2D is the only NMDAR subunit that exhibits significant decline between the first and second postnatal weeks in the lumbar spinal cord, and immunocytochemistry showed that this decline occurs in motoneurons. Since NR2D is known to confer a relatively low sensitivity to Mg$^{2+}$ block to functional NMDARs and was the only subunit found to decrease in the spinal cord during development, we tested whether enhancement of NR2D protein levels using a herpes simplex virus (HSV) viral construct (Finegold et al. 2001; Jakegawa et al. 2003) would restore synaptic function measured at postnatal day 12 (P12) to levels observed earlier in development. Since the action of NT-3 requires functional NMDAR (Arvanian and Mendell 2001b),
we tested the ability of NT-3 to potentiate the synaptic responses associated with these inputs as a further assay of NMDAR function. To help in evaluating the effects of viral treatment, NMDAR-mediated responses and the effect of NT-3 were also examined in spinal cords from E18 animals, a point soon after synaptic responses are initially observed (Kudo and Yamada 1987; Ziskind-Conhaim 1990).

Some of these results have been reported in abstract form (Arvanian et al. 2003b; Mendell and Arvanian 2001).

**Methods**

These studies were performed with the approval of the Institutional Animal Care and Use Committee at SUNY Stony Brook.

**Electrophysiology**

Electrophysiological experiments were carried out in vitro on 67 rat spinal cords removed at E18, P1–P5, or P8–P15. The rats were anesthetized by placing them on a latex glove lying on a bed of ice (P1/P2), by halothane (P3-P14), or by placing the embryo (E18) in chilled aerated artificial cerebrospinal fluid (ACSF) on removal from the halothane-anesthetized mother. The spinal cord was quickly removed from the animal, and the left hemicord was placed in a chamber superfused with ACSF containing (in mM) 117 NaCl, 4.7 KCl, 2.5 CaCl₂, 2.0 MgSO₄, 25 NaHCO₃, 1.2 NaH₂PO₄, and 11 dextrose, aerated with 95% O₂–5% CO₂ (pH 7.4, 30°C), in 30 experiments, corresponding equiosmolar changes in Na/H⁺ ratios, 70–90 amperometric recordings were obtained using sharp microelectrodes (resistance, 70–110 MΩ), filled with 3 M potassium acetate). Electrical stimulation of the DR and/or VLF (50–200 Hz) was at an intensity for evoking the just-maximum monosynaptic response. To help in evaluating the effects of viral treatment, NMDAR-mediated responses and the effect of NT-3 were also examined in spinal cords from E18 animals, a point soon after synaptic responses are initially observed (Kudo and Yamada 1987; Ziskind-Conhaim 1990).

**Determination of Mg²⁺ blockade**

The pharmacologically isolated monosynaptic component of the NMDAR-mediated response was measured in different concentrations of Mg²⁺ in the superfuse (ranging from 50 μM to 5 mM). In these experiments, corresponding equiosmolar changes in Na⁺ concentration were made. The maximum response amplitude at low values of Mg²⁺ was set as 100%, and the smaller responses were expressed as a percentage of this maximum response. This scaling allowed us to average the responses over several cells as a function of [Mg]₀. Previous investigators (Chen and Huang 1992; Kuner and Schoepfer 1996; Liu et al. 2001) have fit these data with similar sigmoidal equations. We used one of these (% of maximum response) = 100/[1 + ([Mg]₀/IC₅₀)ᵃᵇ] (Kuner and Schoepfer 1996), allowing IC₅₀, the half-maximal block by Mg²⁺, and a, the Hill coefficient, to vary freely in fitting the responses at different concentrations of Mg²⁺ (see Fig. 3B) using SigmaPlot (Jandel Scientific). The calculated value of IC₅₀ (here called Kₘg) was used as a measure of the value of the Mg²⁺ block at constant membrane potential. To determine statistical significance we calculated Kₘg separately for the individual cells and compared the means under the different conditions. The mean values of Kₘg obtained in this way were similar to those values obtained by first averaging the amplitudes at each value of [Mg]₀, as in Fig. 3B (always within 5%).

**HSV amplicon vector construction and packaging**

Helper virus-free amplicon packaging and virus purification was performed as previously described (Bowers et al. 2001). Amplicon virus numbers were determined by assessing both expression and transduction titers as previously described (Bowers et al. 2000). Vectors carried the genes for either NR2D (HSVnr2d) or β-galactosidase (HSVlac), as well as the reporter gene, green fluorescent protein (GFP).

**Injection of vectors**

Two-day-old Sprague-Dawley rats were anesthetized by hypothermia as above. Under a dissecting microscope, the skin and muscles overlying the L₁–L₄ vertebrae were separated and retracted, and the underlying spinal cord was exposed. Using a Hamilton microsyringe with a 33-gauge needle, virus (~10⁶ viral particles) was injected directly into the L₆ and L₅ ventral horn (2 injections of 1 μl each) ipsilaterally to the side used for recordings. The muscle and skin were sutured in layers with 5-0 silk sutures. Finally, the skin was closed using Vetbond tissue adhesive (3M Corp.), and the wound was cleaned and covered with sesame oil to prevent rejection of the pup by the mother. Pups were kept warm and were returned to the mother when they became active. Ten days after surgery, the rats were prepared for intracellular recordings as described above.

**Gene chip array**

To compare the expression of a large population of genes in the L₁–L₄ region of spinal cord in 2- and 12-day-old rats, we employed Affymetrix (Santa Clara, CA) oligonucleotide arrays (Zhao et al. 2001). Each group was represented by two replicate samples, using five L₁–L₄ sections of dissected rat spinal cord per sample and two microarrays. All procedures were performed at the Christopher Reeve Paralysis Foundation/Salk Functional Genomics Laboratory. Total RNA (10 μg) was converted into double-stranded cDNA using an oligo-dT primer with a T7 promoter (Life Technologies). Double-stranded cDNA was extracted with phenol/chloroform and used for in vitro transcription with a T7 RNA polymerase. The labeled cRNA was purified with RNeasy columns. After calculating the cRNA concentration by using a 260A spectrometer, cRNA was fragmented and used for in vivo hybridization cocktail containing oligonucleotides. The oligonucleotides were hybridized, and the hybridization fluorescence results were captured with Affymetrix equipment and protocols. To correct for minor differences in overall chip fluorescence, data from each microarray were normalized (Zhao et al. 2001). For analyzing the scanned data and calculations of gene expression levels, we used GeneChip (version 4.1, Affymetrix) and model-based dChip (Li and Hung Wong 2001) software. It has been established that a change of expression level of 1.2 or greater with P ≤ 0.05 (dChip analysis) is significant (Li and Hung Wong 2001). This study used a stricter threshold of at least a 1.5-fold change with P ≤ 0.05 scored by dChip software. The accession number of our GEO-deposited data is GSE1130.

**Real-time quantitative RT-PCR array**

Changes in expression level for certain genes in the L₁–L₄ region of rat spinal cord in 2- and 12-day-old rats, as well as in 12-day-old rats treated at P2 with HSVnr2d or HSVlac, were confirmed by real-time RT-PCR. One microgram of total RNA was reverse transcribed using...
random hexamer priming and AMV reverse transcriptase according to manufacturer protocol (Invitrogen). cDNA pools were diluted 1:5, and 2.5 µl of these diluted samples was analyzed in a standard PE7700 quantitative primer/probe combination multiplexed with an 18S rRNA-specific primer/probe set (internal control). The NR1 probe sequence was 5'-CGCTGGCAACAGTGTACCGCGC-3' and the NR1 sense primer sequence was 5'-TCTTCTTCTGAAGGACGAG-3'; and the NR1 antisense primer sequence was 5'-GCCGTCATGTTACAGCATT-3'. The NR2D probe sequence was 5'-CCCTCAAAAATGACCTGCAACTG-3' and the NR2D sense primer sequence was 5'-CGGCTACACATTCACAGGAA-3'; and the NR2D antisense primer sequence was 5'-GCTGGAAATTACCCTGCAACT-3'; 2.5 µl of diluted cDNA pool, 900 nM of each primer, 50 nM of each probe, and 12.5 µl of 2× Perkin-Elmer Master Mix (PE-Applied Biosystems). Following a 2-min 50°C incubation and 2-min 95°C denaturation step, the samples were subjected to 40 cycles of 95°C for 15 s and 60°C for 1 min. Fluorescence intensity of each sample was detected automatically during the cycles by the Perkin-Elmer Applied Biosystems). Following a 2-min 50°C incubation and 2-min 95°C denaturation step, the samples were subjected to 40 cycles of 95°C for 15 s and 60°C for 1 min. Fluorescence intensity of each sample was detected automatically during the cycles by the Perkin-Elmer Applied Biosystems BioQuantification software. Each PCR run included the following: no-template control samples, no-reverse transcriptase controls, and standard curve dilution series for each of the target sequences. Following the PCR run, “real-time” data were analyzed using Perkin-Elmer Sequence Detector Software version 1.6.3, and regression analyses were performed to calculate relative levels of NR1 or NR2D transcript in each test sample.

Immunocytochemistry

For the developmental study, P2 and P12 rat pups (n = 3 each) from the same litter were used. For the viral vector study, pups were injected on P3 with either HSVnr2d (n = 3) or HSVlac (n = 2) and allowed to survive until P10. The pups were killed with halothane and injected on P3 with either HSVnr2d (3×) or HSVlac (3×) (J Neurophysiol 1990; 1990; Gorham et al. 1990; Terao et al. 2000). The resulting outline template of identified motoneurons was applied to the corresponding NR2D images (and GFP images for the HSV study) to measure the signal density (total pixel intensity/number of pixels). In the HSV study, only motoneurons that were infected by the vector were included in the NR2D density measurements. Criterion for infection was a GFP density that was 2 SD greater than the mean density of the background signal, measured from multiple areas of ventrolateral white matter.

Statistics

Data from prenatal, first postnatal week, and second postnatal week were compared first by carrying out one-way ANOVA. If significant differences were observed between the groups, the Student-Newman-Keuls test was used for pairwise multiple comparisons between them. Pairs of means (e.g., from cells treated with β-galactosidase and NR2D viruses) are presented as means ± SD and were compared using the appropriate Student’s t-test. For anatomical data in stained motoneurons, we compared the mean values (e.g., staining density) from the individual animals, i.e., degrees of freedom were derived from the number of animals, not the total number of cells.

Results

Mg²⁺ sensitivity of the monosynaptic NMDA response increases in motoneurons between E18 and the second postnatal week

We initially examined connections to motoneurons in spinal cords taken from E18 animals (Ziskind-Conhaim 1990). Responses were judged to be monosynaptic if latency and amplitude fluctuations of successive responses were negligible (Fig. 1; see also Fig. 1 of Arvanian et al. [2003a]). Bath administration of NT-3 (0.2 µg/ml) for 15 min induced facilitation of both DR- and VLF-evoked monosynaptic responses in motoneurons of E18 rats (Fig. 1), with no change in resting membrane potential. In contrast, during the first postnatal week, NT-3 potentiated inputs from DR but not VLF, and in the second week, neither input was potentiated by NT-3 (Arvanov et al. 2000; see Fig. 2A for display of mean values). Statistical treatment of these results showed significant decline in NT-3-induced potentiation of the DR response between the first and second postnatal weeks (P < 0.001), whereas a significant decline in NT-3-induced potentiation of the VLF response occurred earlier, between E18 and the first postnatal week.
week ($P < 0.001$), but not between the first and second postnatal weeks.

To determine if NT-3–induced facilitation of VLF responses at E18 was associated with the presence of an NMDAR-mediated component, we examined these synaptic responses under conditions where all known non-NMDA inputs to the motoneurons were blocked pharmacologically. Note that after antagonists of AMPA/kainate, GABA$_A$, GABA$_B$, and glycine receptors were added to the superfuse, both DR and VLF stimulation elicited a response in 2 mM Mg$^{2+}$ that was similar in amplitude to the response recorded in the same motoneuron before the antagonists were applied (Fig. 3A). This suggests that NMDA receptors mediate a large fraction of the monosynaptic input to motoneurons from DR and VLF in prenatal rats, as previously reported for DR inputs (Ziskind-Conhaim 1990), and that NT-3 enhances these NMDAR-mediated responses. This was confirmed by complete abolition of the potentiated response by APV (data not shown).

An NMDAR-mediated response was detectable at P2 in 2 mM Mg$^{2+}$, but only in response to DR stimulation, whereas neither DR nor VLF stimulation elicited such responses at P12 at resting membrane potential, as previously reported (Arvanian and Mendell 2001b) (see Methods and Fig. 3B; numbers of observations given in Fig. 2C). The value of this parameter during the first postnatal week was about three times larger for the response to DR stimulation (1,209 µM; Fig. 3B) than for the response evoked by VLF stimulation (417 µM) in the same motoneurons. In addition, $K_{Mg}$ for DR-evoked responses decreased by nearly 50% between the first and second postnatal weeks (from 1,209 to 671 µM), whereas $K_{Mg}$ for VLF-evoked responses did not change (from 417 to 442 µM). We found that the VLF response was considerably less sensitive to Mg$^{2+}$ at E18 ($K_{Mg} = 1,012$ µM) than in the first postnatal week ($K_{Mg} = 417$ µM). The DR response, relatively insensitive during the first postnatal week ($K_{Mg} = 1,209$ µM), was even less sensitive at E18 ($K_{Mg} = 1,840$ µM).

\[ \text{FIG. 1.} \text{ At E18, neurotrophin-3 (NT-3) facilitates both dorsal root (DR) and ventrolateral funiculus (VLF)-evoked responses. Responses of the same motoneuron to DR and VLF stimulation in normal artificial cerebrospinal fluid (ACSF) and 15 min after bath administration of 0.2 µg/ml NT-3 are superimposed (each trace is the average of 10 consecutive traces). Insets: superimposed single sweeps taken on a faster time base from the records making up the associated DR and VLF control responses. Note the constant latency and amplitude of the initial component used to identify it as monosynaptic.} \]

\[ \text{FIG. 2.} \text{ Changes in function of synapses on motoneurons during development and after treatment with HSVVn2d viruses.} \text{ A and C:} \text{ Addition of NT-3 to normal ACSF facilitates both DR- and VLF-evoked responses (A) only when the inputs have NMDA receptor (NMDAR)-mediated components (C), which were measured in ACSF in the presence of non-NMDA antagonists and 2 mM Mg$^{2+}$. NT-3 had no effect on either DR or VLF responses at P12, when the NMDAR component was absent at both inputs.} \text{ B and D:} \text{ treatment of neonatal rats with HSVVn2d, but not HSVVlac, induced the appearance of NMDAR-mediated responses (D) and reinstated the ability of NT-3 to facilitate DR- and VLF-evoked EPSPs in P12 rats to the degree seen in E18 controls (B). Data for non-treated P2 and P12 rats taken from results presented earlier (Arvanian and Mendell 2001b; Arvanov et al. 2000). Numbers above the bars indicate number of recorded cells.} \]
Statistical testing of these differences using values of $K_{Mg}$ for the individual experiments (see METHODS) revealed a significant decline for the DR input from E18 to P1–P5 ($P < 0.02$) and again from P1–P5 to P8–P15 ($P < 0.03$). $K_{Mg}$ for VLF declined significantly from E18 to P1–P5 ($P < 0.001$), but not from P1–P5 to P8–P15. Thus it appears that the NMDA receptors on motoneurons associated with DR input become progressively more sensitive to Mg$^{2+}$ block from E18 into the second postnatal week, whereas those associated with VLF input reach a plateau during the first postnatal week. This increasing susceptibility of NMDARs associated with DR and VLF synaptic inputs to Mg$^{2+}$ block parallels the reduction in NT-3 effects on the AMPA/kainate receptor-mediated monosynaptic responses evoked by these inputs (see Fig. 2A).

The peak amplitude of the monosynaptic NMDAR-mediated response in solutions containing 100 μM (or lower) concentrations of Mg$^{2+}$ (Fig. 3A, arrowhead) for both DR and VLF stimulation did not change appreciably over the immediate postnatal period [DR (in mV): E18, 6.1 ± 1.5; P1–P5, 7.4 ± 1.4; P8–P15, 6.6 ± 1.7; VLF: E18, 5.6 ± 1.5; P1–P5, 5.7 ± 1.1; P8–P15, 6.1 ± 1.7]. However, at all ages tested, the VLF NMDAR-mediated response was more sensitive to increasing [Mg$^{2+}$]$_0$ than the DR response in the same motoneuron (Fig. 3B).

In addition to blocking NMDAR, increasing extracellular Mg$^{2+}$ decreases presynaptic release of glutamate that may have contributed both to the observed decline in the synaptic response with increasing [Mg$^{2+}$]$_0$ (Fig. 3) (Kuno and Takahashi 1986) and the differences between the Mg$^{2+}$ sensitivity of the responses to DR and VLF. We evaluated this possibility by determining the effect of varying Mg$^{2+}$ concentration on the AMPA/kainate-mediated monosynaptic EPSPs elicited by DR and VLF stimulation at P2. We chose P2 because this is the age at which significant AMPA/kainate receptor-mediated responses are first observed (Fig. 3A) and at which the differences in Mg$^{2+}$ sensitivity of NMDA responses to DR and VLF stimulation were greatest (Fig. 3B). We found that these AMPA/kainate responses were equally diminished by increasing Mg$^{2+}$ in the range used for the NMDAR experiments (Fig. 4), but by an amount that was considerably less than that observed for the NMDAR-mediated response at P2 (Fig. 3B). Thus, although some of the measured decline in the NMDA response was likely due to reduced transmitter release, the differential effects of Mg$^{2+}$ on NMDAR-mediated responsiveness is determined primarily by the magnitude of the Mg$^{2+}$ block of NMDARs.

Decline of NR2D subunit in the perinatal period

The degree of Mg$^{2+}$ blockade of the NMDAR is dependent on its subunit composition (Burnashev et al. 1995; Monyer et al. 1994). NMDA receptors are composed of NR1, NR2A-D, and NMDAR-L (NR3A, B) subunits (Abdrachmanov et al. 2002; Cull-Candy et al. 2001; Sucher et al. 1995). The soma and dendrites of neonatal rat motoneurons express these subunits (Jamon and Clarac 1998). NR1 subunits are essential for the formation of functional NMDA receptors, whereas the other subunits modify receptor properties, including Mg$^{2+}$ block (Burnashev et al. 1995; Momiyama et al. 1996).

To address possible molecular mechanisms of age-dependent changes in the degree of Mg$^{2+}$ blockade and corresponding decrease in NT-3–induced plasticity during initial postnatal period, we examined whether the elevated level of Mg$^{2+}$ blockade of NMDAR in 2-wk-old animals was associated with a change in expression of NMDAR subunits. We initially used

![FIG. 3. Effects of decreasing concentration of Mg$^{2+}$ on NMDAR-mediated synaptic potentials in motoneurons as a function of age and synaptic input. A: traces show DR and VLF responses in the same motoneurons from E18, P2, and P12 preparations. Numeric labels for P12 DR (bottom left): (1) AMPA/kainate monosynaptic responses in normal ACSF (2 mM Mg$^{2+}$; dashed); (2) 15 min after administration of non-NMDAR antagonists (AMPA/kainate antagonist CNQX 10 μM, GABA$_A$ antagonist bicuculline 5 μM, GABA$_B$ antagonist CGP35348 5 μM, glycine antagonist strychnine 5 μM); (3 and 4) NMDA responses with monosynaptic component (at arrow) after decreasing Mg$^{2+}$ concentration to 500 and 100 μM, respectively. The remaining 5 groups of 4 records in A are organized in the same way: record 1 is dashed; peak (record 2) < peak (record 3) < peak (record 4). B: Mg$^{2+}$ sensitivity of NMDAR-mediated responses changes during the perinatal period. Percentage change in the peak amplitude of monosynaptic NMDAR-mediated responses evoked by stimulation of DR or VLF in E18, P1–P5, and P8–P15 rats is expressed as a function of extracellular Mg$^{2+}$. Data points are mean ± SE of responses recorded from 6–8 cells for each case and normalized to the maximum (100%) response obtained in 50 μM Mg$^{2+}$ solution. IC$_{50}$ ($K_{Mg}$) for Mg$^{2+}$ determined by regression analysis (see METHODS).](http://jn.physiology.org/Downloadedfrom)
a microarray to compare mRNA content in the L4–L6 spinal cord between P2 and P12 rats. Of 8,728 genes assayed, 246 showed statistically significant changes in mRNA expression (criteria described in METHODS). Among these was the transcript encoding the NR2D subunit, which decreased between the first and second postnatal week. The expression of other NMDAR subunits did not change significantly during the first 2 postnatal weeks (Table 1). Quantitative RT-PCR analysis confirmed a decrease in the level of NR2D mRNA expression during the first 2 postnatal weeks, with no significant change in NR1 transcript levels (Table 1). The expression of NT-3, as well as the high-affinity trkC and low-affinity p75 receptors, did not change (Table 1), suggesting that developmental changes in NT-3 or its receptor were not responsible for the decay of NT-3-induced plasticity during this postnatal period unless the changes are local ones restricted to terminals of the presynaptic fibers that would not be detected by these techniques.

To determine whether NR2D protein decreased specifically in motoneurons during this period, the density of NR2D protein signal in peripherin-positive ventral horn neurons (motoneurons) was measured in samples from both P2 and P12 rat pups. This assay revealed that motoneurons expressed NR2D at both time points examined, but the mean density of signal decreased significantly with development (P2 = 36.7 ± 7.4; P12 = 20.4 ± 4.7; P < 0.05).

Replacement of NR2D using HSV viral vectors

Considering that the presence of NR2D subunits has been reported to make the NMDAR relatively resistant to Mg2+ blockade (Momiyama et al. 1996), we hypothesized that the decreased expression of NR2D subunits resulted in the development of NMDAR Mg2+ blockade and the decay of NT-3–induced facilitation during the first postnatal week. To test this hypothesis, we next examined whether increasing NR2D expression would prevent or delay development of Mg2+ blockade at both synaptic inputs and reestablish the ability of NT-3 to acutely potentiate transmission to motoneurons. A Herpes simplex virus type-1 amplicon (Bowers et al. 2000, 2001) carrying either NR2D (HSVnr2d) or Escherichia coli–derived -galactosidase (as a control; HSVlac) was injected directly into the ventral horn of P2 rats. Cords injected with HSVnr2d or HSVlac were removed at P12. Some were prepared for determination of NR2D mRNA expression by RT-PCR, or NR2D protein expression by immunocytochemistry. Others were prepared for electrophysiological examination of the degree of Mg2+ blockade of NMDAR-mediated responses and the action of NT-3 on monosynaptic AMPA/kainate transmission at DR and VLF synapses.

TABLE 1. NMDA receptor subunit and neurotrophin-related mRNA expression in lumbar L4–L6 region of spinal cord were compared between P2 and P12 rats, using Affymetrix Gene Chip arrays and RT-PCR

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<th>Genbank Number</th>
<th>Common Name</th>
<th>Gene Chip RT-PCR</th>
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<tr>
<td>L08228</td>
<td>NR1</td>
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<td>AF001423</td>
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Neurotrophin

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<td>X05137</td>
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Fold change refers to the ratio of RNA expression at P12 to that at P2 ("−" indicates decrease). Significance (*) was established by the following criteria: 1) dChip software fold change of “normalized” mean ≥1.5 and P ≤ 0.05; and 2) RT-PCR analyses of fold change ≥1.2 and P ≤ 0.05. FC, fold change.

FIG. 4. Percentage change in the peak amplitude of monosynaptic AMPA/kainate-mediated responses evoked by stimulation of DR or VLF in normal P2 rats is expressed as a function of extracellular Mg2+ (n = 5).

FIG. 5. Real-time quantitative RT-PCR analysis showed enhanced expression of NR2D subunit mRNA in the L4–L6 region of P12 rat spinal cord after treatment at P2 with HSVnr2d. Controls were treated with HSVlac.
As shown in Fig. 5, delivery of HSVnr2d enhanced the level of expression of NR2D mRNA at P12 by 1.2-fold ($P < 0.001$) compared with controls injected with HSVlac. NR2D protein level and localization were assayed as in the developmental study, with the additional limitation of examining NR2D density only in motoneurons that were shown also to have been infected with HSV vector (Fig. 6). Infection by the virus was determined arbitrarily by the presence of GFP in peripherin-positive cells at a level 2 SD over the mean of the background signal. By this criterion, HSV was shown to infect approximately one-half of the motoneurons (HSVlac = 51%; HSVnr2d = 51%) and clearly infected peripherin-negative neurons as well. Mean NR2D protein levels were shown to be significantly elevated in motoneurons from HSVnr2d-injected cords compared with those from HSVlac cords (HSVlac = 95 ± 6.2; HSVnr2d = 148.7 ± 2.6; $P < 0.001$).

**Functional changes induced by HSVnr2d**

HSV-mediated delivery of NR2D induced marked functional changes of synaptic input to motoneurons from treated P12 animals (see Table 2 for list of observed values). The mean amplitude of NMDAR-mediated responses elicited by both DR and VLF in 2 mM Mg$^{2+}$ was significantly increased compared with control [HSVlac; Fig. 2D; DR (in mV): 4.1 ± 2.0 vs. 0.5 ± 0.6, $P < 0.005$; VLF: 2.8 ± 1.5 vs. 0.7 ± 1.0, $P < 0.015$]. The values after HSVnr2d treatment were similar to those observed at E18, although because of differences in motoneuron size and the number of afferent terminals at these two ages, it is difficult to compare these two sets of results. The enlarged NMDAR-mediated EPSPs measured in 2 mM Mg$^{2+}$ were apparently due to a decreased Mg$^{2+}$ blockade (larger $K_{Mg}$) of the NMDAR-mediated response (DR and VLF) after treatment with HSVnr2d [Fig. 7B; DR (in $\mu$M): 1,233 ± 298 vs. 729 ± 206, $P < 0.015$; VLF: 884 ± 237 vs. 453 ± 137, $P < 0.01$]. As anticipated, the enhanced NMDA responsiveness due to decreased Mg$^{2+}$ blockade after HSVnr2d compared with HSVlac was accompanied by an enhanced potentiation of AMPA/kainate monosynaptic responses by NT-3 [Figs. 2D and 7A; DR (%): 76 ± 33 vs. 5 ± 4, $P < 0.001$; VLF:

**TABLE 2.** Response of individual cells to presence of HSVnr2d or HSVlac

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<th>NMDAR Response (mV)</th>
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<tr>
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Three separate measures are shown: % facilitation by NT-3; magnitude of NMDAR-mediated response in 2 mM Mg$^{2+}$; and $K_{Mg}$ (the IC$_{50}$ for Mg$^{2+}$ sensitivity of the response). The response to DR and VLF stimulation is displayed for each of these measures. Note that 1 or 2 cells in each group of 5 or 6 exhibits little or no response to HSVnr2d treatment (asterisk values) as determined by comparison with the results obtained in different motoneurons after HSVlac treatment. Statistical comparisons in text.
mediated EPSPs in the absence of NT-3 (DR: 5.2 ± 1.3 mV, P > 0.6) unlike its effect on NMDA receptor-mediated EPSPs.

It is noteworthy that individual cells in animals treated with HSVnr2d varied in their values of percent facilitation (of the AMPA response by NT-3), NMDAR-mediated response, and $K_{\text{Mg}}$. For each measurement, cells were observed with values similar to the more uniform values found in HSVlac-treated animals (Table 2). This agrees qualitatively with our anatomical data, which suggest that not all motoneurons were infected by the HSV vector. Quantitatively, the infectivity based on the physiological data were about 70–80% compared with our estimate of 50% from the anatomical data. This difference is not surprising given the assumptions necessary to estimate the anatomical infectivity (signal/noise) and the relatively small numbers of cells available for the physiological determination.

**DISCUSSION**

The major finding of this study is that intraspinal delivery of viral vectors carrying the signal for NR2D, but not β-galactosidase, prevented the age-associated increase in Mg$^{2+}$ block of the NMDA receptors, the decline in amplitude of the NMDAR-mediated responses, and the diminished NT-3–induced enhancement of sensory and central AMPA/kainate receptor-mediated EPSPs in motoneurons. This finding is entirely consistent with previous results from this laboratory, in which NMDAR function could be restored acutely during the second postnatal week by placing the cord in a low Mg$^{2+}$ solution, thereby removing the Mg$^{2+}$ block (Arvanian and Mendell 2001b). It remains to be determined whether the enhanced NMDAR function may persist beyond 2 wk and whether injection of the vectors into older animals would have the same functional effects, although there is evidence for the latter from studies carried out in the cerebellum and hippocampus (Kakegawa et al. 2003; Okada et al. 2003).

The ability of NMDAR to respond in relatively high levels of Mg$^{2+}$ has been shown to require the presence of either the NR2C or NR2D subunit, with the latter being more effective (Cull-Candy et al. 2001). Our attention was focused on NR2D because the initial survey of NMDA subunit expression using gene chip analysis showed that NR2D alone declined in the spinal cord during the period when Mg$^{2+}$ block of NMDAR increased. This decline was confirmed at the mRNA level using RT-PCR. The decline in NR2D was further confirmed in motoneurons at the protein level with immunocytochemistry, although a portion of the signal may have been due to NR2C (Marvizon et al. 2002). Other studies have shown NR2D expression in neonatal motoneurons (Abdrachmanova et al. 2000; Shibata et al. 2003; Stegenga and Kalb 2001) as well as a decrease in NR2D, but not NR1, after P7 in preganglionic parasympathetic neurons (Shibata et al. 2003).

The presence of GFP immunoreactivity in many spinal neurons provides evidence that the HSV vectors delivered their genes, and the presence of GFP in peripherin expression neurons shows that motoneurons were infected. However, it was apparent from immunostaining for GFP that a substantial fraction of spinal cells was not infected. This would dilute numbers of cells available for the physiological determination.

The anatomical data indicated quite clearly that the NR2D subunit was introduced into cells other than motoneurons (i.e., into peripherin-negative cells) as well as motoneurons. Nonetheless, we contend that our physiological results indicate a change in NMDA receptor properties in the motoneuron. The changes in Mg$^{2+}$ sensitivity, in NT-3 potentiation of the EPSP, and in the magnitude of the NMDAR-mediated response were all observed for the monosynaptic EPSP, suggesting that interneurons interposed between Ia fibers and motoneurons were not involved. Another possibility is a direct change in NMDA receptors in presynaptic afferent terminals or in interneurons making axo-axonal...
contacts on Ia fiber afferent terminals. However, reducing the susceptibility of either of these NMDA receptors to Mg$^{2+}$ block would be expected to enhance presynaptic inhibition, which would reduce the size of monosynaptic EPSPs in motoneurons (Arvanian and Mendell 2001a). We found exactly the opposite, that NMDAR-mediated EPSPs were enhanced in amplitude after HSVnr2d treatment. We interpret our data to mean that the presence of additional NR2D subunits in the motoneuron reduced the susceptibility of associated NMDA receptors to Mg$^{2+}$ block, which enhanced the NMDAR-mediated response and the effect of NT-3 on the associated AMPA/kainate receptor-mediated response, known to require functional NR2D receptors in the motoneuron (Arvanian and Mendell 2001b; Arvanov et al. 2000). However, it has been reported that second messengers such as PKC can reduce the Mg$^{2+}$ block of NMDA receptors of trigeminal neurons in nucleus caudalis (Chen and Huang 1992); therefore we must consider that contributions to our results from such indirect mechanisms are possible.

Recent experiments in the mouse cerebellum have led to the conclusion that although NR2D could be shown in the postsynaptic cell membrane by exogenous glutamate application, it was not present directly at the synapse since there was no evidence for the characteristic NR2D synaptic response (a PSP with a long decay time constant) (Brickley et al. 2003). Our own functional evidence for participation of NR2D in synaptic NMDAR-mediated responses in motoneurons is based on observing changes in sensitivity of both DR- and VLF-evoked synaptic responses to Mg$^{2+}$ block, rather than differences in EPSP decay time or the low conductance known to be associated with NR2D expression (Cull-Candy et al. 2001). We have no ready explanation for our failure to find prolonged decay of the EPSP, except for the possibility that the NR2D subunits in motoneurons may have co-assembled with other subunit types, which would have altered their decay properties (Cull-Candy et al. 2001; Dunah et al. 1998) while perhaps maintaining their ability to function at relatively high levels of Mg$^{2+}$. It must also be kept in mind that the present experiments were carried out in current clamp, and that polysynaptic pathways were also activated, which might have obscured the expected difference in decay time.

The gene chip and RT-PCR experiments revealed no change in NR1 gene expression between the first and the second postnatal weeks (Table 1). We also found no significant differences in the NMDAR-mediated EPSP elicited from either VLF or DR when measured in low Mg$^{2+}$, i.e., with no Mg$^{2+}$ block. This suggests that the additional synaptic current required to match the increased electrical load due to the increase in motoneuron size between the first and second weeks (Seebach and Mendell 1996) does not require an increase in receptor gene expression. One possibility is that gene expression is maximal initially, with some receptors, initially extrasynaptic, being incorporated into synapses as the afferent fiber terminals expand and branch to make more synapses on the growing postsynaptic cells.

One of the important conclusions from this and previous experiments in this series (Arvanian and Mendell 2001b; Arvanov et al. 2000) is that the properties of NMDAR on single motoneurons can differ according to their synaptic input. Throughout the perinatal period studied here, NMDARs associated with VLF input are more susceptible to Mg$^{2+}$ block than those associated with DR input, presumably due to differences in NMDAR subunit composition (i.e., NR2D). Although our data confirm developmental and input-specific differences in the degree of Mg$^{2+}$ block (Fig. 3), the precise values of $K_{\text{Mg}}$ remain uncertain because of the effect of varying [Mg$^{2+}$], on transmitter release (Fig. 4). Synapse-specific differences in NMDAR subunits have been reported previously in CA3 pyramidal cells (Ito et al. 1997) and on pyramidal cells in neocortex (Kumar and Huguenard 2003). The finding that NMDAR associated with DR and VLF inputs in the same motoneuron undergo changes at a different developmental age most likely means that the conversion of synaptic NMDAR on motoneurons requires an instructive signal from the presynaptic fibers rather than being determined entirely by the motoneuron itself. We cannot presently determine whether this signal is activity driven, activity itself, or some trophic factor that is released in a time-dependent manner from the central and segmental inputs to affect the motoneurons in a synapse-specific manner.

Enhancement of the Mg$^{2+}$ block of NMDAR in motoneurons during the first 2 postnatal weeks contrasts with findings in lamina II of the dorsal horn, where a small NMDAR-mediated response is observed at a membrane potential of $-70$ mV throughout the first 2 postnatal weeks (Bardoni et al. 1998; Garraway et al. 2003). This correlates with expression of the NR2D regulatory subunit in adult rats that has been reported to be absent in motoneurons but weakly expressed in lamina II (Tolle et al. 1993).

Although the functional assays in this study were of motoneurons from rats younger than 15 days old, the results provide "proof of principle" that the age-related conversion of NMDAR to a "silent" mode can be delayed and neurotrophin-induced plasticity of synaptic inputs can be extended by increasing NR2D levels. NT-3 is produced in neonatal motoneurons (Yan et al. 1993), and its chronic removal in vivo using trkC-IgG reduces the size of the monosynaptic EPSP produced by the DR (Seebach et al. 1999). Thus we can infer that NT-3 is likely to contribute to synaptic development in the spinal cord. The relative extent to which these postnatal developmental effects depend on the NMDAR-dependent mechanisms reported here or on NMDAR-independent mechanisms (Arvanian et al. 2003a) remains to be determined.

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