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NMDA receptors and Synaptic Plasticity in Lumbar Motoneurons of Neonatal Rats

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By

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Abstract of the Dissertation

NMDA receptors and Synaptic Plasticity in Lumbar Motoneurons of Neonatal Rats

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Motoneurons in the lumbar spinal cord are responsible for enabling skeletal muscles to carry out precise limb and trunk movements. Input to motoneurons comes from both segmental and descending sources. The developing spinal cord is highly plastic presumably to allow coordinated development of these two inputs to motoneurons and to local interneurons. The major excitatory transmitter in the spinal cord is glutamate acting through two major ionotropic receptors, AMPA and NMDA. Synaptic plasticity and receptor trafficking have generally been associated with AMPA receptors, primarily in the context of learning and memory.

The present experiments demonstrate that the NMDA receptors activated monosynaptically in motoneurons by segmental dorsal root (DR) and descending ventrolateral funiculus (VLF) inputs are independent (i.e., no overlap), that the NMDA receptors associated with the DR input are more susceptible to replacement from extrasynaptic sources (i.e., trafficking) than those associated with the VLF input, that the

subunit composition of NMDA receptors associated with these two inputs are dissimilar, and that the subunit composition of NMDA receptors associated with polysynaptic projections from segmental afferents to motoneurons are different from the monosynaptic projections. It is concluded from pharmacological evidence that maturation of connections to motoneurons in the first and second postnatal week is associated with replacement of synaptic diheteromeric NR1/NR2B receptors with more stable diheteromeric NR1/NR2A or triheteromeric NR1/NR2B/NRXX. This is consistent with reports in other regions of the mammalian brain. In addition, receptor modification occurs earlier at VLF synapses than at DR synapses in the same motoneuron. This novel finding of staggered development of NMDA receptors on the same motoneuron from different synaptic inputs is discussed in the context of its developmental and functional implications.

NMDA receptors have multiple functional effects in the central nervous system (CNS). It is therefore difficult to target them with specificity and exploit them as drug or treatment targets without producing a global effect. Understanding the subtle differences in NMDA receptor expression and physiological properties may enable the design of treatment options that are more specific and have a higher efficacy in treating CNS diseases.

Dedication

To my parents Santha Nelson and Samuel Nelson; for being the epitome of unconditional love and unwavering support

Table of Contents

List of Figures

List of Tables

List of Abbreviations

ACSF	Artificial cerebrospinal fluid
AMPA	Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate
APV	2-amino-5-phosphovaleric acid
BDNF	Brain derived nerve growth factor
CAMKII	Ca2+/calmodulin-dependent protein kinase II
CGP 46381	Ciba Geigy Product 46381
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CNS	Central Nervous system
DR	Dorsal root
EPSC	Excitatory postsynaptic current
EPSP	Excitatory Postsynaptic potential
ER	Endoplasmic reticulum
GABA	γ-amino butyric acid EPSP- excitatory postsynaptic potential
LTD	Long term depression
LTP	Long term potentiation
MAGUK	Membrane Associated Guanylate Kinase
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
PDZ	The PDZ domain is a common structural domain of 80-90 amino-acids found in the signaling proteins of animals used for anchoring transmembrane proteins to the cytoskeleton and hold together signaling complexes. *PDZ* is an acronym combining the first letters of three proteins — post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (DlgA), and zonula occludens-1 protein (zo-1) — which were first discovered to share the domain.
PSD	Post synaptic density
SAP	Synapse-associated protein
Sch	Schaffer Collateral
VLF	Ventrolateral funiculus

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I thank every member of my dissertation committee, especially for their ability to offer excellent scientific criticisms in a way that truly encourages intellectual curiosity. I thank the chair Dr. Bill Collins, for always being involved in my research and for taking a genuine interest in helping me bring this work to completion; Dr. Craig Evinger for constructive criticisms during and outside committee meetings and Dr. Bob Kalb for taking an active interest in the progress made.

I thank the past and current members of my lab; especially Dr. Jeff Petruska for his genuinely infectious enthusiasm for science; his passion, dedication, and counsel motivated me on many an occasion. I thank Dr. Victor Arvanian who taught me the intracellular recording technique for his scientific acumen. I thank our past technician Alyssa Tuthill for her friendship and support. I thank the current members of my lab Dr. Vanessa Boyce for her timely pep talks and encouragement and Dr. Li Liang and Richard Davila for their friendship.

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And above all, I “give thanks to the Lord for He is good, His mercies endure forever”.

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Chapter I
Introduction

Movement is one of the primary tasks orchestrated by the nervous system. The motor system generates movement that can be classified as reflexive, rhythmic or voluntary. The motor circuits that bring about these movements are organized in the spinal cord, brain stem and the forebrain. The cerebellum and basal ganglia provide feedback that is necessary for the coordination and accuracy of these movements and also for maintaining posture. The spinal cord is primarily responsible for mediating reflexive (eg. stretch reflex) and rhythmic movements (eg. Locomotion).

The rat lumbar spinal cord

The ten laminae of spinal cord grey matter (Rexed, 1952) are occupied by primarily five types of neurons: local interneurons, propriospinal neurons whose axons reach distant spinal segments, projection neurons that ascend to the brain centers, autonomic preganglionic neurons, and the motoneurons whose axons exit the nervous system to innervate skeletal muscles and produce movement. The simplest neural circuit is the monosynaptic reflex, where a single primary sensory axon from the muscle spindle groups (I_a afferents) makes a direct connection with a motoneuron. But most motor reflexes depend on circuitry that is polysynaptic, where the primary axons make a connection with spinal interneurons which in turn synapse onto the motoneuron (Figure 1). Motoneurons make synapses with the motor end plate and also with interneurons that inhibit motoneurons of the antagonistic muscle, thereby rendering precision in movement.

The segmental Ia afferent input (DR) and the descending fibres of the ventrolateral funiculus (VLF) form the most prominent monosynaptic input to the lumbar

motoneuron. Brief stimulation of the VLF produces rhythmic locomotion-like activity (Antonino-Green *et al.*, 2002) . The ventrolateral funiculus therefore carries fibres that are locomotor-related and is mainly composed of the descending fibres of the reticulospinal, anterior corticospinal, vestibulospinal and tectospinal tracts. It also carries the ascending locomotor-related tracts like the spinoreticular and spinoreticulotectal pathway that originate bilaterally in lumbar lamina VII and terminate in the ipsilateral medulla (Antonino-Green *et al.*, 2002).

All motor commands (including those descending from the higher order command centers and those from the segmental sensory input) converge onto motoneurons which together with the premotor interneurons, act as central integrator with the end purpose of bringing about appropriate movement of the target muscle. The spinal motoneurons are the "final common pathway" for motor behaviors (Sherrington, 1906). Motoneurons have therefore been studied extensively due to the central role they play in locomotion.

Background and motivation for this study:

Interest in the study of NMDA receptors in motoneurons was stimulated by their role in mediating the neurotrophin-induced potentiation of the AMPA receptor-mediated response. Neurotrophins have been implicated as survival factors for developing afferents (Goedert *et al.*, 1984; Ruit *et al.*, 1992; Oakley *et al.*, 1995; Wright *et al.*, 1997). In the neonatal CNS, they promote both growth and survival of motoneurons and their inputs (Chen *et al.*, 2002) during the early stages of development and strengthen synaptic connections to them (Seebach *et al.*, 1999; Arvanian *et al.*, 2003) . The presence of their receptors, p75 and trk, in adult animals has further enhanced interest in their role in the

adult CNS. Neurotrophins, particularly NT-3 and BDNF, have functional effects in the postnatal spinal cord (Seebach *et al.*, 1999).

When studying the effect of neurotrophins on DR and VLF synapses of neonatal lumbar motor neurons, it was found that brief acute superfusion of the *in vitro* neonatal spinal cord with NT-3 evokes LTP- like facilitation of the AMPA/kainate receptor mediated excitatory post synaptic potential (EPSP) in motoneurons. There is acute/immediate action after exposure to NT-3 and the facilitation is long-lasting, lasting over 4 hours after NT-3 wash out (Arvanov *et al.*, 2000). The facilitation has the following properties:

- In the first postnatal week it is synapse selective: the facilitation was seen in responses evoked by stimulating sensory DR afferents but not the VLF.
- Activation of NMDA channels is required to initiate facilitation of the AMPAR-mediated response but not to maintain it. Acute NT-3 administration induces facilitation of NMDA-mediated synaptic response by directly potentiating the response of postsynaptic NMDA receptors to glutamate.
- The facilitation is age-dependent. In prenatal animals facilitation occurs for responses to both inputs. In the first postnatal week, only the DR responses show facilitation and in the second post natal week responses from neither input can be facilitated. The removal of Mg^{2+} block of NMDA receptors extends the facilitation by NT-3 past the first post natal week (Arvanian & Mendell, 2001b). Gene chip analysis showed that NMDAR NR2D subunits, which confer resistance to Mg^{2+} block of NMDA channels, were down regulated in the spinal cord after the first post natal week. When NR2D was delivered to the rat cord by viral

vectors, the Mg^{2+} block was reduced and NT-3 induced potentiation of AMPA/kainate responses was restored in maturing motoneurons both at the DR and VLF synapses and past the first postnatal week (Arvanian *et al.*, 2004).

These results point to three major themes:

1. There are synapse specific differences between DR and VLF synapses on the same motoneuron; NT-3 could facilitate responses at the former and not the latter.
2. NMDA receptors at these synapses play a crucial role in allowing the NT-3 action at these synapses and prolonging their functional availability at these synapses extends the window for these effects.
3. The synapses under the DR and VLF inputs follow different time-lines of development. NMDA-mediated responses associated with the DR synapses can be potentiated by NT-3 in the embryonic and first postnatal week. Those associated with VLF synapses are potentiated only in the prenatal cord and by the first postnatal week that ability is already lost. The fact that responses associated with neither input can be potentiated in the second postnatal week shows that the DR synapses also lose their sensitivity to neurotrophins, just at a later time-point. This goes to show that there is a progressive loss of NT-3 induced potentiation as the synapses "mature" and that the VLF synapses reach the "mature stage" earlier. Since altering NMDAR availability seems to reverse age-related loss of action, it was hypothesized that the underlying changes in NMDAR composition and their availability at these synapses might contribute to the observed variability in timing of their maturation.

Given the extensive role that NMDA channels play in neurotrophin action on the spinal cord, it is imperative to understand the diversity, distribution and the distinct roles that their various subunits play in motoneurons of both neonates and adults. Since the observed differences between DR and VLF synapses seem to be associated with NMDA receptors, we wanted to characterize their availability and subunit composition at synapses made by these two major inputs to the motoneuron.

NMDA receptor and its properties:

The NMDA receptor is a ligand-gated ionotropic receptor. The ion channel is subject to a voltage-dependent Mg^{2+} block. NMDA receptor channel opening requires ligand binding (by presynaptic gluatamate release) and removal of Mg^{2+} block (by post synaptic depolarization), thus conferring on the NMDA receptor the ability to function as a molecular coincidence detector (Mayer *et al.*, 1984; Lau & Zukin, 2007). NMDA receptors allow Ca^{2+} influx which results in a cascade of intracellular events that can eventually lead to long term potentiation(LTP) or long term depression(LTD) of the synaptic response. The high permeability to calcium ions confers on NMDARs a central role in both synaptic plasticity under physiological conditions and neuronal death under pathological conditions (excitotoxicity) (Paoletti & Neyton, 2007). The initial activation of NMDA receptors requires 2 molecules of glutamate and 2 molecules of the coagonist glycine. The EPSC that results from NMDAR activation has an exceptionally slow rise time (approximately 10 ms) and decay time (greater than 100 ms) (Mori & Mishina, 1995).

NMDA receptor subunit composition:

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 occur as diheteromers containing two NR1 and two NR2 receptors (Figure 2). However, there is experimental evidence for triheteromers composed of two NR1 and two different NR2 subunits (Chazot & Stephenson, 1997; Tovar & Westbrook, 1999; Chazot *et al.*, 2002; Pina-Crespo & Gibb, 2002; Brickley *et al.*, 2003; Hatton & Paoletti, 2005; Jones & Gibb, 2005; Brothwell *et al.*, 2008). The NR1/NR2 complex forms before leaving the endoplasmic reticulum (ER). The NR1 subunit plays a major role in releasing the complex from the ER. The RXR motif is the ER retention motif in the C-terminal region of NR1. The binding of PDZ proteins in the neighboring PDZ-binding domain on the NR1 masks the RXR motif and facilitates egress of the NR1 subunit from the ER (Wenthold *et al.*, 2003). NR2 subunits are unable to reach the cell surface unless coassembled with the NR1 subunit.

Specific interaction of the NR1 and NR2 subunits of the NMDA channel with post synaptic proteins is required for synaptic localization, binding and stabilizing the NMDA receptor in the membrane and for trafficking of receptors (Wenthold *et al.*, 2003). The interaction is mainly between the C-terminal tail of NR2 and the PDZ domains of intracellular scaffolding proteins like the Membrane-Associated Guanylate Kinases (MAGUKs) (Sans *et al.*, 2003). The well known MAGUK proteins are PSD 95, PSD 93, SAP 97, SAP-102 and chapsyn-110 (Kornau *et al.*, 1995).

NR2 subunits play a major role in establishing the pharmacological (Yamakura & Shimoji, 1999; Paoletti & Neyton, 2007) and physiological (Cull-Candy *et al.*, 2001;

Cull-Candy & Leszkiewicz, 2004) profile of NMDARs. NR2A subunit containing NMDARs produce currents with the fastest decay time, deactivation kinetics, high conductance and high sensitivity to Mg^{2+} block. NR2D subunits containing NMDARs produce currents with exceptionally slow decay times, rapid deactivation kinetics (but slower than NR2A), low conductance and low sensitivity to Mg^{2+}. Both NR2B and NR2C produce currents with moderate decay times, and display rapid deactivation kinetics (slower than seen in NR2A containing channels) although NR2B containing receptors are similar to NR2A in their high conductance and high sensitivity to Mg^{2+} block (Cull-Candy & Leszkiewicz, 2004). NR3A is believed to act as a regulatory subunit during early development where it is involved in surface expression of the receptor, and when expressed it reduces the Ca2+ permeability of the channel (Perez-Otano *et al.*, 2001). Modulating the subunit composition of NMDA receptors therefore alters their current dynamics and thereby produces the required properties of synaptic transmission.

Development of NMDA-mediated responses in the rat spinal cord

The DR afferents first enter the spinal grey matter around embryonic day 15. At embryonic day 16, the dorsal root afferents begin to terminate close to the motoneuron dendritic trees, and the excitatory postsynaptic potentials (EPSPs) that are recorded from the motoneuron by stimulating the DR afferents are mediated solely by NMDA receptors (Ziskind-Conhaim, 1990). Around embryonic day 17, immature synapses with a few synaptic vesicles are present in the ventral horn and AMPA/kainate channels begin to contribute to the short-latency monosynaptic component of the EPSP. After birth and as

the animals increase in age, there is increased contribution of AMPA, and kainate to the short-latency monosynaptic component of the EPSP produced in motoneurons. The long-latency polysynaptic component of the EPSPs is mediated exclusively by NMDA receptors (Konnerth *et al.*, 1990; Ziskind-Conhaim, 1990).

Developmental and Regional Expression of NMDA receptors in the rat lumbar cord

In the rat brain developmental changes in expression of NMDA subunits have been well characterized. NR2B and NR2D occur prenatally, NR2A and NR2C are first detected around birth. All transcripts except NR2D peak around P20 and NR2D peaks around P7 and thereafter decrease to adult levels (Monyer *et al.*, 1994). While the developmental and regional expression of NMDA receptors in the brain and brain stem of the CNS have been studied extensively, their expression in the spinal cord is not as well characterized. Studies with *in situ* hybridization have revealed that NR1 subunit mRNA is expressed throughout the spinal grey matter both in the neonate and adult (Furuyama *et al.*, 1993; Tolle *et al.*, 1993; Luque *et al.*, 1994; Shibata *et al.*, 1999; Nagy *et al.*, 2004). However, the expression of the NR2 subunits is a subject of much controversy. Tolle et al., (1993) detected mRNA for NR2C and NR2D subunits in lumbar spinal cord but not for NR2A and NR2B. Stegenga & Kalb, (2001) could only detect mRNA of NR2A at P2 in the ventral horn, but by P22 it was limited to lamina II and was significantly reduced. They detected very modest levels of NR2B and NR2C in the ventral half of spinal cord at P2, but by P10 they were no longer present. Very modest amounts of NR2 subunits were detected in the adult. Shibata et al., (1999) however, found that in the ventral horn of

young adult rats (age not mentioned, animal weight corresponds to after P20), there is cooexpression of NR2A and NR2B in addition to the high levels of NR1 subunit mRNA.

Another puzzling feature in the *in situ* hybridization data was that there was a large mismatch between the location of the expression of NR1 and that of the members of NR2 family. While an abundant amount of NR1 mRNA was detected throughout the spinal grey matter, very small levels of NR2 subunits were detected. Low levels of NR2C were limited to the substantial gelatinosa and lamina X, only very low levels of NR2D were seen in motoneurons (Tolle *et al.*, 1993), NR2A was concentrated in laminae III-IV and NR2B in laminae I-II, and virtually no NR2A or 2B were present at the ventral horn or more specifically in motoneurons (Nagy *et al.*, 2004). Fully functional NMDA receptors are produced only when the NR1 subunits coassemble with NR2 subunits producing a NMDA receptor with a heteromeric configuration. Functional expression studies have demonstrated that homomeric NR1 or homomeric NR2 channels expressed in oocytes or cell cultures exhibit low or no channel activity (Seeburg, 1993; Hollmann & Heinemann, 1994; Nakanishi & Masu, 1994; Mori & Mishina, 1995). Thus it is perplexing that *in situ* hybridization and receptor autoradiography techniques are unable to establish enough colocalization of NR1 and NR2 subunits to form functional NMDA receptors, and yet, as discussed previously, NMDA receptor- mediated EPSPs are observed in motoneurons throughout the first and second postnatal week.

If NR2D or NR2C mRNA subunits are the only subunits present in the ventral horn motoneurons, as suggested by the *in situ* hybridization data, we would expect that only low-conductance NMDA receptor channels to be detected in the spinal cord motoneurons. Palecek et al., (1999) in their experiments with outside-out patches from

motoneurons isolated from lumbar cord of rats between P4-P14 found at least two types of NMDA receptors, one with a low conductance channel opening and another with high conductance. The low conductance receptor corresponds to those channels produced by recombinant expression of NR1/NR2C or NR1/NR2D subunits and is in agreement with the *in situ* data. The high conductance opening of NMDA receptor was not developmentally regulated and did not gradually disappear between P4-P14 even though the subunits associated with high conductance (NR2A and NR2B) were not detected in the *in situ* experiments after P10. Furthermore, the high conductance NMDA channels were found to exhibit considerably higher conductance than diheteromeric NR1/NR2B channels even though they were powerfully inhibited by antagonists against the NR1/NR2B subunit. They interpreted their results as indicating the presence of triheteromeric NMDA receptors in these motoneurons, in which the total conductance of the channel remained unchanged with development between P4-P14. RT-PCR of the cell content of the motoneurons showed that PCR products of NR1 and all the four NR2(A-D) subunits and NR3A subunits were present in rats between P5-P9 (Abdrachmanova *et al.*, 2000). However, a systematic analysis of the motoneuron content encoding the different NMDAR subunits at each postnatal age was not done and therefore the specific NMDA receptor subunit composition in motoneurons at each age was not determined. Much work still remains to be done in characterizing the availability and expression of NMDARs in neonatal motoneurons.

Why is the study of NMDA receptors an important part of study of plasticity?

Activity-dependent changes in synaptic function (as seen in LTP or LTD, learning and memory) are usually associated with AMPA receptor trafficking (Shi *et al.*, 2001; Malenka & Bear, 2004; Karmarkar & Dan, 2006; McCormack *et al.*, 2006; Derkach *et al.*, 2007; Hall & Ghosh, 2008). In the adult CNS NMDA receptors at resting potentials are only minimally opened due to their magnesium block and synaptic response is usually not observed at synapses containing NMDA receptors alone. These synapses are now commonly known as "silent synapses" and they can be converted to active synapses by functional recruitment of AMPA receptors to these synapses (Isaac *et al.*, 1995; Liao *et al.*, 1995; Durand *et al.*, 1996). Prolonged activity can influence the number of AMPARs at individual synapses and their surface expression (Lissin *et al.*, 1998; O'Brien *et al.*, 1998; Turrigiano *et al.*, 1998; Liao *et al.*, 1999). Rapid redistribution of AMPARs is mandatory for synaptic plasticity and has been shown to occur (Lissin *et al.*, 1999). AMPA receptor trafficking is therefore considered the hallmark of synaptic plasticity and is studied extensively.

Role of NMDA receptors in modulating AMPA receptor numbers at synapses

NMDARs are implicated in AMPAR endocytosis since their activation increases intracellular calcium levels leading to the calcium-dependent protein phosphatase cascade that triggers AMPAR endocytosis (Lisman, 1989; Mulkey *et al.*, 1993; Mulkey *et al.*, 1994; Beattie *et al.*, 2000; Ehlers, 2000), a phenomenon that occurs in LTD. Phosphorylation of NMDARs may be a major mechanism for establishing LTP as well. Association of CAMKII with NMDARs occurs following autophosphorylation of CAMKII due to calcium entry from NMDAR activation. It is believed that this

association between CAMKII and NMDARs brings the CAMKII in close enough proximity to AMPA receptors to cause their phosphorylation, leading to an increase in their single-channel conductance, an increase in synaptic insertion of more AMPA receptors and eventual potentiation at the synapse (Benke *et al.*, 1998). Small amounts of NMDAR- mediated calcium influx produces LTD whereas strong activation of NMDAR leads to LTP (Lisman, 1989; Cummings *et al.*, 1996).

NMDA receptors are found in both synaptic and extrasynaptic sites but are clustered at higher densities at the synapses due to their interaction with proteins of the post synaptic density (PSD) (PSD-95 family of MAGUKs like PSD-95, SPD-93, SAP 97, SAP 102) localized along the postsynaptic membrane of excitatory synapses. Stargazin, a MAGUK mediating synaptic targeting of AMPARs, could interact with NMDA MAGUKs and result in linking of NMDA and AMPA receptors at the synapse (Chen *et al.*, 2000; Lisman *et al.*, 2002).

NMDA receptor trafficking

Until recently, NMDA receptors were considered to be stable at the synaptic plasma membrane and synaptic plasticity was not associated with the movement of NMDA receptors. Recent work has revealed evidence for rapid NMDA receptor trafficking. Rapid movement of NMDA receptors from extrasynaptic to synaptic sites has been described at hippocampal autapses (Tovar & Westbrook, 2002) and in motoneurons (Shanthanelson et al., 2009). Single-particle and molecule tracking have shown that NMDARs exhibit lateral mobility with basal diffusion rates comparable to those of AMPARs (Groc *et al.*, 2004; Groc *et al.*, 2006). Recent work has also shown that NMDA receptors undergo rapid insertion into and removal from postsynaptic membranes

analogous to the AMPAR system (Roche *et al.*, 2001; Snyder *et al.*, 2001; Li *et al.*, 2002; Nong *et al.*, 2003; Lavezzari *et al.*, 2004; Scott *et al.*, 2004; Washbourne *et al.*, 2004). This local exocytic-endocytic trafficking is likely to be shifted by activity.

An important question is whether NMDARs are limited to their role of being molecular triggers of synaptic changes (coincidence detectors that encourage AMPA receptor trafficking and insertion into synapses to produce LTP) or whether changes in the synaptic number and composition of NMDA receptors themselves leading to LTP_{NMDA} and LTD_{NMDA} occurs. This is an important question because it involves potentiation or depression of the molecular trigger itself (Lau & Zukin, 2007). Only a handful of work addresses this question. The contribution of NMDAR trafficking to LTP has been demonstrated at Schaffer collateral (Sch)-CA1 synapses of adult hippocampus. At these synapses NMDAR mediated calcium influx triggers LTP_{NMDA} by a mechanism involving activation of PKC and tyrosine kinase Src and is followed by rapid synaptic insertion of NR2A-containing NMDARs (Grosshans *et al.*, 2002). Other examples of LTP_{NMDA} have been demonstrated in the visual cortex (Watt *et al.*, 2004), medial perforant path-dentate granule cell synapse (O'Connor *et al.*, 1994), and Sch- CA1 synapses (Smith & McMahon, 2005). LTD_{NMDA} mediated responses has been described in the Sch-CA1 synapses (Carroll *et al.*, 2001) and occurs by lateral diffusion of NMDA receptors from synaptic to extrasynaptic sites (Morishita *et al.*, 2005). So even though the contribution of LTP_{NMDA} and LTD_{NMDA} is modest in comparison to AMPA LTP and LTD, it does occur, and its significance is gradually being explored.

While Hebbian forms of plasticity are necessary for wiring the brain during development and for encoding information in an activity-dependent manner, they tend to

destabilize neuronal networks over time, if left unchecked (Turrigiano & Nelson, 2004). Non-Hebbian plasticity, like homeostatic plasticity, might provide the global negative feedback necessary to maintain synaptic strength and plasticity within a functional range, by scaling the strength of all synaptic inputs up or down while preserving their relative weights (synaptic scaling) or by increasing the threshold that needs to be met for subsequent Hebbian plasticity to occur (metaplasticity)(Abraham & Tate, 1997; Turrigiano *et al.*, 1998) (for review see (Perez-Otano & Ehlers, 2005).

Synaptic scaling is achieved by altering the number of receptors at the synapse and was originally said to be mediated by AMPA receptors. It is now known that NMDAR currents can be co regulated with AMPA currents; Hebbian plasticity that increases the AMPA receptor component of the excitatory synaptic transmission is followed by a delayed potentiation of NMDA current (Watt *et al.*, 2004).

Metaplasticity is the plasticity in which prior activity shifts the *threshold* for subsequent Hebbian plasticity, without affecting synaptic efficacy (Perez-Otano & Ehlers, 2005). For example during development of the visual cortex, high levels of coordinated activity such as eye opening or light exposure shifts the modification threshold; light frequencies that previously elicited LTP no longer result in potentiation of the response and result in LTD instead (Kirkwood *et al.*, 1995; Kirkwood *et al.*, 1996). Calcium entry through NMDARs is responsible for establishing metaplasticity (Shouval *et al.*, 2002; Philpot *et al.*, 2003). Shifting the threshold required for LTP can be achieved by modifying NMDAR conductance and calcium influx by altering their subunit composition. NR2B subunit containing receptors have slow deactivation kinetics which promotes temporal summation of calcium currents. In the brain, after activity and as the

synapses mature, NR2A subunits replace NR2B (Kew *et al.*, 1998; Cathala *et al.*, 2000; Lopez de Armentia & Sah, 2003; Liu *et al.*, 2004b) . Replacing NR2B with NR2A results in NMDA receptors with faster deactivation kinetics and shortens the duration of its currents (Cull-Candy & Leszkiewicz, 2004). Higher stimulation frequencies are now required to produce the same amount of calcium signal as before and the threshold for LTP is raised (Flint *et al.*, 1997). There are suggestions that NR1/NR2A mediate LTP whereas NR1/NR2B mediates LTD (Liu *et al.*, 2004a; Massey *et al.*, 2004). In the context of synaptic plasticity, the contribution of NMDA receptor trafficking and changes in their postsynaptic insertion to Hebbian and homeostatic plasticity can no longer be ignored.

NMDA receptors and spinal plasticity

The field of NMDAR mediated synaptic plasticity is relatively new and has been studied only in the past decade. As discussed previously, major strides have been made in understanding the role of NMDAR trafficking and plasticity in the adult brain, but very little is known about NMDA receptor's contribution to plasticity in the spinal cord. There are several types of spinal cord plasticity. For the purposes of this introduction, they are divided into activity- dependent plasticity, long-term effects of neurotrophins, and processes triggered by spinal cord injury.

Activity-dependent plasticity

NMDA receptor activity promotes activity-dependent reorganization of the dendritic arbors in motoneurons (Kalb, 1994; Inglis *et al.*, 1998). Overexpression of NR3B subunit increases the length and branching of the dendrites (Prithviraj & Inglis,

2008). In the prenatal period and first two postnatal weeks, NMDA receptors have an important role in proper motoneuron development and establishment of appropriate motor behavior.

Activity-dependent plasticity is not limited to the neonatal period but occurs in the spinal cord throughout life. Driven by input from the periphery and the brain, it has a central role in acquisition and maintenance of motor skills (Wolpaw & Tennissen, 2001). The most common example of simple activity-dependent plasticity produced by activation of the sensory input is seen in dorsal horn neurons. Dorsal horn neurons in nociceptive pathways receiving input from C-fibres exhibit action potential wind-up, a short-term plasticity seen in the adult spinal cord, consisting of a progressive increase in neuronal response during repetitive stimulation of the inputs (Mendell, 1966). Windup requires the activation of L-type calcium channels that establish intrinsic plateau potentials. NMDA receptors provide the critical excitatory component required for the activation of the calcium channel by causing the summation of spinal neurons responses (since they have a very long channel open time) (Fossat *et al.*, 2007). NMDARs, more specifically those containing NR2B subunits, are required to elicit wind-up and the development of central sensitization of the nociceptive pathways (Kovacs *et al.*, 2004) and NR2B antagonism is a strong candidate treatment regime for alleviation of chronic pain (Qu *et al.*, 2009; Zhuo, 2009).

Another input to the spinal cord through which activity-dependent plasticity occurs is the descending input from the brain. Motor skills like walking, writing and specialized skills such as dancing are acquired through prolonged practice and probably maintained through-out life (in spite of peripheral and central changes associated with

growth and aging) by continuous rearrangement of the descending pathways (Wolpaw & Tennissen, 2001). The strength of a spinal reflex depends on physical activity and training (Meyer-Lohmann *et al.*, 1986; Nielsen *et al.*, 1993). Experiments with NMDAR knock-out animals have linked the proper expression of the NMDA receptor with normal acquisition of motor skills. NR2B knock-out results in a lethal phenotype. Loss of both NR2A and NR2C leads to motor discoordination and NR2D knockout mice have deficits in certain locomotor activities. (for review see (Sprengel & Single, 1999)).

Long-term effects of neurotrophins

A second major type of plasticity seen in the spinal cord is through the action of neurotrophins (NGF, BDNF, NT-3 and NT-4/5). Neurotrophins are the differentiation and survival factors for sensory afferents and motoneurons during development (Mendell *et al.*, 1995; Mendell, 1996), and in adult they encourage the growth of damaged axons and have an important implication for regeneration ((Schnell *et al.*, 1994; Xu *et al.*, 1995). As discussed previously, acute NT-3- induced potentiation of synaptic responses requires the activation of NMDARs and is lost after the first postnatal week. Viral delivery of NR2D subunits reduces Mg^{2+} block of NMDA receptors and restores NT-3-induced potentiation of AMPA-kainate responses in maturing rat motoneurons past their normal window of action (Arvanian *et al.*, 2004).

Plasticity after injury

The third major type of spinal plasticity is that which occurs after spinal cord injury. Given that a substiantial reduction in the number of NMDA receptors expressed in the spinal cord occurs past the third postnatal week of age, their role in synaptic plasticity in the adult after injury is debatable. However, recent data argue to the contrary.

Following a C2 hemisection in adult rats there is spontaneous functional recovery of the paralyzed hemidiaphragm (Alilain & Goshgarian, 2008). Western blot analysis of the phrenic nucleus motoneurons in the cervical cord, showed a concurrent upregulation in NR2A and AMPA GluR1 subunit proteins and a downregulation of AMPA GluR2 subunit proteins. The GluR2 subunit is the calcium gate of the AMPAR (Bassani *et al.*, 2009) and its downregulation promotes an increase in intracellular calcium. The calcium influx and the concurrent increase in NR2A expression (possibly increasing NMDA receptor expression at the synapses) could result in unsilencing of the normally silent synapses in these adult animals. This is proposed to have led to the plasticity observed and the associated functional recovery (Alilain & Goshgarian, 2008).

Treadmill training in humans and animals with complete or incomplete spinal cord injuries improves locomotion, produces greater speed, strength, coordination and endurance (for review (Wolpaw & Tennissen, 2001). The alleviation of symptoms with treatment options like treadmill step training are associated with the restoration of the electrophysiological parameters of the motoneurons in the transected spinal cord (Petruska *et al.*, 2007). Combined delivery of NT-3 and NMDA NR2D causes strengthening of synaptic transmission in two different spinal cord injury models (Arvanian *et al.*, 2006). NMDARs therefore play a role in the plasticity seen after spinal cord injury and should be a subject of more intense study in the context of functional synapses established by regenerating fibres.

Experimental plan and justification:

Since NMDA receptors have multiple functional effects in the central nervous system (CNS), it is quite difficult to target them with specificity and exploit them as drug or treatment targets without producing a global effect. However, there are subtle differences in their expression in terms of their location, subunit composition, time period during development when they are expressed, and in their physiological properties. Understanding these subtle differences may enable the design of treatment options that are more specific and have a higher efficacy.

Very little is known about the NMDA receptors that mediate excitatory transmission in the lumbar spinal cord. While we know that their activation is required for motor neuron growth and dendritic arborization during a critical time window in development (Kalb, 1994), and that they mediate the polysynaptic component of the synaptic EPSP exclusively (Ziskind-Conhaim, 1990), and are intimately involved with the synaptic plasticity that occurs at the synapses in the motoneurons (Wenthold *et al.*, 2003; Perez-Otano & Ehlers, 2005), much work still needs to be done to tease out the roles of their subunits and characterization of their synaptic location in the motoneuron. The purpose of this dissertation is two-fold: to first appreciate the complexity of NMDA subunit expression in the neonatal spinal cord and their differential expression at synapses formed by the two main monosynaptic inputs to the lumbar motor neuron. The second purpose is to understand the role of NMDA receptor subunits in the synaptic plasticity that is observed in neonates. The ultimate aim is to explore whether the principles of plasticity observed in the neonate can be extended to the adult, e.g. after spinal injury.

Chapter II will provide evidence for independence of synapses under the DR and VLF input and the presence of NMDAR trafficking at these synapses. Of particular interest is the assertion that these synapses mature at different rates and that one shows a greater affinity for trafficking NMDA receptors than the other, at the ages studied. It will address both Specific Aim 1 and 2. Chapter III will address the underlying differences in the subunit composition of NMDA receptors that comprise these synapses. The idea that this subunit differences is the most likely explanation for the observed differences between these synapses will be explored and this will address Specific Aim 3. Chapter II is published work and Chapter III has been submitted for review; they are presented here with slight modifications to their original form. They are cited appropriately in the reference list. Chapter IV will mention additional experiments that were done to explore further the topics that were raised in Chapter II and III. The objective of this chapter is to explain directions that were explored and point to future experiments that could be pursued. Chapter V contains the general discussion of all results in the context of their general implication and importance.

Specific Aims Outline

SA I. Independence of NMDA receptors activated by DR & VLF inputs

Experiment i) Blocking of receptors under one input using activity dependent irreversible NMDA blocker MK-801 does not block receptors under the other input

SA II. NMDA receptor trafficking- differences between DR & VLF synapses in recovery from MK-801 blockade

Experiment i) Recovery of NMDA EPSPs from irreversible MK-801 block when MK-801 is bath applied

Experiment ii) Lack of recovery with bath application of NMDA

Experiment iii) Viral delivery of NR2D (Herpes Simplex Virus- HSV NR2D)

SA III. Differences in NMDAR subunit populations between synapses formed by DR & VLF inputs

Experiment i) Difference in susceptibility to NR2B subunit specific blockers- Ifenprodil

Experiment ii) Difference in susceptibility to NR2C/2D subunit blocker- *cis* PPDA

Note about the experimental technique:

The experimental protocol is explained in detail in the following sections. As mentioned previously, lumbar motor neurons have two main monosynaptic inputs, one from the segmental Ia afferent input and the other through the descending fibres of the ventrolateral funiculus. The isolated neonatal cord preparation involves recording intracellularly from a L5 motoneuron while stimulating its DR and VLF inputs (Figure 3). There are two reasons that make this technique relevant to the study at hand. Its significance lies in:

1. The relative simplicity with which this system can be manipulated, both pharmacologically and electrophysiologically. Bath application of drugs allows immediate access to the motoneurons and their physiological output (EPSP) is recorded in real time.

2. The motoneuron and its synaptic inputs are both anatomically intact. Unlike cell cultures or autapses, the isolated neonatal cord has synapses that are probably very close to the same as those seen in an intact animal. Their stage of development can be reliably expected be at the stage of development of the rat. This provides more confidence in the reliability of the dynamic changes that are observed in NMDAR properties in these preparations.

Figure 1: Lumbar spinal cord

Shows the two main inputs to the ventral horn alpha motoneurons: segmental afferent input from the muscle spindles enter the spinal grey matter at the dorsal horn and synapse directly onto the motoneuron or onto to local interneurons. The descending motor control arrives from higher order brain command centres through the descending fibres of the Ventrolateral funiculus (in blue). The axons of the alpha motoneuron exit the ventral horn as a ventral root bundle and innervate the effector skeletal muscle at the motor endplates.

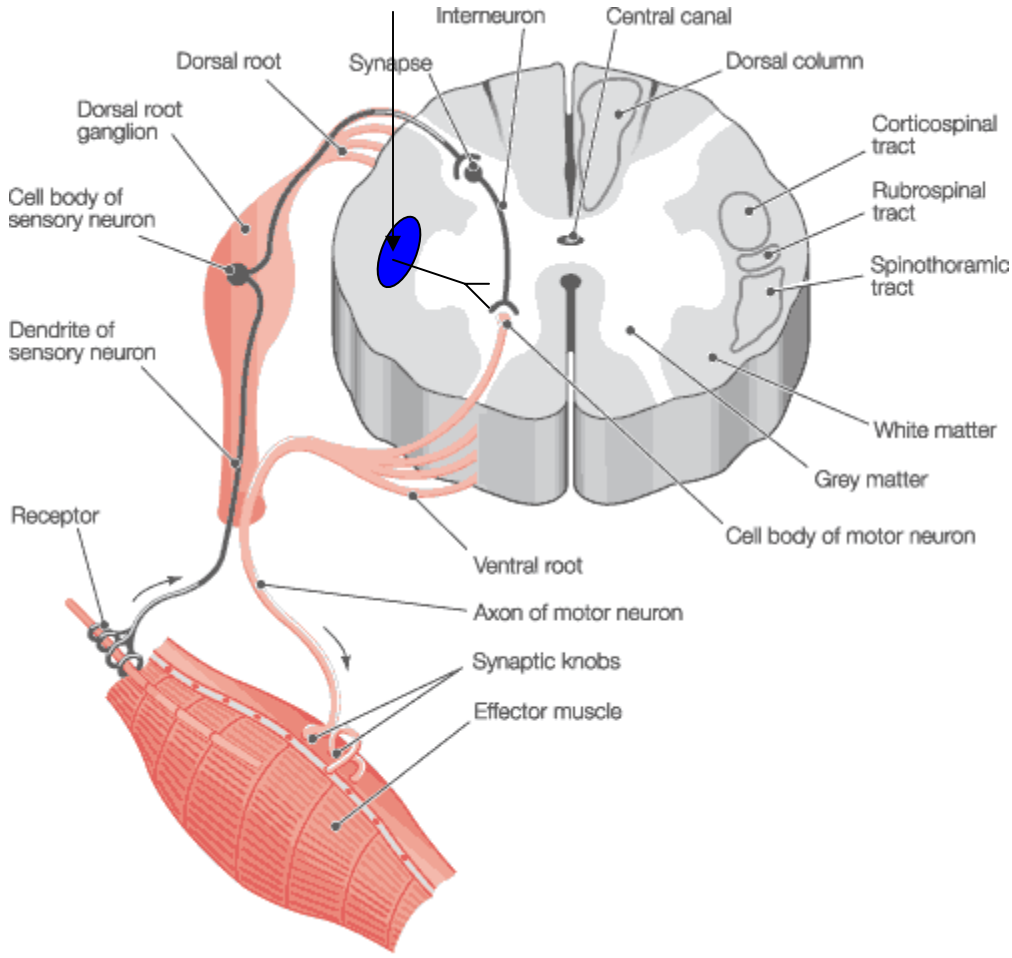
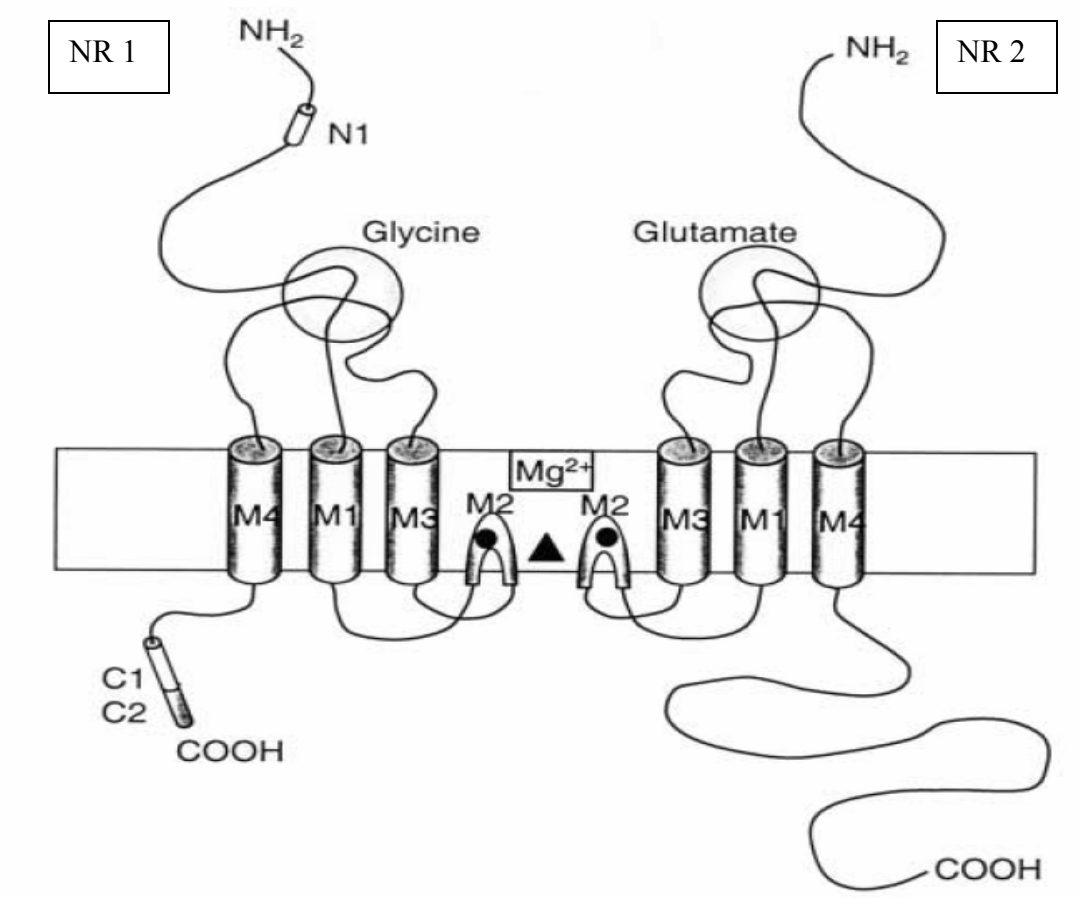


Image credit and ©: Dorling-Kindersley, The Internet Encyclopedia of Science- modified.

Figure 2: NMDA receptor

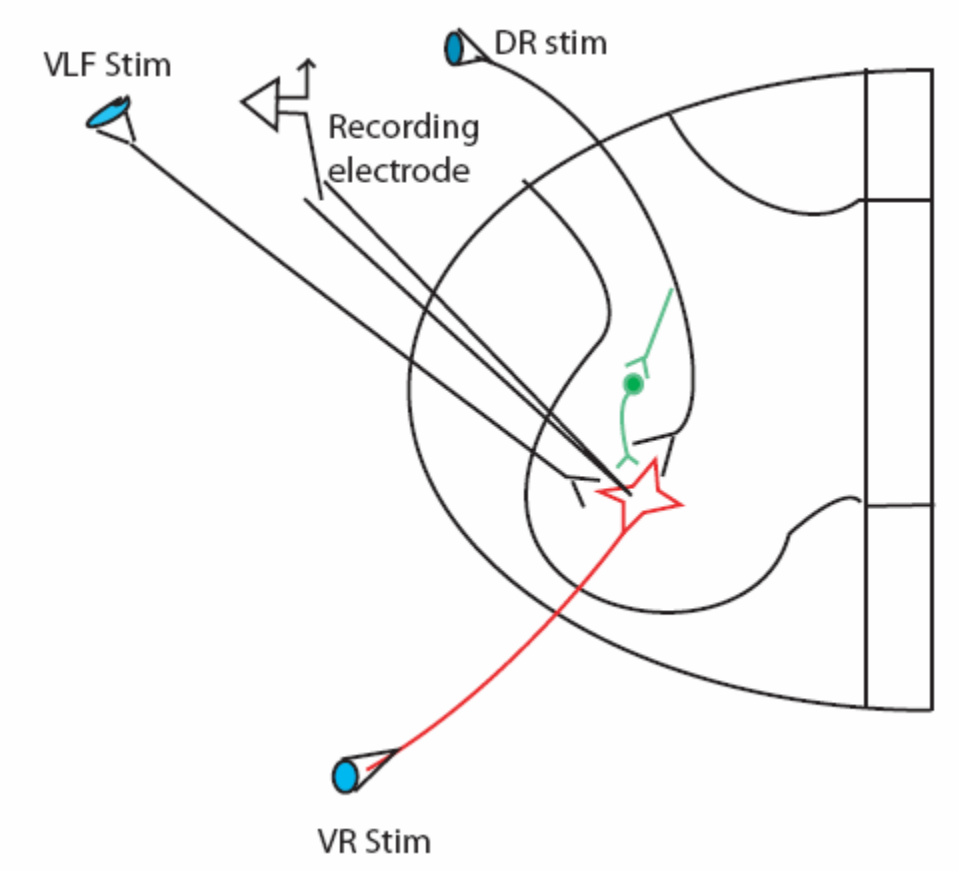
A schematic diagram showing 2 of the four subunits of the NMDA receptor tetramer. The N-terminal region is extracellular. M1, M2, M3, M4 are the transmembrane domains. M2 from all four subunits come together to form the re-entrant loop and channel pore. Glycine and Glutamate refer to the respective ligand binding domains. M2 contains the asparagine residue (represented as a dot) that is requisite for the Ca^{2+} permeability and Mg^{2+} blockade. The MK-801 binding site is represented as a triangle in the channel pore.



Adapted from (Stephenson, 2001)

Figure 3: Experimental set-up

Pictoral of a hemisected rat spinal cord showing dorsal and ventral root suction electrodes attached to the roots, VLF suction electrode attached to the VLF fibres that were dissected out at the T2 region and a glass sharp recording electrode. Depicted in red is a L5 motoneuron and in green is an interneuron. Note the connections can either be monosynaptic where the input makes a direct synapse with the motoneuron or polysynaptic (through the local interneurons).



Chapter II

Independence of NMDA receptors activated by DR & VLF inputs

NMDA receptor trafficking- differences between DR & VLF synapses in recovery from MK-801 blockade

Abstract

Lumbar motoneurons can be activated monosynaptically by two glutamatergic synaptic inputs: segmental dorsal root (DR) and descending ventrolateral funiculus (VLF). To determine if their N-methyl-D-aspartate (NMDA) receptors are independent, we used (5R,10S)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine-hydrogen-maleate (MK-801), known to induce a use-dependent irreversible block of NMDA receptors (NMDAR). In the presence of MK-801 (in bath) and non-NMDA antagonists (in bath, to isolate NMDA receptors pharmacologically) we first stimulated DR. After MK-801 blockade of DR synaptic input, the VLF was stimulated. Its response was found to be not significantly different than its control value suggesting that the DR stimulus activated very few if any receptors also activated by VLF stimulation. Similar findings were made if the stimulation order was reversed. Both inputs also elicited a polysynaptic NMDA receptor- mediated response. Evoking the DR polysynaptic response in the presence of MK-801 eliminated the corresponding VLF response; the reverse did not occur. Surprisingly, when MK-801 was washed from the bath, both DR and VLF responses could recover although the recovery of the DR monosynaptic and polysynaptic responses was reliably greater than those associated with VLF. Recovery was prevented if extrasynaptic receptors were activated by bath applied NMDA in the presence of MK-801 consistent with the possibility that recovery was due to movement of extrasynaptic receptors into parts of the membrane accessible to transmitter released by DR and VLF stimulation. These novel findings suggest that segmental glutamatergic inputs to motoneurons are more susceptible to plastic changes than those from CNS white matter inputs at this developmental stage.

Introduction

Developmental studies in the *in vitro* spinal cord conditions indicate that excitatory glutamatergic transmission to motoneurons undergoes substantial changes during the perinatal period (Arvanian *et al.*, 2004). In evaluating glutamatergic transmission, it is important to recognize that the initial response measured intracellularly from motoneurons consists of 2 components, one mediated by alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) /Kainate receptors and a slightly slower one mediated by NMDA receptors(Ziskind-Conhaim, 1990; Arvanian & Mendell, 2001b; a). A third component mediated by metabotropic glutamate receptors appears considerably later, and requires high intensity stimuli activating C- fibers (Arvanian *et al.*, 2005).

Application of pharmacological blockers of AMPA/kainate transmission and the inhibitory transmitters glycine and GABA isolates the NMDA receptor-mediated responses (Arvanian & Mendell, 2001a). This NMDA component declines in amplitude during the initial 2 postnatal weeks (Arvanian *et al.*, 2004) due to its increasing sensitivity to Mg^{2+} block (Arvanian & Mendell, 2001a) rather than loss of NMDA receptors themselves. During the first postnatal week the VLF NMDA receptor- mediated response resembles that of adults more than the DR response in the same motoneuron in being more sensitive to Mg^{2+} block (Arvanian & Mendell, 2001a).

The fact that DR and VLF NMDA receptor-mediated responses mature at different rates suggests that the receptors are under the control of their presynaptic inputs rather than solely determined by the motoneuron. This predicts very little cross talk between the monosynaptic responses to these 2 synaptic inputs as might occur, for example, from spillover of transmitter from one input to the receptors normally activated

by the other (Kullmann & Asztely, 1998). In order to confirm this, we employed the use-dependent, irreversible NMDA receptor blocker MK-801 (Foster & Wong, 1987; Huettner & Bean, 1988; Kloog *et al.*, 1988; Lipton, 2004) to block the responses to one of these inputs and determined whether it would diminish the initial response to the unstimulated input. If these were independent, stimulation of one input would have little effect on the response to the other (see also (Atasoy *et al.*, 2008)).

An additional question motivating these experiments was the reversibility of MK-801 blockade of NMDA receptors. MK-801- induced blockade of depolarization elicited by repetitive bath-applied NMDA cannot be reversed (Arvanov *et al.*, 2000). However, recent studies in dissociated hippocampal neurons have shown that blockade of NMDA synaptic responses can be reversed after MK-801 (Tovar & Westbrook, 2002; Zhao *et al.*, 2008). This recovery was attributed to movement of unstimulated functional NMDA receptors from the extrasynaptic regions into the synapse. Here, we observed a similar phenomenon in intact spinal tissue, but surprisingly DR responses displayed more recovery than those made by VLF on the same motoneuron. These findings suggest the possibility that NMDA receptors are mobile in neonatal motoneurons in the intact spinal cord, but more importantly that the sites associated with the different synaptic inputs may vary in their susceptibility to accumulate receptors from extrasynaptic sites.

Some of these studies have been presented in abstract form (Shanthanelson et al., 2005).

Materials and 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 neonatal rat spinal cords removed from Sprague Dawley rats (Taconic, Rensselaer, NY) aged P1-11 as previously described (Seebach *et al.*, 1999). 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 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, 30^0 C) at 10 ml/min. The VLF was dissected free of the spinal cord at T2 (Pinco & Lev-Tov, 1994). Suction stimulating electrodes were attached to peeled VLF axon bundles for activation of central inputs to motoneurons, to the cut L5 dorsal root for activation of segmental inputs to motoneurons, and to the L5 ventral root for identification of recorded cells as motoneurons by antidromic activation. Intracellular recordings (Axoclamp 2A amplifier, Molecular Devices, Inc. Sunnyvale, CA) were obtained using sharp microelectrodes (resistance 60-80 MΩ, filled with 3 M potassium acetate). Electrical stimulation of the DR and/or VLF (70 μs duration at a rate of 0.025 Hz) was at an intensity sufficient to evoke the just-maximum monosynaptic potential .

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 CGP 35348 (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 & Mendell, 2001a) for details).

Protocol: Only cells displaying a stable resting membrane potential greater than -60 mV were included in this study. Membrane potential was monitored throughout the recording and corrected by subtracting the value observed after withdrawing the electrode from the motoneuron. Initial AMPA/kainate responses were obtained from both synaptic inputs in the absence of any pharmacological blockers. The AMPA/kainate and inhibitory transmitter antagonist cocktail was then introduced into the bath, and at least 30 min later, control NMDA responses from DR and VLF were obtained at a stimulus intensity sufficient to evoke a maximum monosynaptic response. For both of these control response measures (AMPA/kainate and NMDA) 10 stimuli were delivered at the same intensity with a 40s interval to one input and then the stimulation was switched to the other, and then back, until a total of at least 40 stimuli each had been delivered to DR and VLF. The activity- dependent irreversible NMDAR blocker MK-801 (10 μM) was then added to the bath while continuing administration of the antagonist cocktail. In the presence of MK-801, one input was stimulated exclusively until maximum blockade of the EPSP was achieved, typically after 30- 40 min; with stimulation at 1/ 40s. The other input was then stimulated exclusively until its response was also blocked maximally, i.e., DR and VLF stimulation was not interdigitated as for control measurements (Fig 4a).

For experiments where reversibility of MK-801 was studied, the MK-801 was then washed from the bath (still containing the AMPA/kainate and inhibitory transmitter antagonist cocktail) for 45 minutes. The inputs were not stimulated during the 45 minute wash period. Following the wash, the NMDA receptor- mediated response was measured

for both inputs, again without alternation. 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 AMPA/kainate responses to verify viability of the cell and its synaptic inputs (Fig 5).

For offline analysis the responses to each stimulus were analyzed using Clampfit 8.2 (Molecular Devices, Inc. Sunnyvale, CA) either in single sweeps, in superimposed sweeps or after averaging. The monosynaptic component of the EPSP from successive stimuli overlaps consistently while the polysynaptic component of the EPSP varies. To obtain the peak of the monosynaptic component, the traces were superimposed and the peak was measured at the latest time where consistent overlap of traces was observed (Fig. 6).

In most experiments DR was stimulated before VLF for each test (Fig. 7). In several additional experiments the order was reversed and VLF was stimulated before DR (Fig. 8) (protocol in Fig 4b).

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. Means are reported with standard deviations.

Results

Receptors under the DR and VLF inputs are largely independent

In order to study whether NMDA receptors activated by DR and VLF are independent of each other, we used MK-801's property of blocking only those NMDA channels that have been activated by neurotransmitter (Foster & Wong, 1987; Huettner & Bean, 1988; Kloog *et al.*, 1988; Lipton, 2004). The first NMDA EPSP elicited after MK-801 administration is not reduced. However, during this initial activation of the NMDA receptor-channel complex, MK-801 inserts into the activated NMDA channel and blocks responses induced by subsequent synaptic activation of the same channel.

After achieving a suitable penetration of a motoneuron, we first recorded DR and VLF-evoked responses in normal ACSF (Fig. 7- bottom graphs). The stimuli to the 2 synaptic inputs were interdigitated, 10 consecutive stimuli to the DR followed by 10 consecutive stimuli to the VLF, with an interval of 40s between each stimulus. Consistent with our previous observations, DR and VLF responses, both largely AMPA receptor-mediated (Arvanian *et al.*, 2004), exhibit a short latency monosynaptic component (Arvanov *et al.*, 2000) of uniform amplitude followed by later highly variable polysynaptic components. We then added non-NMDA antagonists to the perfusing solution to block the AMPA/kainate-, GABA- and glycine-mediated responses and recorded the NMDA receptor-mediated control DR and VLF responses (Arvanian & Mendell, 2001a). Again, these NMDA receptor- mediated control responses were obtained by interdigitating the stimulation of both synaptic inputs. Under these conditions we observed a small monosynaptic response with latency 5-7 ms from the stimulus that was generally similar or slightly longer than that associated with the shortest latency

AMPA/kainate response; the rise time, particularly of the VLF response, was longer than the AMPA/kainate mediated response (Fig. 7, control). The amplitude of this presumed monosynaptic response increased as the stimulus intensity was raised. Maximum peak amplitude of the uniform monosynaptic NMDA receptor-mediated EPSPs measured 25-30 ms after the stimulus (Fig. 7, Control) was 1.9 ± 1.4 mV (n=21) and 1.8 ± 2.2 mV (n=25) for DR and VLF responses, respectively. Only cells exhibiting both DR and VLF evoked monosynaptic NMDA receptor- mediated responses were studied. Typical monosynaptic AMPA responses in these cells measured in control ACSF were considerably larger, averaging 5-7 mV (Arvanov *et al.*, 2000).

As reported previously (Arvanian & Mendell, 2001a), stimulation of DR and VLF in the presence CNQX and antagonists of glycine and GABA transmission elicited a second NMDA receptor- dependent depolarization at longer latency that often led to an all-or-none plateau potential with superimposed action potentials (Figs. 7 & 8). The DR-evoked long latency potential has a higher stimulus threshold than the monosynaptic component (Fig. 1A1 in (Arvanian & Mendell, 2001a)) suggesting that smaller afferent fibers are responsible for eliciting this response, probably via interneurons. The all-or-none behavior of the plateau potential resembles findings with directly applied NMDA in the neonatal spinal cord (MacLean *et al.*, 1997). The late DR- evoked plateau potential occurred reliably at the low frequency of stimulation used here. Although the initial portion of the late VLF response also began reliably soon after the peak of the initial response, the all- or- none plateau potential was initiated at highly irregular intervals due to the variability in the time required to reach its threshold voltage (Fig. 8, Control). We speculate that this may have been the result of a very slow, asynchronous volley arriving

from interneurons activated by VLF; this was not studied systematically. The initial component of the late response is not triggered by the monosynaptic EPSP because it often begins on the descending portion of the EPSP, well after the peak of the early response, (see Figs. 1 and 3 of (Arvanian & Mendell, 2001a)), and in a few cells studied here DR stimulation elicited a late response with no evidence of an early monosynaptic response. As stated above, such cells were not included in the results.

After a stable NMDA receptor- mediated response to DR and VLF was achieved, we introduced the use-dependent NMDA channel blocker MK-801 into the perfusing solution and stimulated DR (but not VLF) repetitively every 40s (Fig. 7, MK-801; see Fig. 8 and below for experiments where VLF was stimulated before DR in MK-801), until maximum blockade of the DR-response was achieved as determined by EPSP amplitude reaching a steady state value. This generally took about 25 minutes. The monosynaptic NMDA-mediated DR-response gradually decreased in amplitude with repeated stimulation and eventually declined to less than half initial value (average 38 $\pm$ 21%); only rarely was it completely eliminated (in contrast to results with 2-amino-5-phosphonopentanoic acid (APV), where it was always completely blocked (Arvanian & Mendell, 2001a). The late component also became progressively smaller and slower, i.e., it was no longer all-or-none, and was invariably abolished after repeated stimulation (Fig. 7, MK-801). The finding that the late response declined gradually during MK-801 application (Fig. 7, DR in MK-801; Fig. 8, DR during washout) suggests that the channels responsible for the late depolarization were NMDA channels rather than other channels, e.g., Ca^{2+} channels, triggered by NMDA receptor- mediated depolarization.

After maximum blockade of the DR-evoked NMDAR-mediated responses by MK-801 was achieved, we stimulated VLF still in the presence of MK-801 (n=20). In most cases the monosynaptic response to the first VLF stimulus in the presence of MK-801 was similar in amplitude to the mean value obtained before MK-801 administration despite prior stimulation of DR in the presence of MK-801. In other cases this response was diminished to a limited extent. On average, the initial NMDA-mediated monosynaptic VLF-response (Fig. 9) was about 85% of the control amplitude ($p > 0.05$, rank sign test), and this response was further gradually blocked by MK-801 over the next 40 minutes of VLF stimulation to about 52$\pm$ 17% (Fig. 9). Unlike the monosynaptic response, the polysynaptic component, including the plateau potential, in response to VLF was never observed if DR had previously been stimulated in the presence of MK-801 (Fig. 7, MK-801).

In 6 cells the experiment was performed in reverse order (Fig. 8, protocol in Fig 4b), i.e., we first stimulated VLF in the presence of MK-801, and after VLF-evoked NMDA-mediated responses were blocked we stimulated DR until the response declined to a steady level indicating maximum antagonism by MK-801. The initial monosynaptic DR response (2.7 ± 1.5 mV) after VLF stimulation in the presence of MK-801 was elevated from the control value (2.2 ± 1.1 mV) (Fig. 9), but this difference was not significant ($n=6$; $p > 0.5$). Again, the plateau potentials occurred irregularly in response to the initial stimuli of the series, but eventually they ceased. The response to the initial DR stimuli after VLF stimulation always included a late component which gradually decreased in amplitude and increased in rise time until it disappeared. This was in

contrast to the VLF response after DR stimulation in which the late component was always abolished from the start (Fig. 7, MK-801—see above).

The finding that the initial monosynaptic response to either synaptic input was virtually unaffected after the other had been stimulated in the presence of MK-801 suggests that relatively few individual NMDA receptors on the motoneuron are activated monosynaptically by both synaptic inputs. A different finding was made with regard to the late response, namely that prior stimulation of DR in the presence of MK-801 almost always abolished the initial VLF late response, but the reverse never occurred.

EPSPs elicited by DR and VLF stimulation recover from "irreversible" MK-801 blockade but the DR EPSP recovers to a greater degree than VLF EPSP

Blockade by MK-801 is described as irreversible, because at -70mv MK-801 unbinds very slowly from NMDA receptors (Huettner & Bean, 1988), Thus we were surprised that the EPSP that had been blocked previously in a use-dependent manner by MK-801 could "recover" from the blockade (Figs. 7 and 8). In this study synaptic NMDA responses were blocked first by repetitive stimulation of one and then the other synaptic input in the presence of MK-801 added to the non-NMDA receptor antagonists cocktail (see above). After achieving maximal block of both DR- and VLF-evoked responses, repetitive stimulation was stopped and MK-801 was washed out by bath application of a solution still containing the non-NMDA antagonists. Following a 45 min period of no-stimulation and wash out of MK-801, DR and VLF inputs were stimulated again.

We found several indications that the recovery differs for DR and VLF inputs on the same population of motoneurons. The monosynaptic DR response virtually always

exhibited some degree of recovery from the decrease observed in MK-801; VLF often did not recover but could occasionally display a small recovery (Figs 7, 8 and 10). These differences were independent of the order of stimulation (Figs. 7 and 8). In addition, they occurred despite the similarity in the degree of depression by MK-801 (Fig. 9). Recovery of the initial monosynaptic component was calculated as the percent of the initial decline after MK-801 that recovered when MK-801 was washed out. A recovery of 0% indicated that the EPSP amplitude did not change after removal of MK-801, and 100% indicated recovery to the value before MK-801 was added, i.e., control value. Negative values of recovery were indicative of a response that declined further after washout of MK-801. We observed a significantly greater recovery for monosynaptic DR inputs than for those made by VLF ($p=0.002$; Signed Rank test; $n=10$, Fig. 5). A further indication of differences between recovery of these inputs during MK-801 wash was the finding in some cases (4 of 10) that the DR response recovery was more than 100% indicating that the response became larger than the original value. This was never observed for VLF responses in the same cells. Similarly, in 3 of 10 cases the VLF responses declined after removal of MK-801 (negative value of % recovery), and this was never observed for DR responses in the same cells. In only 1 case did the VLF response display more recovery than the DR response, and this difference was very small.

We also noted that the late DR response, including plateau potentials, generally recovered in contrast to the late VLF response which never recovered. These differences were observed regardless of the order of stimulation in MK-801 (Figs. 7 and 8). We cannot specify the location of the NMDA receptors responsible for the recovery of these responses, i.e., whether they were restricted to the motoneuron, to the intercalated

interneurons, or involved NMDA receptors on both cell types.

As the experimental protocol required a long period (45 min.) of no stimulation while the MK-801 was being washed out, we considered the possibility that the initial stimulus after the long quiescent period resulted in the release of massive amounts of neurotransmitter which would exaggerate the apparent recovery. This would be true for glutamatergic synapses from DR and not VLF. In control experiments with no blockers, we tested the effect of a 45 min. interval between successive DR stimuli and did not find a large increase in the response after the period of inactivity.

In all of these experiments the state of the motoneuron and its synaptic inputs was determined at the end of the manipulations by perfusing with ACSF for 45 min. to wash all antagonists from the bath Following this 45 min wash with no stimulation, both DR and VLF inputs were stimulated to verify that the AMPA responses had recovered. This further proved that the cell and its inputs had remained viable through out the entire course of the experiment and that there were no substantial changes in excitability of the spinal cord.

The responses to repeated stimuli delivered after the washout of MK-801 declined in amplitude, even though MK-801 was no longer in the perfusing solution (Figs. 7 and 8). The percent decrease of the response (49.4 ± 15.1% for DR and 31.6 ± 8.43% for VLF, n=10) was similar to that observed when the inputs were stimulated in the presence of MK-801. This suggests that MK-801 remained in the spinal cord despite being washed from the bath. Further evidence suggesting that MK-801 remained available to block NMDA transmission was obtained from the following experiment (Fig. 11). After blocking the non NMDA receptors with antagonists and demonstrating the stationarity of

the monosynaptic NMDA response, MK-801 was applied to the bath. No synaptic pathways were stimulated during MK- 801 application, and 45 minutes later, while maintaining the motoneuron penetration the MK-801 was washed out of the bath for 45 minutes, again with no stimulation. At the end of this period, the DR stimulation was begun and, unlike what had been observed before MK-801 application, the monosynaptic NMDA receptor- mediated EPSP declined with successive stimuli similar to what had been observed in other motoneurons during application of MK- 801 (Protocol in Fig 12). Similar findings were made for the response to VLF stimulation in the same motoneuron. This suggests that MK-801 had not been washed out and remained available to block the NMDA channel upon stimulation (see Discussion).

The recovery of responses from "irreversible" blockade by MK-801 was not dependent on which of these synaptic inputs was stimulated first following wash of MK-801: in 16 spinal cords, DR was stimulated before VLF, and in 6 other spinal cords VLF was stimulated first. In both cases we observed the greater recovery of the response to DR stimulation when MK-801 was washed out.

The EPSP did not recover from "irreversible" MK-801 blockade when NMDA was bath applied

The recovery of the synaptic response after washout of MK-801 can be attributed to several possible mechanisms (see Discussion). One mechanism demonstrated previously in dissociated neurons is the insertion of new NMDA receptors into the synapse from intracellular sources or by lateral diffusion of receptors into the synapse from extrasynaptic regions (Tovar & Westbrook, 2002). To investigate the possible

contribution of extrasynaptic receptors to recovery of the response, we carried out a manipulation to inactivate all extrasynaptic NMDA receptors and determined whether recovery from MK-801 blockade would still take place.

After the DR NMDA receptor- mediated EPSP (Fig. 13A) had declined to a minimum value during repetitive stimulation of DR in the presence of MK-801 (Fig. 13B), a drop of NMDA (10 μl of 10mM NMDA) was applied to the recording chamber from a micropipette. This induced a depolarizing response since receptors extrasynaptic to DR and VLF inputs had not been blocked by MK-801 (Fig. 13C; (Arvanov *et al.*, 2000)). The response to subsequent NMDA drops was smaller and by the second or third drop the response was completely abolished. Stimulating DR (and VLF) immediately after the NMDA drop applications always elicited no response (Fig. 13D) and there was no recovery after MK-801 and NMDA were washed from the bath for 45 min with a solution containing only the non-NMDA receptor antagonists (Fig. 13E). This was in sharp contrast to the recovery of the response, particularly to DR stimulation, observed in the absence of the NMDA drop (Figs. 7, 8 and 10). Similar findings were made in a total of 6 motoneurons for both DR and VLF synaptic inputs (Protocol in Fig 14). The DR-evoked late response also never recovered after the NMDA drop in contrast to its recovery in the absence of NMDA (see above). The interpretation of this experiment is that the blockade of the non-DR and non-VLF NMDA receptors by bath applied NMDA in the presence of MK-801 prevented them from contributing to the recovery of the DR- and VLF- responses.

In all of these experiments, at the end of all manipulations, all the antagonists were washed out from the bath for 45 min using ACSF without any stimulation.

Following the 45 min wash period, AMPA responses were obtained by first stimulating the DR and then the VLF. The recovery of the AMPA response indicated that the motoneuron and its synaptic inputs were still viable (Fig. 13F).

Discussion

These experiments were undertaken because previous work had shown that DR and VLF NMDA receptor- mediated synapses on the *same* motoneuron exhibit different properties during early postnatal development (Arvanian & Mendell, 2001a; Arvanian *et al.*, 2004). The expectation from these findings was that there would be little or no overlap between the receptors activated by these different inputs. We began by taking advantage of the use- dependence of MK-801- elicited blockade to determine whether stimulating one input thereby blocking the receptors associated with that input would have any effect on the response to the other input. We found that blocking the monosynaptic response to either input first had no significant effect on the monosynaptic response to the other input. The long latency responses displayed a different pattern; stimulation of DR during MK-801 administration always resulted in elimination of the late VLF response (Fig. 7), but never vice versa (Fig. 8).

One implication of these findings is convergence between pathways activated by VLF and DR. These experiments do not permit us to specify the location of such interactions except to say that they can occur on interneurons, on motoneurons or both. (Petruska *et al.*, 2007) demonstrated in adult rats that individual ascending axons in the VLF have cell bodies in the vicinity of L5 motoneurons and send collaterals to terminate on these motoneurons. Thus at the level of the motoneuron, some of the interaction may

take place between inputs from muscle spindle afferents in the dorsal root and *ascending* axons in the VLF activated antidromically. The present findings suggest that there are also interneurons projecting to motoneurons that receive inputs from spinal white matter axons and segmental inputs. The general concept of common interneurons activated by descending and segmental projections and projecting to motoneurons has been described (rev. by (Jankowska, 2008)) and interneurons influencing motoneuron activity that are activated by NMDA have been described (Hochman *et al.*, 1994; Kiehn *et al.*, 1996). Thus neural elements and circuits that could mediate the interactions described here appear to exist although at present we cannot specify their identity and location.

The fact that the interaction occurs from DR to VLF but not in the opposite direction suggests that these 2 inputs normally do not activate common NMDA receptors and that the interaction may occur by a process of "spillover" (Diamond, 2002) whereby transmitter spreads to activate receptors beyond its normal postsynaptic zone of influence (Huang & Bordey, 2004). Spillover is normally limited by transmitter uptake mechanisms (Diamond & Jahr, 1997; Diamond, 2001), particularly into glia (Bergles & Jahr, 1997). Evidence for such a mechanism has been obtained recently by decreasing transmitter uptake using inhibitors of glutamate transporters (Harney *et al.*, 2008; Hires *et al.*, 2008). We speculate that the much larger, faster polysynaptic EPSPs we observed in the same motoneuron from electrical stimulation of DR inputs compared to VLF inputs might be associated with a higher local density of synapses which would result in a higher concentration of glutamate in the synaptic cleft. This might overwhelm mechanisms for transmitter reuptake and result in activation of non DR receptors. However, under normal physiological conditions, the asynchronous discharge of inputs in

the DR pathway may cause very little spillover because of lower concentrations of transmitter in the synaptic cleft although a physiological role for spillover has been suggested (Jahr, 2003).

Recovery from MK-801 blockade occurred for both the short latency monosynaptic and longer latency, presumed polysynaptic NMDA receptor- mediated responses. The recovery was uniformly greater for responses elicited by DR than VLF. This was ascertained quantitatively for the monosynaptic response and qualitatively for the late polysynaptic response. The fact that the differences were also observed for polysynaptic responses suggests that NMDA receptors on interneurons (Hochman *et al.*, 1994) associated with DR and VLF inputs may differ in the same way as those on motoneurons, i.e., NMDA receptor properties may differ according to their synaptic input (see below).

The recovery from MK-801 blockade was unexpected in view of the literature suggesting that this agent is a non- competitive, irreversible inhibitor of NMDA receptor-mediated transmission (Wong *et al.*, 1986). The possibility that MK-801 becomes unbound from the receptors that it had previously blocked during the wash period seems very unlikely since it unbinds very slowly from NMDA receptors at -70 mV (Huettner & Bean, 1988) which is very close to the membrane potential of cells recorded in these preparations. Furthermore, our finding that the MK-801 appears to remain associated with the NMDA receptor after an extended washout period, even in the absence of stimulation throughout its time of application, argues that unbinding did not occur. Another possible explanation is that a population of initially desensitized receptors at the activated synapses was not blocked by MK-801 (Dzubay & Jahr, 1996) and became

functional during the washout of MK-801 from the bath when synaptic inputs to the motoneuron were not activated. According to this hypothesis, one would have also expected these receptors to also become active during washout in experiments where NMDA and MK-801 were co-applied because no stimulation was given during this period. The lack of recovery under these conditions suggests that inability to block a population of desensitized receptors is not the explanation.

Other possible explanations for the apparent recovery of NMDA transmission include increased presynaptic release probability due to the long period of inactivity during washout. Although we cannot completely rule out this possibility, the similarity of the monosynaptic AMPA component after recovery to that observed at the onset of the pharmacological manipulations (Fig. 7) suggest that large changes in transmitter release probability did not occur. Motoneuron membrane potential was monitored throughout and remained relatively constant (typically < 5 mV variation) and so fluctuations in Mg^{2+} block known to affect NMDA transmission (Nowak *et al.*, 1984) were very unlikely to account for our findings.

(Tovar & Westbrook, 2002) observed similar recovery of synaptic responses after MK-801 blockade in autapses made by hippocampal neurons studied in culture. These findings were interpreted as NMDA receptor trafficking from extrasynaptic regions based on the abolition of the recovery by application of NMDA in the presence of MK-801, a finding also made in the present experiments. The similarity of our results to those observed in the earlier experiments, suggests that similar mechanisms are operating in both situations. We speculate that the greater recovery of DR responses compared to VLF reflects greater ability of trafficking receptors to be incorporated at those synapses. This

might reflect differences in factors such as scaffolding proteins or the composition of the NR2 regulatory subunits (El-Husseini *et al.*, 2000; Wenthold *et al.*, 2003) associated with these synaptic inputs. The similarity in recovery from MK-801 of polysynaptic responses from DR and VLF might reflect differences in properties of DR and VLF synapses on interneurons similar to those on motoneurons although this would require direct recording from these cells for verification.

The possibility that the receptors came from intracellular rather than membrane sources (Groc & Choquet, 2006) cannot be discounted, but seems unlikely because bath application of NMDA, which should not affect intracellular sources of NMDA receptors, prevented the recovery from MK-801 blockade. However, if receptors cycle rapidly through the cytoplasm and the plasma membrane as suggested by (Washbourne *et al.*, 2004), the outcome of NMDA drop experiment would be more difficult to predict, particularly in the present experiment where it was applied to an intact spinal cord.

A surprising finding was that MK-801 generally did not completely abolish the synaptic response. This raises the possibility that transmitters other than glutamate were released by the presynaptic fibers or that MK-801 failed to penetrate to all the NMDA receptors. The former seems very unlikely because the reversible antagonist APV completely blocks the response remaining after the non NMDA antagonist cocktail under conditions identical to those in the present experiments (Arvanian & Mendell, 2001a). The latter is contradicted by our finding that MK-801 completely blocked the response produced by the second or third bath applied NMDA. The suggestion that the synaptic response remaining after exposure to MK-801 for 45 minutes and of the order of 50 DR stimuli was mediated by another transmitter also seems unlikely because the response

was permanently abolished when a drop of NMDA was added in the presence of MK-801. This would not have been expected to affect transmission at another receptor unless the NMDA drop elicited presynaptic inhibition of Ia transmission to the motoneuron thereby blocking all transmitter release. NMDA receptors are present on the presynaptic terminals of afferent fibers, and NMDA does elicit presynaptic inhibition of transmitter release from the terminals of dorsal root afferent fibers as monitored via the AMPA/kainate receptor response (Arvanian & Mendell, 2001a; Bardoni *et al.*, 2004). However, it only partially depressed transmitter release, and so this could not account for the uniform total loss of NMDA receptor responsiveness observed after the NMDA drop. Furthermore, VLF transmission was not inhibited by exogenous NMDA (Arvanian & Mendell, 2001a) in contrast to the strong depression observed in the current experiments (not illustrated). Together these comparisons indicate that the NMDA effects on the recovery from MK-801 were not the exclusive result of presynaptic inhibition of glutamate release.

The failure of bath applied MK-801 to completely block the NMDA receptor-mediated synaptic response might also 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). In agreement with this hypothesis, when all NMDA receptors were inactivated by exogenous NMDA in the presence of MK-801, replacement by functional NMDA receptors was not possible and the synaptic response disappeared. NMDA receptor transport within neurons is fast enough to be consistent with such a mechanism (Washbourne *et al.*, 2002; Guillaud *et al.*, 2003; Groc *et al.*, 2004). This replacement hypothesis might also help to explain the

variable recovery of synaptic transmission to levels above or below the original response after synapse specific MK-801 block in the absence of NMDA.

These experiments were based on the use dependence of MK-801 blockade, but the results also revealed that this antagonist can be bound in the absence of synaptic activity. MK-801 blocks transmission when administered (45 min) and washed out (45 min.) in the absence of stimulation. Previous biochemical work has indicated that MK-801 binds to NMDA receptors in cortical membrane preparations via 2 distinct processes: a kinetically fast (half time 10 min.) binding requiring the presence of a ligand for the NMDA receptor, e.g., glutamate, and a much slower effect not requiring the presence of any agonist (half time 2-3 hours) (Javitt & Zukin, 1989). It remains to be determined precisely where the MK-801 is located before inserting into the pore as a result of the initial stimulus. The biochemical experiments suggested that the slow accumulation of MK-801 is hydrophobic raising the possibility that it is lipophilic and remains associated with the membrane.

Although the *in vitro* experiments provide important guidance for interpreting the current experiments mechanistically, the present experiments in a largely intact hemicord preparation permit an additional important functional implication to be drawn, namely, that different classes of NMDA receptor- mediated connections on the same motoneuron, that is, from spindle afferent fibers in the dorsal root and from fibers in the VLF, differ in their properties. Whether these differences are related to the increased susceptibility of VLF NMDAR- mediated synapses to Mg^{2+} block in neonatal rat motoneurons (Arvanian *et al.*, 2004) is not presently known. Another unknown is whether the reduced ability of NMDA receptors to traffic to VLF synapses contributes to the relative lack of sensitivity

of these synapses to the sensitizing effects of neurotrophins (Arvanov *et al.*, 2000; Arvanian & Mendell, 2001b).

Other interesting questions remain. It will be important to determine whether adult rats exhibit similar evidence for membrane trafficking, whether it is synapse specific, and whether it is related to the susceptibility to synaptic plasticity. The finding that recovery of responses at VLF synapses was less than that associated with DR synapses suggests that maturation might be a factor since we have previously shown that VLF synapses on motoneurons display more signs of maturation than those from DR in the immediate postnatal period (Arvanian *et al.*, 2004). It will be important to understand at what factors underlie the apparent difference in ability of synapses under different presynaptic inputs to stabilize trafficking NMDA receptors. There is evidence that the molecular composition of the postsynaptic density as well as the composition of the NMDA receptor itself may be important contributors to the stabilization of the postsynaptic receptors. In view of the well documented role of NMDA receptors in strengthening excitatory connections during development (Aamodt & Constantine-Paton, 1999), the solution to these problems at the molecular and cellular level could improve the ability to regulate the formation of new synaptic connections in the adult nervous system which could help improve the outcome after injury and degenerative diseases.

Figure 4(a): Experimental protocol to study the independence of DR and VLF synapses

The first three bars (ACSF, cocktail and MK-801) represent the composition of the bath. The last bar (Time) is the time for which the experimental manipulation was carried out. DR and VLF stimulation refer to when the respective inputs were stimulated. DR and VLF were alternatively stimulated in ACSF for the first 45 mins, followed by 45 min alternate stimulation of DR and VLF in antagonistic cocktail to obtain control NMDA-mediated EPSPs. After MK-801 was added, first the DR was stimulated for 30 mins and then the VLF was stimulated for 15mins.

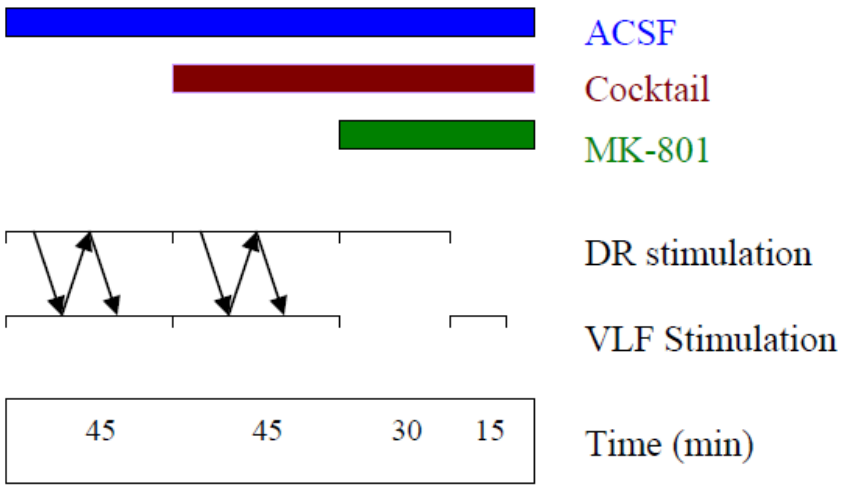


Figure 4(b): VLF was stimulated first in MK-801

The experimental protocol was same as above, with the exception VLF was stimulated first in MK-801

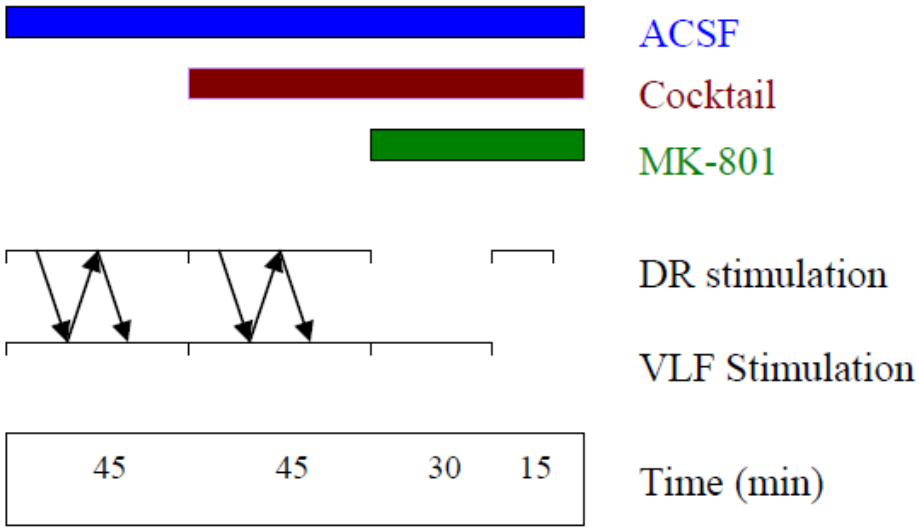


Figure 5: Experimental protocol to study recovery of EPSP from "irreversible" MK-801 blockade

DR and VLF were alternatively stimulated in ACSF for the first 45 mins, followed by 45 min alternate stimulation of DR and VLF in antagonistic cocktail. After MK-801 was added, first the DR was stimulated for 30 mins, with no stimulation of the VLF. It was then followed by a 15 min stimulation of VLF. MK-801 was washed out of the bath for 45 minutes, when neither of the input was stimulated. After obtaining DR and VLF wash responses, all antagonists were washed from the bath for 45 minutes and DR and VLF were stimulated in ACSF to ensure that the responses were still present and that the cell was still viable.

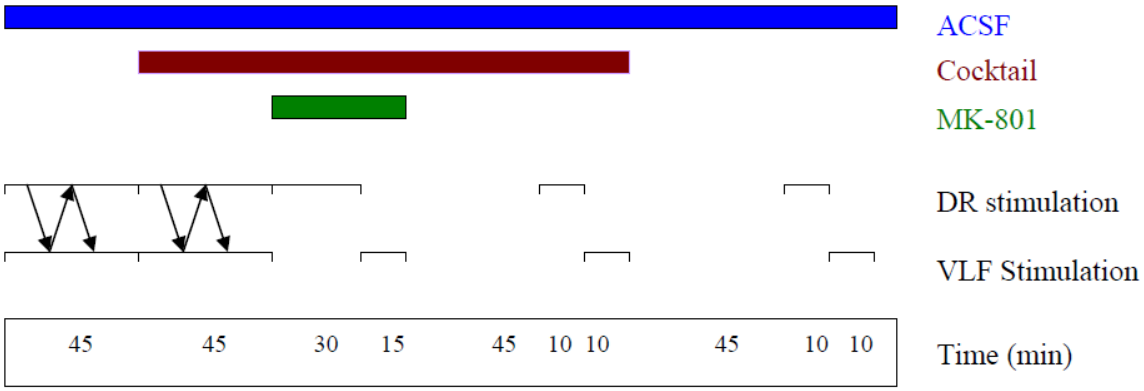


Figure 6: NMDA-mediated responses of the DR EPSP

Several superimposed responses to DR stimulation in a motoneuron. Note that the early monosynaptic responses are superimposed while the later polysynaptic responses are highly variable although they have a similar latency. Stimulation rate was 1/40s.

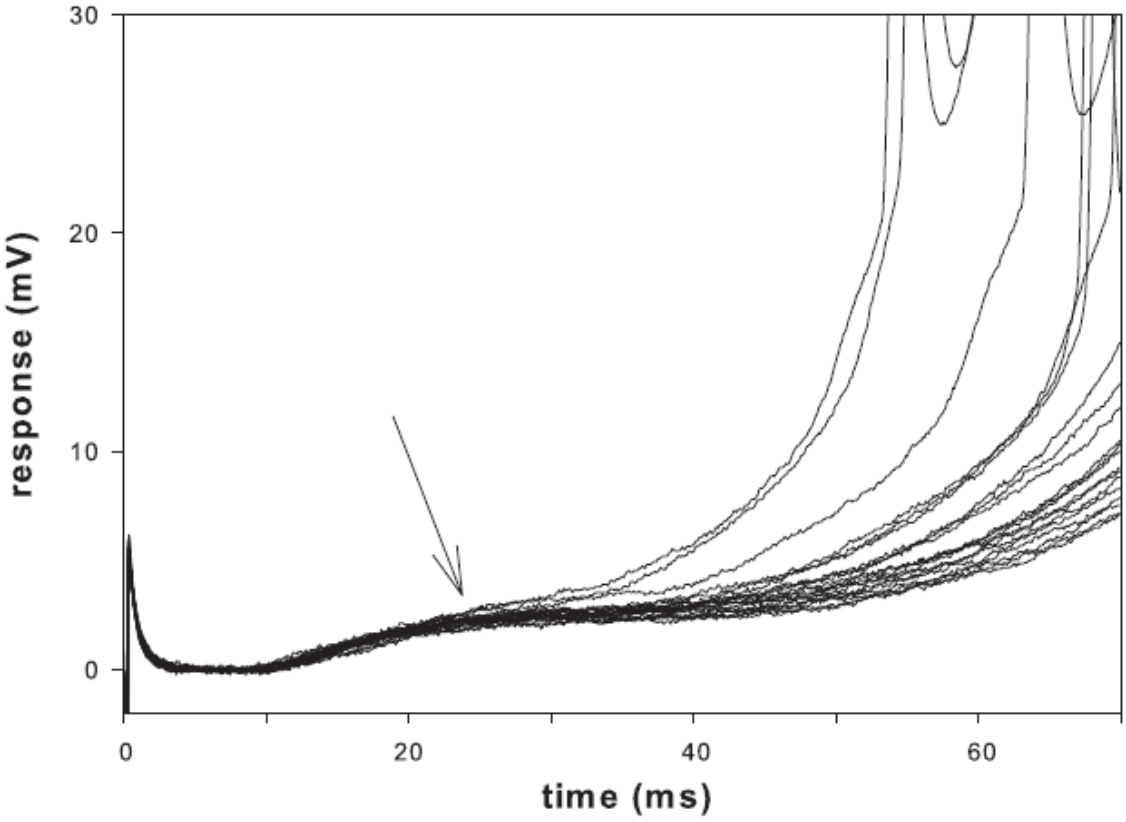


Figure 7: Illustration of the procedures carried out on an individual motoneuron and its inputs to study trafficking of NMDA receptors.

Spinal cord obtained from a P2 rat. Data from a single motoneuron obtained over a 4 hour and 30 min recording period. V_m was about -75 mV throughout. Stimulation rate 1/40s. *Control*: Blockade of all non NMDA receptors with CNQX, bicuculline, strychnine and CGP 35348 (CNQX Cocktail) administered to the bath. Ten consecutive responses to DR and then to VLF are displayed. Note the reliable responses to DR and the irregular responses to VLF. "Insets" placed at top are average monosynaptic responses at higher gain and faster sweep speed. *MK-801*: The NMDA receptor-mediated response 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 μM) into the bath 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. Insets display the initial response (black) and the last response (red) in MK-801. MK-801 was then washed from the bath with ACSF for 45 min. in the absence of stimulation while continuing to apply the non NMDA antagonists. *Wash*. Superimposed responses (n=10 for both DR and VLF) after MK-801 washout are displayed along with the initial response at high gain (insets). Note the recovery of the DR NMDA response followed by further blockade when repetitively stimulated despite absence of MK-801 in the bath. Note also the lesser recovery of VLF NMDA response- the late response never reappeared in contrast to DR. The bottom graphs are average AMPA/kainate responses before CNQX cocktail (black) and at the very end of the experiment after the CXQX cocktail was washed out for about 45 min. (green). Note the similarity in the responses before and after (except for the briefer time course of the DR response which elicited spikes after the wash phase).

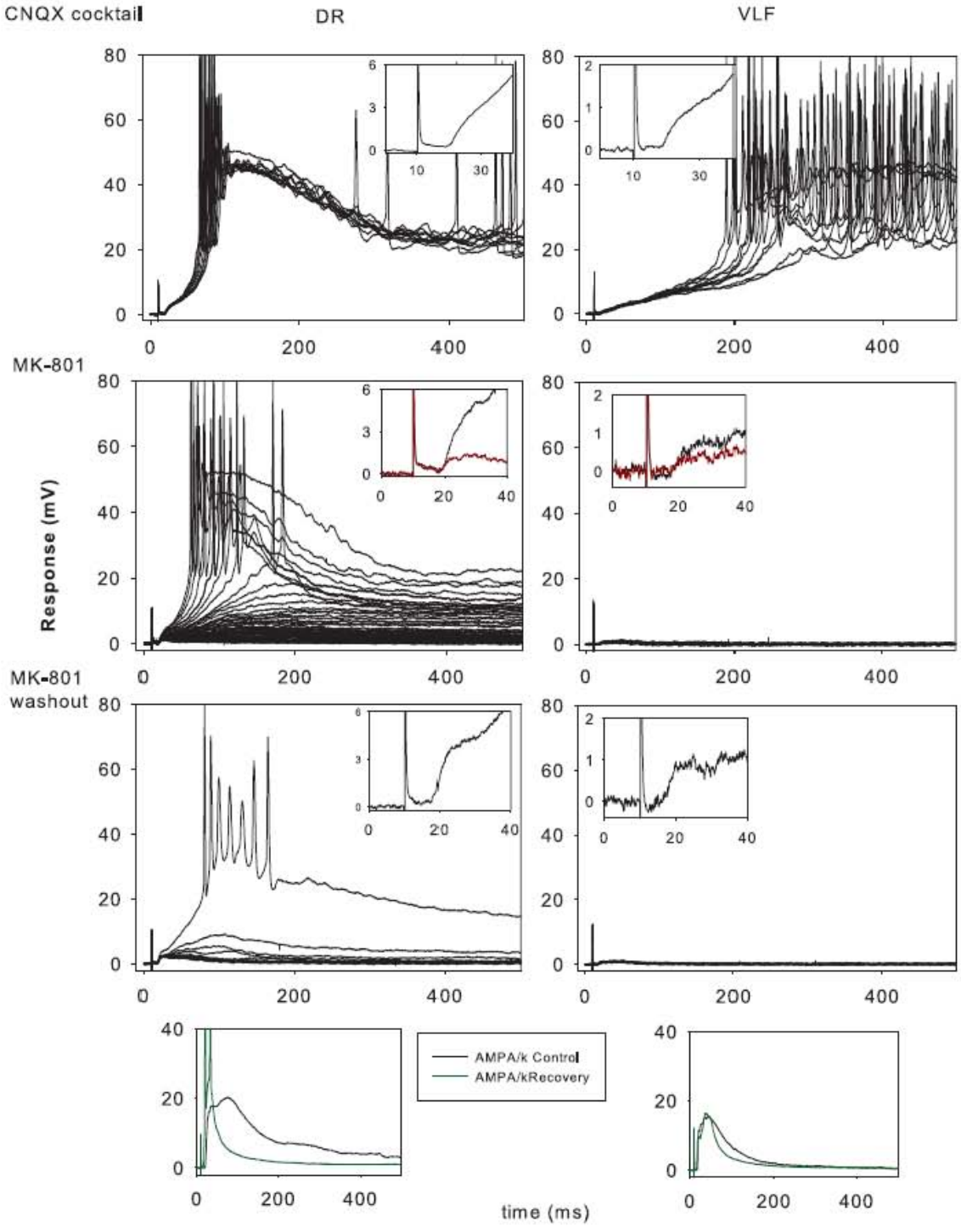

CNQX cocktail
DR
VLF
MK-801
MK-801
washout
Response (mV)
AMPA/k Control
AMPA/kRecovery
time (ms)

Figure 8: Illustration of the procedures carried out on an individual motoneuron and their inputs to study trafficking of NMDA receptors; order of stimulation in MK-801 was reversed – VLF stimulated first

Organization and protocol similar to figure 2 except for the order of stimulation. P4 rat. *Control*: NMDA receptor-mediated response (10 consecutive stimuli for DR and VLF); *MK-801*: response in MK-801 (VLF: 80 stimuli; DR: 60 stimuli); *Wash*: response after 45 min. washout of MK-801 in the absence of stimulation (20 stimuli for both VLF and DR). Insets in the bottom row display the last monosynaptic response in MK-801 (black) and first response after washout of MK-801 (red). Note recovery of DR response and lack of recovery of VLF response.

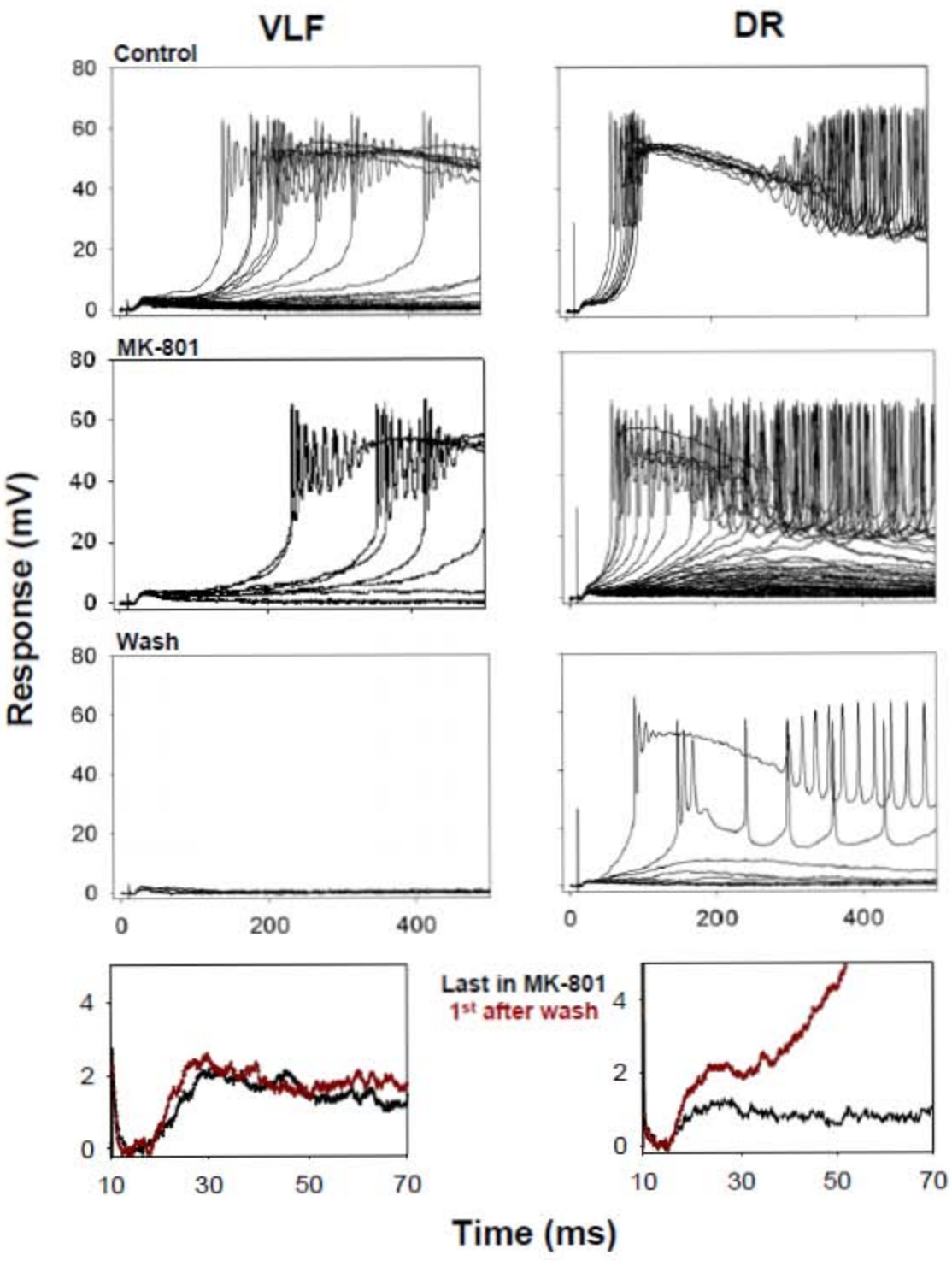

VLF
DR
Control
MK-801
Wash
Response (mV)
80
60
40
20
0
0
200
400
Last in MK-801
1st after wash
4
2
0
10
30
50
70
Time (ms)

Figure 9: Independence of NMDA receptors in DR and VLF synapses

Bar graphs displaying the mean amplitude of the NMDA receptor- mediated monosynaptic EPSPs elicited in the same motoneurons by DR and VLF stimulation. Left: DR (white) (n= 20) was stimulated before VLF (grey) during MK-801. The first bar (vertical lines) is the mean NMDA control response averaged over all cells. The second bar (horizontal lines) is the mean response at the onset of MK-801, and the third bar (diagonals) is mean final response in MK-801. DR and VLF stimuli in control conditions were interdigitated, but in MK-801, DR was stimulated until steady state was reached after which VLF was stimulated. Note that the initial VLF response in MK-801 was similar to the control response, i.e., stimulating DR in MK-801 did not significantly affect the response to VLF. Right: The same was observed when the experimental protocol was reversed (n=6), i.e., stimulating VLF first in MK-801 did not significantly affect the initial response to DR. Note also the depression of all responses after stimulation in MK-801. Further details in the text.

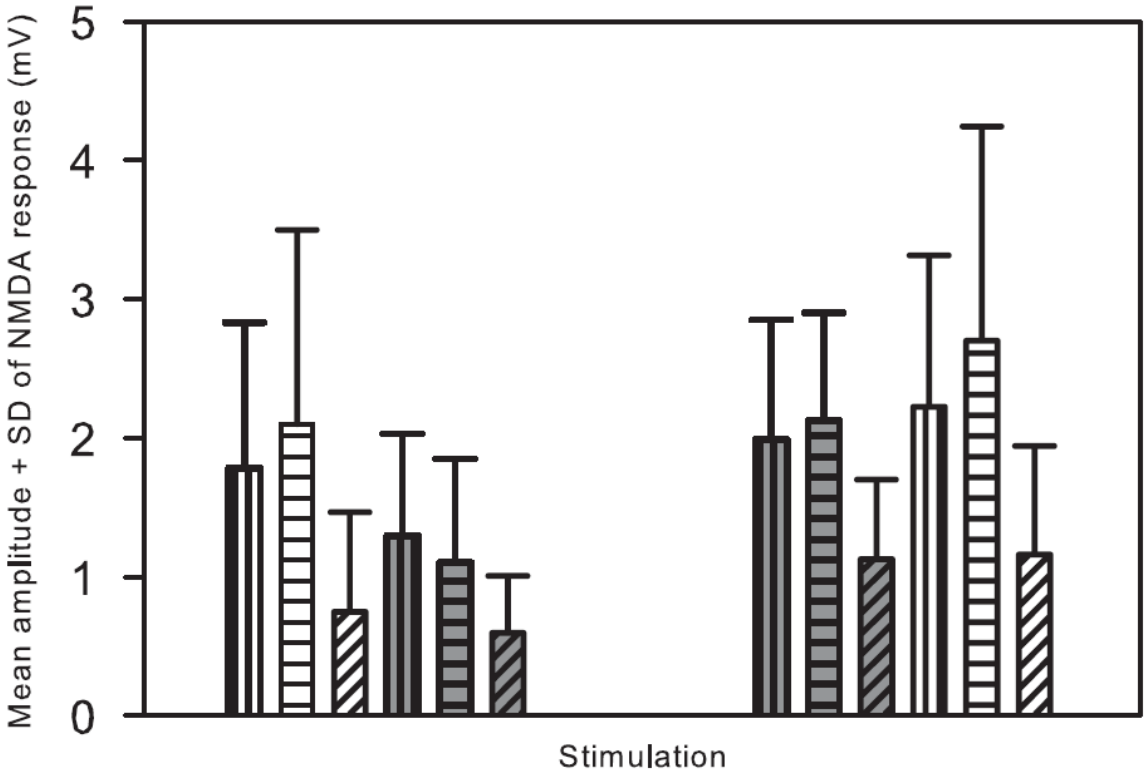


Figure 10: Comparison of DR and VLF recovery from MK-801 blockade

Percent recovery of DR and VLF from MK-801 block when DR was stimulated before VLF. See text for definition of % values. Line connects data from same motoneurons. Note the negative slope for 9 of 10 cells indicating that recovery of response to DR was greater than recovery of response to VLF. Further detail in text.

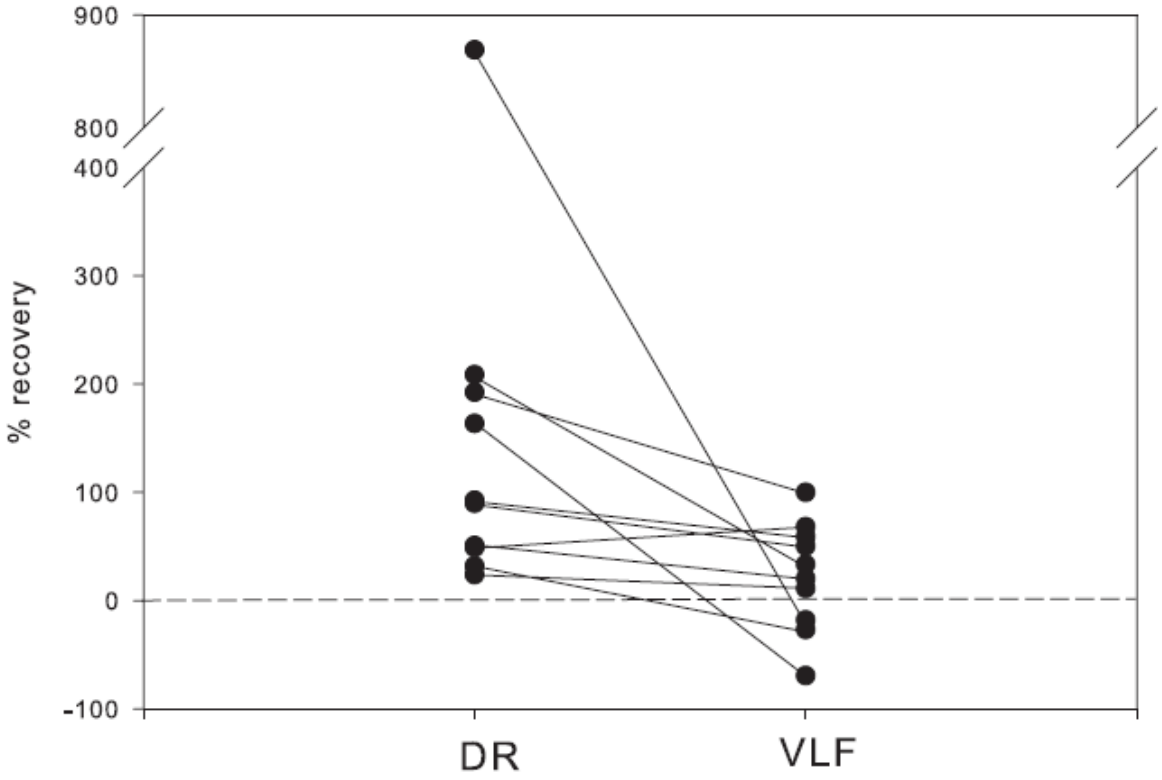


Figure 11: Effects of MK-801 in the absence of any stimulation

Control: NMDA receptor mediated EPSPs recorded in CNQX – cocktail in response to 10 consecutive DR stimuli with the initial 5 in red and the final 5 in black. Inset displays average response to the initial 4 VLF stimuli (red) and the last 4 VLF stimuli (black). Stimulation rate 1/40s. Note the uniform amplitude of the monosynaptic component. *MK-801 wash:* similar records after exposure to MK-801 for 45 min *and* washout for 45 min., both in the absence of any stimulation throughout this 90 minute period. Red traces are progressively decreasing responses to 1st 10 stimuli; black traces are the last 10 responses (of 50). Note that the monosynaptic EPSP declined progressively in amplitude as if MK-801 was present at the synapse. Inset displays the response to VLF as in the Control records above. Note that unlike controls, the VLF EPSPs exhibited a decline in amplitude with repetitive stimulation.

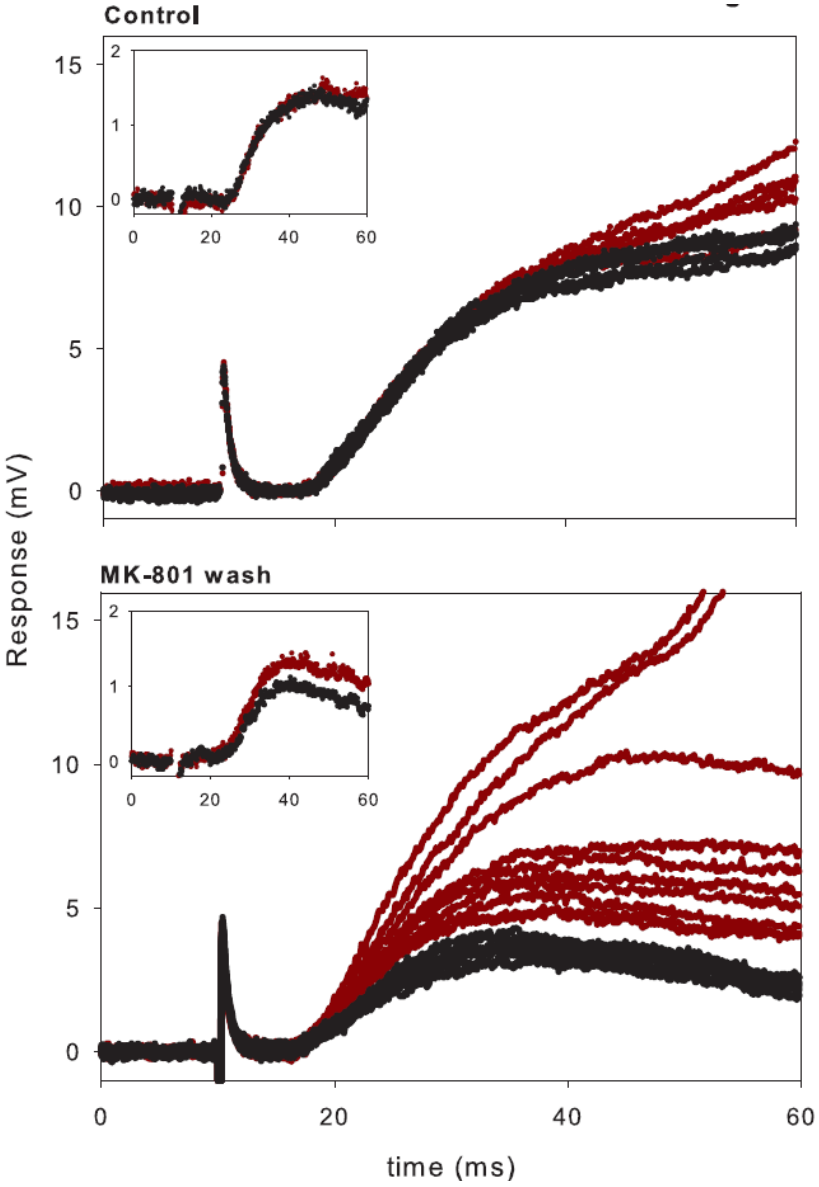


Figure 12: Experimental Protocol to study the lipophilic nature of MK-801

DR and VLF were alternatively stimulated in ACSF for the first 45 mins, followed by 45 min alternate stimulation of DR and VLF in antagonistic cocktail. After MK-801 was added, neither input was stimulated for 45 minutes. MK-801 was washed out of the bath for 45 minutes, during which none of the inputs were stimulated. After obtaining DR and VLF wash responses, all antagonists were washed from the bath for 45 minutes and DR and VLF responses in ACSF were obtained to ensure viability of the cell.

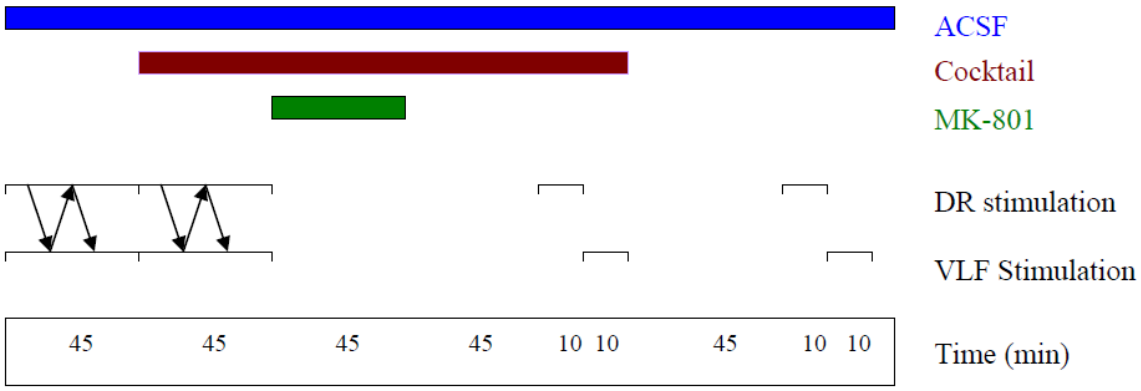


Figure 13: Simultaneous bath application of NMDA and MK-801 prevents recovery from MK-801 blockade

Effects of NMDA drop on recovery of synaptic response after MK-801. P2 rat. DR stimulation. A. NMDA receptor- mediated response to DR stimulation. Note the monosynaptic response followed by the late polysynaptic response. B. NMDA response after DR stimulation for 1 h with MK-801 in the bath illustrating the blockade of the synaptic response. C. Response to two bath applied NMDA drops in the presence of MK-801. Note the depolarizing response obtained to the initial NMDA drop despite the presence of MK-801 beginning about 1 h previously. The second drop elicited no response. D. Response to DR after immediately after NMDA administration. E. Response to DR after 1 hour MK-801 washout without any stimulation. Note the lack of the usual recovery of the early or late DR- mediated NMDA receptor- mediated response after MK-801 washout after NMDA drop is delivered (compare to recovery in Figs 2 and 3). F. Despite lack of recovery of NMDA response, AMPA response recovered after washout of all non NMDA antagonists indicating that failure for NMDA recovery was not due to loss of synaptic input.

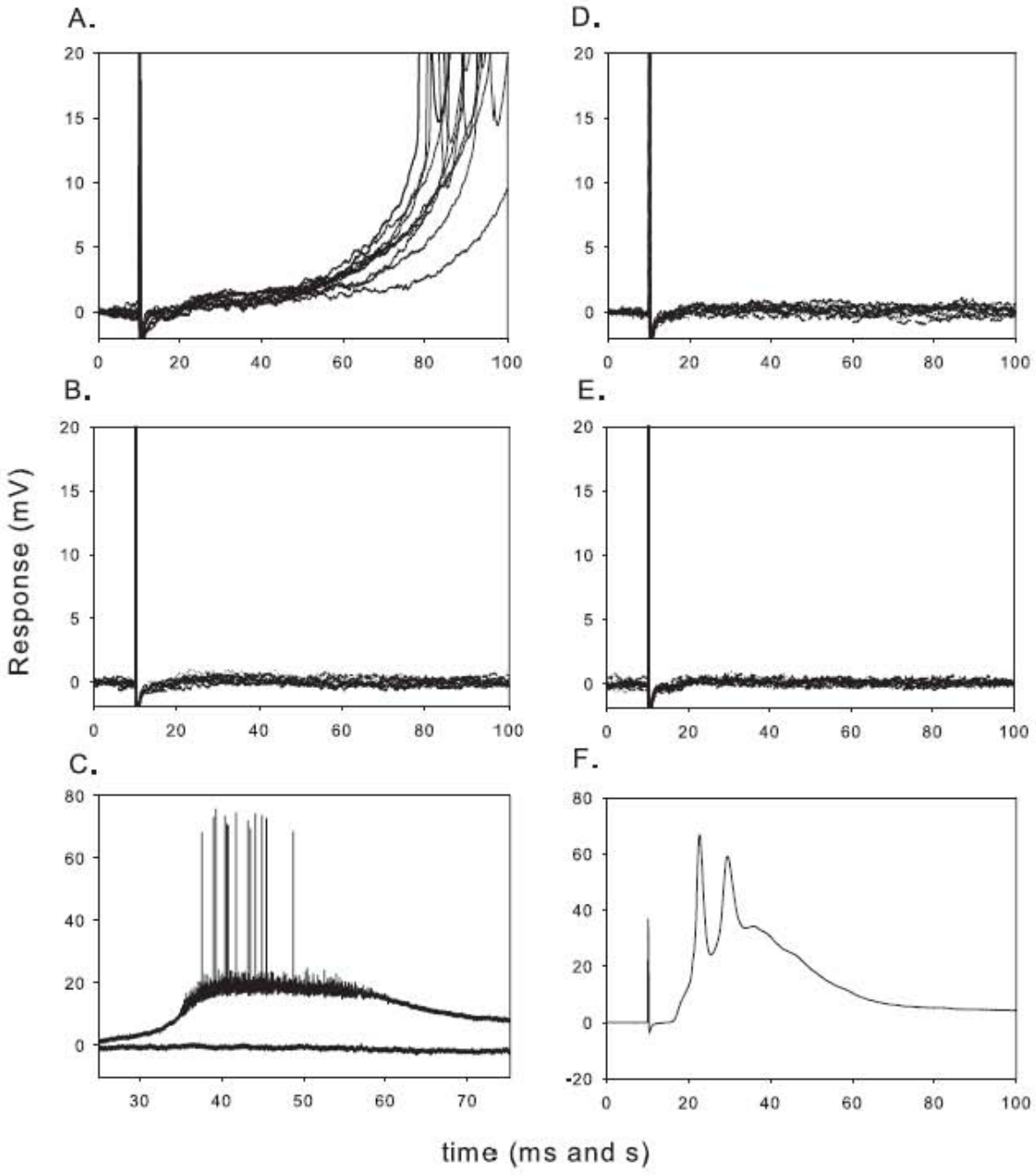
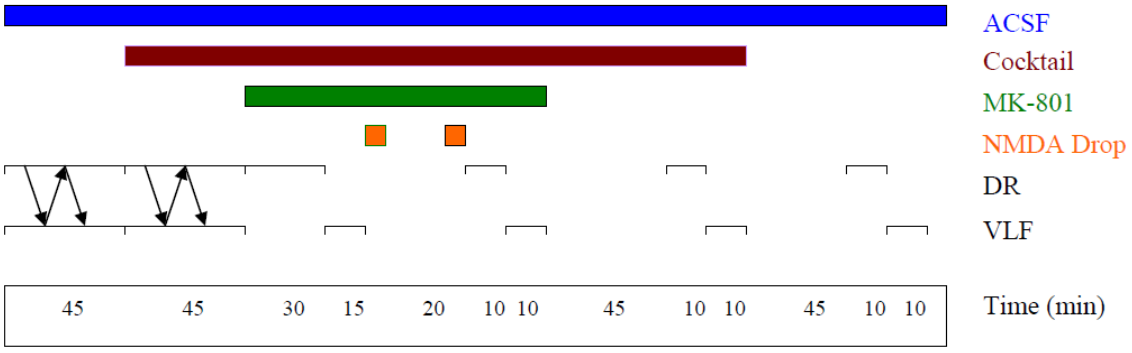

A.
B.
C.
D.
E.
F.
Response (mV)
time (ms and s)

Figure 14: Experimental protocol to study elimination of trafficking ability with coapplication of NMDA and MK-801

DR and VLF were alternatively stimulated in ACSF for the first 45 mins, followed by 45 min alternate stimulation of DR and VLF in antagonistic cocktail. After MK-801 was added, first the DR was stimulated for 30 mins, followed by a 15 min stimulation of VLF. Two drops of NMDA were bath applied in the presence of MK-801 with an interval of 20 minutes between the applications. Both inputs were stimulated to obtain responses. MK-801 was washed out of the bath for 45 minutes, when neither of the input was stimulated. After obtaining DR and VLF wash responses, all antagonists were washed from the bath for 45 minutes and responses in ACSF were obtained by DR and VLF stimulation.



Chapter III

Differences in NMDAR subunit populations between synapses formed by DR & VLF inputs

Abstract

Synapse specific differences in NR2 subunit expression exist in several systems within the mammalian central nervous system. In these experiments we have used ifenprodil's ability to inhibit voltage-, use- and glycine- independent responses mediated by NR2B containing N-methyl-D-aspartate receptors (NMDARs) with high specificity. We show that in a neonatal rat, the synapses made by the DR and VLF inputs onto the same lumbar motoneuron exhibit differences in their susceptibility to ifenprodil blockade. The DR synapses, which have been previously shown to "mature" at a slower rate and have a greater ability to accumulate trafficking NMDARs in comparison to VLF synapses, contain more synaptic diheteromeric NR1/NR2B containing NMDAR. We suggest that as the rats increase in age, these diheteromeric NR1/NR2B receptors are being increasingly replaced with their ifenprodil-insensitive counterparts and show that this switch occurs earlier in the VLF synapses in comparison to their DR counterparts. In the DR synapses, there also exists a difference in the subunit composition between synapses made monosynaptically and polysynaptically through segmental interneurons. The DR synapses on motoneurons display a greater concentration of functional NR1/NR2B containing NMDARs. This novel finding of staggered development of NMDA receptors on the *same* motoneuron from different synaptic inputs is discussed in the context of its developmental and functional implications.

Introduction

In order to further characterize the differences that exist between DR and VLF synapses and to identify key mechanisms responsible for the difference in their time-line of "maturity", the following questions need to be addressed. Is there an intrinsic difference in NMDAR subunit expression between DR and VLF synapses? Are more NR2B and NR2D subunits (associated with immature and plastic synapses in amygdala (Lopez de Armentia & Sah, 2003) expressed at "plastic" synapses made by DR in comparison to the less "plastic" VLF synapses? Is there a time line for expression of specific NMDA receptor subunits and do they coincide with the time-line for maturation of the DR and VLF synapses? In other words, when are the NR2A receptors (associated with mature synapses (Monyer *et al.*, 1994; Stegenga & Kalb, 2001) expressed? Does the developmental switch of NR2B to non-NR2B expressing synapses described in other systems (Kew *et al.*, 1998; Cathala *et al.*, 2000; Lopez de Armentia & Sah, 2003; Liu *et al.*, 2004b) occur in motoneurons? Also does the "developmental switch" occur earlier for VLF NMDA receptors in comparison to the DR receptors?

The intrinsic differences in NMDAR NR2B subunit expression between DR and VLF synapses were determined using ifenprodil which is known to be the most selective among all blockers of NMDA receptor subunits (Neyton & Paoletti, 2006). Ifenprodil is a reversible, noncompetitive, and specific antagonist for NR2B subunit containing NMDARs (Legendre & Westbrook, 1991; Williams, 1993; 2001; Perin-Dureau *et al.*, 2002). In oocytes, ifenprodil at low concentrations (<10μM) 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). By studying the differences in the ifenprodil blockade of DR and VLF excitatory postsynaptic potentials (EPSPs), 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), NaH_2PO_4 (1.2), dextrose (11), aerated with 95% O_2 / 5% CO_2 (pH 7.4, 30^0 C) at 10 ml/min. The VLF was dissected free of the spinal cord at T2 (Pinco & 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 (70 μs duration at a rate of 0.025 Hz for NMDAR- mediated responses) was at an intensity sufficient to evoke the just-maximum monosynaptic potential.

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 35348) (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.

Protocol: Only cells displaying a stable resting membrane potential greater than -60 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. 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 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. The two inputs were alternately stimulated, 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 40 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 then 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.

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.

For offline analysis the responses to each stimulus were analyzed using Axoclamp 9.0 either in single sweeps, in superimposed sweeps or after averaging. The peak of the monosynaptic component was measured as described previously. (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

After achieving a suitable penetration of a L5 lumbar motoneuron, identified by the antidromic action potential elicited with segmental ventral root stimulation, we first recorded DR and then VLF-evoked responses in normal ACSF (Fig 15&16, ACSF). The stimuli to the 2 synaptic inputs were given as 10 consecutive stimuli to the DR followed by 10 consecutive stimuli to the VLF, with a 10 second interval between each stimulus. The amplitude of the monosynaptic DR response obtained was found to average 4.9 mV ± 0.7 SE (n=14), similar to the mean amplitude of the VLF response 4.4 mV ± 0.8 SE (n=14).

We then added non-NMDA antagonists to the perfusing solution to block the AMPA/kainate, GABA, and glycine mediated responses. (Arvanian & Mendell, 2001a). The NMDA responses were obtained by stimulating the DR 10 consecutive times followed by VLF stimulation at 0.025 Hz.(Fig 15, 16 Control). The initial component of the response displayed very little fluctuation and is assumed to be monosynaptically generated; the later component was highly variable especially in the VLF response and was assumed to be polysynaptic (see Chapter II or (Shanthanelson *et al.*, 2009) for further discussion). The peak of the monosynaptic component was measured at the latest time point where there was consistent overlap of successive responses. The polysynaptic component of the NMDA responses was measured at the plateau potential that appeared after the initial part of late response. The mean amplitude of the DR monosynaptic NMDA-mediated response was 1.9 mV ± 0.4 SE (n=14) and the VLF monosynaptic response averaged 1.7 mV ± 0.3 (n=14). The mean amplitude of DR and VLF polysynaptic responses were 42.4 mV ± 7.4 SE and 7.7 mV ± 4.1 SE.

NMDA synapses under the DR inputs are more sensitive to the blockers of NR2B subunits than in comparison to the NMDARs under the VLF inputs

After the NMDA control responses were obtained, 3uM ifenprodil was added to the bath containing the antagonist cocktail. Stimulation of the DR and VLF were alternated, a single DR stimulus was followed by a single VLF stimulus and then back to DR etc., with a 40s interval between stimuli. About 40 stimuli were delivered to each input. Maximal blockade was achieved when no further decrease in response was observed (Fig 15, 16, Ifenprodil). Percent residual was defined as the response remaining after ifenprodil, expressed as a percentage of the control NMDA response obtained initially in the antagonist cocktail. Percent blockade was calculated as (100- %residual).

The mean monosynaptic response blockade of the DR response averaged over all preparations ranging in age from P1 to P9 was 72.8 ± 3.5% SEM (n=14) and the mean VLF monosynaptic response blockade in the same preparations was 48.4 ± 4.4% SE (n=14) (Holm-Sidak ANOVA $p<0.001$). Fig 16 shows a graphical representation of the % blockade of the monosynaptic DR and VLF responses by ifenprodil in each cell (a single cell/spinal cord). The DR and VLF pair from the same cell are connected by a line. Note that in almost all cases the blockade of the DR monosynaptic EPSP by ifenprodil was greater than that of its VLF counterpart in the same cell, suggesting that DR synapses on motoneurons are composed of a greater proportion of functional NR2B containing NMDARs than VLF synapses. The only cell where this was not true was obtained from a P9 rat. Although this is only a single rat, it suggests that the difference in the NMDA

subunit composition between the DR and VLF synapses of these rats might be lost as the rats mature in the second postnatal week (see Discussion).

Comparison of blockade of the monosynaptic and polysynaptic component of the response

As reported previously (Arvanian & Mendell, 2001a), stimulation of the DR and VLF in the presence of the CNQX and antagonists of glycine and GABA transmission elicited a second NMDA receptor-dependent depolarization at longer latency that often led to an all-or-none plateau potential with superimposed action potentials. (Figs. 15 and 16; CNQX cocktail). Although the polysynaptic late component of the DR response was reduced by ifenprodil to a slight degree, it exhibited much less blockade than the monosynaptic component (Fig 15 (ifenprodil)).

The late DR-evoked plateau potential occurred reliably at the low frequency of stimulation used here. Fig 18 compares the blockade by ifenprodil of the early monosynaptic and the late polysynaptic components of responses elicited by DR stimulation . Ifenprodil blocks the early DR monosynaptic component to a significantly greater extent than the polysynaptic component (72.8% vs. 6.1%; Kruskal-Wallis ANOVA on ranks $p < 0.001$; n=14) suggesting that monosynaptic synapses that the DR fibres make directly onto the motoneuron have a greater proportion of NR1/NR2B-containing NMDA receptors. Although the monosynaptic and early portion of the late VLF response also began reliably, the all-or-none late plateau potential was initiated at highly irregular latency due to the variability in time required to reach its threshold voltage (Shanthanelson *et al.*, 2009). The late polysynaptic component of the VLF (Fig

16- ifenprodil) was abolished after repeated stimuli in ifenprodil, but because of its irregular latency even under control conditions, it was impossible to determine the effect of ifenprodil reliably. Therefore the action of ifenprodil on the monosynaptic and polysynaptic components of the VLF response is not compared.

In order to determine whether the reduced blockade of these DR polysynaptic late responses was due to insufficient amount of ifenprodil in the bath, 10μM ifenprodil was used in some experiments. The blockade of polysynaptic late component was not increased under these conditions (-2.7 ± 5.3% (n= 7) in 10μM vs 6.1 ± 14.3% (n= 14) in 3 μM). However, the general NMDAR antagonist MK-801 eliminated the polysynaptic response, showing that these responses were NMDA-mediated. Together, these findings suggest that the NMDA receptors associated with the polysynaptic connections between DR afferents and motoneurons differ from those associated with the monosynaptic connections to motoneurons in having fewer or less ifenprodil- sensitive NMDA receptors. There was no significant increase in the blockade of the monosynaptic component of the DR response by 10μM ifenprodil (74.2 % ± 1.3; n=8 in 10 μM vs. 75.8 ± 3.5%; n= 14 in 3μM) .In contrast to the monosynaptic response elicited by DR that did not show increased blockade, the monosynaptic component of the VLF response was blocked to a greater extent with 10μM ifenprodil (76.6 ± 4.1%, n=12 in 10μM vs. 48.4 ± 4.4% n=14 in 3μM ifenprodil; t-test, $p<0.001$). (See Discussion)

Are NR2B subunits being exchanged for other NR2 subunits?

The finding that ifenprodil did not completely block the response elicited by DR 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 used the activity-dependent, irreversible NMDA antagonist, MK-801.

After maximal blockade with ifenprodil was achieved, we introduced 10μM 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. The DR input alone was stimulated first (i.e no alternation with VLF) repetitively every 40s (Fig 15, 16 MK-801) until maximum blockade of the DR-response was achieved as determined by the EPSP amplitude reaching a steady state value. This generally took about 30 minutes. The VLF input was then stimulated until maximal blockade of that response was achieved.

The action of MK-801 varied depending somewhat on the age of the animal used and the input being stimulated. DR monosynaptic responses for rats age P1-4 on average did not display further blockade, showing that the response that remained after ifenprodil action was not caused by transmission through NMDARs composed of some other NR2 subunit in the place of NR2B (Fig 19). In rats P8 and older, the DR response displayed approximately 15% further blockade with MK-801, suggesting that there were other non-NR2B subunit containing NMDA channels at these synapses in addition to NR2B containing diheteromers. This is consistent with the observation that rat motoneurons at this age have NMDAR channels that are strongly inhibited by ifenprodil but have a higher conductance and a higher susceptibility to Mg^{2+} blockade than exclusively NR2B-containing diheteromers, arguing for the presence of triheteromeric populations (Palecek *et al.*, 1999).

As noted previously the blockade of the VLF monosynaptic response by ifenprodil was considerably less than the blockade of DR monosynaptic response. However, at all ages studied here, MK-801 blocked these VLF monosynaptic responses by about an additional 25% (Fig 19). (Holm- Sidak ANOVA p= 0.003). This provides further evidence that VLF NMDA receptors in motoneurons have a different subunit composition than those associated with DR (see Discussion).

In all of these experiments the state of the motoneuron and its synaptic inputs were determined at the end of the manipulations by perfusing with ACSF for 45 minutes to wash all antagonists from the bath. Following this 45 minute wash with no stimulation, both DR and VLF inputs were stimulated to verify that the AMPA responses had recovered. This further proved that the cell and its inputs had remained viable throughout the entire course of the experiment (5 hours in motoneuron) and that there was no substantial change in excitability of the spinal cord.

Discussion

We examined the ifenprodil sensitivity of NMDA receptor- mediated EPSPs produced by DR and VLF stimulation in neonatal rat lumbar motoneurons. First, we pharmacologically isolated the NMDA-mediated responses using an antagonist cocktail against CNQX, GABA and glycine in the bath. We showed that the remaining monosynaptic EPSP elicited by DR stimulation was consistently reduced to a greater degree by ifenprodil than the EPSP elicited by VLF stimulation. There was also a difference between the sensitivity of the monosynaptic and polysynaptic components of

the DR EPSP; the monosynaptic component was much more sensitive to ifenprodil blockade.

The interpretation of the data obtained depends heavily on ifenprodil's reported ability to act as a high affinity specific blocker of NR2B subunit containing diheteromeric NMDARs, at a concentration of 3μM (Legendre & 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, Lawrence et al. 2002; Hatton and Paoletti 2005). The inability of ifenprodil to block the entire response, even in primarily diheteromeric NR1/NR2B expressing oocytes, was attributed to the fact that ifenprodil alters the gating properties of these receptors by reducing channel open time and frequency, but the channel opening itself is not abolished by the antagonist and therefore some of the response still remains. (Williams 1993). If all receptors are triheteromeric with a NR1/NR2B/ NRXX subunit composition, ifenprodil action results in a 21% inhibition of the response (Tovar & Westbrook, 1999; Hatton & Paoletti, 2005). A mixed subunit population expression (di- and triheteromeric) results in ifenprodil inhibition intermediate between 0 and 90% depending on the available proportion of NR2B subunit containing receptors (Tovar & Westbrook, 1999; Hatton & Paoletti, 2005) . Coassembly of NR2B subunits with other subunits such as NR2A (Chazot & Stephenson, 1997; Tovar & Westbrook, 1999; Chazot *et al.*, 2002; Hatton & Paoletti, 2005) or NR2D (Pina-Crespo & Gibb, 2002; Brickley *et al.*, 2003; Jones & Gibb, 2005; Brothwell *et al.*, 2008) has been described previously. At 3μM, ifenprodil is identified to be a nearly saturating concentration for NR1/NR2B wild type receptors expressed in Xenopus oocytes (Perin-Dureau *et al.*, 2002). Thus by using

3μM of ifenprodil in our experiments, we ensured a selective high affinity blockade that would be maximum for NR1/NR2B diheteromers.

Ifenprodil did not completely block the DR or VLF monosynaptic responses; about 20-30% of the monosynaptic DR control response was ifenprodil-insensitive. Such incomplete blockade has been consistently reported in literature (Legendre and Westbrook 1991). One obvious explanation is the presence at these synapses of other subunit-containing diheteromeric NMDA receptors or triheteromeric NMDARs that are less sensitive to ifenprodil. Because ifenprodil consistently blocked DR monosynaptic responses to a greater extent than those from VLF on the same motoneuron, it seems likely that they have a different subunit composition, with DR synapses having a greater proportion of NR1/NR2B diheteromers. The general NMDAR antagonist MK-801, when applied alone (Fig 20), inhibited responses of DR and VLF to almost the same extent (61.6 ± 6.4 % SEM vs 69.5 ± 9.9% SEM; n=8; age matched data from (Shanthanelson *et al.*, 2009)), further showing that the difference between these synapses lies in the subunit composition of its receptors, and not in the availability of NMDA receptors at the synapses.

The proportion of NR2B containing diheteromeric NMDARs changes during postnatal development

At DR synapses of rats between P1-P4, a majority of the NMDA-mediated response is mediated by diheteromeric NR2B-containing NMDARs since most of it is blocked by ifenprodil. The 25% that remained was generally not reduced in amplitude by MK-801. Despite this observation, we believe that this remaining response was mediated

by NMDA receptors. In previous work we suggested that 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). We also showed that the 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. The extrasynaptic NMDARs would therefore no longer be inhibited by ifenprodil, and would not be blocked by MK-801 since by definition they were not activated synaptically. These receptors would be functional because the action of ifenprodil is fully reversible (Williams, 1993), and 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 remains after ifenprodil and MK-801 action.

The inhibition at VLF- activated synapses, across all ages, was 48.4± 4.4% (n=14), consistent with (although not proving) the idea that a greater proportion NMDARs comprising these synapses have a triheteromeric subunit configuration since these would experience a 50% inhibition by ifenprodil (see above). However, it is not possible to specify the identity of fourth subunit that is part of the NMDAR tetramer. These VLF receptors would nonetheless exhibit further blockade by MK-801 because VLF synapses are less able to accumulate trafficking receptors and are therefore less

susceptible to recover from MK-801 blockade than DR synapses (Shanthanelson *et al.*, 2009). An additional observation that supports the idea that VLF synapses have a greater proportion of triheteromeric or NR1/NR2A receptors is that 10μM ifenprodil blocked the VLF monosynaptic response (76.6 ± 4.1%, n=12) to a greater degree than 3μM ifenprodil (48.4% ± 4.4, n=14), although the blockade of the DR monosynaptic response was unchanged. It has been reported previously that ifenprodil at concentrations 10μM 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). The fact that 10 μM ifenprodil increased the blockade of VLF monosynaptic responses only indicates that VLF synapses express triheteromeric and/or non NR2B diheteromeric NMDA receptors.

Input dependent differences in NR2A/B subunit expression on a common target neuron have also 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 & Huguenard, 2003), interneuron-CA1 synapses (Maccaferri & Dingledine, 2002), mossy fiber-CA3 synapses (Lei & McBain, 2002), Schaffer collateral (Sch)- CA1 pyramidal neuron-perforant path synapses (Otmakhova *et al.*, 2002) etc. In these systems differential expression of these subunits has been proposed to play roles in synapse strengthening, in identification of origin of response and in coincidence detection.

Monosynaptic connections from DR fibres onto motoneurons contain a greater proportion of NR2B- containing dihereromeric NMDARs than those formed polysynaptically through interneurons

Our results also point to a difference between monosynaptic and polysynaptic connections made by the DR fibres onto the motoneuron. The monosynaptic responses of the DR are much more susceptible to ifenprodil blockade (72.8%) than their polysynaptic counterparts (6.1%). The polysynaptic connections of the DR fibres are established through interneurons that synapse onto the motoneuron. A lesser blockade of the polysynaptic responses suggests that the synapses at both DR- interneuron and interneuron- motoneuron synapses have a smaller proportion of diheteromeric NR2B subunit containing NMDARs than DR- motoneuron synapses.

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 synapses. In the brain and brainstem this switch from primarily NR2B containing to non-NR2B (mainly NR2A) containing NMDARs usually occurs later than the second postnatal week (Kew *et al.*, 1998; Cathala *et al.*, 2000; Lopez de Armentia & Sah, 2003; Liu *et al.*, 2004b). However, in rat lumbar motoneurons the non-NR2B containing NMDARs are expressed as early as P1 in the VLF synapses. Early onset of NR2A expression and their postsynaptic localization have

been reported previously in developing motoneurons of the mouse hyoglossal nucleus. Expression in these motoneurons was said to peak between P1-P7 (Oshima *et al.*, 2002). In spinal motoneurons, NR2B is expressed during embryonic period and NR2A in the first and second postnatal week (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 of the requirement for motor activity immediately after birth.

In other neural systems, there is well documented evidence for the activity-dependent regulation of the developmental switch of NR2 subunits. In the context of ocular dominance plasticity, eye opening leads to the replacement of synaptic NR2B containing NMDAR with those with NR2A (Ramoa & Prusky, 1997; Roberts & Ramoa, 1999). The activity dependent redistribution of postsynaptic density 95 (PSD-95) and synapse associated protein (SAP-102) is said to enhance synaptic localization of NR2A (Sans *et al.*, 2000; Yoshii *et al.*, 2003; van Zundert *et al.*, 2004).

In the mammalian spinal cord polysynaptic reflexes develop 1-2 days before the establishment of the monosynaptic reflexes (Saito, 1979; Kudo & Yamada, 1987; Ziskind-Conhaim, 1990). Our results showing that the polysynaptic responses are less affected by ifenprodil than monosynaptic responses suggest that an activity dependent switch from primarily NR2B to non-NR2B containing NMDARs might occur sooner at synapses that begin functioning earlier in development.

Conclusion

NR2B subunits have been associated with "immature" synapses and reportedly are more prevalent in extrasynaptic sites in comparison to synaptic sites of newly formed receptors(Tovar & Westbrook, 1999) . We have previously shown that DR synapses mature at a significantly slower rate than VLF synapses (Arvanian *et al.*, 2004). We have also shown that trafficking of NMDA receptors from extrasynaptic to synaptic sites, a phenomenon associated with synaptic plasticity, occurs more readily at synapses formed by the DR inputs onto the motoneuron than those formed by the VLF. NMDA receptor surface mobility has been shown to depend on NR2 subunits; NR2B containing NMDARs spend less time at synapses and have a greater ability to traffic (Groc *et al.*, 2006). The current finding that there is an increased proportion of NR2B subunit-containing diheteromeric NMDARs at DR synapses is consistent with their later maturation and the increased trafficking of NMDA receptors.

In the early stages of development, NR1/NR2B subunits may play a significant role in synaptic plasticity. In dentate gyrus granule cells they play a pruning role in dendrite development, where they regulate dendrite patterning but not growth or branching ensuring that sensory information is properly represented in the cortex (Espinosa *et al.*, 2009). In the context of synaptic plasticity during development, it is beneficial to have synapses composed of receptors that enhance and encourage synaptic changes.

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 (less than seen in NR2A containing

channels), high conductance and high sensitivity to Mg^{2+} block. NR2A subunit containing NMDARs produce currents with the fastest decay time, deactivation kinetics, high conductance and high sensitivity to Mg^{2+} block (Cull-Candy & 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.

Figure 15: Action of ifenprodil and MK-801 on DR NMDAR-mediated 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. VLF responses described in this legend are displayed in Fig. 2. *Control*: After blockade of all non NMDA receptors with CNQX, bicuculline, strychnine and CGP 35348 (CNQX Cocktail) administered to the bath. Ten consecutive responses to DR and then to VLF (Fig. 2) are displayed. Note the reliable responses to DR and the irregular responses to VLF (Fig. 2). Traces on the right are averaged monosynaptic responses at higher gain and faster sweep speed. *Ifenprodil:* The NMDAR- mediated response was then blocked with ifenprodil (3μM). Alternate stimulation of DR (Fig. 1) and VLF (Fig. 2) (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 (right) display the initial response (black) and the last response (red) in ifenprodil. *MK-801*: 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 μM) 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 (Fig. 2). Single traces (right) display the initial response (red) and the last response (green) in MK-801. The bottom traces are averaged AMPA/kainate responses before CNQX cocktail (black) and at the very end of the experiment after the CXQX cocktail was washed out for about 45 min. (blue). Note the similarity in the responses before and after.

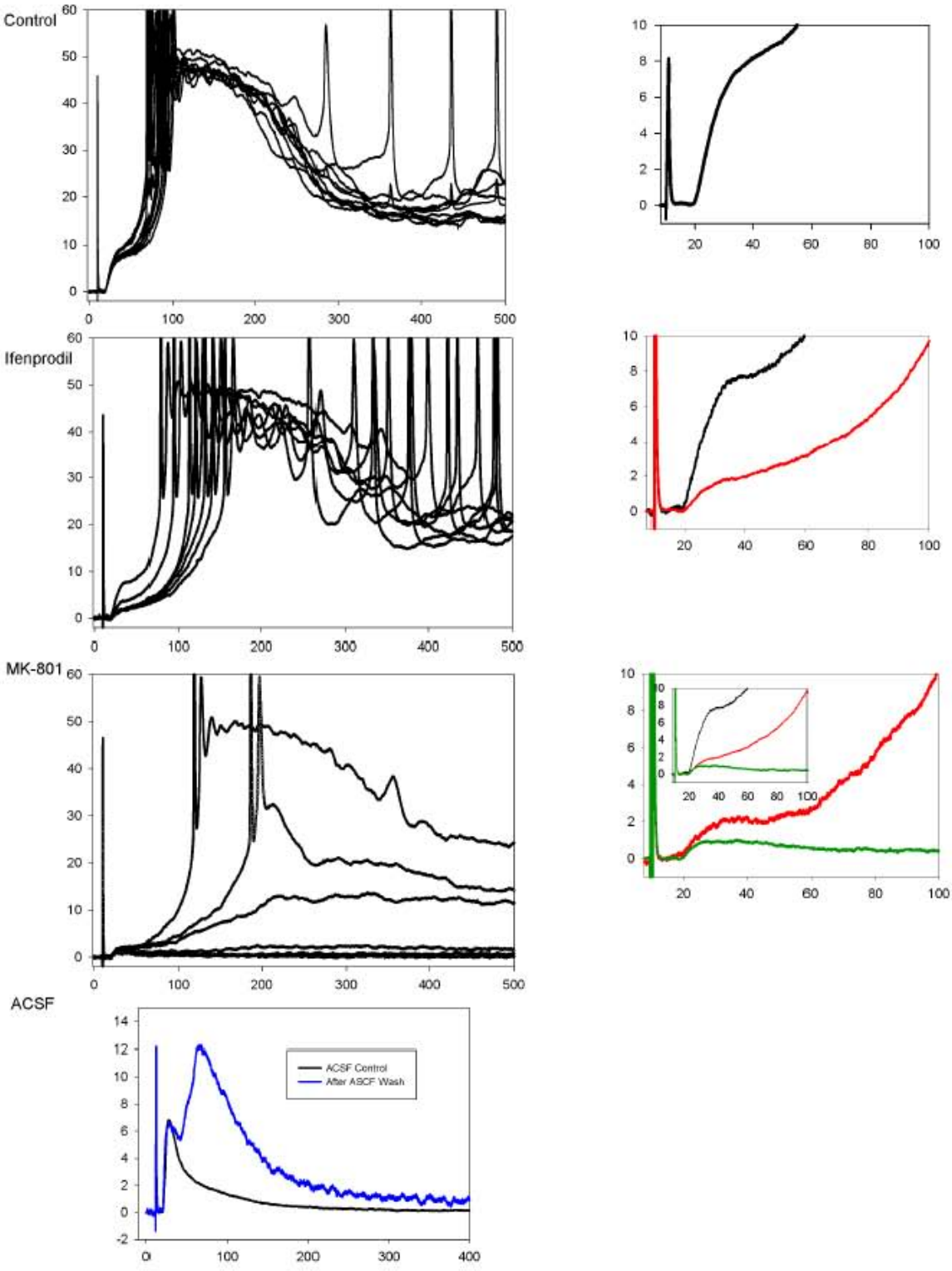

Control
Ifenprodil
MK-801
ACSF
ACSF Control
After ASCF Wash

Figure 16: Action of ifenprodil and MK-801 on VLF NMDAR-mediated EPSP

Protocol explained in legend to figure 1 except that these show the VLF synaptic responses that were obtained in the same cell as in Fig. 1. *Control*: NMDA receptor-mediated response to VLF stimulation (10 consecutive stimuli for DR and VLF); Ifenprodil: VLF response in ifenprodil (DR and VLF alternated; 40 stimuli each) *MK-801*: response in MK-801 (DR: 40 stimuli; DR: 60 stimuli). The bottom graphs are averaged AMPA/Kainate responses

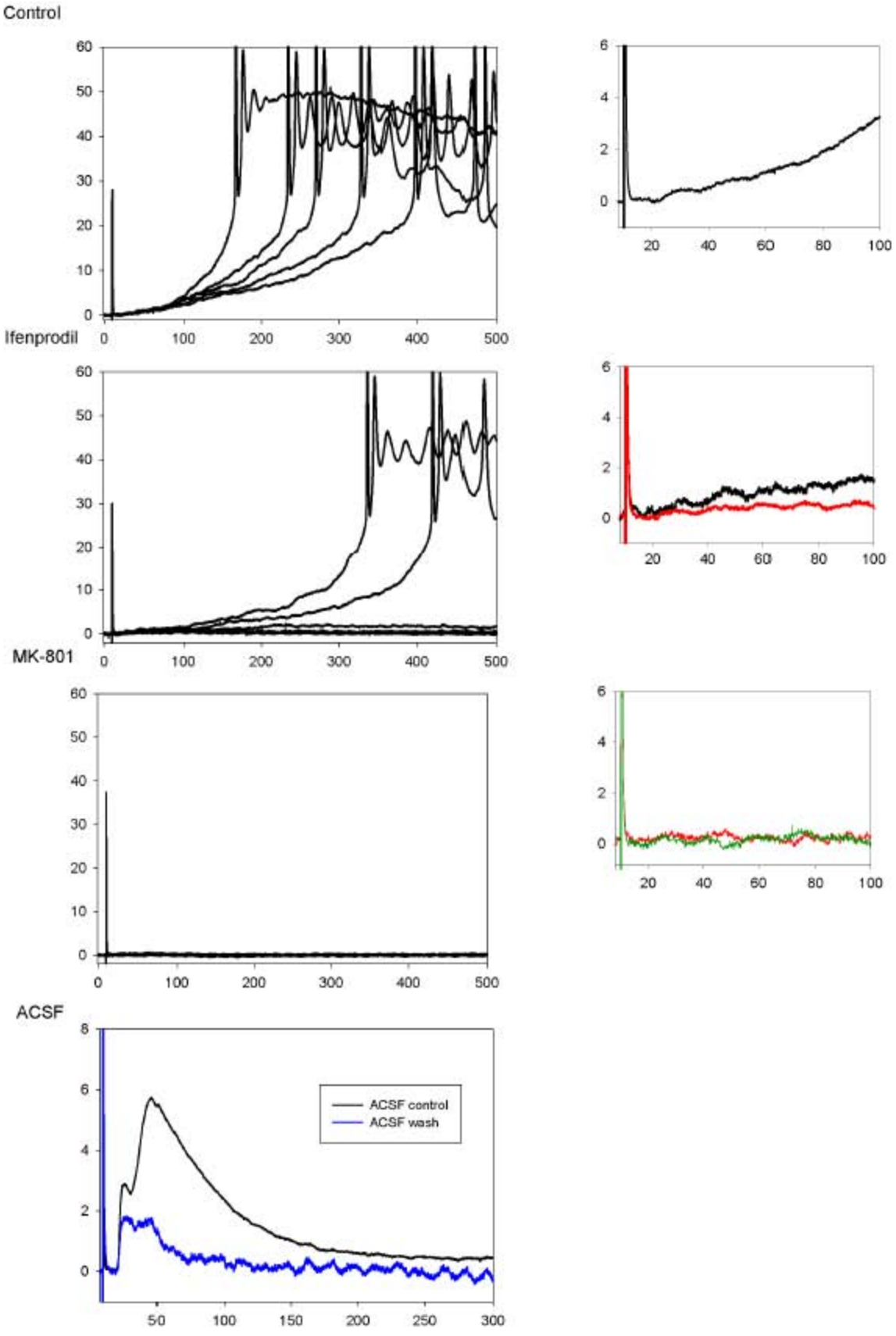

Control
Ifenprodil
MK-801
ACSF
ACSF control
ACSF wash

Figure 17: Comparison of sensitivity of DR and VLF NMDAR-mediated EPSP to ifenprodil

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 9 of 10 cells indicating that the blockade of DR was greater than blockade of response to VLF. Further details in text.

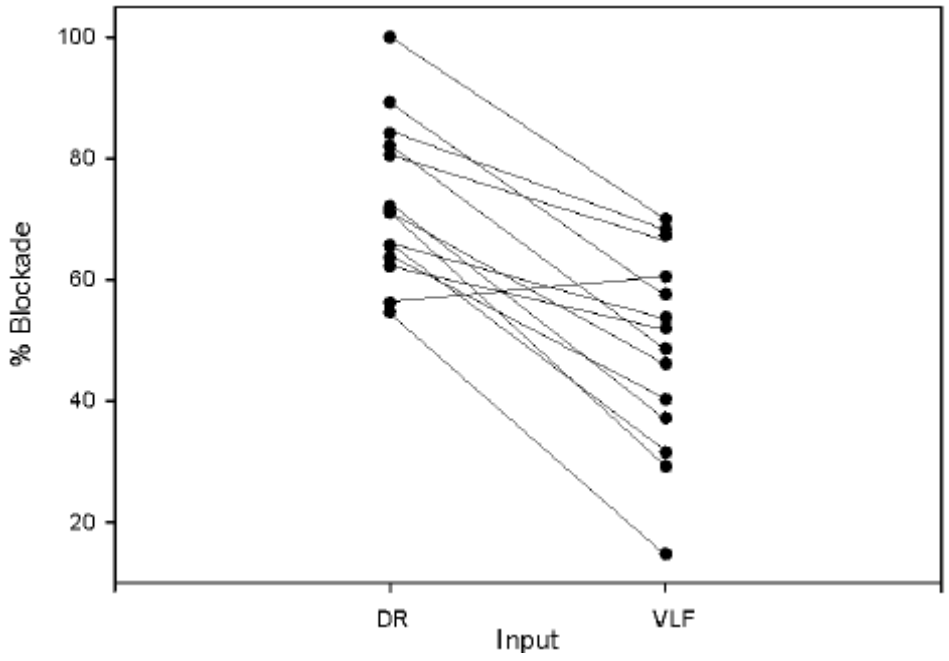


Figure 18: Monosynaptic component of DR EPSP is more sensitive to ifenprodil inhibition than the polysynaptic component

Bar graph displaying the mean percent blockade of the NMDA receptor- mediated monosynaptic and polysynaptic EPSPs elicited in the same motoneurons by DR stimulation. Left: DR (n= 14) was stimulated in ifenprodil. The first bar is the mean percent blockade of the monosynaptic component of the DR EPSP averaged over all cells. The second bar is the mean percent blockade of the polysynaptic component of the DR EPSP. Note that ifenprodil blocks the monosynaptic component significantly more than the polysynaptic component. No such difference is observed between the two components of the VLF response.

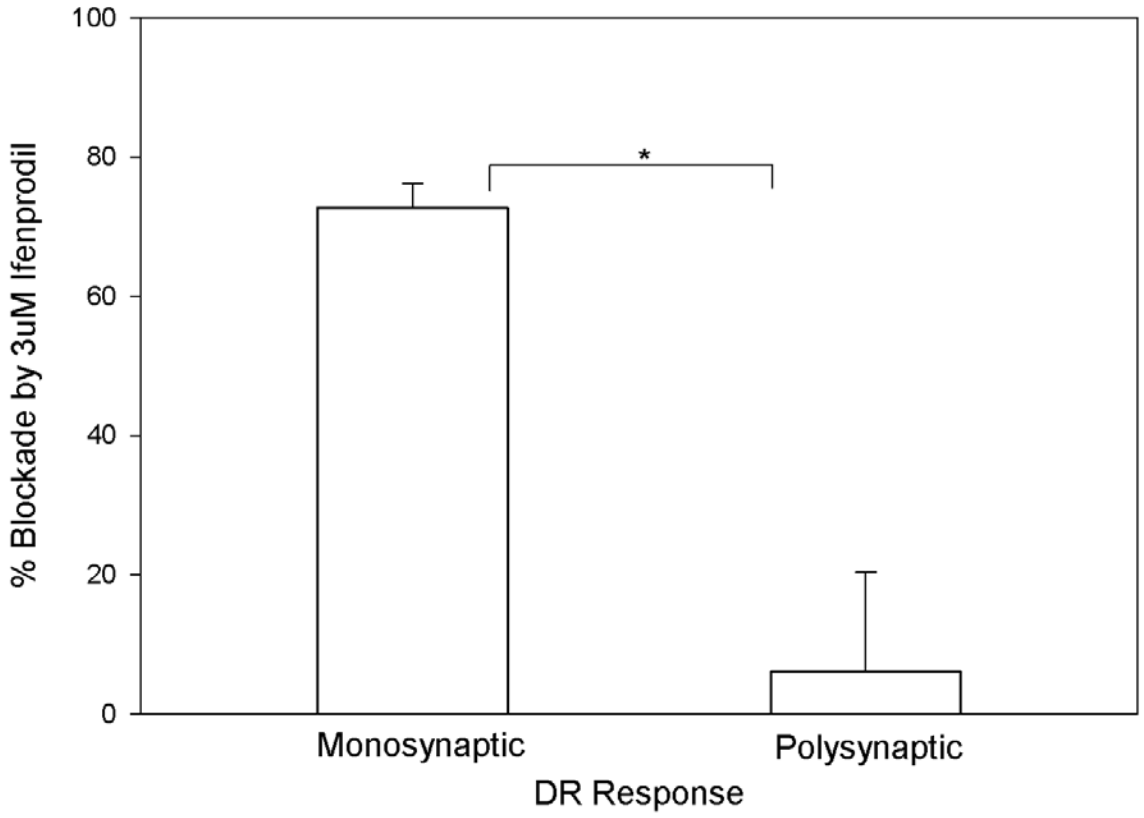


Figure 19: Contribution of Non-NR1/NR2B to DR and VLF NMDAR-mediated responses

Percent residual (see text for definition) of the DR (white) and VLF (grey) responses after maximal inhibition in ifenprodil (clear) and subsequent treatment with MK-801 (diagonals). Note that the DR monosynaptic response in P1-P4 rats that remains after ifenprodil treatment is not blocked further by MK-801. In rats P8 and older, the response that remains after ifenprodil treatment is further blocked by MK-801 although this was not significant. A greater percentage of the VLF response remains after ifenprodil treatment across all ages and are further blocked by subsequent MK-801 treatment.

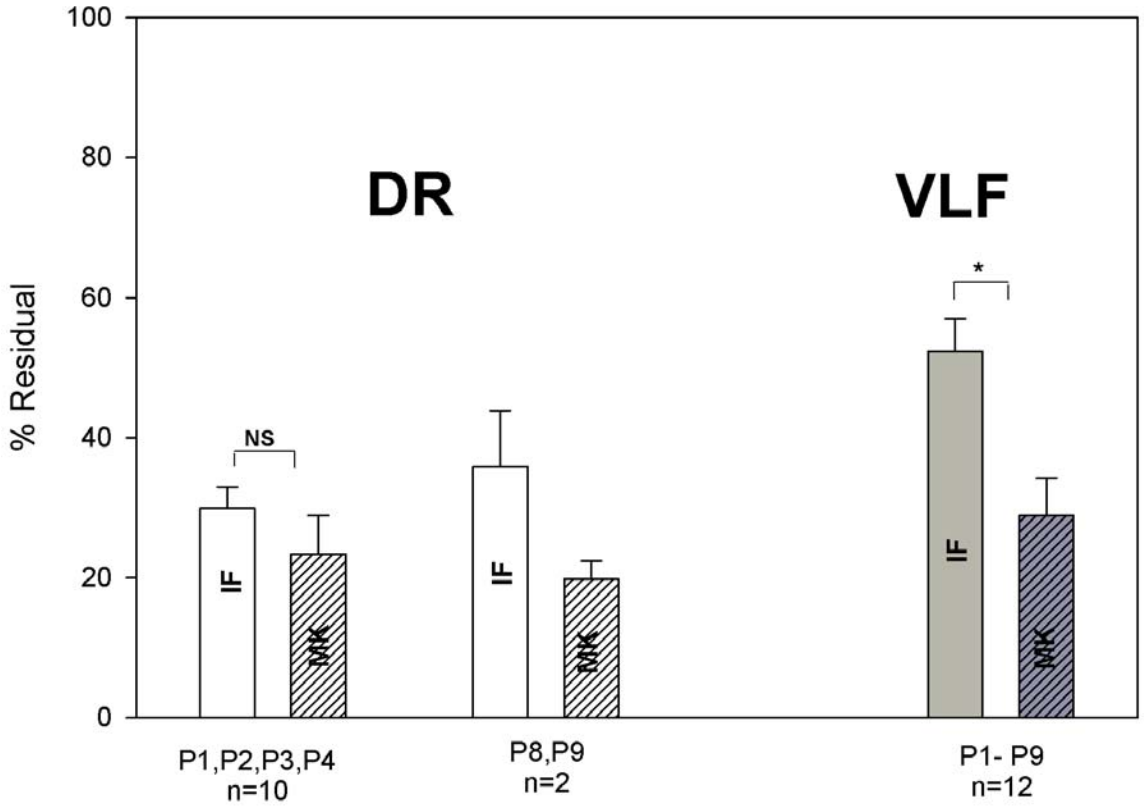
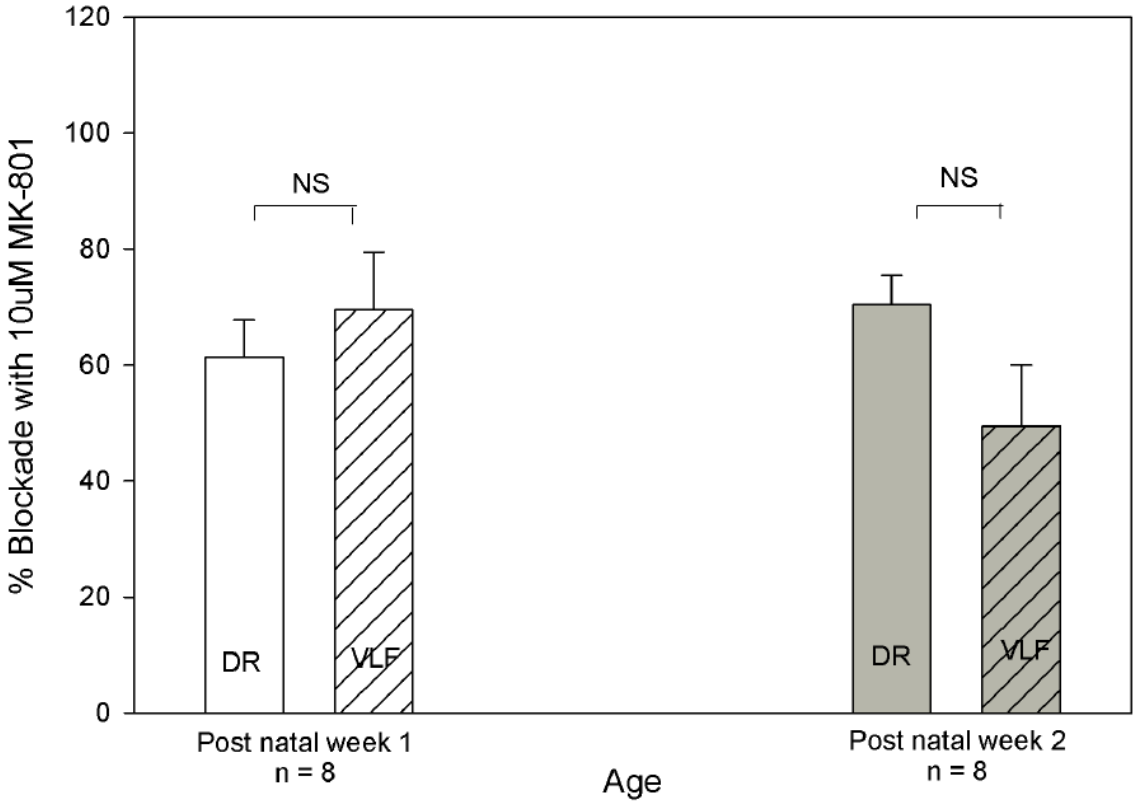


Figure 20: DR and VLF NMDAR-mediated EPSP are inhibited to the same extent by MK-801

NMDA mediated responses both in the first and second postnatal week were inhibited to the same extent by MK-801. Furthermore, there was no statistically significant difference between the action of MK-801 on DR and VLF EPSPs.



Chapter IV
NR2D subunits in motoneurons

Introduction

Chapter IV is supplemental material to the experiments that were addressed in Chapters II and III. It is composed of additional experiments that were done in order to explore the topics at hand further.

The objective of this chapter is to lead to future experiments that need to be pursued, with the background of what's been done already. This section is organized as a list of experiments that were performed and the discussion of results immediately follows the result section from each experiment.

Pretreatment with NR2D using HSV viral vectors improves recovery of VLF EPSPs from MK-801 blockade in rats in the second postnatal week

NMDA responsiveness decreases in the second post natal week. RT-PCR and gene chip analysis showed that NR2D subunit of the NMDAR, which confers resistance to Mg^{2+} blockade (Momiyama *et al.*, 1996) was the only subunit that was significantly down regulated in the second post natal week in motoneurons (Arvanian *et al.*, 2004).

We hypothesized that the decrease in the ability of VLF to recover from MK-801 blockade might be related to the down regulation of NR2D. In order to test this hypothesis, Herpes simplex virus type-1 amplicon (Bowers et al. 2000; 2001) carrying either NR2D (HSVnr2d) or *E. coli*-derived ß-galactosidase (as a control) (HSVlac) was injected directly into the ventral horn of P2 rats. Cords injected with HSVnr2d or HSVlac were removed at P8-11. They were prepared for electrophysiological examination of the ability to recover from MK-801 blockade as described previously in Chapter II.

Results

HSV-mediated delivery of NR2D induced marked functional changes of synaptic input to motoneurons from treated P8-11 animals. Table 1 shows that VLF EPSPs of the rats in the second postnatal week that underwent HSVnr2d pretreatment in the first post natal week recovered considerably more from MK-801 blockade (91.8 ± 29% SE n= 7) than those animals that either had not undergone NR2D pretreatment (-165.1 ± 124 % SE; n=8) or undergone the control β-Gal treatment (-7.8 ± 13.4% SE; n= 6). (Holm-Sidak method of ANOVA; p=0.005).

The DR EPSPs in the second postnatal week also showed a similar trend of increase in recovery from blockade with NR2D pretreatment although the improvement was not as extensive as that was seen in the VLF EPSPs.

Discussion

In the brain NR2B and NR2D are the two NMDA receptor subunits that occur prenatally and NR2D peaks around P7 and thereafter decreases to adult levels while the other subunits peak around P20 (Monyer *et al.*, 1994). Since NR2D subunits are progressively lost as the animals develop, and VLF synapses appear to "mature sooner" (trafficking of NMDARs does not occur at VLF synapses during the same developmental period when it does in DR synapses in the same motoneuron), it is possible that the loss of NR2D subunits occurs sooner in VLF synapses in comparison to DR synapses. Restoring NR2D subunits could confer on VLF synapses the ability to return to their earlier more plastic state.

The current result shows that increasing the availability of NR2D subunits

enhances the ability of VLF EPSPs to recover from "irreversible" MK-801 blockade, an ability neither the sham operated, nor the rats without surgical manipulations have. Reintroduction of NR2D has therefore conferred upon these VLF synapses an increased ability to accept trafficking receptors.

NR2D subunits decrease the magnesium blockade of NMDARs, and it is possible that their increased availability would allow more calcium influx and an increased chance of plasticity at these synapses. We cannot presently describe the mechanism involved in encouraging receptor trafficking. While NR2B subunits are simply less efficiently attached to the postsynaptic density and hence are naturally more mobile, there isn't enough information about the trafficking ability of NR2D subunit containing NMDARs. Whether increasing NR2D availability results in the mobility of NR1/NR2D or indirectly increases mobility of NR1/NR2B at these synapses, is unknown. The extra-synaptic receptor pool is primarily composed of NR2B and some NR2D containing NMDARs, even in adults (Momiyama, 2000; Harney *et al.*, 2008). Increasing the availability of the receptors at the extrasynaptic pool by HSV-NR2D delivery could result in an increase in trafficking.

It should be noted that individual cells in animals treated with HSV-NR2D varied in their values of recovery from MK-801 blockade. Previous anatomical data shows that not all motoneurons were infected by HSV vector. HSV infects approximately half of the motoneurons and some peripherin negative neurons (i.e interneurons), but a significant elevation of NR2D protein was seen in the infected motoneurons. Delivery of HSVnr2d resulted in a 1.2 fold enhancement of expression of NR2D mRNA at P12 compared to controls injected with HSVlac (Arvanian *et al.*, 2004). The difference in infectivity

between motoneurons might offer an explanation for why some cells showed better recovery than others from MK-801 blockade.

We did not, however, treat cords with HSVnr2b. One question that needs to be addressed is whether increasing the availability of other subunits at these synapses would also result in increased trafficking of receptors or does trafficking ability depend exclusively on the NR2D and NR2B subunits. Another experiment to be considered is siRNA interference studies. Currently available techniques do not allow siRNA interference in intact spinal tissue. If siRNA could be tagged to lenti-virus vector (Lenti-viruses are transported in the cord faster than HSV) knock-out of these subunits in the first postnatal week could possibly be achieved. It would be interesting to see if lack of the NR2B and NR2D subunits in the first postnatal week would result in an earlier replacement with NR2A at these synapses and whether that would result in decreased trafficking of NMDARs at these synapses during a time period when trafficking normally occurs readily. The alternate possibility is that these motoneurons may simply die due to the loss of these subunits at this critical developmental stage. These would be complex experiments whose results would be difficult to interpret in the neonatal preparation and hence were not pursued.

The NR2D subunit blocker cis-PPDA eliminated both DR and VLF NMDA mediated responses

In order to study the contribution of other NMDA receptor subunits to the NMDA-mediated responses of both the DR and VLF during the first postnatal week, we employed the NR2C/NR2D preferring NMDA receptor antagonist cis-PPDA. K_i values

of cis-PPDA are 0.096 μM, 0.125 μM, 0.55 μM and 0.31 μM for NR2C, NR2D, NR2A and NR2B subunits respectively. Numerical values of K_i reciprocally reflect inhibiting potency; that is, low values of K_i indicate high inhibiting potency. Cis-PPDA is 1.3, 3.2 and 6 times more potent at binding NR1/NR2C in comparison to NR2D, NR2B and NR2A respectively (Morley *et al.*, 2005).

Results

The experimental protocol is similar to the one described in section II which was used for the trafficking studies. NMDA-mediated responses were first isolated in a cocktail of antagonists against AMPA, $GABA_A$, $GABA_B$ and glycine that were added to the bath. In the place of MK-801, 10 μM *cis*-PPDA was used. All other parts of the protocol remain unchanged. It should be noted that *cis*-PPDA is insoluble in water and its final concentration at the motoneuron could not be reliably established, and could have been slightly less or more than 10 μM.

Figure 21 and 22 show DR and VLF responses respectively, obtained from a P4 rat. *Cis*-PPDA, unlike ifenprodil eliminated both the DR and VLF NMDA-mediated EPSPs (Fig 21, 22 *cis* PPDA) and resulted in a 100% blockade. After *cis*-PPDA was washed from the bath, the NMDA mediated responses of the DR recovered to above control values (Fig 21; wash). While the monosynaptic component of the VLF response recovered modestly, the polysynaptic component did not (Fig 22; wash). DR and VLF responses obtained after wash-out of all antagonists showed that the motoneuron and its inputs had remained viable through-out the experiment (Fig 21, 22; Wash).

Discussion

Cis-PPDA completely eliminated both DR and VLF NMDA-mediated responses. It is, however, a very modestly selective antagonist for the NR2C/2D subunits. It has a 6 fold increased affinity for NR1-NR2C/D over NR1/NR2A while ifenprodil has a 400 fold increased affinity for NR1/NR2B over NR1/NR2A. It however has a 100-fold higher affinity for NMDA receptors vs AMPA receptor binding sites, and therefore selectively blocks NMDA receptors (Morley *et al.*, 2005). In addition to its lack of subunit specificity, its concentration at the motoneuron could not be reliably established because of its water insolubility. It is also possible that at approximately 10 μM, the concentration used was greater than what would be appropriate for ideal resolution between subunits. Therefore the complete blockade of the NMDA-mediated response does not indicate that the NMDA mediated response is completely mediated by NR2C and NR2D containing NMDARs. It is far more likely that the response was completely eliminated because cis-PPDA is relatively non-specific and blocked all available NMDA receptors in the motoneurons irrespective of their subunit composition. Its action, in this case, could be likened to that of a general NMDAR antagonist like APV.

Complete blockade of the NMDA-mediated response could not be achieved with MK-801 when used alone, raising the question of whether these responses were indeed completely NMDA-mediated. Chapter II addresses this question and argues that the persistent response, which is not blocked by MK-801 is NMDA-mediated and should be attributed to the dynamic trafficking of receptors from the extra-synaptic to synaptic sites. Since MK-801 is activity dependent, these extra-synaptic receptors were unblocked and available to move into synaptic regions. *Cis*-PPDA however blocks all available

NMDARs (both synaptic and extrasynaptic) since it is not activity dependent. The lack of the persistent response after cis-PPDA action provides further evidence that it is completely NMDA mediated and when trafficking of unblocked receptors is eliminated, the persistent response is also abolished.

Wash out of the reversible cis-PPDA results in complete recovery of the DR monosynaptic and polysynaptic responses. While the VLF monosynaptic component recovers modestly, the polysynaptic component of the EPSP does not, once more indicating a difference between the DR vs VLF synapses and monosynaptic vs polysynaptic synapses. The recovered response of the DR synapses recovered to a much higher value than their control value. The AMPA response obtained after the wash-out of all antagonists also increased to a significantly larger value than its control. It should be noted that there was no difference in the latency between the response after recovery and the control. One of the hallmark features of synaptic plasticity is rapid recruitment of receptors to a synapse which is activated but has no functional receptors available. The higher than control values of recovery of both NMDA and AMPA responses at DR synapses could be caused due to rapid recruitment of these receptors. The VLF synapses, as established in Chapter II, have a lesser ability to accumulate trafficking NMDA receptors and their responses do not show an appreciable recovery after wash-out. However AMPA responses at VLF synapses recovered to values larger than control values, possibly suggesting that VLF synapses might recruit AMPA receptors more efficiently than NMDA receptors.

Given the unpredictability of the concentration of *cis*-PPDA available at the motoneuron and its non-specificity, conclusive remarks cannot be made about the

expression of NR2C/NR2D subunit containing NMDA receptors in the motoneurons of these rats. Further experiments were therefore discontinued.

Table 1: % Recovery of EPSP from MK-801 blockade; with or without viral injections

% Recovery of EPSPs from blockade by MK-801, in the control animals, animals treated with HSV-NR2D and animals treated with HSV-βGal. The viral treatment was given at P2 and the animals were allowed to survive. The recovery from MK-801 blockade was measured in the second postnatal week. All animals are age matched.

	% RECOVERY (UNTREATED CORD)	**% RECOVERY (HSV-NR2D)**	**% RECOVERY (HSV- BGAL)**
DR Monosynaptic EPSP	66.8 ± 42.5 % SE (n= 8)	124.1 ± 10.8 % S.E (n=5)	34 ± 51.7 % SE (n=6)
VLF Monosynaptic EPSP	-165.1 ± 124 % SE (n=8)	91.8 ± 29 % SE (n=7)	- 7.8 ± 13.4 % SE (n= 6)

Figure 21: Illustration of procedures to study effect of cis-PPDA on NMDAR-mediated responses of DR

Illustration of the procedures carried out on an individual motoneuron from a P4 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. VLF responses described in this legend are displayed in Fig. 2. *Control*: After blockade of all non NMDA receptors with CNQX, bicuculline, strychnine and CGP 35348 (CNQX Cocktail) administered to the bath. Ten consecutive responses to DR and then to VLF (Fig. 2) are displayed. Note the reliable responses to DR and the irregular responses to VLF (Fig. 2). Traces on the right are averaged monosynaptic responses at higher gain and faster sweep speed. *Cis-PPDA:* The NMDAR- mediated response was then blocked with cis-PPDA (10μM). Alternate stimulation of DR (Fig. 1) and VLF (Fig. 2) (a total of 40 stimuli to each input at 0.025 Hz). Trial 1 was obtained immediately after introducing cis-PPDA into the bath and the decline of monosynaptic response occurred immediately. The stimulation was stopped when steady state was reached. Single traces (right) display the initial response (black) and the last response (red) in cis-PPDA. Cis-PPDA was then washed from the bath with ACSF for 45 min. in the absence of stimulation while continuing to apply the non NMDA antagonists. *Wash*. Superimposed responses after cis-PPDA washout are displayed along with the initial response at high gain (on the right). Note the lesser recovery of VLF NMDA response- the late response never reappeared in contrast to DR. The bottom traces are averaged AMPA/kainate responses before CNQX cocktail (black) and at the very end of the experiment after the CXQX cocktail was washed out for about 45 min. (blue).

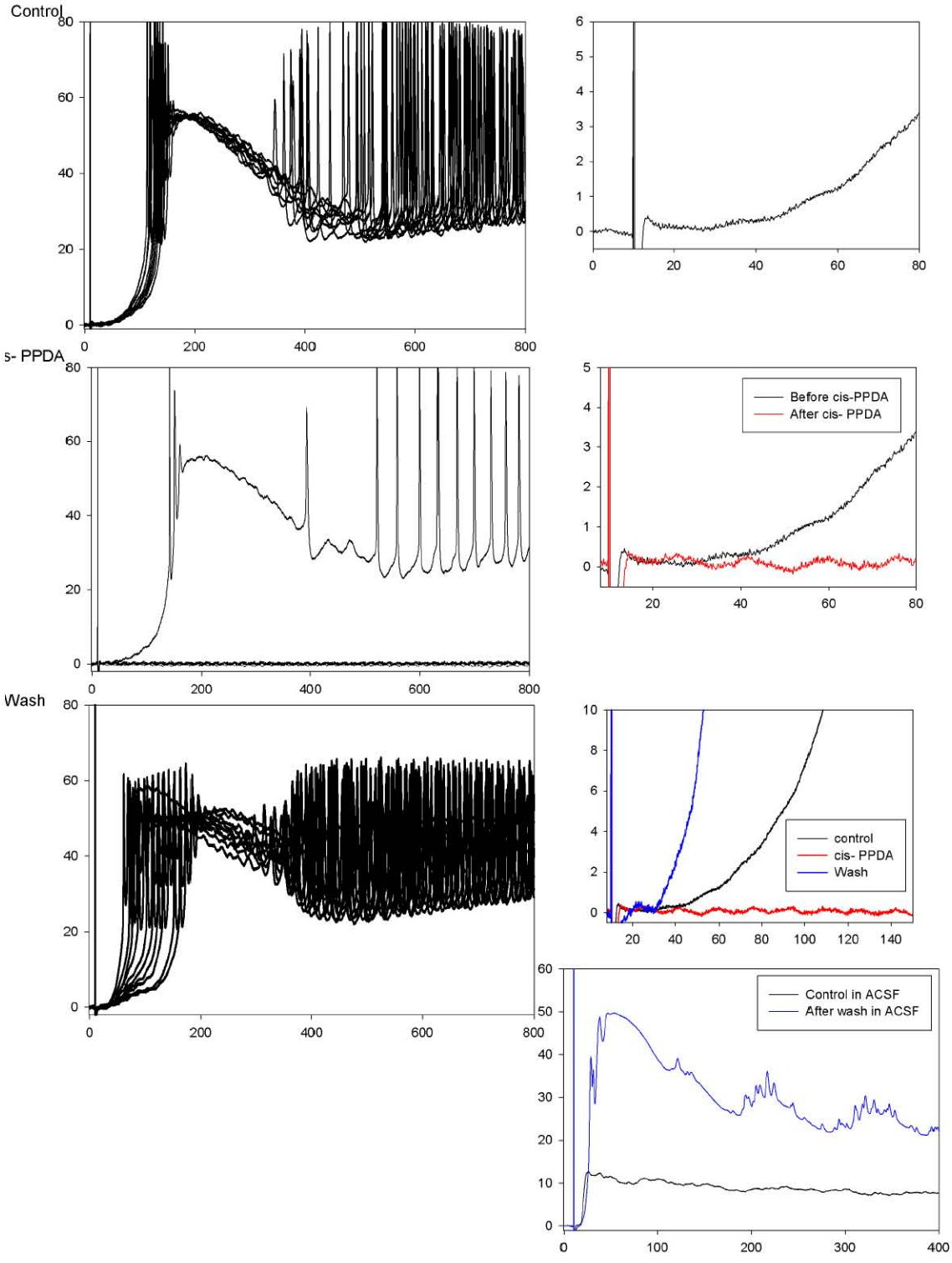
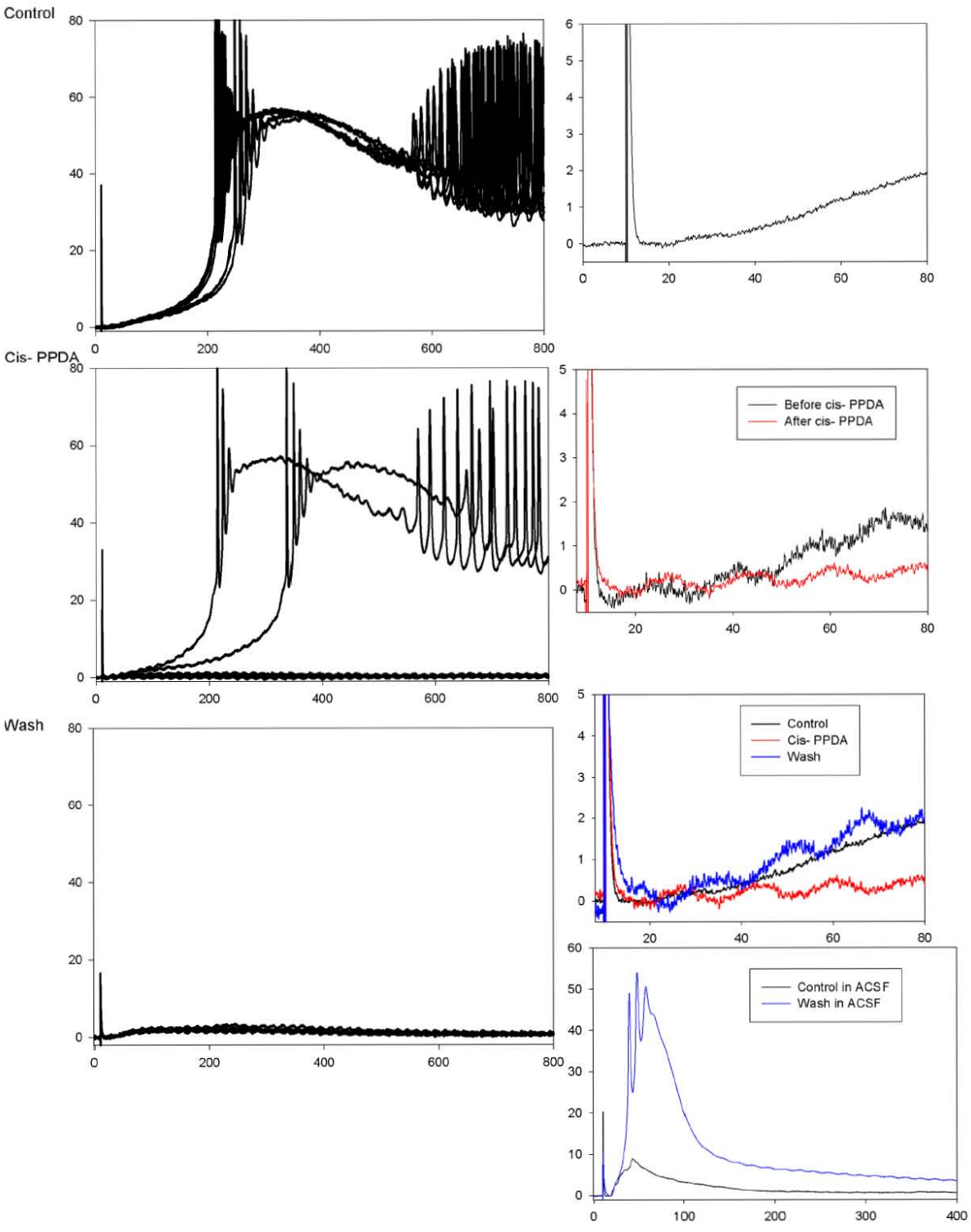

Control
s- PPDA
Wash
Before cis-PPDA
After cis- PPDA
control
cis- PPDA
Wash
Control in ACSF
After wash in ACSF

Figure 22: Illustration of procedures to study effect of cis-PPDA on NMDAR-mediated responses of VLF

Protocol explained in legend to figure 1 except that these show the VLF synaptic responses that were obtained in the same cell as in Fig. 1. *Control*: NMDA receptor-mediated response to VLF stimulation (10 consecutive stimuli for DR and VLF); *cis-PPDA*: VLF response in cis-PPDA (DR and VLF alternated; 40 stimuli each) *Wash*: response after 45 minute, no stimulation wash-out period to remove cis-PPDA (DR: 40 stimuli; DR: 60 stimuli). The bottom graphs are averaged AMPA/Kainate responses


Control
Cis- PPDA
Wash
Before cis- PPDA
After cis- PPDA
Control
Cis- PPDA
Wash
Control in ACSF
Wash in ACSF

CHAPTER V

General Discussion

Using electrophysiological and pharmacological techniques we have demonstrated that NMDA receptors associated with synapses of the DR and VLF are independent of each other. DR synapses have a greater proportion of NR1/NR2B, and the NMDA receptors associated with them show an increased ability to traffic from extrasynaptic to synaptic sites. VLF synapses have a greater proportion of non-NR2B containing diheteromeric and triheteromeric NMDA receptor populations, and there is very little trafficking observed in receptors associated with them. The trafficking ability, however, can be restored at VLF synapses by increasing the availability of NR2D subunits. The developmental switch from primarily diheteromeric NR1/NR2B to triheteromeric NR1/NR2B/NRXX occurs earlier in development at synapses of the VLF than of the DR.

The results of this work should be interpreted in the context of development that is occurring in the rat pup during the first and second postnatal week when these experiments were undertaken. Motoneurons are produced between E11-12 in the cervical cord and E13-14 in the lumbo-sacral cord (Altman & Bayer, 1984). Motor behavior of the rat is immature at birth and rapid maturation occurs in the first and second postnatal weeks. It is at the end of the 1st postnatal week that animals are able to lift their trunk from the floor and to walk spontaneously (Geisler *et al.*, 1993; Jamon & Clarac, 1998). Although the adult pattern of locomotion does not emerge until P16, during the early postnatal period the rat is capable of spontaneous locomotor-like activity like air stepping (McEwen *et al.*, 1997; Jamon & Clarac, 1998) and swimming (Cazalets *et al.*, 1990; McEwen *et al.*, 1997)

The greatest growth of soma area of the motoneuron occurs during the second postnatal week. A 4 fold increase in soma area of the soleus ankle extensor motoneuron occurs during the first 3 postnatal weeks, with the greatest growth occurring in the second postnatal week. At P21 they reach about 70% of their adult size (Westerga & Gramsbergen, 1992). The characteristic dendritic arbor of the motoneurons develops predominantly during the postnatal period; dendritic branches increase in length during the first two months after birth (Dekkers *et al.*, 1994).

Maturation of membrane properties of motoneurons also occurs during this period. Motoneurons develop excitability at about E15. At E17 NMDA, AMPA and kainate channels contribute to the short-latency monosynaptic component of the EPSP produced in motoneurons. The long-latency polysynaptic component of the DR EPSPs is mediated exclusively by NMDA receptors (Konnerth *et al.*, 1990; Ziskind-Conhaim, 1990). In the first and second postnatal week there is a large increase in density of synaptic voltage gated ion channels (Gao & Ziskind-Conhaim, 1998). Adult expression levels of the L-type calcium channels are reached at P18; the plateau potentials they mediate might have relevance for the development of posture and locomotion (Jiang *et al.*, 1999). Large increases in noninactivating delayed rectifier type- current has also been observed right after birth (Gao & Ziskind-Conhaim, 1998).

Since the first two postnatal weeks are critical in the development of motoneurons and motor behavior and the motoneuron properties that are established during this time point are carried through to adult life, our experiments examined NMDA-mediated responses during this critical time window. Expression of NMDA receptors and their subunits, staggered development of the DR and VLF synapses, and the synaptic plasticity

mediated by NMDA receptor trafficking that occurs at these synapses and the implication that these changes have for the acquisition of motor skills during this critical time window, are investigated.

Contrary to immunocytochemistry results, functional NMDA receptors exist in neonatal motoneurons in the first and second postnatal week

In situ hybrization data suggests that functional NMDA receptors are gradually lost from the spinal cord starting after the first postnatal week (since the only globally available NMDAR subunit detected in the spinal ventral horn is NR1 (Kalb *et al.*, 1992; Stegenga & Kalb, 2001) and monomeric NMDARs do not form functional channels (Seeburg, 1993; Hollmann & Heinemann, 1994; Nakanishi & Masu, 1994; Mori & Mishina, 1995)). However, functional NMDA receptors persist during this period. Our electrophysiological experiments show the presence of NMDA-mediated responses at these synapses during both the first and second postnatal week (Table 2). The presence of these responses demonstrates that NMDA receptors are expressed in their di or triheteromeric configurations that allow them to be functional at these synapses.

Why then do *in situ* hybridization which detects specific mRNA sequences by hybridizing the complementary strand of a nucleotide probe to the sequence of interest, and conventional immunocytochemistry not detect much NR2 subunits in the ventral horn of the neonatal cord? There are two immediate possibilities: the mRNA to be detected is at too low a concentration (enough to form subunits after translation but not enough to detect) or NMDA receptors at synapses are being masked by protein meshworks associated with the postsynaptic membrane (Watanabe *et al.*, 1998). NR1

subunits are synthesized in a large excess (approximately 10 fold more) when compared to the NR2 units (Huh & Wenthold, 1999) and their mRNA is present at very high levels and would be detected more easily than NR2 subunit mRNA. Associated proteins also become more complex as the animal ages and this may also contribute to making NR2 subunits of synaptic receptors difficult to detect (Watanabe *et al.*, 1998). Increasing the permeability of the cell and making the nucleotide sequence more available to bind to the probe (Nagy *et al.*, 2004) may provide a more accurate picture of the expression of these subunits.

Another possibility is that the gradual reduction in NR2 signal may be due to a general loss of motoneurons that occurs during this time. The literature suggests that about 50% of the motoneurons are lost during a critical period from embryonic day 14 until postnatal day 3 (Sendtner *et al.*, 2000). Motoneuron cell death as seen throughout the first and second postnatal week is said to correlate with loss of NMDA receptors which have been suggested to play a role in regulation of motoneuron survival in the early postnatal period (Hori *et al.*, 2002). The reduction in NR2 signal strength that occurs as the rats develop, as detected with *in situ* hybridization and immunocytochemistry, may therefore be a reflection of the NMDARs that are lost with the motoneurons that die, and may not be a measure of the loss of NMDA receptors in viable motoneurons that survive this critical period.

The concentration of Mg^{2+} in the bath had to be reduced to enable us to see the NMDA-mediated component of these responses. This suggests that the gradual disappearance of the NMDA-mediated response in motoneurons during this time period, as suggested by electrophysiological work from other labs (Hori & Kanda, 1996; Hori *et*

al., 2002), may be partly due to the increased magnesium blockade of the receptors and not necessarily due to a complete loss of NMDA receptors from the motoneurons (Arvanian & Mendell, 2001b; Arvanian *et al.*, 2004).

Furthermore, if an appreciable decrease in synaptic NMDA receptor number between first and second postnatal week occurs, we would expect a concurrent decrease in the amplitude of the NMDA-mediated response. However, our results indicate that neither the amplitude of the monosynaptic response nor the contribution of NMDA mediated response to the monosynaptic response changes within the first and second postnatal week (Table 2). These results are consistent with what was observed for the total synaptic response (mainly AMPA) by (Seebach & Mendell, 1996). They noticed that as the animals develop, rheobase, and input conductance increases, but there are no changes in the amplitude of the EPSP suggesting that "some aspect of these synapses underwent a compensatory change" as the motoneuron becomes larger.

Even though the mean amplitude of NMDA-mediated EPSP does not change over the first and second postnatal week, there is much variability in the amplitude of DR (or VLF) responses within the group of animals in the same age. This may be because the individual motoneurons belonged to different motor pools. In our experiments motoneurons were identified to be in the L5 segmental region whose axons traveled in the L5 ventral root bundle, and produced antidromic action potentials in response to its stimulation; the motor pools they belonged to were not identified. Differential maturation of membrane properties and of the postsynaptic distribution of receptors between motoneurons belonging to different motor pools have been described (Vinay *et al.*, 2000a).

Developmental changes in the subunit composition of NMDA receptors in lumbar motoneurons

Developmentally regulated expression of subunits of ionotropic glutamate receptors is well documented. In the AMPA receptor complex not all GluR subunits are expressed similarly over development; for example, the GluR4 subunit is expressed only during early postnatal period (Wenthold *et al.*, 1996; Zhu *et al.*, 2000; Petralia *et al.*, 2005). It is not surprising that the NMDA receptors follow a similar type of subunit regulation to allow maturation of synapses. In the rat brain and brainstem NR2B and NR2D occur prenatally, NR2A and NR2C are first detected around birth. NR2D peaks around P7 and the others peak around P20 and thereafter decrease to adult levels (Monyer *et al.*, 1994). In adulthood there is spatial regulation of subunits, i.e specific subunits are restricted to specific regions of the brain. For example NR2C is present in the cerebellum, NR2D is restricted to diencephalic, mesencephalic and brainstem structures (Wenzel *et al.*, 1996). A somewhat similar trend in the developmental regulation of NMDA receptor subunit expression occurs in motoneurons of the lumbar spinal cord and is discussed below.

NR2B subunits:

Figure 23 in the Supplementary Figures section shows a trend that was observed in ifenprodil's inhibitory action on NMDA-mediated responses in the motoneurons during the first and postnatal week. Expression of NR2B subunits, which are among the most prevalent subunits during the embryonic period, peaks around P3 (seen as the age point where maximum blockade with ifenprodil occurred) and is subsequently reduced. That both DR and VLF synapses showed a similar trend gives credence to this

observation, although there was not enough power to establish statistical significance. Anatomical data in rats show that the outgrowth of dorsal root fibres and formation of monosynaptic contacts begins at E15 and lasts until P2. From P2 onwards stabilization and refinement of connections with elimination of redundant and possibly inappropriate synapses occurs (Kudo & Yamada, 1987). The downregulation of diheteromeric NR2B after P3 is consistent with the idea that replacement of synaptic NR2B with NR2A or other non-NR2B containing channels which is required for the stabilization of receptors at the synapse (Groc *et al.*, 2006) occurs at this developmental stage.

NR2D subunits:

While it was not possible to derive any conclusions about the expression of NR2D subunits in the motoneurons from the cis-PPDA experiments, RT-PCR results showed that NR2D mRNA is present at high levels at P2 and substantially down regulated at P12 in L4-L6 lumbar cord (Arvanian *et al.*, 2004). Reintroduction of NR2D subunits in the motoneurons resulted in reversing age related loss of trafficking ability in the second postnatal week and offers further support that these subunits are present during the first postnatal week and are subsequently down-regulated. Our results are consistent with the observations made by others, namely that NR2D peaks around P7 in most other CNS regions (Monyer *et al.*, 1994).

NR2A, NR2C, and NR3 subunits:

Experiments to directly measure the availability of these subunits were not undertaken due to lack of pharmacological antagonists that have a high specificity for these subunits (Neyton & Paoletti, 2006). To date, ifenprodil is the only antagonist with a high enough (it has a 400 fold higher affinity for NR2B over all other NMDA subunits

(Williams, 1993; 2001)) specificity to selectively target a specific NMDA subunit. While Zn^{2+} is a highly potent inhibitor of NR1/NR2A, it interacts with many other synaptic targets besides NMDA receptors (Smart *et al.*, 2004), a property that restricts its usefulness as a pharmacological tool to study NMDA receptors in slices or in vivo (Neyton & Paoletti, 2006). The presence of non-NR2B subunits was therefore measured indirectly in the experiments that were described in Chapter II.

Figure 24 in the Supplementary Figures is an expanded version of figure 19 from Chapter II. The NMDA-mediated response that was ifenprodil-insensitive was subjected to MK-801 blockade. Additional blockade of the ifenprodil-insensitive response with MK-801 would indicate the presence of non-NR2B containing NMDARs at these synapses (see Chapter III for discussion). At DR synapses almost all NMDA-mediated response was mediated by NR2B-containing NMDARs until P3. However starting at P4, there was an increased contribution of non-NR2B diheteromers and possibly triheteromeric channels with NR1/NR2B/NRXX configuration to these NMDA-mediated responses. While we did not conclusively determine the identity of the fourth subunit in the triheteromeric complex, we speculate, based on the expression patterns seen in other CNS regions, that the fourth subunit is most likely NR2A.

Newly formed synapses initially acquire NR1/NR2B in a manner that does not require glutamatergic transmission (Barria & Malinow, 2002). Experiments done in hippocampal slices tagging NMDARs showed that the synaptic insertion of NR2B-containing receptors does not does not require ligand binding. Furthermore, it does not increase if NR2B subunit expression is increased. It is possible that other events such as apposition of the presynaptic terminal or release of other substances can control synaptic

insertion of NR1/NR2B (Dalva *et al.*, 2000), but they are not dependent on activity. In contrast, synaptic insertion of NR1/NR2A does require activity and is promoted by increased levels of NR2A expression (Barria & Malinow, 2002) which occurs as the animals develop. The best studied model of this is ocular dominance plasticity where the synaptic replacement of NR2B with NR2A occurs after eye opening (Ramoa & Prusky, 1997; Quinlan *et al.*, 1999; Roberts & Ramoa, 1999).

Ligand binding alone is sufficient to bring about replacement of NR2B with NR2A. The implication is that spontaneous, incoherent synaptic activity like the rhythmic locomotor-like activity in the neonatal cord that releases glutamate into the synaptic cleft can effect the switch from NR2B to NR2A, and is quite unlike the coincident pre and post synaptic activity that is required for Hebbian synaptic plasticity. Both synaptic activity and NR2A subunit expression progressively increase in the lumbar spinal cord in the first and second postnatal week, and results in the synaptic replacement of NR1/NR2B with NR1/NR2A.

There are two readily apparent functional reasons why this receptor switch is favorable in development. First, NR2A containing NMDARs are more stable than NR2B containing ones and overexpression of NR2A stabilizes trafficking NR2B containing receptors at synapses (Groc *et al.*, 2006). NR1/NR2B receptors have a greater tendency to be lost from synaptic sites either through lateral movement out of the synapse or by receptor internalization. Postsynaptic increase in Reelin concentration (an extracellular matrix protein important in development of laminar structures (D'Arcangelo & Curran, 1998)) that occurs in development preferentially increases the surface mobility of synaptic NR1/NR2B and not NR1/NR2A leading to a preferential loss of NR1/NR2B

from the synapse (Groc *et al.*, 2007). NR1/NR2B is also especially prone to receptor internalization (Lavezzari *et al.*, 2004). Two domains in the C terminus of the NR2 subunit are involved in synaptic localization of the NMDA receptors. A preferential interaction of the C terminus of the NR2B and not NR2A with a clathrin adaptor protein results in the subsequent release of the receptor from the synapse and this underlies the higher rate of internalization of NR1/NR2B (Groc & Choquet, 2006). The developmental switch from NR2B diheteromers to triheteromers or non-NR2B containing diheteromers, may therefore be required to allow NMDA receptors to stabilize within the synapses.

A second functional advantage of the NR2B to NR2A subunit switch at the synapse is that NR2A subunit containing NMDARs produce currents with the fastest decay time, deactivation kinetics, high conductance and high sensitivity to Mg^{2+} block (Cull-Candy & Leszkiewicz, 2004). Decay times and deactivation kinetics of NR1/NR2B are much slower and they therefore lead to long lasting responses. Expression of NR2A subunits allows refining of the synaptic current time course and ensures faster and more precise synaptic transmission.

Figure 25 in the Supplementary Figures is a summary of the information presented above and offers a synopsis for the timing of expression of NMDA receptor subunits in motoneurons based on what was observed from our experiments and what is known in the literature. Note that we did not probe the expression patterns of all the subunits at all the ages mentioned in the model; the objective of the model is only to give a visual reference for the general trend that is observed in the expression of these subunits in lumbar motoneurons.

Differences between monosynaptic synapses of DR and VLF fibres on motoneurons

One of the major findings of this work is that synapses under the afferent and those under the descending inputs are independent of each other. There is very little cross-talk between these synapses. The topic of independence of these receptors is described in detail in Chapter II. Not only are the synapses of DR and VLF independent of each other, the NMDA mediated responses associated with them also have characteristically different properties. These experiments to characterize the properties of NMDA receptors associated with these two inputs to the motoneuron were undertaken because previous work in this lab (Arvanov *et al.*, 2000; Arvanian & Mendell, 2001b; Arvanian *et al.*, 2004) established that DR and VLF synapses on the motoneuron differed in two major ways: 1.A progressive increase in magnesium sensitivity of the NMDA receptors occurs with development. NMDA-mediated responses of VLF synapses are much more sensitive to magnesium blockade than those of DR synapses and visualizing these responses requires relieving the magnesium block by decreasing concentration of magnesium in the bath. VLF synapses exhibit an earlier gain of Mg^{2+} sensitivity than DR synapses 2. NT-3 was able to potentiate NMDA mediated responses at DR and not at VLF synapses, and reintroduction of NR2D subunits restored NT-3 induced facilitation at VLF synapses. These were the first indication that NMDA receptors at DR and VLF synapses were different from each other.

This work suggests that DR synapses contain a larger proportion of diheteromeric NR1/NR2B. VLF synapses are composed of a mixture of receptors possibly some diheteromeric ones and more triheteromeric NR1/NR2B/ NRXX receptors. The switch

from a primarily diheteromeric NR1/NR2B receptor population to other subunit-containing NMDARs at synapses that occurs in development, occurs around P3 at DR synapses, and occurs much earlier at VLF synapses. At the first age studied here, P1, VLF synapses already have a large population of triheteromeric NMDA receptors. This is further evidence that the VLF synapses follow an earlier time-line for maturation than DR synapses. Experiments with ifenprodil blockade should be carried out in the embryonic period to further confirm this idea of an earlier time-line for VLF synaptic maturation. As mentioned previously, ligand binding alone is enough to cause subunit switch and synaptic receptor maturation. Spontaneous activity like the rhythmic locomotor-like activity occurs early in development, the neural network underlying locomotion are available at birth (Brocard *et al.*, 1999). As a consequence, descending motor information probably arrives at the VLF synapses much earlier in development than sensory information that arrives at the DR synapses from the peripheral muscles. In other words synaptic activation perhaps occurs earlier at VLF synapses than DR synapses in the same motoneuron thereby orchestrating their early time-line for maturation.

NMDA receptor trafficking from extrasynaptic to synaptic sites also occurs more readily at DR synapses. However reintroduction of NR2D subunits using HSV viral vectors results in increasing the ability of VLF synapses to accumulate trafficking receptors (see Chapter III). The topic of NMDA receptor trafficking in dealt with in greater detail later in this discussion.

Differences between monosynaptic and polysynaptic synapses

Development of descending projections from spinal projecting brainstem nuclei starts between E11-15. They arrive at the cervical cord around E13-14, at lower thoracic levels at E14-15 and lower lumbar levels immediately before birth (Lakke, 1997; Vinay *et al.*, 2000b). However, the brain stem nuclei exerts their influence upon the spinal cord through polysynaptic connections well before they make monosynaptic connections on motoneurons (Floeter & Lev-Tov, 1993; Brocard *et al.*, 1999) and a considerable number of axons continue to arrive in the lumbar enlargement postnatally (Leong *et al.*, 1984). Establishment of polysynaptic connections to motoneurons precedes that of monosynaptic connections.

Similarly, polysynaptic reflexes from sensory fibres to motoneurons also develop 1-2 days before the establishment of the monosynaptic reflexes (Saito, 1979; Kudo & Yamada, 1987; Ziskind-Conhaim, 1990). Our results showing that the polysynaptic responses are less affected by ifenprodil than monosynaptic responses suggest that an activity dependent switch from primarily NR2B to non-NR2B containing NMDARs might occur sooner at these synapses in the polysynaptic pathway that begin functioning earlier in development. NMDA receptor trafficking also never occurs at VLF polysynaptic synapses.

NMDA receptor trafficking in synaptic plasticity

Our experiments with the activity dependent blocker MK-801 shows that NMDA-mediated responses are able to recover from “irreversible” MK-801 blockade. Chapter II discusses in detail why this recovery can be best explained by trafficking of unblocked

extrasynaptic NMDA receptors to synaptic sites. Trafficking of NMDA receptors, although not as well studied as trafficking of AMPA receptors, has been reported in dissociated cells of the mammalian nervous system (Tovar & Westbrook, 2002).

One of our novel findings in this experiment is that NMDA receptors associated with different synapses in the same motoneuron can differ in their trafficking ability. In the first postnatal week, the NMDA receptors associated with the DR synapses traffic to a greater extent than those associated with VLF synapses, as seen by the consistently greater recovery of DR NMDA EPSP from MK-801 blockade. In the first postnatal week DR synapses also have a larger proportion of NR2B containing diheteromeric NMDARs than VLF synapses. Based on these observations we suggest that availability of NR2B in motoneuron synapses encourages receptor trafficking, consistent with what has been described in other systems (Groc *et al.*, 2006).

Extrasynaptic NR1/NR2B replace synaptic NR1/NR2B but not NR1/NR2A

Trafficking occurs due to lateral diffusion of receptors from extrasynaptic sites to synaptic regions (Choquet & Triller, 2003; Groc *et al.*, 2004; Triller & Choquet, 2005; Cognet *et al.*, 2006). Synaptic and extrasynaptic receptors differ in their subunit composition; study of current kinetics and antagonist sensitivity shows that extrasynaptic receptor pools are composed mainly of NR1/NR2B and some NR1/NR2D and NR1/NR2A (Rumbaugh & Vicini, 1999; Tovar & Westbrook, 1999; Momiyama, 2000; Thomas *et al.*, 2006) even during the developmental stage when their synaptic receptors are composed of NR1/NR2A.

As shown by (Barria & Malinow, 2002), NR1/NR2B only replaces other NR1/NR2B containing synaptic receptors and not NR1/NR2A. In contrast, after ligand

binding (as caused by synaptic activity) NR1/NR2A can replace synaptic NR1/NR2B especially if NR2A subunits are available in large quantities (Barria & Malinow, 2002). Since a majority of the extrasynaptic receptors are composed of NR1/NR2B which can only traffic to and replace other NR1/NR2B containing receptors, it is possible that trafficking occurs more readily at DR synapses and during the first postnatal week, when a greater proportion of synaptic receptors is composed of NR1/NR2B. A loss of synaptic NR2B with maturation would then lead to a related loss of trafficking ability; since the loss occurs earlier at VLF synapses, we speculate that the related loss of trafficking ability also occurs earlier at these synapses. Studies have shown that NR1/NR2A can also diffuse laterally but more slowly(2×10^{-4} $\mu m^2 sec^{-1}$) than NR1/NR2B(500×10^{-4} $\mu m^2 sec^{-1}$) (Groc *et al.*, 2006). This could be another reason why lateral mobility decreases with maturation, with the inclusion of NR2A at these synapses.

However, trafficking ability cannot be attributed exclusively to the presence of synaptic diheteromeric NR1/NR2B since increasing the availability of NR2D subunits also resulted in increased NMDA receptor trafficking (increased recovery from MK-801 blockade). Similarily, DR polysynaptic responses also recovered from MK-801 blockade, even though the synapses associated with them are composed primarily of non-diheteromic NR1/NR2B. Therefore trafficking of NMDA receptors may be facilitated by other subunits as well, although it occurs more readily at synapses composed of NR1/NR2B receptors.

NR1/NR2B mediated AMPA receptor insertion at postsynaptic membrane

NT-3 at DR synapses causes LTP-like potentiation of both AMPA- and NMDA-mediated responses (Arvanov *et al.*, 2000). The potentiation is long lasting, as seen in

LTP (still present 4 hrs following brief NT-3 application). Most synaptic effects of neurotrophins are accounted for by presynaptic modification of transmitter secretion (Poo, 2001); however, instances where neurotrophins were found to modify properties of postsynaptic transmitter channels have also been reported (Levine *et al.*, 1995; Wang & Poo, 1997; Balkowiec *et al.*, 2000). NT-3 action at DR synapses was attributed to the presence of NR2D subunits in the postsynaptic motoneuron especially since increasing NR2D availability increased NT-3 induced facilitation (Arvanian *et al.*, 2004). An interesting question is whether NT-3 induced facilitation at DR synapses is partly mediated by insertion of new receptors at these synapses.

The presence of NR1/NR2B diheteromers in a synapse not only encourages NMDA receptor trafficking but also increases synaptic insertion of AMPA receptors. Phosphorylation of NMDARs may be a major mechanism for regulating receptor trafficking at synapses. Association of CAMKII with NMDARs occurs following autophosphorylation of CAMKII due to calcium entry from NMDAR activation. It is believed that this association between CAMKII and NMDARs brings the CAMKII in close enough proximity to AMPA receptors to cause their phosphorylation, leading to an increase in their single-channel conductance, an increase in synaptic insertion of more AMPA receptors, and eventual potentiation of the synaptic response (Benke *et al.*, 1998). CAMKII forms a stable complex with NR1/NR2B and not with NR1/NR2A and stimulation of NMDARs enhances this association (Leonard *et al.*, 1999). NR1/NR2B are therefore much more efficient than NR1/NR2A (although NR1/NR2A-mediated LTP does occur in adults (Grosshans *et al.*, 2002)) in bringing about CAMKII phophorylation-mediated insertion of AMPA receptors at the post synaptic membrane and leading

eventually to LTP (Barria & Malinow, 2005). Furthermore, as shown in the hippocampus, CaMKII competes for the same binding site as post synaptic density components like PSD-95 on the NR2A subunit (Gardoni *et al.*, 2001). These sites which are used for anchoring NR1/NR2A to the PSD are therefore not readily available for CaMKII activity further limiting the possibility of phophorylation mediated LTP at these synapses.

The availability of NR1/NR2B at synapses therefore encourages AMPA receptor insertion and plasticity at these synapses. Their availability in large quantities in the synapses of neonate in comparison to adult animals, probably contributes to make the neonatal cord more plastic than the adult spinal cord. New connections are being actively made during the embryonic and first postnatal week and it is conceivable that the high mobility associated with NR1/NR2B is crucial for maintaining the requirement for plasticity at these synapses. The loss of synaptic NR2B from VLF synapses and the associated loss of trafficking ability at these synapses could perhaps also play a role in limiting the ability of NT-3 to potentiate the VLF responses. Experiments testing trafficking ability after increasing availability of NR2B subunits should be undertaken to further address this question.

Hebbian plasticity of NMDA-mediated responses

When NT-3-induced facilitation of synaptic responses in the neonate occurs facilitation of both AMPAR-mediated and NMDAR-mediated responses is observed (Arvanov *et al.*, 2000). The previous section proposes that the facilitation of AMPA-mediated responses could be caused by NMDAR-mediated (specifically NR1/NR2B – mediated) AMPA receptor insertion at these synapses. Facilitation of NMDAR-mediated

responses can be brought about by, among other possibilities, changes in synaptic number and composition of NMDA receptors. While there is an explosion of studies that document the requirement for AMPA trafficking in LTP and LTD, very few groups have studied the requirement for NMDA receptor trafficking.

In the dentate gyrus, NR2D containing extrasynaptic receptors are selectively recruited to the synapse during LTP (Harney *et al.*, 2008). Activation of extra-synaptic NR2D is not required for induction of LTP_{NMDA}, but after LTP induction, the extra-synaptic NR1/NR2D traffic to the synapse to sustain the LTP. Other examples of LTP_{NMDA} have been demonstrated in the visual cortex (Watt *et al.*, 2004), medial perforant path-dentate granule cell synapse (O'Connor *et al.*, 1994), and Schaffer(Sch) collateral - CA1 synapses (Smith & McMahon, 2005). LTD of NMDA mediated responses has been described in the Sch-CA1 synapses (Carroll *et al.*, 2001) and occurs by lateral diffusion of NMDA receptors away from the synapse i.e from synaptic to extrasynaptic sites (Morishita *et al.*, 2005).

LTP_{NMDA} might also have particular significance in the adult. It is suggested that unlike in the neonate where there is a large pool of intracellular AMPA receptors that could be actively recruited to the synapse during activity, in the adult most AMPA receptors are already inserted in synaptic locations (Grosshans *et al.*, 2002). There is, however, a large source of intracellular NMDA receptors that could be recruited to increase the synaptic response.This was first demonstrated in Sch-CA1 synapses in adult hippocampus. At these synapses NMDAR-mediated calcium influx triggers LTP of the NMDAR- mediated response by a mechanism involving activation of PKC and tyrosine kinease Src and is followed by rapid synaptic insertion of NR2A-containing NMDARs.

The very same synapses in the neonate show LTP by AMPA receptor synaptic insertion (Grosshans *et al.*, 2002).

The mechanisms that underlie the requirement for NMDAR trafficking in synaptic plasticity are still largely unknown. Metabotropic glutamate receptor activation (O'Connor *et al.*, 1994), PKC- and Src-family tyrosine kinase activation (Grosshans *et al.*, 2002) have been implicated in establishing LTP $_{NMDA}$ and calcium-dependent actin depolymerization has been implicated in establishing LTD $_{NMDA}$ (Morishita *et al.*, 2005).

NMDA receptor trafficking in homeostatic plasticity

In the postsynaptic membrane, the average number of NMDARs present is about 50, and the number of AMPARs ranges from 0 (silent synapses) to about 50 for a PSD with a diameter of 400 nm (Kennedy, 2000). The NMDA-AMPA ratio at functional synapses is remarkably constant (Umemiya *et al.*, 1999; McAllister & Stevens, 2000; Watt *et al.*, 2000; Groc *et al.*, 2002). Even in LTP this ratio is only transiently perturbed (Watt *et al.*, 2004). There is work that suggests that the original NMDA-to-AMPA ratio of the current is restored within 2 hours of LTP induction and rapid LTP of AMPA currents is followed by a delayed potentiation of NMDA currents (Watt *et al.*, 2004). This restoration of balance by activity dependent scaling is most likely achieved by NMDAR trafficking to these synapses thereby maintaining a constant ratio between these two receptors (Watt *et al.*, 2000). In conclusion, NMDAR trafficking has strong implications for synaptic plasticity, both Hebbian and homeostatic.

Conclusion

With synaptic activity comes the related subunit switch and the inevitable decrease in plasticity. The compromise is the establishment of transmission that is

reliable. As in all of biology, this is the story of a precarious balance between two opposing forces. The question of consequence is whether these principles that have been studied in early stages of development can be extended to the adult. Does increasing availability of NMDA receptor subunits, enhancing NMDAR trafficking and increasing neuronal activity (with step training or electrical stimulation) result in plasticity in the adult spinal cord? And if it does, does it have functional implications for axonal recovery and rewiring after spinal cord injury? And do they then lead to functional recovery in motor behavior? While many questions remain, the demonstration that NMDA receptor trafficking occurs in motoneurons opens new vistas. The idea of tapping into this system, in combination with other treatment regimes to promote plasticity that strengthens synaptic transmission after spinal cord injury is tantalizing.

Not much is known about the way in which differential assembly of NR2 or NR1 subunits occurs; whether this process depends on neuronal activity or subunit availability is still an open question even though we know that the synthesis and ER exit of NMDA receptor subunits can be altered by the level of neuronal activity. Overexpression of the NR2B subunit in the forebrain produced the "smart mouse" which showed improved performance on memory tasks and had larger NMDA-mediated currents in the hippocampus (Tang *et al.*, 1999). This mouse, however, also had an increased sensitivity to pain (Wei *et al.*, 2001). Complex systems control NMDA-mediated transmission and any manipulation of the subunit composition should be seen in the context of the global effects it will produce.

Section VI

Supplemental Figures

Response	Input	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12
Total Monosynaptic Response	DR	5.5 ± 2.8 (n=4)	7.4 ± 4.6 (n=17)	5.9 ± 3.9 (n=10)	7.2 ± 4.3 (n=10)	4.5 ± 0.35 (n=5)				4.9 ± 3.2 (n= 6)			
	VLF	5.2 ± 1.3 (n=4)	4.6 ± 1.8 (n=15)	1.92 ± 1.6 (n=7)	4.6 ± 3.3 (n=10)	4.6 ± 1.9 (n=5)		6.8		3.3 ± 1.2 (n=6)	10.5		1.9
NMDA Monosynaptic Response	DR	1.2 ± 0.9 (n=4)	3.5 ± 2.8 (n=16)	2.1 ± 1.8 (n=9)	2.7 ± 3.8 (n=9)	1.4 ± 0.7 (n=6)		1.5 ± 0.4 (n=2)	1.9 ± 1.1 (n=3)	2.5 ± 1.8 (n=6)	4.6		0.9
	VLF	4.1 ± 4.8 (n=4)	1.8 ± 1.4 (n=15)	1.3 ± 0.6 (n=13)	1.6 ± 1.0 (n=9)	2.0 ± 0.9 (n=7)		1.7 ± 0.7 (n=2)	1.2 ± 0.6 (n=3)	1.3 ± 1.2 (n=6)	5.8		

Table 2: The amplitude of monosynaptic responses obtained in ACSF (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, 30^0 C) at 10 ml/min) and in cocktail of antagonists (AMPA antagonist CNQX (10 µM), $GABA_A$ receptor antagonist bicuculline (5 µM), $GABA_B$ receptor antagonist CGP 35348 (10 µM) and glycine receptor antagonist strychnine (5 µM)) from L5 motoneurons of rats in the first and second postnatal week of age. The values are given as (mV ± SD).

Figure 23: Age dependent susceptibility of DR and VLF EPSP to ifenprodil

Ifenprodil inhibition of the responses was seen in cords from rats of ages P1-P9 suggesting that NR2B containing NMDAR remain at these synapses throughout this period. Percent blockade of both the DR and VLF responses was found to correlate with the age of the animal used. DR (circles) responses of P3 animals were blocked maximally (82.1 % ± 9.9 SE). Responses in rats P1, P2, P4 and rats older than P8 were blocked to a lesser degree (64.6% ± 0.9, 68.2% ± 2.9, 73.61% ± 9.5, 64.1% ± 7.9 respectively).
A similar trend was observed at synapses made by VLF (triangles). VLF responses of P3 animals were blocked maximally (63.7% ± 5.0). Responses of rats P1, P2, P4 and rats older than P8 were blocked to a lesser degree (35.9% ± 4.3, 42.4% ± 6.8, 43.9% ± 15.6, 48.9% ± 11.7 respectively).

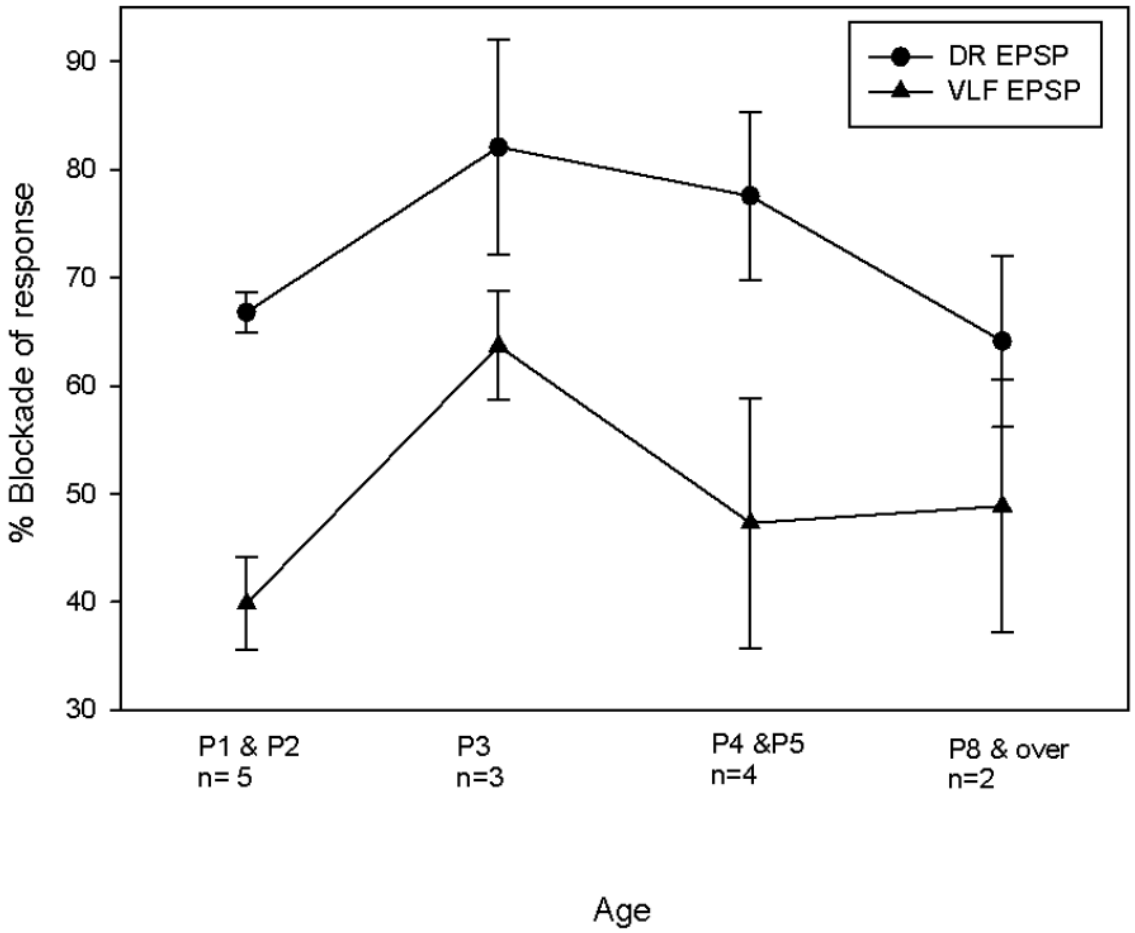


Figure 24: Contribution of non-NR2B receptors to NMDA-mediated responses of the DR and VLF (Expanded)

Percent residual (see text for definition) of the DR (white) and VLF (grey) response that remains after maximal inhibition was achieved in ifenprodil (empty) and followed by subsequent treatment with MK-801 (diagonals). Note that in the DR monosynaptic responses, in rats of ages between P1-P3 the response that remains after ifenprodil treatment is not blocked further by MK-801. In rats P4 and older, the response that remains after ifenprodil treatment is further blocked by MK-801. A greater percentage of the VLF response remains after ifenprodil treatment across all ages and across ages they are further blocked by subsequent MK-801 treatment.

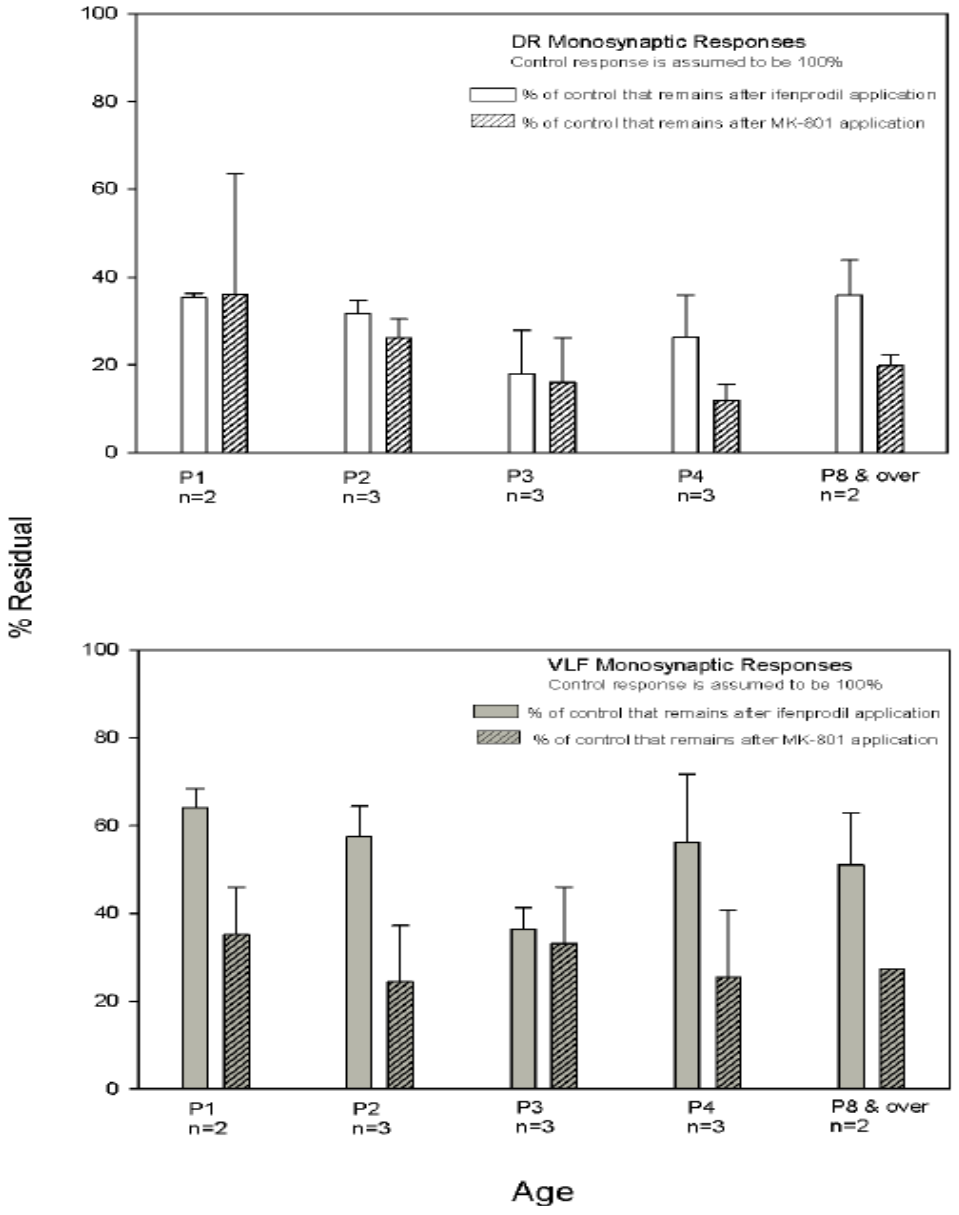
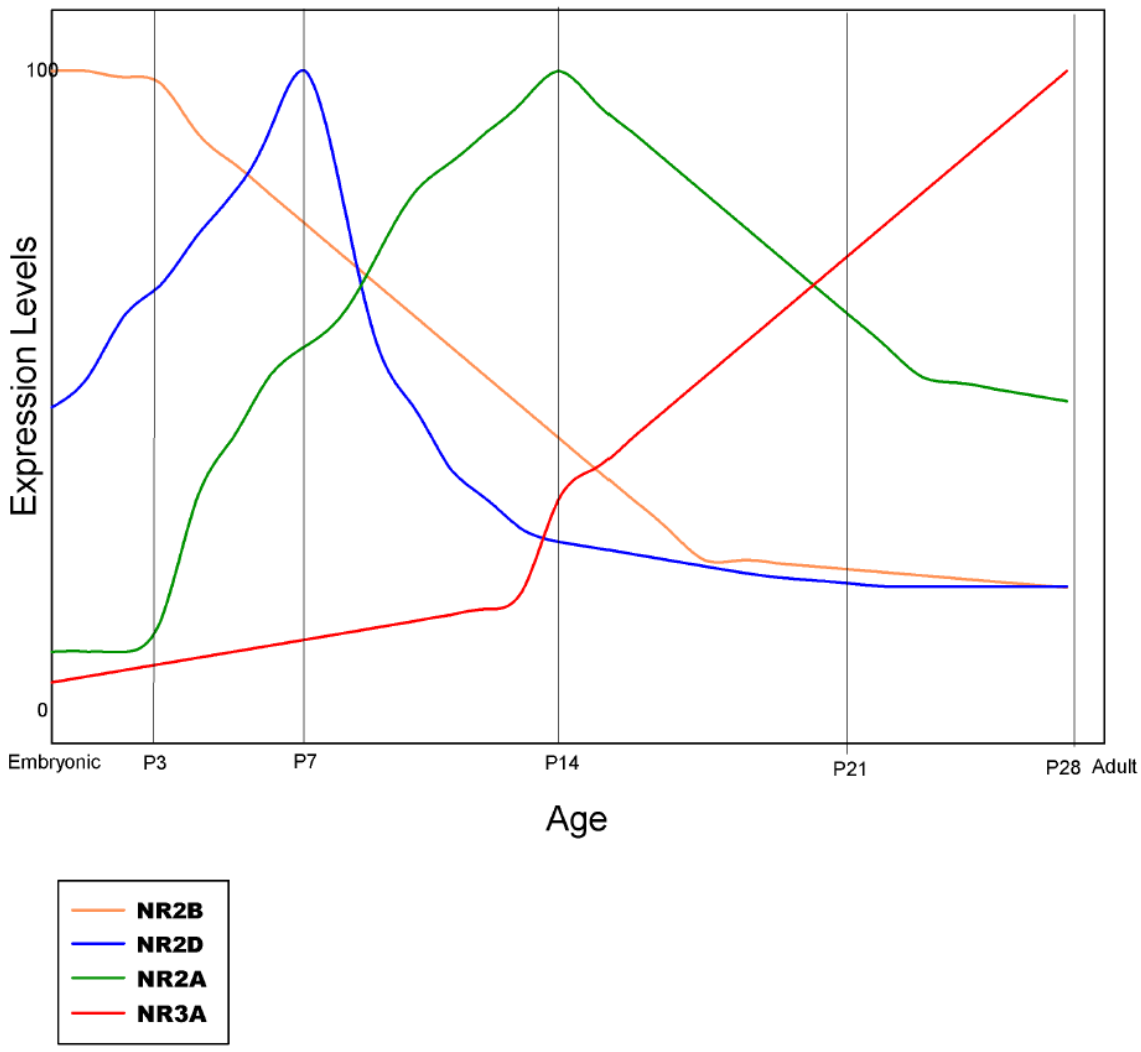


Figure 25: Model of expression of NR2 subunits in the neonatal L5 lumbar motoneuron

NR2B and NR2D subunits are expressed at high concentrations in the embryonic period. The expression of NR2B peaks around P3 and thereafter continues to decrease to adult levels. NR2D peaks around P7. The expression of NR2A increases starting P4, peaks around P14 and then decreases to adult levels. NR3A expression begins at P14 and peaks around P28 and decreases to adult levels after that.



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