REGULATION OF N-METHYL-D-ASPARTATE RECEPTOR ACTIVITY IN CULTURED RAT HIPPOCAMPAL NEURONS

by

BO LI

B.Sc., Jining Medical College, 1992
M.Sc., Institute of Psychology, Chinese Academy of Sciences, 1997

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Department of Program in Neuroscience

The University of British Columbia
Vancouver, Canada

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ABSTRACT

NMDA receptors (NMDARs) play a crucial role in neuronal development, synaptic plasticity, and excitotoxicity, therefore regulation of NMDAR function is important in both physiological and pathological conditions. In this study, the regulation of the properties of NMDARs, including rundown and desensitization, in cultured rat hippocampal pyramidal neurons was investigated by electrophysiological, immunocytochemical, and biochemical approaches. Novel mechanisms for regulating rundown and desensitization of NMDARs were identified.

NR2B-subtype NMDARs, which are mainly extrasynaptic, showed faster and more extensive peak current rundown in response to repeated agonist applications compared with NR2A-containing NMDARs, which are mainly synaptic. Moreover, rundown of the extrasynaptic, 2B-subtype receptors was largely independent of Ca\(^{2+}\) and dependent on tyrosine dephosphorylation, whereas rundown of the synaptic, 2A-containing receptors was Ca\(^{2+}\)-dependent and regulated by F-actin. The differences in rundown of the two subpopulations of NMDARs were determined by subcellular localization rather than the subunit composition, since synaptic 2B-subtype and 2A-containing receptors were resistant to Ca\(^{2+}\)-independent rundown, and extrasynaptic 2B-subtype and 2A-containing receptors were vulnerable to Ca\(^{2+}\)-independent rundown. Furthermore, an increase in receptor internalization and resulting decrease in numbers of NMDARs available on the cell surface were closely correlated with the Ca\(^{2+}\)-independent rundown of extrasynaptic NMDARs.
Subcellular localization also regulates NMDAR desensitization. The glycine-independent desensitization of NMDARs in rat cultured hippocampal neurons decreases during development. This decrease was not dependent on a switch in subunit composition, nor was it due to a change in the sensitivity of NMDARs to agonist or zinc during development. Instead, the developmental decrease in glycine-independent desensitization correlated with the synaptic localization of the receptor. Furthermore, overexpression of PSD-95 in immature neurons reduced NMDAR desensitization, and dispersion of PSD-95 away from synapses or manipulations that induced movement of NMDARs away from synapses increased NMDAR desensitization in mature neurons.

We conclude that synaptic localization increases stability of hippocampal neuronal NMDAR responses to sustained agonist exposure. Our results elucidate mechanisms for regulating NMDAR function that tune receptor activity in neurons of different developmental stages, or the response of subpopulations of NMDARs in a single neuron to different stimuli.
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LIST OF ABBREVIATIONS

°C  Degrees centigrade
μm  micrometer
μM  micromolar
AKAP  A kinase anchoring protein
AMPA  α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate
APV  DL-2-amino-5-phosphonovaleric acid
BDNF  brain-derived neurotrophic factor
BME  β-2-Mercapto-ethanol
BpV(phen)  potassium bisperoxo(1,10-phenanthroline)oxovanadate(V)
CAKβ  cell adhesion kinase β
CaMKII  calcium-calmodulin-dependent protein kinase II
cAMP  cyclic adenosine 5′-monophosphate
cDNA  complementary deoxyribonucleic acid
CNS  central nervous system
CREB  cAMP response element binding protein
DARPP-32  dopamine- and cyclic adenosine 3′, 5′-monophosphate-regulated phosphoprotein of 32 kDa
DIV  day in vitro
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>DLG</td>
<td>the Drosophila septate junction protein Discs-large</td>
</tr>
<tr>
<td>EBSS</td>
<td>Eagle's balanced salt solution</td>
</tr>
<tr>
<td>EC(_{50})</td>
<td>effective concentration to achieve 50 percent maximal response</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethyleneglycol-bis[(\beta)-aminoethyl ether]-N,N,N’N’-tetraacetic acid</td>
</tr>
<tr>
<td>EPSC</td>
<td>excitatory postsynaptic current</td>
</tr>
<tr>
<td>F-actin</td>
<td>filamentous actin</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FUDR</td>
<td>5-fluoro-2’deoxyridine</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GK</td>
<td>guanylate kinase</td>
</tr>
<tr>
<td>GKAP</td>
<td>guanylate kinase-associated protein</td>
</tr>
<tr>
<td>GluR</td>
<td>glutamate receptor</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hanks’ Balanced Salt Solution</td>
</tr>
<tr>
<td>HEK293 cell</td>
<td>human embryonic kidney 293 cell</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-Hydroxy-ethyl)-piperazine-1-ethane-sulfonic acid</td>
</tr>
<tr>
<td>KN-93</td>
<td>2-[N-(2-hydroxyethyl)-N-(4-methoxybenzenesulfonyl)]amino-N-(4-chlorocinnamyl)-N-methylbenzylamine</td>
</tr>
<tr>
<td>LIVBP</td>
<td>leucine, isoleucine, valine binding protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>LTD</td>
<td>long-term depression</td>
</tr>
<tr>
<td>LTP</td>
<td>long-term potentiation</td>
</tr>
<tr>
<td>MAGUK</td>
<td>membrane-associated guanylate kinases</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum Essential Medium</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>MK-801</td>
<td>dizocilpine or (5R, 10S)-(+-)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate</td>
</tr>
<tr>
<td>mM</td>
<td>millimolar</td>
</tr>
<tr>
<td>ms</td>
<td>millisecond</td>
</tr>
<tr>
<td>mV</td>
<td>millivolt</td>
</tr>
<tr>
<td>NF-L</td>
<td>neurofilament-L</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>NMDAR</td>
<td>NMDA receptor</td>
</tr>
<tr>
<td>nNOS</td>
<td>neuronal nitric oxide synthase</td>
</tr>
<tr>
<td>NR</td>
<td>NMDA receptor</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PDL</td>
<td>Poly-D-lysine</td>
</tr>
<tr>
<td>PDZ</td>
<td>PSD-95/SAP90, DLG (the Drosophila septate junction protein Discs-large), and ZO-1 (the epithelial tight junction protein)</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>PKI</td>
<td>protein kinase A inhibitor 14-22 amide</td>
</tr>
<tr>
<td>PP1</td>
<td>protein phosphatase 1</td>
</tr>
<tr>
<td>PP2</td>
<td>4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidin</td>
</tr>
<tr>
<td>PP2A</td>
<td>protein phosphatase 2 A</td>
</tr>
<tr>
<td>PP2B</td>
<td>protein phosphatase 2 B, or calcineurin</td>
</tr>
<tr>
<td>PSD</td>
<td>postsynaptic density</td>
</tr>
<tr>
<td>PSD-95</td>
<td>postsynaptic density protein of 95 kDa</td>
</tr>
<tr>
<td>PTPα</td>
<td>protein tyrosine phosphatase alpha</td>
</tr>
<tr>
<td>PTX</td>
<td>picrotoxin</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene difluoride</td>
</tr>
<tr>
<td>SAP</td>
<td>Synaptic Associated Protein</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>sec</td>
<td>second</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SH3</td>
<td>Src homology 3</td>
</tr>
<tr>
<td>SynGAP</td>
<td>synaptic GTPase-activating protein</td>
</tr>
<tr>
<td>TTX</td>
<td>tetrodotoxin</td>
</tr>
</tbody>
</table>
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CHAPTER I

Introduction

Glutamate is the major neurotransmitter in the mammalian central nervous system (CNS) that mediates fast excitatory synaptic transmission. Excitatory amino acids were first suggested to be involved in the modulation of neuronal activity by a study showing the convulsant properties of L-glutamate in the cerebral cortex (Hayashi, 1954). Ionophoretic studies later confirmed the excitatory actions of L-glutamate and L-aspartate on spinal and virtually all neurons in the CNS (Curtis et al., 1959, 1960). The idea that glutamate might be a neurotransmitter was initially doubted since it is too intimately involved in general metabolic pathways and cannot be distinguished from the “metabolic” pool of substances in neurochemical studies (Watkins and Evans, 1981). Nevertheless, the role of glutamate as a neurotransmitter was firmly established by subsequent progress in mainly three types of investigations including: first, the development of specific agonists and antagonists to study the pharmacological properties of different types of receptors for excitatory amino acids; second, the neurochemical studies on regional distribution of amino acids and their enzymes, as well as the release and uptake/inactivation of excitatory amino acids; third, the use of radioactive-labeled ligands to study the membrane binding sites for excitatory amino acids (reviewed by Watkins and Evans, 1981).

The action of glutamate in fast synaptic transmission is mediated by its activation of ionotropic glutamate receptors. Here I review the function, distribution, trafficking, and many aspects of regulation of the N-methyl-D-aspartate type of glutamate receptor that are relevant to this study.
1.1 An overview of ionotropic glutamate receptors

Fast excitatory synaptic transmission in the mammalian CNS is mediated predominantly by three types of ionotropic glutamate receptors including α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), N-methyl-D-aspartate (NMDA), and kainate receptors (Monaghan et al., 1989). The three types of ionotropic glutamate receptors were initially classified pharmacologically according to their relatively selective activation by AMPA, NMDA, and kainate, respectively (McBain and Mayer, 1994; Dingledine et al., 1999). Cloning of genes encoding individual glutamate receptor subunits helped to define glutamate receptor subunits as products of six gene families (Hollmann and Heinemann, 1994). AMPA receptor subunits GluR1-4 were encoded by a single gene family, kainate receptor subunits GluR5-7 and KA-1/KA-2, respectively, were encoded by two gene families, whereas NMDA receptor subunits NR1, NR2A-D, and NR3, respectively, were encoded by three gene families (Dingledine et al., 1999).
Figure 1-1. Diagram of glutamate receptor subunit membrane topology
(Modified from Dingledine et al., 1999; Brauner-Osborne et al., 2000)

(a) The transmembrane topology of a single subunit is shown linearly with the two ligand-binding domains S1 and S2, transmembrane domains M1, M3, and M4, the re-entrant loop M2, as well as the N/Q/R site. (b) The transmembrane topology of a single subunit is shown "three dimensionally". The N-terminal domain (gray) is folded as a periplasmatic binding protein (leucine, isoleucine, valine binding protein; LIVBP) that contains the high affinity zinc binding site (for NR2A; see 1.5.2.) and a domain that controls glycine-independent desensitization (for NR2A and NR2B; see 1.9.4.). The agonist-binding sites S1 and S2 are blue and red, respectively. The flip/flop domain (for AMPA receptor subunits) is green.
Modified from Dimgledine et al., 1999
and Brauner-Osborne et al., 2000
Subunits of the three types of ionotropic glutamate receptors have a similar transmembrane topology: three transmembrane domains including M1, M3, and M4, and a re-entrant membrane loop M2, with the agonist binding domains S1 and S2 just preceding M1 and following M3 respectively (see Figure 1-1) (Dingledine et al., 1999). Despite these structural similarities, one subunit co-assembles only with others from the same glutamate receptor type (i.e. AMPA, NMDA, or kainate) to form a functional receptor (Dingledine et al., 1999). Recent studies suggested that glutamate receptors are tetrameric complexes of subunits, and that the subunit composition regulates channel properties (reviewed by Dingledine et al., 1999). Native AMPA receptors usually have very low permeability to calcium due to the presence of the GluR2 subunit, whereas kainate receptors show relatively high calcium permeability compared with AMPA receptors (Ozawa et al., 1998). NMDARs, on the other hand, display high Ca\(^{2+}\) permeability and voltage-dependent block by extracellular Mg\(^{2+}\) (McBain and Mayer, 1994). Compared with NMDARs, AMPA and kainate receptors show lower affinity to glutamate (EC\(_{50} \geq 500 \mu M\)) and faster kinetics, including activation (\(\tau=0.2\)-0.4 ms), deactivation (\(\tau=0.8\)-2.5 ms), and desensitization (\(\tau=0.9\)-8.4 ms) (reviewed by Dingledine et al., 1999). The different properties of AMPA and NMDA receptors and the frequent (but not universal) colocalization of the two types of receptors in a single CNS synapse (Kharazia et al., 1996) well fit their different roles in synaptic transmission. AMPA receptors mediate most of the fast synaptic response. NMDA receptors, which have slow kinetics and high permeability to calcium, are opened only upon sufficient membrane depolarization (to relieve Mg\(^{2+}\) blockade due to repeated AMPA receptor activation) and mediate synaptic plasticity (reviewed by Nusser, 2000 and Dingledine et al., 1999).
1.2. Functional importance of NMDA Receptors

Compared with AMPA and kainate receptors, NMDA receptors (NMDARs) show unique properties such as high glutamate affinity (EC50=0.4-1.8 μM depending on different subtypes), high Ca2+ permeability, voltage-dependent block by extracellular Mg2+, and slow kinetics, including activation (τ=10-50 ms), deactivation (τ ranging from 70 to 4000 ms depending on different subtypes), and desensitization (τ=649-750 ms for NR1/NR2A, other subtypes are even slower) (McBain and Mayer, 1994; Dingledine et al., 1999). These unique properties may underlie the crucial roles that NMDARs play in processes underlying synaptic plasticity (Bliss and Collingridge, 1993; Malenka, 1994; Malenka and Nicoll, 1999), neural development (Rabacchi et al., 1992; Fox et al., 1996; Katz, 1999; Luthi et al., 2001), as well as excitotoxicity (Choi, 1994; Choi, 1995; Lipton, 1999).

1.2.1. Synaptic plasticity

During the past two decades synaptic plasticity has been the subject of intensive study. Two forms of synaptic plasticity, long-term potentiation (LTP; Bliss and Lomo, 1973) and long-term depression (LTD), are believed to provide critical clues to the cellular and molecular mechanisms that underlie learning and memory (reviewed by Malenka, 1994; Malenka and Nicoll, 1999). It has been well established that NMDARs are required for the induction of LTP at most glutamatergic excitatory synapses in the mammalian brain, including the most-studied synapses between Schaffer collateral or commissural axons and the apical dendrites of CA1 pyramidal neurons (Malenka and Nicoll, 1999). During high frequency stimulation that induces LTP, activation of AMPA
receptors depolarizes the postsynaptic membrane and release Mg$^{2+}$ block of NMDARs, resulting in Ca$^{2+}$ influx through NMDARs. The localized Ca$^{2+}$ rise within the spine is the key factor in triggering LTP (Malenka, 1994).

The importance of NMDARs in LTP is supported by a variety of studies. Specific NMDAR antagonists completely block the induction of LTP whereas they have little effect on basal synaptic transmission (Collingridge et al., 1983). Introducing Ca$^{2+}$ chelators into the postsynaptic cell to prevent the increase in intracellular Ca$^{2+}$ concentration prevents LTP, whereas a rise in intracellular Ca$^{2+}$ induced by other sources, such as influx through voltage-gated calcium channels or by uncaging Ca$^{2+}$, can mimic LTP (reviewed by Malenka and Nicoll, 1999).

Paradoxically, NMDARs are also required for the induction of LTD at the same synapses that show NMDAR-dependent LTP (Malenka, 1994). Studies in this field suggest that the distinct spatial and temporal changes of Ca$^{2+}$ signals induced by either LTP or LTD stimulation are decoded by two different sets of enzyme, which lead to either LTP or LTD respectively. Specifically, stimuli that induce brief (~3 sec), large (~10 μM) increases in intracellular Ca$^{2+}$ selectively activate CaMKII, leading to LTP, whereas stimuli that induce long (~1 min), low (<1 μM) increases in intracellular Ca$^{2+}$ selectively activate calcineurin, leading to LTD (reviewed by Malenka, 1994; Zucker, 1999; Franks and Sejnowski, 2002).

1.2.2. Learning and memory

If learning and memory share mechanisms with synaptic plasticity, it is conceivable that NMDAR function should also be critical for the learning process and the
formation of memory. Indeed, pharmacological studies showed that systematic or brain-
region specific application of NMDAR-specific antagonists impairs memory in animals
tested in various tasks (Castellano et al., 2001). While targeted disruption of NR1 or
NR2B is lethal (Forrest et al., 1994; Li et al., 1994; Kutsuwada et al., 1996), NR2A
knockout mice develop normally, but show reduced LTP at the hippocampal CA1
synapse and impaired spatial learning (Sakimura et al., 1995). With the advance in
molecular biology and genetics, spatial- and temporal-specific knockout of specific
NMDAR subunits that have minimal influence on normal brain development becomes a
feasible and powerful way to examine the role in learning and memory of NMDARs
expressed in different subsets of neurons. As expected, specific ablation of NMDARs in
CA1 or CA3 pyramidal cells in adult mice impairs different forms of learning and
memory tasks (Huerta et al., 2000; Rondi-Reig et al., 2001; Nakazawa et al., 2002). On
the other hand, transgenic mice that overexpress the NR2B subunit in the forebrain show
enhanced learning and memory ability correlating with increased activation of NMDARs
and facilitated LTP (Tang et al., 1999).

1.2.3. Neuronal development

Data accumulated from a variety of studies indicate that NMDAR activity is
critical for the formation and maturation of excitatory synapses during development, as
well as the induction of structural changes in spines of more mature neurons (Durand et
al., 1996; Wu et al., 1996; Isaac et al., 1997; Constantine-Paton and Cline, 1998; Engert
and Bonhoeffer, 1999; Liao et al., 1999; Maletic-Savatic et al., 1999). However, other
studies demonstrated that the initial formation of a synaptic connection does not require
NMDAR activity (Friedman et al., 2000b; Gomperts et al., 2000; Verhage et al., 2000). Instead, NMDAR activity may be critical for the refinement of established connections by inducing the trimming of excessive neurite branches or elimination of existing synapses (Rabacchi et al., 1992; Fox et al., 1996; Katz, 1999; Luthi et al., 2001). Indeed, chronic block of NMDARs in vivo prevents the regression of functional climbing fiber synapses in developing cerebellar Purkinje cells (Rabacchi et al., 1992), while temporary block of NMDA and non-NMDA glutamate receptors during the critical period disrupts the topographic refinement of thalamocortical connectivity and columnar organization (Fox et al., 1996). As well, chronic blockade of NMDARs during the first two weeks of postnatal development results in a dramatic increase in the number of synapses and more complex dendritic arborization in hippocampal CA1 pyramidal neurons (Luthi et al., 2001).

1.2.4. Pathophysiology

NMDARs have been implicated in a number of pathological conditions including ischemia, Huntington’s disease, Parkinson’s disease, Alzheimer’s disease, epilepsy, acquired immunodeficiency syndrome (AIDS), as well as chronic pain (Brauner-Osborne et al., 2000). NMDAR overactivation-mediated calcium overload and the resulting excitotoxicity has been well established as an important factor in mediating neuronal death in ischemia and some neuronal degenerative diseases. In ischemia, for example, deprivation of oxygen and glucose leads to the buildup of extracellular glutamate, which in turn over-activates ionotropic glutamate receptors. The resulting excessive calcium influx mainly through NMDAR channels overloads the neuron and induces further
metabolic damage (Lee et al., 1999). It has been shown that NMDAR antagonists largely prevent the Ca\(^{2+}\) influx induced by glutamate application and markedly reduce the neuronal death resulting from glutamate application or oxygen and glucose deprivation in neuronal cultures (reviewed by Lee et al., 1999). It has also been shown that NMDAR antagonists reduce neuronal death and infarct volume in animal models of ischemia (Simon et al., 1984; Wieloch, 1985; Albers et al., 1992; Hewitt and Corbett, 1992; Lee et al., 1999). It should be noticed, however, the protective effects of NMDAR antagonists on animal models of ischemia may be partly due to the drug-induced hypothermia (Corbett et al., 1990; Hewitt and Corbett, 1992).

1.3. NMDAR subunit composition and distribution

1.3.1. Subunit composition

Recent studies suggest that neuronal NMDARs are heterotetrameric structures composed of NR1 subunits in combination with one or more of NR2A, NR2B, NR2C or NR2D (Dingledine et al., 1999), or with the recently identified NR3 subunits NR3A and NR3B (Nishi et al., 2001; Chatterton et al., 2002). The NR1 subunit is essential in forming a functional NMDAR, and is encoded by a gene with three alternatively spliced exons (exons 5, 21, and 22) giving rise to eight isoforms (Dingledine et al., 1999) (see Figure 1-2). NR2 subunits A-D are encoded by four different genes, and are important in determining the channel properties (Dingledine et al., 1999) (see 1.4. for details).
Figure 1-2. Alternative splicing of NMDAR NR1 subunit

(Dingledine et al., 1999)

Alternative splicing of NMDAR NR1 subunit exons 5, 21, and 22 gives rise to eight isoforms including NR1-1a or b, NR1-2a or b, NR1-3a or b, and NR1-4a or b (nomenclature of Hollmann et al., 1993), which contain different combination of the cassettes N1, C1, C2, and C2'.

From Dingledine et al., 1999
Unlike AMPA and kainate receptors, which need only glutamate as an agonist, opening of NMDARs requires binding of both glutamate and glycine, with glycine binding to NR1 and glutamate binding to NR2 subunits (Dingledine et al., 1999). However, the recently described NR3 subunits NR3A and NR3B co-assemble with NR1 to form excitatory receptors that use only glycine as the agonist, and receptor activity is unaffected by glutamate or NMDA (Chatterton et al., 2002).

1.3.2. Subunit distribution

The NR1 subunit is expressed ubiquitously throughout the CNS during development at both mRNA and protein levels. The eight alternatively spliced variants of NR1 subunits show regionally specific expression patterns that do not exhibit much change during development (Laurie and Seeburg, 1994; Laurie et al., 1995) except that in the cerebellum, expression of the alternatively spliced N1 cassette (encoded by exon 5) is developmentally regulated. More than 80% of NR1 subunits contain N1 in the adult cerebellum, whereas only 20% contain this cassette early in development (Prybylowski et al., 2000). In the adult, the NR1-1a and NR1-2 variants (nomenclature of Hollmann et al., 1993) are highly expressed in most of the brain regions, whereas NR1-1b is highly expressed in cerebellum, caudate, substantia nigra, cortex, and thalamus. The highest expression of NR1-4 signal is in approximately complementary areas in the brain to that of NR1-1, except that NR1-1b and NR1-4 overlap in areas such as parietal cortex, hippocampus CA3, thalamus, inferior colliculus, and cerebellar granule cells. The NR1-3
variants are not highly expressed in any brain regions (Laurie and Seeburg, 1994; Laurie et al., 1995).

The expression of NR2 subunits is regulated both spatially and temporally (Hollmann and Heinemann, 1994; Monyer et al., 1994; Ozawa et al., 1998). In the adult brain, NR2A mRNA is widely expressed, with high levels in the cerebral cortex and cerebellum. The NR2B mRNA is more selectively expressed in the forebrain, with high levels in the cerebral cortex, septum, caudate-putamen and olfactory bulb. Both NR2A and NR2B mRNAs are highly expressed in hippocampal pyramidal cells. Expression of NR2C mRNA, on the other hand, is mainly in the cerebellar granule cell layer, and also in the olfactory bulb and thalamus at low levels. NR2D mRNA is expressed at low levels in regions including olfactory bulb, thalamus, and brain stem. NR2C and NR2D mRNAs can also be detected in subsets of hippocampal interneurons (Hollmann and Heinemann, 1994, and reviewed by Ozawa et al., 1998). Expression of NR2 subunits is also developmentally regulated, with NR2B and NR2D being expressed prenatally, whereas expression of NR2A and NR2C begins around birth and peaks around P20 (Monyer et al., 1994, reviewed by Ozawa et al., 1998).

In vitro studies also showed that cultured hippocampal or cortical neurons express mainly NR2B subunits early in development, whereas the expression of NR2A subunit increases during maturation (Williams et al., 1993; Zhong et al., 1994; Li et al., 1998; Rao et al., 1998; Stocca and Vicini, 1998; Tovar and Westbrook, 1999). NMDARs in immature neurons, which are mainly the NR1/NR2B subtype, are also predominantly non-synaptic (Li et al., 1998; Rao et al., 1998; Tovar and Westbrook, 1999). During maturation, the majority of NR2A subunits are incorporated into synapses, probably
through the replacement of existing NR2B subunits (Barria and Malinow, 2002), while
the NR2B subunits continue to predominate at extrasynaptic sites (Li et al., 1998; Stocca
and Vicini, 1998; Tovar and Westbrook, 1999, and reviewed by Cull-Candy et al., 2001)
(see Figure 1-3).
**Figure 1-3. Subcellular distribution of NMDAR subunits**

In mature hippocampal pyramidal neurons, NR2A (red) subunits are mainly synaptic. NR2B (green) subunits are predominant at the extrasynaptic site. For simplicity, the possible synaptic NR1/NR2A/NR2B heterotrimeric NMDARs (Tovar and Westbrook, 1999) are not shown.
Presynaptic

Active zone

NMDAR (2A-containing)

Postsynaptic

NMDAR (2B-subtype)

Extrasynaptic

NMDAR (2B-subtype)
1.4. Regulation of NMDAR function by subunit composition

1.4.1. NR2 subunits

Studies of recombinant receptors expressed in heterologous systems have shown that NR2 subunits modulate several critical receptor-channel properties, including agonist and antagonist sensitivity, Mg\(^{2+}\) block, single channel conductance, channel open probability, sensitivity to zinc inhibition, and channel kinetics (Chen et al., 1999; Dingledine et al., 1999). Specifically, the presence of different NR2 subunits dramatically influences NMDAR channel deactivation, desensitization, and inactivation. The deactivation time constant is about 50 ms for NR1/NR2A, 300-500 ms for NR1/NR2B or NR1/NR2C, and 2-4 s for NR1/NR2D (Dingledine et al., 1999; Cull-Candy et al., 2001). The influence of NR2 subunits on NMDAR desensitization and inactivation is discussed in 1.9.

NR1/NR2B has a higher sensitivity for glutamate than NR1/NR2A (peak current EC\(_{50}\) = ~1 and ~8 μM, respectively) (Dingledine et al., 1999; Chen et al., 2001), but has lower open probability than NR1/NR2A (Chen et al., 1999). Both NR1/NR2B and NR1/NR2A have a higher single channel conductance and higher sensitivity to Mg\(^{2+}\) blockade than either NR1/NR2C or NR1/NR2D (Dingledine et al., 1999; Cull-Candy et al., 2001). Other effects of NR2 subunits on NMDAR channel properties are discussed in 1.5.

1.4.2. NR3 subunits

The recently described NR3 subunits NR3A or NR3B can assemble with either NR1 alone or together with NR1 and NR2 subunits to generate functional receptors (Das
et al., 1998; Perez-Otano et al., 2001; Chatterton et al., 2002). NR1/NR3 receptors are excitatory glycine receptors that are not activated by glutamate or NMDA, and are relatively Ca\(^{2+}\)-impermeable and insensitive to Mg\(^{2+}\) or the use-dependent channel blocker MK801 (Chatterton et al., 2002). On the other hand, addition of NR3 subunits into NR1/NR2 complexes results in channels with reduced conductance and dramatically decreased Ca\(^{2+}\) permeability (Das et al., 1998; Cull-Candy et al., 2001; Perez-Otano et al., 2001).

1.4.3. NR1 subunits

NR1 splice variants also modulate NMDAR function. It has been shown that the presence of the alternatively spliced N1 cassette reduced the sensitivity of NMDARs to inhibition by protons and zinc, as well as to potentiation by polyamines (Traynelis et al., 1995; Traynelis et al., 1998) (also see 1.5.).

A recent study showed that the N1 cassette regulates NMDAR deactivation (Rumbaugh et al., 2000). The presence of the N1 cassette in NR1 (i.e. NR1-1b) greatly accelerated deactivation of NR1/NR2B expressed in HEK cells in response to glutamate (Rumbaugh et al., 2000), whereas N1 did not influence deactivation of NR1/NR2A (Vicini et al., 1998).

1.5. Regulation of NMDAR function by endogenous extracellular modulators

1.5.1. Ca\(^{2+}\) and Mg\(^{2+}\)

As mentioned above, two key properties of NMDARs are their high Ca\(^{2+}\) permeability and voltage-dependent block by Mg\(^{2+}\). An asparagine (N) at the tip of the
M2 segment of NMDAR subunits confers both Ca$^{2+}$ permeability and Mg$^{2+}$ blockade to NMDARs (Ozawa et al., 1998; Dingledine et al., 1999). Replacing asparagine with arginine at this site almost completely abolishes both Ca$^{2+}$ permeability and Mg$^{2+}$ block (Burnashev et al., 1992; Sakurada et al., 1993). This site, the so-called Q/R/N site (see Figure 1-1 a), has an arginine (converted from glutamine by RNA editing) in the AMPA receptor GluR2 subunit, which makes AMPA receptors impermeable to Ca$^{2+}$ (Dingledine et al., 1999).

The sensitivity to Mg$^{2+}$ block is regulated by NR2 subunits, with NR1/NR2A and NR1/NR2B more sensitive to Mg$^{2+}$ block than NR1/NR2C and NR1/NR2D (Monyer et al., 1992; Monyer et al., 1994). At physiological concentration of Mg$^{2+}$ (1 mM), the current responses are largest at -25 mV for NR1/NR2A and NR1/NR2B, but at -45 mV for NR1/NR2C and NR1/NR2D. Moreover, the Mg$^{2+}$ blockade of the channel is much stronger at membrane potentials ranging from -25 to -80 mV for NR1/NR2A and NR1/NR2B than NR1/NR2C and NR1/NR2D (Monyer et al., 1994). The voltage-dependent block by Mg$^{2+}$ makes NMDAR a coincidence detector that senses both presynaptic transmitter release and postsynaptic depolarization. Due to the weaker Mg$^{2+}$ blockade, neurons expressing NR2C or NR2D may be able to detect presynaptic activities causing relatively small postsynaptic depolarizations. This together with the slow deactivation that permits prolonged Ca$^{2+}$ influx (see 1.4.1) for NR2D suggest an important role for its early expression during the development (Monyer et al., 1994).

The Ca$^{2+}$ permeability among different NMDAR subtypes is similar. While being the mediator for NMDAR function in development, synaptic plasticity, or excitotoxicity, Ca$^{2+}$ influx through NMDARs also down-regulates NMDAR function in a negative
feedback manner through Ca\(^{2+}\)-dependent inactivation, Ca\(^{2+}\)-dependent rundown, or activation of calcineurin (see 1.9. for detailed information). As well, extracellular Ca\(^{2+}\) binds to a site that is near the entrance of the NMDAR channel pore. Binding of Ca\(^{2+}\) to this site partially blocks the channel to effectively decrease the channel conductance to Na\(^{+}\), probably by an electrostatic blocking mechanism (Premkumar and Auerbach, 1996). This blockade of Na\(^{+}\) conductance may contribute to determining the Ca\(^{2+}\)-selectivity of NMDARs (Premkumar and Auerbach, 1996).

1.5.2. Zinc

Zinc is released from synaptic vesicles at synapses such as the mossy fiber-CA3 synapses (Charton et al., 1985; Aniksztejn et al., 1987; Slomianka, 1992). Upon high rates of neuronal firing, zinc can reach a peak concentration of 300 μM in the extracellular space (Assaf and Chung, 1984; Howell et al., 1984) that inhibits NMDAR activity as Zn\(^{2+}\) has been shown to inhibit both NR1/NR2A and NR1/NR2B mediated current in a voltage-independent manner, with IC\(_{50}\) values around 5 nM and 10 μM, respectively (Chen et al., 1997; Paoletti et al., 1997). Zn\(^{2+}\) also blocks the channel of either type of NMDAR in a voltage-dependent manner, with an IC\(_{50}\) value of about 80 μM (Chen et al., 1997; Paoletti et al., 1997). The high affinity Zn\(^{2+}\) binding domain was mapped to the LIVBP-like domain (LIVBP for leucine, isoleucine, valine binding protein) in the extracellular N-terminal region of NR2A subunit (see Figure 1-1 b), and the amino acids H44, D102, D105, H128, K233, and E266 have been found to be critical for high affinity binding (Choi and Lipton, 1999; Paoletti et al., 2000). The mechanism of high-affinity Zn\(^{2+}\) inhibition of the NMDAR was suggested to involve enhancement of
proton inhibition (Choi and Lipton, 1999). As a result, inclusion of the N1 cassette in NR1, which reduces NMDAR sensitivity to protons (see 1.5.3.), also reduces NMDAR sensitivity to Zn$^{2+}$ (Traynelis et al., 1998). Zn$^{2+}$ also regulates NMDAR desensitization (Chen et al., 1997; Zheng et al., 2001) (see 1.9.). Interestingly, while the acute effect of Zn$^{2+}$ on NMDAR activity is inhibitory, longer exposures to Zn$^{2+}$ have been shown to potentiate NMDAR-mediated current through activation of intracellular Src family kinases (Manzerra et al., 2001).

1.5.3. Protons

Unlike AMPA receptors and kainate receptors, which are inhibited by extracellular protons at IC$_{50}$ values of pH 6.3 and 5.7 respectively (McBain and Mayer, 1994), the IC$_{50}$ value for extracellular proton inhibition of NMDARs ranges from pH 6.6 to 7.3 (McBain and Mayer, 1994), suggesting that NMDARs are partially inhibited under physiological conditions and that further acidification will strongly inhibit NMDAR activity. The importance of proton regulation of NMDAR activity is apparent as acidification can occur during either physiological conditions such as high frequency synaptic transmission or during pathological conditions such as ischemia (McBain and Mayer, 1994; Dingledine et al., 1999). Protons inhibit NMDAR activity in a voltage-independent manner, by changing the channel opening frequency but not single-channel conductance (McBain and Mayer, 1994; Dingledine et al., 1999). Both polyamines and inclusion of the N1 cassette in the NR1 subunit reduce proton inhibition (Traynelis et al., 1995). Mutagenesis studies have identified many residues, on both NR1 and NR2
subunits, which influence NMDAR sensitivity to protons (reviewed by Dingledine et al., 1999).

1.5.4. Polyamines

Extracellular polyamines such as spermidine and spermine can regulate NMDAR function in at least three ways: voltage-dependent block of the channel pore, glycine-dependent potentiation, and voltage- and glycine-independent potentiation (reviewed by Dingledine et al., 1999). Similar to Mg\(^{2+}\) block, the voltage-dependent block of NMDAR channel by polyamines is subunit dependent, with NR1/NR2A and NR1/NR2B more sensitive to polyamines than NR1/NR2C (Dingledine et al., 1999). Glycine-dependent potentiation is due to an increase in glycine affinity of NMDARs in the presence of polyamine (Benveniste and Mayer, 1993). Both NR1/NR2A and NR1/NR2B are sensitive to glycine-dependent potentiation, whereas only receptors containing NR2B show glycine-independent potentiation (Williams, 1994; Zhang et al., 1994; Williams, 1995). Glycine-independent potentiation of NMDAR function by polyamines has been proposed to be due to the relief of tonic proton inhibition, which is similar to the effect of including the N1 cassette in the NR1 N-terminus on NMDAR proton sensitivity (Traynelis et al., 1995; Kumamoto, 1996). This is supported by a study showing that glycine-independent potentiation is abolished when N1 cassette is present in NR1 (Durand et al., 1993). However, this mechanism cannot explain why NR2A containing NMDARs, which are highly sensitive to proton inhibition, are not potentiated by polyamines (Dingledine et al., 1999).
Spermine has also been shown to change NMDAR kinetics. That spermine decreases NMDAR desensitization is probably due to its inhibition of glycine-dependent desensitization by increasing glycine affinity (Lerma, 1992). Spermine also greatly accelerated deactivation in response to glutamate of NR1-a/NR2B expressed in HEK cells (Rumbaugh et al., 2000).

It is important to note that intracellular polyamines can up-regulate NMDAR function indirectly via activation of casein kinase II (Lieberman and Mody, 1999), given that the majority of spermine in the CNS is localized intracellularly (Shaw and Pateman, 1973).

1.5.5. Redox modulation

Neuronal NMDARs are very sensitive to redox modulation (reviewed by McBain and Mayer, 1994; Dingledine et al., 1999). Studies on recombinant NMDARs showed that two cysteine residues (Cys744 and Cys798) of NR1 subunit are critical for this modulation, and that NR2 subunits also play a regulatory role (McBain and Mayer, 1994; Dingledine et al., 1999). When these cysteines are reduced, the NMDAR-mediated responses are increased, mainly due to an increase in channel open frequency but not single-channel conductance. On the other hand, when the cysteines are oxidized the NMDAR-mediated responses are decreased (Dingledine et al., 1999). Interestingly, mutation of these two cysteines also eliminated spermine potentiation of NMDR function and shifted the IC$_{50}$ for proton inhibition of NMDARs (Sullivan et al., 1994), suggesting that the same residues contribute to NMDAR regulation by protons, polyamines, and reducing/oxidizing agents.
1.5.6. Mechanosensitivity

Mechanical stretch has been shown to potentiate NMDAR-mediated responses (Paoletti and Ascher, 1994). Mechanical stretch may happen in excitotoxic cell death, during which neurons may swell (Choi, 1988), or in neuronal regions of high mobility such as spines or filopodia.

1.6. Regulation of NMDAR by phosphorylation

NMDAR subunits can be phosphorylated at either serine/threonine or tyrosine residues within the C-terminal intracellular domain, and many kinases or phosphatases have been shown to modify the functional properties of NMDARs (Dingledine et al., 1999).

1.6.1. Serine/threonine phosphorylation

1.6.1.1. PKA

Both NR1 and NR2 subunits can be phosphorylated by PKA (Dingledine et al., 1999). Specifically PKA phosphorylates serine 897 of NR1 (Tingley et al., 1997). PKA activation has been shown to enhance NMDAR-mediated responses by either overcoming the effects of calcineurin (Raman et al., 1996) or inhibition of protein phosphatase 1 (PP1) and/or protein phosphatase 2A (PP2A) mediated by the protein phosphatase inhibitor DARPP-32 (dopamine- and cyclic adenosine 3’, 5’-monophosphate-regulated phosphoprotein of 32 kDa) (Blank et al., 1997; Snyder et al., 1998). Moreover, recent studies showed that the physical coupling of PKA to NMDARs by scaffolding proteins
such as yotiao (see Figure 1-4 b) or PSD95/AKAP (postsynaptic density-95/A kinase anchoring protein) complex facilitates PKA phosphorylation of NMDARs and regulation of NMDAR function (Westphal et al., 1999; Colledge et al., 2000). In addition, a PKA-dependent down-regulation of NMDAR activity has also been reported. This is possibly an indirect effect due to the activation of a tyrosine phosphatase, since mutation of a tyrosine residue on the C-terminus of NR2A (Y842) abolished the effects of PKA activation (Woodward, 2002). Mutation of this tyrosine residue has been shown to prevent the tyrosine dephosphorylation-dependent and use-dependent down-regulation of NMDAR activity (Vissel et al., 2001) (also see 1.9.1.).

1.6.1.2. PKC

PKC can also phosphorylate both NR1 and NR2 subunits, but the PKC phosphorylation sites are different from those of PKA (Leonard and Hell, 1997). Specifically PKC phosphorylates Serine 890 and 896 of NR1 (Tingley et al., 1997). PKC activation has been shown to potentiate NMDAR activity (reviewed by Dingledine et al., 1999). Specifically, phosphorylation by PKC increased the channel open probability, decreased the affinity for extracellular Mg$^{2+}$ block (Chen and Huang, 1992), increased the channel open rate, and promoted receptor surface expression (Lan et al., 2001a; Lan et al., 2001b). A reduction of NMDAR-mediated synaptic current after PKC activation was also reported (Markram and Segal, 1992), possibly because phosphorylation of serine 890 in the NR1 C1 cassette can inhibit NR1 clustering (Ehlers et al., 1995; Tingley et al., 1997). PKC activation has also been shown to increase Ca$^{2+}$-dependent inactivation of
NMDARs, presumably through phosphorylation of the C1 cassette in NR1 (Lu et al., 2000) (see also 1.9.).

Recent reports suggest that the potentiation of NMDAR activity by PKC activation may not be due to the direct phosphorylation of NMDARs. For example, PKC-dependent up-regulation of NMDAR function is absent in neurons from src-/- mice (Lu et al., 1999), suggesting that the effect of PKC is mediated by the activation of the non-receptor tyrosine kinase (Src). A recent study showed that activation of PKC results in increased tyrosine phosphorylation of both NR2A and NR2B, which was blocked by PKC inhibitor or Src family tyrosine kinase inhibitor (Grosshans and Browning, 2001). As well, PKC-mediated NMDAR potentiation is still observed for NMDARs carrying mutations of all known PKC phosphorylation sites (Zheng et al., 1999). These data suggest that the effect of PKC is correlated with a change in NMDAR tyrosine phosphorylation.

1.6.1.3. α-calcium-calmodulin-dependent protein kinase II

α-calcium-calmodulin-dependent protein kinase II (CaMKII) has been shown to phosphorylate serine1303 in the NR2B subunit and a homologous site on NR2A (Omkumar et al., 1996), and the interaction with the NR2B subunit locks CaMKII in an active conformation (Bayer et al., 2001) (see also 1.7.1.). Exogenous intracellular application of CaMKII enhances NMDAR-mediated current (Kolaj et al., 1994), whereas inhibition of endogenous CaMKII decreases NMDAR channel open probability (Lieberman and Mody, 1999).
1.6.1.4. Casein kinase-II

Another serine/threonine kinase casein kinase-II has recently been shown to tonically regulate NMDAR activity, since application of casein kinase-II inhibitor decreases, whereas application of its activator increases NMDAR channel open probability, and the inhibition of casein kinase-II also inhibits synaptic NMDAR-mediated responses (Lieberman and Mody, 1999). Casein kinase-II can balance the effect of calcineurin (Lieberman and Mody, 1999), but whether its effects are through direct phosphorylation of NMDARs remains to be determined.

While most of the studies showed that phosphorylation by serine/threonine kinases enhance NMDAR activity, serine/threonine phosphatases PP1, PP2A, or PP2B (calcineurin) have been shown to inhibit NMDAR activity (Raman et al., 1996; Westphal et al., 1999, and reviewed by Dingledine et al., 1999). For example, NMDAR mediated current is potentiated by intracellular application of PP1 and/or PP2A inhibitors (Blank et al., 1997). A recent study showed that PP1 may tonically down-regulate NMDAR activity (Westphal et al., 1999). Calcineurin has been shown to negatively regulate NMDAR activity through desensitization (Sather et al., 1992; Tong and Jahr, 1994; Tong et al., 1995; Krupp et al., 2002) (see also 1.9.4.).

1.6.2. Tyrosine phosphorylation

A previous study has shown that the NR2B subunit is the major tyrosine-phosphorylated protein in the postsynaptic density (PSD) (Moon et al., 1994). A subsequent study showed that both NR2A and NR2B are tyrosine-phosphorylated at low levels (2% for NR2A and 4% for NR2B) in synaptic membranes, but NR1 or subunits of
AMP A and kainate receptors are not tyrosine phosphorylated (Lau and Huganir, 1995). In addition, endogenous tyrosine kinases increase the phosphorylation of NR2A, but not NR2B, by 6-8-fold (Lau and Huganir, 1995). Endogenous Src and NMDARs have also been shown to co-precipitate (Yu et al., 1997), suggesting a close physical interaction between the two. Furthermore, exogenous application of Src family tyrosine kinases, including Src and Fyn, to isolated postsynaptic membranes results in phosphorylation of NR2A and NR2B subunits (Suzuki and Okumura-Noji, 1995).

Src and Fyn also regulate NMDAR function (reviewed by Ali and Salter, 2001). Exogenous intracellular application of Src or Fyn potentiates NR1/NR2A but not NR1/NR2B, NR1/NR2C, or NR1/NR2D mediated current (Kohr and Seeburg, 1996). Exogenously applied Src enhances NMDAR-mediated current in neurons (Wang and Salter, 1994), whereas endogenous Src regulates the function of synaptic NMDARs (Yu et al., 1997). A recent study showed that coupling of Fyn to NR2A by PSD-95 (see Figure 1-4 a) promotes tyrosine phosphorylation of NR2A by Fyn (Tezuka et al., 1999).

For recombinant NR1/NR2A expressed in HEK293 cells the effects of tyrosine kinases on NMDAR function can be explained by relief of high-affinity Zn$^{2+}$ inhibition by ambient zinc in the recording solution (Zheng et al., 1998). However, Src kinase potentiated neuronal NMDAR-mediated current even in the absence of ambient Zn$^{2+}$ contamination (Xiong et al., 1999), suggesting other mechanism are involved in Src regulation of neuronal NMDAR activity.

Regulation of NMDAR function by Src family kinases is physiologically important, as studies have indicated that Src family kinases, especially Src itself, play an important role in the induction of LTP, probably via potentiation of NMDAR function.
A model has thus been proposed for the role of Src kinase in the induction of LTP based on those studies. Upon high frequency stimulation that induces LTP, Src is activated, which enhances NMDAR function. The enhanced NMDAR responses together with depolarization-induced Mg\(^{2+}\) unblock then induce the potentiation of AMPAR-mediated synaptic responses (reviewed by Ali and Salter, 2001). Activation of Src during LTP induction is mediated by CAK\(\beta\) (cell adhesion kinase \(\beta\)), which can be activated by raising intracellular Ca\(^{2+}\) (Huang et al., 2001).

As tyrosine kinases up-regulate NMDAR function, tyrosine phosphatases may down-regulate NMDAR activity. This was confirmed by intracellular application of a tyrosine phosphatase, which decreases NMDAR-mediated whole-cell responses, or by intracellular application of a tyrosine phosphatase inhibitor, which increases NMDAR-mediated current (Wang and Salter, 1994; Wang et al., 1996). However, tyrosine phosphatase may also up-regulate NMDAR function indirectly, as a recent study showed that protein tyrosine phosphatase alpha (PTP\(\alpha\)) promotes NMDAR function, probably via activation of Src (Lei et al., 2002).

1.7. NMDAR interaction with intracellular proteins: implications in anchoring and signalling

Recent studies on NMDAR interaction with intracellular signaling and scaffolding proteins have great influence on our understanding of how NMDARs are anchored in the PSD compartment, how activation of NMDARs is coupled to specific
downstream signaling cascades, and how NMDAR function is fine-tuned to different patterns and intensity of stimuli.

1.7.1. Interaction of NR2 subunits

NR2 subunits NR2A-D of NMDARs all contain long intracellular C-terminal tails (≤644 amino acids) that are crucial for the signalling and anchoring of NMDARs. It was found that the synaptic localization of NR2B is impaired in mice carrying deletion of the NR2B C-terminal tail (Mori et al., 1998) and that the synaptic targeting of the NMDAR is also impaired by C-terminal truncation of NR2A (Steigerwald et al., 2000). The synaptic NMDAR-mediated responses decreased in both cases. As a result, either LTP (Sprengel et al., 1998) or LTD (Mori et al., 1998) was impaired in those mice.

One key feature of the NR2 C-terminal tails is that they all contain conserved sequences at the very end: ESDV for NR2A and NR2B, and ESEV for NR2C and NR2D. These short sequences are known ligands for PDZ domains (Sheng and Pak, 2000), which mediate the interaction between NMDARs and PSD-95 (see Figure 1-4 a). The PDZ domain was initially recognized as an ~90 amino acid-long conserved sequence in three proteins including PSD-95/SAP90 (PSD for Postsynaptic Density, SAP for Synaptic Associated Protein), Dlg (the Drosophila septate junction protein Discs-large), and ZO-1 (the epithelial tight junction protein). PSD-95, Discs-large, and ZO-1 belong to a superfamily of membrane-associated guanylate kinases (MAGUKs). The PSD-95 subfamily of MAGUKs includes PSD-95/SAP90, PSD-93/Chapsyn-110, SAP97/hDlg, and SAP102, each of which contains three N-terminal PDZ domains, an SH3 domain, and a C-terminal guanylate kinase (GK)-like domain (Figure 1-4 a) (reviewed by Garner
et al., 2000; Sheng and Pak, 2000; Sheng and Sala, 2001; Hung and Sheng, 2002). Every member of the PSD-95 family of proteins except SAP97 is expressed in the PSD and interacts with NMDA NR2 subunits (Sheng and Pak, 2000).

Genetic studies have shown that Dlg, the only homolog of PSD-95 in Drosophila, is critical for synaptic targeting of the Shaker K⁺ channel and Fasciclin II (Tejedor et al., 1997; Thomas et al., 1997; Zito et al., 1997). In COS-7 cells transfected with NMDARs, overexpression of PSD-95 is sufficient to induce NMDAR clustering in the membrane (Kim et al., 1996). However, the role of PSD-95 in NMDAR synaptic targeting has yet to be established. PSD-95 knockout mice showed normal synaptic localization of NMDARs (Migaud et al., 1998). On the other hand, PSD-95 has been recently shown to target AMPA receptors to the synapse, through interaction with a transmembrane AMPA receptor interacting protein stargazin (Chen et al., 2000; Schnell et al., 2002).

Although its role in NMDAR targeting is still not known, PSD-95 is clearly important in NMDAR-mediated signal transduction, as PSD-95 knockout mice display severely impaired NMDAR-dependent synaptic plasticity and learning ability (Migaud et al., 1998). The effects of PSD-95 on NMDAR-mediated signalling may be explained by recent discoveries that, in addition to interaction with NMDARs, PSD-95 PDZ domains also directly bind to many signalling molecules, including neuronal nitric oxide synthase (nNOS), SynGAP (GTPase-activating protein for the small GTPase Ras), citron (effector for the small GTPase Rho), and Src family nonreceptor tyrosine kinases (see Figure 1-4 a) (reviewed by Sheng and Pak, 2000).

The GK-like domain of PSD-95 provides another site for interaction with proteins, including guanylate kinase-associated protein (GKAP)/SAPAP/DAP, BEGAIN,
and the microtubule-binding protein MAP1A (Figure 1-4 a) (Sheng and Pak, 2000). The physiological significance of these interactions remains unclear.

In addition to those signalling proteins, PSD-95 also links NMDARs to the cytoskeleton. The third PDZ domain of PSD-95 interacts with CRIPT (Figure 1-4 a), a small protein that associates with microtubules (Passafaro et al., 1999). PSD-95 also binds to MAP1A through its GK domain (Brenman et al., 1998). A recent study showed that, by interaction with GKAP, a new PDZ-domain containing protein Shank interacts with PSD-95 to form a ternary complex of Shank/GKAP/PSD-95. Shank directly binds to cortactin, which links the NMDAR/PSD-95 complex to F-actin, the major cytoskeletal element within the spine (Naisbitt et al., 1999).

In addition to the interactions mediated by PSD-95 family proteins, NR2 subunits can also directly interact with signalling molecules. For example, CaMKII has been reported to bind to the C-terminus of NR2B (see Figure 1-4 b) (Strack and Colbran, 1998; Leonard et al., 1999; Strack et al., 2000). Ca$^{2+}$ influx via NMDARs is sufficient to target CaMKII to the C-terminus of NR2B, and the interaction with the NR2B subunit locks CaMKII in an active conformation (Bayer et al., 2001). This interaction appears to be physiologically important since it puts CaMKII in an optimum location to respond to Ca$^{2+}$ influx due to NMDAR activation and suppresses inhibitory autophosphorylation of CaMKII (Bayer et al., 2001).

1.7.2. Interactions of the NR1 subunit

The NMDAR NR1 subunit C-terminus contains three cassettes C0, C1, and C2. The C1 and C2 cassettes, which are encoded by exons 21 and 22, undergo alternative
splicing (Figure 1-2). Together with the alternative splicing of the N-terminal N1 cassette (encoded by exon 5), alternative splicing gives rise to eight NR1 isoforms (see Figure 1-2) (Dingledine et al., 1999). The C-terminal tails of NR1 subunits are shorter (~100 amino acids) than those of NR2 and usually do not interact with PSD-95, except for splice variants containing the C2' cassette (Kornau et al., 1995b; Sheng and Pak, 2000). However, the C-terminus of NR1 directly binds to a number of proteins that can regulate NMDAR function (see Figure 1-4 b). The actin-binding protein α-actinin binds to the CO domain of NR1 competitively with Ca²⁺/calmodulin (Wyszynski et al., 1997).

Replacement of α-actinin by Ca²⁺/calmodulin upon a rise in intracellular Ca²⁺ causes Ca²⁺-dependent inactivation of the NMDAR (Ehlers et al., 1996; Zhang et al., 1998; Krupp et al., 1999) (see 1.9. for details). Since α-actinin is enriched in the spine, this also provides NMDARs with another anchor to the cytoskeleton (Figure 1-4 b) (Sheng and Pak, 2000).

The C1 cassette of the NR1 C-terminus contains protein kinase C (PKC) phosphorylation sites. Phosphorylation of C1 by PKC inhibits clustering of NR1 expressed in fibroblasts (Ehlers et al., 1995). A recent study showed that a novel protein, yotiao, binds to the C1 cassette (Figure 1-4 b) (Lin et al., 1998). Yotiao also binds to both protein kinase A (PKA) and protein phosphatase 1 (PP1) (Figure 1-4 b). Thus yotiao targets PKA and PP1 to NMDARs containing the NR1-2 and NR1-3 splice variants (see 1.3.2.) to facilitate their regulation of NMDAR function (Westphal et al., 1999).

Neurofilament L has also been shown to bind the C1 cassette (Figure 1-4 b) (Ehlers et al., 1998), but the functional consequences of this interaction is unclear.
Another actin-binding protein, spectrin, has been reported to bind the C-terminal intracellular tails of NR1, NR2A, and NR2B via sites different from the binding sites for α-actinin or PSD-95 (Figure 1-4 b) (Wechsler and Teichberg, 1998). The interaction of spectrin and NR2B is inhibited by calcium and Fyn phosphorylation of NR2B, whereas the interaction between spectrin and NR1 is antagonized by calmodulin and PKC/PKA phosphorylation of NR1 (Wechsler and Teichberg, 1998). Thus, spectrin appears to be another anchor to actin cytoskeleton for both NR1 and NR2 subunits.

Anchoring of NMDARs to the cytoskeleton is important for their synaptic localization, as disruption of F-actin by latrunculin moves NMDARs along with PSD-95 away from synapses, resulting in decreased NMDAR-mediated synaptic current amplitude with unchanged whole-cell current amplitude (Allison et al., 1998; Sattler et al., 2000).
Figure 1-4. Interaction of NMDAR subunits with intracellular scaffolding and signalling proteins

(Modified from Sheng and Pak, 2000)

(a) The NMDAR NR2 subunits C-terminal tails bind to the first two PDZ domains of PSD-95. Some of the identified proteins that bind to PSD-95 and their binding sites are shown. PSD-95 is clustered via its N-terminus that is palmitoylated (Craven and Bredt, 1998; Craven et al., 1999). The PDZ domains are labeled 1, 2, and 3, respectively. S, SH3 domain; GK, guanylate kinase-like domain; nNOS, nitric oxide synthase; GKAP, guanylate kinase-associated protein; SynGAP, synaptic GTPase-activating protein. (b) The NR1 subunit C-terminus contains three segments: C0, C1, and C2. The C1 and C2 cassettes are alternatively spliced. C0 and C1 mediate binding with different set of proteins. Black filled circles represent actin-binding domains of α-actinin and spectrin. CaM, Ca$^{2+}$/calmodulin; CaMKII, calmodulin-dependent kinase type II; PP1, protein phosphatase 1; PKA, protein kinase A; NF-L, neurofilament-L.
Modified from Sheng and Pak, 2000
1.8. NMDAR Trafficking

A great progress in our understanding of the mechanisms of synaptic plasticity is the finding that AMPA receptors undergo rapid cycling between the synaptic membrane and the intracellular pool in response to different patterns of stimulation that induce synaptic plasticity (reviewed by Malinow and Malenka, 2002). Stimulation that induces LTP leads to the rapid insertion of AMPA receptors to the synaptic membrane (Shi et al., 1999; Hayashi et al., 2000; Shi et al., 2001), whereas stimulation that induces LTD results in the rapid removal of AMPA receptors from the synaptic membrane by endocytosis (Carroll et al., 1999a; Carroll et al., 1999b; Luscher et al., 1999; Heynen et al., 2000; Lin et al., 2000; Man et al., 2000; reviewed by Carroll et al., 2001). Whereas the trafficking of AMPA receptors gained most of the attention over the past few years, NMDA receptors have been suggested to be less mobile (Luscher et al., 1999; Ehlers, 2000), and its movement can only be resolved at a longer time scale. For example, NMDARs move into the synapse during chronic inhibition (2-3 weeks) of neuronal activity (Rao and Craig, 1997). Recently, however, there is growing evidence to suggest that NMDARs are also capable of rapid movement.

1.8.1. Insertion

At developing excitatory synapses, NMDARs and AMPARs are clustered at the postsynaptic side within 1-2 hr after initial contact between axons and dendrites (Friedman et al., 2000a). In adult hippocampal slices, LTP stimulation rapidly (30 min after stimulation) promotes NMDAR insertion into cell surface membrane and increases
NMDAR-mediated synaptic responses in a tyrosine phosphorylation-dependent manner (Grosshans et al., 2002). Interestingly, activation of dopamine D1 receptor for 10 min markedly increases the insertion of NMDARs to the postsynaptic membrane in rat striatal slices, and this effect is also mediated by tyrosine phosphorylation (Dunah and Standaert, 2001).

An in vitro study showed that NR2A subunits are inserted into synapses in an activity-dependent manner (Barria and Malinow, 2002) and an in vivo study also showed that experience-driven synaptic insertion of new NR2A subunits occurs rapidly within hours (Quinlan et al., 1999b). Chronic treatment of neuronal cultures with either the NMDAR antagonist APV or sodium channel blocker TTX dramatically increases the synaptic expression of NMDARs (Rao and Craig, 1997; Liao et al., 1999; Watt et al., 2000). Since insertion of NR2A, but not NR2B, subunits into synapses depends on agonist binding of NMDARs (Barria and Malinow, 2002), chronic blockade of synaptic transmission may result in a preferential accumulation of NR2B subunits at synapses, as shown by a recent study (Chavis and Westbrook, 2001).

PKC activation has been shown to promote surface expression and synaptic targeting of NMDARs (Lan et al., 2001a; Lan et al., 2001b). However, other studies showed that PKC phosphorylation of serine890 within the C1 cassette of NR1 results in dispersion of surface associated clusters of the NR1 subunits expressed in fibroblasts (Ehlers et al., 1995; Tingley et al., 1997), and that PKC treatment of cultured rat hippocampal neurons rapidly (within 45 min) drives synaptic NMDARs to the extrasynaptic sites (Fong et al., 2002).
The effects of PKC on NMDAR trafficking may involve multiple mechanisms. First, an ER retention motif, RXR (X is any amino acid), has been identified for the NR1 subunit in the alternatively spliced C1 cassette (Standley et al., 2000; Scott et al., 2001). PKC phosphorylation of Ser 896, which is close to the ER-retention motif, promotes exit of NR1 subunits from the ER, and thus mediates the relatively slow (within 2-3 hr) insertion of NMDARs into the cell membrane (Scott et al., 2001). Second, rapid (within 10 min) insertion of NMDARs into cell membrane may involve phosphorylation of SNAP (synaptosome-associated protein)-25 (Lan et al., 2001a; Lan et al., 2001b, and reviewed by Carroll and Zukin, 2002). Third, the effect of PKC may be mediated by activation of tyrosine kinases (see 1.6. for details), which have been shown to be involved in NMDAR trafficking (Dunah and Standaert, 2001; Grosshans et al., 2002). The mechanism by which PKC activation disperses NMDAR clusters or moves synaptic NMDARs to extrasynaptic sites is unknown. It is possible that phosphorylation by PKC disrupts the interaction between NMDARs and some scaffolding proteins and thus promotes dispersion of NMDARs.

1.8.2. Internalization

Previous studies suggested that, compared with AMPA receptors, NMDARs are more tightly anchored in the postsynaptic membrane and may not undergo rapid regulated internalization (Luscher et al., 1999; Ehlers, 2000). Using different experimental paradigms, however, recent studies indicated that NMDARs also rapidly internalize in response to a variety of stimuli. Activation of group I mGluRs for 15 min induces marked internalization of synaptic NMDARs (Snyder et al., 2001). NMDAR-
dependent LTD might also induce NMDAR internalization (Montgomery and Madison, 2002). Binding of agonist to recombinant NR1/NR2A expressed in HEK293 cells induces a rapid (within 10 min) down-regulation of NMDAR activity that is dependent on the clathrin-adaptor protein AP-2, indicating that this down-regulation is likely due to NMDAR endocytosis (Vissel et al., 2001). The replacement of NR2B subunits with NR2A at the synapse triggered by agonist binding to NMDARs is probably preceded by internalization of NR2B subunits (Barria and Malinow, 2002). Interestingly, in contrast to the tyrosine phosphorylation-dependence of NMDAR insertion (Dunah and Standaert, 2001; Grosshans et al., 2002), the use-dependent internalization of NMDARs found in transfected non-neuronal cells depends on the dephosphorylation of a tyrosine residue just following the M4 transmembrane domain (Y842 for NR2A) (Vissel et al., 2001). Mutation of this tyrosine residue blocks the use-dependent down-regulation of NMDARs (Vissel et al., 2001). Using an electrophysiological method, another group showed that activation of PKA also induces rapid (within 6 min) down-regulation of recombinant NMDARs expressed in HEK cells (Woodward, 2002). The PKA activation induced NMDAR down-regulation is blocked by either mutation of the Y842 or by hypertonic sucrose application, suggesting that NMDAR internalization may be involved in this NMDAR down-regulation (Woodward, 2002).

NMDARs in immature, but not mature, neurons also undergo rapid (within 15 min) constitutive internalization (Roche et al., 2001). An internalization motif (YEKL) that is close to the PSD-95 binding sequence is defined on the distal C-terminus of NR2B. Interaction of NR2B with PSD-95 inhibited constitutive NR2B internalization,
which may explain why NMDARs in mature neurons, most of which are synaptic and interact with PSD-95, do not internalize (Roche et al., 2001).

1.8.3. Lateral movement

Shuttling of receptors in and out of synapses by lateral diffusion provides another way to control synaptic strength or replace nonfunctional receptors at the synapse (reviewed by Carroll and Zukin, 2002). It has been shown that acetylcholine receptors can rapidly diffuse in and out of the neuromuscular junction and produce immediate changes in synaptic responses (Young and Poo, 1983; Akaaboune et al., 1999). Recently glycine and AMPA receptors have also been shown to move laterally within the membrane on a time scale of seconds (Meier et al., 2001; Borgdorff and Choquet, 2002), and that the mobility of AMPA receptors decreases during maturation of the neuron (Borgdorff and Choquet, 2002).

Lateral movement of NMDARs is suggested by a study showing that PKC treatment of cultured rat hippocampal neurons disperses synaptic NMDARs to extrasynaptic sites within 45 min (Fong et al., 2002). Using electrophysiological and pharmacological methods, a recent study showed that NMDARs are also capable of more rapid lateral movements into synapses (Tovar and Westbrook, 2002). The authors showed that, after blockade of synaptic NMDARs by the use-dependent channel blocker MK801, the synaptic NMDAR-mediated response recovers to about 30% of control amplitude within 7 min. The recovery of synaptic NMDAR-mediated response is not due to the unbinding of MK801 or insertion of new receptors into the synapses, suggesting
that it is due to the diffusion of extrasynaptic receptors into the synapse (Tovar and Westbrook, 2002).

It has been well established that NMDARs play a key role in the induction of synaptic plasticity at certain synapses. The fact that NMDARs also undergo rapid regulated insertion, internalization, and lateral movement at the synapse suggests that NMDAR may also directly contribute to the modification of synaptic strength and participate in the expression of synaptic plasticity (Carroll and Zukin, 2002).

1.9. Negative feedback regulation of NMDARs

After NMDAR is activated, its channel activity is limited in a negative feedback manner via a number of processes including (1) peak-current rundown (Rosenmund and Westbrook, 1993b; Rosenmund and Westbrook, 1993a; Wang et al., 1996; Norenberg et al., 1999; Price et al., 1999; Vissel et al., 2001; Li et al., 2002; Woodward, 2002), (2) Ca\(^{2+}\)-dependent inactivation (Legendre et al., 1993; Medina et al., 1995; Ehlers et al., 1996; Krupp et al., 1996; Wyszynski et al., 1997; Zhang et al., 1998; Krupp et al., 1999), (3) glycine-dependent desensitization (Mayer et al., 1989; Vyklicky et al., 1990), and (4) glycine-independent desensitization (Krupp et al., 1998; Villarroel et al., 1998). A description of each process including the different time courses, the proposed mechanisms, as well as the optimal protocols for induction is given in table 1.
Table 1. Different forms of negative feedback NMDAR regulation

<table>
<thead>
<tr>
<th>Process</th>
<th>Optimal protocol for induction</th>
<th>Time-course</th>
<th>Mechanism(s)</th>
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<tr>
<td></td>
<td></td>
<td>Onset (s)</td>
<td>Recovery (s)</td>
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<tr>
<td>Rundown</td>
<td></td>
<td>~100-1000</td>
<td>~100-1000</td>
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<tr>
<td></td>
<td>Conventional whole-cell</td>
<td>~100-1000</td>
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<tr>
<td></td>
<td>recording: 10-30 μM glycine, 10 μM of NMDA (applied every 50s for 3s), 2 mM Ca²⁺</td>
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<td></td>
<td>Nystatin-perforated</td>
<td>~100-1000</td>
<td>Not determined</td>
</tr>
<tr>
<td></td>
<td>recording: 10-30 μM glycine, 10 μM of NMDA (applied every 30-60s for 10-15s), 2 mM Ca²⁺</td>
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<tr>
<td></td>
<td>Ca²⁺-independent</td>
<td>~100-1000</td>
<td>Not determined</td>
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<td></td>
<td>(Wang et al., 1993; Wang et al., 1996; Vissel et al., 2001)</td>
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<tr>
<td></td>
<td>1-100 μM glycine, 0.1-1 mM NMDA (applied every 30-120 s for 5-10s), 0 mM Ca²⁺</td>
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<tr>
<td>Ca²⁺-dependent inactivation</td>
<td>10-30 μM glycine, 0.1-1 mM of NMDA (2-5 s), 2 mM Ca²⁺</td>
<td>~0.1-1</td>
<td>~1-10</td>
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<td></td>
<td>(Legendre et al., 1993; Medina et al., 1995; Ehlers et al., 1996; Krupp et al., 1996; Wyszynski et al., 1997; Zhang et al., 1998; Krupp et al., 1999)</td>
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<td></td>
<td>30 nM-0.3 μM of glycine</td>
<td>≥ 5 (for 30 nM of glycine)</td>
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<tr>
<td>Glycine-independent desensitization</td>
<td>10-30 μM glycine, 0.1-1 mM of NMDA (2-5 s), 0 mM Ca²⁺</td>
<td>~0.1-1</td>
<td>~0.5-2</td>
</tr>
<tr>
<td>Sather et al., 1990; Sather et al., 1992; Tong and Jahr, 1994; Tong et al., 1995; Krupp et al., 1998; Villarroel et al., 1998)</td>
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1.9.1. Rundown

Repeated activation of NMDARs induces a reduction of the peak current amplitude over time, which is known as “rundown”. Rundown may occur by more than one mechanism, since reports differ with respect to its Ca\(^{2+}\) dependence (Rosenmund and Westbrook, 1993a; Wang et al., 1996). Ca\(^{2+}\)-dependent rundown is due to the Ca\(^{2+}\) influx from NMDARs and subsequent depolymerization of F-actin (Rosenmund and Westbrook, 1993a; Rosenmund and Westbrook, 1993b). Ca\(^{2+}\)-dependent rundown is blocked by including an ATP-regenerative system in the electrode solution, by eliminating extracellular Ca\(^{2+}\), by high intracellular Ca\(^{2+}\) buffering, or by stabilizing F-actin (Rosenmund and Westbrook, 1993a; Rosenmund and Westbrook, 1993b).

Ca\(^{2+}\)-independent rundown depends on tyrosine dephosphorylation (Wang et al., 1993; Wang et al., 1996; Vissel et al., 2001; Woodward, 2002), which is probably through tyrosine dephosphorylation of the NMDAR itself, as mutation of certain tyrosine residues in the C-termini of NR1 or NR2A blocks rundown (Vissel et al., 2001). The Ca\(^{2+}\)-independent rundown is best explained by NMDAR endocytosis following tyrosine dephosphorylation, since there is a reduction of the number of NMDARs on the cell surface (Vissel et al., 2001), and rundown can be blocked by a dominant-negative subunit of AP-2, which is a clathrin adaptor protein (Vissel et al., 2001), or by hypertonic sucrose, which inhibits the assembly of the clathrin coat (Woodward, 2002).

1.9.2. Ca\(^{2+}\)-dependent inactivation

Ca\(^{2+}\)-dependent inactivation depends on Ca\(^{2+}\) influx through NMDARs or voltage-dependent Ca\(^{2+}\) channels (Legendre et al., 1993; Medina et al., 1995). Studies on
recombinant NMDARs showed that NR1/NR2A and NR1/NR2D have a greater inactivation than NR1/NR2B, whereas NR1/NR2C does not inactivate (Krupp et al., 1996). Since Ca$^{2+}$/calmodulin and α-actinin bind competitively to the C0 cassette of NR1 (see Figure 1-4 b) (Wyszynski et al., 1997), Ca$^{2+}$/calmodulin formed upon Ca$^{2+}$ influx through activated NMDARs replaces α-actinin binding on the C-terminus of NR1 (Zhang et al., 1998). Because α-actinin attaches NMDARs to F-actin, which is required for the fully functioning of NMDARs (Rosenmund and Westbrook, 1993a), the replacement of α-actinin by Ca$^{2+}$/calmodulin leads to Ca$^{2+}$-dependent inactivation (Ehlers et al., 1996; Zhang et al., 1998). Furthermore, α-actinin can also bind directly to Ca$^{2+}$, which decreases its affinity for NR1 and thus contributes to inactivation (Krupp et al., 1999). A recent study showed that PKC activation enhances Ca$^{2+}$-dependent inactivation, probably due to a phosphorylation dependent regulation of the interaction between NR1 and calmodulin (Lu et al., 2000).

1.9.3. Glycine-dependent desensitization

Glycine-dependent desensitization is due to the negative allosteric interaction between the glutamate and glycine binding sites. Binding of glutamate reduces the receptor’s affinity for glycine, leading to a reduction of current during agonist application (Mayer et al., 1989; Vylický et al., 1990; Dingledine et al., 1999). Glycine-dependent desensitization can be eliminated by a saturating concentration of glycine.

1.9.4. Glycine-independent desensitization
Glycine-independent desensitization was initially described for cultured neurons, in which NMDAR currents decay during sustained application of glutamate or NMDA in spite of a saturating concentration of glycine (Sather et al., 1990; Sather et al., 1992). Unlike the Ca\(^{2+}\)-dependent inactivation (see 1.9.2.), the molecular determinants of glycine-independent desensitization have recently been mapped on NR2 subunits using mutagenesis techniques on recombinant NMDARs (Krupp et al., 1998; Villarroel et al., 1998). Glycine-independent desensitization is subtype dependent. Both 2B and 2A subtype show desensitization, whereas 2C and 2D subtype do not desensitize (Krupp et al., 1996; Krupp et al., 1998; Villarroel et al., 1998). The two domains flanking the putative agonist-binding domain S1 (see Figure 1-1 a) in the N-terminus of the NR2A subunit are both necessary and sufficient for determining glycine-independent desensitization (Krupp et al., 1998; Villarroel et al., 1998).

Glycine-independent desensitization is modulated by calcineurin activity, which appears as a time-dependent increase in desensitization (Sather et al., 1992; Tong and Jahr, 1994; Tong et al., 1995; Krupp et al., 2002). The effects of calcineurin are modulatory rather than mandatory, because 1) inhibition of calcineurin activity only partially blocks glycine-independent desensitization (Tong and Jahr, 1994), 2) calcineurin should exert its effects on the intracellular C-terminus of NR2A (Krupp et al., 2002). However, chimeric receptor containing the extracellular N-terminus of non-desensitizing NR2C and the intracellular C-terminus of desensitizing NR2A does not desensitize even after prolonged recording (Krupp et al., 1998).

1.10. Rationale of research
1.10.1. Hypothesis

Extensive studies on recombinant NMDARs expressed in heterologous systems have shown that NR2 subunits modulate several critical receptor-channel properties (see 1.4. for details). Specifically, NR2 subunits dramatically influence NMDAR deactivation, glycine-independent desensitization, and Ca\(^{2+}\)-dependent inactivation. Similar studies on neuronal NMDARs have been lacking, partly because more than one of the four possible NR2 subunits are expressed in individual neurons. Moreover, different subunits of NMDARs have different subcellular localization so that in mature cultured hippocampal neurons the NR2B subunit is mainly extrasynaptic, whereas NR2A is mainly synaptic (see 1.3.). The synaptic NMDARs reside in the PSD, a highly specialized apparatus concentrated with scaffolding proteins, cytoskeleton, and numerous signalling molecules (see 1.7.). A recent study identified 77 proteins in the NMDAR complex (Husi et al., 2000), many of which regulate NMDAR activity (see 1.6. and 1.7.). Extrasynaptic NMDARs, including NMDARs in immature neurons and 2B-subtype of NMDARs in mature neurons, apparently interact less with those proteins enriched in the PSD and are coupled to different signalling pathways (Lu et al., 2001; Hardingham and Bading, 2002; Hardingham et al., 2002). Therefore extrasynaptic NMDARs are probably also regulated differently compared with synaptic NMDARs.

Based on all these findings our general hypothesis is that synaptic and extrasynaptic NMDARs are regulated differently by distinct intracellular processes based on their specific subcellular localization. As a result, these two subpopulations of NMDARs, when stimulated, will respond differently with respect to their degree of rundown or desensitization. Since NMDARs are gradually incorporated into the synapse
during development, with the majority of NMDARs being extrasynaptic early in development and becoming increasingly synaptic during maturation (see 1.3.), there should also be a developmental change in NMDAR properties that correlates with the transition in subcellular localization.

To test our hypotheses we have focused on two well studied NMDAR properties: peak current rundown and glycine-independent desensitization (see 1.9. for details).

1.10.1. Physiological relevance

Regulation of NMDAR activity is believed to be important in both physiological and pathological conditions. Synaptic NMDAR desensitization during repetitive firing contributes to shaping synaptic responses and neuronal activity (Tong et al., 1995; Jones and Westbrook, 1996). Manipulations that suppress Ca\(^{2+}\)-dependent rundown of NMDARs enhance NMDA- or glutamate-induced excitotoxicity (Furukawa et al., 1995; Abdel-Hamid and Baimbridge, 1997; Furukawa et al., 1997), whereas conditions that promote Ca\(^{2+}\)-dependent rundown protect from glutamate- or OGD (oxygen/glucose deprivation)-induced excitotoxicity (Furukawa et al., 1995; Sattler et al., 2000). Rapid desensitization of non-NMDARs is protective against AMPA-induced excitotoxicity (Zorumski et al., 1990; May and Robison, 1993; Brorson et al., 1995; Raymond et al., 1996). Desensitization and inactivation of NMDARs, although slower and more limited in extent compared with non-NMDA receptors (McBain and Mayer, 1994; Dingledine et al., 1999), may also be protective during a sustained glutamate insult such as that occurs in ischemia, by limiting calcium influx through its own channel.
Differential regulation of synaptic versus extrasynaptic NMDARs is of great interest considering that they are functionally distinct. Calcium influx through synaptic NMDA receptors induces CREB (cAMP response element binding protein) function and BDNF (brain-derived neurotrophic factor) gene expression, and is anti-apoptotic. In contrast, calcium entry through extrasynaptic NMDARs shuts off the CREB pathway, blocks BDNF expression, causes loss of mitochondrial membrane potential and cell death (Hardingham and Bading, 2002; Hardingham et al., 2002). Also, selective activation of synaptic NMDARs induces synaptic insertion of new AMPA receptors and LTP, whereas activation of extrasynaptic NMDARs results in LTD in cultured hippocampal neurons (Lu et al., 2001). Activation of extrasynaptic NMDARs may occur by “spillover” due to enhanced transmitter release (Lozovaya et al., 1999), which may happen during repeated firing or pathological conditions such as ischemia. Conceivably differential regulation of synaptic versus extrasynaptic NMDAR function may contribute to adapting them to the distinct roles they play in different situations.
CHAPTER II

Materials and methods

2.1. Standard embryonic hippocampal culture and transfection

2.1.1. Culture

Embryonic hippocampal cultures were prepared based on a protocol modified from that described previously (Brewer et al., 1993; Brewer, 1995). The detailed protocol is described at below.

Solutions

1. Poly-D-lysine (PDL, Sigma P-6407): 50 μg/ml.

2. Eagle’s balanced salt solution (EBSS) (1 L): EBSS salts (Sigma E-6132) 8.7 g, glucose (Sigma G-5767) 2.01 g, NaHCO₃ (Sigma S-5761) 2.2 g.

3. EBSS+ (13.3 ml): EBSS solution 13.3 ml, L-cysteine HCl 2.34 mg, EDTA (50 mM) 0.133 ml.

4. Papain (Sigma P-3125) solution: 20 U/ml (diluted with EBSS+). Papain solution was kept in 37°C until clear, and oxygenated for 3 min just before use.

5. 10/10 solution (20 ml): EBSS 20 ml, BSA (Sigma A-3350) 200 mg, ovomucoid (trypsin inhibitor, Sigma T-9253) 200 mg. The solution was made just before use and kept at 37°C for 1 hr.

6. DNAase stock solution (1 ml): DNAase (Sigma DN25) 5 mg, EBSS 1 ml. The stock solution was stored at –20°C.
7. DNAase working solution in (5 ml): EBSS 4.5 ml, 10/10 solution 0.5 ml, DNAase stock solution 100 µl. This solution was made immediately before use.

8. BME stock (10 ml): BME (2-Mercapto-ethanol, sigma M-7522) 7.2 µl, Neurobasal medium 10 ml.

9. Plating and feed medium (500 ml): B27 (Gibco 17504-044) 10 ml, glutamine 0.5 mM, penicillin/streptomycin (10 U/ml), BME 1.25 ml, Neurobasal Medium (NBM, Gibco 21103-049) 500 ml.

**Procedure**

1. The following solutions were prepared (all sterile) before making culture.
   a. poly-D-lysine (PDL): 50µg/ml
   b. sterile Milli-Q H₂O
   c. EDTA stock (50 mM)
   d. PBS
   e. Eagle’s balanced salt solution (EBSS)
   f. 10/10 solution (see below)
   g. DNAase (5mg/ml in EBSS, stored at -20 °C)
   h. Plating medium (keep no longer than 14 days)

2. The coverslips were coated with 50µg/ml PDL overnight in 24-well plates. The PDL working solution was diluted from the stock solution with sterile Milli-Q H₂O.
3. PDL was aspirated from the plate. The plates were air-dried for at least 1 hour (could be stored for up to one week).

4. Plating medium was prepared (if not made the day before).

5. The following materials were prepared in the dissection hood:
   a. petri dishes (100, 65 & 35mm)
   b. conical tubes (50 & 15 ml)
   c. syringes (30 & 10 ml) & syringe filters (0.22 μm Millex-GV low-binding)
   d. dissection tools (in 70% ethanol for sterilization)
   e. under-pad for initial dissection
   f. cold PBS

6. Papain solution (15 ml) was made at a concentration of 20 U/ml in EBSS+. The solution was kept in 37 °C water bath until clear (~30min), and was then oxygenated for 3 min. The solution was filtered (with 0.22 μm filter) to sterilize before use.

7. While the papain solution was heating, the anesthetized 17-18-day gestation rat was decapitated. The dissection was immediately performed in the dissection hood.

8. Dissection:
   a. The rat was put on a fresh under-pad in the hood, with ventral side up. The abdomen was sprayed well with 70% ethanol to sterilize.
   b. Cold PBS was prepared in two 10-cm plates, with each about half full.
   c. The skin of the abdomen was lifted using heavy forceps, and was cut open using heavy scissors.
d. The uterus was lifted using heavy forceps and was cut free at the base and from the mesenteries. The uterus was then placed in a dish of cold PBS.

e. The uterus was cut open, and fetuses were removed (without placenta) to another dish of cold PBS.

f. Cold PBS was prepared in two 65-mm dishes.

g. Fetuses were decapitated, and the heads were placed in one 65-mm dish with fresh PBS, rinsed and transferred to another dish of PBS.

h. Additional cold PBS was prepared in several 35-mm dishes, which were placed on ice.

i. Brains were removed using two pairs of fine forceps. To do this, the head was oriented with the crown up and nose to left (if you are right-handed). The points of the left hand forceps were placed into the eye sockets to hold the head steady. One prong of the right hand forceps was used to slit the skull along centerline in the nose-to-nape direction. Flaps of the skull were peeled back, and the brain was scooped out using the closed tips of the right hand forceps. The brains were placed in one of the 35-mm dishes with cold PBS (on ice).

j. The hippocampi were removed under a dissection microscope, and were placed in another 35-mm dish of cold PBS on ice.

9. Digestion:

a. Hippocampi were added to the sterile, warm papain solution, and were triturated for 2-3 times with a 10 ml pipette to break up the tissue.
b. The hippocampi were then incubated for 5-10 min at 37 °C water bath with mixing every 3 min.

c. The tissue was gently centrifuged for 2-3 min (1000 rpm) in one 15 ml tube.

d. DNAase working solution was prepared while spinning. The stock DNAase aliquots were used directly from the −20 °C freezer. 200 µl of DNAase stock solution was diluted into 5 ml of 1/1 solution, which was diluted 10 times from the 10/10 solution. The solution was filtered to sterilize.

e. The hippocampi pellet was resuspended in the DNAase solution. A 10-ml pipette was used to triturate the tissue for 4-5 times, and then a fire-polished, cotton-plugged Pasteur pipette was used to triturate for about ten times until tissue was broken up (do not over-triturate or make a lot of air bubbles).

f. 3-5 ml of 10/10 solution was added to the tube to create gradient (drip 10/10 solution slowly down along the side of a slanted tube).

g. Cells were gently centrifuged to form pellet (1000 rpm, 30 seconds).

10. The supernatant solution was removed from the tube without the pelleted cells intact. The pellet was washed once gently with 5 ml of plating medium.

11. The pellet was resuspended in 6 ml of plating medium and cells were counted using a hemacytometer. All living cells in the 16 squares (4X4 bounded by triple lines) were counted. The number of cells/ml = the number of cells in the 16 squares X 25 X 10^4.
12. The cells were plated on the dry, PDL-coated coverslips in the 24-well-plate at a desired density. Excellent long-term viability can be achieved after 4 weeks in culture with greater than 90% viability for cells plated at 640 cells/mm$^2$ (2.26X$10^5$ cells/ml if you plate 0.5 ml of cells into one well of 24-well-plate) and greater than 50% viability for cells plated at 160 cells/mm$^2$ (0.57X$10^5$ cells/ml) (Brewer et al., 1993; Brewer, 1995). For my experiments, the cells were plated at a density of about 300-400 cells/mm$^2$.

13. One-half of the medium was replaced with fresh medium at 4-day in vitro. One-half of the medium was changed twice a week thereafter.

2.1.2. Transfection

Neurons were transfected with either PSD-95-GFP or GFP construct by lipid-mediated gene transfer kit (DOTAP, Roche Diagnostics, Quebec, Canada) as described (Craven et al., 1999), or by Effectene Transfection Reagent (QIAGEN, Hilden, Germany).

For transfection with DOTAP, neurons were transfected just before plating in Hanks’ Balanced Salt Solution (HBSS, Invitrogen Canada, Ontario) at 1X$10^6$ cells/0.25 ml. Two µg of DNA and 10 µl of DOTAP were mixed in 25 µl of Heps-buffered saline (HBS; NaCl 150 mM, HEPES 20 mM [pH 7.4]) and added to the 0.25 ml cells with immediate and gentle mixing. After 1 hr of incubation at 37°C, cells were plated at 500-600 cells/mm$^2$ on PDL coated glass coverslips in 24-well plates. Cells were used for patch-clamp recording at 4-7 days in vitro (DIV).
For transfection with Effectene Transfection Reagent, neurons were transfected at 2-4 DIV. For transfection of 8 wells (in a 24-well plate), 2 µg DNA was mixed with 150 µl EC buffer and 16 µl enhancer. After 5 min of incubation at room temperature, 25 µl Effectene was added. The resulting solution was mixed gently and incubated for another 10 min at room temperature. 1 ml fresh Neurobasal medium was then added to the solution. 135 µl of this DNA-effectene mix solution was added into each well of neurons containing 200 µl of medium, and incubated at 37 °C for 3-5 hrs. DNA-effectene mix solution was then replaced with 0.5 ml medium (contained 50% conditioned and 50% fresh medium) for each well. Neurons were used for patch-clamp recording 2-3 days after transfection.

2.2. Microisland (autaptic) culture

Microisland (autaptic) cultures were made as previously described (Bekkers and Stevens, 1991). The detailed protocol is described at below.

2.2.1. Plate Preparation

1. Round coverslips (12 mm diameter, Marienfeld #1 thickness) were soaked in 70% HNO₃ for at least 24hr.
2. The coverslips were then rinsed with MilliQ H₂O and autoclaved.
3. Agarose solution (0.15%) was prepared by adding 15 mg agarose powder (Sigma A9918) to 10 ml MilliQ H₂O. The mixture was heated to almost boiling in a beaker on a hot plate until dissolved.
4. Each coverslip was coated with about 20µl of the agarose solution.
5. The coated coverslips were allowed to air-dry in the hood for about 30 min.
6. A collagen/PDL mixture (see below) was sprayed over the agarose-coated coverslips using a custom-designed sprayer. The sprayer was made from a syringe needle connected to a duster. Micro-dots dry within one min.
7. The coverslips were exposed to UV light for 30 min to sterilize.
8. The coverslips were then placed into 24-well plates.
9. The plates (with coverslips) were stored in the 37°C, 5% CO₂ cell-culture incubator until use.
10. The coverslips were washed with Minimum Essential Medium (MEM) once immediately before plating of cells.

Solution needed:

**Collagen/PDL mixture** (can be stored at 4°C for one month):

a. PDL (Sigma P6407) solution (1 mg/ml) was prepared with sterile MilliQ H₂O and stored in 0.5 ml aliquots in −20 °C freezer.

b. Collagen solution (Upstate biotechnology 08-115) was diluted with the PDL solution to ~2 mg/ml.

2.2.2. Glia island Preparation

1. After being kept for 3 days *in vitro*, the glial culture (see 2.1.1.2.3) was rinsed with culture medium using a 10-ml pipette until the neurons in the culture come off.
2. The medium was replaced with 9 ml fresh plating medium.
3. After 6 days *in vitro* (~90% confluent), the culture was rinsed with the culture medium to remove debris.

4. The medium was aspirated and 1 ml trypsin-EDTA solution (Gibco 25200) was added into the glial dish.

5. The culture was incubated in the cell culture incubator for a few minutes until the cells were suspended.

6. 1 ml plating medium was added to stop the enzymatic activity. The suspension solution (with glia in it) was removed and placed in a sterile 15 ml tube.

7. The dish was washed with 2 ml plating medium to obtain as many cells as possible and the solution was added to the 15 ml tube.

8. The solution (with cells in it) was centrifuged for 2 min at 1000 rpm.

9. The supernatant was discarded and the cells were resuspend in the plating medium.

10. Cells were plated at a density of 5-6×10⁴ cells/ml into the previously prepared plates at 0.5 ml/well.

11. FUDR (5 μl/well) was added 2 days later.

**Solutions needed:**

**Plating medium (100ml)**

- Minimum Essential Medium (MEM) (Gibco 5120-038) 87.5 ml
- FBS 10.0 ml
- 200 mM Glutamine (Sigma G5763) 1.0 ml
- Pen/Strep (Gibco 15140) 0.2 ml
30% glucose 1.2 ml
0.5% phenol red 0.1 ml

The solution was filtered (0.22 μm filter) and stored at 4°C.

**FUDR**

5-fluoro-2’deoxyridine (Sigma F0503) 8.3 mg
uridine (Sigma U3003) 20.83 mg
MEM 4.17 ml

The solution was filtered and stored in 200 μl aliquots in the −20 °C freezer.

### 2.2.3. Dissection procedure

1. Postnatal rats (P0-3) were anesthetized. After sterilized with 70% ethanol, the head was removed, and the whole brain was dissected out. The brain was placed in a 10-cm dish containing dissection solution.
2. Hippocampi were removed as in 2.1.1.1.2.
3. Each hippocampus was chopped into 3 pieces and the dentate gyrus (DG) was removed.
4. The resulting CA1- and CA3-enriched hippocampi were transferred to a 15 ml tube containing 5 ml papain solution and were incubated at 32°C for 15 min.
5. The tissue was transferred to a new 15 ml tube containing 5 ml papain solution and was incubated at 32°C for another 15 min.
6. The tissue was then transferred to a new 15 ml tube containing 5 ml inactivation solution, and was allowed to settle for 1 min.
7. The supernatant was discarded with the tissue untouched and 1 ml triturating solution was added.

8. The tissue was gently triturated with a 1 ml pipett for 10 times, and was further triturated with a fire-polished, cotton-plugged Pasteur pipette until the tissue was completely broken up.

9. The cells were resuspended in the plating medium and were counted using a hemacytometer. The neurons were plated onto the pre-made glia island at a density of ~1.2x10^4 cell/ml (0.5-0.6ml/well for the 24-well plate).

10. The remaining cells were plated into a 10-cm dish (9 ml per dish) at a density of ~8x10^5 cells/dish (the dish was not coated with PDL so that neurons could not grow) to make glial culture. This glial culture was used for glia island preparation (see 2.1.1.2.2.1).

11. The plating medium was completely changed to feed medium on day 1. A portion (~0.2 ml) of the medium was replaced with fresh feed medium thereafter every other day.

Solutions needed:

**10×Dissociation medium (DM)**

<table>
<thead>
<tr>
<th></th>
<th>grams/100ml</th>
<th>Concentration in 10XDM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na₂SO₄</td>
<td>12.78</td>
<td>900 mM</td>
</tr>
<tr>
<td>K₂SO₄</td>
<td>5.23</td>
<td>300 mM</td>
</tr>
<tr>
<td>MgCl₂-6H₂O</td>
<td>1.18</td>
<td>58 mM</td>
</tr>
<tr>
<td>CaCl₂-2H₂O</td>
<td>0.037</td>
<td>2.5 mM</td>
</tr>
</tbody>
</table>
HEPES 0.381 16 mM
0.5% Phenol red 2.0 ml 0.01%
1 M NaOH

Note: CaCl₂ was dissolved first, then the other Components. NaOH was added last to adjust pH to 7.4. The solution was warmed up to facilitate dissolve. The 10×DM solution was filtered (with 0.22 µm filter) and stored at 4°C. When the 10×solution was diluted to 1×, the measured osmolarity was 285 mOsm.

**Dissection solution (20ml)**

10X DM 2.0 ml
30% glucose 0.24 ml
100 mM Kynurenic Acid 0.2 ml
MilliQ H₂O 17.56 ml

Filtered (with 0.22 µm filter)

**Papain solution (10ml)**

10X DM 1 ml
MilliQ H₂O 8.5 ml
30% glucose 0.12 ml
100 mM Kynurenic Acid (Sigma K3375) 0.1 ml
5 mg/ml DNase (Sigma DN25) (dissolved in EBSS) 0.08 ml
L-cysteine 1.6 mg
papain (Sigma P3125) 200 units
pH was adjusted to 7.4 with 1M NaOH (~30 µl) and the solution was filtered (0.22 µm filter) immediately before use.

**Triturating solution (25ml)**

30 ml Plating medium

**Inactivation solution (5ml)**

BSA (Sigma A2153) 12.5mg
trypsin inhibitor (Sigma T9253) 12.5mg
Plating medium 5 ml

This solution was prepared during the 2nd papain treatment and filtered with 0.22 µm filter.

**Feed medium (50ml)**

Neurobasal–A medium (Gibco 10888) 46.925 ml
B27 (Gibco 17504) 1.0 ml
30% glucose 0.6 ml
200 mM Glutamine 0.125 ml
Pen/Strep 0.1 ml
FBS 1.25 ml

Filtered with 0.22 µm filter and stored at 4°C.

2.3. HEK293 cell culture and transfection
2.3.1. Culture

HEK (human embryonic kidney) 293 cells (CRL 1573; American Type Culture Collection, Rockville, MD) were maintained at 37° and 5% CO₂ in minimum essential medium containing Earle's salts and supplemented with L-glutamine (2 mM), sodium pyruvate (1 mM), penicillin/streptomycin (100 units/ml), and 10% fetal bovine serum. The cells were passaged every 3-4 days and plated at a density of ~1 × 10⁶/ml at 10-24 hr before transfection.

2.3.2. Transfection

Cells were transfected according to the method of calcium phosphate precipitation, as previously described (Chen and Okayama, 1988; Raymond et al., 1996). Briefly, a total of 12 μg of plasmid DNA was used for each 10-cm plate. Cells were transfected with a 1:1:1 ratio of cDNAs encoding NR1-1a (nomenclature of (Hollmann et al., 1993)), NR2 (A or B), and GFP (to allow for identification of transfected cells during recording) or PSD-95-GFP (as indicated). After precipitation with 3 M sodium acetate and 100% ethanol, the plasmid DNAs were resuspended in 450 μl 0.1 X TE, and mixed with CaCl₂ (2.5 M, 50 μl) and BES (2 X, 500 μl). The mixture was incubated for 20 min at room temperature. During this time, cells (in 10-cm plates) were placed in the 3% CO₂, 37 °C incubator for 20 min. The mixture containing DNAs was then added to each plate. After incubation for 8-10 hrs in the 3% CO₂, 37 °C incubator, the cell medium containing the transfection reagents was replaced by fresh medium. NMDAR antagonist D,L-APV (1 mM, for NR1/NR2A transfections) or memantine (100 μM, for NR1/NR2B transfections) was included in the medium to protect the cells from excitotoxicity.
(Raymond et al., 1996). Because 2B is agonist-preferring and APV is a competitive antagonist, we found memantine (a use-dependent blocker) was more effective in protecting cells expressing NR1/NR2B from excitotoxicity. Transfected cells were maintained on glass coverslips and used at 20-36 hr after the start of transfection.

2.4. Electrophysiology

Neurons from standard hippocampal neuronal cultures as well as autaptic cultures were used for recording at different developmental stages as indicated. Transfected HEK293 cells were used at 20-36 hr after the start of transfection. Cells were transferred to the stage of an inverted microscope (Axiovert 100, Carl Zeiss, Thornburg, NY) equipped with fluorescence detection. Patch clamp recording in the whole-cell mode was performed using either the nystatin-perforated patch clamp recording method (Akaike and Harata, 1994) to maintain intracellular contents, as previously described (Li et al., 2002), or conventional whole-cell patch clamp recording method (Hamill et al., 1981).

2.4.1. Internal solutions

For nystatin-perforated path clamp recording, a 100 mg/ml stock of nystatin was prepared fresh daily in dimethyl sulfoxide; 5 µl of this stock was added to 0.5 ml of the electrode solution, which contained 150 mM CsCl and 10 mM HEPES (pH 7.2 with Tris-OH). CsCl was replaced by KCl for recording on autaptic cells. The resulting suspension was vortexed and then bath-sonicated for 30 s. When filled with this solution, electrodes had resistances of 1-3 MΩ. Upon sealing the electrode to the cell, nystatin partitioning was allowed to proceed until the access resistance decreased and stabilized at < 30 MΩ.
For conventional whole-cell recording, the electrode solution contained Cs-methansulfonate 115 mM, HEPES 10 mM, K₂-creatine phosphate 20 mM, creatine phosphokinase 50 U/ml, MgATP 4 mM, BAPTA 10 mM, pH 7.26 (KOH), 310 mOsm/kg of water. For recording on autaptic neurons, the electrode solution contained 112.5 mM K-methansulfonate, 8 mM NaCl, 5 mM MgATP, 20 mM HEPES, 0.2 mM BAPTA, 20 mM K₂-creatine phosphate, 50 U/ml creatine phosphokinase (pH 7.2).

### 2.4.2. External solutions

The cells were continuously superfused with the external recording solution. For HEK293 cells, the external recording solution contained (in mM): NaCl 145, KCl 5.4, glucose 11, CaCl₂ 2, HEPES 10 (pH 7.3). Glycine (30 µM) was added just before use. For neurons the external recording solution contained (in mM): NaCl 167, KCl 2.4, HEPES 10, glucose 10, CaCl₂ 2, MgCl₂ either 0 or 1 as indicated (325 mOsm/kg of water, pH 7.3). TTX (300 nM), strychnine (2 µM), and glycine (30 µM or 100 µM as indicated) were added just before use. TTX was omitted for autaptic culture recording.

As previously described (Chen et al., 1997), agonist or drug application was gravity fed to the cells using a θ-tube. Glutamate (1 mM) or NMDA (100 µM or 1-2 mM, as indicated) was included only in the agonist side of the θ-tube, which contained the same solution as the control side except sometimes with reduced CaCl₂ concentration as indicated (0.2 mM CaCl₂ for the desensitization experiments). For the Ca²⁺-independent rundown experiments as indicated, 0 mM CaCl₂ plus 100 µM EGTA with 1 mM MgCl₂ were used in both agonist side and control side. MgCl₂ was included to maintain stable long-term recording (Wang et al., 1996). The solution in the control side of the θ-tube
was of the same ionic composition as the external bath solution (unless otherwise indicated) and was continuously superfused onto the cells.

Drugs other than the agonists were included in both the control and agonist side of the θ-tube. All drugs, including NMDA, glutamate, glycine, ifenprodil (10 or 3 μM), MK-801 (20μM), as well as many other drugs as indicated in the text, were diluted into the recording solution from stock solutions (most of which were made as aqueous solutions and kept at −20 °C) just before use.

Rapid switching between the control and agonist solutions was achieved by using computer-controlled solenoid-driven valves for neurons, or a piezo-driven switch (Burleigh, Fishers, NY, U.S.A.) for HEK293 cells (Chen et al., 1997), which were lifted from the bottom of the chamber.

2.4.3. Recording protocol

For rundown experiments, the recording protocol consisted of 10-s agonist applications at a rate of 2/min. For the experiments indicated, cells were incubated with either 50 μM genistein or 2 μM jasplakinolide for ~ 15 min before the beginning of recording. For experiments using 100 μM bpV(phen), this reagent was applied during recording.

For desensitization experiments, the recording protocol consisted of 10-s (or 3-s for exogenous agonist application on autaptic cells) agonist applications at a rate of 1/min, unless otherwise indicated.

For recording on autaptic neurons, EPSCs were evoked by action potentials triggered by depolarizing the cells from −65 mV to 0 mV for 1.5 ms, and the whole-cell
responses were evoked by applying 1 mM NMDA in the external solution in which CaCl$_2$ was replaced with 1 mM SrCl$_2$ to minimize Ca$^{2+}$-dependent inactivation (Legendre et al., 1993; Krupp et al., 1996).

All recordings were made in voltage-clamp mode. The membrane holding potential was –70 (for nystatin-perforated patch clamp recording) or –65 mV (for conventional whole-cell recording) when Mg$^{2+}$ was not present in the recording solutions, and was +40 mV when 1 mM Mg$^{2+}$ was present. Data were acquired using the Axopatch 200B (Axon Instruments, Foster City, CA) patch clamp amplifier. Currents were filtered at 5 kHz and digitized at 10 kHz. pCLAMP 6.1 or 8.1 software (Axon Instruments) was used for data acquisition and analysis. Access resistance and cell capacitance were monitored by applying a brief, 5 mV hyperpolarizing voltage step just before each agonist application (Li et al., 2002). The series resistance was compensated 60-70%.

2.5. Immunocytochemistry

To characterize the subcellular localization of NMDARs in cultured hippocampal neurons, cells were double-labelled with antibodies against a presynaptic marker synaptophysin and NR1. Briefly, neuronal cultures were fixed with methanol at –20 °C for 10 min. After washing with PBS, the cultures were blocked with 1% BSA for 30 min. A mouse monoclonal NR1 antibody (Pharmingen, CA, USA; 1:1000) and a rabbit polyclonal synaptophysin antibody (Zymed Laboratories, Inc., CA, USA; 1:1000) were then incubated with the cultures at 4 °C for overnight. After washing with PBS, the cultures were further incubated with green fluorescent anti-rabbit (Alexa 488, Molecular Probes, OR, USA; 1:1000) and red fluorescent anti-mouse (Cy3, Jackson
Secondary antibodies at room temperature for 1 hr. After thorough washing with PBS, the cultures were mounted onto slides with fluoromount-G (Southern Biotechnology Associates, Inc., Alabama, USA). To do this, one drop of fluoromount-G was added directly to the cell preparation, coverslips were mounted onto slides and pressed gently with a gauze sponge to remove excess mounting medium and to seal the coverslip. Mounted preparations were allowed to air-dry for 5 minutes before examination under the fluorescence microscope. Images were acquired using a CCD camera affixed to a Zeiss inverted microscope with AxioVision software.

2.6. Biotinylation and western blot assay of receptor surface expression

Surface expression of NMDARs was determined using a biotinylation assay as described previously (Chen et al., 1999). Briefly, hippocampal cultures were first treated with one of three different conditions at 37 °C: a) 1mM NMDA and 100 μM glycine (3 min), b) 50 or 100 μM genistein (15 min), or c) control condition (15 min). All pharmacological reagents were dissolved in recording solution (see above) in the absence of Ca^{2+}, with 100 μM EGTA and 300 nM TTX. Cultures were washed once with PBS/Ca^{2+}/Mg^{2+} and then incubated with 1.5 mg/ml NHS-SS-biotin (Pierce, Rockford, IL) in PBS/Ca^{2+}/Mg^{2+} at 4 °C while being shaken gently for 12 min. Cultures were washed three times with PBS/Ca^{2+}/Mg^{2+} + 0.1% BSA to remove any NHS-SS-biotin not bound to protein.
Cells were then harvested and membrane proteins were collected as described (Chen et al., 1999). Briefly, after washing the cells with Mg$^{2+}$/Ca$^{2+}$-containing PBS three times, cells were scraped with 1 ml of ice-cold harvest buffer (1 mM EGTA, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotonin, 20 μg/ml leupeptin, and 20 μg/ml pepstatin in PBS) into eppendorf tubes prechilled on ice, sonicated (10 sec), and centrifuged (14,000 rpm for 30 min at 4°C). The precipitates, which correspond to the membrane fraction, were redissolved by sonication (10 sec) in solubilization buffer (harvest buffer with 1% Triton X-100) followed by end-over-end mixing for 30 min in 4°C cold room. After centrifugation (14,000 rpm, 30 min at 4°C), supernatants were saved and stored at −80°C for further analysis.

Protein concentrations were determined by bicinchoninic acid protein assay (BCA kit; Pierce). For analysis of NMDA receptor overall expression, identical amounts (4, 8, 16, and 32 μg) of protein from the membrane fraction of each cell lysate were loaded to 8% SDS-PAGE. For the assessment of surface receptor expression level, identical amounts of the remainder of each cell lysate protein were incubated with 100 μl (spun down from 200 μl suspension) neutravidin-linked beads (Pierce) by end-over-end rotation for 2 hr at 4°C. Beads were extensively centrifuged and washed to isolate bead-bound proteins. These proteins were eluted by incubating the beads with dithiothreitol-containing SDS-PAGE loading buffer and loaded to 8% SDS-PAGE.

After overnight transfer of gels to PVDF membranes, the membranes were probed with antibodies against the NMDAR NR1 subunit (rabbit polyclonal, 1 μg/ml, Upstate, Waltham, MA) or else the NMDAR NR2B subunit (1:1000 of affinity purified rabbit polyclonal, a generous gift of Dr. Richard Huganir, Johns Hopkins Medical Institutions).
The membranes were then incubated with horseradish peroxidase-conjugated donkey anti-rabbit secondary antibody (Amersham, Arlington Heights, IL) diluted 1:5000. Bands were visualized by enhanced chemiluminescence (ECL; Amersham). Band intensities were determined by densitometry, and protein quantity-band density relation standard curves were generated from measurements of bands representing NMDAR (NR1 or NR2B) from the 4, 8, 16, 32 and 64 μg aliquots of the total membrane lysates. Surface NMDAR (NR1 or NR2B) protein expression levels were also determined by densitometry, and calculated according to the standard curve as a percentage of total NMDAR protein.

The percentage of receptors remaining on the cell surface after treatment with different conditions was determined.

2.7. Immunofluorescence internalization assay

After washing with recording solution (see above), live hippocampal neurons at either < 7 or >13 d.i.v. were labelled for 30 min at room temperature with an antibody directed against an extracellular region of NR1 (MAB363 from Chemicon, Temecula, CA or 556308 from Pharmingen, San Jose, CA). Primary antibodies were diluted into recording solution with 2 mM of CaCl₂, 1 μM TTX, and 100 μM of glycine, and used at 10 μg/ml. Neurons were then treated with either control condition (salt solution), NMDA, or bpV(phen) + NMDA for 10 min. Neurons were then fixed for 15 min with 4% paraformaldehyde/4% sucrose in PBS without permeabilization and incubated with FITC-labeled secondary antibodies (goat anti-mouse at 1:1000; Molecular Probes, Eugene OR) for 1 h at room temperature to label surface NMDARs. Then neurons were
permeabilized for 1 min with 100% methanol at -20 °C and treated with cy3-labeled secondary antibodies (donkey anti-mouse at 1:200; Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 h at room temperature to recognize pre-labeled internalized NMDARs (Lin et al., 2000). Images were acquired using a CCD camera affixed to a Zeiss inverted microscope with AxioVision software. Fluorescence intensities at both somatic and dendritic areas were measured using IDL software (Research Systems Inc., Boulder, Colo.) and quantified as red-to-green signal ratio to determine the degree of internalization.

2.8. Materials

Genistein, bpV(phen), PKI, and KN93 were from Calbiochem-Novabiochem Co. (San Diego, CA, USA), jasplakinolide was from Molecular Probes (Eugene, OR, USA), Latrunculin A or B was from BIOMOL Research Laboratories, Inc. (PA, USA). 2-bromopalmitate was a gift from Dr. A.E. El-Husseini (UBC). Tissue culture material was obtained from Invitrogen Canada Inc (Burlington, Ontario, Canada), unless otherwise indicated. All other chemicals were purchased from Sigma (St. Louis, MO, USA).

Anti-NR1 C-terminus antibody was purchased from Upstate (Waltham, MA). Anti-NR2B C-terminus antibody was a gift from Dr. R. Huganir. Antibodies directed against an extracellular region of NR1 were purchased from either Chemicon (Temecula, CA) or Pharmingen (San Jose, CA).
NR1-la and NR2B cDNAs were gifts from Dr. Nakanishi (Kyoto University, Kyoto, Japan), and NR2A (e1) was a gift from Dr. Mishina (Tokyo University, Tokyo, Japan). These cDNAs were subcloned into a mammalian expression vector with a CMV promoter, as previously described (Raymond et al., 1996; Chen et al., 1997). PSD-95-GFP construct was a gift from Dr. D. S. Bredt and was described previously (Craven et al., 1999).

2.9. Data analysis

Results are presented as mean ± standard error. Sets of different results were compared using one-way ANOVA, two-way ANOVA, or Student’s t test as appropriate, and significant differences were determined at the 95% confidence intervals. For rundown experiments, the peak-current amplitude at different time point was normalized to the first response. For desensitization experiments, 3-10 responses of each cell were averaged for estimation of Iss/IP ratio. In some cases only the first response was measured since the Iss/IP ratio was fairly stable over time during our recording conditions (see Figure 4-11).
CHAPTER III

Differential regulation of synaptic and extrasynaptic NMDA receptor rundown by calcium and tyrosine phosphorylation

A variety of processes limit NMDAR activity in response to agonist exposure, including rundown -- the decline of peak current with repeated, sustained agonist application (see 1.9.). Rundown may occur by more than one mechanism, since reports differ with respect to its Ca\(^{2+}\) dependence (Rosenmund and Westbrook, 1993a; Wang et al., 1996; Dingledine et al., 1999; Vissel et al., 2001).

In the present experiments, we further investigated the mechanisms of rundown of NMDAR-mediated current using cultured hippocampal neurons and tested the hypothesis that subcellular localization (synaptic versus extrasynaptic) may account, at least in part, for the differences in the reported properties.

3.1. Characterization of NMDAR composition and subcellular distribution in cultured hippocampal neurons

We first characterized components of the NMDAR current from both ‘immature’ (4-7 DIV) and ‘mature’ (>13 DIV) cultured hippocampal neurons. We chose these two ages since NMDARs in 4-7 DIV neurons are mainly extrasynaptic, whereas in >13 DIV neurons synaptic NMDARs become prominent in our cultures, as indicated by double labeling with antibodies against NR1 and the presynaptic marker synaptophysin (Figure 3-1). This result is consistent with previous studies (Rao et al., 1998; Tovar and
Westbrook, 1999; Dalva et al., 2000; Crump et al., 2001). Previous studies have also indicated that hippocampal or cortical neurons express mainly 2B-subtype receptors during early development in vitro and in vivo, and that NR2A expression increases dramatically with maturation (Williams et al., 1993; Zhong et al., 1994; Li et al., 1998; Rao et al., 1998; Stocca and Vicini, 1998; Tovar and Westbrook, 1999). In the presence of 3-10 μM ifenprodil >90% of current mediated by NR1/NR2B (i.e., “2B-subtype” NMDARs) is inhibited (Williams, 1993; Tovar and Westbrook, 1999); therefore any NMDA-evoked current remaining in neuronal recordings should be mediated predominantly by 2A-containing NMDARs, comprised of diheteromeric NR1/NR2A and/or triheteromeric NR1/NR2A/NR2B. In our experiments, 76 ± 4% of the NMDA-evoked current recorded from immature neurons was inhibited by 10 μM ifenprodil (n = 9; Figure 3-2 a & c) whereas only 18 ± 4% of the NMDAR current from mature neurons was inhibited (n = 13; Figure 3-2 a & c), consistent with the increasing expression of NR2A during development.

We also examined the subcellular distribution of 2B-subtype and 2A-containing receptors in the more mature (>13 DIV) neurons. To isolate the activity of extrasynaptic NMDARs, we incubated neuronal cultures for 15 min in 100 μM glycine and 20 μM of the non-reversible open-channel blocker MK-801 (Halliwell et al., 1989), followed by extensive wash-out. The high glycine concentration facilitates activation of synaptic, but not extrasynaptic, NMDARs (Lu et al., 2001) which are then blocked by MK-801. After this treatment, we observed a 43 ± 8% reduction in the peak current evoked by exogenous application of 100 μM NMDA (n = 8; Figure 3-2 b). It is possible that not all of the synaptic NMDARs were blocked using this protocol, since a previous study
indicated that ~70% of NMDARs were synaptic in cultured hippocampal neurons after 9-14 DIV (Rosenmund et al., 1995). Nevertheless, after partial block of synaptic NMDARs, 48 ± 5% of the remaining current was inhibited by 10 μM ifenprodil (n = 10; Figure 3-2 b & c), as compared with 18% ifenprodil inhibition before MK-801 and glycine treatment (Figure 3-2 c; p<0.001 by unpaired t-test). These data suggest preferential localization of 2B-subtype receptors to extrasynaptic sites and 2A-containing receptors to the synapses in these neurons, consistent with previous studies (Li et al., 1998; Stocca and Vicini, 1998; Rumbaugh and Vicini, 1999; Tovar and Westbrook, 1999).
Figure 3-1. Developmental change in NMDAR subcellular distribution

Representative photomicrographs of double labeling of NR1 (red) and synaptophysin (Syn; green) in neurons of 7 DIV and 19 DIV respectively. NR1 staining in the 19 DIV neuron is more punctate and co-localized with synaptophysin compared with that in the 7 DIV neuron. Scale bar: 10 μm.
Figure 3-2. Characterization of NMDAR composition and distribution

(a) Representative current responses of neurons of 6 DIV or 17 DIV to 100μM NMDA, with (+ifen) or without 10μM ifenprodil. (b) Representative 100μM NMDA-evoked current responses before and after the blockade of synaptic NMDARs, and the subsequent inhibition by 10μM ifenprodil. Broken lines indicate the application of 100μM glycine together with 20μM MK-801 for 15 minutes in the absence of NMDA to block only synaptic NMDARs. (c) Pooled data showing mean ± SE of the current sensitive to 10μM ifenprodil inhibition (4-7 DIV: n = 9; >13 DIV: n = 13; >13 DIV, post MK801: n = 10). *** p<0.001 compared with >13 DIV group, ### p<0.001 compared with 4-7 DIV group; unpaired t-test.
3.2. Rundown of 2B-subtype of NMDARs differs from that of 2A-containing NMDARs

To compare rundown of extrasynaptic NMDARs with that of synaptic NMDARs, we first recorded from ‘immature’ (4-7 DIV) neurons to measure currents mediated largely by extrasynaptic, 2B-subtype NMDARs, and used the NR1/NR2B-selective antagonist ifenprodil (10μM) for recordings on ‘mature’ (>13 DIV) neurons to enrich the 2A-containing synaptic component of NMDAR current (Hardingham et al., 2002).

Conventional whole-cell patch clamp recording may disrupt cytoskeleton integrity and dialyze intracellular contents, including ATP and soluble protein kinases and phosphatases, factors that have been shown to regulate NMDAR activity (see Chapter 1; for review see Dingledine et al., 1999). To circumvent this problem, we employed the nystatin-perforated patch clamp recording technique (Akaike and Harata, 1994; Price et al., 1999) to study NMDAR rundown.

Rundown of NMDAR-mediated peak current induced by repeated application of 100 μM NMDA (10s, 2 per min, in the presence of 30 μM glycine) was apparent for both immature and mature neurons in the presence of 2 mM CaCl₂ (Figure 3-3 a & b). Importantly, rundown was NMDAR use-dependent under both conditions since no decrease in peak current was observed when brief agonist pulses were given at 10 min intervals (not shown).

Compared with 2A-containing (mainly synaptic) NMDARs, rundown in immature neurons evolved faster (the time constant for loss of peak current amplitude was 5.2 min and 2.5 min for NMDARs recorded from mature vs. immature neurons) and was more extensive (n = 12, P<0.0001; Figure 3-3 b). This difference in rundown could
not be attributed to differences in Ca\textsuperscript{2+} influx since the mature neurons had larger peak current amplitudes (even in the presence of ifenprodil) than immature neurons (2014 ± 187 pA, n = 10 and 788 ± 225 pA, n = 9, respectively). Thus, we observed a dramatic difference in rate and extent of rundown between neurons differing in level of \textit{in vitro} development (4-7 DIV vs. >13 DIV).

To determine whether 2A-containing and 2B-subtype NMDARs in neurons of the same age differ in their rate and extent of rundown, we isolated 2B-subtype receptor-mediated current in recordings from mature neurons using ifenprodil protection from MK-801 block. In the presence of both ifenprodil and MK-801, repeated applications of NMDA resulted in selective activation of 2A-containing receptors that were then irreversibly blocked by MK-801. After approximately 5-7 ten-second applications of NMDA in the presence of the two antagonists, the current was almost completely blocked (peak current was 6 ± 1 % of control, n = 6). We then removed ifenprodil and MK-801 and washed extensively with control solution to recover the 2B-subtype receptor current, which showed stable peak amplitude in response to 3 – 4 brief (500 ms) applications of agonist (not shown), indicating complete wash-out of MK-801.

Neurons with enriched 2B-subtype mediated current were then subjected to the rundown protocol. As shown in Figure 3-2b, rundown of this current was faster and more extensive, than that mediated by 2A-containing NMDARs in neurons of the same age (n = 7, P<0.0001; Figure 3-3 a and b). In fact, rundown was faster and more extensive than that measured in the 4-7 DIV neurons, likely because recordings from the immature neurons were contaminated by currents from 2A-containing receptors, as supported by the incomplete block of NMDAR current by ifenprodil (76 %; Figure 3-2 a, c). These
data suggest that the rate and extent of NMDAR rundown does not correlate simply with the level of *in vitro* neuronal development.
Figure 3-3. Rundown of peak current mediated by 2B-subtype differs from that of 2A containing NMDARs

(a) Representative responses evoked by NMDA recorded from neurons at 5 DIV (upper), 14 DIV with 10µM ifenprodil (middle), and 15 DIV following isolation of 2B-subtype current by pre-treatment with 20µM MK-801 and 10µM ifenprodil (lower). Extracellular Ca$^{2+}$ was 2 mM. The 1$^{st}$ and 20$^{th}$ responses are shown. (b) Pooled data showing development of rundown. Peak current amplitude was normalized to first response.
a 100 μM NMDA, 30 μM glycine, 2 mM CaCl₂

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<th>DIV</th>
<th>Current</th>
<th>Time</th>
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<td>5</td>
<td>200 pA</td>
<td>4 s</td>
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<tr>
<td>14</td>
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<tr>
<td>15</td>
<td>100 pA</td>
<td>4 s</td>
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b 2 mM CaCl₂

Normalized peak current vs. Time (min)

- ○ 4-7 DIV
- □ >13 DIV, 2A
- △ >13 DIV, 2B
3.3. Ca$^{2+}$-independent NMDAR current rundown

Both Ca$^{2+}$-dependent (Rosenmund and Westbrook, 1993a) and Ca$^{2+}$-independent (Wang et al., 1996; Vissel et al., 2001) forms of NMDAR rundown have been reported; therefore, we made recordings in external solution lacking Ca$^{2+}$ and containing 100μM EGTA ("zero Ca$^{2+}$e"; Figure 3-4 a and b). At a holding potential of either -70 or +40 mV, zero Ca$^{2+}$e only partially inhibited rundown of the mainly 2B-subtype, predominantly extrasynaptic receptors (4-7 DIV, n = 9 and 11 respectively) but abolished rundown of 2A-containing, mainly synaptic receptors (n = 5; P<0.0001; Figure 3-4 a and b).

It has been reported that recombinant NR1/NR2B is more sensitive to glutamate than NR1/NR2A and that the potency of NMDA is lower than that of glutamate (Dingledine et al., 1999). For example, studies of NMDARs expressed in cortical neurons showed that the EC$_{50}$ for NMDA and glutamate were 57 and 16.5 μM, respectively (Sather et al., 1992). It is possible that 100 μM NMDA used in our experiments saturated, or nearly saturated, 2B-subtype NMDARs but effected submaximal activation of 2A-containing NMDARs, and that could account for the observed differences in rundown of these two NMDAR subtypes. To address this question, we repeated the experiments in zero extracellular calcium using 2 mM NMDA and 100 μM glycine to stimulate NMDARs, which should saturate both receptor subtypes. Under these conditions, currents mediated by 2A-containing NMDARs remained stable while the 2B-subtype of NMDAR-mediated currents again showed significant rundown (Figure 3-4 c; 4-7 DIV: n = 15; >13 DIV, 2A: n =8; p<0.0001 by two-way ANOVA).
Figure 3-4. Ca^{2+}-independent NMDAR current rundown

(a) Representative responses evoked by NMDA recorded from neurons at 6 DIV (upper), 5 DIV (middle), and 16 DIV with 10μM ifenprodil (lower). Recordings were made in zero Ca^{2+}. Recordings at +40 mV were made in 1 mM external Mg^{2+}. The 1st and 20th responses are shown. (b) Pooled data showing Ca^{2+}-independent rundown. (c) Same as b, except 2 mM NMDA and 100 μM glycine were used.
100 μM NMDA, 30 μM glycine, 0 CaCl₂

6 DIV, \( V_h = -70 \text{ mV} \)

5 DIV, \( V_h = +40 \text{ mV} \)

16 DIV, 2A, \( V_h = +40 \text{ mV} \)

0 CaCl₂

Normalized peak current

\[ \text{Time (min)} \]

2 mM NMDA
0 CaCl₂

Normalized peak current

\[ \text{Time (min)} \]
3.4. Synaptic NMDARs are more stable than extrasynaptic NMDARs

The differences we observed in Ca\(^{2+}\)-independent rundown for 2A-containing versus 2B-subtype NMDARs could be due to intrinsic subunit-dependent receptor properties or to their synaptic versus extrasynaptic localization. Against the first possibility, we have previously published that rundown of both NR1/NR2A and NR1/NR2B peak currents in HEK293 cells is eliminated in the absence of extracellular Ca\(^{2+}\), using the nystatin perforated patch technique for whole-cell recording (Price et al., 1999). However, this study used a relatively low concentration of glutamate and glycine (10 μM for both). Therefore we repeated this experiment using high concentrations of glutamate and glycine (1 mM and 100 μM, respectively) to stimulate NMDARs. We found that under similar conditions (absence of extracellular Ca\(^{2+}\), Nystatin patch recording) peak currents for both recombinant subtypes were stable in response to a rundown protocol (10s, 2/min; P>0.05 between the two types of receptors by two-way ANOVA; n = 3 for NR1/NR2A, n = 6 for NR1/NR2B) (Figure 3-5). Therefore, regulation of peak current rundown for the two NMDAR subtypes in HEK293 cells is similar.
Figure 3-5. Both subtypes of recombinant NMDARs expressed in HEK293 cells do not show Ca\textsuperscript{2+}-independent rundown

NR1/NR2A or NR1/NR2B expressed in HEK293 cells was stimulated (10s, 2/min) by glutamate and glycine (1 mM and 100 μM, respectively) in the absence of extracellular Ca\textsuperscript{2+}. Nystatin patch recording was used. NMDAR-mediated currents by both subtypes were stable under these conditions.
1 mM glutamate
100 μM glycine
0.2 CaCl₂

NR1/NR2B
NR1/NR2A

Time (min)

(normalized peak current)
To test the possibility that synaptic versus extrasynaptic localization is responsible for the observed difference, we directly compared the NMDAR component of synaptic current with the whole-cell current (mediated by both synaptic and extrasynaptic NMDARs) in hippocampal microisland (autaptic) cultures. As before, we repeatedly applied 100μM NMDA (10s, 2/min; with 30 μM glycine) to induce Ca\(^{2+}\)-independent rundown of NMDA-evoked current. Sr\(^{2+}\) (0.2 or 1 mM) were added to stabilize recordings in 0 Ca\(^{2+}\)+100 μM EGTA with minimum effects on NMDAR activity (Umemiya et al., 2001). Synaptic currents were evoked by applying brief depolarization to 0 mV for 3 ms. We recorded the synaptic NMDAR current from 5-6 DIV neurons before and after the rundown protocol. After 5-8 min of the rundown protocol, the whole-cell NMDAR-mediated response showed significant rundown, whereas the synaptic NMDAR-mediated current was stable (Figure 3-6 a-c). At this stage in culture, both synaptic and extrasynaptic NMDARs were mainly 2B-subtype since 73 ± 2 % of whole-cell and 82 ± 8 % of synaptic NMDAR-mediated current was inhibited by ifenprodil (10 μM; \( n = 5 \) and \( 3, p > 0.05 \) by t-test). The mean decay time constant for synaptically-evoked NMDAR currents was 90 ms without ifenprodil and 60 ms with the drug (\( n = 3 \)), consistent with block of a slower NR2B component.
Figure 3-6. Synaptic 2B-subtype of NMDARs are more stable than extrasynaptic 2B-subtype

(a) NMDA-evoked whole-cell currents recorded from a neuron in microisland culture (6 DIV). The 1st and 10th responses are superimposed for comparison. (b) Synaptic NMDAR-mediated responses were recorded from the same cell as in (a) before and after rundown protocol and are superimposed for comparison. (c) Mean residual current (normalized to 1st response) after 5-8 min of recording with rundown protocol (n = 4; ** P < 0.005).
5-6 DIV Autaptic cultures, 2B-subtype NMDARs

a
0 CaCl₂, 1 mM SrCl₂
100 μM NMDA

b
1 mM CaCl₂
5 μM CNQX

After rundown
200 pA
2 s

Whole-cell

10th
1st
200 pA

Before rundown
100 ms

Synaptic

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<th>after rundown</th>
<th>before rundown</th>
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<td>Whole-cell</td>
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<tr>
<td>Synaptic</td>
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To determine whether synaptic localization also conferred stability to 2A-containing NMDARs, experiments were repeated in older (7-8 DIV) microisland cultures with ifenprodil to isolate the 2A-containing NMDAR current. Again, the agonist-evoked current showed significant rundown, whereas the synaptic current was stable (Figure 3-7 a-c). To determine the ratio of extrasynaptic to synaptic 2A-containing NMDAR current in 7-8 DIV neurons, we measured the NMDA-evoked whole-cell current (in the presence of 10 μM ifenprodil) before and after blockade of synaptically stimulated NMDARs with 20μM MK801. After repeated synaptic stimulation with 20-40 action potentials that blocked >90% of the synaptic NMDARs, MK801 was washed out. The NMDA-evoked peak-current amplitude after MK801 wash-out compared with the initial peak current revealed that 71± 4 % of 2A-containing NMDARs were extrasynaptic at this stage of development (n = 3).

These data indicate that for either 2B-subtype or 2A-containing NMDARs, synaptic localization is sufficient to confer resistance to Ca\(^{2+}\)-independent rundown.
Figure 3-7. Synaptic 2A-containing NMDARs are more stable than extrasynaptic 2A-containing NMDARs

(a) NMDA-evoked whole-cell currents recorded from a neuron in microisland culture (8 DIV) in the presence of 10 μM ifenprodil. The 1st and 10th responses are superimposed for comparison. (b) Synaptic NMDAR-mediated responses were recorded in the presence of 10 μM ifenprodil from the same cell as in (a) before and after rundown protocol and are superimposed for comparison. (c) Mean residual current (normalized to 1st response) after 5-8 min of recording with rundown protocol (n = 5; ** P < 0.005).
7-8 DIV Autaptic cultures, 2A-containing NMDARs

(a) 0 CaCl₂, 1 mM SrCl₂, 100 μM NMDA, 10 μM ifen
(b) 1 mM CaCl₂, 5 μM CNQX, 10 μM ifen

Whole-cell

Synaptic

**
3.5. Different mechanisms underlying Ca$^{2+}$-independent and -dependent rundown

Previous reports indicate that Ca$^{2+}$-independent NMDAR rundown is dependent on tyrosine dephosphorylation (Wang et al., 1996; Vissel et al., 2001). If ongoing tyrosine kinase activity is required to maintain activity of extrasynaptic NMDARs, blockade of this class of kinases in immature neurons should produce rundown and occlude the effect of subsequent repeated agonist application. As predicted, a 15-min preincubation with the tyrosine kinase inhibitor genistein (50μM) decreased NMDAR peak current to 60 ± 3.1 % of control (n = 7) and occluded the subsequent development of Ca$^{2+}$-independent rundown (Figure 3-8 a; n = 11, p<0.0001). Incubation with 100μM bpV(phen), a tyrosine phosphatase inhibitor (Posner et al., 1994), during the rundown protocol inhibited Ca$^{2+}$-independent rundown (Figure 3-8 a; n = 9, P<0.0001), whereas treatment with bpV(phen) for 10 min without the rundown protocol did not alter peak current amplitude (98 ± 3 % of control, n = 5). BpV(phen) had no effect on Ca$^{2+}$-dependent rundown of 2A-containing NMDARs in mature neurons (69 ± 4 % and 70 ± 3 % residual current at 8 min for control and bpV(phen)-treated groups, respectively, n = 12 and 9). These data support the idea that Ca$^{2+}$-independent NMDAR rundown is dependent on tyrosine dephosphorylation, perhaps of the NMDAR itself (Wang et al., 1996; Vissel et al., 2001).

Recent data from NMDAR-transfected HEK 293 cells suggested that Ca$^{2+}$-independent rundown is due to receptor internalization following tyrosine dephosphorylation (Vissel et al., 2001). To determine whether a decrease in neuronal NMDAR surface expression may underlie Ca$^{2+}$-independent rundown or the decrease in peak current observed after a 15-min incubation with genistein, we measured the fraction
of surface NMDARs by biotinylation (Chen et al., 1999) in 6-7 DIV cultures. Following sustained treatment with NMDA/glycine (3-5 min) in zero Ca\(^{2+}\), which resulted in a 30 ± 8 % (n=4) decrease in peak current, or genistein (15 min), the percentage of surface NR2B expression was decreased by 35% compared with control (salt solution) treatment (Figure 3-8 b, c), correlating well with our electrophysiological data.
Figure 3-8. Ca$^{2+}$-independent rundown correlated with tyrosine dephosphorylation and decreased surface NMDAR level

(a) Pre-incubation of immature neurons with genistein before recording occluded, whereas incubation with bpV(phen) during recording inhibited, Ca$^{2+}$-independent rundown. (b) Western blots showing change in surface NR2B after treatment with 1mM NMDA/100μM glycine for 3-5 min (NMDA) or incubation with 50μM genistein for 10 min (genistein). Numbers indicate μg protein loaded. Incremental amounts of total cell lysate (total) were loaded to generate an internal standard curve for calculating percentage of surface receptors (surf). (c) Pooled data from n = 3-5 different experiments. Bars represent mean percentage surface receptors. * $P < 0.05$. Similar results were obtained by probing for NR1 (not shown).
a 4-7 DIV, 0 CaCl₂

![Graph showing normalized peak current over time with different treatments: control, bpv, and genistein.](image)

b 6 DIV, 0 CaCl₂

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<tr>
<th></th>
<th>Total</th>
<th>Surf</th>
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<td>8</td>
</tr>
<tr>
<td>NMDA</td>
<td>16</td>
<td>32</td>
</tr>
<tr>
<td>Genistein</td>
<td>75</td>
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![Western blot images with anti-NR2B antibody.](image)

C Percent surface NR2B

![Bar graph showing percent surface NR2B with treatments: Control, NMDA, Genistein.](image)
To determine whether the change in surface NMDAR expression correlated with enhanced internalization or reduced insertion, we used a fluorescent anti-NR1 antibody-based internalization assay (Man et al., 2000) in 7-9 DIV cultures; green staining labels surface NMDARs while red staining labels internalized NMDARs (Figure 3-9; see 2.7 for detailed method). After a 10-min NMDA treatment in zero Ca\(^{2+}\), intensity of green staining decreased while that of red staining increased markedly compared to control (no NMDA). The increase of NMDAR internalization was inhibited by adding bpV(phen) during treatment with NMDA (Figure 3-9). The red-to-green ratio, which represents the degree of internalization, for control, NMDA, and NMDA plus bpV(phen) groups was 1.8 ± 0.2 (n = 170 regions of interest / 4 experiments), 9.8 ± 1.9 (n = 170 / 4), and 1.3 ± 0.1 (n = 120 / 2), respectively (P < 0.0001). Note that at this stage in culture, NMDAR staining shows a less punctate pattern than found in mature neurons (Figure 3-1 and Figure 3-11), consistent with fewer synaptic clusters. NMDARs in >13 DIV neurons showed only little internalization after NMDA treatment (Figure 3-11).
Figure 3-9. Internalization of NMDARs in immature neurons in response to sustained NMDA application

Live neurons of 6-8 DIV were labeled with antibodies recognizing an extracellular region of NR1 and then treated with either control condition (salt solution), NMDA (as in Figure 3-8 b), or 200μM bpV(phen) + NMDA for 10 min (see 2.7. for detailed method). Representative images from neurons of 8 DIV are shown.
8 DIV

Control  NMDA  NMDA+bpV(phen)
To determine whether the Ca\(^{2+}\)-dependent rundown is affected by the integrity of F-actin (Rosenmund and Westbrook, 1993a), we used the cell permeable, F-actin stabilizing agent jasplakinolide. This agent suppressed the rundown of 2A-containing NMDARs in the presence of Ca\(^{2+}\) (Figure 3-10 a), consistent with previous reports (Rosenmund and Westbrook, 1993a). In contrast, jasplakinolide had no effect on Ca\(^{2+}\)-independent rundown in 4-7 DIV neurons (69 ± 6 % and 69 ± 9 % residual current at 8 min for control and jasplakinolide treated neurons, respectively, n = 4 for both groups), indicating little role for actin depolymerization. Moreover, Ca\(^{2+}\)-dependent rundown in mature neurons did not correlate with decreased surface NMDAR level as determined by biotinylation assay and internalization assay (Figure 3-10 b, c and Figure 3-11).
Figure 3-10. Ca$^{2+}$-dependent rundown did not correlate with a decrease in surface NMDAR level

(a) Rundown of 2A-containing receptors in >13 DIV neurons with Ca$^{2+}$ was partially inhibited by pre-incubation with 2μM jasplakinolide (jasp, n = 8; control: n = 5; P<0.0001). (b) Western blots showing the surface NR1 level after treatment with 1mM NMDA/100μM glycine for 3-5 min (NMDA) or control (salt solution only) condition. All treatments were done with 2 mM CaCl$_2$. Numbers indicate μg protein loaded. Incremental amounts of total cell lysate (total) were loaded to generate an internal standard curve for calculating percentage of surface receptors (surf). (c) Pooled data from n = 5 different experiments. Bars represent mean percentage surface receptors. $P > 0.05$. 
(a) >13 DIV, 2A
2 mM CaCl₂

Normalized peak current

Time (min)

(b) 16 DIV, 2 mM CaCl₂
Total | Surf
-----|-----
4     | 8   
16    | 32  
64    | 128 
Control

NMDA

Anti-NR1

(c) Percent surface NR1

Control | NMDA
Figure 3-11. NMDARs in mature neurons were resistant to internalization in response to sustained NMDA application

Live neurons of >13 DIV were labeled with antibodies recognizing an extracellular region of NR1 and then treated with either control condition (salt solution), NMDA (as in Figure 3-9) (see 2.7. for detailed method). Representative images from neurons of 20 DIV are shown.
3.6. Summary

In this study we have shown for the first time that subpopulations of NMDAR expressed in a single hippocampal neuron differ with respect to activity-dependent regulation. Moreover, these data were obtained using the method of nystatin perforated patch recording to minimize disturbances of the intracellular milieu and mimic physiological conditions as closely as possible. We conclude from our results that hippocampal neuronal NMDARs composed of NR1/NR2B and localized mainly to extrasynaptic sites exhibit extensive peak current rundown that is independent of Ca\(^{2+}\) influx. In contrast, 2A-containing hippocampal neuronal NMDARs, which reside predominantly at synapses, show stable peak current in the absence of Ca\(^{2+}\) influx. The differences we observed in rundown between these neuronal NMDAR subtypes could not be attributed to intrinsic differences in receptor properties, since synaptic localization is both sufficient and necessary to render either 2A- or 2B-subtype of NMDARs resistant to rundown, and recombinant NR1/NR2B and NR1/NR2A expressed in HEK293 cells showed similar peak current rundown. Moreover, these differences were not due to use of a subsaturating agonist concentration (100 μM NMDA) for stimulating neuronal 2A-containing NMDARs, since the same effects were observed using 2 mM NMDA.

In agreement with a previous study (Rosenmund and Westbrook, 1993a), we found that rundown of 2A-containing NMDAR peak current in the presence of Ca\(^{2+}\) was, at least in part, dependent on the polymerization state of F-actin. Our data support a role for tyrosine dephosphorylation in Ca\(^{2+}\)-independent rundown of hippocampal NMDARs. A similar phenomenon of Ca\(^{2+}\)-insensitive rundown that was dependent on tyrosine dephosphorylation has been described, using the conventional whole-cell recording
method, for spinal dorsal horn neurons (Wang et al., 1996) and more recently for recombinant NR1/NR2A expressed in HEK cells (Vissel et al., 2001). The latter study, showing Ca\(^{2+}\)-independent rundown of recombinant NR1/NR2A in HEK293 cells, contrasts with our findings that peak current mediated by either NR1/NR2A or NR1/NR2B in HEK cells is resistant to rundown in the absence of Ca\(^{2+}\). Because we used the nystatin perforated patch method instead of the conventional whole-cell recording configuration used by Vissel et al. (Vissel et al., 2001), it is possible that cytoplasmic factors (e.g., soluble protein tyrosine kinases) responsible for stabilizing the NR1/NR2A peak current response in HEK cells were retained in our experiments to account for the difference in results. The similarity of our results for extrasynaptic hippocampal NMDARs with those of Wang et al. (Wang et al., 1996) for NMDAR currents recorded from spinal dorsal horn neurons suggest that NMDARs expressed in the latter preparation resemble extrasynaptic hippocampal NMDARs, at least with regard to Ca\(^{2+}\)-independent rundown.

It has been proposed that dephosphorylation of a ring of tyrosine residues located at the interface between the intracellular C-terminus and the last transmembrane domain leads to NMDAR internalization, which accounts for Ca\(^{2+}\)-independent rundown (Vissel et al., 2001). Substantial evidence has emerged recently indicating that NMDARs, like AMPA receptors, can rapidly shuttle between the intracellular compartment and the cell surface membrane (see 1.8.). Importantly, NMDARs in immature, but not mature, neurons undergo rapid constitutive internalization (Roche et al., 2001). Here, we have provided evidence in the immature neuronal cultures that tyrosine kinase inhibition, as well as sustained exposure to high concentrations of NMDA and glycine, are associated
with the loss of surface NMDARs and increased internalization, whereas NMDARs in mature neurons are resistant to NMDA induced internalization. It is possible that NMDARs in mature neurons, most of which are synaptic, are highly tyrosine phosphorylated due to their close interaction with tyrosine kinases that are concentrated in the postsynaptic density, and thus resistant to NMDA induced internalization. This is supported by studies showing that PSD-95, the postsynaptic density enriched protein that interacts with NMDARs (see 1.7.), targets src family kinases to NMDARs and promotes tyrosine phosphorylation of NMDARs by either fyn or src (Tezuka et al., 1999; Liao et al., 2000). Importantly, a recent study showed that internalization of NMDARs is inhibited by PSD-95 (Roche et al., 2001).

In summary, we conclude that NMDARs in immature neurons or extrasynaptic NMDARs display Ca\textsuperscript{2+}-independent and tyrosine dephosphorylation dependent rundown, which can be explained by higher rates of use-dependent NMDAR turnover from the surface. NMDARs in mature neurons or synaptic NMDARs, on the other hand, are either highly tyrosine phosphorylated or stabilized by direct physical coupling with scaffolding proteins, showed much lower rate of turnover from the surface, and thus less peak-current rundown.
Developmental change in NMDA receptor desensitization determined by receptor subcellular localization

In the last chapter we showed that the subcellular localization (synaptic versus extrasynaptic) regulates the stability of NMDAR peak current and surface expression. Since many molecules concentrated in the PSD regulate NMDAR function (see Chapter 1 for details), other properties of NMDARs may also be regulated based on the receptor’s subcellular (synaptic versus extrasynaptic) localization. As well, since the subcellular localization of NMDARs changes during development from predominantly extrasynaptic to mainly synaptic, it is conceivable that a developmental change in NMDAR properties may also occur due to the transition in NMDAR subcellular localization during development. To test this possibility we recorded NMDAR-mediated whole-cell responses in cultured hippocampal neurons to study the glycine-independent desensitization during neuronal development.

4.1. Neuronal NMDARs show developmental change in desensitization independent of subunit composition

NMDAR glycine-independent desensitization is involved in limiting receptor activation during repeated or sustained exposure to glutamate (McBain and Mayer, 1994; Tong et al., 1995). To identify factors regulating this process in cultured hippocampal neurons we measured desensitization of NMDAR currents in response to 10s applications
of 100 μM NMDA in the continuous presence of 30 μM glycine, using the Nystatin perforated patch recording technique to preserve intracellular contents and endogenous signaling mechanisms. Since NMDAR subunit composition is an important determinant of receptor function and modulation, we began by comparing desensitization of 2A-containing NMDARs (recorded from >13 DIV neurons bathed in ifenprodil) to that of NMDARs in 4-7 DIV neurons, that mainly express the 2B-subtype, and to 2B-subtype NMDARs isolated pharmacologically in >13 DIV neurons using a combination of MK801 and ifenprodil (see 3.1. and 3.2.). Strikingly, the 2B-subtype NMDARs in both 4-7 DIV and >13 DIV neurons showed a higher degree of glycine-independent desensitization compared with 2A-containing NMDARs in >13 DIV neurons when recorded in 2 mM calcium (Figure 4-1 a; \( P<0.001 \) between >13 DIV, 2A and other two groups). Removal of calcium from the external solution and the use of positive holding potential resulted in less extensive desensitization for both subtypes, but the difference in steady-state to peak current ratio (I\(_{ss}/I_p\)) remained significant (Figure 4-1 b; \( P<0.001 \)).

Since the 2B-subtype is more sensitive to agonist than the 2A-subtype of NMDARs (Dingledine et al., 1999) and NMDA is less potent than glutamate (Sather et al., 1992), it is possible that 100 μM NMDA saturated 2B- but not 2A-subtype NMDARs, and thus caused different degrees of desensitization. When we repeated our experiment using 2 mM NMDA to saturate both classes of receptors, we still observed significantly more extensive desensitization for 2B-subtype NMDARs in 4-7 DIV neurons than 2A-containing NMDARs in >13 DIV neurons (Figure 4-2; \( P<0.001 \)). These results indicate that differential sensitivity to NMDA cannot explain the observed differences in NMDAR desensitization.
Figure 4-1. Neuronal 2B-subtype of NMDARs showed more glycine-independent desensitization than 2A-subtype

(a) Left: representative currents mediated by NMDARs in a 6 DIV neuron and in a 15 DIV neuron in the presence of 10 μM ifenprodil, in response to 100 μM NMDA (30 μM glycine) in the presence of 2 mM calcium. Right: quantification data showing that NMDARs in 4-7 DIV neurons or 2B-subtype of NMDARs in >13 DIV neurons have higher degree of desensitization than 2A-subtype of NMDARs in >13 neurons. (b). Same as A, except Ca$^{2+}$ was replaced by 1 mM Mg$^{2+}$ and holding potential was +40 mV. All recording was performed using nystatin-perforated patch technique.
Figure 4-2. Neuronal 2B-subtype of NMDARs showed more glycine-independent desensitization than 2A-subtype in response to saturating concentration of agonists. Left: representative currents mediated by NMDARs in a 6 DIV neuron and in a 14 DIV neuron in the presence of 10 μM ifenprodil, in response to 2 mM NMDA (100 μM glycine) in the absence of calcium. Right: quantification data showing that NMDARs in 4-7 DIV neurons have higher degree of desensitization than 2A-subtype of NMDARs in >13 DIV neurons; N=25 and 12 respectively; P<0.001. All recording was performed using nystatin-perforated patch technique.
To test the possibility that the marked difference in desensitization observed for 2A-containing NMDARs in >13 DIV neurons compared with 2B-subtype NMDARs in 4-7 or >13 DIV neurons was due to an intrinsic difference in receptor properties due to subunit composition, we compared desensitization of recombinant NR1/NR2A with NR1/NR2B in transfected HEK293 cells using the same Nystatin perforated patch recording technique. In contrast to our results for hippocampal neuronal NMDARs, NR1/NR2A receptors desensitized more extensively than NR1/NR2B receptors in response to 1 mM glutamate (with 100 μM glycine) in the absence of calcium (Figure 4-3; P<0.01), consistent with a previous study using conventional whole-cell recording (Krupp et al., 1996). These data suggested that the observed differences in NMDAR desensitization were independent of receptor subunit composition.
Figure 4-3. Recombinant NR1/NR2B showed less glycine-independent desensitization than NR1/NR2A

Left: representative currents mediated by NR1/NR2A or NR1/NR2B expressed in HEK293 cells, in response to 1 mM glutamate (50 μM glycine) in the absence of calcium. Peak currents were normalized for comparison of desensitization. Right: quantification data showing NR1/NR2A receptors have higher degree of desensitization than NR1/NR2B. Iss/Ip for NR1/NR2A and NR1/NR2B was 48±10% and 77±2% respectively (n=7 and 10 respectively, P<0.01). All recording was performed using nystatin-perforated patch technique.
To define the macroscopic kinetics of the NMDAR response more precisely and to further investigate the factors regulating differences in neuronal NMDAR desensitization during development, we also used conventional whole-cell recording. In these experiments the intracellular pipette solution contained 10 mM BAPTA to minimize Ca\(^{2+}\)-dependent inactivation (Zheng et al., 2001), and saturating concentrations of NMDA (1 mM) and glycine (100 \(\mu\)M) were used to isolate glycine-independent desensitization. The extracellular recording solution contained 0.2 mM calcium for most experiments. Sometimes 1 mM calcium was used, which did not significantly change the Iss/IP and thus the data were combined. Under these conditions, neuronal NMDARs showed a clear decrease in desensitization during development (Figure 4-4 A). The Iss/IP ratio was 34±3% (N=25), 47±5% (N=12), and 74±2% (N=30) for neurons of 4-7, 10-11, and >13 DIV respectively (P<0.0001 by one-way ANOVA followed by Bonferroni’s multiple comparison test).

To determine whether the NR2A-containing NMDARs also undergo these developmental changes, we repeated the experiments in the presence of the NR2B-subtype selective inhibitor ifenprodil (3 \(\mu\)M). Ifenprodil markedly increased desensitization at each developmental stage, which is consistent with a previous study showing that ifenprodil increases desensitization of recombinant NMDARs expressed in HEK 293 cells by an allosteric interaction between the ifenprodil and glutamate binding sites (Zheng et al., 2001). Nonetheless, the developmental decrease in desensitization was still apparent (Figure 4-4 b). The Iss/IP ratio was 7±3% (N=5), 25±2% (N=5), and 43±3% (N=16) for neurons of 4-7, 10-11, and >13 DIV respectively (P<0.0001 by one-way ANOVA followed by Bonferroni’s multiple comparison test). These results provide
further evidence to support the conclusion that the developmental change in NMDAR
desensitization was not dependent on the change in subunit composition.
Figure 4-4. NMDAR desensitization showed a developmental change independent of subunit composition

(a) Left: Representative responses of NMDARs in a 6 DIV, 11 DIV, and a 16 DIV neuron in response to 1 mM NMDA (100 μM glycine) in 0.2 mM extracellular Ca\(^{2+}\). Electrode solution contained 10 mM BAPTA. Peak currents were normalized for comparison of desensitization. Right: quantification of desensitization showing a developmental change. (b) Same as A, except 3 μM ifenprodil was present during recording to isolate the current mediated by NR2A-containing receptors.
4.2. Developmental change in desensitization not due to change in NMDAR zinc sensitivity

Zinc in the submicromolar range has been shown to increase NMDAR desensitization for recombinant receptors expressed in HEK293 cells (Chen et al., 1997; Zheng et al., 2001). Similar to the effect of ifenprodil on desensitization of NR1/NR2B, zinc increases desensitization of NR1/NR2A via an allosteric interaction between the glutamate and zinc binding sites (Zheng et al., 2001). As a result, binding of glutamate increases NMDAR’s affinity for zinc, leading to an apparent increase in desensitization (Zheng et al., 2001). Since salt solutions often contain submicromolar zinc concentrations because of contamination (Paoletti et al., 1997), we therefore investigated whether a change in sensitivity to ambient zinc during development might explain our results. To test this possibility, we used 2 μM TPEN to chelate ambient zinc in the recording solution (Paoletti et al., 1997). Surprisingly, removal of zinc did not decrease the desensitization of NMDARs at any developmental stage (Figure 4-5), indicating that ambient zinc did not contribute to the developmental change in NMDAR desensitization. Removal of zinc also had no effect on the developmental change in desensitization in the presence of ifenprodil (Figure 4-5). On the other hand, removal of zinc dramatically inhibited the effects of ifenprodil on desensitization at all developmental stages such that in the absence of zinc ifenprodil did not increase NMDAR desensitization (Figure 4-5; P>0.05 at each development stage between control+TPEN and ifenprodil+TPEN conditions). Another zinc chelator, EDTA, had similar effects (data not shown).
Figure 4-5. Zinc sensitivity did not mediate the developmental change in NMDAR desensitization

Inclusion of 2 μM TPEN to remove ambient zinc (control+TPEN) did not significantly change NMDAR desensitization at each developmental stage compared with control condition. In the presence of ifenprodil, removal of zinc (ifenprodil+TPEN) markedly decreased NMDAR desensitization at each developmental stage, but the developmental change in desensitization persisted. In the absence of zinc ifenprodil did not significantly change NMDAR desensitization at each developmental stage. Iss/Ip for the control groups was reconstructed from Figure 4 a; Iss/Ip for the control+TPEN groups was 34±2%, 39±3%, and 66±3%; N=6, 4, and 5 respectively for 4-7, 10-11, and >13 DIV neurons; P>0.05 at each development stage between control and TPEN conditions. Iss/Ip for the ifenprodil groups was reconstructed from Figure 4 b; Iss/Ip for the ifenprodil+TPEN groups was 36±3%, 49±4%, and 61±5%; N=5, 12, and 5 respectively for 4-7, 10-11, and >13 DIV neurons; P<0.01 at each development stage between ifenprodil and any other condition.
These data support the conclusion that the developmental change in desensitization of neuronal NMDARs is not due to a change in receptor sensitivity to zinc during development. Although a zinc chelator can act to reverse ifenprodil’s effect of increasing desensitization of neuronal NMDARs, ambient zinc alone was not sufficient to regulate desensitization. The latter result contrasts with the effects of zinc and ifenprodil on desensitization of recombinant NMDARs expressed in HEK cells (Zheng et al., 2001). However, our data showing that neuronal NMDARs are apparently less sensitive to ambient zinc than is reported for recombinant NR1/NR2A (Williams, 1996; Chen et al., 1997; Paoletti et al., 1997), are in agreement with results of two previous studies (Zheng et al., 1998; Xiong et al., 1999).

4.3. NMDAR desensitization is regulated by receptor subcellular localization

One major difference between NMDARs in 4-7 DIV and those in >13 DIV neurons is their subcellular localization. NMDARs expressed in immature neurons are mainly extrasynaptic, whereas the majority are localized to synapses in more mature neurons (Figure 3-1) (Li et al., 1998; Rao et al., 1998; Tovar and Westbrook, 1999). It is possible that synaptic localization renders NMDARs more resistant to desensitization. This explanation would also be consistent with our results showing that 2B-subtype NMDARs in >13 DIV neurons desensitize more extensively than 2A-subtype NMDARs (Figure 4-1 a), since the majority of NR2A subunits are incorporated into synapses while NR2B subunits continue to predominate at extrasynaptic sites (Figure 1-3 and Figure 3-2).
(Li et al., 1998; Stocca and Vicini, 1998; Tovar and Westbrook, 1999; Barria and Malinow, 2002; Li et al., 2002).

To test whether extrasynaptic NMDARs desensitize more extensively than synaptic NMDARs, we chronically treated >13 DIV neurons with picrotoxin (PTX), which decreases inhibitory input thereby increasing activation of NMDARs and driving them away from synapses (Crump et al., 2001). Treatment with PTX (100 µM) for 1 week indeed decreased the synaptic localization of NMDARs in >13 DIV neurons, as assessed by immunocytochemical colocalization with synaptophysin (not shown), and also markedly increased NMDAR desensitization (Figure 4-6). Recordings made from outside-out patches from the soma of >13 DIV neurons, in which NMDARs should be extrasynaptic, also showed dramatic desensitization that was even more pronounced than that observed in 4-7 DIV neurons (Figure 4-6), consistent with a previous study (Sather et al., 1990).
Figure 4-6. Extrasynaptic NMDARs showed higher degree of desensitization than synaptic NMDARs in standard neuronal cultures

Recording was made on mature cultured neurons treated with vehicle alone (>13 DIV), picrotoxin (>13 DIV, PTX), or patches drawn from the cell soma (>13 DIV, patch).

$\text{I}_{\text{ss}}/\text{I}_p=74\pm2\%$ and $44\pm2\%$, $N=30$ and 12 for control and PTX group, respectively;

$P<0.0001$. $\text{I}_{\text{ss}}/\text{I}_p=22\pm2\%$, $N=5$ for NMDAR currents recorded from outside-out patches;

$P<0.0001$ compared with control.
To test whether extrasynaptic NMDARs within a single cell desensitize more readily than synaptic NMDARs, we used autaptic-cell cultures (Tovar and Westbrook, 1999; Li et al., 2002) to directly compare extrasynaptic NMDARs with the total surface NMDARs. Autaptic synapses in 11-14 DIV single island cultures were stimulated with short depolarizations to generate action potentials in the presence of the use dependent blocker MK801 (20 μM). Under these conditions, synaptic NMDARs were selectively activated and blocked (Figure 4-7 a). Desensitization before and after blockade of the synaptic NMDARs of a representative neuron is shown in Figure 4-7 b. Responses mediated by extrasynaptic NMDARs spared from activity-dependent MK801 block clearly showed a higher degree of desensitization compared with the whole-cell responses mediated by the combined pool of synaptic and extrasynaptic NMDARs (Figure 4-7 c). About 60% of the NMDARs in these autaptic neurons were synaptic, as the extrasynaptic NMDAR-mediated response was 39±4% of the whole-cell response (N=7).
Figure 4-7. Extrasynaptic NMDARs showed higher degree of desensitization than synaptic NMDARs in autaptic neuronal cultures

(a) NMDAR-mediated synaptic responses were recorded from an autaptic neuron in the presence of 20 μM MK801. 5 μM of CNQX and 2 mM Ca\(^{2+}\) were present in the extracellular recording solution. 1\(^{st}\), 10\(^{th}\), 20\(^{th}\), and 44\(^{th}\) responses of an autaptic neuron in the presence of MK801 are shown. (b) Responses of the same neuron as that in (a) to 1 mM NMDA (100 μM glycine) before and after blockade of synaptic NMDARs. Ca\(^{2+}\) was replaced with 1 mM Sr\(^{2+}\). Peak currents were normalized for comparison of desensitization. (c) Quantification showing extrasynaptic NMDAR-mediated responses had higher degree of desensitization than whole-cell responses. Iss/IP=71±4% and 41±2% for responses before and after block of synaptic receptors, respectively; N=8; P<0.0001, paired t-test.
4.4. Effect of PSD-95 on NMDAR desensitization

Unlike extrasynaptic NMDARs, synaptic NMDARs are directly associated with a variety of proteins that are enriched in the postsynaptic density (PSD) compartment, including the major scaffolding protein PSD-95 (Kornau et al., 1995a; Niethammer et al., 1996). To determine whether PSD-95 can play a role in mediating regulation of NMDAR desensitization, we first treated the >13 DIV neurons with 2-bromopalmitate, a potent palmitoylation inhibitor that can disperse clusters of PSD-95 away from synapses (El-Husseini et al., 2002). Incubation with 100 μM 2-bromopalmitate for 6 hrs increased NMDAR desensitization (Figure 4-8). Treatment with latrunculin B (5 μM) to depolymerize F-actin (Allison et al., 1998), however, had no effect on desensitization (Figure 4-8); similarly, latrunculin A had no effect and data were combined. Although depolymerization of F-actin has been shown to reduce the number of synaptic NMDARs (Allison et al., 1998; Sattler et al., 2000), immunocytochemical staining has demonstrated that NMDARs remain clustered and colocalized with PSD-95 even after latrunculin treatment (Allison et al., 1998). In contrast, treatment with PTX reduces synaptic levels of NMDARs without influencing the synaptic clustering of PSD-95 (Crump et al., 2001), indicating that this treatment uncouples NMDARs from PSD-95. Together with our data, these results suggest that PSD-95, or proteins recruited by PSD-95, may be critical components that lead to a reduction in NMDAR desensitization.
Figure 4-8. NMDAR desensitization was regulated by PSD-95 in mature neurons

Treatment with 2-bromopalmitate to disperse PSD-95, but not latrunculin to disperse F-actin or vehicle only (control), increased NMDAR desensitization in >13 DIV neurons. Iss/Ip=49±2% and 68±3%, N=5 and 12 for 2-bromopalmitate and control, respectively; P<0.001. Iss/Ip=70±5%, N=11 for latrunculin-treated neurons; P>0.05 compared with control.
In immature (4-7 DIV) neurons, on the other hand, overexpression of PSD-95-GFP decreased NMDAR desensitization (Figure 4-9 a; Iss/Ip=64±3%, 46±2%, and 39±2%; N=8, 8, and 4 for PSD-95-GFP, GFP, and non-transfected group respectively; P<0.001 between PSD-95-GFP and other groups). To determine if, under more physiological conditions, overexpression of PSD-95 can still change the kinetics of NMDAR-mediated responses, we recorded currents evoked by 100 μM NMDA and 30 μM glycine in the presence of 2 mM calcium using the nystatin patch-clamp method. Again, overexpression of PSD-95 decreased NMDAR desensitization (Figure 4-9 b; Iss/Ip=47±4% and 34±2% for PSD-95 and control non-transfected group respectively; N=7 for both groups; P<0.01).

These data confirm that association of NMDARs with PSD-95 is necessary to mediate a dramatic decrease in receptor desensitization during development. Notably, co-expression of PSD-95 with either NR1/NR2A or NR1/NR2B in HEK293 cells did not significantly alter desensitization (Figure 4-10), indicating that binding of PSD-95 with NMDARs is not sufficient to alter receptor desensitization.
Figure 4-9. NMDAR desensitization was regulated by PSD-95 in immature neurons
(a) Overexpression in 4-7 DIV neurons of PSD-95-GFP (PSD-95) decreased NMDAR
desensitization compared with overexpression of GFP or with control neurons (not
transfected). (b) Same as (a), except responses were recorded using 100 μM NMDA (30
μM glycine) in the presence of 2 mM Ca\(^{2+}\), and nystatin-perforated patch recording
technique was used.
Figure 4-10. PSD-95 did not influence desensitization of recombinant NMDARs expressed in HEK293 cells

NR1/NR2B or NR1/NR2A was co-expressed with either PSD-95-GFP or GFP in HEK 293 cells. Responses of those receptors to 1mM glutamate and 100 μM glycine (10 s) in the presence of 100 μM EGTA and absence of CaCl₂ were recorded. The Iss/Ip value was 48±10% (n=7) and 57±15% (n=5) for NR1/NR2A/GFP and NR1/NR2A/PSD95, respectively (P>0.05). The Iss/Ip value was 77±2% (n=10) and 70±10% (n=5) for NR1/NR2B/GFP and NR1/NR2B/PSD95, respectively (P>0.05).
4.5. Effects of Kinase/phosphatase activity

Since PSD-95 most likely regulated NMDAR desensitization indirectly, it is possible that its effect was exerted by recruiting kinases and/or phosphatases to the NMDAR-complex, and thereby altering the balance between phosphorylation and dephosphorylation of either the NMDAR itself or a closely related protein. To test this hypothesis, we examined the effects of some kinases and phosphatases that have been implicated in regulating NMDAR function.

Glycine-independent desensitization is promoted by calcineurin activity (Lieberman and Mody, 1994; Tong and Jahr, 1994; Tong et al., 1995), an effect that appears as a time-dependent increase in desensitization associated with prolonged whole-cell dialysis and an apparent increase in \([\text{Ca}^{2+}]_i\) (Sather et al., 1992; Tong and Jahr, 1994; Krupp et al., 2002). Under our experimental conditions using either the Nystatin perforated or the conventional whole-cell patch clamp recording method, we found that the Iss/Ip ratio was fairly stable over time for either 4-7 DIV neurons or >13 DIV neurons (Figure 4-11 a, b), suggesting that calcineurin did not play a large role in our experiments. This result was not surprising since we used conditions to promote maintenance of low \([\text{Ca}^{2+}]_i\), such as a low extracellular calcium concentration and high intracellular calcium buffering, thus minimizing calcineurin activity. To examine further a role for calcineurin, we applied a calcineurin inhibitor cyclosporin A (500 nM) for 5-15 min, which did not alter the desensitization of NMDARs in 4-7 DIV neurons (Figure 4-12 a; Iss/Ip = 40±3%, 40±2%; N=13 and 6 for control and cyclosporin A respectively; \(P>0.05\)). Previous studies also showed that the effect of calcineurin was inhibited by blocking calcium entry (Lieberman and Mody, 1994), and that under conditions of high...
intracellular calcium buffering (10 mM EGTA), calcineurin-mediated dephosphorylation was slow and did not appreciably contribute to fast synaptic NMDAR desensitization (Raman et al., 1996).

To address further the role of protein phosphorylation in regulating NMDAR desensitization we applied a variety of protein kinase and phosphatase inhibitors/activators to neuronal cultures. For NMDARs in 4-7 DIV neurons, application of forskolin (50 μM, 5-15 min), a PKA activator that augments NMDAR-mediated responses by overcoming the effect of calcineurin (Raman et al., 1996), or the PKA inhibitor PKI (Raman et al., 1996), had no effect on Iss/IP (Figure 4-12 a). Similarly, a CaMKII inhibitor KN93 (10 μM, 5-15 min) and a tyrosine phosphatase inhibitor bpV(phen) (100μM, 5-15 min) had no significant effect on desensitization when all treatment groups were compared using one-way ANOVA followed by Bonferroni’s multiple comparisons test (Figure 4-12 a; P>0.05 for comparison among all groups). On the other hand, the tyrosine phosphatase inhibitor bpV(phen) resulted in a small increase in Iss/IP that was significantly different when compared to the control (vehicle treatment) group alone (Iss/IP = 49±3%, N=12 and 40±3%, N=13 for bpV and control groups, respectively; P<0.05, unpaired t-test).

Results for mature (>13 DIV) neurons were similar to those of experiments with immature neurons. Treatment of >13 DIV neurons with either the tyrosine kinase inhibitor genistein (50 μM, 5-15 min) or Src family tyrosine kinase inhibitor PP2 (10 μM, 5-15 min) or KN93, bpV, forskolin or PKI, all failed to significantly affect NMDAR desensitization, using the one-way ANOVA followed by Bonferroni’s multiple comparison for all treatment groups (P>0.05; Figure 4-12 b). However, incubation with
forskolin resulted in a small increase in NMDAR desensitization that was significantly different when compared directly with the control group alone, and its effect was blocked by co-incubation with PKI (Iss/Ip = 65±3%, 77±4%, and 78±3%; N=11, 13 and 7 for control, forskolin and forskolin+PKI, respectively; P<0.05 between forskolin and control, or between forskolin and forskolin+PKI, by unpaired t-test). Co-incubation with bpV(phen) did not block the effect of forskolin on desensitization (Figure 4-12 b; Iss/Ip=65±3%; N=7; P>0.05 between forskolin+bpV and control; P>0.05 between forskolin alone and forskolin+bpV), suggesting the effect of forskolin occurred through the activation of PKA and not via cross-talk between PKA and tyrosine kinases or phosphatases as proposed by a recent study (Woodward, 2002).

We conclude from these data that the activity of the protein kinases and phosphatases tested in our experiments cannot fully account for the dramatic difference in extent of NMDAR desensitization observed in immature versus mature neurons.
Figure 4-11. Neuronal NMDARs did not show time-dependent increase in desensitization

(a) Desensitization of NMDARs in 4-7 DIV or >13 DIV neurons was stable over time, suggesting calcineurin did not regulate desensitization. Recording was made using 100 μM NMDA (30 μM glycine) in the absence of Ca$^{2+}$. 10 μM of ifenprodil was included for recording on >13 DIV neurons. Nystatin-perforated patch recording technique was used. (b) Same as (a), except that 1 mM NMDA (100 μM glycine) was used (without ifenprodil for either ages), recording was made in the absence of ifenprodil, and conventional whole-cell recording technique was employed with 10 mM BAPTA in the electrode solution.
Figure a: Graph showing the ratio of I$_{ss}$/I$_{p}$ versus time for Nystatin treatment. The graph shows two types of data points: circles for 4-7 DIV and squares for >13 DIV.

Figure b: Graph showing the ratio of I$_{ss}$/I$_{p}$ versus time for Whole-cell treatment. The graph shows two types of data points: circles for 4-7 DIV and squares for >13 DIV.
Figure 4-12. Effects of Phosphorylation / dephosphorylation on NMDAR desensitization

(a) Treatment of 4-7 DIV neurons with forskolin, PKI, cyclosporin A, KN93, or bpV(phen) all had no significant effect on desensitization when all treatment groups were compared using one-way ANOVA followed by Bonferroni’s multiple comparisons test \( (P>0.05) \). On the other hand, bpV(phen) resulted in a small increase in Iss/IP that was significantly different when compared to the control (vehicle treatment) group alone \( (\text{Iss/IP} = 49\pm3\%, \text{N=12} \) and \( 40\pm3\%, \text{N=13} \) for bpV and control groups, respectively; \( P<0.05 \), unpaired \( t \)-test). (b) Treatment of >13 DIV neurons with genistein, PP2, KN93, bpV, forskolin or PKI, all failed to significantly affect NMDAR desensitization, using the one-way ANOVA followed by Bonferroni’s multiple comparison for all treatment groups \( (P>0.05) \). However, incubation with forskolin resulted in a small increase in NMDAR desensitization that was significantly different when compared directly with the control group alone, and its effect was blocked by co-incubation with PKI \( (\text{Iss/IP} = 65\pm3\%, 77\pm4\%, \text{and} 78\pm3\%; \text{N=11, 13 and 7} \) for control, forskolin and forskolin+PKI, respectively; \( P<0.05 \) between forskolin and control, or between forskolin and forskolin+PKI, by unpaired \( t \)-test). Co-incubation with bpV(phen) did not block the effect of forskolin on desensitization \( (\text{Iss/IP}=65\pm3\%; \text{N=7}; P<0.05 \) between forskolin+bpV and control; \( P>0.05 \) between forskolin alone and forskolin+bpV).
4.6. Summary

We report a developmental change in NMDAR desensitization, which was not dependent on the switch in NMDAR subunit composition or a change in the sensitivity to ambient zinc, but correlated with the change in receptor subcellular localization during development. A major difference between synaptic and extra-synaptic NMDARs is that synaptic NMDARs colocalize and interact with many signalling proteins that are concentrated within the PSD compartment (Ziff, 1997). Of the many PSD proteins that can potentially modulate NMDAR properties, PSD-95 has gained the most attention.

Here, we provide several lines of evidence to suggest that co-localization of PSD-95 with NMDARs can largely account for the developmental change in NMDAR desensitization. First, PSD-95 is co-clustered with NMDARs largely at synapses and the two are rarely co-localized on dendritic shafts or the soma (Rao et al., 1998). Consistent with this, NMDAR desensitization in outside-out patches pulled from the soma of mature neurons was extensive and similar to that found in immature neurons. Second, inhibition of palmitoylation in mature neurons increased NMDAR desensitization. Inhibitors of PSD-95 palmitoylation have been shown to decrease PSD-95 clusters at synapses and increase diffuse immunostaining of PSD-95 throughout dendritic shafts and the soma, indicating a shift from synaptic to non-synaptic localization (El-Husseini et al., 2002). However, NMDARs remain clustered at synapses under these conditions (El-Husseini et al., 2002), suggesting that NMDARs and PSD-95 become “uncoupled”. Similarly, PTX treatment of mature neurons has been shown to result in a shift of NMDARs away from synapses, whereas PSD-95 synaptic clusters remain intact (Crump et al., 2001). Again, with PTX treatment NMDARs and PSD-95 are largely uncoupled, and under these
conditions we found that NMDAR desensitization was increased markedly to levels not
significantly different from those found in immature (4 – 7 DIV) neurons. On the other
hand, treatment with latrunculin, resulting in actin depolymerization, causes movement of
both NMDARs and PSD-95 away from synapses, where the two remain co-clustered
(Allison et al., 1998). Consistent with this, latrunculin treatment of mature neurons had
no effect on NMDAR desensitization. Finally, when PSD-95 expression was increased
by transfecting immature neurons with PSD-95 plasmid DNA, NMDAR desensitization
was dramatically reduced, and not significantly different from that found in mature (>13
DIV) neurons. It should be noted that treatments that altered NMDAR desensitization in
mature neurons also disrupted association of PSD-95 and NMDARs by shifting one or
both away from synapses, rather than changing overall expression levels of PSD-95. It is
interesting that in a previous study, suppression of PSD-95 protein levels in cultured
cortical neurons using an antisense oligonucleotide approach did not result in a change in
macroscopic kinetics of NMDAR-mediated currents (Sattler et al., 1999). It is possible
that those results were due to compensation by other signalling molecules during chronic
suppression of PSD-95 expression.

PSD-95 has been shown to cluster kainate receptors and inhibit kainate receptor
desensitization when co-expressed in HEK293 cells or COS-1 cells (Garcia et al., 1998).
Overexpression of PSD-95 in HEK cells co-transfected with NMDARs did not change
NMDAR desensitization (Figure 4-10), suggesting that the effect of PSD-95 on NMDAR
desensitization was neuron-specific and not simply due to direct binding of PSD-95 to
NMDARs. NMDARs in 4-7 DIV neurons are mostly extrasynaptic and do not interact
with PSD-95. Overexpression of PSD-95 in those neurons may regulate NMDAR
desensitization by recruiting tyrosine kinases and promoting tyrosine-phosphorylation of NMDARs (Tezuka et al., 1999; Liao et al., 2000). This is supported by the fact that inhibition of tyrosine phosphatase activity by bpV(phen) decreased NMDAR desensitization in 4-7 DIV neurons (Figure 4-12). Consistently, bpV(phen) also decreased the desensitization of recombinant NMDARs expressed in HEK cells (Krupp et al., 2002). It must be emphasized, however, that an altered balance of tyrosine phosphorylation due to PSD-95 recruitment of protein tyrosine kinases may not fully explain the difference in NMDAR desensitization between immature and mature neurons, since this difference is large compared with the effect of tyrosine phosphatase inhibition on NMDAR desensitization in immature neurons. In addition, desensitization of NMDARs in >13 DIV neurons was not influenced by incubation with either PP2 or genistein, agents that inhibit tyrosine kinases, suggesting that synaptic NMDARs are resistant to tyrosine dephosphorylation or else that factors other than tyrosine kinases/phosphatases contribute to regulation of NMDAR desensitization in mature neurons versus immature neurons.

PSD-95 can also induce the assembly of signalling molecules other than tyrosine kinases in the PSD to regulate synaptic NMDAR function. For example, PKA is targeted to NMDARs via an interaction between PSD-95 and A-kinase anchoring protein (AKAP; Colledge et al., 2000), and we found that activation of PKA by forskolin resulted in a small increase in desensitization of NMDARs in >13 DIV neurons. This effect of PKA was somewhat surprising, since previous studies indicate that PKA activation can overcome the effects of calcineurin (Raman et al., 1996) and prevent calcineurin-dependent increase in desensitization (Krupp et al., 2002). However, the effects of PKA
on NMDAR function are complicated, both up-regulation (Blank et al., 1997; Westphal et al., 1999) and down-regulation (Woodward, 2002) of NMDAR function have been reported. Moreover, mutation of serine 900 on NR2A has been shown to increase desensitization, whereas mutation of serine 929 suppresses desensitization (Krupp et al., 2002). These studies suggest that phosphorylation of NR2A by PKA at different sites can have opposite effects. Our data showed that PKA or its inhibitor did not regulate NMDAR desensitization in 4-7 DIV neurons, which mainly have extrasynaptic NMDARs. This can be explained by recent studies showing that the regulation of glutamate receptors by PKA requires that PKA be targeted to the receptors by “bridging” proteins including yotiao, PSD-95/AKAP, and SAP97/AKAP, all of which are enriched in the PSD (Lin et al., 1998; Westphal et al., 1999; Colledge et al., 2000).

Zinc has been shown to increase desensitization of NR1/NR2A expressed in HEK293 cells (Chen et al., 1997; Zheng et al., 2001). Zinc increases desensitization of NR1/NR2A via an allosteric interaction between the glutamate and zinc binding sites. As a result, binding of glutamate increases NMDAR’s affinity for zinc, leading to an apparent increase in desensitization during continuous presence of agonist (Zheng et al., 2001). Similarly, ifenprodil increases desensitization of NR1/NR2B by an allosteric interaction between the ifenprodil and glutamate binding sites (Zheng et al., 2001).

Surprisingly, removal of zinc by zinc chelators did not decrease the desensitization of NMDARs at any developmental stage in our experiments, indicating that ambient zinc did not contribute to the developmental change in NMDAR desensitization. These data also suggested that the effect of low zinc concentrations on neuronal NMDARs was different from that on recombinant NMDARs expressed in a
heterologous system so that although zinc chelators can act to reverse ifenprodil’s effect of increasing desensitization of neuronal NMDARs, ambient zinc alone was not sufficient to regulate desensitization. The latter result contrasts with the effects of zinc on desensitization of recombinant NMDARs expressed in HEK cells (Zheng et al., 2001). Our data suggested that hippocampal neuronal NMDARs are less sensitive to ambient zinc than is reported for recombinant NR1/NR2A (Williams, 1996; Chen et al., 1997; Paoletti et al., 1997). This is in agreement with results of two previous studies, which showed that recombinant NR1/NR2A expressed in HEK293 cells are tonically inhibited by ambient zinc (Zheng et al., 1998), whereas hippocampal neuronal NMDARs are only minimally affected by ambient zinc (Xiong et al., 1999).

A developmental change in the decay of NMDAR-mediated synaptic response has been reported to depend on the developmental switch in subunit composition (Carmignoto and Vicini, 1992; Hestrin, 1992; Monyer et al., 1994; Flint et al., 1997). Here we showed that the function of NMDAR is also regulated developmentally on a slower time scale through desensitization, and this change is associated with the recruiting of NMDARs into the synapses during development. In Chapter III we have shown that the activity of extrasynaptic NMDARs was more readily down regulated through a process called “rundown” compared with synaptic NMDARs. Together with the current findings, our results indicated that the function of NMDARs within a single neuron is fine-tuned based on their localization. The activity of synaptic NMDARs, which is resistant to down-regulation by either rundown or desensitization, keeps a high fidelity transmission in the synapse to fulfill its role in synaptic plasticity during repeated firing. The activity of extrasynaptic NMDARs, on the other hand, is readily shut down to
limit neuronal damage by either rundown or desensitization during pathological conditions such as ischemia, where sustained glutamate release and spill-over can occur.
CHAPTER V

General discussion

We have shown, for the first time, that the subcellular localization (synaptic versus extrasynaptic) regulates the stability as well as the function of NMDARs. Compared with extrasynaptic NMDARs, synaptic NMDARs show less Ca\(^{2+}\)-independent rundown that can be explained by their resistance to internalization in response to sustained or repeated agonist application. As well, synaptic NMDARs show reduced glycine-independent desensitization in the continuous presence of agonist, which can be largely accounted for by the association of PSD-95 with NMDARs in the postsynaptic density.

5.1. Ca\(^{2+}\)-independent rundown

The subcellular localization of NMDARs in hippocampal pyramidal neurons regulates NMDAR rundown. Predominantly extrasynaptic NR2B-subtype NMDARs showed faster and more extensive peak current rundown in response to repeated, sustained agonist application compared with mainly synaptic NR2A-containing NMDARs. Moreover, rundown of 2B-subtype receptors was largely Ca\(^{2+}\)-insensitive and dependent on tyrosine dephosphorylation whereas rundown of 2A-containing receptors was Ca\(^{2+}\)-dependent and partly mediated by actin depolymerization. The Ca\(^{2+}\)-independent rundown appears to be related to the extrasynaptic localization but not the subunit composition, since synaptic 2B-subtype receptors were resistant to Ca\(^{2+}\).
independent rundown, whereas 2A-containing receptors located extrasynaptically were vulnerable to Ca\textsuperscript{2+}-independent rundown. We also found that a decrease in the number of NMDARs on the cell surface due to an increase in receptor internalization could explain the Ca\textsuperscript{2+}-independent rundown of extrasynaptic NMDARs. Based on these data we propose that interactions with synaptic proteins stabilize NMDARs and prevent receptor down-regulation in response to frequent or sustained agonist exposure. Consistent with this idea, a recent study showed that overexpression of PSD-95 inhibits, and that deletion of the PDZ-binding domain of NR2B increases, the constitutive internalization of a NR2B chimeric protein expressed in neurons (Roche et al., 2001).

Future studies should be carried out to determine whether the use-dependent or agonist binding induced internalization of the full-length or endogenous NMDAR subunits is also regulated by PSD-95 or other scaffolding proteins in the PSD, and whether this internalization uses the classic clathrin coat mediated endocytotic pathway. A reduction in the number of surface NMDARs should result in reduced calcium influx, which may be protective of the neurons in excitotoxicity conditions. The effects of inhibition of NMDAR internalization on excitotoxicity-induced cell death should be tested.

5.2. Glycine-independent desensitization

Previous studies have shown that deactivation of NMDAR-mediated synaptic responses change during development due to a switch in receptor subunit composition, and this change has been proposed to underlie developmental changes in plasticity (Kirkwood et al., 1995; Kirkwood et al., 1996; Quinlan et al., 1999a; Quinlan et al.,
We have found that glycine-independent desensitization of NMDARs in rat cultured hippocampal neurons decreases during development. This decrease was not dependent on a switch in subunit composition, but was correlated with synaptic localization of the receptor. Furthermore, overexpression of PSD-95 in immature neurons reduced NMDAR desensitization, whereas agents that interfere with synaptic targeting of PSD-95 or that induce movement of NMDARs away from synapses increased NMDAR desensitization in mature neurons.

Since PSD-95 recruits protein kinases/phosphatases into the PSD (see Chapter 1), we examined the effect of a variety of phosphatase and kinase inhibitors. However, the activity of the kinases/phosphatases tested so far could not fully account for the difference between immature and mature neurons. Given that PSD-95 recruits many scaffolding and signaling molecules into PSD (see Chapter 1), our screening of candidate factors was far from thorough. PSD-95 has been recently shown to promote synaptogenesis and recruit AMPA receptors to synapses (Chen et al., 2000; El-Husseini et al., 2000; El-Husseini et al., 2002; Schnell et al., 2002). It is also possible that factors that do not directly interact with PSD-95, but are recruited to the PSD during synaptogenesis, participate in regulating NMDAR desensitization.

Additional approaches could be taken to screen for other proteins involved in regulating NMDAR desensitization. For example, NR2 subunit C-terminal peptides that interfere with NMDAR interaction with specific proteins (see Chapter 1) could be introduced intracellularly during whole-cell patch clamp recording to test for effects on NMDAR desensitization. Additional factors that can promote synaptogenesis could also be tested on immature neurons. For example, a recent study showed that EphrinB-EphB
signaling promotes synaptogenesis and that EphB directly bind to NMDARs via an interaction between extracellular domains of the two receptors (Dalva et al., 2000). Therefore, it is possible that Ephrin signaling is involved in regulating NMDAR desensitization.

5.3. Functional relevance

5.3.1. Pathophysiology

It has been established that NMDARs are critically involved in both pathological and physiological conditions (see Chapter 1). Specifically, NMDAR-mediated excitotoxicity is believed to be an important factor in mediating neuronal death in brain stroke. In ischemic conditions, deprivation of oxygen and glucose leads to depletion of ATP and accumulation of the neurotransmitter glutamate in the extracellular solution, which in turn over-activates ionotropic glutamate receptors. The resulting excessive calcium influx mainly through NMDAR channels overloads the neuron and induces further metabolic damage (Lee et al., 1999). It has been shown that NMDAR antagonists prevent the Ca\(^{2+}\) overload and markedly reduce the neuronal death in \textit{in vitro} ischemia models as well as reduce neuronal death and infarct volume in animal models of ischemia (Simon et al., 1984; Wieloch, 1985; Albers et al., 1992; Lee et al., 1999; Brauner-Osborne et al., 2000).

Clinical trials using NMDAR antagonists for stroke, however, have been disappointing, partly because blockade of NMDARs causes serious unwanted side-effects, such as the induction of psychotomimetic effects, respiratory depression, or
cardiovascular dysregulation (Brauner-Osborne et al., 2000). An alternative way to protect neurons from excitotoxicity is to take advantage of the negative-feedback regulation of NMDARs (see Chapter 1). It has been shown that suppression of Ca\(^{2+}\)-dependent rundown of NMDARs enhances NMDA- or glutamate-induced excitotoxicity (Furukawa et al., 1995; Abdel-Hamid and Baimbridge, 1997; Furukawa et al., 1997), whereas increasing Ca\(^{2+}\)-dependent rundown protects from glutamate- or OGD (oxygen/glucose deprivation)-induced excitotoxicity (Furukawa et al., 1995; Sattler et al., 2000). As well, rapid desensitization of non-NMDARs is protective against AMPA-induced excitotoxicity (Zorumski et al., 1990; May and Robison, 1993; Brorson et al., 1995; Raymond et al., 1996). Although the desensitization of NMDARs is slower and more limited in extent compared with non-NMDA receptors (McBain and Mayer, 1994; Dingledine et al., 1999), it may also be protective during a sustained glutamate insult such as that which occurs in ischemia, by limiting calcium influx through its own channel.

The accumulated glutamate in the extracellular compartment during ischemia activates not only the synaptic NMDARs but also extrasynaptic NMDARs. Importantly, activation of extrasynaptic NMDARs is more toxic than activation of synaptic NMDARs, as a recent study showed that calcium entry through synaptic NMDARs induces CREB activity and BDNF (brain-derived neurotrophic factor) gene expression, and thus has anti-apoptotic activity. In contrast, calcium entry through extrasynaptic NMDARs shuts off CREB expression, blocks the induction of BDNF expression, and causes loss of mitochondrial membrane potential and cell death (Hardingham et al., 2002). Thus, the specific down-regulation of extrasynaptic NMDARs via either rundown or
desensitization may help minimize neuronal damage during ischemic insults while maintaining relatively stable synaptic transmission mediated by synaptic NMDARs.

5.3.2. Physiology

A variety of studies indicate that NMDAR activity is also critical for the formation and maturation of excitatory synapses during development (Durand et al., 1996; Wu et al., 1996; Isaac et al., 1997; Constantine-Paton and Cline, 1998; Engert and Bonhoeffer, 1999; Liao et al., 1999; Maletic-Savatic et al., 1999). Prior to synapse formation, neurotransmitter signaling may act to influence cell proliferation, migration, and differentiation (Nguyen et al., 2001; Owens and Kriegstein, 2002). A recent study showed that glutamate is released early in development in an unconventional manner that does not depend on vesicle fusion (Demarque et al., 2002). This study indicates that NMDARs, presumably extrasynaptic at this developmental stage, can be activated by the unconventionally released glutamate, suggesting an important role for those NMDARs in neural development.

NMDARs are also crucial for synaptic plasticity, including both LTP and LTD (reviewed by Malenka, 1994; Malenka and Nicoll, 1999) (see Chapter 1). It has been well established that NMDARs are required for the induction of LTP at most glutamatergic excitatory synapses in the mammalian brain (Malenka and Nicoll, 1999). NMDARs are also required for the induction of LTD at the same synapses that show NMDAR-dependent LTP (Malenka, 1994). The information encoded by the distinct spatial and temporal changes in Ca^{2+} dynamics induced by either LTP or LTD stimulation is the key in dictating whether LTP or LTD will be induced (reviewed by Malenka, 1994; Zucker,
1999; Franks and Sejnowski, 2002) (see Chapter 1). Interestingly, selective activation of synaptic NMDARs induces increased synaptic insertion of AMPA receptors and LTP, whereas activation of extrasynaptic NMDARs results in LTD in cultured hippocampal neurons (Lu et al., 2001). Conceivably this is due to the different patterns of calcium signal profile induced by the activation of either synaptic or extrasynaptic NMDARs.

Thus, the differential regulation of synaptic and nonsynaptic NMDAR activity may be crucial for normal physiological processing, synaptogenesis and synaptic plasticity.

5.4. Concluding remarks

We conclude that synaptic localization increases the stability of hippocampal neuronal NMDAR responses to sustained agonist exposure. Our results elucidate mechanisms for regulating NMDAR function that tune receptor activity in neurons of different developmental stages, or the response of subpopulations of NMDARs in a single neuron to different stimuli. The fact that extrasynaptic and synaptic NMDARs serve different functions in both physiological and pathological conditions suggests that the differential regulation of the two subpopulations of receptors is functionally important.
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