Published in final edited form as:

Neuroscience. 2010 March 17; 166(2): 730–737. doi:10.1016/j.neuroscience.2009.12.025.

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 central nervous system. 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.

Keywords

MK-801; EPSP; NMDA receptor; Ifenprodil; NR2B

Introduction

These experiments characterize properties of 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

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responses are observed in motoneurons at physiological Mg^{2+} concentration. In the first postnatal week, DR-evoked NMDA receptor-mediated 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^{++} blockade (Arvanian and Mendell, 2001). NMDA-mediated responses at VLF synapses develop Mg^{++} blockade at resting membrane potential immediately after birth, earlier than DR synapses which display Mg^{++} 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 (Legendre and Westbrook, 1991; Williams, 1993, 2001; Perin-Dureau et al., 2002). In oocytes, ifenprodil at low concentrations ($<10\mu M$) 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.

Materials and Methods

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),

NaH₂PO₄ (1.2), dextrose (11), aerated with 95% O₂ / 5% CO₂ (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.

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 40s 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 40s 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 40s. 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 minutes 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 hours, in general this was more difficult in cells from older neonates (> P5).

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 Figure 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 2 major components, a short latency monosynaptic component followed by a large plateau- like potential with superimposed spikes (see(Arvanian and Mendell, 2001)). The amplitude of the monosynaptic DR response obtained averaged $1.9 \text{ mV} \pm 0.4 \text{ SE}$ ($n=14$), similar to the mean amplitude of the VLF response ($1.7 \text{ mV} \pm 0.3 \text{ SE}$ ($n=14$)).

The bottom 2 panels illustrate the effects on the same cell after administering ifenprodil ($3 \mu\text{M}$). 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, i.e., no further decrease in 3 successive trials. Insets display the monosynaptic responses to the first and last stimulus of each series on a faster sweep speed and at higher gain where it can be seen that the DR response was more completely blocked than the VLF response. This difference was observed in most cases (Fig. 2). The blockade of the monosynaptic response to DR averaged over all preparations ranging in age from P1 to P9 was $72.8 \pm 3.5\%$ SEM ($n=14$), greater than the mean VLF monosynaptic response blockade in the same preparations ($48.4 \pm 4.4\%$ SE ($n=14$)) (Holm-Sidak ANOVA $p<0.001$). The time course of the decline for both the DR and VLF monosynaptic responses is illustrated in Fig 3. It can be seen that the reduction in both inputs began at about the same time and the decay was in parallel. A Mann-Whitney Rank sum test revealed no statistically significant difference in the time required for the response to DR and VLF to begin declining after ifenprodil was introduced into the bath ($p > 0.5$; $n= 13$). Thus ifenprodil at $3 \mu\text{M}$ has equal access to synapses made by DR and VLF on motoneurons.

The plateau potential in response to DR was only marginally affected by the ifenprodil. Its onset became more delayed with progressive stimulation and it exhibited somewhat more decay, particularly at longer times after the stimulus ($> 250 \text{ ms}$). The plateau potential response to VLF was labile even in the absence of ifenprodil, and so it was difficult to characterize its response to ifenprodil accurately.

In order to determine whether the reduced blockade of these DR polysynaptic late responses was due to insufficient amount of ifenprodil in the bath, $10 \mu\text{M}$ ifenprodil was used in some experiments. The plateau potential exhibited somewhat more decay but the main effect was on the latency of the onset which tended to reach an even longer onset latency. There was no significant increase in the blockade of the monosynaptic component of the DR response by $10 \mu\text{M}$ ifenprodil ($74.2 \% \pm 1.3$; $n=8$ in $10 \mu\text{M}$ vs. $75.8 \pm 3.5\%$; $n= 14$ in $3 \mu\text{M}$). However, the monosynaptic component of the VLF response was blocked to a greater extent with $10 \mu\text{M}$ ifenprodil ($76.6 \pm 4.1\%$, $n=12$ in $10 \mu\text{M}$ vs. $48.4 \pm 4.4\%$ $n=14$ in $3 \mu\text{M}$ ifenprodil; t-test, $p<0.001$) (See Discussion)

Effects of MK-801

The finding that ifenprodil did not completely block the response elicited by DR and VLF stimulation in the presence of a cocktail of non-NMDAR antagonists raised the question of whether the remaining response was indeed NMDA-mediated. We therefore compared the effect of the activity-dependent, irreversible NMDA antagonist MK-801 in some cells with the effect of 3 μ M ifenprodil. In addition to determining whether these remaining responses are NMDA receptor-mediated, we wished to determine the proportion of synaptic NMDA receptors associated with input that was not NR2B.

Fig. 4 displays the response of one motoneuron treated with ifenprodil and MK-801. After maximal blockade with ifenprodil was achieved, we introduced 10 μ M MK-801 into the perfusing solution containing the CNQX and antagonists against GABA and glycine. Ifenprodil application was discontinued, and so its concentration in the bath was gradually reduced as the concentration of MK-801 was increased (see Discussion).

Because MK-801 is a use-dependent antagonist, the DR input alone was stimulated (i.e., no alternation with VLF) repetitively every 40s until maximum blockade of the DR-response was achieved as determined by the EPSP amplitude reaching a steady state value (Shanthanelson et al., 2009). This generally took about 30 minutes. The VLF input was then stimulated repetitively every 40s until maximal blockade of its response was achieved. The first and maximally depressed response of each series are displayed in the insets at a faster sweep speed and higher gain. Note that the DR response was blocked by ifenprodil but not more by MK-801. The VLF response was blocked to a lesser extent by ifenprodil and then to a greater extent by MK-801.

The mean effect of MK-801 varied depending on the input being stimulated (Fig. 5). VLF responses exhibited significantly more depression after MK-801 than after ifenprodil (Holm-Sidak ANOVA $p=0.003$) indicating the presence of non NR2B subunits at these receptors. DR monosynaptic responses on average were more completely inhibited by ifenprodil than VLF responses, but did not display further significant blockade with MK-801, showing that the response that remained after ifenprodil action was not mediated by transmission through NMDARs composed of a different NR2 subunit in the place of NR2B. However, these responses remaining after ifenprodil were likely mediated by NMDA receptors despite the lack of any significant effect of MK-801 (see Discussion, and Shanthanelson et al., 2009). This pattern was particularly evident for the bulk of the cells recorded in younger animals (P1 to P4). In 2 rats P8 and older (not included in this figure), the DR response displayed approximately 15% further blockade with MK-801, suggesting the presence of other non-NR2B subunit-containing NMDA channels at these synapses in older neonates, but we could not establish significance due to the small sample size. However, this finding is consistent with previous observations that rat motoneurons studied in the second postnatal week have NMDA receptors whose properties indicate the presence of triheteromers (Palecek et al., 1999).

In agreement with previous observations (Shanthanelson et al, 2009) MK-801 substantially reduced the amplitude of the plateau potential. This decline was substantially larger than that observed in ifenprodil even at 10 μ M.

Discussion

We examined the ifenprodil-sensitivity of the pharmacologically isolated NMDA receptor-mediated EPSPs produced by DR and VLF stimulation in neonatal rat lumbar motoneurons. The NMDA receptor-mediated monosynaptic EPSP elicited by DR stimulation was consistently reduced to a significantly greater extent by ifenprodil (3 μ M) than that elicited by VLF stimulation. The time of onset of inhibition by ifenprodil was similar for both synaptic

inputs indicating that there was no difference in its access to these synapses. These considerations also strongly indicate that the increased blockade of VLF synapses by 10 μ M ifenprodil was not simply due to attaining a concentration at VLF synapses equivalent to that achieved at DR synapses with 3 μ M ifenprodil, i.e., the VLF and DR synapses on motoneurons have a true difference in sensitivity to ifenprodil at 3 μ M that is absent at 10 μ M.

Differences in NMDA receptor subunits associated with DR and VLF inputs to neonatal motoneurons

The interpretation of these results depends heavily on ifenprodil's action as a high affinity specific blocker of NR2B subunit containing diheteromeric NMDARs at a concentration of 3 μ M (Legendre and Westbrook, 1991; Perin-Dureau et al., 2002). Maximal inhibition by ifenprodil is about 90% and requires that both NR2 subunits in diheteromers be NR2B (Chazot and Stephenson, 1997; Tovar and Westbrook, 1999; Chazot et al., 2002; Hatton and Paoletti, 2005). If all receptors are triheteromeric with a NR1/NR2B/ NRXX subunit composition, ifenprodil action results in only a 21% inhibition of the response (Tovar and Westbrook, 1999; Hatton and Paoletti, 2005). A mixed subunit population expression (di- and triheteromeric) results in ifenprodil inhibition intermediate between 0 and 90% depending on the proportion of NR2B subunit containing receptors (Tovar and Westbrook, 1999; Hatton and Paoletti, 2005). Coassembly of NR2B subunits with other subunits such as NR2A (Chazot and Stephenson, 1997; Tovar and Westbrook, 1999; Chazot et al., 2002; Hatton and Paoletti, 2005) or NR2D (Pina-Crespo and Gibb, 2002; Brickley et al., 2003; Jones and Gibb, 2005; Brothwell et al., 2008) has been described previously. At 3 μ M, ifenprodil is identified to be a nearly saturating concentration for blockade of NR1/NR2B wild type receptors expressed in *Xenopus* oocytes (Perin-Dureau et al., 2002) and has virtually no effect on NR1/NR2A wild type receptors. At 10 μ M ifenprodil blocks about 40% of the response of heterologous NR1/ NR2A receptors at a membrane potential of -70 mV. Thus by using 3 μ M of ifenprodil in our experiments, we ensured a selective high affinity blockade for NR1/NR2B diheteromers.

At DR synapses on motoneurons in rats from P1–P4, a majority of the NMDA-mediated response is mediated by diheteromeric NR2B-containing NMDARs since it is almost completely blocked by ifenprodil. The inhibition at VLF- activated synapses in the postnatal period studied here was significantly less, consistent with (although not proving) the idea that a greater proportion NMDARs comprising these synapses have a triheteromeric subunit configuration (see above). However, it is not possible to specify the identity of fourth subunit that is part of the NMDAR tetramer. Additional support for the idea that VLF synapses have a greater proportion of triheteromeric or NR1/NR2A receptors is that 10 μ M ifenprodil blocked the VLF monosynaptic response ($76.6 \pm 4.1\%$, $n=12$) to a greater degree than 3 μ M ifenprodil ($48.4\% \pm 4.4$, $n=14$), with no change in blockade of the DR monosynaptic response. It has been reported previously that ifenprodil at concentrations 10 μ M or greater, acts as a weak voltage-dependent, open-channel blocker of NR1/NR2A/NR2B and to some extent of NR1/NR2A, in addition to blocking NR1/NR2B receptors (Kew et al., 1998; Perin-Dureau et al., 2002).

Although the DR response remaining after ifenprodil was generally not further reduced in amplitude by MK-801 in cords from rats from P1 to P4, we believe that this remaining response was mediated by NMDA receptors. The failure of the bath applied MK-801 to completely block the NMDA receptor-mediated synaptic responses might be explained by a constant replacement of NMDA receptors at the synapse by independently functioning unblocked extrasynaptic receptors (Clark et al., 1997; Momiyama, 2000; Thomas et al., 2006; Shanthanelson et al., 2009). Complete elimination of this response can be achieved only when both synaptic and extrasynaptic NMDARs are blocked (by coapplication of NMDA and MK-801), thereby preventing the accumulation of functional NMDA receptors from extrasynaptic regions (Shanthanelson et al., 2009). In the present experiments, ifenprodil

application was discontinued as the MK-801 was applied and since the action of ifenprodil is fully reversible (Williams, 1993), the extrasynaptic NMDARs would no longer be inhibited by ifenprodil. These functional receptors would be free to move into the synaptic region that was previously occupied by NMDARs that were inhibited by the activity dependent block of MK-801. This could account for the persistent NMDA-mediated response that remained after ifenprodil and MK-801 action.

Input-dependent variation in NR2A/B subunit expression on a common target neuron has been reported in other systems. Examples include spiny stellate cells in layer 4 of the mouse barrel cortex (Fleidervish et al., 1998), associated fibre-CA3-commisural synapses (Ito et al., 2000), intracortical- neocortical layer 5 pyramidal neuron- callosal synapses (Kumar and Huguenard, 2003), interneuron-CA1 synapses (Maccaferri and Dingledine, 2002), mossy fiber-CA3 synapses (Lei and McBain, 2002), and Schaffer collateral- CA1 pyramidal neuron-perforant path synapses (Otmakhova et al., 2002). In these systems differential expression of these subunits has been proposed to play roles in synapse strengthening, in identifying the origin of the input and in coincidence detection.

Action of Ifenprodil on the plateau potential

Ifenprodil had a relatively minor effect in the plateau potential in contrast to its effect on the monosynaptic potential. It appeared to delay the onset of the plateau and tended to accelerate the decay of the potential. The latter effect was similar though much smaller than what occurs after MK-801. Both ifenprodil and MK-801 are known to block the HV-activated Ca channels and thereby retard the entry of Ca into cells (Church et al., 1994). This would be expected to delay the onset and attenuate the amplitude of the plateau potential.

Developmental and functional implications

NR2A- containing NMDARs are more stable than NR2B- containing ones and overexpression of NR2A stabilizes the trafficking of NR2B containing receptors at synapses (Groc et al., 2006). The developmental switch from NR2B diheteromers to triheteromers or non-NR2B-containing diheteromers may therefore be required to allow these receptors to stabilize within the synapse. In the brain and brainstem this switch usually occurs later than the second postnatal week (Kew et al., 1998; Cathala et al., 2000; Lopez de Armentia and Sah, 2003; Liu et al., 2004) in contrast to rat lumbar motoneurons where the non-NR2B containing NMDARs are expressed as early as P1 at synapses made by VLF fibers. Early onset of NR2A expression and its postsynaptic localization have been reported previously in developing motoneurons of the mouse hypoglossal nucleus (Oshima et al., 2002; Fukaya et al., 2005). This early onset and synaptic stabilization of non-NR2B containing NMDARs may therefore be specific to motoneuron development and maturation, perhaps because motor activity is required immediately after birth.

Conclusion

We have previously shown that DR synapses mature at a significantly slower rate than VLF synapses (Arvanian et al., 2004), and also that trafficking of NMDA receptors from extrasynaptic to synaptic sites, a phenomenon associated with synaptic plasticity, occurs more readily at DR synapses on motoneurons than those formed by VLF (Shanthanelson et al., 2009). NMDA receptor surface mobility has been shown to depend on the expression of NR2 subunits; NR2B- containing NMDARs spend less time at synapses and have a greater ability to traffic (Groc et al., 2006). The current finding of an increased proportion of NR2B subunit-containing diheteromeric NMDARs at DR synapses is consistent with their later maturation and the increased trafficking of their NMDA receptors.

NR2 subunits play a major role in establishing the pharmacological and physiological profile of NMDARs. NR2B- containing NMDARs produce currents with moderate decay times, rapid deactivation kinetics, high conductance and high sensitivity to Mg^{2+} block. NR2A- containing NMDARs produce currents with the fastest decay time, slower deactivation kinetics, high conductance and high sensitivity to Mg^{2+} block (Cull-Candy and Leszkiewicz, 2004). As the synapses mature, there is a need for NMDA-mediated transmission with high through-put, rapid decay and deactivation to enable precise relay of information, and with increased voltage sensitivity (in the form of Mg^{2+} block) to allow proper coincidence detection. Modulating the subunit composition of NMDARs is an efficient way to alter their current dynamics and achieve the desired physiological properties of transmission.

Acknowledgments

We thank Dr. Victor L. Arvanian for helpful discussion. The research was supported by a grant from the NIH to LMM (5 RO1 NS16996) as well as the Christopher and Dana Reeve Foundation (LMM).

List of Abbreviations

ACSF	Artificial cerebrospinal fluid
AMPA	Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate
CGP 46381	Ciba Geigy Product 46381
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
DR	Dorsal root
EPSP	Excitatory Postsynaptic potential
GABA	γ -amino butyric acid 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

Literature cited

- Arvanian VL, Mendell LM. Removal of NMDA receptor $Mg(2+)$ block extends the action of NT-3 on synaptic transmission in neonatal rat motoneurons. *J Neurophysiol* 2001;86:123–129. [PubMed: 11431494]
- Arvanian VL, Bowers WJ, Petruska JC, Motin V, Manuzon H, Narrow WC, Federoff HJ, Mendell LM. 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. *J Neurophysiol* 2004;92:2394–2404. [PubMed: 15152019]

- Arvanov VL, Seebach BS, Mendell LM. NT-3 evokes an LTP-like facilitation of AMPA/kainate receptor-mediated synaptic transmission in the neonatal rat spinal cord. *J Neurophysiol* 2000;84:752–758. [PubMed: 10938302]
- Brickley SG, Misra C, Mok MH, Mishina M, Cull-Candy SG. NR2B and NR2D subunits coassemble in cerebellar Golgi cells to form a distinct NMDA receptor subtype restricted to extrasynaptic sites. *J Neurosci* 2003;23:4958–4966. [PubMed: 12832518]
- Brothwell SL, Barber JL, Monaghan DT, Jane DE, Gibb AJ, Jones S. NR2B- and NR2D-containing synaptic NMDA receptors in developing rat substantia nigra pars compacta dopaminergic neurones. *J Physiol* 2008;586:739–750. [PubMed: 18033813]
- Cathala L, Misra C, Cull-Candy S. Developmental profile of the changing properties of NMDA receptors at cerebellar mossy fiber-granule cell synapses. *J Neurosci* 2000;20:5899–5905. [PubMed: 10934236]
- Chazot PL, Stephenson FA. Molecular dissection of native mammalian forebrain NMDA receptors containing the NR1 C2 exon: direct demonstration of NMDA receptors comprising NR1, NR2A, and NR2B subunits within the same complex. *J Neurochem* 1997;69:2138–2144. [PubMed: 9349560]
- Chazot PL, Lawrence S, Thompson CL. Studies on the subtype selectivity of CP-101,606: evidence for two classes of NR2B-selective NMDA receptor antagonists. *Neuropharmacology* 2002;42:319–324. [PubMed: 11897110]
- Church J, Fletcher EJ, Baxter K, MacDonald JF. Blockade by ifenprodil of high voltage-activated Ca²⁺ channels in rat and mouse cultured hippocampal pyramidal neurones: comparison with N-methyl-D-aspartate receptor antagonist actions. *Br J Pharmacol* 1994;113:499–507. [PubMed: 7834201]
- Clark BA, Farrant M, Cull-Candy SG. A direct comparison of the single-channel properties of synaptic and extrasynaptic NMDA receptors. *J Neurosci* 1997;17:107–116. [PubMed: 8987740]
- Cull-Candy SG, Leszkiewicz DN. Role of distinct NMDA receptor subtypes at central synapses. *Sci STKE* 2004;2004:re16. [PubMed: 15494561]
- Fleidervish IA, Binshtok AM, Gutnick MJ. Functionally distinct NMDA receptors mediate horizontal connectivity within layer 4 of mouse barrel cortex. *Neuron* 1998;21:1055–1065. [PubMed: 9856461]
- Fukaya M, Hayashi Y, Watanabe M. NR2 to NR3B subunit switchover of NMDA receptors in early postnatal motoneurons. *Eur J Neurosci* 2005;21:1432–1436. [PubMed: 15813953]
- Groc L, Heine M, Cousins SL, Stephenson FA, Lounis B, Cognet L, Choquet D. NMDA receptor surface mobility depends on NR2A-2B subunits. *Proc Natl Acad Sci U S A* 2006;103:18769–18774. [PubMed: 17124177]
- Hatton CJ, Paoletti P. Modulation of triheteromeric NMDA receptors by N-terminal domain ligands. *Neuron* 2005;46:261–274. [PubMed: 15848804]
- Ito I, Kawakami R, Sakimura K, Mishina M, Sugiyama H. Input-specific targeting of NMDA receptor subtypes at mouse hippocampal CA3 pyramidal neuron synapses. *Neuropharmacology* 2000;39:943–951. [PubMed: 10727704]
- Jones S, Gibb AJ. Functional NR2B- and NR2D-containing NMDA receptor channels in rat substantia nigra dopaminergic neurones. *J Physiol* 2005;569:209–221. [PubMed: 16141268]
- Kalb RG, Lidow MS, Halsted MJ, Hockfield S. N-methyl-D-aspartate receptors are transiently expressed in the developing spinal cord ventral horn. *Proc Natl Acad Sci U S A* 1992;89:8502–8506. [PubMed: 1356265]
- Kew JN, Richards JG, Mutel V, Kemp JA. Developmental changes in NMDA receptor glycine affinity and ifenprodil sensitivity reveal three distinct populations of NMDA receptors in individual rat cortical neurons. *J Neurosci* 1998;18:1935–1943. [PubMed: 9482779]
- Kumar SS, Huguenard JR. Pathway-specific differences in subunit composition of synaptic NMDA receptors on pyramidal neurons in neocortex. *J Neurosci* 2003;23:10074–10083. [PubMed: 14602822]
- Laube B, Kuhse J, Betz H. Evidence for a tetrameric structure of recombinant NMDA receptors. *J Neurosci* 1998;18:2954–2961. [PubMed: 9526012]
- Legendre P, Westbrook GL. Ifenprodil blocks N-methyl-D-aspartate receptors by a two-component mechanism. *Mol Pharmacol* 1991;40:289–298. [PubMed: 1715017]
- Lei S, McBain CJ. Distinct NMDA receptors provide differential modes of transmission at mossy fiber-interneuron synapses. *Neuron* 2002;33:921–933. [PubMed: 11906698]

- Liu XB, Murray KD, Jones EG. Switching of NMDA receptor 2A and 2B subunits at thalamic and cortical synapses during early postnatal development. *J Neurosci* 2004;24:8885–8895. [PubMed: 15470155]
- Lopez de Armentia M, Sah P. Development and subunit composition of synaptic NMDA receptors in the amygdala: NR2B synapses in the adult central amygdala. *J Neurosci* 2003;23:6876–6883. [PubMed: 12890782]
- Maccaferri G, Dingledine R. Control of feedforward dendritic inhibition by NMDA receptor-dependent spike timing in hippocampal interneurons. *J Neurosci* 2002;22:5462–5472. [PubMed: 12097498]
- Momiyama A. Distinct synaptic and extrasynaptic NMDA receptors identified in dorsal horn neurones of the adult rat spinal cord. *J Physiol* 2000;523(Pt 3):621–628. [PubMed: 10718742]
- Monyer H, Burnashev N, Laurie DJ, Sakmann B, Seeburg PH. Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. *Neuron* 1994;12:529–540. [PubMed: 7512349]
- Oshima S, Fukaya M, Masabumi N, Shirakawa T, Oguchi H, Watanabe M. Early onset of NMDA receptor GluR epsilon 1 (NR2A) expression and its abundant postsynaptic localization in developing motoneurons of the mouse hypoglossal nucleus. *Neurosci Res* 2002;43:239–250. [PubMed: 12103442]
- Otmakhova NA, Otmakhov N, Lisman JE. Pathway-specific properties of AMPA and NMDA-mediated transmission in CA1 hippocampal pyramidal cells. *J Neurosci* 2002;22:1199–1207. [PubMed: 11850447]
- Palecek JJ, Abdrachmanova G, Vlachova V, Vyklick L Jr. Properties of NMDA receptors in rat spinal cord motoneurons. *Eur J Neurosci* 1999;11:827–836. [PubMed: 10103076]
- Perin-Dureau F, Rachline J, Neyton J, Paoletti P. Mapping the binding site of the neuroprotectant ifenprodil on NMDA receptors. *J Neurosci* 2002;22:5955–5965. [PubMed: 12122058]
- Pina-Crespo JC, Gibb AJ. Subtypes of NMDA receptors in new-born rat hippocampal granule cells. *J Physiol* 2002;541:41–64. [PubMed: 12015419]
- Pinco M, Lev-Tov A. Synaptic transmission between ventrolateral funiculus axons and lumbar motoneurons in the isolated spinal cord of the neonatal rat. *J Neurophysiol* 1994;72:2406–2419. [PubMed: 7884467]
- Seebach BS, Arvanov V, Mendell LM. Effects of BDNF and NT-3 on development of Ia/motoneuron functional connectivity in neonatal rats. *J Neurophysiol* 1999;81:2398–2405. [PubMed: 10322075]
- Shanthanelson M, Arvanian VL, Mendell LM. Input-specific plasticity of N-methyl-D-aspartate receptor-mediated synaptic responses in neonatal rat motoneurons. *Eur J Neurosci* 2009;29:2125–2136. [PubMed: 19490018]
- Stegenga SL, Kalb RG. Developmental regulation of N-methyl-D-aspartate- and kainate-type glutamate receptor expression in the rat spinal cord. *Neuroscience* 2001;105:499–507. [PubMed: 11672615]
- Thomas CG, Miller AJ, Westbrook GL. Synaptic and extrasynaptic NMDA receptor NR2 subunits in cultured hippocampal neurons. *J Neurophysiol* 2006;95:1727–1734. [PubMed: 16319212]
- Tovar KR, Westbrook GL. The incorporation of NMDA receptors with a distinct subunit composition at nascent hippocampal synapses in vitro. *J Neurosci* 1999;19:4180–4188. [PubMed: 10234045]
- Williams K. Ifenprodil discriminates subtypes of the N-methyl-D-aspartate receptor: selectivity and mechanisms at recombinant heteromeric receptors. *Mol Pharmacol* 1993;44:851–859. [PubMed: 7901753]
- Williams K. Ifenprodil, a novel NMDA receptor antagonist: site and mechanism of action. *Curr Drug Targets* 2001;2:285–298. [PubMed: 11554553]

Figure 1. Action of Ifenprodil on DR and VLF synaptic EPSP

Illustration of the procedures carried out on an individual motoneuron from a P2 rat. Data from a single motoneuron obtained over a 3 hour and 30 min recording period. V_m was about -70 mV throughout. Stimulation rate 1/40s. Top Row: Ten consecutive NMDA-mediated responses to DR and to VLF obtained after blockade of all non NMDA receptors with CNQX, bicuculline, strychnine and CGP 35348 (CNQX Cocktail) administered to the bath (not shown). Note the reliable responses to DR and the irregular responses to VLF. Insets are averaged monosynaptic responses at higher gain and faster sweep speed. Bottom row: The NMDAR-mediated response was then blocked with ifenprodil ($3\mu\text{M}$). Alternate stimulation of DR and VLF (a total of 40 stimuli to each input at 0.025 Hz). Trial 1 was obtained immediately after introducing ifenprodil into the bath and the decline of monosynaptic response occurred progressively. The stimulation was stopped when steady state was reached. Single traces (inset) display the initial response (black) and the last response (red) in ifenprodil.

Figure 2. EPSPs produced by DR stimulation are more sensitive to ifenprodil inhibition than those produced by VLF stimulation

Percent blockade of DR and VLF with ifenprodil. See text for definition of % values. Lines connect data from same motoneuron. Note the negative slope for 13 of 14 cells indicating that the blockade of DR was greater than blockade of response to VLF. Further details in text.

Figure 3.

Decline of NMDA-mediated responses from DR and VLF in the same motoneuron during 45 minute application of ifenprodil (3 μ M). The lines represent the averaged data normalized to 100% at the beginning of the ifenprodil application and smoothed by a process of running averages (Sigmaplot 11; 2-D smoothing; running average; sampling proportion =0.3).

Individual DR and VLF EPSPs displayed on the same scale. Note that the reduction in the responses to both inputs began at about the same time, that the decline for both inputs occurred in parallel both reaching a steady state, and that the DR response declined more than the VLF response.

Figure 4. Action of MK-801 on the ifenprodil-insensitive component of DR and VLF EPSP

Illustration of the procedures carried out on an individual motoneuron from a P2 rat. Data from a single motoneuron obtained over a 4 hour and 30 min recording period. V_m was about -70 mV throughout. Stimulation rate 1/40s. *Top Row:* After blockade of all non NMDA receptors with the CNQX cocktail administered to the bath (not shown), the NMDAR-mediated response was then blocked with ifenprodil. *Bottom Row:* After maximal blockade with ifenprodil was achieved, the ifenprodil-insensitive response that remained was then blocked with MK-801, stimulating only DR (60 trials at 0.025 Hz). Trial 1 was obtained immediately after introducing MK-801 ($10 \mu\text{M}$) into the bath (ifenprodil application was discontinued) and the decline of the late response took place progressively as stimulation was continued. Stimulation was stopped when steady state was reached. VLF was then stimulated ($n=20$) until its response declined to steady state. Single traces (inset) display the initial response (black) and the last response (red) in MK-801.

Figure 5. Comparison of decline of DR and VLF NMDA receptor- mediated EPSPs in ifenprodil and MK- 801

Percent residual (see text for definition) of DR (white) and VLF (grey) responses after maximal inhibition in ifenprodil (clear) and subsequent treatment with MK-801 (diagonals) in P1–P4 neonatal rats. Note that the DR monosynaptic response that remains after ifenprodil treatment does not change in MK-801 indicating a similar level of blockade. A greater percentage of the VLF response remains after ifenprodil treatment and is blocked to a greater extent by subsequent treatment with MK-801.