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Postnatal switching of NMDA receptor subunits from NR2B to NR2A in rat facial motor neurons

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Abstract

The subunit composition of *N*-methyl-D-aspartate (NMDA) receptors affects their function under normal and pathological conditions. Functional NMDA receptors are expressed in lower motor neurons, but their subunit composition has not been defined. Here, we employed electrophysiology, quantitative PCR, and immunohistochemistry to investigate the subunit composition of NMDA receptors in postnatal motor neurons of the Wistar rat facial nucleus (FN). Whole-cell patch clamp recordings of acutely dissociated motor neurons from postnatal days 3 and 4 (P3–P4) showed that ifenprodil, a specific antagonist of the NMDA receptor 2B (NR2B) subunit, inhibited 91.62% ± 2.02% of NMDA-induced current, whereas NVP-AAM007, a specific antagonist of the NMDA receptor 2A (NR2A) subunit, inhibited much less of the current (16.69% ± 3.28%). Starting from P5, the inhibitory effects of ifenprodil and NVP-AAM007 gradually decreased and increased, respectively, such that the effect of NVP-AAM007 exceeded that of ifenprodil by P10. At P14, most of the NMDA-induced current was inhibited by NVP-AAM007 (84.59% ± 3.35%). Consistent with this, NR2B mRNA and protein were expressed highly at P3 and then gradually decreased by more than 75% by P14 in FN motor neurons, while NR1 was expressed stably over the same ages. However, NR2A mRNA and protein showed relatively constant levels between P3–P10 and decreased to 45% and 75% of the P3 level, respectively, by P14. Thus, analysis of functional NMDA receptors is critical to revealing subunit switching, which may be an important step in postnatal development of FN motor neurons.

Introduction

N-methyl-D-aspartate receptors (NMDARs) are characterized by high Ca^{2+} permeability and voltage-dependent regulation of their activity and are involved in important neural functions, such as synapse development, synaptic plasticity, and learning and memory (Mayer & Westbrook, 1987; Nakanishi *et al*., 1998; Seeburg *et al*., 1998; Dingledine *et al*., 1999; Yamakura & Shimoji, 1999), as well as in pathophysiological processes (Choi, 1988; Lipton & Rosenberg, 1994).

Three gene families encoding seven NMDA receptor subunits have been identified: NMDA receptor subunit 1 (NR1), NR2A–D, and NR3A–B (Moriyoshi *et al*., 1991; Meguro *et al*., 1992; Monyer *et al*., 1992; Ciabarra *et al*., 1995; Sucher *et al*., 1995; Nishi, 2001; Chatterton *et al*., 2002). Functional NMDARs are composed of an obligatory NR1 subunit and one type or more of the NR2A–D and NR3A–B subunits (Moriyoshi *et al*., 1991; Kutsuwada *et al*., 1992; Meguro *et al*., 1992; Monyer *et al*., 1992; Chatterton *et al*., 2002). The diverse physiological and pharmacological properties of NMDARs (Kutsuwada *et al*., 1992; Monyer *et al*., 1992; Stern *et al*., 1992; Monyer *et al*., 1994; Wyllie *et al*., 1996; Premkumar *et al*., 1997; Dingledine *et al*., 1999; Yamakura & Shimoji, 1999) are largely determined by the type(s) of NR2 and/or NR3 subunits that they contain, although splice variants of the NR1 subunit also contribute to functional diversity. The surface expression and structural modification of NMDARs also depend on their subunit composition (Lau & Huganir, 1995; Barria & Malinow, 2002; Lavezzari *et al*., 2004). Furthermore, the expression of various NR2 and NR3 subunits is regionally and developmentally regulated in the postnatal brain (Moriyoshi *et al*., 1991; Meguro *et al*., 1992; Monyer *et al*., 1992; Ciabarra *et al*., 1995; Sucher *et al*., 1995; Nishi, 2001; Chatterton *et al*., 2002).

Lower motor neurons in the brainstem and spinal cord express various NMDAR subunits (Wenzel *et al*., 1995; Laurie *et al*., 1997; Sundstrom *et al*., 1997; Oshima *et al*., 2002; Fukaya *et al*., 2005; Paarmann *et al*., 2005) and manifest NMDA receptor-mediated responses at embryonic and postnatal stages (Konnerth *et al*., 1990; Ziskind-Conhaim, 1990; Hori & Kanda, 1996; Palecek *et al*., 1999; Arvanov *et al*., 2000). Which of these subunits form functional receptors has not yet been examined and the role of NMDARs in these neurons during development is not completely understood. Defining the role of NMDARs in motor neurons in this time period will be important for understanding how lower motor neurons receive synaptic input from the brain and become the 'final common pathway' for executing motor tasks. Here, we investigated the subunit composition of NMDA receptors in postnatal motor neurons of the rat facial nucleus (FN). NR2B-containing NMDARs predominated in the early postnatal stage and were gradually replaced by NR2A-containing NMDARs during the second postnatal week. Such NR2B-to-NR2A switching may play an important role in the development of motor neuron functions.

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Materials and methods

Animals

Wistar rats from postnatal day 2 to day 14 (P2–P14) were used. All procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and the Society for Neuroscience Guidelines for the Use of Animals in Neuroscience Research, using protocols approved by the Burnham Institute Animal Research Committee.

Acute dissociation of FN motor neurons

Rats were anaesthetized with isoflurane and decapitated. The brain stem was rapidly removed into ice-cold sucrose-enriched artificial cerebral spinal fluid (ACSF; in mM: 128 sucrose, 10 D-glucose, 64 NaCl, 2.5 KCl, 0.65 NaH_2PO_4, 26 $NaHCO_3$, 2 $MgSO_4$, 2 $CaCl_2$, pH 7.4). The ventral region of the upper medulla containing the FN was isolated, treated with 20 units/mL papain for 50 min, and mechanically dissociated. The cell suspension was plated into a culture dish well containing a poly lysine-coated glass coverslip. Cells were maintained for 1–3 h before recording at 37 °C in an incubator (95% O_2, 5% CO_2) in complete Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum, 2 mM L-glutamine, and 10 mM HEPES.

Electrophysiological recordings

For electrophysiological studies, the dye Δ^9-DiI (Invitrogen, Carlsbad, CA, USA) was applied to the exposed facial nerve at P2 to retrogradely label FN motor neurons. After cell dissociation, FN motor neurons were identified by their red fluorescence, and whole-cell patch clamp recordings were performed at room temperature (RT) with an EPC7 amplifier (Heka Instruments, Heidelberg, Germany), Digidata 1322A analogue-to-digital (A/D) converter interface, and pClamp9.0 software (Axon Instruments, Foster City, CA, USA). Signals were filtered at 2 kHz, digitized at 10 kHz, and stored in a computer for analysis. Recording pipettes were made from borosilicate capillary tubes (Warner Instruments, Hamden, CT, USA) on a Sutter P-97 puller (Sutter Instrument Company, Novato, CA, USA), with a tip resistance of 2.5–5 MΩ when filled with pipette solution containing (in mM) 120 $CsCl_2$, 2.25 EGTA, 10 HEPES, 2 $MgCl_2$, 1 $CaCl_2$, 20 TEA-Cl, adjusted to pH 7.2 with CsOH. The bath solution contained (in mM) 138 NaCl, 5 KCl, 0.5 $CaCl_2$, 10 HEPES, 10 glucose, 0.4 KH_2PO_4, 0.3 Na_2HPO_4, adjusted to pH 7.4 with NaOH. Drugs were prepared in the bath solution and delivered by a gravity-fed multibarrel perfusion system (VC-6 six channel valve controller, Warner Instrument Corp. Hamden, CT, USA). Only one motor neuron from each coverslip was used for drug tests and the recording chamber was washed thoroughly between tests. All drugs were purchased from Tocris (Ellisville, MO, USA) except for NVP-AAM007, which was a gift from Novartis (St. Louis, MO, USA). Current traces were initially analysed using the Clampfit subroutines of pClamp 9.0 and then imported into Prism 4.0 software for statistical analysis. All data are presented as mean ± SEM. Linear regression analysis was performed using Prism 4.0 and statistical significance was defined as $P < 0.05$.

Isolation of FN motor neurons with laser capture microdissection (LCM)

Postnatal rats at P3–P14 were anaesthetized with isoflurane and perfused transcardially with cold RNase-free phosphate-buffered saline (PBS; 10 mM sodium phosphate and 138 mM sodium chloride, pH 7.4). The brain was isolated, embedded in Tissue-Tek O.C.T. compound (Sakura Finetek, Torrance, CA, USA), and snap-frozen in 2-methyl butane containing dry ice. Cryostat sections (8 μm) were collected onto Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA, USA) and stained with a HistoGene LCM Frozen Section Staining Kit (Arcturus Bioscience, Mountain View, CA, USA). LCM was performed using a PixCell II LCM system (Arcturus). Cells were captured with the 7.5-μm laser setting and CapSure HS LCM Caps (Arcturus). FN motor neurons were selectively captured from each section on the basis of their large size.

Real-time PCR analysis

Total RNA was extracted from LCM-isolated FN motor neurons (~200 for each sample) within 1 h following capture using the Ambion RNAqueous-Micro Kit (Ambion, Austin, TX, USA). The manufacturer's protocol was followed, including the DNA removal step. Purified total RNA was reverse transcribed in a 20-μL reaction mixture containing 0.25 mM oligo(dT) by SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Quantitative real-time PCR was performed with the Stratagene Mx3000p QPCR system (Stratagene, La Jolla, CA, USA) using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Each sample was analysed in triplicate. At the end of the PCR, baseline and threshold values were set in the software and the cycle threshold values were exported to Microsoft Excel for analysis. Gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to correct for RNA input variability. The primers designed using the Primer3 software (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_http://www.cgi) were NR2A (NM_012573), 5′-TTTCCTCCCTTCTGGTGATG-3′ (forward), 5′-TGCTAGCCTTGGACACACAG-3′ (reverse); NR2B (NM_012574), 5′-ACCCTCAAAGCCCGACTAAT-3′ (forward), 5′-GTTGGCAAAGGAGCTCTCAC-3′ (reverse); and GAPDH (NM_017008), 5′-GGCATTGCTCTCAATGACAA-3′ (forward), 5′-TGTGAGGGAGATGCTCAGTG-3′ (reverse).

Immunohistochemistry

Postnatal rats at P3–P14 were anaesthetized with isoflurane and perfused transcardially with chilled PBS followed by phosphate-buffered 4% paraformaldehyde solution. The brain was dissected immediately, postfixed for 2 h, and cytoprotected in 25% sucrose overnight. Sagittal cryostat sections (14 μm) were processed for immunohistochemistry as described previously (Wang & Zhang, 2005). In brief, sections were incubated at 4 °C with rabbit anti-NR2A (1 : 1000; Upstate, Lake Placid, NY, USA) or rabbit anti-NR2B (1 : 500; Chemicon, Temecula, CA, USA) for 24 h, followed by incubation with a biotinylated goat anti-rabbit antibody (1 : 200, Vector Laboratories, Burlingame, CA, USA). The detection of NR2A or NR2B immunoreactivity was performed using a Vectastain Elite ABC kit (Vector Laboratories) and visualized with a DAB substrate solution (Roche Applied Science, Penzberg, Germany). The intensity of immunostaining in the FN motor neurons was analysed with Adobe Photoshop CS 8.0. Briefly, the grayscale image was first inverted by choosing Image > Adjustments > Invert. Areas without any cells in the image were randomly selected by Lasso tool and the mean of the pixels was obtained from the Histogram palette. This value was used as background of the image.

The mean of the pixels of an individual FN motor neuron was also obtained by using Lasso tool and Histogram palette. The intensity of immunostaining of this motor neuron was then computed by subtracting the background from its mean of the pixels. Ten FN motor neurons at each age were randomly selected and the average intensity of immunostaining was then calculated. Relative intensity was expressed as a percentage by normalizing the average intensity at each age to that at P3.

Results

The effects of various drugs on NMDA-evoked currents were examined by whole-cell patch clamp recording at a holding potential of −70 mV in acutely dissociated FN motor neurons retrogradely labelled *in vivo* with Δ^9-DiI. From P3 to P14, application of 100 μM NMDA supplemented with 10 μM glycine evoked an inward current in all motor neurons recorded (56/56); 100 μM 2-amino-5-phosphonopentanoic acid (APV) completely blocked this current (Fig. 1A).

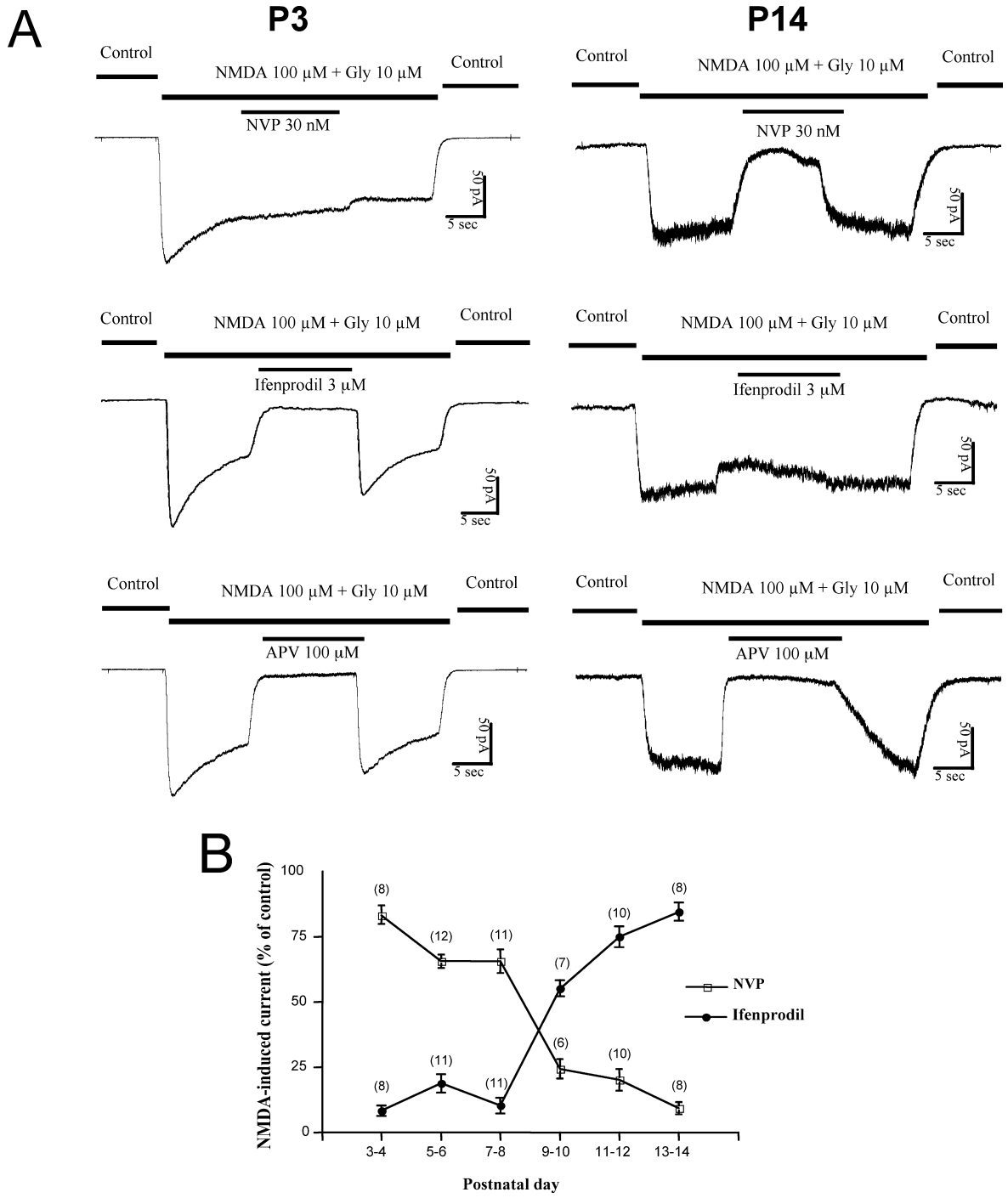

FIG. 1. (A) Recordings were made in FN motor neurons at P3 (left) and P14 (right). Currents induced by 100 μM NMDA and 10 μM glycine were differentially inhibited by 30 nM NVP-AAM007 (top trace), 3 μM ifenprodil (middle trace), and 100 μM APV (bottom trace) in the same neuron. (B) Plots summarizing the inhibitory effects of NVP-AAM007 and ifenprodil on NMDA-induced currents over time (the number of neurons is indicated above each data point). Linear regression analysis showed a statistically significant negative and positive correlation between NMDA-induced currents and ages in the presence of NVP-AAM007 ($r = -0.9614$, $P < 0.01$) and ifenprodil ($r = 0.9373$, $P < 0.01$), respectively.

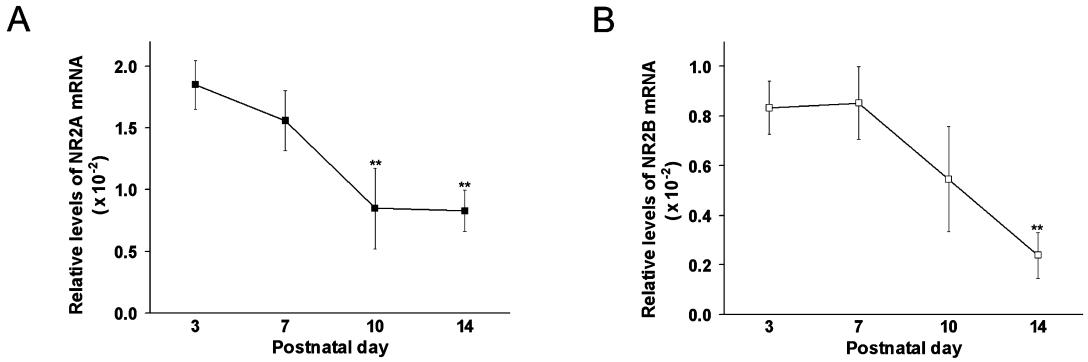

FIG. 2. Quantification of NR2A (A) and NR2B (B) mRNA expression in rat FN motor neurons at P3, P7, P10, and P14. The mRNAs were measured by real-time PCR and normalized to GAPDH mRNA expression. Data are given as means ± SD ($n = 3$). The significance of difference between each age and P3 was assessed by one-way ANOVA, Dunnett's test (**$P < 0.01$).

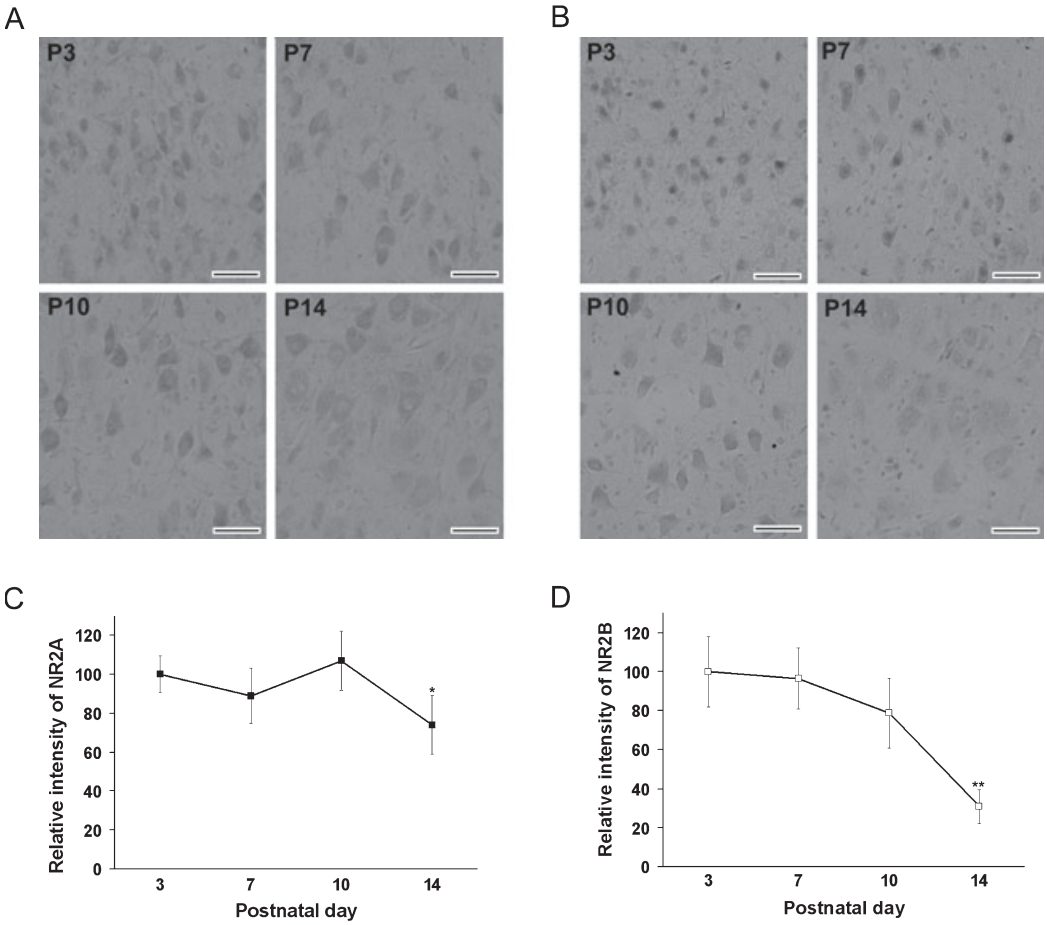

FIG. 3. Expression of NR2A (A and C) and NR2B (B and D) protein in rat FN motor neurons at P3, P7, P10, and P14. Both NR2A (A) and NR2B (B) immunoreactivities were detected in the cell bodies of FN motor neurons at all ages tested. The relative intensities of NR2A (C) and NR2B (D) in FN motor neurons at each age were calculated by comparing their average intensities at each age to those at P3 and expressing the ratio as a percentage. The significance of difference between each age and P3 was analysed by Student's *t*-test ($n = 10$, *$P < 0.05$, **$P < 0.01$). Scale bar, 50 μm.

The contribution of NR2A- and NR2B-containing NMDARs was assessed by comparing NMDA-induced currents in the absence and presence of 30 nM NVP-AAM007, a selective NR2A antagonist, or 3 μM ifenprodil, a selective NR2B antagonist. Control values were defined as the peak amplitude of the NMDA-induced current prior to administration of antagonist. From P3 to P4, ifenprodil inhibited 91.62% ± 2.02% of the NMDA-induced current, whereas NVP-AAM007 had a smaller effect (16.69% ± 3.28% inhibition; Fig. 1B). Starting from P5, the inhibitory effect of ifenprodil on NMDA-induced currents gradually decreased, while the effect of NVP-AAM007 gradually increased (Fig. 1B). At P14, the NMDA-induced currents were almost completely inhibited by NVP-AAM007 (84.59% ± 3.35%), but were inhibited to only a small degree by ifenprodil. Linear regression analysis showed a statistically significant

negative and positive correlation between NMDA-induced currents and ages in the presence of NVP-AAM007 ($r = –0.9614$, $P < 0.01$) and ifenprodil ($r = 0.9373$, $P < 0.01$), respectively, from P3 to P14. There was a relatively large deviation at the P7–8 point, suggesting the expression of NR2A and NR2B may not be strictly linear around this age period.

Several studies have reported the early expression of NR2A mRNA in embryonic and early postnatal lower motor neurons (Oshima *et al*., 2002; Fukaya *et al*., 2005). We therefore investigated the expression level of NR2A and NR2B mRNAs in FN motor neurons obtained from P3 to P14 using the LCM method. Quantitative real-time PCR showed the expression of both NR2A and NR2B mRNAs in these FN motor neurons. When normalized to GAPDH mRNA, NR2A was expressed at higher levels than NR2B at each age tested (Fig. 2). Consistent with the results from the electrophysiological studies above, the expression of NR2B mRNA was maximal between P3 and P7, dropped at P10, and at P14 was less than 25% of its expression at P3 (Fig. 2B). However, the highest level of NR2A mRNA was also detected at P3, after which it decreased to less than 45% of that at P3 (Fig. 2A). NR1 mRNA was expressed stably at all postnatal stages investigated (P0 to P14, not shown).

Immunohistochemical staining confirmed the presence of NR2A and NR2B proteins in postnatal FN motor neurons (Fig. 3), mainly in the cell body (Fig. 3A and B). The expression of NR2B protein was maximal at P3 and decreased until it reached approximately 25% of the P3 level at P14 (Fig. 3B and D). NR2A protein was present at high levels from P3 to P10, and had dropped by only approximately 25% at P14 (Fig. 3A and C).

Discussion

We have shown for the first time that NR2B-containing NMDARs in FN motor neurons are largely replaced by NR2A-containing NMDARs during the second postnatal week. Although this is consistent with the developmental scheme of other CNS neurons (Monyer *et al*., 1994; Sheng *et al*., 1994; Liu *et al*., 2004), NR2B-to-NR2A switching in lower motor neurons was unexpected because of the current knowledge about the developmental features of these neurons. Lower motor neurons are among the first projection neurons to develop in the CNS, and their programmed cell death is largely completed at birth in rodents (Lance-Jones, 1982; Harris & McCaig, 1984; Oppenheim, 1986). In agreement with this, previous studies of NMDAR subunit mRNAs emphasized the earlier expression of NR2A in motor neurons (Oshima *et al*., 2002; Fukaya *et al*., 2005), implicating NR2A-containing receptors in the early development of these neurons. Although the expression of NMDAR mRNAs and proteins in FN motor neurons agrees with these and other previous studies (Watanabe *et al*., 1992; Tolle *et al*., 1993; Monyer *et al*., 1994; Watanabe *et al*., 1994), our results point to a greater functional importance of NR2B-containing receptors in the early development of motor neurons. In addition, as NMDAR-mediated responses in FN motor neurons were largely inhibited by an NR2B-selective antagonist at early postnatal stages and by an NR2A-selective antagonist at later postnatal stages, other types of NMDARs such as NR1/NR2C and NR1/NR2D are unlikely to be major components of these neurons, even though triheteromeric receptors like NR1/NR2/NR2C or NR1/NR2/NR2D may still exist. Our studies clearly demonstrate the importance of identifying functional receptors rather than just expression patterns in order to assess the roles of subunits in motor neurons.

Because NR2A- and NR2B-containing NMDARs act through distinctive signal transduction pathways to regulate neuronal functions in normal and pathological conditions (Krapivinsky *et al*., 2003; Kim *et al*., 2005; Zhou & Baudry, 2006), the time-dependent switching between the two is likely to be linked to specific developmental requirements of postnatal motor neurons. Furthermore, the switching itself may also be tightly controlled by these developmental events, because replacement of NR2B- with NR2A-containing receptors is activity-dependent (Barria & Malinow, 2002). Interestingly, the same study showed that the NR2B-to-NR2A switching does not depend on the expression level of NR2A protein, which may explain the mismatch of NR2A mRNA and protein with NR2A-containing functional receptors in FN motor neurons. Our results suggest that the process from transcription to translation, and finally to the assembly of functional receptors, may be quite prompt for NR2B, but lags behind for NR2A in FN motor neurons. Alternatively, NR2A may prefer to form NR1/NR2A/NR2B triheteromeric receptors in the presence of NR1 and NR2B, which may be more sensitive to ifenprodil but not NVP-AAM007 under our recording conditions.

Developing motor neurons undergo many physiological and anatomical changes in the perinatal period in order to accomplish a series of time-dependent tasks; many of these tasks require the activation of NMDARs (Kalb, 1994; Inglis *et al*., 1998; Llado *et al*., 1999). Many important physiological properties of NMDARs depend on their subunit composition, for example the Mg^{2+} sensitivity, which has been proposed to result from a switch between NR2C- or NR2D- to NR2A-containing receptors in postnatal spinal motor neurons (Arvanian & Mendell, 2001; Arvanian *et al*., 2004). Based on the temporal pattern of functional NMDAR expression found in this study, we speculate that NR2B-containing NMDARs play a key role in the regulation of motor neuron numbers and the elimination of inappropriate connections, which occur at late embryonic and early postnatal stages, whereas NR2A-containing NMDARs play a role in later postnatal functions, such as maturation of the dendritic tree. Further studies will be necessary to elucidate the role of NR2A- and NR2B-containing NMDARs in both normal development and early onset diseases of motor neurons.

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Abbreviations

FN, facial nucleus; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LCM, laser capture microdissection; NMDA, *N*-methyl-D-aspartate; NMDAR, *N*-methyl-D-aspartate receptor; NR1, NR2A–D, and NR3A–B, *N*-methyl-D-aspartate receptor subunit 1, 2A–D, and 3A–B.

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