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DIFFERENTIAL NR2B SUBUNIT EXPRESSION AT DORSAL ROOT AND VENTROLATERAL FUNICULUS SYNAPSES ON LUMBAR MOTONEURONS OF NEONATAL RAT

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Abstract—Synapse specific differences in NR2 subunit expression exist in several systems within the mammalian CNS. Here we have studied such differences on motoneurons in the neonatal rat cord using ifenprodil known to inhibit voltage-, use- and glycine-independent responses mediated by NR2B-containing N-methyl-D-aspartate receptors (NMDARs) with high specificity. In neonatal rats (P1–P9), the synapses made by the dorsal root (DR) fibres were more sensitive to ifenprodil than ventrolateral funiculus (VLF) connections on the same motoneuron. DR connections exhibited very little additional blockade to bath-applied MK-801 whereas VLF connections displayed a further decrease in amplitude. This suggests that at this immediate postnatal age, DR synapses on motoneurons contain a higher proportion of ifenprodil-sensitive diheteromeric NR1/NR2B receptors than VLF synapses. Since DR synapses have been shown in other studies to be less mature than VLF synapses on the same motoneuron at this developmental stage, these data are interpreted as indicating that less mature NMDA receptors feature a higher proportion of NR2B subunits which declines as the synapse matures. This novel finding of staggered development of NMDA receptors from different synaptic inputs on the same motoneuron is discussed in the context of its developmental and functional implications. Published by Elsevier Ltd on behalf of IBRO.

Key words: MK-801, EPSP, NMDA receptor, ifenprodil, NR2B.

These experiments characterize properties of N-methyl-D-aspartate (NMDA) receptors associated with two major inputs to the motoneurons, dorsal root afferent fibers (DR) and fibers in the ventrolateral funiculus of the spinal cord (VLF). They were undertaken because previous work (Arvanov et al., 2000; Arvanian and Mendell, 2001; Arvanian et al., 2004) established that the properties of the NMDAR-mediated responses of DR and VLF synapses on the same motoneuron differ. During the prenatal period both DR- and VLF-evoked monosynaptic NMDA-mediated responses are observed in motoneurons at physiological Mg^{2+} concentration. In the first postnatal week, DR-evoked NMDA receptor-mediated excitatory postsynaptic potentials (EPSPs) are observed, but they are absent at VLF synapses. In the second postnatal week, NMDA receptor-mediated responses are absent at synapses made by both inputs (Arvanian et al., 2004). The progressive decrease in the NMDA-mediated response at these synapses is not due to a loss of NMDA receptors but rather to a developing Mg^{2+} blockade (Arvanian and Mendell, 2001). NMDA-mediated responses at VLF synapses develop Mg^{2+} blockade at resting membrane potential immediately after birth, earlier than DR synapses which display Mg^{2+} blockade only after the first postnatal week (Arvanian et al., 2004). These observations provided the first hint that NMDA receptors of DR and VLF synapses on the same motoneuron have a different timetable of maturation. We therefore set out to identify key elements associated with this staggered development of NMDA receptors from different synaptic inputs on the same motoneuron.

NMDA receptors are tetrameric, with four subunits (Laube et al., 1998). The three main families of NMDA subunits are NR1, NR2 (A–D) and NR3 (A, B). Native NMDA receptors usually occur as diheteromers containing two NR1 and two identical NR2 subunits. However, there is also experimental evidence for triheteromers composed of two NR1 and two different NR2 subunits (Chazot et al., 2002; Pina-Crespo and Gibb, 2002; Brickley et al., 2003; Hatton and Paoletti, 2005; Brothwell et al., 2008). The expression of NMDA receptor subunits changes with development (Kalb et al., 1992; Monyer et al., 1994; Stegenga and Kalb, 2001). For example NR2B and NR2D subunits, associated with immature and plastic synapses (Lopez de Armentia and Sah, 2003) are expressed early in development in the central amygdala and are gradually replaced with NR2A subunits that are associated with mature synapses (Monyer et al., 1994; Stegenga and Kalb, 2001).

The purpose of our experiments was to determine whether there is an intrinsic difference in NMDAR subunit expression between DR and VLF synapses in the first postnatal week. Do “less mature” synapses formed by the DR have a greater proportion of NR2B subunits at this age than “more mature” synapses formed by the VLF? We approached this by studying the differences in blockade of DR and VLF NMDA receptor-mediated EPSPs by ifenprodil. Ifenprodil is a highly selective, reversible, noncompetitive, antagonist for NR2B subunit-containing NMDARs

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Abbreviations: ACSF, artificial cerebrospinal fluid; CGP 46381, Ciba Geigy Product 46381; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DR, dorsal root; EPSP, excitatory postsynaptic potential; MK-801, (5R,10S)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10 imine-hydrogen-maleate; NMDA, N-methyl-D-aspartate; NMDAR, N-methyl-D-aspartate receptor; NR2, subunit 2 of the NMDA receptor; NR1/NR2X, NMDA receptor containing NR2 subunit; NT-3, neurotrophin-3; PSD, post synaptic density; SAP, synapse-associated protein; VLF, ventrolateral funiculus.

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(Legendre and Westbrook, 1991; Williams, 1993, 2001; Perin-Dureau et al., 2002). In oocytes, ifenprodil at low concentrations (<10 μM) has a 400-fold increased affinity for diheteromeric NMDARs containing NR2B over those containing NR2A. The inhibitory action of ifenprodil is not activity or voltage dependent. The binding site is outside the ion channel pore, and its effect is described as distinct from the effects of non-competitive open-channel blockers such as (5R,10S)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine-hydrogen-maleate (MK-801) (Williams, 1993, 2001). Using ifenprodil we were able to infer differences in the subunit composition of the NMDARs that comprise these synapses.

EXPERIMENTAL PROCEDURES

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

Electrophysiology

Electrophysiological experiments were carried out *in vitro* on neonatal rat spinal cords removed from rats aged P1–9 as previously described (Seebach et al., 1999; Shanthanelson et al., 2009). The rats were anesthetized by placing them on a latex glove lying on a bed of ice (P1/P2), or by halothane (P3–P11). The spinal cord was quickly removed from the animal and the left hemicord was placed in a chamber superfused with artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl (117), KCl (4.7), $CaCl_2$ (2.5), $MgSO_4$ (800 μM), $NaHCO_3$ (25), NaH_2PO_4 (1.2), dextrose (11), aerated with 95% O_2/5% CO_2 (pH 7.4, 30 °C) at 10 ml/min. The VLF was dissected free of the spinal cord at T2 (Pinco and Lev-Tov, 1994). Suction stimulating electrodes were attached to peeled VLF axon bundles for activation of this fiber tract, to the cut L5 dorsal root for activation of segmental inputs, and to the L5 ventral root for identification of recorded cells as motoneurons by antidromic activation. Intracellular recordings were obtained using sharp microelectrodes (resistance 60–80 MΩ, filled with 3 M potassium acetate). Electrical stimulation of the DR and/or VLF was at an intensity sufficient to evoke the just-maximum monosynaptic response. All efforts were made to minimize the number of animals used and their suffering.

Use of pharmacological blockers

The AMPA/kainate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (10 μM), $GABA_A$ receptor antagonist bicuculline (5 μM), $GABA_B$ receptor antagonist Ciba Geigy Product 46381(CGP 46381) (10 μM) and glycine receptor antagonist strychnine (5 μM) were added to the perfusion solution to isolate NMDAR-mediated responses pharmacologically (stimulation rate was 0.025 Hz when NMDAR-mediated responses were studied (see; Arvanian and Mendell, 2001 for details)). The reversible antagonist ifenprodil was used to selectively block NMDA NR2B subunits. MK-801, an irreversible activity dependent antagonist, was used to block all activated synaptic NMDA channels.

CNQX, ifenprodil and MK-801 were obtained from Sigma (St. Louis, MO, USA). CGP 46381 and bicuculline methochloride was purchased from Tocris Bioscience (Bristol, UK). Strychnine HCl was obtained from Research Biochemical International (Natick, MA, USA).

Protocol

Only cells displaying a stable resting membrane potential greater than −65 mV were included in this study. Initial AMPA/kainate responses were obtained from both synaptic inputs in the absence of any pharmacological blockers. The cord was then exposed to the AMPA/kainate and inhibitory transmitter antagonist cocktail by superfusion for at least 30 min, after which control NMDA responses from DR and VLF were obtained (Shanthanelson et al., 2009). For control stimulation 10 stimuli were delivered at the same intensity with a 40 s interval to each input and then the stimulation was switched to the other input, and then back, until a total of approximately 40 stimuli each had been delivered to both DR and VLF. The NR2B antagonist ifenprodil (3 μM) was then added to the bath while continuing the administration of the antagonist cocktail. During ifenprodil administration, the two inputs were stimulated alternately, a single stimulation of DR first, followed by a single VLF stimulation, and then back, with a 40 s interval between each stimulus. A total of at least 30 stimuli each were delivered to both DR and VLF. After maximal blockade by ifenprodil was achieved, the activity-dependent irreversible NMDAR blocker MK-801 (10 μM) was added to the bath while continuing administration of the antagonist cocktail but discontinuing ifenprodil administration. In the presence of MK-801, one of the inputs was stimulated exclusively until maximal blockade of the EPSP was achieved, typically after 30 min with stimulation at 1 every 40 s. The other input was then stimulated exclusively until its response was also blocked maximally (Shanthanelson et al., 2009).

As a final step, all antagonists were washed out for 45 min in ACSF. The inputs were not stimulated during this time. DR and VLF were then stimulated in the same order as before to obtain responses to ensure viability of the cell and their synaptic inputs throughout the experiment (Shanthanelson et al., 2009). These experiments required long-lasting recordings from individual cells, typically 4–5 h, in general this was more difficult in cells from older neonates (>P 5).

For offline analysis the responses to each stimulus were analyzed using Axoclamp 9.0 in single sweeps, in superimposed sweeps or after averaging. The peak of the monosynaptic component was measured at the latest time point where there was consistent overlap of successive responses (See Shanthanelson et al., 2009).

Statistics

Only a single cell was studied in each cord, and so the number of observations is the number of cells. The specific tests are described in the Results.

RESULTS

The effects of ifenprodil on the DR- and VLF-NMDA receptor-mediated synaptic responses are illustrated in Fig. 1. The top panels display superimposed sweeps from successive trials after the AMPA/kainate response had been blocked with non NMDA antagonists. Note that the NMDA response consisted of two major components, a short latency monosynaptic component followed by a large plateau-like potential with superimposed spikes (seeArvanian and Mendell, 2001). The amplitude of the monosynaptic DR response obtained averaged 1.9 mV±0.4SE (n=14), similar to the mean amplitude of the VLF response (1.7 mV±0.3SE (n=14)).

The bottom two panels illustrate the effects on the same cell after administering ifenprodil (3 μM). The monosynaptic response was gradually diminished in amplitude, with the DR response decreasing more than the VLF response. This was continued until maximum blockade was observed, that is, no further decrease in three successive trials. Insets display the monosynaptic responses to the