NMDA RECEPTOR DESENSITIZATION REGULATED BY DIRECT BINDING TO PDZ1-2 DOMAINS OF PSD-95

by

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ABSTRACT

Regulation of NMDA receptor (NMDAR) activity by desensitization is important in physiological and pathological states; NMDAR desensitization contributes in shaping synaptic responses and may be protective by limiting calcium influx during sustained glutamate insults. We previously reported that glycine-independent desensitization decreases during hippocampal neuronal development, correlating with NMDAR synaptic localization and association with post-synaptic density 95 (PSD-95). PSD-95/Discs large/zona occludens (PDZ) domains 1 and 2 of PSD-95 bind to the C-terminus of NMDAR NR2 subunits. The role of PSD-95 in anchoring signaling proteins in close proximity to NMDARs is well documented. To determine if changes in NMDAR desensitization occur because of the direct binding of PSD-95 to NMDARs or due to PSD-95 recruitment of regulatory molecules, we tested the effects of various PSD-95 constructs on NMDAR currents in human embryonic kidney 293 (HEK293) cells and neurons. In HEK cells wild-type PSD-95 markedly reduced NMDAR desensitization, and the N-terminus of PSD-95 truncated after the PDZ1-2 domains was sufficient for this effect even when multimerization of PSD-95 was abolished. In immature neurons where PSD-95 expression is low, overexpression of either PSD-95 or PSD-95 PDZ1-2 was sufficient to significantly decrease desensitization. In mature neurons, disruption of PSD-95/NMDAR interaction through protein kinase C (PKC) activation increased desensitization to levels found in immature neurons, and this effect was not due to direct regulation of NMDAR activity by PKC. We conclude that direct binding of PSD-95 increases stability of NMDAR responses to sustained agonist exposure in neuronal and non-neuronal cells.

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CHAPTER I

Introduction

1.1. Ionotropic glutamate receptor overview

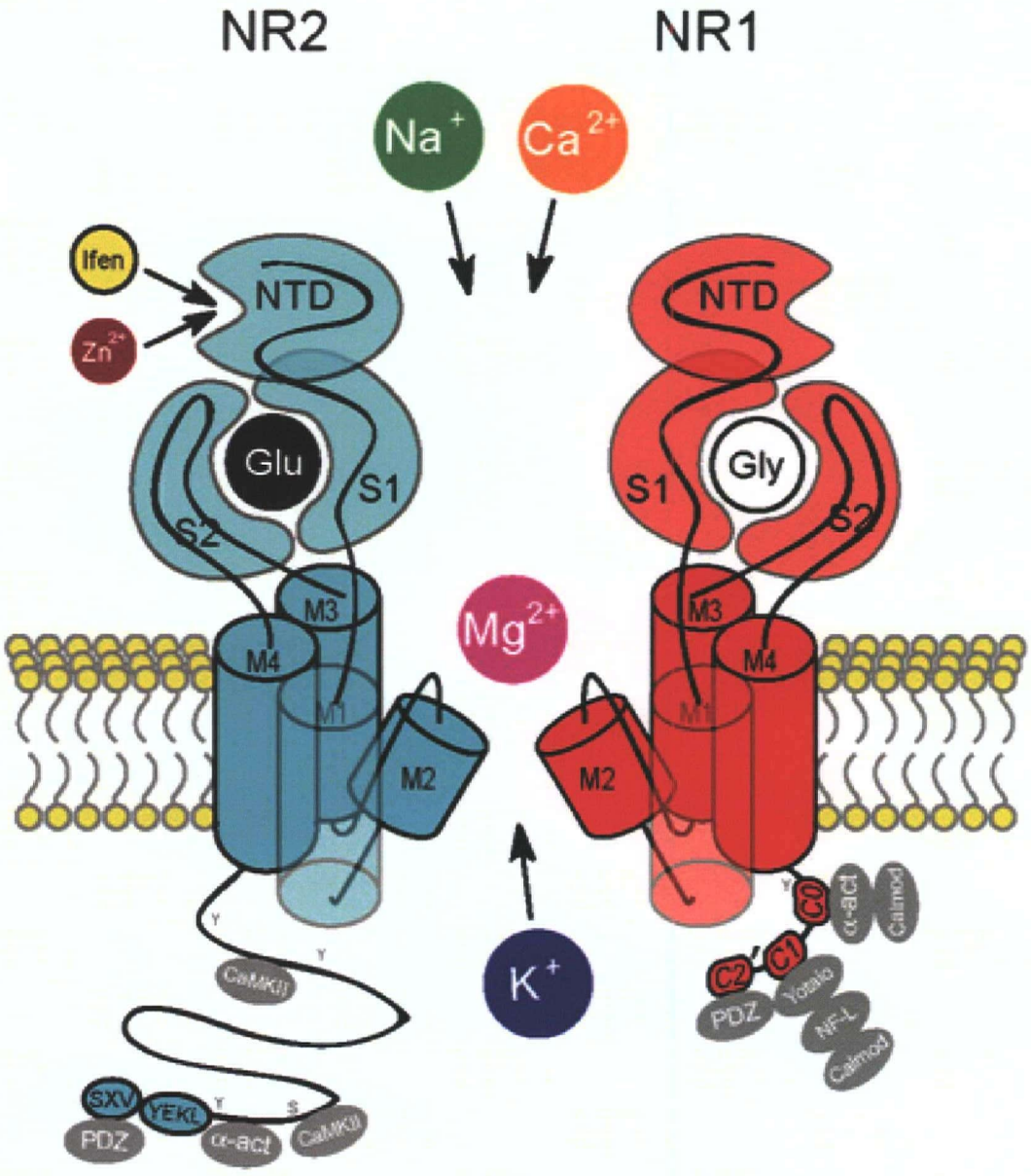
Ionotropic glutamate receptors are ligand-gated ion channels that mediate the majority of excitatory neurotransmission in the central nervous system. Three pharmacologically distinct classes of ionotropic glutamate receptors exist named after partially selective agonists: N-methyl-D-aspartic acid r, α-amino-3-hydroxy-5-methylisoxazole-4- propionic acid (AMPA) and kainate receptors. Cloning of genes encoding individual glutamate receptor subunits identified six gene families. There is one gene family for AMPA receptors – GluR1,2,3,4; two for kainate receptors – GluR5,6,7 and KA-1,2; and three for NMDARs – NR1, NR2A,B,C,D and NR3A,B (Hollmann and Heinemann, 1994; Dingledine et al., 1999). Similarity in sequence suggests a common evolutionary origin for all ionotropic glutamate receptor genes; however, only subunits within the same glutamate receptor type assemble to form functional receptors (Suchanek et al., 1995; Dingledine et al., 1999).

The three ionotropic glutamate receptor classes share similar structural topology. There are three transmembrane domains (M1, M3 and M4) and a re-entrant loop on the cytoplasmic facing membrane -- M2. Preceding the M1 and following the M3 regions are the S1 and S2 domains respectively, which are responsible for agonist binding. The N-terminus is located extracellularly while the C-terminus resides intracellularly (Dingledine et al., 1999, Figure 1-1). Early studies of conductance states suggested that NMDA receptors existed as pentamers of three NR1 and two NR2 subunits (Premkumar and Auerbach, 1997; Dingledine et al., 1999), however, more recent studies propose that glutamate receptors are tetrameric complexes of two NR1 with two NR2, or a mix of one NR2 and one NR3, subunits (Dingledine et al., 1999).

The three classes of receptors display distinct responses in the presence of agonist (Dingledine et al., 1999). NMDA receptors display high affinity for glutamate, which binds to the NR2 subunits, and activation requires the co-agonist glycine, which binds to NR1 subunits (Figure 1-1). These receptors exhibit relatively slow activation, deactivation and desensitization. AMPA and kainate receptors show lower affinity for glutamate, along with faster activation, deactivation and desensitization than NMDA receptors. AMPA receptors generally have low calcium permeability unless they lack the GluR2 subunit (Hollmann et al., 1991; Ozawa, 1998; Dingledine et al., 1999). Kainate receptors show moderate calcium permeability, which is higher than that observed for AMPA receptors with GluR2 subunits. NMDA receptors display high calcium permeability and voltage dependent block by extracellular magnesium. These differences allow for robust responses with the single endogenous agonist glutamate, and the differing properties of these ionotropic receptors permit specialized roles in synaptic transmission. AMPA receptors mediate the fast synaptic response while also providing the depolarization required to alleviate the magnesium block of NMDA receptors. The slow kinetics and high calcium permeability of NMDA receptors are important in regulating synaptic plasticity under physiological conditions, while overactivation can result in excitotoxity (Dingledine et al., 1999).

Figure 1-1. Membrane topology of NMDARs

The S1 and S2 domains forms the binding sites for glutamate in the NR2 subunits and for glycine in the NR1 subunits. The M2 region is a re-entrant pore loop that confers permeability to Na^+, K^+ and Ca^{2+}. Extracellular Mg^{2+} causes voltage-sensitive block of the receptor by residing within the pore. Intracellularly, the C-terminal tail of both the NR1 and NR2 subunits are regulated by phosphorylation sites (Y,S) as well as association with various intracellular proteins denoted in grey. Adapted from Cull-Candy and Leszkiewicz, 2004.



(Cull-Candy and Leszkiewicz, 2004)

1.2. NMDARs in synaptic plasticity

Following high-frequency stimulation of the presynaptic fiber, NMDA receptor activation induces changes in the postsynaptic terminal that ultimately lead to prolonged increases in the efficiency of synaptic transmission. This modification of synaptic efficiency, termed long-term potentiation (LTP), occurs within milliseconds and persists for hours or days (Bliss and Lomo, 1973). During high frequency stimulation, presynaptic glutamate release activates AMPA receptors, which depolarize the postsynaptic membrane to alleviate the Mg^{2+} block of NMDARs allowing calcium to enter the neuron through the highly permeable NMDARs. Conversely, low frequency stimulation has been shown to decrease the efficiency of synaptic transmission in a process termed long-term depression (LTD). The importance of NMDARs in both LTP and LTD is supported by a variety of studies. Specific NMDAR antagonists block the induction of LTP without affecting basal synaptic transmission (Collingridge et al., 1983). The majority of synapses that undergo LTP do so in an NMDAR-dependent manner; however, rises in intracellular calcium induced by alternate sources, such as voltage-gated calcium channels, can mimic LTP (Grover and Teyler, 1990; Malenka and Nicoll, 1999).

A great deal of evidence implicates that alpha -calcium-calmodulin-dependent protein kinase II (α-CaMKII) is a key component in NMDAR-dependent LTP (Malenka and Nicoll, 1999; Malenka and Bear, 2004). Postsynaptic injection of inhibitors of CaMKII or genetic deletion of a critical CaMKII subunit blocks the ability to generate LTP (Malenka et al., 1989). Paradoxically, NMDAR activation through low frequency stimulation can also lead to the induction of LTD at the same synapses that show NMDAR-dependent LTP (Malenka, 1994; Malenka and Bear, 2004). Many of the same requirements of LTP are found in LTD induction:

dependence on activation of AMPARs to alleviate Mg^{2+} blockade, activation of NMDARs, and rises in postsynaptic intracellular calcium levels (Dudek and Bear, 1992; Mulkey and Malenka, 1992; Malenka, 1994; Malenka and Bear, 2004). The critical components that determine whether LTP or LTD is induced are the presynaptic stimulation paradigm and the subsequent postsynaptic intracellular calcium levels. Studies suggest stimuli that induce brief, large increases in intracellular calcium (~3sec, ~10μM) activate CaMKII which leads to LTP, whereas stimulation protocols that induce prolonged, low levels of calcium (~1min, <1μM) preferentially activate the serine-threonine phosphatase calcinuerin, leading to LTD (Dudek and Bear, 1992; Mulkey and Malenka, 1992; Malenka, 1994; Malenka and Bear, 2004).

1.3. NMDAR involvment in neuronal survival and excitotoxicity

Numerous studies indicate that physiological activity of NMDAR activity is critical for neuronal survival. For example, *in vivo* blockade of NMDA receptors for only a few hours during late fetal or early neonatal life causes extensive apoptosis in many brain regions (Ikonomidou et al., 1999). Studies on cultured neurons suggest a neuroprotective role of synaptic NMDAR activity. Blockade of receptors in spinal cord cultures results in increased neuronal death caused by elimination of synaptic NMDAR activity (Brenneman et al., 1990).

The mechanisms underlying synaptic NMDAR-induced neuroprotection are currently being investigated. Sub-toxic levels of agonist *in vitro* have been shown to mediate phosphorylation and subsequent inactivation of the pro-apoptotic protein BAD by both calcium-mediated activation of CaMKII and activation of the Ras-ERK1/2 pathway (Yano et al., 1998; Bonni et al., 1999; Brunet et al., 2001). Furthermore, synaptic activation of NMDARs *in vivo* results in alterations in gene expression that promote cell survival. Synaptic receptor stimulation, in both *in vivo* and *in vitro* systems, results in activation of the cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) which in turn increases the

transcription of several immediate early genes involved in neuronal survival (Hardingham and Bading, 2003).

Although activation of NMDARs is critical for neuronal survival during development, NMDA receptors can cause cell death when intensely or chronically activated (Lipton and Rosenberg, 1994). This overactivation of NMDARs has been implicated in the etiology of many chronic neurodegenerative disorders, such as Huntington's disease, HIV-associated dementia and Alzheimer's disease (Fan and Raymond 2007 – Progress in Neurobiol – Epub ahead of print; Lipton and Rosenberg, 1994; Lancelot and Beal, 1998; Kaul et al., 2001). Calcium entry through NMDARs activates multiple downstream pathways linked to cell death, including mitochondrial dysfunction, mitogen-activated protein kinase (MAPK) signaling, as well as calcium-activated kinases, phosphatases, and proteases (Lynch and Guttmann, 2002; Waxman and Lynch, 2005).

Interestingly, NMDAR-mediated excitotoxicity appears to exhibit subunit specificity. For example, heterologous cells transfected with recombinant NMDARs show highest levels of death when expressing NR1/NR2A, followed by NR1/NR2B and finally NR1/NR2C (Waxman and Lynch, 2005). This trend is also seen developmentally in cultured neurons. As NR2B levels decrease and NR2A levels increase in neuronal development, toxicity due to NMDAR activation also increases (Waxman and Lynch, 2005). However, recent studies in acute hippocampal slices show increased NMDAR-mediated toxicity in slices from young rats compared to older animals. The authors conclude that the majority of the NMDA-mediated toxicity is caused by activation of NR2B- but not NR2A-containing NMDA receptors leading to calpain activation (Zhou and Baudry, 2006). This seemingly contradictory report may be reconciled in part by the differences in downstream signaling due to subunit dependence and subcellular localization. NR2B-containing receptors predominate at extrasynaptic sites later in development and are implicated in NMDA receptor-dependent CREB shut-off signaling pathways (Li et al., 1998; Hardingham and Bading, 2002; Hardingham et al., 2002).

1.4. NMDAR structural determinants of function

1.4.1. NMDAR subunit composition

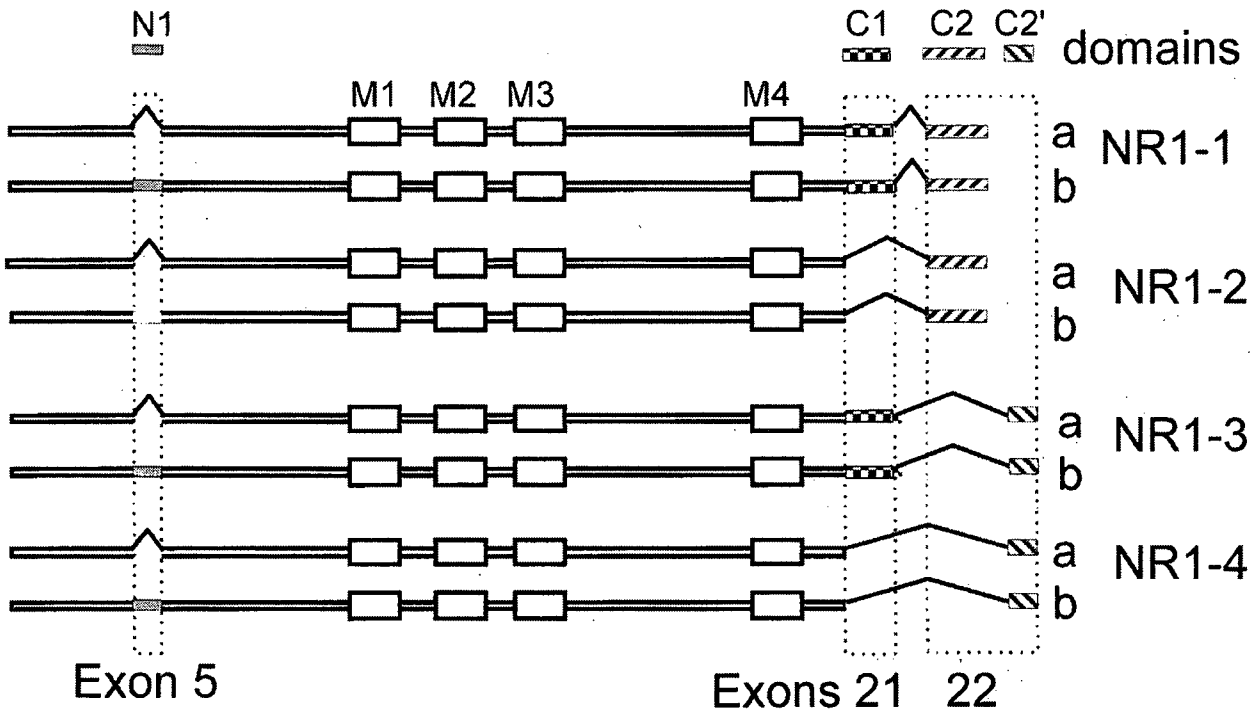
NMDARs are heteromeric protein complexes incorporating 3 types of subunits: NR1, NR2 and NR3. The NR1 subunit is essential in forming a functional channel and is encoded by a single gene with three alternatively spliced exons (5, 21, 22) giving rise to 8 possible isoforms (Figure 1-2). There are four possible NR2 subunits (A, B, C, and D) which are encoded by 4 different genes (Dingledine et al., 1999). NR2 subunits are important in regulating channel properties (Dingledine et al., 1999). NR3 A and B subunits are encoded by two separate genes (Chatterton et al., 2002). Expression of functional recombinant NMDARs in mammalian cells requires the co-expression of NR1 and NR2 subunits. The stoichiometry of NMDARs has not yet been established definitely, but the consensus is that NMDARs are tetramers that most often incorporate two NR1 and two of the same or different NR2 subunits (referred to as diheteromers or triheteromers, respectively) (Dingledine et al., 1999).

Neurons express distinct complements of subunits, which are temporally and spatially regulated. There is evidence for expression of NMDARs that are triheteromeric combinations of NR1 with NR2A and NR2B in cerebellar granule cells and hippocampal pyramidal neurons, NR1 with NR2A and NR2C in cerebellar granule cells, and NR1 with NR2B and NR2D in cerebellar Golgi and stellate cells (Cull-Candy and Leszkiewicz, 2004). The role of NR3 subunits is still under investigation; however, recent evidence suggests NR3 may modulate receptor activity by forming triheteromeric receptors that display decreased NMDAR unitary conductance, decreased Ca^{2+} permeability, reduced Mg^{2+} sensitivity, and slightly increased mean open time compared with NR1/NR2 channels (Ciabarra et al., 1995; Das et al., 1998; Dingledine et al., 1999; Sasaki et al., 2002).

NMDA receptors require binding of both glutamate and glycine to activate; there is one site on the NR1 subunit that binds glycine as a full agonist and D-serine as a partial agonist, and another site on the NR2 subunit that binds glutamate as a full agonist and NMDA as a partial agonist (Dingledine et al., 1999; Gibb, 2004) (Figure 1-1). NMDARs show negative cooperativity between binding of glutamate and glycine, which manifests as glycine-depedent desensitization. However, expression of NR1 together with NR3 A or B subunits results in the formation of excitatory receptors that utilize only glycine as an agonist and are unaffected by glutamate or NMDA; these receptors are inhibited by D-serine, a co-activator of conventional NMDARs (Chatterton et al., 2002).

Figure 1-2. Alternative splice variants of NMDAR NR1 subunit

Alternative splicing of exons 5, 21, and 22 gives arise to 8 possible isoforms which contain different combinations of the N1, C1, C2, and C2' cassettes. From Dingledine et al., 1999



(Dingledine, et al., 1999)

1.4.2. NMDAR subunit composition and spatial-temporal distribution in the CNS

The eight splice variants of NR1 show regionally specific expression and do not exhibit much change during development except in the cerebellum (Laurie and Seeburg, 1994; Laurie et al., 1995; Prybylowski and Wolfe, 2000). In the developing rat brain, NR1 mRNA is barely detectable at embryonic day 14, but increases gradually during development until the third postnatal week, after which it declines slightly to adult levels (Laurie and Seeburg, 1994). The NR1-1 and NR1-2 variants which lack the C2' cassette (see Figure 1-2), are widely and abundantly distributed throughout the brain, except for the inferior colliculus (Laurie et al., 1995). NR1-1b is highly expressed in the sensorimotor cortex, neonatal lateral caudate, thalamus, hippocampal CA3 field, and cerebellar granule cells, but is absent from adult caudate. The NR1-1 and NR1-4 splice variants display complimentary patterns; the NR1-4 splice forms are present in caudal regions such as the thalamus, colliculi and cerebullum whereas the NR1-1 variant is present in rostral structures such as cortex, caudate and hippocampus (Laurie and Seeburg, 1994). The NR1-3 mRNA variant is sparsely expressed in the central nervous system, where very low levels exist in postnatal cortex and hippocampus (Laurie and Seeburg, 1994).

The four NR2 transcripts display distinct expression patterns (Cull-Candy et al., 2001; Cull-Candy and Leszkiewicz, 2004). The NR2A mRNA is distributed widely in the brain but predominates in the cerebral cortex, hippocampus, and cerebellum. In the adult brain, the NR2B subunit is selectively present in the forebrain, where it is highly expressed in the cerebral cortex, hippocampus, septum, caudate-putamen and olfactory bulb. NR2C mRNA is expressed predominantly in the granule cell layer of the cerebellum although lower levels are also detected in the olfactory bulb and thalamus. NR2D transcripts are detected at low levels in the thalamus, brain stem and olfactory bulb. In hippocampal neurons, NR2A and NR2B mRNAs are

prominent however NR2C and NR2D transcripts are also found in subsets of interneurons (Monyer et al., 1994; Ozawa et al., 1998).

The expression of NR2 subunits is also developmentally regulated. In the developing rat brain, NR2B and NR2D mRNAs occur prenatally, whereas NR2A and NR2C mRNAs are first detected near birth. Expression of all NR2 subunit mRNA peaks around postnatal day 20 except NR2D. NR2D mRNA is present predominantly in midbrain structures and peaks around P7, then decreases to adult levels (Monyer et al., 1994). *In vitro* studies have shown a developmental shift in subunit expression in hippocampal neurons. Hippocampal neurons tend to express NR2B subunits early in development; these NR2B-containing receptors are present at the earliest synapses, but predominate at non-synaptic. As the neurons mature, NR2A is incorporated into synapses to form homomers of NR1/2A receptors or triheteromeric NR1/2A/2B receptors whereas homomeric NR1/2B receptors predominate at extrasynaptic sites (Williams et al., 1993; Zhong et al., 1994; Li et al., 1998; Rao et al., 1998; Stocca and Vicini, 1998; Tovar and Westbrook, 1999; Li et al., 2002; Li et al., 2003). However, NR2A-containing receptors can still be found at non-synaptic sites and NR1/NR2B at synapses, and definite determination of the subcellular distribution of NMDAR subunits is complicated by the limitations of the available pharmacological tools (Vicini et al., 1998; Tovar and Westbrook, 1999; Thomas et al., 2006).

1.4.3. NMDAR regulation by NR1 subunits

The NR1 splice variants modulate NMDAR properties. The pH sensitivity of NMDARs is determined by the presence of exon 5; at physiological pH NR1 subunits that contain exon 5 are fully active while NR1 subunits lacking exon 5 are partially blocked. NR1 subunits containing exon 5 also display no potentiation by polyamines or voltage-dependent block by zinc (Traynelis et al., 1995). However these effects are also dependent on the identity of the NR2 subunits present in the NMDAR complex. Assembly of NR1 splice variants that lack exon 5

with NR2C or NR2D subunits produces NMDARs that are insensitive to zinc blockade or proton inhibition (Paoletti et al., 1997; Traynelis et al., 1998). Exon 5 of NR1 can also regulate the deactivation of NMDARs containing NR2B subunits. The presence of exon 5 increases the deactivation rate of recombinant NR1/2B receptors, whereas exon 5 has no effect on NR1/2A receptors (Vicini et al., 1998; Rumbaugh et al., 2000).

The NR1 subunit also plays a critical role in the trafficking of NMDARs from the ER to the surface membrane. NR2A and NR2B subunits are unable to reach the cell surface unless assembled with NR1, yet most splice variants of NR1 can reach the cell surface without NR2 subunits present (Fukaya et al., 2003). The trafficking of NR1 subunits from the ER is regulated by splice variants. ER export is accelerated by NR1 subunits containing the C2' domain and slowed by NR1 subunits expressing the C2 cassette (Mu et al., 2003). Moreover, the process of forward trafficking from the ER to the cell surface is tightly regulated by coordinated PKA and PKC phosphorylation of the sites flanking the RXR motif within the C1 cassette of NR1 subunits (Scott et al., 2001; Scott et al., 2003). Additionally, NR1 splice variants display selective targeting to specific cell regions (Mu et al., 2003).

1.4.4. NMDAR regulation by NR2 subunits

Many of the subunit-dependent properties of NMDARs are controlled by the NR2 subunit. NR2 subunits have been shown to modulate agonist affinity, NMDAR single-channel conductance, channel open probability, sensitivity to zinc inhibition, block by extracellular Mg2+, and channel kinetics (Dingledine et al., 1999; Cull-Candy et al., 2001; Cull-Candy and Leszkiewicz, 2004). Diheteromeric NR1/NR2A receptors show lower sensitivity for glutamate compared to NR1/NR2B receptors but display a higher open probability. NR2A- or NR2B-containing NMDARs generate 'high-conductance' channel openings with a high sensitivity to block by Mg^{2+}, whereas NR2C- or NR2D-containing receptors give rise to 'low-conductance'

openings with a lower sensitivity to extracellular Mg^{2+} (Dingledine et al., 1999; Cull-Candy et al., 2001; Cull-Candy and Leszkiewicz, 2004). Further differences in NR2 subunits with regard to agonist affinity and various forms of desensitization are discussed later.

There is much evidence suggesting populations of NMDARs may exist as two NR1 subunits and two different NR2 subunits to produce a functionally distinct triheteromeric receptor complex (Vicini et al., 1998; Tovar and Westbrook, 1999). Transfection of NR1, NR2A and NR2B subunits into recombinant systems results in receptors that display reduced ifenprodil sensitivity, distinguishing them from homomeric NR1/NR2B receptors that are inhibited by low micromolar concentrations of ifenprodil and infenprodil-insensitive NR1/NR2A receptors (Tovar and Westbrook, 1999). The presence of triheteromeric receptors that exhibit distinct functional and pharamacologial properties increases the diversity of signaling available in the central nervous system.

1.4.5. NR3 Subunits

The recently identified NR3 subunits (A and B) co-assemble with NR1 to form diheteromeric functional excitatory glycine receptors that are unaffected by glutamate or NMDA, inhibited by D-serine, relatively calcium impermeable, and also insensitive to magnesium block, the pore blocker MK-801 or competitive antagonist memantine (Chatterton et al., 2002). Triheteromeric NR1/NR2/NR3 receptors exist early in development and regulate NMDARs by decreasing unitary conductance. Knockout mice lacking the NR3A gene display enhanced NMDA responses and increased dendritic spines in early postnatal cerebrocortical neurons (Das et al., 1998).

1.5. NMDAR regulation by serine/threonine phosphorylation

Most phosphorylation sites on glutamate receptors have been shown to be on the intracellular C-terminal domain (Swope et al., 1999). NMDARs can be phosphorylated by PKA, PKC, casein kinase-2 (CK2) and CaMKII. The balance of phosphorylated and unphosphorylated states is maintained by the aforementioned kinases' activity and the calcium/calmodulin-dependent phosphatase calcineurin.

Twelve isoforms of PKC have been discovered and are categorized into three structurally and functionally distinct groups. Conventional cPKC isoforms (PKCα, PKCβ and PKCγ) are diacylglycerol sensitive and responsive to calcium. The novel nPKC isoforms (PKCδ, PKCε, PKCη and PKCθ) are sensitive to diacylglycerol but calcium insensitive. The atypical aPKC isoforms (PKCζ and PKCι/λ) are insensitive to both calcium and diacylglcerol, and are regulated by the PB1 domain of their N-terminus (Battaini, 2001; Parker and Murray-Rust, 2004).

PKC activity has varied and profound effects on NMDAR function. PKC phosphorylation has been shown to increase open probability, decrease the external magnesium block of NMDARs, inhibit clustering of receptors, regulate release of receptors from the endoplasmic reticulum and promote surface expression (Chen and Huang, 1992; Kelso et al., 1992; Ehlers et al., 1995; Tingley et al., 1997; Dingledine et al., 1999; Lu et al., 1999; Zheng et al., 1999; Lan et al., 2001; Scott et al., 2001; Scott et al., 2003). Various metabotropic receptors have been shown to potentiate NMDAR current through PKC activation (Markram and Segal, 1990; Aniksztejn et al., 1992; Chen and Huang, 1992; Dildy-Mayfield and Harris, 1994). However, PKC augmentation of NMDAR current does not completely depend on phosphorylation of the receptor itself as experiments involving NMDARs assembled from subunits lacking all known sites of PKC phosphorylation can still exhibit PKC potentiation (Zheng et al., 1999). These data suggest that PKC-induced potentiation of NMDAR activity does not occur by direct phosphorylation of the receptor protein but rather through downstream

signaling. In support of this, PKC potentiation of NMDAR current is absent in neurons from knockout mice lacking src kinase, suggesting that the effect of PKC is through tyrosine phosphorylation (Lu et al., 1999).

Trafficking and clustering of NMDARs subunits are heavily regulated by PKC activity. PKC activation and subsequent phosphorylation of Ser890 of the C1 cassette of NR1 can inhibit clustering of NR1 (Ehlers et al., 1995; Tingley et al., 1997). NR1 subunit phosphorylation also regulates forward trafficking of NMDARs from the ER; PKC phosphorylation sites flanking the RXR motif of NR1 subunits expressing the C1 cassette suppress ER retention (Scott et al., 2001; Scott et al., 2003). As well, activation of PKC allows for the rapid delivery of NMDARs from vesicular, plasma membrane-proximal pools in a SNARE-dependent, exocytotic manner resulting in increased surface expression (Lan et al., 2001).

Compared to the work on PKC modulation of NMDARs, little is known about PKA, CK2, and CAMKII phosphorylation of NMDARs. The NR1 C1 cassette contains a PKA phosphorylation site that is involved in forward trafficking from the ER. Coordinate phosphorylation of sites flanking the RXR motif of NR1 promotes release of receptors from the ER for trafficking to the plasma membrane. In hippocampal neurons PKA activation by β-adrenergic receptors potentiated NMDAR activity indirectly through calcineurin inhibition (Raman et al., 1996). CK2 activity can potentiate NMDAR responses by increasing NMDAR channel open probability, although whether these effects are through direct phosphorylation of NMDARs has yet to be determined (Lieberman and Mody, 1999). On the other hand, CK2 phosphorylation of site 1480 in NR2B reduces interaction with PSD-95 and other PDZ domain-containing proteins (see next section), decreasing NMDAR surface expression (Chung et al., 2004).

Along with kinase activity, the level of serine/threonine phosphorylation is also controlled by activity of phosphatases PP1, PP2A or PP2B. Inhibition of PP1 and/or PP2A with

the use of the specific inhibitor calyculin A occluded the PKA-mediated potentiation of striatal NMDAR current (Blank et al., 1997). Calcineurin activity has been shown to down-regulate NMDAR activity through desensitization at the C-terminus of the NR2A subunit in a dephosphorylation dependent manner (Tong et al., 1995; Krupp et al., 2002).

1.6. NMDAR regulation by MAGUKs

The NR2 subunits of NMDARs contain intracellular C-terminal domains that are critical for signaling and anchoring of NMDARs. One crucial feature of the NR2 C-terminus in regulating NMDAR function is the terminal, conserved sequence ESDV of NR2A and NR2B, and ESEV for NR2C and NR2D. These sequences are known ligands for PSD-95/Dlg/ZO-1 (PDZ) domains which are modular protein interaction domains specialized for binding to short peptide motifs in over 400 proteins in mice and humans (Kim and Sheng, 2004). Typically, the PDZ scaffold and its associated multiprotein complex are targeted to a specific subcellular site to perform a specialized local function. The PDZ domain was originally recognized in three proteins, postsynaptic density 95kDa (PSD-95), ZO-1 and Dlg that are all members of a superfamily of membrane associated guanylate kinases (MAGUKs). The PSD-95 family of MAGUKs includes PSD-93/Chapsyn-110, synapse associated protein 102 kDa (SAP-102), and synapse associated protein 102 kDa (SAP-97). These proteins are roughly 70% homologous to each other, and all contain three N-terminal PDZ domains, an SH3 domain and a C-terminal guanylate kinase-like domain (Kim and Sheng, 2004).

1.6.1. SAP-102

Studies from intact brains early in development suggest expression of PSD-95 and PSD-93 are low in the hippocampus whereas SAP-102 is highly expressed at synapses (Sans et al., 2000). The high level of SAP-102 at immature synapses coincides with the early developmental

expression of the NR2B subunit, whereas the later expression of NR2A coincides with the prevalence of PSD-93 and PSD-95 at synaptic sites. Furthermore, co-immunoprecipitation studies suggest a preference for NR2A-containing receptors in complexes with PSD-95 or PSD-93 whereas SAP-102 is largely associated with NR2B-containing receptors (Sans et al., 2000). In support of SAP-102's role in developing synapses, acute knockdown in immature hippocampal slices resulted in a 50% reduction of AMPAR EPSCs while acute knockdown in mature slices caused no noticeable alterations in synaptic transmission (Elias et al., 2006). SAP-102 may also play a role in trafficking NMDARs from the endoplasmic reticulum and golgi apparatus to the synapse in a PDZ dependent manner as a part of an exocyst complex (Sans et al., 2003).

1.6.2. PSD-95

PSD-95 is the best characterized of the MAGUK family of proteins and has been shown to have varied effects in synaptic regulation. Deletion mutagenesis studies established that the N-terminal region of PSD-95, containing palmitoylated Cys-3 and Cys-5, regulates both membrane targeting and multimerization (Craven et al., 1999; Hsueh and Sheng, 1999; Christopherson et al., 2003). Expression of PSD-95 in non-neuronal cell lines has been shown to cluster Shaker type K^+ channels and reduces the single-channel conductance of the Kir2.3 inward rectifier K^+ channnel (Kim et al., 1995; Kim and Sheng, 1996; Nehring et al., 2000). PSD-95 regulates AMPA receptor clustering in neurons although it is not required for proper NMDA receptor trafficking and clustering (El-Husseini et al., 2000). Evidence suggests PSD-95 is involved in surface delivery of NMDARs, expression of NMDARs, and stabilization of NR2B-containing receptors at synaptic sites (Lin et al., 2004; Prybylowski et al., 2005). Additionally, PSD-95 has been implicated in regulating glycine-independent desensitization; overexpression of PSD-95 in immature neurons results in a decrease in desensitization, and

conditions that shift either PSD-95 or NMDA receptors away from synaptic sites in mature neurons result in increased NMDAR desensitization (Li et al., 2003).

Despite the large body of evidence suggesting a role for PSD-95 in regulating NMDAR receptor function, overexpression of PSD-95 selectively enhances AMPAR mediated EPSCs while NMDAR-mediated EPSCs remain unaltered (Schnell et al., 2002; Rumbaugh et al., 2003; Elias et al., 2006). Furthermore, PSD-95 knockout mice show no alterations in synaptic transmission (Elias et al., 2006). These surprising results may partly be explained by functional redundancy and compensation by other members of the MAGUK family of proteins; acute knockdown of PSD-95 results in a reduction of AMPAR mEPSC frequency similar to the PSD-95/PSD-93 double knockout mice (Elias et al., 2006).

Problems arise when isolating whether the effects of PSD-95 are through a direct interaction or are secondary to its role as a scaffolding molecule. Figure 1-3 displays a non-exhaustive list of PSD-95 binding partners and to which domains they interact with. The complexity that arises in the neuronal post-synaptic density from the wide network of molecules PSD-95 can recruit and interact with causes difficulty in directly assessing the role of PSD-95 in a neuronal preparation (Figure 1-4).

Figure 1-3. Table of proteins that interact with PSD-95

By binding polypeptides through its various domains, PSD-95 can assemble large macromolecular complexes. From Kim and Sheng, 2004.

Table 1 | **Proteins that interact with PSD-95 family scaffolds**

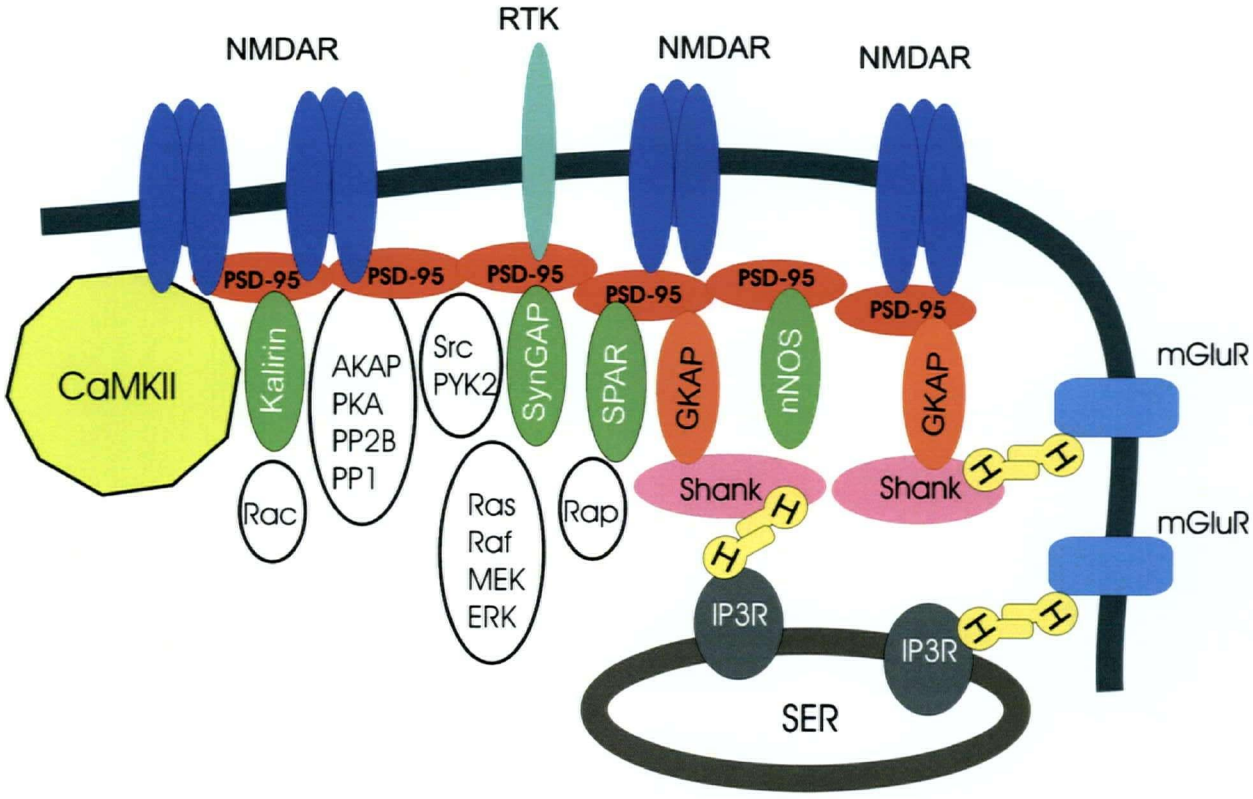
Interacting protein	Comments on the interacting proteins	References
PDZ domains		
NR2A–D	Subunits of NMDA receptors	131
GluR6	Subunit of kainate receptors	132
δ2 GluR	Subunit of δ-ionotropic glutamate receptors	133
β1-adrenergic receptor	G-protein-coupled receptor	25
nAChRc	Subunit of neuronal nicotinic acetylcholine receptor	76,77
5-HT2A and 5-HT2C Rc	Subunits of 5-HT (serotonin) receptors	134
ErbB4	A receptor tyrosine kinase for neuregulin	135,136
Kv1	Voltage-gated potassium channel	14
Kir2, Kir3, Kir4 and Kir5	Inward-rectifying potassium channels	32,124
Neuroligin	A postsynaptic membrane protein that binds to β-neurexins and regulates synaptic adhesion and development	26,28,30
Stargazin family proteins	Tetra-spanning transmembrane proteins required for surface and synaptic expression of AMPA receptors	64
nNOS	Neuronal nitric oxide synthase	3,33
SynGAP	An abundant RasGAP of the PSD that regulates synaptic plasticity	35,36,39,40
Kalirin-7	A guanine nucleotide exchange factor for Rac1 that regulates spine morphogenesis	53
Fyn, Lyn, Src and Yes	Src family non-receptor protein tyrosine kinases; might also interact with the SH3 domain of PSD-95	48,49
Cypin	A cytosolic protein that regulates dendrite patterning by promoting microtubule assembly	137
CRIPT	A microtubule-binding protein	18
Sec8	A subunit of the exocyst complex involved in protein and vesicle trafficking	115
KIF1Bα	A motor of the kinesin superfamily	111
SH3 domain		
Pyk2	A non-receptor tyrosine kinase regulated by calcium and PKC and required for LTP induction	50,138
GK domain		
GKAP/SAPAP	An abundant multi-domain scaffold of PSD that links PSD-95 with Shank	139
SPAR	A postsynaptic RapGAP that regulates spine morphogenesis	57
SH3 and GK domains		
KA2 GluR	Subunit of kainate receptors	132
AKAP79/150	An anchoring protein that binds to protein kinase A and protein phosphatase 1	41
L27 domain		
CASK	Mammalian homologue of LIN2	140,141
Myosin VI	A minus-end-directed actin-based motor	113

Only proteins that interact directly with PSD-95 family scaffolds are listed. These interactions might not apply to all members of the PSD-95 family. Owing to space limitations, this list is not comprehensive and not all relevant references are cited. AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; GK, guanylate kinase-like domain; LTP, long-term potentiation; NMDA, *N*-methyl-D-aspartate; PKC, protein kinase C; PSD-95, postsynaptic density protein 95; Rac, Rap and Ras, small monomeric G-proteins; RapGAP, Rap GTPase-activating protein; RasGAP, Ras GTPase-activating protein; SH3 domain, Src homology 3 domain; Shank, SH3 and ankyrin repeat-containing protein.

(Kim and Sheng, 2004)

Figure 1-4. Schematic representation of NMDAR-associated protein complex

Schematic representation of NMDAR-associated protein complex. The cytoplasmic tails of NMDARs also bind directly to cytoskeletal and signaling proteins, including α-actinin, calmodulin and PSD-95. PSD-95 interacts with a multitude of cytoplasmic signaling molecules thereby connecting NMDARs to multiple signal transduction pathways. From Sheng and Kim, 2002.



(Sheng and Kim, 2002)

1.6.3. SAP-97 and PSD-93

Much less is known about SAP-97 or PSD-93 in the regulation of receptor function. SAP-97 has been found to be present in both axons and postsynaptically at excitatory synapses (Muller et al., 1995; Valtschanoff et al., 2000; Aoki et al., 2001; Rumbaugh et al., 2003). The differential targeting of SAP-97 is accomplished through splice variations; SAP-97 expressing the I3 splice insert target preferentially to postsynaptic spines whereas splice forms lacking this motif are localized diffusely (Rumbaugh et al., 2003). Overexpression of synaptically targeted SAP-97 in neurons results in altered spine morphology, increased surface AMPA receptors, and increased AMPAR mEPSC frequency (Rumbaugh et al., 2003). SAP-97 is unique in that it is the only MAGUK to bind directly to AMPAR through a PDZ interaction with the GluR1 subunit (Leonard et al., 1998).

PSD-93 is homologous to the previously described MAGUKs but displays some unique characterisitics. PSD-93 is largely localized to post-synaptic densities although it has been detected utilizing electron microscopy diffusely on pre-synaptic membranes, in the cytoplasm of axons and in dendritic shafts (Sans et al., 2000; Aoki et al., 2001). Although PSD-93 is N-terminally palmitoylated, it does not require palmitoylation for membrane targeting (Firestein et al., 2000). Overexpression of PSD-93 results in an increase in AMPAR-mediated evoked EPSC amplitude while leaving NMDAR-mediated EPSCs unaffected in cultured hippocampal neurons (Schnell et al., 2002; Elias et al., 2006). However, PSD-93 knockout mice show decreased surface expression of NR2A and NR2B subunits, reduced NMDAR-mediated mEPSCs and attenuated evoked NMDAR-dependent EPSCs without altering AMPAR surface expression or synaptic function in neurons from the spinal dorsal horn and forebrain (Tao et al., 2003).

1.7. NMDAR regulation by glycine-dependent desensitization

In the continuous presence of glutamate (or NMDA) and low concentrations of co-agonist glycine, NMDAR current responses diminish over time. This form of desensitization is indicative of the negative allosteric coupling between the glutamate binding sites of the NR2 subunits and the glycine binding sites on the NR1 subunits, and manifests as a decrease in glycine affinity. This form of desensitization can be overcome by high glycine concentrations, on the order of 50 μM (Mayer et al., 1989; McBain and Mayer, 1994; Dingledine et al., 1999; Gibb, 2004).

1.8. NMDAR regulation by calcium-dependent inactivation

Intracellular calcium can reversibly inhibit NMDAR receptor activity through the process of calcium-dependent inactivation (CDI) (Rosenmund and Westbrook, 1993). CDI is sensitive to influx of calcium through the NMDAR and it can be abolished by high intracellular calcium buffering (Rosenmund and Westbrook, 1993). Recombinant NMDA receptor studies have shown CDI to be NR2 subunit dependent; inactivation of recombinant NMDA receptors requires either the NR2A or NR2D subunits, whereas inactivation of diheteromeric NR1/NR2B or NR1/NR2C receptors does not occur (Krupp et al., 1996). The mechanism for CDI relies on the calcium binding protein calmodulin, which interacts with the NR1 subunit near the membrane-proximal region (also called "C0") when bound to calcium (Wyszynski et al., 1997; Zhang et al., 1998). The inactivating effect of calmodulin is prevented by overexpressing a region of the cytoskeletal protein α-actinin known to interact with the C0 region of NR1 (Zhang et al., 1998). α-actinin anchors NMDARs to F-actin, which when depolymerized, has been shown to inhibit NMDAR channel activity in a calcium- and ATP-dependent manner (Rosenmund and Westbrook, 1993; Zhang et al., 1998). Furthermore, calcium can bind directly to α-actinin and decrease its affinity for NR1 binding (Krupp et al., 1999). Together, these data suggests that the

displacement of α-actinin by calcium/calmodulin leads to CDI by disrupting NMDAR anchoring to the actin cytoskeleton. This process can be modulated by second messenger systems, PKC activity, and calcineurin activity, and is thought to be important in regulating synaptic transmission (Tong et al., 1995; Dingledine et al., 1999; Lu et al., 2000).

1.9. NMDAR regulation by glycine-independent desensitization

Glycine-independent desensitization is characterized by the decay in NMDAR current during sustained, saturating concentrations of both agonists. Glycine-independent desensitization is NR2 subtype-dependent; NR2A and NR2B subtypes exhibit this form of desensitization whereas NR2C and NR2D subtypes do not (Villarroel et al., 1998). The structural determinants of glycine-independent desensitization were proposed to be located on the N terminus of the NR2 subunits. It has been suggested that two regions in particular of the NR2 subunits are critical for two distinct components of glycine-independent desensitization. Mutations of the pre-M1 region of NR2A selectively eliminated the slow component of desensitization while substitution of the amino terminal domain (ATD) of NR2A with the corresponding region of NR2C eliminated the fast component of glycine-independent desensitization (Krupp et al., 1998; Villarroel et al., 1998). However, recent studies suggest these two domains, are involved in zinc-dependent desensitization and not glycine-independent desensitization. The fast component of desensitization of NMDARs, previously ascribed to glycine-independent desensitization, is caused by a negative allosteric interaction between the zinc binding site in the ATD and the glutamate binding site in the S1/S2 domain rather than true glycine-independent desensitization (Zheng et al., 2001). In a follow-up study, alterations in either the pre-M1 or ATD sites were found to be critical for the component of desensitization that is caused by ambient zinc acting at the extracellular, high affinity site of the NR2A subunit. However, mutation of the lurcher motif, located near the extracellular end of the M3 region, of

either NR1 or NR2 subunits reduces glycine-independent desensitization as well as proton sensitivity and the deactivation rate (Hu and Zheng, 2005). The role of the lurcher motif in desensitization may be a common feature of all ionotropic glutamate receptors; recent studies have shown mutations in analogous regions of the GluR1 AMPA receptor subunit alter the desensitization properties as well (Klein and Howe, 2004).

1.10. Hypothesis

The extent of glycine-independent NMDAR desensitization significantly decreases over the course of neuronal development when comparing NMDAR current recorded from cultured immature neurons (4-7 days *in vitro*) to that in mature neurons (14-23 days *in vitro*) (Li et al 2003). Several lines of evidence indicate that the decrease in desensitization with development is correlated with the localization of NMDARs to the synapse and their association with PSD-95 (Li et al 2003). Over-expression of PSD-95 in immature neurons decreased the amount of NMDAR desensitization. Chronic treatment of mature neurons with picrotoxin, which causes chronic NMDAR activation by decreasing inhibitory input, leads to a decreased number of synaptic NMDARs while PSD-95 distribution remains unaffected (Crump et al., 2001). Treatment of mature neurons with 2-bromopalmitate results in PSD-95 dispersion from the synapse by inhibiting palmitoylation while NMDA receptor clustering remains intact. In both conditions, NMDAR desensitization in mature neurons increased to levels found in immature neurons (Li et al., 2003). However, it is not clear whether the effect of PSD-95 is secondary to its role as a scaffolding protein, anchoring protein kinases and phosphatases in close proximity to NMDARs, or if PSD-95 binding to NMDARs could directly alter receptor desensitization. In this thesis, I test the hypothesis that direct binding of NMDARs to PSD-95 and not synaptic localization is important in regulating glycine-independent desensitization.

CHAPTER II

Materials and Methods

2.1. Neuronal cell culture and transfection

2.1.1. Culture

Embryonic hippocampal culture was 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): 50μg/ml.
2. Eagle's balanced salt solution (EBSS) (1 L): EBSS salts (Sigma E-6132) 8.7 g, glucose (Sigma G-5767) 2.01g, 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 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. 2-mercapto-ethanol (BME, Sigma, M-7522) stock (10 ml): BME 7.2 μl, Neurobasal medium10 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. ethylenediaminetetraacetic acid (EDTA) stock (50 mM)

d. Phosphate buffered solution (PBS

e. EBSS

f. 10/10

g. DNAase (5mg/ml in EBSS, stored at -20 °C)

h. Plating medium (keep no longer than 14 days)

2. The plates (24-well-plate, with coverslips in it) were coated with 50μg/ml PDL overnight. 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 a couple of hours.

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 one65-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 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 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 by (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 resuspend 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 104.

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/mm2 (2.26 X 105 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/ mm2 (0.57 X 105 cells/ml) (Brewer et al., 1993; Brewer, 1995). For my experiments, the cells were plated at a density of about 300-400 cells / mm2.

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

2.1.2. Neuronal transfection

Neurons were transfected with 1.2ug of DNA per 24 well of either PSD-95-green fluorescent protein (GFP), PSD-95 PDZ1-2 GFP or GFP alone, using a calcium phosphate kit (Clontech, Mountain View, CA). Briefly, neurons were transfected at 4 DIV and were used for patch-clamp recording 2-3 d after transfection. The detailed procedure follows for transfection of 4 wells in a 24 well plate:

Solutions:

1. 2M calcium solution (Clontech)
2. 2X Hepes-buffered saline (HBS, Clontech)

Procedure

1. Add 8μg of DNA along with 12.4 μL of 2M calcium solution and add sterile water to make 100μL of final volume
2. Add DNA solution to 100μL 2X HBS while vortexing gently
3. Leave solutions at room temperature for 10 minutes
4. Add 50μL of solution drop-wise into each well while rocking gently into 500μL of conditioned media

5. Incubate at 37°, 5% CO2 for 10 minutes

6. Wash with and replace wells with fresh media

2.2. HEK293 cell culture and transfection

2.2.1. Culture

Human embryonic kidney (HEK) 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.

2.2.2. Transfection

Cells were transfected according to the method of calcium phosphate precipitation, as previously described (Chen and Okayama, 1988; Li et al., 2003). Briefly, a total of 12 µg of plasmid DNA was used for each 10-cm plate. Cells were transfected with a 1:1:2 ratio of cDNAs encoding NR1-1a, NR2 (A or B), and either GFP, PSD-95-GFP, PSD-95 PDZ1-2-GFP, GAP14 PDZ1-2-GFP, Prenylated PSD-95-GFP, or GAP14.

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 (250 M, 50 µl) and N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (Aarts et al.) (2 X, 500 µl). The mixture was incubated for 20 min at room temperature. During this time, cells (in 10-cm plates) were 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 memantine (50 uM) was included in the medium to protect the cells from excitotoxicity (Raymond et al., 1996).

Transfected cells were maintained on glass coverslips and used at 20-36 hr after the start of transfection.

2.3. Electrophysiology

Cultured neurons were used for recording at 4-7 DIV ("immature"), or >13 DIV ("mature"). Recordings from HEK293 cells were made 12-24 hours following the end of transfection. Conventional whole-cell patch clamp recording was conducted as previously described (Hamill et al., 1981). Electrodes were fabricated from borosilicate glass (Warner Instruments, Hamden, CT) using a Narashige (Tokyo, Japan) PP-83 electrode puller. Open tip resistance was 5-6 MΩ for electrodes containing (in mM) 115 Cs-methanesulfonate, 10 HEPES, 20 K2-creatine phosphate, 4 MgATP, 10 BAPTA, as well as 50 U/ml creatine phosphokinase, pH 7.26 , 310 mOsm.

Both neurons and HEK293 cells were superperfused with external recording solution, containing (in mM): 167 NaCl, 2.4 KCl, 10 HEPES, 10 glucose, 0.2 CaCl2, pH 7.3 (325 mOsm). Tetrodotoxin (TTX) (300 nM) and glycine (50 μM) were added just before use. Agonist (1 mM NMDA) was dissolved in the same solution used to bathe the cells and gravity fed to the cells through one side of a theta-tube (Chen et al., 1997). All other drugs, dissolved in the external bathing solution, were included in both the control and agonist side of the theta-tube. Computer-controlled solenoid-driven valves were used to rapidly switch between the two solutions. Agonist was applied for 10 sec at 1-min intervals. For experiments in cultured hippocampal neurons, large, pyramidal-shaped neurons were selected for recording.

All recordings were made in voltage-clamp mode at a holding potential of -70 mV. Data were acquired using the Axopatch 200B patch-clamp amplifier (Axon Instruments, Foster City, CA). Currents were filtered at 1 kHz and digitized at 10 kHz. pClamp 8.1 software (Axon

Instruments) was used for data acquisition and analysis. Access resistance and cell capacitance were regularly monitored and recordings were abandoned if access resistance exceeded 20 MΩ.

2.4. Co-immunoprecipatition and western blot analysis

Batches of 14-17 DIV hippocampal neuronal cultures in 10 cm dishes were treated with drugs or vehicle as described in Results, then each dish was collected in 1 ml Harvest buffer containing 1 mM EGTA, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 20 μg/ml pepstatin A, and 20 μg/ml leupeptin in PBS. Cell suspensions were processed as described previously (Li et al., 2003). Briefly, after centrifugation, pellets were lysed and solubilized in Harvest buffer containing 0.1% Sodium dodecyl sulfate (SDS) and 0.8% Triton X-100 (0.5 ml final volume). One-tenth of the lysate was reserved for input loading, and the remainder was incubated with protein-A and protein-G beads then briefly centrifuged to remove nonspecifically bound proteins. The supernatant was incubated with 10 μg of rabbit polyclonal anti-NR2A antibody (Upstate Biotechnology, Chicago, IL) for 1 hr at 4°C, then Protein-A and protein-G beads were added for another 1 hr incubation period. Beads were washed three times with 50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, and 1% Triton X-100. Proteins were eluted from the beads and denatured by boiling in loading buffer for 5 min, then loaded to SDS-PAGE. Proteins from each group of different treatment conditions (vehicle, 2-bromopalmitate (2-BP), 12-O-tetradecanoylphorbol-13-acetate (TPA), and TPA with 2-BP; or vehicle, RO-320432 (RO), and TPA) were loaded to the same gel. After transfer, membranes were probed with antibodies against NR2A (the same as used for immunoprecipitation, at 1 μg/ml) and PSD-95 (mouse monocolonal; 5 μg/ml; Chemicon). Bands were visualized using Enhanced Chemiluminescence (Amersham, Piscataway, NJ) and densities were quantified by densitometric analysis (Li et al., 2003). The PSD-95 to NR2A band-density ratio was calculated to determine the amount of PSD-95 co-immunoprecipated with NR2A.

2.5. Materials

All chemicals were purchased from Sigma (St. Louis, MO). All PSD-95 constructs were described previously (Craven et al., 1999; El-Husseini et al., 2000; Schnell et al., 2002; Christopherson et al., 2003; Prange et al., 2004). NR1-1a (Dingledine et al., 1999), NR2B and NR2A (ε1) cDNAs were described previously (Chen et al., 1997). Tissue culture reagents were obtained from Invitrogen (Carlsbad, CA). Stock solutions of 1 mM NMDA and 100 μM glycine were each stored in individual aliquots for up to 6 weeks at -200C. TPA was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 1mM and diluted to a final concentration of 100nM. A 200mM stock solution of 2-BP in ethanol was diluted to a final concentration of 100μM. RO was dissolved in DMSO for a stock concentration of 2mM and used at 1μM.

2.6. Data analysis

Results are presented as mean ± SE. Sets of different results were compared using one-way ANOVA followed by Bonferroni post-test or Student's t-test as appropriate, and significant differences were determined at the 95% confidence intervals unless otherwise indicated. Three to 10 responses of each cell were averaged for estimation of steady-state to peak current (Iss/Ip).

CHAPTER III

Results

3.1. Age-dependent desensitization

NMDAR desensitization attenuates receptor activation during sustained exposure to agonists (Tong et al., 1995). Glycine-independent desensitization can be isolated in cultured hippocampal neurons by recording NMDAR currents in response to 10-sec applications of saturating concentrations of agonist -- 1 mM NMDA with 50 μM glycine -- in low external calcium and a high intracellular concentration of BAPTA (Mayer et al., 1989; Tong and Jahr, 1994). Previously, we demonstrated a developmental decrease in this form of NMDAR desensitization recorded from cultured hippocampal neurons, which could be explained by receptor localization to the synapse; however, the reduction in desensitization also correlated with co-association of NMDARs with PSD-95 (Li et al., 2003).

3.2. PKC regulates NMDAR glycine-independent desensitization in mature neurons

3.2.1. PKC uncouples PSD-95 from NMDARs

Previously we have shown that treatments that disrupt NMDAR/PSD-95 interactions result in increased desensitization in mature neurons (Li et al 2003). Treatment of mature hippocampal neurons with phorbol esters to activate PKC increases diffuse staining of NMDAR clusters throughout dendrites, indicating a shift from synaptic to non-synaptic localization; under these conditions, PSD-95 staining remained punctate and clustered at the synapse suggesting that NMDARs and PSD-95 become uncoupled after PKC activation (Fong et al., 2002).

To confirm that treatment with phorbol esters results in dissociation of NMDARs from PSD-95, we treated neurons with 100 nM TPA for 10, 30 or 60 minutes and examined the interaction between NMDARs and PSD-95 by co-immunoprecipitation with an anti-NR2A

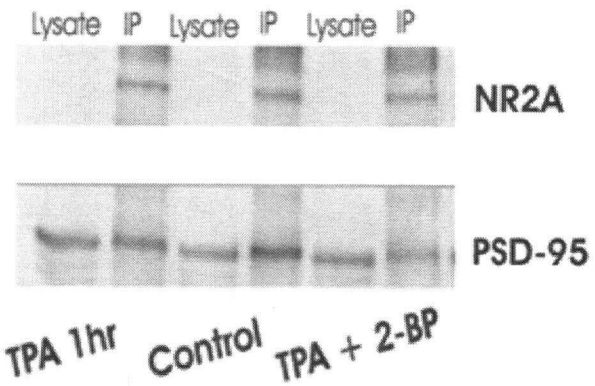
antibody. TPA significantly decreased the amount of PSD-95 co-immunoprecipitated with NR2A compared with control after a 60-min treatment (PSD-95/NR2A ratio scaled to control was 77 ± 13%, 67 ± 15%, and 51 ± 13% after 10-, 30- and 60-min TPA treatments, respectively; n=5 independent experiments with all four conditions done in parallel; $P < 0.01$ for control vs. 60-min TPA). Although phorbol esters activate PKC over the course of minutes, prolonged treatment can reduce PKC activity in many cell types (Wagey et al., 2001). To confirm that the effect of a 60-min TPA treatment was not a result of down-regulation of PKC activity, we treated neurons with 1 μM RO, which inhibits PKC activity by binding to the catalytic region ATP binding cassette (Birchall et al., 1994). Consistent with our hypothesis that NR2A and PSD-95 co-association was reduced because of PKC activation by TPA, treatment with RO for 10 minutes had no effect on the co-immunoprecipitation of NR2A and PSD-95 (PSD-95/NR2A ratio scaled to control was 98 ± 19% and 64 ± 12% after 10-min RO and 60-min TPA treatments, respectively; n=5 independent experiments with all three conditions done in parallel; $P<0.05$ for control vs. 60-min TPA).

Previous studies have shown that inhibition of PSD-95 palmitoylation by treatment with 2-BP shifts PSD-95 away from synapses and reduces co-localization with NMDARs (Craven et al., 1999). Indeed, 6-hr treatment with 100 μM 2-BP caused a significant reduction in co-immunoprecipitation of PSD-95 with NR2A (Figure 3-1), similar to previously published results (Li et al., 2003). Co-treatment of neurons with 2-BP (6 hours) and TPA (60 min) resulted in a reduction in the association of PSD-95 with NR2A that was not significantly different than the decrease found for either treatment alone (Figure 3-1). Together, these results indicate that PKC activation by TPA results in a partial uncoupling of NR2A and PSD-95 that is similar, and not additive, to that produced by 2-BP.

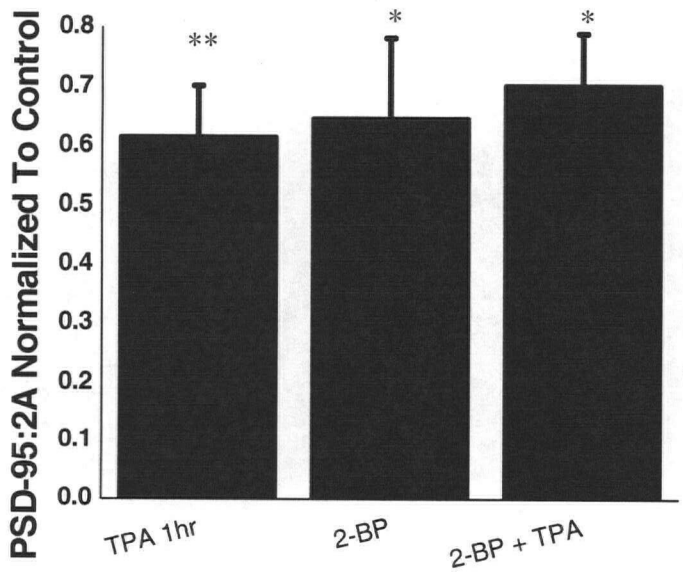
Figure 3-1. PKC activation with 2-BP treatment does not produce additive uncoupling

(a) Representative blot of co-immunoprecipitation. PSD-95 band density was significantly reduced in IP lanes with TPA alone or TPA plus 2-BP treatments compared to control. Combined 1-hour TPA and 6-hour 2-BP treatment did not produce additive reduction in NR2A/PSD-95 association. **(b)** Pooled data showing mean ± SE of PSD-95/NR2A band density normalized to control (n = 8 independent experiments for all conditions). $^{**}P < 0.01$ for control vs. 60-min TPA. $^{*}P < 0.05$ for control vs. 2-BP and 2-BP + 60-min TPA. Figure contributed by Lily Zhang.

a


Lysate
IP
Lysate
IP
Lysate
IP
NR2A
PSD-95
TPA 1hr
Control
TPA + 2-BP

b


PSD-95:2A Normalized To Control
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
**
*
*
TPA 1hr
2-BP
2-BP + TPA

3.2.2. Uncoupling of NMDARs/PSD-95 reduces receptor desensitization in mature hippocampal neurons

To determine whether PKC-induced dissociation of PSD-95 from NMDARs could alter desensitization, we recorded NMDA-evoked current from cultured mature hippocampal neurons after a 60-min treatment with 100 nM TPA or control solution. TPA treatment caused a marked increase in desensitization to levels similar to those found in immature neurons (Figure 3-2), whereas shorter treatments with TPA (10 min) or inhibition of PKC with RO – both treatments that did not affect coupling between PSD-95 and NR2A – did not alter NMDAR desensitization (Figure 3-2). This is another argument in favor of PSD-95 uncoupling being responsible for the TPA effect on NMDAR desensitization; if the PKC –mediated increase in NMDAR desensitization was due to receptor phosphorylation alone, should see it after 10 min. As a control, incubation with an inactive phorbol ester, 4α-TPA (100 nM), for 1 hour did not alter NMDAR current desensitization (Figure 3-2). Consistent with the similar reduction in co-association of NR2A and PSD-95 produced by treatment with TPA or 2-BP or the two together, these treatments also produced a similar, non-additive, reduction in NMDAR current Iss/Ip (Figure 3-2). Both treatments result in a dissociation of NMDARs from PSD-95: TPA treatment results in extrasynaptic localization of NMDARs while PSD-95 distribution remains unaltered at synaptic sites (Fong et al., 2002); 2-BP treatment results in removal of PSD-95 from synaptic sites while NMDAR distribution is unaltered at the synapse (Craven et al., 1999). The increase in desensitization that results from either treatment suggests binding of PSD-95 to NMDARs is critical in regulating this process.

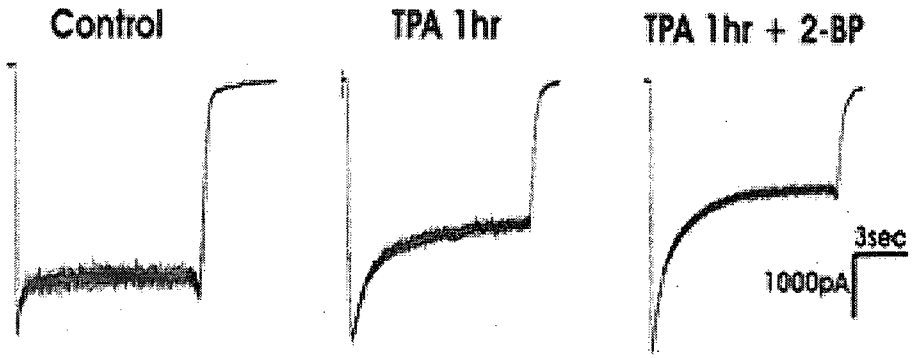
Previous work indicates that PKC activation increases NMDAR peak current and surface expression in *Xenopus* oocytes (Lan et al., 2001). However, we found no change in peak NMDAR current density after a 60-min treatment with 100nM TPA (Figure 3-3), consistent with

a previous study using a biochemical approach to show that NMDAR surface expression in cultured hippocampal neurons was unchanged following an identical TPA treatment (Fong et al., 2002). TPA has also been shown to enhance NMDAR currents in hippocampal slices (Chen and Huang, 1992) and cultures (Xiong et al., 1998). To further test whether TPA directly alters channel gating under our experimental conditions, we determined whether TPA treatment had any effect on NMDAR current in immature hippocampal neurons that express low levels of PSD-95. TPA did not significantly change the NMDAR Iss/Ip, peak current or current density compared with the control treatment (control vs. 60-min TPA showed: Iss/Ip of 34 ± 9% vs. 29 ± 11% and peak current density of 31 ± 10 pA/pF vs. 37 ± 13 pA/pF; n=15 for control n=7 for 60-min TPA; recordings were made from neurons at 4-5 DIV, Figure 3-4). These results indicate that the effect of TPA on NMDAR desensitization is not a result of direct PKC modulation of NMDAR current or surface expression. Instead, they suggest that association of PSD-95 with NMDARs, disrupted by PKC activation, underlies the reduced NMDAR desensitization found in mature compared with immature neurons.

Figure 3-2. PKC activation alters desensitization in mature neurons.

(a) Representative traces of mature neurons under control conditions, treated with TPA for 1 hour, and treated with TPA for one hour and 6-hour 2-BP. **(b)** 1-hour TPA treatment reduced Iss/Ip of mature (>14 DIV) neurons. Shorter 10-minute TPA treatments, 10-minute incubation with a PKC inhibitor (RO), or 1-hour treatment with an inactive phorbol (4αTPA) did not alter desensitization of mature neurons. Combined TPA and 2-BP treatments did not further increase the extent of desensitization. Control, n=23; 60-min TPA, n=9; 10-min TPA, n=7; 10-min RO, n=9; 1-hr αTPA, n=8; 2-BP + TPA, n=9. $^{**}P<0.01$ by One way ANOVA followed by Bonferroni post-test.

a


Control
TPA 1hr
TPA 1hr + 2-BP
3sec
1000pA

b

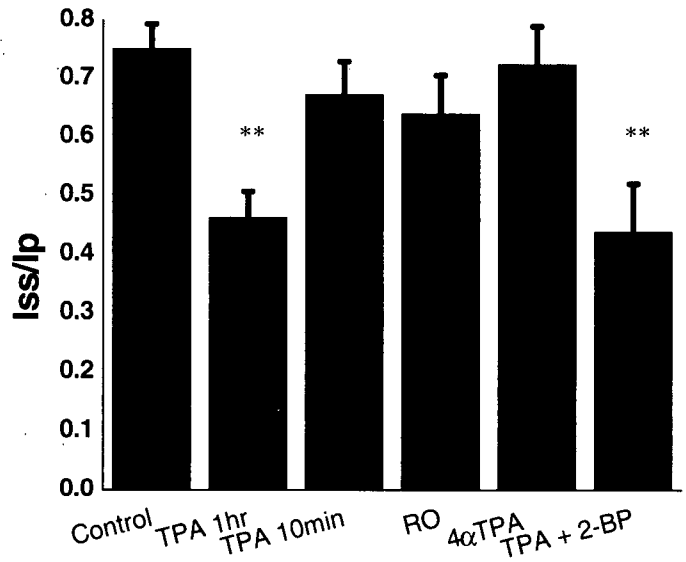

Iss/Ip
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
**
**
Control
TPA 1hr
TPA 10min
RO 4αTPA
TPA + 2-BP

Figure 3-3. Change in desensitization in mature neurons not due to increased peak current but altered steady state.

Mature neurons displayed no changes in current density with any treatment. Control, n=12; 60-min TPA, n=9; 10-min TPA, n=7; 10-min RO, n=9; 1-hr 4αTPA, n=8; 2-BP + TPA, n=9. $P>0.05$ by One way ANOVA.

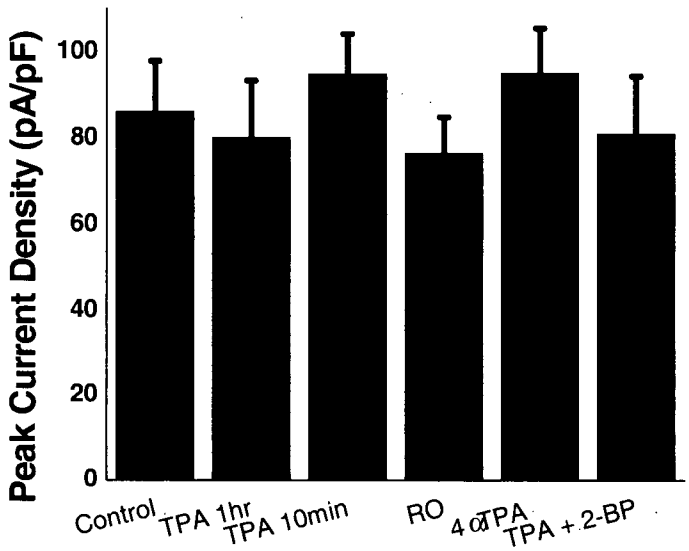
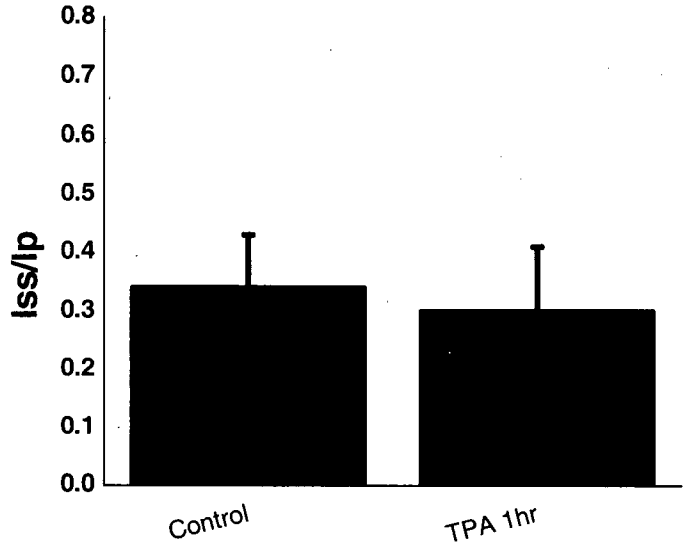

Peak Current Density (pA/pF)
100
80
60
40
20
0
Control
TPA 1hr
TPA 10min
RO 4 αTPA
TPA + 2-BP

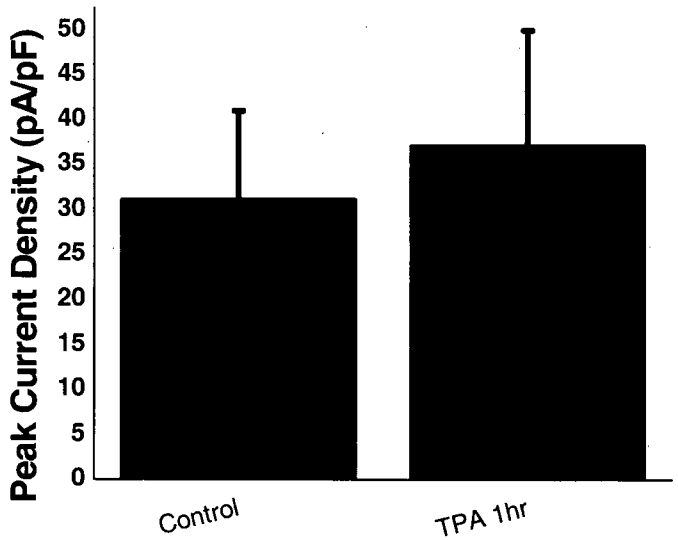
Figure 3-4. PKC activation does not alter desensitization in immature neurons.

(a) Immature neurons displayed no changes in Iss/Ip with 60-min TPA treatment. Control, n=15; 60-min TPA, n=7; .P>0.05 by student's t-test. **(b)** TPA does not alter peak current density in immature neurons. Immature neurons displayed no changes in current density with any treatment. Control, n=15; 60-min TPA, n=7. P>0.05 by student's t-test.

a


0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Iss/Ip
Control
TPA 1hr

b


50
45
40
35
30
25
20
15
10
5
0
Peak Current Density (pA/pF)
Control
TPA 1hr

3.3. PSD-95 Regulates glycine-independent desensitization of NMDARs

3.3.1. Coexpression of PSD-95 with NMDAR subunits decreases desensitization

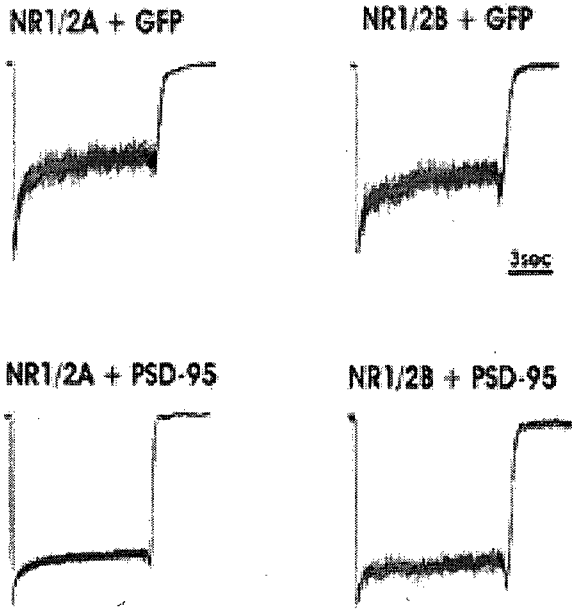
PSD-95 recruits many scaffolding and signaling molecules to the post-synaptic density directly through its various domains or indirectly through macromolecular MAGUK-AKAP complexes (Colledge et al., 2000; Sheng and Pak, 2000; Lim et al., 2002; Kim and Sheng, 2004). To assess whether the previously reported effect of PSD-95 on NMDAR desensitization (Li et al., 2003) occurred as a result of neuronal-specific mechanisms, we compared NMDAR currents from HEK293 cells transfected with either GFP (control) or PSD-95, in combination with different subtypes of NMDARs. HEK293 cells expressing GFP and NR1/NR2A showed significantly greater desensitization compared to cells co-expressing GFP and NR1/NR2B (Figure 3-5), as previously reported (Krupp et al., 1996; Dingledine et al., 1999). Strikingly, immature neurons that predominantly express NR1 and NR2B subunits exhibit more extensive NMDAR desensitization than recombinant NR1/NR2B in HEK293 cells (compare Figure 3-2 with 3-5). Furthermore mature hippocampal neurons that express largely NR2A-containing receptors (Li et al., 2002) show significantly less desensitization compared with either NMDARs in immature neurons or NR1/NR2A expressed in non-neuronal cells (compare Figure 3-2 with 3-5). The co-expression of PSD-95 significantly reduced the extent of NMDA-evoked current desensitization in both NR1/NR2A- and NR1/NR2B-expressing cells (Figure 3-5). Moreover, the steady-state to peak ratio for NMDAR current in PSD-95-expressing cells was similar for NR1/NR2A and NR1/NR2B, and also resembled the ratio found in mature cultured hippocampal neurons (for NMDAR current in PSD-95-expressing HEK293 cells was 64 ± 7%, n=11 for NR1/NR2A and 71 ± 7%, n=12 for NR1/NR2B, whereas this ratio was 75 ± 9%, n=23 in mature hippocampal neurons; $P>0.05$, by one-way ANOVA, Figure 3-2,3-5). These data demonstrate that the role of PSD-95 in regulating NMDAR desensitization is independent of other neuronal-

and synapse-specific proteins and suggest that direct binding of PSD-95 to NMDARs is sufficient to alter glycine-independent desensitization.

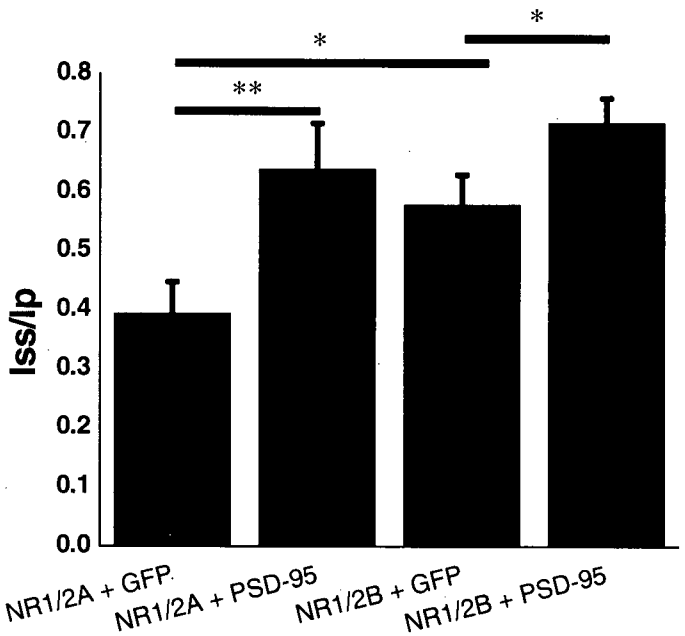
Figure 3-5. Expression of PSD-95 regulates glycine-independent desensitization of recombinant NMDARs.

(a) Representitive traces of NMDAR currents in HEK293 cells expressing NMDAR subunits with GFP or PSD-95-GFP. Currents were normalized for comparison of desensitization. **(b)** Expression of PSD-95 significantly reduced the extent of NMDA-evoked current desensitization in both NR1/NR2A- and NR1/NR2B-expressing cells. NR1/2A + GFP, n=13; NR1/2A + PSD-95-GFP, n=11; NR1/2B + GFP, n=11; NR1/2B + PSD-95; $*P<0.05$, $**P<0.01$; One way ANOVA followed by Bonferroni post-test.

a


NR1/2A + GFP
NR1/2B + GFP
3sec
NR1/2A + PSD-95
NR1/2B + PSD-95

b


*
*
**
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Iss/Ip
NR1/2A + GFP
NR1/2A + PSD-95
NR1/2B + GFP
NR1/2B + PSD-95

3.3.2. PDZ1-2 domains of PSD-95 are sufficient to alter NMDAR desensitization in HEK293 cells; multimeriazation not required for altered NMDAR desensitization

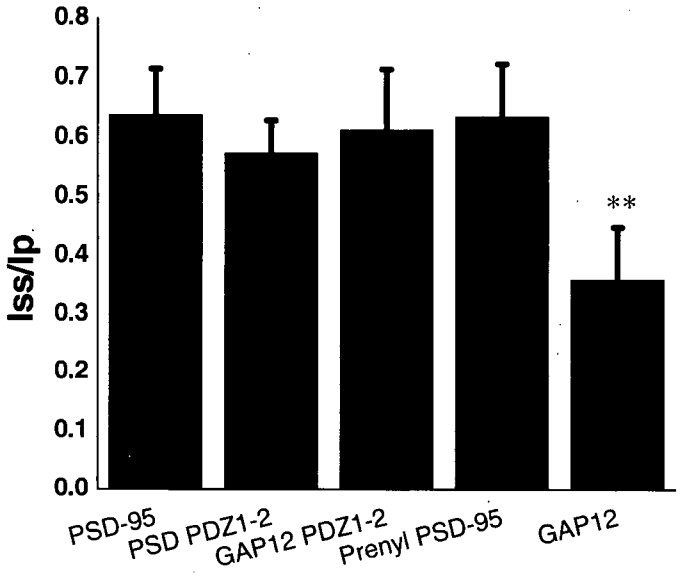
PSD-95 contains three N-terminal PDZ domains, a central Src homology 3 (SH3) domain, and a C-terminal guanylate kinase-like (GK) domain, which can each recruit various proteins to the membrane (Cho et al., 1992; Kim et al., 1995; Kornau et al., 1995). NMDARs directly interact with PDZ1 and PDZ2 of PSD-95 through the C-terminal PDZ-binding motif (ESDV) of the NR2 subunit (Kornau et al., 1995). Deletion mutagenesis studies established that the N-terminal region of PSD-95, containing palmitoylated Cys-3 and Cys-5, regulates both membrane targeting and multimerization (Craven et al., 1999; Hsueh and Sheng, 1999; Christopherson et al., 2003).

In order to isolate which domains of PSD-95 are required for regulating NMDAR desensitization, we utilized PSD-95 constructs with deletions of specific domains and/or with mutations that eliminated PSD-95 multimerization. In HEK293 cells expressing NR1/NR2A, co-transfection of PSD-95 truncated after domain PDZ2 (PSD-95 PDZ1-2-GFP) resulted in significantly reduced NMDA-evoked current desensitization compared to cells co-expressing only GFP, and an Iss/Ip ratio similar to that found with full-length wild-type PSD-95 (Figure 3-6). Although the PSD-95 PDZ1-2 construct lacks many of the domains involved in recruiting kinases and phosphatases to the membrane, it can multimerize, allowing proteins such as GluR1 to co-cluster via free PDZ domains (Schnell et al., 2002). To assess the role of PSD-95 multimerization in the regulation of NMDAR desensitization we used two different PSD-95 mutants that targeted to the membrane but remained in monomeric form: a variant of the truncated PSD-95 PDZ1-2-GFP construct, in which the N-terminal 13 amino acids were replaced with the first 12 amino acids of growth associated protein-43 (GAP12 PDZ1-2-GFP); and a variant of the C3,5S mutant of full-length PSD-95, in which the prenylation motif of paralemmin

was added to the C terminus (Prenyl-PSD-95-GFP). The Iss/Ip of NMDA-evoked currents recorded from HEK293 cells expressing these multimerization-deficient constructs was not significantly different from that observed in cells expressing full-length wildtype PSD-95-GFP, indicating that multimerization does not play a role in regulating glycine-independent NMDAR desensitization (Figure 3-6). Although mutating the N-terminus of PSD-95 should block multimerization, these constructs were GFP-tagged and GFP may form oligomers (Jain et al., 2001). However, we transfected the same constructs without the GFP tag and found no significant difference in the Iss/Ip (data not shown). As a control, we recorded NMDAR current from cells expressing the first 12 amino acids of GAP43 fused to GFP only (GAP12-GFP) together with NR1/NR2A. This construct had no effect on NMDAR desensitization (Figure 3-6; Iss/Ip was not significantly different from that found in cells co-expressing GFP and NR1/NR2A), confirming that it is the PDZ domains of the GAP12 PDZ1-2-GFP protein that are responsible for altering NMDAR current desensitization. This evidence strongly supports the idea that PSD-95 regulation of NMDAR desensitization occurs through direct binding of PDZ1-2 domains to the NMDAR and does not require domains involved in scaffolding other proteins in proximity to NMDARs.

Figure 3-6. PDZ1-2 domains are sufficient to alter NMDAR desensitization; multimerization of PSD-95 and protein targeting to the membrane are not required in regulating glycine-independent desensitization.

NR1/2A + PSD-95-GFP, n=11; NR1/2A + PSD PDZ1-2-GFP, n=11; NR1/2A + GAP12 PDZ1-2, n=7; NR1/2A + Prenyl PSD-95, n=8; NR1/2A + GAP12, n=8; $^{**}P<0.01$. One way ANOVA followed by Bonferroni post-test.


0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Iss/Ip
**
PSD-95
PSD PDZ1-2
GAP12 PDZ1-2
Prenyl PSD-95
GAP12

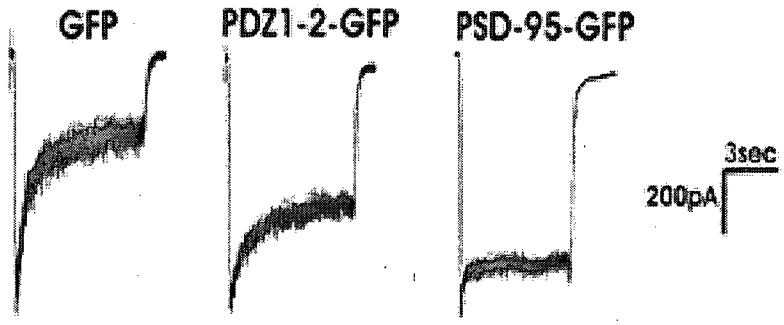
3.3.3. Overexpression of PDZ1-2 domains decreases desensitization in immature neurons

To determine whether expression of PSD-95 PDZ1-2-GFP, including only the N-terminal region through PDZ1 and PDZ2, in immature cultured hippocampal neurons is sufficient to reduce NMDA-evoked current desensitization to levels found in mature neurons, we transfected GFP, PSD-95-GFP, or PSD-95 PDZ1-2-GFP plasmids into 4 DIV neurons and compared NMDA-evoked currents recorded at 6 DIV. Expression of PSD-95-GFP in immature neurons resulted in a decrease in NMDA-evoked current desensitization compared to GFP-transfected controls and an Iss/Ip similar to that observed in mature neurons (compare Figures 3-3 with 3-7). Notably, there was no significant difference in peak current between neurons transfected with GFP and PSD-95 (1093 ± 205 pA, n=7 and 1264 ± 148 pA, n=12, respectively). Transfection of PSD-95 PDZ1-2-GFP also significantly reduced NMDAR desensitization compared to GFP-transfected neurons, although the effect was smaller than that observed for full-length PSD-95-GFP (Figure 3-7). From these data, our observations in HEK293 cells, and our previously published results (Li et al., 2003), we conclude that binding of PSD-95 PDZ1-2 domains to NMDARs plays a critical role in reducing receptor desensitization over the course of neuronal development.

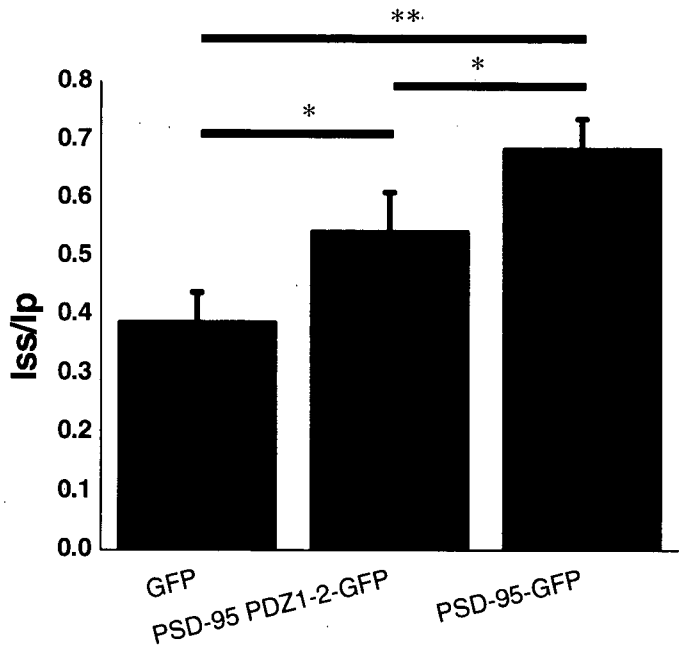
Figure 3-7. Overepression of PSD-95 in Immature Neurons Decreases Glycine-Independent Desensitization.

(a) Representitive traces of NMDAR responses in immature neurons overexpressing GFP, PSD-95 PDZ1-2-GFP or PSD-95-GFP. **(b)** Overexpression of PSD-95 PDZ1-2-GFP or PSD-95-GFP in immature hippocampal neurons results in decreased desensitization compared to GFP transfected controls. GFP n=7; PSD-95 PDZ1-2-GFP, n=6; PSD-95-GFP, n=12; $*P<0.05$, $**P<0.01$. One way ANOVA followed by Bonferroni post-test.

a


GFP
PDZ1-2-GFP
PSD-95-GFP
3sec
200pA

b


**
*
*
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Iss/Ip
GFP
PSD-95 PDZ1-2-GFP
PSD-95-GFP

CHAPTER IV

General Discussion

The major findings of this study indicate that direct binding of PSD-95 to NMDARs decreases the extent of glycine-independent desensitization in HEK293 cells and neurons. The first two PDZ domains of PSD-95 were sufficient to mediate this effect, which did not require PSD-95 multimerization. Moreover, desensitization can be regulated by PKC activity by altering the interaction between PSD-95 and NMDARs: mature neurons expressing high levels of PSD-95 show little desensitization, however PKC activation uncouples PSD-95 from NMDARs and increases desensitization to levels observed in immature neurons.

Interestingly, binding of PSD-95 to the C-terminus of NR2 subunits modulates NMDAR desensitization gating that is largely determined by amino acid residues in the lurcher motif, part of the channel vestibule (Hu and Zheng, 2005). However, there is precedence for changes in C-terminal domains of NMDAR subunits affecting function of distant domains. For example, src-mediated phosphorylation of NR2A C-terminal residues relieves NMDAR zinc inhibition, which is determined by the amino terminal domain (Zheng et al., 1998).

4.1. Phorbol ester-induced PKC activation and regulation of neuronal NMDAR function

Our data demonstrate that one-hour TPA treatment of mature neurons results in increased NMDAR desensitization, without altering peak current, through PKC activation and subsequent uncoupling of PSD-95 from NMDARs; as well, TPA did not alter NMDAR peak current in immature neurons expressing only low levels of PSD-95. A previous study using immunocytochemical techniques also showed dissociation of PSD-95 and NMDARs, without alterations in NMDAR surface expression, by one-hour TPA treatment in mature hippocampal neurons (Fong et al., 2002).

In contrast to results with chronic (1-hour) phorbol ester treatments, acute phorbol ester treatments have been shown to alter NMDAR peak current by increasing surface number, with a trend towards increasing channel opening rate (Xiong et al., 1998; Zheng et al., 1999; Lan et al., 2001); however, it may be that these effects of phorbol esters are observed under basal conditions only for NMDARs containing NR1-4b splice variants, which exhibit the highest surface expression and undergo the largest PKC potentiation of all NR1 splice variants when expressed in in *Xenopus* oocytes (Kelso et al., 1992; Okabe et al., 1999; Lan et al., 2001). Interestingly, in HEK293 cells expressing NR1-1a and NR2A or NR2B, PKC activation enhanced glycine-independent desensitization without altering the peak current (Jackson et al., 2006), consistent with the idea that the acute effects of phorbol esters on NMDAR function are specific to NMDARs composed of certain NR1 splice variants and/or that these effects differ between heterologous systems. In the latter study, the effects of phorbol esters on NMDAR desensitization were still evident despite mutagenesis of the major PKC sites on the NR1 subunit, and the removal of the C-terminal tail of NR1 or NR2A (Jackson et al., 2006). In contrast, our data indicates that TPA enhances NMDAR desensitization by disrupting PSD-95 binding to the C-terminus of NR2; differences between our result and that found by (Jackson et al.) may be, in part, accounted for by differences in PKC isoform expression and activity between HEK293 cells and hippocampal neurons. In other studies in neurons, an increase in the NMDAR surface expression occurred after acute TPA treatment in conjunction with the phosphatase inhibitor okadaic acid (Lan et al., 2001), which has been shown to substantially augment the peak current potentiation produced by phorbol ester treatment (Lu et al., 1999; Lan et al., 2001). Loading neurons with the constitutively active form of PKC also resulted in NMDAR peak current potentiation (Xiong et al., 1998). Our stimulation conditions activated only endogenous PKC without inhibition of phosphatases, which may not be sufficient to mediate increases in neuronal NMDAR peak current density and surface expression.

A recent study has shown that PKC potentiation of NMDAR currents in *Xenopus* oocytes can be occluded by co-expression of PSD-95, and is dependent on phosphorylation of Serine-1462, which lies within the PDZ-binding motif of NR2A (Liao et al., 2000; Lin et al., 2006). These results suggest that the lack of NMDAR current potentiation in mature neurons following phorbol ester treatment may be explained by the high levels of co-association of PSD-95 with NMDARs, which are largely localized to synapses (Li et al., 2003). However, when we uncoupled PSD-95 from NMDARs with 2-bromopalmitate and subsequently activated PKC, we still found no potentiation of NMDAR peak current amplitude or density. Moreover, the lack of potentiation of NMDAR current density by TPA in immature neurons, which express low levels of PSD-95, also indicates that regulation of NMDAR function by phosphorylation is different in heterologous compared with neuronal cells.

4.2. Role of PSD-95 direct binding to NMDARs on current desensitization

In a previous report (Li et al., 2003), several lines of evidence indirectly implicated the interaction of PSD-95 with NMDARs in regulating glycine–independent desensitization; conditions where PSD-95 targeting to the synapse was disrupted by inhibition of palmitoylation, or where NMDARs were removed from the synapse by chronic treatment with picrotoxin, both resulted in an increase of NMDAR desensitization (Li et al., 2003). The complexity of interactions that occur between PSD-95 and NMDARs makes it difficult to isolate which mechanisms may be involved in regulating NMDAR desensitization in neurons. In HEK293 cells, expression of either wild-type PSD-95 or the N-terminal region of PSD-95 truncated after the PDZ1-2 domains reduced NMDAR desensitization, to levels similar to those found in mature hippocampal neurons, indicating other neuronal-specific proteins and signaling pathways are not required for the effect of PSD-95 on NMDAR desensitization. However, neuronal-specific mechanisms could still regulate NMDAR desensitization. For example, immature hippocampal

neurons that express predominantly NR2B-containing receptors and have lower levels of PSD-95 in early development show more NMDAR desensitization than HEK293 cells expressing NR1/NR2B. The extent of neuronal NR1/NR2B current desensitization in the absence of PSD-95 expression may be regulated by cell-specific receptor phosphorylation, since desensitization is enhanced by phosphorylation of NR2B Ser1303 (Sessoms-Sikes et al., 2005) and reduced by tyrosine phosphatase inhibition in NR2B-containing immature neurons (Li et al., 2003). Conversely, mature neurons exhibit low levels of desensitization while expressing high levels of NR2A-containing receptors, which desensitize extensively in HEK293 cells. This may be explained by a direct interaction with PSD-95: NMDARs in mature neurons are largely synaptic and colocalize and presumably interact with PSD-95, while PSD-95 coexpression with NR1/NR2A in HEK293 cells was sufficient to decrease desensitization.

Co-expression of PSD-95 with NR1/NR2A in HEK293 cells was previously reported to increase the number of functional channels at the cell surface (Lin et al., 2004). We did not directly address this question in our electrophysiological recordings from transfected HEK293 cells, due to the high cell-to-cell variability in NMDAR expression. Notably, the Iss/Ip did not correlate with the peak current amplitude in HEK293 cells. In neurons, however, we did not observe any change in peak current after overexpression of PSD-95 or with dissociation of NMDARs and PSD-95, suggesting other neuronal-specific mechanisms may contribute to regulating NMDAR surface expression and occlude the effect of PSD-95. Interestingly, PDZ interactions have also been shown to stabilize NMDARs at synaptic sites. Previously, it has been shown that overexpression of PSD-95 in immature cerebellar granule cells resulted in a decrease in current density (Losi et al., 2003). PSD-95 cotransfection can retard the internalization rate of chimeras containing the C-terminus of the NR2B subunit (Roche et al., 2001). PSD-95 also has been shown to stabilize NR2B-containaing receptors at the synapse while trafficking of NR2A- or NR2B-containing receptors to the surface occurred in a PSD-95

independent manner (Prybylowski et al., 2005). This evidence suggests the larger role of PSD-95 regulation of NMDARs in neurons may be in maintenance of surface receptors rather than through forward trafficking and surface delivery.

With overexpression of full-length PSD-95 in immature neurons, we observed a significant decrease in NMDAR glycine-independent desensitization compared to GFP-transfected controls, an effect that was attenuated though still significant with expression of the truncated mutant, PSD-95 PDZ1-2-GFP. PSD-95 PDZ1-2-GFP has been shown to cluster at synaptic sites, although less efficiently than full-length PSD-95 (Craven et al., 1999). Although the PSD-95 PDZ1-2-GFP construct contains the N-terminal palmitoylation sites involved in membrane targeting, a second signal found in the last 13–25 amino acids of PSD-95 is also required for proper synaptic targeting (Craven et al., 1999); absence of this region in the truncated mutant results in a "semi-clustered" phenotype. Furthermore, the PDZ3 domain binds the postsynaptic protein CRIPT, linking PSD-95 to the microtubule cytoskeleton. Disruption of the CRIPT/PSD-95 interaction prevented the association of PSD-95 with microtubules and inhibited synaptic targeting in hippocampal neurons (Passafaro et al., 1999). In HEK293 cells, expression of truncated mutants did not show a significant difference in NMDAR current desensitization compared to full-length PSD-95. As targeting or clustering of NMDARs is independent of PSD-95 in neurons (El-Husseini et al., 2000), an inefficient synaptic targeting or clustering of PSD-95 PDZ1-2-GFP suggests that NMDARs and PSD-95 PDZ1-2-GFP could be in non-overlapping subcellular compartments, resulting in less effective interaction. This might, at least in part, explain the discrepancy between the overexpression in neurons versus HEK293 cells, where targeting to the plasma membrane is sufficient to colocalize NMDARs and PSD-95.

PSD-95 exists predominantly in an α isoform containing two cysteine residues that can be palmitoylated to regulate membrane targeting as well as multimerization, however a rarer β isoform containing an L27 domain instead of a palmitoylation motif is also present in neurons.

The β isoform has been shown to regulate PSD-95 clustering as well as AMPA receptor function in an activity-dependent manner through the CaMKII pathway (Chetkovich et al., 2002; Schluter et al., 2006). In our overexpression studies we have not utilized any constructs containing the N-terminal domain of the β isoform, and in mature neurons expressing endogenous PSD-95, the α isoform has been shown to account for more than 80% of the total PSD-95 expression. In support of this, mature neurons exhibit high sensitivity to treatments that interfere with palmitoylation of PSD-95 (Li et al., 2003; Schluter et al., 2006).

4.3. Effects of PSD-95/NMDAR interaction on NMDAR function in neurons

Previous studies investigating the role of PSD-95 in modulating neuronal NMDAR currents yielded apparently conflicting results. In mice expressing the truncated C-terminus NR2A subunit ($NR2A^{\Delta C/\Delta C}$), which is unable to bind PSD-95, hippocampal neurons in brain slice have reduced NMDAR spontaneous and evoked excitatory postsynaptic currents (EPSCs) and dramatic reductions in hippocampal CA1 long-term potentiation (LTP) (Sprengel et al., 1998; Steigerwald et al., 2000); consistent with our findings, NMDAR current desensitization was increased in whole-cell patches from these neurons (Steigerwald et al., 2000). Also consistent with our results, in mice carrying a targeted mutation in the PSD-95 gene that leaves the first two PDZ domains intact by introducing a stop codon in the PDZ3 domain, there were no alterations of whole-cell NMDAR current in cultured neurons or gross differences in synaptic transmission compared to wild-type (Migaud et al., 1998).

On the other hand, hippocampal slices from PSD-95 knock-out mice ($PSD\text{-}95^{-/-}$), or after acute knock-down of PSD-95 using short hairpin RNAs (shRNA), showed no difference in NMDAR-mediated EPSCs (Nakagawa et al., 2004; Elias et al., 2006). Furthermore, acute disruption of PSD-95/NMDAR interactions utilizing peptides that competitively bind to the first two PDZ domains of PSD-95 in hippocampal slices showed no effect on NMDA receptor-

mediated EPSC amplitude (Lim et al., 2003). However, these studies did not directly examine NMDAR desensitization, and glycine-independent desensitization may be difficult to assess in slice preparations, since single evoked responses reflect transient agonist exposure. Desensitization has been shown to shape synaptic responses, but this was observed during repetitive stimulation (Tong et al., 1995). We postulate that disruption of the NMDAR/PSD-95 interaction shifts the relationship between NMDAR activation and activity-dependent plasticity to the right, such that higher NMDAR activity levels are required to induce long-term potentiation. In this case, acute interference with the NMDAR/PSD-95 interaction may result in no apparent change in LTP at stimulation frequencies that were formerly in the saturating range for inducing LTP (Migaud et al., 1998; Lim et al., 2003; Malenka and Bear, 2004).

4.4. PSD-95/NMDAR association may regulate synaptic plasticity and excitotoxicity

NMDAR desensitization limits calcium influx during periods of repeated synaptic stimulation or prolonged neuronal exposure to glutamate as may occur during ischemia. PSD-95 binding to NR2 subunits to reduce NMDAR desensitization may contribute to the shift in stimulation frequencies required to induce NMDAR-dependent synaptic plasticity that occur during brain development (Philpot et al., 2003), with consequences for new learning. As well, the effect of NMDAR/PSD-95 binding on the integrated calcium current in response to sustained glutamate insults may contribute to neuronal sensitivity to excitotoxicity. It has been shown that NMDAR antagonists prevent toxicity in *in vitro* models of ischemia however clinical trials utilizing NMDAR antagonists have largely been disappointing due to troubling, unwanted side effects (Lee et al., 1999; Brauner-Osborne et al., 2000). Regulating desensitization by disrupting NMDAR/PSD-95 interactions may provide a novel approach for protection from excitotoxicity.

4.5. Future directions

We have shown here that direct binding of PSD-95 to NMDARs regulates glycine-independent desensitization. Although this finding is interesting there remains much to be studied. Members of the PSD-95 family of MAGUKs share approximately 70% homology, and a large body of evidence suggests a degree of functional redundancy exists within the family (Kim and Sheng, 2004). PSD-95 and PSD-93 knock-out mice show no alterations in synaptic transmission; however, acute knockdown of either protein or creation of a double knockout results in severely altered neurotransmission (Elias et al., 2006). It would be important to determine whether other members of the MAGUK family of proteins such as PSD-93 or SAP-102 confer altered desensitization profiles to NMDARs through direct binding to the C-terminus of the NR2 subunit.

Although we suggest alterations in desensitization may be relevant in both learning/memory and excitotoxicity, we do not address these processes directly in this thesis. It has been previously published that disruption of binding between NR2B-containing NMDARs and PSD-95 resulted in no alterations in calcium entry or whole-cell responses while decreasing NMDA-induced toxicity through an uncoupling of NMDARs from downstream toxic signaling pathways (Sattler et al., 1999; Aarts et al., 2002). One possible explanation for the unaltered whole-cell currents and calcium signal may be the small differences between the integrated current in cells expressing NR2B-containing NMDARs with and without PSD-95. Although PSD-95 significantly decreased the extent of desensitization of NR2B-containing NMDARs in HEK293 cells in our study, the differences are much less robust compared to cells expressing NR2A-containing NMDARs with and without PSD-95. This compounded with the possibility that uncoupling caused by the peptide may be incomplete could easily result in little alteration in whole cell currents or calcium signals. Peptides targeted for the disruption of the PDZ binding domain of NR2A have shown limited success in uncoupling the NMDAR/PSD-95 complex due

to the higher affinity of NR2A compared to NR2B in binding PSD-95 (Sans et al., 2000; Lim et al., 2003). To provide more evidence that the alterations in desensitization are dependent on direct binding of PSD-95 to the C-terminus of NR2A- or NR2B-containing receptors, truncation of the PDZ binding domain (ESDV) of either NR2 subunit should be tested to determine if these mutations negate the decrease in desensitization seen with co-expression of PSD-95 in HEK293 cells.

REFERECES

Aarts M, Liu Y, Liu L, Besshoh S, Arundine M, Gurd JW, Wang YT, Salter MW, Tymianski M (2002) Treatment of ischemic brain damage by perturbing NMDA receptor- PSD-95 protein interactions. Science 298:846-850.

Aniksztejn L, Otani S, Ben-Ari Y (1992) Quisqualate Metabotropic Receptors Modulate NMDA Currents and Facilitate Induction of Long-Term Potentiation Through Protein Kinase C. Eur J Neurosci 4:500-505.

Aoki C, Miko I, Oviedo H, Mikeladze-Dvali T, Alexandre L, Sweeney N, Bredt DS (2001) Electron microscopic immunocytochemical detection of PSD-95, PSD-93, SAP-102, and SAP-97 at postsynaptic, presynaptic, and nonsynaptic sites of adult and neonatal rat visual cortex. Synapse 40:239-257.

Battaini F (2001) Protein kinase C isoforms as therapeutic targets in nervous system disease states. Pharmacol Res 44:353-361.

Birchall AM, Bishop J, Bradshaw D, Cline A, Coffey J, Elliott LH, Gibson VM, Greenham A, Hallam TJ, Harris W, et al. (1994) Ro 32-0432, a selective and orally active inhibitor of protein kinase C prevents T-cell activation. J Pharmacol Exp Ther 268:922-929.

Blank T, Nijholt I, Teichert U, Kugler H, Behrsing H, Fienberg A, Greengard P, Spiess J (1997) The phosphoprotein DARPP-32 mediates cAMP-dependent potentiation of striatal N-methyl-D-aspartate responses. Proc Natl Acad Sci U S A 94:14859-14864.

Bliss TV, Lomo T (1973) Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path. J Physiol 232:331-356.

Bonni A, Brunet A, West AE, Datta SR, Takasu MA, Greenberg ME (1999) Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and -independent mechanisms. Science 286:1358-1362.

Brauner-Osborne H, Egebjerg J, Nielsen EO, Madsen U, Krogsgaard-Larsen P (2000) Ligands for glutamate receptors: design and therapeutic prospects. J Med Chem 43:2609-2645.

Brenneman DE, Forsythe ID, Nicol T, Nelson PG (1990) N-methyl-D-aspartate receptors influence neuronal survival in developing spinal cord cultures. Brain Res Dev Brain Res 51:63-68.

Brewer GJ (1995) Serum-free B27/neurobasal medium supports differentiated growth of neurons from the striatum, substantia nigra, septum, cerebral cortex, cerebellum, and dentate gyrus. J Neurosci Res 42:674-683.

Brewer GJ, Torricelli JR, Evege EK, Price PJ (1993) Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination. J Neurosci Res 35:567-576.

Brunet A, Datta SR, Greenberg ME (2001) Transcription-dependent and -independent control of neuronal survival by the PI3K-Akt signaling pathway. Curr Opin Neurobiol 11:297-305.

Chatterton JE, Awobuluyi M, Premkumar LS, Takahashi H, Talantova M, Shin Y, Cui J, Tu S, Sevarino KA, Nakanishi N, Tong G, Lipton SA, Zhang D (2002) Excitatory glycine receptors containing the NR3 family of NMDA receptor subunits. Nature 415:793-798.

Chen CA, Okayama H (1988) Calcium phosphate-mediated gene transfer: a highly efficient transfection system for stably transforming cells with plasmid DNA. Biotechniques 6:632-638.

Chen L, Huang LY (1992) Protein kinase C reduces Mg2+ block of NMDA-receptor channels as a mechanism of modulation. Nature 356:521-523.

Chetkovich DM, Bunn RC, Kuo SH, Kawasaki Y, Kohwi M, Bredt DS (2002) Postsynaptic targeting of alternative postsynaptic density-95 isoforms by distinct mechanisms. J Neurosci 22:6415-6425.

Cho KO, Hunt CA, Kennedy MB (1992) The rat brain postsynaptic density fraction contains a homolog of the Drosophila discs-large tumor suppressor protein. Neuron 9:929-942.

Christopherson KS, Sweeney NT, Craven SE, Kang R, El-Husseini Ael D, Bredt DS (2003) Lipid- and protein-mediated multimerization of PSD-95: implications for receptor clustering and assembly of synaptic protein networks. J Cell Sci 116:3213-3219.

Chung HJ, Huang YH, Lau LF, Huganir RL (2004) Regulation of the NMDA receptor complex and trafficking by activity-dependent phosphorylation of the NR2B subunit PDZ ligand. J Neurosci 24:10248-10259.

Ciabarra AM, Sullivan JM, Gahn LG, Pecht G, Heinemann S, Sevarino KA (1995) Cloning and characterization of chi-1: a developmentally regulated member of a novel class of the ionotropic glutamate receptor family. J Neurosci 15:6498-6508.

Colledge M, Dean RA, Scott GK, Langeberg LK, Huganir RL, Scott JD (2000) Targeting of PKA to glutamate receptors through a MAGUK-AKAP complex. Neuron 27:107-119.

Collingridge GL, Kehl SJ, McLennan H (1983) The antagonism of amino acid-induced excitations of rat hippocampal CA1 neurones in vitro. J Physiol 334:19-31.

Craven SE, El-Husseini AE, Bredt DS (1999) Synaptic targeting of the postsynaptic density protein PSD-95 mediated by lipid and protein motifs. Neuron 22:497-509.

Crump FT, Dillman KS, Craig AM (2001) cAMP-dependent protein kinase mediates activity-regulated synaptic targeting of NMDA receptors. J Neurosci 21:5079-5088.

Cull-Candy S, Brickley S, Farrant M (2001) NMDA receptor subunits: diversity, development and disease. Curr Opin Neurobiol 11:327-335.

Cull-Candy SG, Leszkiewicz DN (2004) Role of distinct NMDA receptor subtypes at central synapses. Sci STKE 2004:re16.

Das S, Sasaki YF, Rothe T, Premkumar LS, Takasu M, Crandall JE, Dikkes P, Conner DA, Rayudu PV, Cheung W, Chen HS, Lipton SA, Nakanishi N (1998) Increased NMDA current and spine density in mice lacking the NMDA receptor subunit NR3A. Nature 393:377-381.

Dildy-Mayfield JE, Harris RA (1994) Activation of protein kinase C inhibits kainate-induced currents in oocytes expressing glutamate receptor subunits. J Neurochem 62:1639-1642.

Dingledine R, Borges K, Bowie D, Traynelis SF (1999) The glutamate receptor ion channels. Pharmacol Rev 51:7-61.

Dudek SM, Bear MF (1992) Homosynaptic long-term depression in area CA1 of hippocampus and effects of N-methyl-D-aspartate receptor blockade. Proc Natl Acad Sci U S A 89:4363-4367.

Ehlers MD, Tingley WG, Huganir RL (1995) Regulated subcellular distribution of the NR1 subunit of the NMDA receptor. Science 269:1734-1737.

El-Husseini AE, Schnell E, Chetkovich DM, Nicoll RA, Bredt DS (2000) PSD-95 involvement in maturation of excitatory synapses. Science 290:1364-1368.

Elias GM, Funke L, Stein V, Grant SG, Bredt DS, Nicoll RA (2006) Synapse-Specific and Developmentally Regulated Targeting of AMPA Receptors by a Family of MAGUK Scaffolding Proteins. Neuron 52:307-320.

Firestein BL, Craven SE, Bredt DS (2000) Postsynaptic targeting of MAGUKs mediated by distinct N-terminal domains. Neuroreport 11:3479-3484.

Fong DK, Rao A, Crump FT, Craig AM (2002) Rapid synaptic remodeling by protein kinase C: reciprocal translocation of NMDA receptors and calcium/calmodulin-dependent kinase II. J Neurosci 22:2153-2164.

Fukaya M, Kato A, Lovett C, Tonegawa S, Watanabe M (2003) Retention of NMDA receptor NR2 subunits in the lumen of endoplasmic reticulum in targeted NR1 knockout mice. Proc Natl Acad Sci U S A 100:4855-4860.

Gibb AJ (2004) NMDA receptor subunit gating--uncovered. Trends Neurosci 27:7-10; discussion 10.

Grover LM, Teyler TJ (1990) Two components of long-term potentiation induced by different patterns of afferent activation. Nature 347:477-479.

Hardingham GE, Bading H (2002) Coupling of extrasynaptic NMDA receptors to a CREB shut-off pathway is developmentally regulated. Biochim Biophys Acta 1600:148-153.

Hardingham GE, Bading H (2003) The Yin and Yang of NMDA receptor signalling. Trends Neurosci 26:81-89.

Hardingham GE, Fukunaga Y, Bading H (2002) Extrasynaptic NMDARs oppose synaptic NMDARs by triggering CREB shut-off and cell death pathways. Nat Neurosci 5:405-414.

Hollmann M, Heinemann S (1994) Cloned glutamate receptors. Annu Rev Neurosci 17:31-108.

Hollmann M, Hartley M, Heinemann S (1991) Ca2+ permeability of KA-AMPA--gated glutamate receptor channels depends on subunit composition. Science 252:851-853.

Hsueh YP, Sheng M (1999) Requirement of N-terminal cysteines of PSD-95 for PSD-95 multimerization and ternary complex formation, but not for binding to potassium channel Kv1.4. J Biol Chem 274:532-536.

Hu B, Zheng F (2005) Molecular determinants of glycine-independent desensitization of NR1/NR2A receptors. J Pharmacol Exp Ther 313:563-569.

Ikonomidou C, Bosch F, Miksa M, Bittigau P, Vockler J, Dikranian K, Tenkova TI, Stefovska V, Turski L, Olney JW (1999) Blockade of NMDA receptors and apoptotic neurodegeneration in the developing brain. Science 283:70-74.

Jackson MF, Konarski JZ, Weerapura M, Czerwinski W, MacDonald JF (2006) Protein kinase C enhances glycine-insensitive desensitization of NMDA receptors independently of previously identified protein kinase C sites. J Neurochem 96:1509-1518.

Jain RK, Joyce PB, Molinete M, Halban PA, Gorr SU (2001) Oligomerization of green fluorescent protein in the secretory pathway of endocrine cells. Biochem J 360:645-649.

Kaul M, Garden GA, Lipton SA (2001) Pathways to neuronal injury and apoptosis in HIV-associated dementia. Nature 410:988-994.

Kelso SR, Nelson TE, Leonard JP (1992) Protein kinase C-mediated enhancement of NMDA currents by metabotropic glutamate receptors in Xenopus oocytes. J Physiol 449:705-718.

Kim E, Sheng M (1996) Differential K+ channel clustering activity of PSD-95 and SAP97, two related membrane-associated putative guanylate kinases. Neuropharmacology 35:993-1000.

Kim E, Sheng M (2004) PDZ domain proteins of synapses. Nat Rev Neurosci 5:771-781.

Kim E, Niethammer M, Rothschild A, Jan YN, Sheng M (1995) Clustering of Shaker-type K+ channels by interaction with a family of membrane-associated guanylate kinases. Nature 378:85-88.

Klein RM, Howe JR (2004) Effects of the lurcher mutation on GluR1 desensitization and activation kinetics. J Neurosci 24:4941-4951.

Kornau HC, Schenker LT, Kennedy MB, Seeburg PH (1995) Domain interaction between NMDA receptor subunits and the postsynaptic density protein PSD-95. Science 269:1737-1740.

Krupp JJ, Vissel B, Heinemann SF, Westbrook GL (1996) Calcium-dependent inactivation of recombinant N-methyl-D-aspartate receptors is NR2 subunit specific. Mol Pharmacol 50:1680-1688.

Krupp JJ, Vissel B, Heinemann SF, Westbrook GL (1998) N-terminal domains in the NR2 subunit control desensitization of NMDA receptors. Neuron 20:317-327.

Krupp JJ, Vissel B, Thomas CG, Heinemann SF, Westbrook GL (1999) Interactions of calmodulin and alpha-actinin with the NR1 subunit modulate Ca2+-dependent inactivation of NMDA receptors. J Neurosci 19:1165-1178.

Krupp JJ, Vissel B, Thomas CG, Heinemann SF, Westbrook GL (2002) Calcineurin acts via the C-terminus of NR2A to modulate desensitization of NMDA receptors. Neuropharmacology 42:593-602.

Lan JY, Skeberdis VA, Jover T, Grooms SY, Lin Y, Araneda RC, Zheng X, Bennett MV, Zukin RS (2001) Protein kinase C modulates NMDA receptor trafficking and gating. Nat Neurosci 4:382-390.

Lancelot E, Beal MF (1998) Glutamate toxicity in chronic neurodegenerative disease. Prog Brain Res 116:331-347.

Laurie DJ, Seeburg PH (1994) Regional and developmental heterogeneity in splicing of the rat brain NMDAR1 mRNA. J Neurosci 14:3180-3194.

Laurie DJ, Putzke J, Zieglgansberger W, Seeburg PH, Tolle TR (1995) The distribution of splice variants of the NMDAR1 subunit mRNA in adult rat brain. Brain Res Mol Brain Res 32:94-108.

Lee JM, Zipfel GJ, Choi DW (1999) The changing landscape of ischaemic brain injury mechanisms. Nature 399:A7-14.

Leonard AS, Davare MA, Horne MC, Garner CC, Hell JW (1998) SAP97 is associated with the alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor GluR1 subunit. J Biol Chem 273:19518-19524.

Li B, Otsu Y, Murphy TH, Raymond LA (2003) Developmental decrease in NMDA receptor desensitization associated with shift to synapse and interaction with postsynaptic density-95. J Neurosci 23:11244-11254.

Li B, Chen N, Luo T, Otsu Y, Murphy TH, Raymond LA (2002) Differential regulation of synaptic and extra-synaptic NMDA receptors. Nat Neurosci 5:833-834.

Li JH, Wang YH, Wolfe BB, Krueger KE, Corsi L, Stocca G, Vicini S (1998) Developmental changes in localization of NMDA receptor subunits in primary cultures of cortical neurons. Eur J Neurosci 10:1704-1715.

Liao GY, Kreitzer MA, Sweetman BJ, Leonard JP (2000) The postsynaptic density protein PSD-95 differentially regulates insulin- and Src-mediated current modulation of mouse NMDA receptors expressed in Xenopus oocytes. J Neurochem 75:282-287.

Lieberman DN, Mody I (1999) Casein kinase-II regulates NMDA channel function in hippocampal neurons. Nat Neurosci 2:125-132.

Lim IA, Hall DD, Hell JW (2002) Selectivity and promiscuity of the first and second PDZ domains of PSD-95 and synapse-associated protein 102. J Biol Chem 277:21697-21711.

Lim IA, Merrill MA, Chen Y, Hell JW (2003) Disruption of the NMDA receptor-PSD-95 interaction in hippocampal neurons with no obvious physiological short-term effect. Neuropharmacology 45:738-754.

Lin Y, Skeberdis VA, Francesconi A, Bennett MV, Zukin RS (2004) Postsynaptic density protein-95 regulates NMDA channel gating and surface expression. J Neurosci 24:10138-10148.

Lin Y, Jover-Mengual T, Wong J, Bennett MV, Zukin RS (2006) PSD-95 and PKC converge in regulating NMDA receptor trafficking and gating. Proc Natl Acad Sci U S A 103:19902-19907.

Lipton SA, Rosenberg PA (1994) Excitatory amino acids as a final common pathway for neurologic disorders. N Engl J Med 330:613-622.

Losi G, Prybylowski K, Fu Z, Luo J, Wenthold RJ, Vicini S (2003) PSD-95 regulates NMDA receptors in developing cerebellar granule neurons of the rat. J Physiol 548:21-29.

Lu WY, Jackson MF, Bai D, Orser BA, MacDonald JF (2000) In CA1 pyramidal neurons of the hippocampus protein kinase C regulates calcium-dependent inactivation of NMDA receptors. J Neurosci 20:4452-4461.

Lu WY, Xiong ZG, Lei S, Orser BA, Dudek E, Browning MD, MacDonald JF (1999) G-protein-coupled receptors act via protein kinase C and Src to regulate NMDA receptors. Nat Neurosci 2:331-338.

Lynch DR, Guttmann RP (2002) Excitotoxicity: perspectives based on N-methyl-D-aspartate receptor subtypes. J Pharmacol Exp Ther 300:717-723.

Malenka RC (1994) Synaptic plasticity in the hippocampus: LTP and LTD. Cell 78:535-538.

Malenka RC, Nicoll RA (1999) Long-term potentiation--a decade of progress? Science 285:1870-1874.

Malenka RC, Bear MF (2004) LTP and LTD: an embarrassment of riches. Neuron 44:5-21.

Malenka RC, Kauer JA, Perkel DJ, Mauk MD, Kelly PT, Nicoll RA, Waxham MN (1989) An essential role for postsynaptic calmodulin and protein kinase activity in long-term potentiation. Nature 340:554-557.

Markram H, Segal M (1990) Acetylcholine potentiates responses to N-methyl-D-aspartate in the rat hippocampus. Neurosci Lett 113:62-65.

Mayer ML, Vyklicky L, Jr., Clements J (1989) Regulation of NMDA receptor desensitization in mouse hippocampal neurons by glycine. Nature 338:425-427.

McBain CJ, Mayer ML (1994) N-methyl-D-aspartic acid receptor structure and function. Physiol Rev 74:723-760.

Migaud M, Charlesworth P, Dempster M, Webster LC, Watabe AM, Makhinson M, He Y, Ramsay MF, Morris RG, Morrison JH, O'Dell TJ, Grant SG (1998) Enhanced long-term potentiation and impaired learning in mice with mutant postsynaptic density-95 protein. Nature 396:433-439.

Monyer H, Burnashev N, Laurie DJ, Sakmann B, Seeburg PH (1994) Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. Neuron 12:529-540.

Mu Y, Otsuka T, Horton AC, Scott DB, Ehlers MD (2003) Activity-dependent mRNA splicing controls ER export and synaptic delivery of NMDA receptors. Neuron 40:581-594.

Mulkey RM, Malenka RC (1992) Mechanisms underlying induction of homosynaptic long-term depression in area CA1 of the hippocampus. Neuron 9:967-975.

Muller BM, Kistner U, Veh RW, Cases-Langhoff C, Becker B, Gundelfinger ED, Garner CC (1995) Molecular characterization and spatial distribution of SAP97, a novel presynaptic protein homologous to SAP90 and the Drosophila discs-large tumor suppressor protein. J Neurosci 15:2354-2366.

Nakagawa T, Futai K, Lashuel HA, Lo I, Okamoto K, Walz T, Hayashi Y, Sheng M (2004) Quaternary structure, protein dynamics, and synaptic function of SAP97 controlled by L27 domain interactions. Neuron 44:453-467.

Nehring RB, Wischmeyer E, Doring F, Veh RW, Sheng M, Karschin A (2000) Neuronal inwardly rectifying K(+) channels differentially couple to PDZ proteins of the PSD-95/SAP90 family. J Neurosci 20:156-162.

Okabe S, Miwa A, Okado H (1999) Alternative splicing of the C-terminal domain regulates cell surface expression of the NMDA receptor NR1 subunit. J Neurosci 19:7781-7792.

Ozawa S (1998) [Ca2+ permeation through the ionotropic glutamate receptor]. Tanpakushitsu Kakusan Koso 43:1589-1595.

Ozawa S, Kamiya H, Tsuzuki K (1998) Glutamate receptors in the mammalian central nervous system. Prog Neurobiol 54:581-618.

Paoletti P, Ascher P, Neyton J (1997) High-affinity zinc inhibition of NMDA NR1-NR2A receptors. J Neurosci 17:5711-5725.

Parker PJ, Murray-Rust J (2004) PKC at a glance. J Cell Sci 117:131-132.

Passafaro M, Sala C, Niethammer M, Sheng M (1999) Microtubule binding by CRIPT and its potential role in the synaptic clustering of PSD-95. Nat Neurosci 2:1063-1069.

Philpot BD, Espinosa JS, Bear MF (2003) Evidence for altered NMDA receptor function as a basis for metaplasticity in visual cortex. J Neurosci 23:5583-5588.

Premkumar LS, Auerbach A (1997) Stoichiometry of recombinant N-methyl-D-aspartate receptor channels inferred from single-channel current patterns. J Gen Physiol 110:485-502.

Prybylowski K, Chang K, Sans N, Kan L, Vicini S, Wenthold RJ (2005) The synaptic localization of NR2B-containing NMDA receptors is controlled by interactions with PDZ proteins and AP-2. Neuron 47:845-857.

Prybylowski KL, Wolfe BB (2000) Developmental differences in alternative splicing of the NR1 protein in rat cortex and cerebellum. Brain Res Dev Brain Res 123:143-150.

Raman IM, Tong G, Jahr CE (1996) Beta-adrenergic regulation of synaptic NMDA receptors by cAMP-dependent protein kinase. Neuron 16:415-421.

Rao A, Kim E, Sheng M, Craig AM (1998) Heterogeneity in the molecular composition of excitatory postsynaptic sites during development of hippocampal neurons in culture. J Neurosci 18:1217-1229.

Raymond LA, Moshaver A, Tingley WG, Huganir RL (1996) Glutamate receptor ion channel properties predict vulnerability to cytotoxicity in a transfected nonneuronal cell line. Mol Cell Neurosci 7:102-115.

Roche KW, Standley S, McCallum J, Dune Ly C, Ehlers MD, Wenthold RJ (2001) Molecular determinants of NMDA receptor internalization. Nat Neurosci 4:794-802.

Rosenmund C, Westbrook GL (1993) Calcium-induced actin depolymerization reduces NMDA channel activity. Neuron 10:805-814.

Rumbaugh G, Prybylowski K, Wang JF, Vicini S (2000) Exon 5 and spermine regulate deactivation of NMDA receptor subtypes. J Neurophysiol 83:1300-1306.

Rumbaugh G, Sia GM, Garner CC, Huganir RL (2003) Synapse-associated protein-97 isoform-specific regulation of surface AMPA receptors and synaptic function in cultured neurons. J Neurosci 23:4567-4576.

Sans N, Petralia RS, Wang YX, Blahos J, 2nd, Hell JW, Wenthold RJ (2000) A developmental change in NMDA receptor-associated proteins at hippocampal synapses. J Neurosci 20:1260-1271.

Sans N, Prybylowski K, Petralia RS, Chang K, Wang YX, Racca C, Vicini S, Wenthold RJ (2003) NMDA receptor trafficking through an interaction between PDZ proteins and the exocyst complex. Nat Cell Biol 5:520-530.

Sasaki YF, Rothe T, Premkumar LS, Das S, Cui J, Talantova MV, Wong HK, Gong X, Chan SF, Zhang D, Nakanishi N, Sucher NJ, Lipton SA (2002) Characterization and comparison of the NR3A subunit of the NMDA receptor in recombinant systems and primary cortical neurons. J Neurophysiol 87:2052-2063.

Sattler R, Xiong Z, Lu WY, Hafner M, MacDonald JF, Tymianski M (1999) Specific coupling of NMDA receptor activation to nitric oxide neurotoxicity by PSD-95 protein. Science 284:1845-1848.

Schluter OM, Xu W, Malenka RC (2006) Alternative N-terminal domains of PSD-95 and SAP97 govern activity-dependent regulation of synaptic AMPA receptor function. Neuron 51:99-111.

Schnell E, Sizemore M, Karimzadegan S, Chen L, Bredt DS, Nicoll RA (2002) Direct interactions between PSD-95 and stargazin control synaptic AMPA receptor number. Proc Natl Acad Sci U S A 99:13902-13907.

Scott DB, Blanpied TA, Ehlers MD (2003) Coordinated PKA and PKC phosphorylation suppresses RXR-mediated ER retention and regulates the surface delivery of NMDA receptors. Neuropharmacology 45:755-767.

Scott DB, Blanpied TA, Swanson GT, Zhang C, Ehlers MD (2001) An NMDA receptor ER retention signal regulated by phosphorylation and alternative splicing. J Neurosci 21:3063-3072.

Sessoms-Sikes S, Honse Y, Lovinger DM, Colbran RJ (2005) CaMKIIalpha enhances the desensitization of NR2B-containing NMDA receptors by an autophosphorylation-dependent mechanism. Mol Cell Neurosci 29:139-147.

Sheng M, Pak DT (2000) Ligand-gated ion channel interactions with cytoskeletal and signaling proteins. Annu Rev Physiol 62:755-778.

Sprengel R, Suchanek B, Amico C, Brusa R, Burnashev N, Rozov A, Hvalby O, Jensen V, Paulsen O, Andersen P, Kim JJ, Thompson RF, Sun W, Webster LC, Grant SG, Eilers J, Konnerth A, Li J, McNamara JO, Seeburg PH (1998) Importance of the intracellular domain of NR2 subunits for NMDA receptor function in vivo. Cell 92:279-289.

Steigerwald F, Schulz TW, Schenker LT, Kennedy MB, Seeburg PH, Kohr G (2000) C-Terminal truncation of NR2A subunits impairs synaptic but not extrasynaptic localization of NMDA receptors. J Neurosci 20:4573-4581.

Stocca G, Vicini S (1998) Increased contribution of NR2A subunit to synaptic NMDA receptors in developing rat cortical neurons. J Physiol 507 (Pt 1):13-24.

Suchanek B, Seeburg PH, Sprengel R (1995) Gene structure of the murine N-methyl D-aspartate receptor subunit NR2C. J Biol Chem 270:41-44.

Swope SL, Moss SJ, Raymond LA, Huganir RL (1999) Regulation of ligand-gated ion channels by protein phosphorylation. Adv Second Messenger Phosphoprotein Res 33:49-78.

Tao YX, Rumbaugh G, Wang GD, Petralia RS, Zhao C, Kauer FW, Tao F, Zhuo M, Wenthold RJ, Raja SN, Huganir RL, Bredt DS, Johns RA (2003) Impaired NMDA receptor-mediated postsynaptic function and blunted NMDA receptor-dependent persistent pain in mice lacking postsynaptic density-93 protein. J Neurosci 23:6703-6712.

Thomas CG, Miller AJ, Westbrook GL (2006) Synaptic and extrasynaptic NMDA receptor NR2 subunits in cultured hippocampal neurons. J Neurophysiol 95:1727-1734.

Tingley WG, Ehlers MD, Kameyama K, Doherty C, Ptak JB, Riley CT, Huganir RL (1997) Characterization of protein kinase A and protein kinase C phosphorylation of the N-methyl-D-aspartate receptor NR1 subunit using phosphorylation site-specific antibodies. J Biol Chem 272:5157-5166.

Tong G, Jahr CE (1994) Regulation of glycine-insensitive desensitization of the NMDA receptor in outside-out patches. J Neurophysiol 72:754-761.

Tong G, Shepherd D, Jahr CE (1995) Synaptic desensitization of NMDA receptors by calcineurin. Science 267:1510-1512.

Tovar KR, Westbrook GL (1999) The incorporation of NMDA receptors with a distinct subunit composition at nascent hippocampal synapses in vitro. J Neurosci 19:4180-4188.

Traynelis SF, Hartley M, Heinemann SF (1995) Control of proton sensitivity of the NMDA receptor by RNA splicing and polyamines. Science 268:873-876.

Traynelis SF, Burgess MF, Zheng F, Lyuboslavsky P, Powers JL (1998) Control of voltage-independent zinc inhibition of NMDA receptors by the NR1 subunit. J Neurosci 18:6163-6175.

Valtschanoff JG, Burette A, Davare MA, Leonard AS, Hell JW, Weinberg RJ (2000) SAP97 concentrates at the postsynaptic density in cerebral cortex. Eur J Neurosci 12:3605-3614.

Vicini S, Wang JF, Li JH, Zhu WJ, Wang YH, Luo JH, Wolfe BB, Grayson DR (1998) Functional and pharmacological differences between recombinant N-methyl-D-aspartate receptors. J Neurophysiol 79:555-566.

Villarroel A, Regalado MP, Lerma J (1998) Glycine-independent NMDA receptor desensitization: localization of structural determinants. Neuron 20:329-339.

Wagey R, Hu J, Pelech SL, Raymond LA, Krieger C (2001) Modulation of NMDA-mediated excitotoxicity by protein kinase C. J Neurochem 78:715-726.

Waxman EA, Lynch DR (2005) N-methyl-D-aspartate receptor subtypes: multiple roles in excitotoxicity and neurological disease. Neuroscientist 11:37-49.

Williams K, Russell SL, Shen YM, Molinoff PB (1993) Developmental switch in the expression of NMDA receptors occurs in vivo and in vitro. Neuron 10:267-278.

Wyszynski M, Lin J, Rao A, Nigh E, Beggs AH, Craig AM, Sheng M (1997) Competitive binding of alpha-actinin and calmodulin to the NMDA receptor. Nature 385:439-442.

Xiong ZG, Raouf R, Lu WY, Wang LY, Orser BA, Dudek EM, Browning MD, MacDonald JF (1998) Regulation of N-methyl-D-aspartate receptor function by constitutively active protein kinase C. Mol Pharmacol 54:1055-1063.

Yano S, Tokumitsu H, Soderling TR (1998) Calcium promotes cell survival through CaM-K kinase activation of the protein-kinase-B pathway. Nature 396:584-587.

Zhang S, Ehlers MD, Bernhardt JP, Su CT, Huganir RL (1998) Calmodulin mediates calcium-dependent inactivation of N-methyl-D-aspartate receptors. Neuron 21:443-453.

Zheng F, Gingrich MB, Traynelis SF, Conn PJ (1998) Tyrosine kinase potentiates NMDA receptor currents by reducing tonic zinc inhibition. Nat Neurosci 1:185-191.

Zheng F, Erreger K, Low CM, Banke T, Lee CJ, Conn PJ, Traynelis SF (2001) Allosteric interaction between the amino terminal domain and the ligand binding domain of NR2A. Nat Neurosci 4:894-901.

Zheng X, Zhang L, Wang AP, Bennett MV, Zukin RS (1999) Protein kinase C potentiation of N-methyl-D-aspartate receptor activity is not mediated by phosphorylation of N-methyl-D-aspartate receptor subunits. Proc Natl Acad Sci U S A 96:15262-15267.

Zhong J, Russell SL, Pritchett DB, Molinoff PB, Williams K (1994) Expression of mRNAs encoding subunits of the N-methyl-D-aspartate receptor in cultured cortical neurons. Mol Pharmacol 45:846-853.

Zhou M, Baudry M (2006) Developmental changes in NMDA neurotoxicity reflect developmental changes in subunit composition of NMDA receptors. J Neurosci 26:2956-2963.