STUDIES ON THE ROLE OF THE N-TERMINAL DOMAIN OF NR1 IN THE REDOX MODULATION OF NR2A-CONTAINING NMDA RECEPTORS

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The NMDA receptor is the subject of intense study due to its critical role in many neuronal processes and neuropathologies. This receptor is modulated by a wide variety of endogenous and exogenous agents, including reducing and oxidizing (redox) agents. Despite a wealth of physiological information, details of the structural basis of modulation are only beginning to emerge. It has been proposed that the amino terminal domain (ATD) of NMDA receptor subunits may serve as a modulatory domain, as several agents appear to have sites of action in this region of the receptor. NR1/NR2A receptors contain cysteines in the ATD of both NR1 and NR2 that confer unique redox sensitivity to these receptors; however, the ATD redox sensitivity of NR1/NR2A receptors remains largely unexplored. The goal of this dissertation was to explore the impact of reducing and oxidizing agents on NMDA receptor function, focusing on the amino terminal domain redox sites. Here we demonstrate that a clinically efficacious neuroprotective agent, ebselen, is active as an oxidizing agent of the NMDA receptor. Additionally, these studies demonstrate a novel modulation of NR1/NR2A redox mutants by the polyamine spermine and explore a relationship between redox and spermine modulation of NR1/NR2A mutant receptors.
Dedication and Acknowledgements

This work is dedicated to my mother, Karen Sue Seals Herin (1946-2001). The losing battle she fought against leukemia was the most profound experience of my graduate training. The price that Mom paid in enduring the rigors of chemotherapy and hospitalization in order to grant us a few more months with her was very high indeed. For that I am very deeply grateful. The apostle Paul writes to the Romans (Chapter 5), "Not only so, but we also rejoice in our sufferings, because we know that suffering produces perseverance; perseverance, character; and character, hope. And hope does not disappoint us." Without my mother's loving influence, I could not have developed the tenacity required for undertaking a dissertation.

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The study of the brain is no less than the search for the composition of our souls. Neurobiologists, educators, and social scholars furiously debate the relative importance of nurture versus nature in determining one's abilities. My humble opinion (and that of a few prominent scholars) is that one cannot meaningfully separate talent from training. I am most thankful for the high quality of training I received here in the Department of Neurobiology and the Center for Neuroscience at the University of Pittsburgh. Foremost, I owe a debt of gratitude
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To everyone I have mentioned here: I hope for the opportunity someday to
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1. Introduction

1.1. Composition of NMDA receptors

Ionotropic glutamate receptors (iGluRs) mediate excitatory neurotransmission in the central nervous system through the ligand-induced opening of ion channels (reviewed in Dingledine et al., 1999; McBain and Mayer, 1994). Activation of the NMDA subtype of iGluR has been linked to long-term potentiation and depression, as well as neuronal development (Dingledine et al., 1999). Additionally, overactivation of the NMDA receptor is a well-documented causative factor in pathological processes such as ischemia-induced excitotoxicity (Lee et al., 1999) and seizures (Loscher, 1998). Therefore, there is great scientific and clinical interest in elucidating NMDA receptor structure-function relationships in order to develop novel pharmaceutical strategies aimed at modifying NMDA receptor activity.

Despite intense investigation, many questions remain unanswered regarding NMDA receptor composition, structure, and the molecular mechanisms of gating and modulation. NMDA receptors are most likely tetramers (Schorge and Colquhoun, 2003). The best studied of the NMDA receptors are those composed of NR1 and NR2 subunits. Cloning of NMDA receptor subunits has revealed that the NR1 subunit is a single gene product that has at least eight splice variants, a-h, while the NR2 subunits are the products of four different genes NR2A-NR2D (McBain and Mayer, 1994). Differential expression of NR1 splice variants and NR2 subunits occurs spatially and developmentally, conferring temporal and regional specificity of NMDA receptor composition.
(Monyer et al., 1994; Paupard et al., 1997; Prybylowski et al., 2001). In addition to NR1 and NR2 subunits, the NR3 subunit has been cloned and studied in heterologous expression systems, and may form receptors with unique properties, albeit yet to be extensively characterized (Al-Hallaq et al., 2002; Matsuda et al., 2002; Perez-Otano et al., 2001).

NMDA receptor subunit and splice variant composition determines the sensitivity of receptors to modulation by a wide variety of compounds. Exogenous agents such as ifenprodil (Masuko et al., 1999; Williams, 1993), and cyanide (Arden et al., 1998) have subunit-specific actions on NMDA receptors. More importantly, endogenous agents modulate NMDA receptors at physiological ranges and in a subunit-specific manner (McBain and Mayer, 1994). These modulatory agents include protons (Traynelis et al., 1995), polyamines (Williams, 1997b), zinc (Paoletti et al., 1997; Zheng et al., 2001), and sulfhydryl reducing and oxidizing agents (Aizenman, 1994; Brimecombe et al., 1997).

Homology modeling (Fayyazuddin et al., 2000; Masuko et al., 1999; Zheng et al., 2001) and structural studies (Armstrong et al., 1998; Furukawa and Gouaux, 2003; Jin et al., 2003; Jin et al., 2002) reveal ionotropic glutamate receptor subunits to be modular proteins (Figure 1; Wo and Oswald, 1995). There appear to be discrete ligand binding domains which also bind competitive antagonists such as 5,7-dichlorokyurenate (DCK; Furukawa and Gouaux, 2003) and L(+)-2-Amino-5-phosphonopentanoic acid (L-AP5; Tikhonova et al., 2002). It appears that a pore forming domain in which channel blockers such as Mg (Wollmuth et al., 1998a; Wollmuth et al., 1998b) and MK-801 (Elhallaoui et al.,
bind and inhibit NMDA mediated responses, and an amino terminal domain which has been called a "regulatory domain" by some groups to show that this domain appears to be an important site of binding for allosteric modulators (Masuko et al., 1999).

Figure 1. Schematic of NMDA receptor subunit putative domain structure.
Schematic adapted from (Zheng et al., 2001). NMDA receptor subunits appear to be modular proteins with functional domains. The amino terminal domain (approximately 400 amino acids), ligand-binding domain, pore-forming domain, and carboxyl-terminal domain are illustrated.
1.2. Modulation of NMDA receptor-mediated responses by redox agents

1.2.1. The ligand binding domain-associated redox site of NR1

Physiological responses in neurons mediated by NMDA receptors are potentiated by disulfide reducing agents such as dithiothreitol (DTT; Aizenman et al., 1989; Tang and Aizenman, 1993b). Conversely, sulfhydryl oxidants such as 5,5'-dithio-bis(2-nitrobenzoic acid) (Aizenman et al., 1989; Brimecombe et al., 1997), lipoic acid (Tang and Aizenman, 1993a), and pyrroloquinoline quinone (PQQ; Aizenman et al., 1992) are able to reverse DTT potentiation. Oxidants can also depress NMDA receptor function from baseline levels, depending on the native redox state of the protein (Aizenman et al., 1989; Colton et al., 1989; Gozlan and Ben-Ari, 1995; Sinor et al., 1997). Indeed, it was demonstrated that PQQ, previously shown to be neuroprotective in vitro and in vivo (Aizenman and Reynolds, 1992; Jensen et al., 1994), was able to reverse the chemical reduction of the NMDA receptor redox site that occurred as a result of seizure activity (Sanchez et al., 2000).

In heterologous systems, NMDA receptors containing the NR1 subunit and either the NR2B, NR2C, or NR2D subunits are rendered relatively insensitive to potentiation by reducing agents when NR1a cysteines 744 and 798 are mutated (Sullivan et al., 1994). A high resolution crystal structure of the NR1 ligand-binding domain reveals a hinged clamshell-like structure that closes upon ligand binding (Furukawa and Gouaux, 2003). Cysteines 744 and 798 of NR1 are located at the hinge of the cleft of NR1, and therefore, Furukawa and Gouaux (2003) postulate that the oxidation state of the disulfide bond in the NMDA
receptor determines the flexibility of the hinge. The degree of closure of the ligand-binding clamshell correlates with the activity of the channel (Jin et al., 2003). Hypothetically, when NMDA receptor disulfide bond c744-c798 is oxidized, the flexibility of the ligand-binding hinge is decreased, and closure of the clamshell domain is inhibited. Conversely, in the absence of the disulfide bond, the constraint is relieved, and the clamshell closes more fully.

1.2.2. The Amino Terminal Domain-associated redox sites

Although redox sensitivity is nearly abolished when the mutant NR1 subunit NR1a(c744a,c798a) is co-expressed with NR2B,NR2C, and NR2D receptors, NR1a(c744a,c798a)/NR2A receptors remain sensitive to reducing agents to the same extent as that of wild-type NR1a/NR2A receptors (Brimecombe et al., 1999; Kohr et al., 1994). Choi et al. (2001) demonstrated that the remaining DTT sensitivity of NR1a(c744a,c798a)/NR2A NMDA receptors is attributable to cysteines in the N-terminal domains of both NR1a(c79,c308) and NR2A(c87,c320). Mutation of all six of these cysteines renders NR1/NR2A receptors insensitive to modulation by DTT and the oxidizing agent DTNB (Choi et al., 2001). This implies that that cysteines NR1a(c79,c308) and NR2A(c87, c320) dominate redox sensitivity in wild-type NR1/NR2A receptors.

1.3. Modulation at the ATD domain of NMDA receptor subunits

Interestingly, the amino terminal domain (ATD) of glutamate receptor subunits bears a weak homology to the bacterial amino-acid binding protein
leucine-isoleucine-valine binding protein (LIVBP), which has a clamshell-like structure analogous to the ligand binding domain (Sutcliffe et al., 1996). Modeling and mutagenesis studies have suggested that several modulatory sites are located in the ATD domain. For example, fast desensitization of NMDA receptors is present only in NR1/NR2A recombinant receptors (Zheng et al., 2001). The molecular basis of this desensitization was linked to a high affinity zinc binding site in the ATD in the NR2A subunits (Krupp et al., 1998). There is an allosteric interaction between the zinc binding site in the ATD and the glutamate binding site such that glutamate binding results in a higher affinity of the receptor for zinc (Zheng et al., 2001). Residues that bind zinc in the ATD domain of the NR2A subunit are arranged facing each other across a central cleft, and cysteines engineered into the inside of the cleft are inaccessible to modification by the cysteine modifying agent 2-trimethylammonioethylmethane thiosulfonate (MTSET) in the presence of zinc (Paoletti et al., 2000). This suggests that zinc binding in the cleft of this domain induces a molecular "motion" that transduces the allosteric effect of zinc.

Phenylethanolamines such as ifenprodil inhibit NMDA responses in an NR2B-specific manner (Dingledine et al., 1999). Data suggest that the ifenprodil binding site may be located in the ATD domain of NR2B (Paoletti et al., 2000; Perin-Dureau, 2001; Perin-Dureau et al., 2002; but see Masuko et al., 1999). Ifenprodil produces desensitization of NR1/NR2B receptors in a manner that is analogous to zinc-induced desensitization of NR1/NR2A receptors (Zheng et al., 2001). Mutagenesis studies based on the sequence alignment of NR2B and
LIVBP reveal that residues critical to ifenprodil inhibition are located in the cleft of the clamshell-like domain (Perin-Dureau, 2001; Perin-Dureau et al., 2002). This evidence regarding zinc and ifenprodil is highly suggestive that binding of modulatory compounds to the ATD induces a conformational change that allosterically affects the ligand binding domain and ultimately the gating mechanism (Masuko et al., 1999; Paoletti et al., 2000; Perin-Dureau et al., 2002; Zheng et al., 2001).

Although ligands for ATD domains in NR2A and NR2B have been suggested, potential modulatory agents that bind to the analogous region of NR1 are controversial. A potential ligand of the ATD-domain of NR1 is the endogenous polyamine spermine. Spermine has several effects on the NMDA receptor (Williams, 1997b). These include voltage-dependent channel block at high concentrations of spermine as well as current enhancement due to an increase in the affinity of the receptor for glycine. At low concentrations of spermine (3-100 µM) and saturating concentrations of glycine, an additional enhancement of NMDA-induced currents is observed in NR1/NR2B receptors, simply referred to as spermine potentiation (Williams, 1997b). Mutation of residues in the N-terminus of NR1 that block spermine potentiation, concomitantly decreases proton inhibition (Masuko et al., 1999; Williams, 1997b). This is in agreement with earlier work demonstrating pharmacologically that spermine potentiation of NR1/NR2B receptors was via relief of proton inhibition (Traynelis et al., 1995).
Interestingly, NMDA-mediated currents are inhibited by protons with an IC$_{50}$ that corresponds to physiological pH (Traynelis and Cull-Candy, 1990; Traynelis and Cull-Candy, 1991). This implies that variations in extracellular pH not only can have a profound effect on NMDA receptor function *per se*, but can also dramatically alter pH-dependent modulation of the receptor by polyamines and other modulatory agents. Recent studies have suggested that the structural components of pH sensitivity are localized very close to the putative gate in NR1 and NR2 subunits (Low *et al.*, 2003). This might explain the common pH dependence of many agents that act at the ATD of NMDA receptor subunits. Potentially, conformational changes in the ATD may be allosterically linked to gating through a pH sensitive structural element.

Splice variants of the NR1 subunit that contain the 21-amino acid insert exon 5 (NR1b) are not inhibited as strongly by protons (Traynelis *et al.*, 1995). Exon 5 contains several positively charged residues that may resemble the structure of spermine (Traynelis *et al.*, 1995). Additionally, a model of the predicted secondary structure of NR1, based on the structure of LIVBP, places two residues critical for spermine potentiation (e181,e185) very close to the site of the exon 5 insert, supporting the evidence that exon 5 masks or constitutively occupies a spermine binding site in the NR1 ATD domain.

A growing body of work reveals that modulators of NMDA receptor function acting at ATD modulatory sites have pleiotropic allosteric interactions with each other. Zinc inhibits NR1/NR2A receptors at very low concentrations by enhancing proton inhibition (Choi and Lipton, 1999; Low *et al.*, 2000; Paoletti *et
Amino acid substitutions in the cleft of the ATD domain of NR2A that most strongly affect zinc binding are also critical to pH dependency of zinc inhibition (Low et al., 2000). This implies that the zinc binding domains may interact functionally and/or structurally with the putative proton sensor.

It is believed that ifenprodil and derivatives bind to the cleft of the ATD domain of NR2B (Perin-Dureau, 2001) and enhance proton inhibition of the receptor (Mott et al., 1998). Additionally, there appears to be a negative allosteric interaction between ifenprodil and spermine, such that binding of spermine to the NMDA receptor results in a reduction of affinity for ifenprodil (Kew and Kemp, 1998). It is worthy to note that concentrations of spermine (1 and 3 mM) used in the Kew and Kemp (1998) study have been shown to inhibit NMDA receptors in a voltage-dependent manner (Williams, 1997b). Therefore it is unclear whether the actions of spermine in this study are due to the binding of spermine to a site in the ATD domain, in the channel pore itself, or due to an unspecified mechanism. However, Mott et al., (1998) show that the presence of 100 μM spermine reduced inhibition of NR1/NR2B receptors by the ifenprodil analogue CP101,606 by 44-fold.

Several themes emerge from examination of ATD-associated modulation of the NMDA receptor. (1) Inhibitory agents such as zinc and ifenprodil produce an incomplete block of receptor function. This may be because their site of action is remote from the ligand-binding domains and the gating element, and therefore these agents have an allosteric mechanism of action. (2) Inhibition by
protons is integral to modulation by ifenprodil, spermine, and zinc. It is likely that each of these agents is acting upstream of a proton-sensitive element at or near the gate of the channel. (3) Most modulatory agents display subunit specificity. Zinc inhibition is present only in NR1/NR2A receptors. Ifenprodil inhibition is present only in NR1/NR2B receptors, and glycine independent spermine potentiation has previously only been observed in this receptor configuration. (4) There are numerous interactions between modulatory sites within the ATD domain, suggesting that macroscopic effects of NMDA receptor modulation may reflect the composite contributions of several different modulatory sites.

1.3.1. Interaction between redox modulation and other modulatory agents

Mutagenesis studies suggest that residues important for redox modulation of NMDA receptors are also required for modulation of NMDA receptors by other agents. This is suggestive of an allosteric interaction between redox sensitive moieties and other modulatory sites within the ATD region. There are several properties of NMDA receptor redox site mutants that support this hypothesis. For example: The NR1a(c79s,c308s,c744a,c798a)/NR2A(c87a,c320a) mutation lacks high affinity zinc block (Choi et al., 2001). Mutation of each of the putative cysteine pairs has a cumulative effect on zinc inhibition (Choi et al., 2001). This implies at least two possibilities: First, that the redox sites of the ATD domain have an effect on the allosteric modulation of NR1/NR2A receptors by zinc. Second, redox modulation of each subunit does not necessarily impact solely the conformation of its own domain, i.e., if zinc binds to the ATD of NR2A, and the NR2A ATD domain cysteines allosterically modulate zinc binding in the same
domain, then one would predict that NR2A(c87a,c320a)-containing receptors would be far less modulated by zinc than NMDA receptors containing other cysteine mutations. This is not the case, implying that the redox state of each redox "site" has a more global allosteric effect. (Choi et al., 2001).

Additional studies of redox mutants suggests allosteric interactions between redox sites and other modulatory sites. Mutation of the ligand-binding domain redox site residues in the relatively redox insensitive NR1a(c744a/c798a)/NR2B receptor abolishes spermine potentiation and proton inhibition (Sullivan et al., 1994). Mutation of c744 and c798 on NR1 also decreases ifenprodil inhibition in NR1/2B receptors (Mott et al., 1998).

Several thorough studies have been performed to elucidate the molecular mechanisms of NMDA receptor modulation by zinc, ifenprodil, spermine, exon 5, and protons at the ATD domains on NMDA receptor subunits (Masuko et al., 1999; Paoletti et al., 2000; Zheng et al., 2001). The presence of redox sensitive cysteines in the same domain has been suggested (Choi and Lipton, 1999), however, very little is known regarding the impact of reducing and oxidizing agents on the ATD domains in NR1/NR2A receptors, and possible interactions among ATD cysteines and other modulatory agents of NMDA receptors. The studies contained herein characterize the actions of an exogenous redox modulator of NMDA receptor, in addition to studies aimed at determining potential structural features of redox modulation of NR1/NR2A NMDA receptors.
2. Oxidation of NMDA receptors by the anti-oxidant drug ebselen

2.1. Abstract

Ebselen is a seleno-organic compound currently in clinical trials for the treatment of ischemic stroke and subarachnoid hemorrhage. Its putative mode of action as a neuroprotectant is via cyclical reduction and oxidation reactions, in a manner akin to glutathione peroxidase. For this reason, we have investigated the effects of ebselen on the redox-sensitive NMDA receptor. We have found that ebselen readily reversed dithiothreitol (DTT) potentiation of NMDA-mediated currents in cultured neurons and in Chinese hamster ovary (CHO) cells expressing wild-type NMDA NR1/NR2B receptors. In contrast, ebselen was unable to modulate NMDA-induced currents in neurons previously exposed to the thiol oxidant 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), or in CHO cells expressing a mutant receptor lacking the NR1 redox modulatory site, suggesting that ebselen oxidizes the NMDA receptor via this site. In addition, ebselen was substantially less effective in modifying NMDA responses in neurons exposed to alkylating agent n-ethylmaleimide (NEM) following DTT treatment. Ebselen also reversed DTT inhibition of carbachol-mediated currents in Cos-7 cells expressing the αβγδ subunits of the acetylcholine receptor, an additional redox-sensitive ion channel. Ebselen was observed to significantly increase cell viability following a 30 min NMDA exposure in cultured neurons. In contrast, other more typical antioxidant compounds did not afford neuroprotection in a similar paradigm. We conclude that ebselen may be neuroprotective in part due to its actions as a modulator of the NMDA receptor redox modulatory site.
2.2. Introduction

Ebselen (2-phenyl-1, 2-benzisoselenazol-3[2H]-one) is a seleno-organic compound that has been shown to be cytoprotective in various ischemia-reperfusion models in both heart (Maulik et al., 1998) and brain (Takasago et al., 1997). Ebselen is currently in clinical trials for the treatment of ischemic stroke (Yamaguchi et al., 1998) and aneurysmal subarachnoid hemorrhage (Saito et al., 1998). Although generally considered to be an antioxidant, ebselen is not a free radical scavenger per se, but mimics the enzyme glutathione peroxidase (Mueller et al., 1984; Sies, 1993; Wendel et al., 1984) and a closely related enzyme, phospholipid hydroperoxide glutathione peroxidase (Maiorino et al., 1988). Ebselen, like these enzymes, acts with glutathione through a selenium core to eliminate hydroperoxides and lipoperoxides. Although the mechanism of ebselen is similar to these macromolecules, ebselen does not have a substrate-binding site, and therefore, has a much wider range of specificity (Schewe, 1995; Sies, 1993). Importantly, ebselen can interact with cysteine residues contained in proteins such as metallothionein (Jacob et al., 1998), glutathione-S-transferase, (Nikawa et al., 1994), and the IP3 receptor (Dimmeler et al., 1991), leading to thiol oxidation and formation of disulfides and an ebselen diselenide product (Schewe, 1995).

Several ligand-gated ion channels contain labile cysteine residues that are modified by redox-active compounds. These include the nicotinic acetylcholine receptor (Karlin and Bartels, 1966), the GABA_A receptor (Pan et al., 1995), and the NMDA subtype of glutamate receptor (Aizenman et al., 1989; Tang and Aizenman, 1993b). Of these, a physiological role for redox modulation has been
evaluated extensively on the NMDA receptor (Aizenman, 1994; Aizenman et al., 1998), due to the importance of this protein in such processes as neuronal development, learning and memory, and excitotoxicity (Dingledine et al., 1999). Physiological responses in neurons mediated by NMDA receptors are potentiated by disulfide reducing agents such as DTT (Aizenman et al., 1989; Tang and Aizenman, 1993b). Conversely, sulfhydryl oxidants such as 5,5'-dithiobis(2-nitrobenzoic acid) (Aizenman et al., 1989), lipoic acid (Tang and Aizenman, 1993a), and PQQ (Aizenman et al., 1992) are able to reverse DTT potentiation. Oxidants can also depress NMDA receptor function from baseline levels, depending on the native redox state of the receptor (Aizenman et al., 1989; Colton et al., 1989; Gozlan et al., 1994; Sinor et al., 1997). Indeed, it was recently demonstrated that PQQ, previously shown to be neuroprotective in vitro and in vivo (Aizenman et al., 1992; Jensen et al., 1994), was able to reverse the chemical reduction of the NMDA receptor redox site that occurred as a result of seizure activity (Sanchez et al., 2000). This condition appeared to induce the release of yet unidentified endogenous reducing agents (Sanchez et al., 2000). As ebselen can alter the function of various proteins via thiol oxidation, we investigated whether this drug could also interact with the redox-sensitive NMDA receptor to modulate its function. Such an interaction would be suggestive of a novel mechanism of ebselen in altering glutamatergic synaptic transmission and associated pathophysiologies.
2.3. Materials and Methods

2.3.1. Tissue Culture

Cortical neurons were dissociated from E16 Sprague-Dawley rats as described previously (Hartnett et al., 1997). Cortices were dissociated in Earle’s balanced salt solution (EBSS) with 0.03% trypsin at 37°C. Plating suspension was adjusted to a density of 335,000 cells/ml growth media. Growth media contained 80% (v/v) Dulbecco’s Modified Eagle Medium (DMEM; Gibco BRL, Rockville, MD) with 10% heat-inactivated, iron-supplemented bovine calf serum (Hyclone, Logan, UT), 10% Ham’s F-12 media (Sigma), 25 mM HEPES, 24 U/ml penicillin, 24 U/ml streptomycin, and 2 mM L-glutamine. Dissociated cells were plated in 6-well plates containing five 12 mm glass coverslips that had been previously treated with poly-L-lysine. Cells were refed on a Monday-Wednesday-Friday basis and maintained in 37°C, 5% CO₂. Two weeks after plating, non-neuronal cell growth was arrested with a 72-hour treatment with 2 µM cytosine arabinoside, after which the growth media contained only 2% serum and no F-12. Cells were used for electrophysiology experiments in the fourth week after dissociation.

For toxicity experiments, forebrain neuronal-enriched cultures were prepared as previously described (Aizenman et al., 2000). Dissociated cells from E17 rat fetuses were plated on poly-L-ornithine-treated tissue culture plates in a growth medium containing 80% DMEM (high glucose with L-glutamine and without sodium pyruvate; Gibco BRL), 10% Ham’s F-12 nutrients, and 10% heat-inactivated bovine calf serum, and 1x anti-mycotic/anti-biotic mixture with amphotericin B and streptomycin sulfate (Gibco BRL). Cultures were maintained
at 37°C in 5% CO₂. Glial cell proliferation was inhibited after 48 hours in culture
with 1-2 µM cytosine arabinoside. Serum-containing medium was replaced after
three days in vitro with a serum-free medium containing Neurobasal medium
(without L-glutamine; Gibco BRL), B27 supplement (Gibco BRL), and anti-
mycotic/anti-biotic mixture as above.

2.3.2. Heterologous Expression Systems

Chinese hamster ovary (CHO) cells were grown in Ham’s F-12 media
containing 10% Fetal Bovine Serum (FBS) and 2 mM L-glutamine. Cells were
passaged (less than 30 times) at a 1:10 dilution when 80% confluent, usually
every two days. The cDNAs for NMDA receptor subunits had been previously
ligated into mammalian expression vectors containing the cytomegalovirus
promoter (Boeckman and Aizenman, 1996; Brimecombe et al., 1999). CHO cells
were seeded at 2.8x10⁵ cells/well into 6-well plates 24 hours previous to
transfection. Cells were transfected in serum-free medium with 6 µL
LipofectAMINE reagent (Gibco BRL) and a total of 1.4 µg of DNA/well. A ratio of
0.3:1:3 Green Fluorescent Protein: NR1: NR2 subunit ratio was employed. Four
hours after transfection, cells were refed with media containing 10% serum, and
24 hours or less after transfection, 300 µM ketamine was added to prevent the
excitotoxic cell death that occurs following functional receptor expression
(Boeckman and Aizenman, 1996).

Cos-7 cells were maintained in DMEM containing 10% FBS and 2 mM L-
glutamine and were passaged at a 1:5 dilution at 70-80% confluency,
approximately every two days. Acetylcholine receptor subunit (α, β, δ and ε)
cDNA in pSM with an SV40 promoter was the kind gift of Dr. Zuo-Zhong Wang (University of Pittsburgh School of Medicine, Pittsburgh, PA). Cos-7 cells were transiently transfected with the following subunit ratios: 1.32 alpha: 0.66 beta: 0.32 delta: 1.00 epsilon. The transfection protocol was similar to that described for CHO cells, without the ketamine addition. Cells were used for recording 40-60 hours after transfection (Gu et al., 1990).

2.3.3. Electrophysiology

Electrophysiological recordings were performed at room temperature (25°C) using the whole-cell configuration of the patch-clamp technique. Cells were bathed in external solution containing (in mM): 150 NaCl, 1.0 CaCl2, 2.8 KCl, 10 HEPES, 10 glycine, 25 tetrodotoxin (Calbiochem, La Jolla, CA) and pH was adjusted to 7.2 with NaOH. Electrodes were pulled on a Sutter P-87 electrode puller (Sutter Instruments, Novato, CA) to a resistance of 1.5-3 MΩ when filled with internal solution containing (in mM): 140 CsF, 10 EGTA/CsOH, 1 CaCl2, and 10 HEPES (pH adjusted to 7.2 with CsOH). Signals were amplified using an Axopatch 200B integrating patch-clamp amplifier (Axon Instruments, Foster City, CA), filtered using an 80 dB/decade filter at 1 kHz, and digitized at 2 kHz with a DigiData 1200b (Axon Instruments) computer interface. The reference electrode was an Ag/AgCl wire bridged with 2M KCl/1% agarose in PE-90 tubing. Drugs were applied via a perfusion system with a stepper motor for fast solution changes (Warner Instruments Corp., Hamden, CT). NMDA, DTT, DTNB, NEM and ebselen (Sigma Chemical Company, St. Louis, MO) were all dissolved in external solution for recording. Ebselen was diluted from a 50 mM
stock solution in DMSO. Data were collected and analyzed using commercially available software (pCLAMP 8, Axon Instruments).

2.3.4. Toxicity Assays

Coverslips containing forebrain neurons were transferred from 6-well plates to 24-well plates and treated in triplicate. Coverslips were maintained in serum-containing media at 37°C until ready for treatment. Cells were gently washed twice with 2 ml of a solution containing minimum essential media (MEM; no phenol red), 0.01% bovine serum albumin (BSA; Sigma), and 25 mM HEPES (MEM/BSA). Drugs were diluted in 1 ml MEM/BSA, and wells were incubated in treatment solutions for the indicated period of time. After treatment, cells were washed as before. Cells were incubated for 20 hours in MEM/BSA, at which point neuronal viability was measured with a lactate dehydrogenase (LDH) -based in vitro toxicology assay kit (Sigma; Hartnett et al., 1997). Media samples (40 µL) were analyzed spectrophotometrically (490:630 nm) according to the manufacturer's protocol, to obtain a measure of cytoplasmic LDH release from dead and dying neurons. Relative toxicity is expressed in optical density (O.D.) units.

2.4. Results

2.4.1. Ebselen modifies NMDA receptor function

Whole-cell responses to 30 µM NMDA were recorded at –60 mV under control conditions, after a 3-min application of 4 mM DTT, and following a 30-sec incubation in varying concentrations of ebselen. Ebselen was able to readily reverse DTT-mediated NMDA current potentiation in a concentration-dependent
manner (Figure 2A). At higher concentrations of ebselen (10-30 µM), post-
ebselen currents were sometimes smaller than control responses (i.e. before
DTT treatment). Cells were treated further with repeated iterations of this
protocol; DTT and ebselen were able to exert opposing effects on each cell for as
long as the recording configuration was held. Furthermore, the effects of ebselen
in depressing NMDA-induced currents could only be reversed by an additional
DTT treatment. This effect of ebselen closely resembles the actions of the
oxidizing agent DTNB (Aizenman et al., 1989; Tang and Aizenman, 1993b),
implying that ebselen is able to oxidize the redox site of the NMDA receptor. We
noted that incubations with ebselen at concentrations in the 100-300 µM range
quickly destabilized the recordings. Therefore, we were unable to determine
whether this compound could oxidize the NMDA receptor to the same extent as
DTNB (500 µM; Figure 2B). Nonetheless, pre-treatment with DTNB (500 µM)
occluded any potentially additional actions of ebselen (30 µM), further
strengthening the notion that the seleno-organic compound acts via the NMDA
receptor redox site (Figure 2C).

An additional set of experiments confirmed that ebselen did not alter
membrane conductance by itself or directly antagonize NMDA responses. No
currents were observed during a 2 min application of 10 µM ebselen (not shown).
In addition, 10 µM ebselen co-applied during application of 30 µM NMDA did not
appreciably alter whole cell responses (Figure 3).
Figure 2. Ebselen reverses NMDA-induced whole-cell current potentiation by the reducing agent DTT.

A: Representative whole-cell recordings from a cortical neuron during activation by 30 µM NMDA at –60 mV. Cells were bathed in 4 mM DTT for 3 min, which potentiated NMDA currents by an average of two-fold. Immediately following DTT, cells were treated with 10 µM ebselen for 30 sec, reversing the actions of DTT. Bar depicting NMDA application is only shown in the first trace in this and subsequent figures for clarity. B: Whole-cell responses to 30 µM NMDA from a cell previously exposed to 4 mM DTT, and following a 30 s exposure to 500 µM
DTNB, and following a wash period, to an additional 30 s treatment with 30 µM ebselen. Note that ebselen does not alter the amplitude of the currents after prior oxidation with DTNB. Similar observations were made in a total of 6 cells.

C: Reversal of DTT potentiation is concentration dependent. Each bar of the histogram represents the mean ratio (± S.E.M. n=4 to 8) between the peak potentiated current (3 min DTT) and the peak current after 30 sec treatment with wash, ebselen, or 500 µM of the oxidizing agent DTNB. A Kruskal-Wallis test revealed a significant treatment effect by ebselen (p< 0.05). Controls utilizing the maximal concentration of DMSO used (0.06%) had no effect on the currents.
Figure 3.  Ebselen does not directly antagonize NMDA receptors.  

**A**: NMDA (30 µM)-induced current elicited from a 4-week old cortical neuron. **B**: When 10 µM ebselen was applied for 4 sec during application of NMDA, ebselen did not alter whole cell responses to agonist. Similar results were obtained in a total of 5 cells.
2.4.2. Ebselen is unable to modulate NR1a(c744a,c798a)/NR2B receptors

To confirm that ebselen reversed the actions of DTT in neuronal receptors by oxidizing the known redox-sensitive site on the NR1 subunit (Sullivan et al., 1994), we transiently expressed cDNAs for both wild-type (NR1/NR2B) and a redox-insensitive double cysteine mutant (NR1a(c744a, c798a)/NR2B; Brimecombe et al., 1999; Sullivan et al., 1994) NMDA receptor in CHO cells. Currents recorded from cells expressing NR1/NR2B NMDA receptors showed properties similar to those obtained from cultured neurons. DTT potentiation was approximately 1.5-2 -fold, and a 30-sec application of ebselen (10-30 µM) reversed this potentiation (Figure 4A). However, in NR1a(c744a,c798a)/NR2B expressing cells, little or no DTT potentiation was observed, and ebselen did not appreciably alter the magnitude of currents (Figure 4B). The lack of effect of ebselen in this redox-insensitive NMDA receptor configuration strongly implicates cysteines 744 and 798 in the modulation of NMDA receptor function by this compound. In an additional set of studies, we incubated neurons with the alkylating agent NEM (300 µM) immediately following a 6 mM DTT treatment. Under these conditions, we have previously shown that the NMDA receptor can be alkylated and rendered relatively insensitive to further reduction or oxidation (Tang and Aizenman, 1993b). Following alkylation, the actions of ebselen in modulating NMDA receptor function were dramatically diminished (Figure 4C).
Figure 4. Modulation of NMDA currents by ebselen requires a functional redox site.

A: Representative whole cell currents recorded from a CHO cell transiently expressing recombinant NR1/NR2B receptors during application of 30 µM
NMDA. Currents were measured under control conditions, following a 3 min application of 4 mM DTT, and a subsequent 30 sec application of 10 µM ebselen. DTT typically potentiated currents 1.5-2 –fold, reversible by ebselen. B: Ebselen has no effect on responses from a CHO cell transfected with NR1a(c744a,c798a)/NR2B receptors, which lack a functional NR1 redox site. Note that DTT does not significantly enhance responses to NMDA, nor does ebselen depress responses from baseline. Similar observations were noted in a total of 9 cells expressing wild-type receptors and in 12 cells expressing the mutated NR1 subunit. C: Treatment with the irreversible alkylating agent N-ethylmaleimide (NEM) causes a permanent potentiation of NMDA-mediated whole-cell currents in cortical neurons. Cells were treated with the reducing agent DTT (6 mM; 6 min), and immediately bathed in 300 µM NEM (1 min), which substantially prevented further peak current modification by ebselen. Changes in the desensitization profile of the response were not reproducible.
2.4.3. Ebselen can oxidize cysteine residues in the nicotinic receptor

We evaluated whether ebselen could modify another redox-sensitive, ligand-gated ion channel. These experiments were performed to confirm that ebselen has no substrate-specificity and that it can readily oxidize cysteine residues in unrelated members of the ligand-gated channel superfamily. Nicotinic acetylcholine receptors (nAChRs) contain a disulfide bond that lies very close to the agonist-binding site of the receptor complex, and reduction of this bond abolishes receptor function (Kao and Karlin, 1986; Karlin and Bartels, 1966). Oxidation can readily restore activity in these channels. Carbachol (10 μM)-induced currents were recorded from Cos-7 cells expressing α2βγδ subunits of the nAChR under control conditions and following incubation with 4 mM DTT (3 min) or 10 μM ebselen. As expected, carbachol-induced currents were unaffected by ebselen (10 μM) but diminished substantially following DTT treatment. Treatment with 10 μM ebselen after DTT returned currents to near control levels (Figure 5). DTNB (500 μM; 7 min) mimicked the actions of ebselen in reversing the effects of DTT (not shown), suggesting that ebselen also acts as an oxidant at the nicotinic receptor.
Figure 5. Ebselen reverses the effects of DTT on carbachol-induced currents in nicotinic acetylcholine receptors.

Responses to 10 μM carbachol obtained from α2βδε recombinant nicotinic acetylcholine receptors transiently expressed in Cos-7 cells were unchanged by a three minute incubation in ebselen prior to treatment with DTT. A subsequent 3 min treatment with 4 mM DTT substantially diminished carbachol-induced currents. Application of 10 μM ebselen (3 min) returned currents to control levels. Similar results were observed in a total of 6 cells.
2.4.4. Ebselen is neuroprotective against NMDA toxicity *in vitro*

We sought to determine whether the interaction of ebselen with the NMDA receptor could protect cells from NMDA in a neurotoxicity paradigm. When applied to neurons overnight, 30 µM ebselen showed no intrinsic toxicity (Figure 6). Exposure of cultured rat cortical neurons to 100 µM NMDA for 30 min caused significant neurotoxicity, reflected as an increase in lactate dehydrogenase release from control. Neuronal toxicity was nearly completely blocked by a co-application with the NMDA receptor blocker MK-801 (10 µM), confirming the NMDA-receptor dependency of the neuronal death observed. Ebselen (10 µM) significantly blocked cell death by approximately 40%, a level of protection similar to what had previously been described for PQQ (Aizenman *et al.*, 1992). In these experiments, ebselen was present 30 min prior to NMDA exposure to ensure that all the receptors were in the oxidized state before activation. Ebselen was also present during and following agonist exposure. The reason for including ebselen following agonist exposure was to eliminate the possibility that secondary glutamate release might obfuscate the neuroprotective actions of ebselen, as this drug does not antagonize the agonist actions of NMDA *per se*. In order to ensure that ebselen induced neuroprotection by oxidizing the NMDA receptor prior to NMDA exposure, we performed additional experiments where 10 µM MK-801 substituted for ebselen in the post-exposure period. Under these conditions, we observed 43% neuroprotection in the ebselen/post-MK-801 treatment group, compared to 19% protection in the post-MK-801 only treatment group (not shown). Hence, the complete neuroprotective effects of ebselen in...
our paradigm are likely mediated by maintenance of the receptors in the oxidized state during the secondary release of glutamate. Ebselen has been shown in several systems to abrogate oxidative injury (Mueller et al., 1984; Sies, 1993; Wendel et al., 1984). In fact, a recent study has suggested that ebselen is protective against glutamate toxicity in cultured rat cerebellar granule cells by acting as an antioxidant (Porciuncula et al., 2001). Therefore, we evaluated whether other, more traditional, antioxidant compounds could also inhibit NMDA toxicity under our exposure conditions. We utilized three different antioxidants: Trolox (100 µM), glutathione methyl ester (1 mM), and N-acetylcysteine (1 mM). None of these agents provided any measurable protection against NMDA-induced neurotoxicity (Figure 6B).
**Figure 6. Ebselen is neuroprotective in an excitotoxicity paradigm.**

**A:** Neuronal cultures were exposed for 30 min to vehicle (control), 100 µM NMDA alone, or in the presence of either 10 µM ebselen or 10 µM MK-801, or ebselen alone. Ebselen was present 30 min prior to, during, and following NMDA exposure. MK-801 was present only during NMDA exposure. The decrease in LDH values from NMDA represents increased neuronal viability, and when normalized against total NMDA toxicity, a significant increase in neuronal survival was observed in the NMDA + ebselen and NMDA + MK801 treatment groups.
(**p < 0.001, ANOVA followed by Bonferroni post-hoc tests). Results represent the mean ± S.D. of a representative experiment performed in triplicate. Similar results were obtained in a total of 6 experiments. B: an additional set of cultures were exposed for 30 min to vehicle (control), 100 µM NMDA alone, or in the presence of 100 µM Trolox, 1 mM glutathione methyl ester (GSH-ME), or 1 mM N-acetylcysteine (NAC). Antioxidants were present 30 min prior to, during, and following NMDA exposure. No observable neuroprotection was afforded by these compounds, nor were they toxic on their own (not shown). Results represent the mean ± S.D. of a representative experiment performed in triplicate. Similar results were obtained in a total of 3 experiments.
2.5. Discussion

We have found ebselen reversed DTT-potentiation of NMDA-induced currents in cortical neurons in a concentration-dependent manner. This effect was also observed in recombinant NR1/NR2B receptors expressed in CHO cells, but not in cells expressing an NR1a(C744A/C798A)/NR2B mutant receptor, which lacks a functional redox modulatory site. Furthermore, alkylation of native receptors also substantially diminished the actions of ebselen. In addition, recombinant nicotinic acetylcholine receptors are modulated by ebselen in a manner analogous to other thiol oxidizing agents such as DTNB. This leads us to conclude that ebselen acts as an oxidant at the NMDA receptor redox modulatory site, a previously undescribed mechanism of action for this drug. We believe that the final oxidation products of the interaction of ebselen with the NMDA receptor are an intramolecular disulfide within the cysteines of the redox site itself and an ebselen diselenide product (Schewe, 1995). This is due to the fact that an ebselen-cysteiny1 adduct would be structurally similar to the alkylated form of the receptor, a conformation that results in a permanent potentiation of NMDA-induced currents (Figure 4C; Tang and Aizenman, 1993b). Whole-cell recordings revealed that ebselen seldom decreased baseline NMDA-induced currents; that is, the actions of ebselen were mostly apparent after the NMDA receptor had been chemically reduced by DTT. This suggests that under our recording configuration, most of the NMDA receptors in the culture system exist primarily in the oxidized state. Although this situation is normally encountered in cultured cells that are being rapidly perfused in the recording setup (Aizenman
and Reynolds, 1992), it may not be reflective of the redox state of NMDA receptors present in neurons in more intact preparations, especially during pathophysiological conditions e.g. NMDA exposure or seizure activity; (Sanchez et al., 2000).

There is a substantial body of evidence linking excessive NMDA receptor-mediated calcium influx to the widespread neuronal death that occurs in primary cultures upon exposure to glutamate (Lee et al., 1999). Due to the high calcium permeability of the receptor, modulation of the receptor by reducing and oxidizing agents accordingly modulates calcium influx (Reynolds et al., 1990; Sucher et al., 1990). As such, DTT and other reducing agents exacerbate excitotoxic neuronal death in culture (Aizenman and Hartnett, 1992; Aizenman et al., 1990), and oxidizing agents that act at NMDA receptors are neuroprotective in vitro (Aizenman and Hartnett, 1992) and in vivo (Jensen et al., 1994). We observed here that ebselen is neuroprotective against NMDA-mediated neurotoxicity in rat cortical cultures. We propose that the observed neuroprotection induced by ebselen in vitro and in vivo (Saito et al., 1998; Takasago et al., 1997; Yamaguchi et al., 1998) may be due, at least in part, to modulation of the NMDA receptor redox site.

We recognize that NMDA receptor oxidation may not fully account for the degree of neuroprotection afforded by ebselen in vivo. For instance, ebselen is cardioprotective against ischemia (Maulik et al., 1998). As NMDA receptors are irrelevant to ischemia/reperfusion-induced cardiopathology, this drug probably exerts its protective effects through some other function. However, hypoxic
neuronal injury in cortical culture is mediated primarily by activation of NMDA receptors (Goldberg and Choi, 1993; Sattler et al., 2000; Sinor et al., 2000). Hence, exposure of cortical neurons to NMDA in culture is a good model of ischemic injury in this system. In addition, a recent study suggests that oxidative stress may not be a primary component in triggering NMDA-mediated excitotoxicity in culture, at least under certain conditions (Rudolph et al., 2000). Therefore, it is likely that neuroprotection by ebselen in our model is due primarily to a direct interaction of this drug with the NMDA receptor redox modulatory site. This is supported by our observation that more typical antioxidants, such as trolox, glutathione-methyl ester, and N-acetylcysteine are not neuroprotective against NMDA toxicity under identical conditions.

NMDA receptors have been implicated in the pathogenesis of neurological disorders including stroke, Parkinson’s disease, chronic pain, and epilepsy (Dingledine et al., 1999). Though under heavy investigation as therapeutic agents, NMDA receptor blockers have met with limited success in clinical trials due to untoward side effects associated with blockade of physiological glutamatergic transmission (Lee et al., 1999). However, redox modulatory agents do not cause total receptor function loss, and thus are less likely to interfere with normal synaptic transmission. In fact, PQQ, an NMDA receptor oxidant, has been shown to minimize epileptic seizures in vitro and in vivo while not significantly affecting LTP in hippocampal slices (Sanchez et al., 2000). However, PQQ may be toxic at high doses (Jensen et al., 1994). In contrast, ebselen shows little toxicity in humans at therapeutic doses (Saito et al., 1998;
Yamaguchi et al., 1998). The findings herein may extend the clinical applicability of this compound beyond stroke to other disorders where the pathophysiological consequences of abnormal glutamatergic transmission have been implicated.
3. Interaction between redox and spermine modulation of NR1/NR2A cysteine mutant receptors

3.1. Abstract

The NMDA subtype of glutamate receptor is sensitive to reducing and oxidizing agents in a subunit specific-manner. Cysteines 744 and 798 on NR1a are responsible for most of the redox sensitivity of NR1a/NR2B, NR1a/NR2C and NR1a/NR2D receptors. However, NR1a(c744a,c798a)/NR2A receptors remain fully sensitive to redox modulation. This has been attributed to additional cysteines in the amino-terminal domain (ATD) of NR1 and NR2A. The NR1a(c744a,c798a) mutation has also been shown to alter spermine and proton modulation of NR1a(c744a,c798a)/NR2B receptors, and zinc modulation in NR1a(c744a,c798a)/NR2A receptors. This implies that structural determinants of redox, spermine, proton, and zinc modulation may be linked in a subunit-specific manner. We sought to determine whether NR1a(c744a,c798a)/NR2A receptors would have altered sensitivity to spermine and if spermine could have an impact on the remaining ATD redox sensitivity of NR1a(c744a,c798a)/NR2A receptors.

We recorded NMDA-induced currents from CHO cells expressing the NR1a(c744a,c798a)/NR2A receptor and related mutant NMDA receptors. We observed that 30 \(\mu\)M spermine triggered a rapid but transient potentiation (175\%) of currents in NR1a(c744a,c798a)/NR2A-expressing cells. In an attempt to determine whether spermine potentiation of NR1a(c744a,c798a)/NR2A receptors was functionally related to glycine-independent potentiation by spermine of NR1a/NR2B receptors, we tested the sensitivity of NMDA-induced currents to spermine in the exon-5 containing NR1b(c765a,c819a)/NR2A construct. We
observed that currents were potentiated by spermine to the same degree as NR1a(c744a,c798a)/2A receptors, although the transient nature of spermine potentiation was eliminated. Likewise, we observed that 30 µM spermine potentiated NR1a(e181q,e185q,c765a,c819a)/NR2A receptors because NR1a(e181) and NR1a(e185) appear to be required for polyamine potentiation of NR1a/NR2B receptors. The ATD redox site mutant NR1a(c79s,c744a,c798a)/NR2A receptor was also sensitive to spermine. Therefore, we conclude that spermine potentiates NR1a(c744a,c798a)/NR2A receptors via a mechanism that is different from spermine potentiation of NR1/NR2B receptors.

In addition, we observed that the presence of spermine (30 µM) inhibits the redox sensitivity of NR1a(c744a,c798a)/NR2A receptors by 38%. Spermine did not interfere with redox sensitivity in NR1a(c79s,c744a,c798a)/NR2A and NR1b(c765a,c819a)/NR2A receptors, suggesting that spermine inhibition of redox sensitivity is qualitatively different than spermine potentiation of NR1a(c744a,c798a)/NR2A receptors. In conclusion, spermine potentiates NR1a(c744a,c798a)/NR2A receptors in a transient manner via a mechanism that does not appear to be analogous to spermine potentiation of NR1a/NR2B receptors. In addition, spermine blocks DTT potentiation in NR1a(c744a,c798a)/NR2A receptors, but not as effectively in NR1b(c765a,c819a)/NR2A and NR1a(c79s,c765a,c819a)/NR2A mutants. From these findings we conclude that structural determinants of spermine and redox modulation of NR1a(c744a,c798a)/NR2A receptors are closely associated.
3.2. Introduction

The NMDA receptor has been intensely studied due to its role in neuropathologies such as pain, ischemic stroke, schizophrenia and epilepsy (reviewed in Dingledine et al., 1999; Lee et al., 1999). To date, three gene families that encode NMDA receptor subunits have been identified: NR1, a single gene product with eight splice variants a-h, NR2, which are comprised of four genes, A-D, and NR3 (Chatterton et al., 2002; Dingledine et al., 1999). Expression of at least one NR1 and NR2 subunit is required to form functional receptors in mammalian heterologous systems. These "binary" NMDA receptors have been studied intensely to determine the functional and pharmacological consequences of the subunit composition (Dingledine et al., 1999). For example, NMDA-induced currents are modulated by a number of exogenous and endogenous agents, including protons (Traynelis et al., 1998; Traynelis et al., 1995), zinc (Low et al., 2000; Paoletti et al., 1997; Traynelis et al., 1998), phenylethanolamines (Mott et al., 1998), polyamines (Williams, 1997b), and reducing and oxidizing agents (Brimecombe et al., 1997; Sullivan et al., 1994) in a subunit-specific manner. Although the structure of the NMDA receptor remains unsolved, functional and biochemical studies of mutant NMDA receptors have yielded insights as to potential binding sites for these modulatory agents. Evidence exists that ifenprodil, an NR2B-sepcific inhibitor, binds with high affinity to a site in the amino terminal domain (ATD) of the NR2B subunit (Perin-Dureau, 2001; Perin-Dureau et al., 2002; Zheng et al., 2001). Likewise, high affinity zinc inhibition of NR2A-containing receptors requires the ATD of NR2A (Fayyazuddin et al., 2000; Zheng et al., 2001). In addition, spermine and redox agents have
putative sites of action in the ATD of NR1 (Choi and Lipton, 1999; Masuko et al., 1999).

Spermine has multiple effects on NMDA-induced currents: at high concentrations, it can block NMDA receptor function in a voltage dependent manner (Williams, 1997b). At lower concentrations, it potentiates receptor function by increasing the affinity of the receptors for glycine. At saturating concentrations of glycine, spermine potentiates NMDA receptors via a voltage independent, proton dependent mechanism that is specific to NR1a/NR2B receptors, hereafter referred to simply as spermine potentiation.

The NR1b splice variant contains exon 5, a 21 amino-acid insert that has been shown to mimic the potentiating effects of spermine in NR1a/NR2B receptors (Durand et al., 1993; Traynelis et al., 1995). For example, spermine potentiates NR1a/NR2B receptors, whereas the exon-5 containing NR1b/NR2B receptors appear already potentiated, and spermine has no further effect (Traynelis et al., 1995). It is thought that exon 5 interacts with two negatively charged residues near its splice site, e181, and e185. These residues may form part of a binding site for spermine. Indeed, NR1a(e181q,e185q)/NR2B receptors are largely insensitive to spermine potentiation (Masuko et al., 1999).

In addition to spermine, reducing and oxidizing (redox) agents may have sites of action in the ATD of NR1. Redox agents modulate NMDA receptors such that reducing agents potentiate and oxidizing agents reverse potentiation of NMDA-induced currents (Aizenman et al., 1989). Mutation of ligand binding cysteines in the NR1 subunit of NR1a(c744a,c798a)/NR2B, NR2C, and NR2D
receptors nearly abolishes redox sensitivity of these receptors. However, NR1a(c744a,c798a)/NR2A receptors remain sensitive to redox modulation (Sullivan et al., 1994). This remaining redox sensitivity has been ascribed to two putative pairs of cysteine residues in the ATD of NR1 and NR2 (Choi et al., 2001). Interestingly, it was observed that mutation of the redox site in NR1a(c744a,c798a)/NR2B receptors also eliminated spermine potentiation and proton sensitivity, implying that redox, spermine, and proton modulation may share a common structural mechanism (Sullivan et al., 1994). Although interactions between spermine and redox modulation have been studied in NR1/NR2B receptors (Sullivan et al., 1994), potential interactions between spermine and redox modulation of NR2A-containing receptors have not been investigated. We report here that although spermine has no impact on the redox sensitivity of NR1/NR2A receptors, we observe a novel form of spermine potentiation in NR1a(c744a,c798a)/NR2A receptors that is not disrupted by perturbations of a putative spermine binding site. As a result of this finding, we sought to determine whether spermine could affect redox sensitivity in NR1a(c744a,c798a)/NR2A receptors. We observed an inhibition of redox sensitivity by spermine that depends in part on NR1a(c79), and can be disrupted by the presence of exon 5.

3.3. Methods

3.3.1. Tissue Culture and Transfection

Tissue culture and other reagents were purchased from Sigma (St. Louis, MO) unless otherwise noted. CHO cells were maintained in Ham’s F-12 media
containing 10% fetal bovine serum (FBS) and 2 mM L-glutamine. Cells were passaged at a 1:10 dilution when 80% confluent, usually every two days, no more than 35 times. CHO cells were seeded at 2.8x10^5 cells/well onto 6-well plates 24 hours previous to transfection. The cDNAs for the NR1a, NR1a(c744a,c798a), NR2A, NR2B and the positive transfection marker, eGFP were previously subcloned in mammalian expression vectors (Boeckman and Aizenman, 1994; Boeckman and Aizenman, 1996; Brimecombe et al., 1997). The cDNAs for NR1b, NR1b(c765a,c819a), and NR1a(c79a) were a kind gift from Dr. F. Zheng (University of Arkansas, Little Rock, AK). Cells were transfected with 5.0 µg total DNA in Opti-MEM medium with 10-15 µL LipofectAMINE reagent (Invitrogen, Carlsbad, CA). cDNAs were transfected at the following ratios: NMDAR:GFP 100:1, and 1:3 NR1/NR2A. Cells were trypsinized and replated onto coverslips four hours later, and treated with 30 µM ketamine to prevent the excitotoxic cell death that occurs following functional receptor expression (Boeckman and Aizenman, 1996). Recordings were obtained 10-30 hours post transfection.

3.3.2. Electrophysiology

Electrophysiological recordings were performed at room temperature using the whole-cell configuration of the patch-clamp technique, as described in Herin et al. (2001). Cells were bathed in external solution containing (in mM): 150 NaCl, 1.0 CaCl₂, 2.8 KCl, 10 HEPES, 10 glycine. pH was adjusted to 7.2 with NaOH. Electrodes were pulled on either a Sutter P-87 or P-97 electrode puller (Sutter Instruments, Novato, CA) to a resistance of 1.5-3 MΩ when filled
with internal solution containing (in mM): 140 CsF, 10 EGTA/CsOH, 1 CaCl₂, and 10 HEPES (pH adjusted to 7.2 with CsOH). Signals were amplified using an Axopatch 200B integrating patch-clamp amplifier (Axon Instruments, Foster City, CA), filtered using an 80 dB/decade filter at 1 kHz, and digitized at 2 kHz with a DigiData 1200b (Axon Instruments) computer interface. The reference electrode was an Ag/AgCl wire bridged with 2 M KCl/1% agarose in PE-90 tubing. Drugs and agonists were applied via a fast perfusion system (Warner Instruments Corp., Hamden, CT). Data were collected and analyzed using commercially available software (pCLAMP 8, Axon Instruments).

3.3.3. Site-directed mutagenesis

Site directed mutagenesis was performed using the Quick-change kit (Stratagene, LaJolla, CA) according to the manufacturer’s directions. The NR1a(e181q,e185q,c744a,c798a) mutant was obtained by amplification of NR1a(c744a,c798) in PRC/CMV as a template with the following primers:

forward gca gaa gcg ctt gca gac gtt gct gca gga acg gga gtc, reverse gac tcc cgt tcc tgc agc aac gtc tgc aag cgc ttc tgc (IDT Technologies, Coralville, IA).

Methylated parental cDNA was digested with DpnI and transformed by electroporation into Top-10 cells (Invitrogen, Carlsbad, CA). Colonies were selected and screened using EcoRI digestion. Mutations were confirmed by sequencing.
3.4. Results

3.4.1. Effects of spermine on NR2A-containing receptors

3.4.1.1. Spermine does not alter NR1a/NR2A currents

NMDA-induced currents were obtained from CHO cells expressing recombinant NMDA receptor subunits using whole-cell patch clamp electrophysiology. After establishment of the whole cell configuration, NMDA (30 µM) was applied to the cell via a fast perfusion system in the presence of saturating concentrations of glycine (10 µM). Currents were measured approximately every 30 seconds until the amplitude of the currents stabilized (approx. 2-4 minutes). Cells were then switched into a bathing solution containing 30 µM spermine and NMDA-induced currents were elicited at the following time points: 10 seconds, 30 seconds, and then every thirty seconds thereafter for up to 8 minutes.

NMDA-elicited currents in CHO cells transfected with NR1/NR2A subunits were unaffected by the presence of spermine (Figure 7A-B). This lack of effect on NR1/NR2A receptors at low concentrations of spermine and saturating glycine concentrations is in agreement with previous reports in Xenopus oocytes (Williams et al., 1994).
Figure 7. Spermine does not alter NR1/NR2A currents.

A: Representative whole cell currents obtained from CHO cells transiently expressing recombinant NR1/NR2A receptors during application of 30 µM NMDA in the presence of 10 µM glycine. Scale bar represents 200 pA and 1 second. Currents were measured for 2-3 minutes in external solution prior to application of 30 µM spermine. Time 0 represents the NMDA application obtained
immediately prior to application of spermine. Cells were switched into external solution containing 30 μM and currents were obtained in spermine-free NMDA containing solution. The diagram is meant to illustrate the putative structure of the NMDA receptor, and to illustrate various mutations (in this case, none).

**B:** Average peak current magnitude represented as a percentage of the time 0 magnitude. N=2.
3.4.1.2. Spermine transiently potentiates NR1a(c744a,c798a)/NR2A receptors

In contrast with the results we obtained in NR1/NR2A, we observed that 30 µM spermine potentiated currents by 173% in CHO cells expressing NR1a(c744a,c798a)/NR2A receptors (Figure 8A). This potentiation was transient; as currents returned to control levels within 4.5 min in the continuous presence of spermine in 5/5 cells (Figure 8A and B; p>0.8 potentiation at 0 min vs 4.5 min). This suggests that cysteines NR1a(c744) and/or NR1a(c798) may mask a spermine binding site, or that a conformational change induced by mutation of these residues confers spermine sensitivity to NR1a(c744a,c798a)/NR2A receptors. As spermine potentiation of currents from wild-type NR1/NR2B receptors has been well studied (Williams, 1997a), we sought to determine whether structural features that are important for spermine potentiation of wild type NR1/NR2B receptors were also important for this novel form of spermine potentiation in NR1a(c744a,c798a)/NR2A receptors.
Figure 8. Spermine transiently potentiates NR1a(c744a,c798a)/NR2A currents.

A: Representative whole cell currents obtained from CHO cells transiently expressing recombinant NR1a(c744a,c798a)/NR2A. Scale bar represents 500 pA and 1 sec. Currents were measured for 2-3 minutes in external solution prior to application of 30 µM spermine. Diagram is meant to illustrate relative location
of mutated residues. **B:** Average peak current magnitude represented as a percentage of the time 0 magnitude. Bars represent average +/- SEM (n=5). A one-way ANOVA reveals that the difference between the pre-spermine value (-0.5 min) and 0.5 min are highly significant (p < 0.001). The value at 4.5 min is not significantly different from -0.5 min (p > 0.05) and significantly different from the point at 0.5 min (p < 0.001).
3.4.1.3. Potentiation of NR1a(c744a,c798a)/NR2A by spermine does not occur via an NR1 "polyamine site"

We were interested in further characterizing the molecular basis for spermine potentiation of NR1a(c744a,c798a)/NR2A receptors. As there appears to be a spermine binding site in the amino terminal of NR1 which could still be present in the spermine-insensitive NR1/NR2A receptors (Masuko et al., 1999), we hypothesized that the NR1a(c744a,c798a) mutation could unmask spermine potentiation. If this were the case, it would be expected that this NR1 spermine binding site might share the same structural determinants as spermine potentiation of NR1a/NR2B receptors. For example, splice variants of NR1 that contain exon 5 are insensitive to the potentiating effects of spermine in NR1/NR2B receptors (Williams et al., 1994), and so we investigated whether the exon 5-containing NR1b(c765a,c819a)/NR2A receptor configuration would be rendered relatively insensitive to the potentiating effects of spermine. Surprisingly, 30 µM spermine significantly potentiated NMDA-induced currents from the cognate exon 5-containing NR1b(c765a,c819a)/NR2A receptors (200%; Figure 9A). This potentiation did not spontaneously reverse as seen in NR1a(c744a,c798a)/NR2A receptors, but was rapidly reversible upon washout of spermine. This result argues against the hypothesis that spermine potentiation of NR1a(c744a,c798a)/NR2A receptors is analogous to spermine potentiation of NR1/NR2B receptors, and suggests that it occurs by a different mechanism.
Figure 9. Spermine potentiates NR1b(c765a,c819a)/NR2A currents.

A: Representative whole cell currents obtained from CHO cells transiently expressing recombinant NR1b(c765a,c819a)/NR2A. Scale bar represents 200 pA and 1 sec. Currents were measured for 2-3 minutes in external solution prior to application of 30 µM spermine. Time 0 represents the NMDA application immediately prior to application of spermine. Spermine was applied for five
minutes, and then the bathing solution was returned to control. Diagram is meant to illustrate relative locations of mutations and exon 5 (purple subdomain).

**B:** Average peak current represented as a percentage of the time 0 magnitude. The point at 6 minutes represents current magnitude after 1 minute washout. Bars represent average +/- SEM (n=4). A one-way ANOVA reveals that the magnitude at -0.5 min and 0.5 min are significantly different (p < 0.05). The value at 4.5 min is significantly different from -0.5 min (p < 0.01) and not different from the point at 0.5 min (p > 0.05).
3.4.1.4. Spermine potentiates NR1a(e181q,e185q,c744a,c798a)/NR2A currents.

To further evaluate structural determinants involved in spermine potentiation of NR1a(c744a,c798a)/NR2A receptors, we tested whether spermine could potentiate NR1a(e181q,e185q,c744a,c798a)/NR2A receptors. The negatively charged residues, e181 and e185, located in the proximity of the exon 5 splice site of NR1, appear to be required for spermine potentiation in NR1a/NR2B receptors. Accordingly, alteration of these residues eliminates spermine potentiation of NR1/NR2B receptors (Masuko et al., 1999). Therefore, we recorded NMDA-induced currents from NR1a(e181q,e185q,c744a,c798a)/NR2A-expressing cells. Currents were potentiated by spermine (206%) in a manner similar to NR1b(c765a,c819a)/NR2A receptors. These results further support the notion that the structural determinants of spermine potentiation of NR1 cysteine mutants are not similar to that of glycine-independent spermine potentiation of NR1/NR2B receptors. It is unclear, however whether potentiation of NR1a(e181q,e185q,c744a,c798a)/NR2A receptors returns to baseline upon continuous spermine exposure, as there was no statistical difference between that pre-spermine (-0.5 min), and 3.0 minutes spermine group.
Figure 10. Spermine potentiates NR1a(e181q,e185q,c744a,c798a)/NR2A currents.

A: Representative whole cell currents obtained from CHO cells transiently expressing recombinant NR1a(e181q,e185q,c744a,c798a)/NR2A receptors. Scale bar represents 100 pA and 1 sec. Currents were measured for 2-3 minutes in external solution prior to application of 30 µM spermine. Spermine
was applied for three minutes. Diagram is meant to illustrate relative locations of mutations; NR1a(e181q,185q) represented as an x in the ATD of NR1, and the redox site mutations in the ligand binding domain of NR1. **B:** Average peak current magnitude represented as a percentage of the time 0 magnitude. Bars represent average +/- SEM (n=4). A two-tailed T test shows a significant difference between the pre-spermine group (-0.5 min) and post-spermine group (0.5 min, p = 0.02). A one way ANOVA showed no significance due to variation within the groups.
3.4.1.5. Spermine potentiates NR1a(c79s,c744a,c798a)/NR2A receptors

In an effort to search for potential sites of action of spermine that could explain its effects on NR1a(c744a,c798a)/NR2A receptors, we became interested in the role of the remaining redox sensitivity of this receptor configuration. We hypothesized that if mutation of the ligand-binding redox site uncovers spermine sensitivity, and spermine and redox modulation in NR1a(c744a,c798a)/NR2A receptors are interrelated, then the remaining redox sensitive cysteines might be important for spermine potentiation. We thus tested the spermine sensitivity of NMDA-induced currents from cells expressing NR1a(c79s,c744a,c798a)/NR2A receptors. This receptor configuration alters a putative ATD redox site and abolishes the ligand binding redox site of NR1. However, spermine (30 µM) also potentiated NMDA induced currents in 4/4 cells an average of 202%. This is indicative that c79 is not important for spermine modulation of NR1a(c744a,c798a)/NR2A receptors.
Figure 11. Spermine potentiates NR1a(c79s,c744a,c798a)/NR2A receptor function.

Average peak current magnitude represented as a percentage of the time 0 amplitude. Bars represent average +/- SEM (n=3). Bars represent average +/- SEM (n=3). A one-way ANOVA shows a significant difference between pre-spermine treatment (time 0) and 0.5 minutes incubation on spermine (p < 0.05) and the washout group (time 6.5) is not significantly different from the pre-spermine group (p > 0.05)
3.4.2. Interaction between redox and spermine modulation of NR1a(c744a,c798a)/NR2A receptors

3.4.2.1. Spermine does not alter redox modulation of NR1/NR2A receptors

Given the observation that mutation of NR1 redox sites conferred spermine sensitivity to an otherwise non-sensitive receptor configuration, we evaluated whether spermine could affect the redox properties of NR1/NR2A receptors. We first tested if spermine could alter the redox sensitivity of wild-type NR1/NR2A receptors. To quantify redox sensitivity, we used a protocol very similar to that used to measure spermine potentiation: we established the whole cell patch clamp configuration, elicited NMDA induced currents every 30 seconds until a stable baseline was established, and then switched to a DTT (4 mM)-containing recording solution. We measured NMDA-induced currents in DTT at 0 seconds, 30 seconds, and then every thirty seconds up to 5 minutes (black line; figure 12A). DTT produced an approximate tripling of NMDA receptor current amplitudes within 5 minutes A), a result that agrees with previous reports in neurons and heterologous expression systems (Arden et al., 1998; Herin et al., 2001; Tang and Aizenman, 1993b).

We then examined whether the presence of spermine would alter the redox sensitivity of wild-type NR1a/NR2A receptors. With 30 µM spermine present in all recording solutions, we tested potentiation by DTT in an identical manner as in the absence of spermine, again, assuring that currents had reached a steady state before switching into DTT containing solutions. Potentiation of NMDA-induced responses after a 5 minute application by DTT
was similar to that seen in the absence of spermine (Figure 12A; blue line; $p=0.87$). This is consistent with previous reports (Gallagher et al., 1997).
Figure 12. Spermine does not alter DTT potentiation of NR1/NR2A currents.

A: NMDA-elicited currents were recorded from CHO cells transfected with NR1a and NR2A subunit cDNA. After current amplitudes stabilized, cells were
switched to external solution containing 4 mM DTT. NMDA-elicited currents were recorded 10 seconds after the onset of DTT, at thirty seconds, and every thirty seconds thereafter. Spermine (30 µM) was present in all solutions in the spermine condition. Points represent the average (+/- SEM) percent of control (pre-DTT) current in 9 (control) and 5 (spermine) cells. B: Bar graph representing means+/- S.E.M for currents at 4.5 minutes compared to the spermine alone condition at 4.5 minutes (from Figure 7B).
3.4.2.2. Spermine inhibits DTT-potentiation of NR1a(c744a,c798a)/NR2A receptors

Since spermine potentiation of NR2A-containing receptors appeared to be manifested only in NR1a(c744a,c798a)/NR2A-related receptor configurations, we investigated whether spermine altered DTT-potentiation in these receptors. In the absence of spermine, we found NR1a(c744a,c798a)/NR2A receptors to be sensitive to DTT-potentiation to the same extent as wildtype receptors at 4-5 minutes (342% ± 43 S.E.M.; Figure 13B black line). The magnitude and time course of potentiation agrees with previous reports in both oocytes (Choi et al., 2001; Sullivan et al., 1994) and mammalian cells (Brimecombe et al., 1999).

In contrast with NR1a/NR2A receptors, when 30 µM spermine was present in all recording solutions, DTT-potentiation of NR1a(c744a,c798a)/NR2A receptors was decreased by approximately 40% (Figure 13B; blue line). Inhibition of redox sensitivity by spermine was compared among varying concentrations at 1.5 minutes of incubation, and though there appeared to be a trend with maximal inhibition at 30 µM, Figure 13C) but this was not statistically significant. These results suggest that spermine can inhibit the effects of DTT, but only when the redox modulatory site on ligand-binding domain of NR1 has been abolished. This is suggestive of an allosteric interaction between a spermine binding site and the redox sites of this mutant, putatively of the ATD redox sites NR1a(c79,c308) and/or NR2A(c87,c320).
Figure 13. Spermine inhibits DTT-potentiation of NR1a(c744a,c798a)/NR2A receptors.

A: NMDA-elicited currents from CHO cells transfected with NR1a(c744a,c798a)/NR2A cDNA. The bar above the first trace represents the duration of application of 30 µM NMDA and is omitted from subsequent traces for clarity. Horizontal dashed lines are for comparison of traces to the peak of the control trace. Traces in black were obtained from cells incubated in external solution and then switched into bath solution containing 4 mM DTT. Traces in blue represent cells that were exposed to DTT as above, however 30µM spermine was present in all solutions. Schematic represents putative domains structure of NR1/NR2A, with redox site mutations represented by an x. B: Points represent the average (+/- SEM) of the following number of cells: black line-
NR1a(c744a,c798a)/NR2A control (5); Blue line- NR1a(c744a,c798a)/NR2A + 30 µM spermine (7). C: Concentration-response relationship for spermine inhibition of DTT potentiation. Points are means ± SEM percent of pre-treatment currents at 1.5 minutes. The first bar represents spermine alone at 1.5 minutes, and the subsequent bars are DTT (4mM) potentiation in the respective spermine concentrations for the following numbers of cells: spermine alone (5); 0 spermine (DTT alone) (4) 10 µM spermine (5); 30 µM spermine (7); 100 µM spermine (3).
3.4.2.3. Redox sensitivity of NR1a(c79s,c744a,c798a)/NR2A receptors

Structural determinants that are important in spermine potentiation of NR1/NR2B receptors are located in the ATD of NR1 (Masuko et al., 1999). In addition, the redox sensitivity of NR1a(c744a,c798a)/NR2A receptors may be due to cysteines in the ATDs of NR1 and NR2 (Choi et al., 2001). As residues important for spermine and redox modulation may be situated near each other in the ATD of NR1, we hypothesized that the NR1 redox-sensitive cysteines may be critical for spermine inhibition of redox modulation of NR1a(c744a,c798a)/NR2A receptors. The NR1a(c79s,c744a,c798a)/NR2A receptor putatively eliminates both the NR1 ligand binding domain redox site and disrupts the ATD redox sensitivity of NR1 (Choi et al., 2001; Sullivan et al., 1994). We predicted that spermine inhibition of DTT potentiation in NR1a(c79s,c765a,c819a)/NR2A expressing cells would be diminished compared to cells expressing NR1a(c744a,c798a)/NR2A receptors.

We obtained recordings from mutant NR1a(c79s,c744a,c798a)/NR2A receptors in an identical experimental paradigm as above. In the absence of spermine, NR1a(c79s,c744a,c798a)/NR2A mutants were potentiated 206% after 4 minutes incubation in DTT (4 mM). This potentiation is diminished from NR1a(c744a,c798a)/NR2A receptors by 40%. These results agree with previous reports of diminished redox sensitivity in multiple NR1 cysteine mutants (Choi et al., 2001). In addition, the time course of redox potentiation is very similar between NR1a(c79s,c744a,c798a)/NR2A and NR1a(c744a,c798a)/NR2A receptors (Figure 14B; black line).
3.4.2.4. The effects of spermine on DTT potentiation of NR1a(c79s,c744a,c798a)/NR2A receptors

Spermine (30 µM) inhibited DTT-potentiation of NR1a(c79s,c744a,c798a)/NR2A receptors in a manner similar to NR1a(c744a,c798a)/NR2a receptors (Figure 14B). However, inhibition by spermine was significantly diminished (22%; Figure 14C). In addition, the time course of DTT potentiation of NR1a(c79s,c765a,c819a)/NR2A receptors was very similar in control and spermine conditions. As spermine inhibition is diminished in NR1a(c79s,c765a,c819a)/NR2A receptors, these data support the hypothesis that NR1a(c79) plays a role, or at least is permissive, in the interaction between spermine and DTT in NR1a(c744a,c798a)/NR2A receptors.
A

Control  Spermine

DTT

30s  1min  2min  5min

B

Percent Pre-DTT current

Minutes DTT incubation

Control  30 µM Spermine
Figure 14. Spermine inhibition of DTT-potentiation is diminished in NR1a(c79s,c765a,c819a)/NR2A receptors.

A: NMDA (30 µM) -elicited currents from CHO cells transfected with NR1a(c79s,c765a,c819a)/NR2A cDNA. Traces in black represent cells bathed in control external solution then switched to 4 mM DTT containing solution. Purple traces are cells that were exposed to 30 µM Spermine prior to, and during exposure to DTT. B: Points represent the average (± SEM) of the following number of cells: NR1a(c79s,c744a,798a)/NR2A DTT alone (4); NR1a(c79s,c744a,798a)2A DTT + spermine (9). C: Bars represent the mean (± SEM) percent inhibition of DTT-potentiation by spermine as follows: % pre-DTT current minus %DTT current in spermine for each time point, and then binned
from 2 to 8 minutes (*P<0.001 two-tailed t-test). Schematic represents putative domains structure of NR1/NR2A, with cysteine mutations represented by an x.
3.4.2.5. Spermine inhibition of redox sensitivity in NR1b(c765a,c819a)/NR2A receptors

We were interested in investigating whether structural determinants of spermine potentiation of NR1a/NR2B receptors were also important in spermine inhibition of DTT potentiation. Given prior evidence that exon 5 mimics the actions of spermine (Rumbaugh et al., 2000; Traynelis et al., 1995), we hypothesized that the exon 5 containing NR1b(c765a,c819a)/NR2A construct would be relatively insensitive to spermine inhibition of redox sensitivity. Alternatively, perhaps the spermine-redox interaction in NR1a(c744a,c798a)/NR2A resembles spermine potentiation of NR1a(c744a,c798a)/NR2A receptors (this report), and is independent of the effects of exon 5.

It appeared that there was a slight decrease in the DTT sensitivity of the NR1b(c765a,c819a)/NR2A receptor, when compared to the NR1a(c744a,c798a)/NR2A (compare third bar with first bar, Figure 15), however this was not statistically significant. DTT (4mM) potentiated NMDA-induced currents from NR1b(c765a,c819a)/NR2A receptors (second bar) by 252%. This figure is not significantly different from DTT potentiation of NR1a(c744a,c798a)/NR2A (fourth bar) receptors in the presence of spermine. In addition, spermine (30 µM) in the recording solutions did not further attenuate DTT potentiation of NMDA-induced currents in NR1b(c765a,c819a)/NR2A expressing CHO cells (Figure 15). This suggests that the presence of exon 5 functionally resembles spermine in modulation of redox sensitivity. The ability of exon 5 to mimic spermine inhibition of DTT potentiation of
NR1a(c744a,c798a)/NR2A receptors is suggestive that the actions of spermine in this paradigm resembles spermine potentiation of wild-type NR1/NR2B receptors as opposed to the novel form of spermine potentiation of NR1a(c744a,c798a)/NR2A receptors outlined above.

Figure 15. The presence of exon 5 mimics the effects of spermine on redox sensitivity.

A comparison of exon 5-lacking and -containing NMDA receptor redox mutants (as denoted below the bar graph) NR1a(c744a,c798a)/NR2A results are shown for comparison (from Figure 13; 0 (first bar) and 30µM spermine (third bar)). Bars represent means ± S.E.M percent pre-DTT current recorded at 4.5-5 minutes of incubation in DTT in the following number of cells:
NR1b(c765a,c819a)/NR2A + 4 mM DTT (12) and NR1b(c765a,c819a)/NR2A + 4 mM DTT + 30µM spermine (6). In NR1a(c744a,c798a)/NR2A receptors, N.S. denotes that these conditions are not statistically different from each other (One way ANOVA, p = 0.22).
3.4.2.6. Elimination of a putative site of spermine binding in NR1a(e181q,e185q,c744a,c798a)/NR2A receptors induces a new phenotype

As the presence of exon 5 in the experiment outlined above mimics the actions of spermine, this suggests that the actions of spermine may be attributable to a putative spermine binding site in the ATD of NR1, analogous to spermine potentiation of NR1a/NR2B receptors (Masuko et al., 1999). It has been shown that that residues NR1a(e181) and NR1a(e185) are important for spermine potentiation of NR1a/NR2B receptors, and may form a spermine binding site in the ATD of NR1 (Masuko et al., 1999). For this reason, we sought to determine whether the NR1a residues e181 and e185 are important in spermine inhibition of redox sensitivity in NR1a(c744a,c798a)/NR2A receptors. We recorded NMDA-induced currents from CHO cells transfected with the NR1a(e181q,e185q,c744a,c798a) construct co-expressed with NR2A. Unexpectedly, DTT sensitivity was considerably diminished in this mutant compared with NR1a(c744a,c798a)/NR2A at 1.5 minutes DTT potentiates NR1a(c744a,c798a)/NR2A receptors 279% (+/-24.2 S.E.M.), but NR1a(e181q,e185q,c765a,c819a)/NR2A receptors 139% (+/- 7.1 S.E.M) (Figure 12 vs Figure 16; p < 0.0001) Despite attenuated DTT potentiation in the absence of spermine, responses in the presence of spermine were greatly potentiated by 4 mM DTT. For example, at 1.5 minutes of DTT application in the presence of 10 µM spermine, currents were potentiated 480%. Interestingly, increasing concentrations of spermine decreased potentiation by DTT, returning to 227% at 100 µM (1.5 minutes of DTT incubation). From these data, we can only
determine that NR1α(e181q,e185q,c744a,c798a)/NR2A receptors have a new, uninterpretable phenotype that needs to be further characterized. Thus, we cannot use this construct to address the hypothesis that residues NR1α(e181) and NR1α(e185) are critical for spermine inhibition of DTT potentiatiuon in NR1α(c744a,c798a)/NR2A receptors.

Figure 16. DTT and Spermine sensitivity of NR1α(e181q,e185q,c744a,c798a)/NR2A receptors

Concentration-response relationship for spermine inhibition of DTT potentiatiuon of NMDA currents in NR1α(e181q,e185q,c744a,c798a)/NR2A expressing cells. Points are mean (± SEM) percent of pre-treatment currents at 1.5 minutes. The first bar represents spermine alone at 1.5 minutes, and the subsequent bars are DTT (4mM) potentiatiuon in the respective spermine.
concentrations. Means represent the following numbers of cells: spermine alone (5); 0 spermine (DTT alone) (8) 10 μM spermine (5); 30 μM spermine (5); 100 μM spermine (4).
3.4.3. **Tricine does not potentiate NMDA–induced currents in our recording conditions**

In our laboratory, the first few currents recorded from CHO cells and neurons are largely insensitive to oxidizing agents, but readily potentiated by reducing agents. This potentiation can be reversed by application of oxidizing agents, and repeated iterations of reducing and oxidizing agents potentiate and decrease currents for the duration of the recording (Arden *et al.*, 1998; Herin *et al.*, 2001). This is highly indicative that in our recording conditions, the population of NMDA receptors exists primarily in the oxidized state prior to application of reducing agents. For this reason, we equate sensitivity of NMDA-induced currents to reducing agents (such as DTT) to their redox sensitivity.

However, studies by other groups have suggested that DTT potentiation may be due to chelation of trace amounts of zinc in recording solutions, as NR2A-containing NMDA receptors are inhibited by nanomolar quantities of zinc (Paoletti *et al.*, 1997). As zinc contamination of our solutions could compound our results, we ascertained whether the heavy metal chelating agent, tricine, could potentiate NMDA-induced currents. Tricine (10 mM) was applied for 30 seconds during a 1.5 minute application of NMDA (30 µM). Currents recorded from CHO cells expressing NR1a/NR2A, NR1a(c744a,c798a)/NR2A, and NR1b(c765a,c819a)/NR2A receptors were insensitive to tricine (Figure 17). This concentration and duration of tricine has been sufficient in potentiating NR1a/NR2A receptors expressed in oocytes in the presence of zinc (Choi *et al.*, 2001; Paoletti *et al.*, 1997). This confirms previous reports from our laboratory using the metal chelator TPEN (Arden *et al.*, 1998). Therefore, chelation of zinc
appears not to be an important consequence of DTT application in this study.

Figure 17. Tricine does not potentiate NMDA-induced currents.

NMDA (30 µM)-induced currents elicited from a CHO cell expressing:

A: NR1/NR2A receptors, B: NR1a(c744a,c798a)/NR2A, or C: NR1b(c765a,c819a)/NR2A receptors. 10 mM tricine applied for 10s during a 30 second application of 30 µM NMDA does not potentiate responses.
3.5. Discussion

3.5.1. Summary

3.5.1.1. Spermine potentiates cysteine mutant NR1/NR2A receptors

The aim of this study was to characterize effects of spermine on NR1/NR2A mutant receptors, and to explore a possible interaction between spermine and redox modulation of NR1/NR2A NMDA receptors. Here we confirmed that spermine does not alter NR1/NR2A receptor function; however, mutations of redox-sensitive residues in the ligand-binding domain of NR1 conferred spermine sensitivity to NR1a(c744a,c798a)/NR2A receptors. This novel form of spermine potentiation was not abolished by receptor configurations and mutations that eliminate spermine potentiation of NR1a/NR2B receptors. This suggests that the mechanism of spermine potentiation of NR1a(c744a,c798a)/NR2A receptors differs from that of spermine potentiation of NR1/NR2B receptors.

3.5.1.2. Interaction between spermine and redox modulation of NR1/NR2A cysteine mutant receptors

We found an additional effect of spermine: spermine (30 µM) inhibited DTT potentiation of NR1a(c744a,c798a)/NR2A receptors by approximately 40%. Mutation of an NR1 amino terminal cysteine, c79, diminished redox sensitivity in NR1a(c79s,c765a,c819a)/NR2A receptors, and relieved spermine inhibition of the remaining redox sensitivity of NR1a(c79s,c765a,c819a)/NR2A receptors. This indicates that spermine inhibition of redox sensitivity is manifested at least in part by the putative ATD redox site of NR1. Inclusion of exon 5 in
NR1b(c765a,c819a)/NR2A receptors mimicked the actions of spermine on redox sensitivity, suggesting that spermine may bind to a putative polyamine binding site that is also implicated in spermine potentiation of NR1a/NR2B receptors (Williams, 1997b). Attempts to alter this putative binding site in NR1a(e181q,e185q,c744a,c798a)/NR2A receptors were uninterpretable. Nonetheless, it appears that the structural determinants for spermine inhibition of redox sensitivity are more related to spermine potentiation of NR1/NR2B receptors than spermine potentiation of NR1a(c744a,c798a)/NR2A receptors. From this we conclude that the structural elements that are responsible for redox modulation and spermine modulation of NMDA receptors may be intimately linked, and/or share important downstream elements.

3.5.2. Possible interpretations of spermine potentiation of NR1a(c744a,c798a)/NR2A receptors

3.5.2.1. NR1a(c744a,c798a)/NR2A receptors form a de novo spermine binding site in the proximity of c744 and/or c798

We have shown that mutation of cysteines NR1a(c744) and NR1a(c798a) alter NR1/NR2A receptors such that they become sensitive to spermine. A possible interpretation is that NR1a(c744a,c798a) forms a new spermine binding site in the proximity of C744 and C798 that is independent of sites previously explored in the literature. Our data do not refute this interpretation: we show that spermine potentiation of NR1a(c744a,c798a)/NR2A receptors is not affected by the presence of exon 5, nor mutations of positively charged residues near the exon-5 slice site (NR1a(e181q,e185q)). However, as spermine is a highly basic molecule, most mutations that eliminate spermine effects in other paradigms
change acidic residues into neutral or basic residues (Kashiwagi et al., 1996; Masuko et al., 1999; Williams et al., 1995). This implies that negatively charged residues are important in the binding and/or actions of the positively charged spermine molecule. In the NR1a(c744a,c798a) mutation cysteines are mutated to alanines. As cysteines are moderately basic residues, and alanines are neutral residues, this mutation could be sufficient to create a locally acidic environment that could bind spermine directly.

Contradictory to this hypothesis, the same mutation has a different behavior in NR1a(c744a,c798a)/NR2B receptors. In this case, spermine sensitivity is abolished in an otherwise sensitive receptor configuration (Sullivan et al., 1994; Traynelis et al., 1998). In addition, NR1a(c744a,c798a)/NR2B receptors are less sensitive to proton and zinc inhibition (Choi et al., 2001; Sullivan et al., 1994; Traynelis et al., 1998). This evidence suggests that NR1a(c744a,c798a) does not form a de novo spermine binding site, but that it alters a downstream element of convergent modulatory mechanisms.

3.5.2.2. NR1a(c744a,c798a)/NR2A uncovers an otherwise masked spermine modulatory site: Potential sites in the ATD of NR2A and NR2B

While this study focuses on a site in NR1 important for spermine modulation of NR1a/NR2B receptors, other potential binding sites for spermine have been proposed. For example, Gallagher et al. (1997) constructed chimeric NR2A/NR2B receptors to localize a putative spermine binding site in NR2B. This group identified a negatively charged residue, e201, that when mutated diminished polyamine potentiation of NR1a/NR2B(e201r) receptors in a proton-dependent manner. In an attempt to determine whether both NR2B(e201) and
its adjacent residue NR2B(e200) were important for spermine stimulation, Gallagher et al. (1997) measured spermine stimulation of NR2B(e200q,e201n) receptors, which retained full polyamine sensitivity. Interestingly, the sequence NR2B(q200,n201) is equivalent to the native NR2A sequence (Gallagher et al., 1997), implying that spermine may bind at the equivalent site in the ATD of NR2A. It is also interesting to note that both NR2B(e201) and NR2A(q201) are analogous to NR1a(e185). Therefore, it is possible that equivalent spermine binding sites exist in the ATD of NR1, NR2A and NR2B, but that NR1/NR2A receptors contain structural elements that mask access of polyamines to this site. Alternatively, the NR2A environment is non-permissive of transduction of polyamine binding to polyamine potentiation. Our data suggest that NR1a cysteines (c744) and (c798) are important in masking either access to a spermine binding site or transduction of the effects of spermine binding in NR1/NR2A receptors.

3.5.2.3. Multiple polyamine mechanisms

If multiple spermine binding sites exist in NR1a(c744a,c798a)/NR2A receptors, each site could have differing effects on NMDA receptor function. For example, our data indicate a complex mechanism for spermine in potentiation of NR1a(c744a,c798a)/NR2A. We have observed a biphasic nature of spermine potentiation of NR1a(c744a,c798a)/NR2A receptors: a "fast potentiation" and a "desensitizing phase." Even though the "fast potentiation" is not dependent on exon 5 or NR1a(e181) and NR1a(e185), the "desensitizing phase" appears to be. This suggests that the mechanism responsible for "fast potentiation" of
NR1a(c744a,c798a)/NR2A receptors is different from the mechanism that determines the "desensitizing phase." Because it appears that alteration of the ATD of NR1 abolishes the "desensitizing phase" but not the "fast potentiation," it is tempting to speculate that a spermine site on NR2A, unmasked by mutation of NR1a(c744) and (c798), is responsible for the "fast potentiation" of NR1a(c744a,c798a)/NR2A receptors, and that the "desensitizing phase" is due to the actions of spermine on a site in NR1a.

3.5.3. Possible interpretations of spermine inhibition of redox sensitivity

3.5.3.1. Spermine and DTT potentiation are additive

Our results indicate that pre-incubation with spermine inhibits DTT-potentiation of NMDA-mediated currents in NR1a(c744a,c798a)/NR2A-expressing CHO cells. Given the evidence that spermine has a potentiating effect on NR1a(c744a,c798a)/NR2A receptor currents, a possible interpretation of these data is that spermine had already potentiated currents before DTT application. If there were a maximum, or ceiling, for allosteric potentiation, then apparent inhibition of DTT potentiation may represent the additive effects of two potentiating agents. There are several lines of evidence that argue against this interpretation. (1) Spermine inhibition of redox sensitivity is still present after spermine potentiation of NR1a(c744a,c798a)/NR2A receptors has returned to baseline. DTT is applied only after currents have stabilized in spermine-containing solutions. For currents from NR1a(c744a,c798a)/NR2A, this would mean that potentiation by spermine alone had reached a steady state close to control levels at or beyond 4 minutes of incubation in spermine (Figure 8B).
While spermine may still be binding to the site or sites responsible for spermine potentiation of NR1a(c744a,c798a)/NR2A receptors, it does suggest that at 4.5 minutes the contribution of spermine to the overall potentiation is close to zero.

(2) The mechanisms appear to differ. Spermine potentiation of NR1a(c744a,c798a)/NR2A receptors is not affected by exon 5, and spermine inhibition of redox sensitivity is exon-5 dependent. Therefore, it is unlikely that spermine inhibition of DTT potentiation is the consequence of spermine potentiation of the same receptors. (3) DTT does not occlude spermine potentiation. If spermine occludes DTT potentiation by an additive mechanism, then one would expect the reverse to be true: DTT would also be expected to occlude spermine potentiation. Preliminary evidence (Figure 18) indicates that DTT potentiation does not occlude spermine potentiation, as would be expected for a "ceiling effect."
Figure 18. DTT potentiation does not preclude spermine potentiation of NR1a(c744a,c798a)/NR2A receptors

Points represent percent pre-DTT current amplitude for 2 cells. Traces were obtained every 30 seconds. NR1a(c744a,c798a)/NR2A cells were incubated in control external solution, and then switched to 4 mM DTT containing external. After DTT potentiation stabilized, cells were switched to a solution containing 30 \( \mu \)M spermine and 4mM DTT. Note that spermine appears to potentiate NR1a(c744a,c798a)/NR2A receptors even after maximal potentiation by DTT. In addition, potentiation of NR1a(c744a,c798a)/NR2A by spermine after potentiation by DTT approximates the time course and magnitude of spermine potentiation in the absence of DTT.
3.5.3.2. Spermine and redox sensitivity share downstream elements

Ifenprodil inhibits NR1/NR2B receptors through an enhancement of proton inhibition (Mott et al., 1998). Similarly, it is believed that spermine potentiates NR1/NR2B receptors via relief of proton inhibition (Kashiwagi et al., 1997; Traynelis et al., 1998). Therefore, if protons are a common determinant of ifenprodil inhibition, zinc inhibition, and spermine potentiation of NR1/NR2B receptors, then one would expect the effects of ifenprodil and zinc to be additive, and spermine to counteract the effects of zinc and ifenprodil on NR1/NR2AB-mediated currents. There is evidence to support this hypothesis (Berger and Rebernik, 1999; Traynelis et al., 1998). Therefore is it possible, if not likely, that spermine inhibition of redox sensitivity of NR1a(c744a,c798a)/NR2A receptors is due to a shared mechanism of allosteric regulation.

3.5.3.2.1. Potential Interactions with zinc

Previous reports have shown that NR1a(c744a,c798a)/NR2B receptors have altered sensitivity to modulatory agents such as zinc, protons, and ifenprodil (Mott et al., 1998; Sullivan et al., 1994; Traynelis et al., 1998; Zheng et al., 1998). This implies that these modulatory agents share a common downstream element or effector in the NMDA macromolecule that ultimately leads to an alteration in gating. It is possible that spermine inhibition of redox sensitivity of NR1a(c744a,c798a)/NR2A receptors could be a secondary effect of reduced or enhanced modulation of zinc, protons, or a yet unknown modulator of NMDA receptor function that may be present in our recording solutions.
For example, Paoletti et al. (1997) observed that NR1/NR2A receptors were inhibited by concentrations of zinc in the nanomolar range. They demonstrated that contaminating levels of zinc in recording solutions could tonically inhibit NR1/NR2A receptor function. This group suggested that some of the effects of DTT on NR1/NR2A were due to chelation of zinc. In addition, Choi et al., (2001) observed that NR1a(c744a,c798a)/NR2A receptors were less sensitive to zinc inhibition than their wild-type counterparts, but only slightly so (IC$_{50}$ 12.3 ± 1 nM for NR1a/NR2A vs. 16.2 ± 3.1 nM for NR1a(c744a,c798a)/NR2A ). Therefore it is possible that DTT is acting both as a zinc chelator and a disulfide reductant in our studies.

Spermine has been shown to relieve zinc inhibition in NR1a/NR2B receptors, a mechanism that appears to be intimately related to the proton dependence of zinc and spermine modulation (Traynelis et al., 1998). If the effects of DTT in our paradigm are compound, then spermine could minimize the effects of inhibition by contaminating zinc without altering the effects of DTT on disulfide bonds, i.e. spermine relief of zinc inhibition would manifest itself as an apparent inhibition of DTT potentiation. For this to be plausible: (1) we would be able to observe tonic modulation of NMDA currents by contaminating zinc, and (2) the effects of spermine should be similar in both NR1/NR2A- and NR1a(c744a,c798a)/NR2A-mediated currents.

To address the first condition, we tested our recording solutions for the presence of contaminating levels of zinc. In our laboratory, chelation of zinc with both TPEN (1 µM; Arden et al., 1998) and 10 mM tricine (this report) does not
potentiate NR1a/NR2A-mediated currents as would be expected if zinc were present at concentrations sufficient to inhibit NMDA receptor function. In addition, reports suggest that DTT-potentiation of NR1/NR2A receptors by chelation of zinc is responsible for the fast component of DTT-potentiation (Choi et al., 2001; Kohr et al., 1994). We do not observe this fast component of DTT potentiation in NR1/NR2A receptors Figure 12A). Secondly, we observe no spermine potentiation of NR1/NR2A receptors, but a considerable degree of spermine inhibition in NR1a(c744a,c798a)/NR2A receptors, which are slightly less sensitive to zinc. Therefore, it seems implausible that spermine inhibition of redox sensitivity is a secondary effect due to contaminating levels of zinc.

3.5.3.2.2. Protons as a common mechanism

Another potential modulatory agent that may be important for the interaction between spermine and redox sensitivity is protons. There is strong evidence that the mechanism of spermine potentiation in NR1/NR2B receptors is due to a relief of proton inhibition (Low et al., 2003; Mott et al., 2003; Traynelis et al., 1998; Traynelis et al., 1995). However, NR1a(c744a,c798a)/NR2B receptors are less sensitive to protons than wild-type receptors (Sullivan et al., 1994). Thus it appears that mutation of NR1a(c744) and NR1a(c798a) residues renders the NR1/NR2B receptor less sensitive to protons. NR1/NR2A receptors are also sensitive to protons (Choi and Lipton, 1999; Low et al., 2000). However, the proton sensitivity of NR1a(c744a,c798a)/NR2A receptors remains unknown. Although DTT potentiation does not appear to be functionally dependent on protons in neurons (Tang and Aizenman, 1993b), the cysteine mutants
NR1a(c744a,c798a)/NR2B have altered proton sensitivity, implying that at least redox sensitivity and proton sensitivity share some structural determinants. If NR1a(c744a,c798a)/NR2A receptors remain sensitive to protons, then hypothetically, spermine could shift the pKa of NR1a(c744a,c798a)/NR2A to more basic values. This would result in a more potentiated receptor at pH 7.2 (our recording conditions). If DTT were acting in the same manner, the ability of DTT to fully potentiate DM receptors through relief of proton inhibition would be diminished. Unfortunately, attempts to characterize pH dependence of NR1a(c744a,c798a)/NR2A during the course of this study met with technical difficulties. Therefore, the proton dependence of redox modulation and spermine modulation of NR1a(c744a,c798a)/NR2A receptors remains to be investigated.
4. **General discussion**

The focus of this dissertation has been to characterize the functional effects of reducing and oxidizing agents at the NMDA receptor; specifically the ATD redox sites. Attempts to isolate the ATD redox sensitivity of NR1/NR2A receptors by mutation of the ligand binding domain redox site in NR1a(c744a,c798a)/NR2A receptors led to the finding that redox site mutations affect spermine sensitivity and that spermine affects redox sensitivity in these receptors, demonstrating the inherent interrelatedness of modulatory agents with putative binding sites within the ATD of NMDA receptor subunits.

4.1. **Relative importance of three redox sites of NR1/NR2A**

NR1/NR2A receptors putatively contain three active redox sites: a ligand binding domain site in NR1 (Sullivan et al., 1994) and two ATD "sites", one in NR1, and another in NR2A (Choi et al., 2001). It is interesting to note that the ATD redox "site" in NR1 is present in NR1/NR2B, NR1/NR2C and NR1/NR2D receptors also, but appears to be inactive or masked in these receptor configurations. We observe a residual redox sensitivity in NR1a(c744a,c798a)/NR2B receptors (Chapter II Figure 3B; Brimecombe et al., 1997; Herin et al., 2001), however, it remains to be investigated whether this residual sensitivity may be due the redox sensitive cysteines in the ATD of NR1.

Studies suggest that the ATD redox sensitivities of NR1 and NR2A may be the dominant redox sites in NR1/NR2A, as the single channel kinetics of NR1a(c744a,c798a)/NR2A receptors are indistinguishable from that of wild-type NR1/NR2A receptors (Brimecombe et al., 1999). However, macroscopic time
courses of DTT potentiation of NR1/NR2A and NR1a(c744a,c798a)/NR2A receptors are considerably different (compare Figure 11A and Figure 12B). The basis of the difference in time course remains to be studied, but it does suggest qualitative differences in redox sensitivity between NR1/NR2A and NR1a(c744a,c798a)/NR2A receptors. Our studies suggest that mutation of the ligand binding domain redox sites causes the most profound changes in NR1/NR2A receptor function, but that alteration of the NR1 redox sensitive cysteine NR1a(c79) brought about more subtle changes in function. Therefore it appears that even though the ligand binding domain redox site may not be dominant in terms of microscopic redox properties, it does appear to be more critical structurally, as mutations of this site have pleiotropic effects.

4.2. The mechanism of redox sensitivity of ligand binding domain redox site vs ATD "sites"

The structure of the NMDA receptor remains unsolved, therefore so do many questions regarding the structural basis for redox modulation. However, the crystal structure of the ligand binding domain of NR1 has been solved, and shows that NR1a(c744) and NR1a(c798) are located in the "hinge" region of the ligand binding domain (Furukawa and Gouaux, 2003). The authors of this study hypothesize that removal of these cysteines in NR1a(c744a,c798a)/NR2A receptors relieves a constraint on clamshell closure and facilitates agonist binding (Furukawa and Gouaux, 2003). As the degree of hinge closure correlates with the degree of receptor activation (Jin et al., 2003), this could explain the profound effect oxidizing and reducing agents have on NMDA receptor function.
Conversely, little is known regarding the localization of the cysteines in the ATD. Although two cysteines in each domain have been shown to be important for ATD redox modulation, there is little evidence that they form "pairs." A model of the ATD of NR1 and NR2A based on the crystal structure of a somewhat homologous bacterial binding protein, LIVBP, suggests that ATD redox sensitive cysteines do not lie across the cleft of the putative ATD clamshell-like structure. Therefore, redox modulation of the NMDA receptors by amino terminal domain cysteines does not appear to be homologous to modulation by the ligand binding domain cysteines. Although Choi and Lipton (2000) postulate that the mechanism of redox modulation of the NR1 and NR2A ATD cysteines is intimately related to high affinity zinc inhibition, our results do not concur. The structural mechanism of redox modulation by ATD cysteines remains to be determined.

4.3. The modular versus macromolecular concepts

The studies in this dissertation have been based on two opposing ideas of the structure-function relationship of the oligomeric NMDA receptor. A review of the literature shows a pervasive concept of the NMDA receptor and other ionotropic glutamate receptors as "modular receptors," namely a collection of four (or five) subunits that act somewhat independently and contribute additively to the total function of the receptor/ion channel. Another idea is that the ionotropic glutamate receptor oligomer is a functional unit, a macromolecule, whose behavior cannot be described simply as the sum of its parts. I will discuss both of these concepts as they relate to this dissertation study.
4.3.1. Evidence for the modular concept

The "modular concept" envisions each subunit of an iGluR as a functional unit that contains all the elements necessary to perform the functions of a ligand-gated ion channel. This would encompass at minimum a ligand binding element and a pore forming element that contains a "gate." The free energy change of ligand binding would be translated from the ligand binding core of each subunit into work, which would act to open a subunit-specific gating element. The additive effect of all four gating elements would result in macroscopic ion flux.

The modular concept resembles most closely the sequential model of allostery of oligomeric proteins as proposed by Koshland et al. (1966), in which a conformational change induced by ligand binding in one subunit (in this case, hemoglobin) influences the affinity of the neighboring subunit to bind ligand. The degree of influence between subunits depends upon the degree of their mechanical coupling. In Koshland's sequential model of allostery, at one extreme there is no mechanical coupling between subunits, and a conformational change in one subunit will have no effect on the other. At the other extreme, that in which there is infinite mechanical coupling between elements, ligand binding in one subunit would result in a completely concerted allosteric effect, outlined further in section 4.3.2.

This concept of modularity of ion channels is supported by studies of voltage-gated potassium channels. Gating models based on high resolution structures of the KvAP channel suggest that each subunit of the channel contains an independent voltage sensor that moves in response to changes in membrane voltage. The independent motion of each of these sensors results in an additive
conformational change that leads ultimately to gating of the channel (Jiang et al., 2003a; Jiang et al., 2003b).

For glutamate receptors, the "modular" concept arises historically from the observation that the ligand binding domain of iGluRs shares homology with bacterial binding proteins (Nakanishi et al., 1990). This suggested that iGluRs subunits could be "pieced together" evolutionarily from functionally distinct elements such as a potassium channel-like pore element, and bacterial amino acid binding protein-like ligand binding elements and modulatory ATD elements (reviewed in Wo and Oswald, 1995).

4.3.1.1. Ligand binding domains of ionotropic glutamate receptors appear to be modular elements

The most extensively studied element of the glutamate receptor ion channels is the ligand binding element. This element is contained in a globular protein domain (ligand binding domain; S1S2) that is somewhat conserved among all glutamate receptors (Dingledine et al., 1999; Nakanishi et al., 1990; Nakanishi and Masu, 1994). This domain appears to function somewhat independently of the rest of the protein structure, as ligand binding domain constructs retain ligand binding activity when expressed in isolation. For example, constructs of the ligand binding domain of NR1 have been expressed in e. coli and bind the competitive antagonist [³H]MDL105,519 with high affinity (Ivanovic et al., 1998; Neugebauer et al., 2003). A ligand binding domain construct of the AMPA receptor subunit GluR2 (Armstrong and Gouaux, 2000; Armstrong et al., 1998; Jin and Gouaux, 2003) and mutants thereof (Armstrong
et al., 2003) have been shown to bind agonists in isolation from the full length receptor.

In addition, domain-swapping studies suggest that the ligand binding domain is a functional unit. Ligand binding domains of kainate receptors can be exchanged with those of non-functional glutamate receptors to produce functional receptors with an agonist EC$_{50}$ resembling that of the kainate receptor (Strutz et al., 2002; Villmann et al., 1997). Glycine dependent desensitization in NMDA receptors can be transferred from the desensitizing NR2A-conataining receptors to non-desensitizing NR2C-conatining receptor by exchanging the amino-terminal domain and a small segment just N-terminal to the first transmembrane domain (Villarroel et al., 1998).

4.3.1.2. Amino terminal domains of ionotropic glutamate receptors could be modular

It appears that the ligand binding domain retains its function separately from the rest of the receptor subunit. Is this true of the ATD? Whether the ATD retains the ability to bind agents such as ifenprodil of zinc in isolation from the rest of the receptor subunit remains to be determined. However, an AMPA receptor construct lacking the amino terminal domain is expressed and fully functional (Pasternack et al., 2002), suggesting that the ATD is not required for AMPA receptor function. Conversely, this appears not to be true of NR1 NMDA receptor subunits, as attempts to express NR1 receptors lacking the amino terminal domain have been unsuccessful thus far (Meddows et al., 2001).

The subunit dependence of NMDA receptor modulatory agents initially led investigators to assume that if, for example, a modulatory agent is specific for
NR2A, that its site of binding and/or action must be present on the NR2A subunit. This approach has proven to be fruitful in determining binding sites in the ATD of NR2 subunits for zinc and ifenprodil (Fayyazuddin et al., 2000; Paoletti et al., 1997; Paoletti et al., 2000; Perin-Dureau et al., 2002; Zheng et al., 2001). Further, Perin-Dureau et al. (2002) have demonstrated that ifenprodil inhibition of NMDA receptors, specific for NR2B-containing receptors, can be transferred to NR2A-containing receptors by swapping the ATDs of the respective NR2 subunits. This suggests that the ATD of NMDA receptors may also function in a modular manner. A modular nature of NMDA receptor modulation would predict that the conformational change induced by binding of agents to the ATD of NR2 would be more likely to affect ligand binding in same subunit. In support of this, (Zheng et al., 2001) provide evidence that zinc and ifenprodil binding to NR2A and NR2B, respectively, caused an allosteric intra-domain interaction with the glutamate binding site.

4.3.1.3. Modularity of gating

A functional study designed to determine the subunit stoichiometry of ionotropic glutamate receptors suggests that ligand binding and "gating" may be linked in a subunit-specific manner (Rosenmund et al., 1998). These investigators obtained single channel recordings from HEK cells expressing GluR3/GluR6 cDNA. Cells were bathed in a high affinity antagonist, and then switched to a solution containing saturating concentrations of agonist. Under these conditions, the authors observed subconductance levels that "stepped" from no current to full current through three steps. Models of waiting times
predicted that GluR3/GluR6 iGluRs require two ligand binding sites to be occupied for activation of the ion-conducting pore, and that further occupation of each ligand binding site results in a discrete conductance. Assuming one ligand binding site per subunit, this is suggestive that occupation of the ligand binding site in each subunit it results in a subunit-specific gating event and that occupation of each binding site is independent of the others.

The evidence outlined above favors a concept of ionotropic glutamate receptors as being modular proteins in which structural domains of each subunit may be specialized to perform an independent function, whether it be gating, ligand binding or allosteric modulation of the receptor, and that these functions are not coupled tightly to other domains of the macromolecule. This would require that the degree of mechanical coupling between domains is fairly low. A minimum degree of coupling would require that ligand binding domains must be coupled to the gate, and that regulatory domains coupled to the ligand binding domain in order to translate ligand binding into ion flux. However, this concept is limited in its ability to explain the physiological functions that have been described in the literature.

4.3.2. Evidence for the macromolecular concept

An opposing view of the "modular concept" of ionotropic glutamate receptor structure is a view in which the allosteric sites in the receptor are all tightly coupled such that the entire molecule functions as a unit. In this view, a conformational change that occurs, for instance, in the c-terminal region of NR1 would elicit a conformational change that not only included the NR1 C-terminal,
but also the ATD of NR2, a concerted conformational change throughout that entire macromolecule. This view resembles the Monod-Wyman-Changeaux model of allostery in hemoglobin (Monod et al., 1965), in which the conformation of a subunit in an oligomer is constrained by the conformation of the other subunits.

Supporting the macromolecular concept of ionotropic glutamate receptors are a myriad of studies that show allosteric interaction not only between subunits, but from intracellular binding sites to extracellular binding sites. These allosteric interactions suggest that occupation of a binding site causes a more global conformational change that may modify the molecular behavior of sites that are distant from the binding site.

4.3.2.1. Intersubunit interaction between co-agonist sites

NMDA receptors are activated by the binding of coagonists glutamate and glycine to sites on NR2 and NR1, respectively (Dingledine et al., 1999). The affinity of the NMDA receptor for glycine depends on the NR2 subunit that it is co-expressed with. The difference in glycine affinity of NR1/NR2C is approximately 10 fold higher than that of NR1/NR2A receptors (Laurie and Seeburg, 1994). Accordingly, a radioligand has been developed that can distinguish between NR1 glycine sites when bound to various subunits (Honer et al., 1998). This suggests that the properties of glycine binding site are not independent, but highly dependent on the environment provided by the NR2 subunit.
In addition, the glutamate and glycine binding sites are coupled allosterically in a negative manner such that binding of glutamate to its site in NR1 decreases the affinity of the NMDA receptor for glycine and vice versa (Lerma et al., 1990; Nahum-Levy et al., 2002). A recent report suggests that a linker in the ATD of NR2 may account for this inter subunit interaction (Regalado et al., 2001). Therefore, it appears that the binding of ligand in one domain causes a considerable change in the environment of the neighboring subunit, at least for NMDA receptors. This suggests a high degree of cooperativity between subunits, which would support the idea that the ionotropic glutamate receptors do not function as a collection of functional domains.

4.3.2.2. Intersubunit interactions in zinc and ifenprodil inhibition of NMDA receptors

Intersubunit allostery is not limited to the ligand binding domains. Subunit-specific modulation allosteric modulation demonstrates a highly interrelated nature of the subunits. Both ifenprodil and zinc inhibit NMDA receptor function in a subunit specific manner via binding sites in the NR2 subunit (Fayyazuddin et al., 2000; Low et al., 2000; Paoletti et al., 2000). However, inhibition by both of these agents is affected by structural elements located in the NR1 subunit. For zinc, the presence or absence of exon 5 on NR1 determines the sensitivity of NR1/NR2A receptors to high affinity zinc inhibition (Traynelis et al., 1998). Additionally, almost all mutations in NR1 previously shown to affect proton sensitivity affect zinc inhibition in parallel (Traynelis et al., 1998). Ifenprodil inhibition also is affected by mutations in the NR1 subunit. One mutation, NR1a(d130n) reduced the sensitivity of NR1/NR2B receptors by 500 fold.
(Masuko et al., 1999). As mentioned in the introduction, alteration of the ligand-binding domain redox site in NR1 abolished ifenprodil inhibition in NR1a(c744a,c798a)/NR2B receptors (Sullivan et al., 1994). This is highly suggestive that structural elements in the NR1 subunit are required for "transduction" of ifenprodil or zinc binding in the ATD of NR2 to the gating element, further arguing against a modular idea of ionotropic glutamate receptor function.

4.3.2.3. "Distant" allosteric interactions

Protein kinases, interacting proteins, and other intracellular agents modulate glutamate receptor function. In general, ionotropic glutamate receptor function is potentiated by phosphorylation (reviewed in Dingledine et al., 1999). Interestingly, evidence suggests that the tyrosine kinase src potentiates NR1/NR2A receptors by relief of high affinity zinc inhibition (Xiong et al., 1999; Yamada et al., 2002; Zheng et al., 1998). As src acts intracellularly, and zinc has a binding site likely located in the ATD of NR2A, it appears that conformational changes induced in an intracellular domain are not independent of the relatively "remote" ATD.

4.3.3. Evidence from this study: modular or macromolecular?

This dissertation adopts the modular view when forming hypotheses about redox and polyamine modulation of NR1 receptors. The hypothesis that a polyamine binding site may have an allosteric effect on the redox modulation of the same domain assumed a modular nature of the ATD of NR1. In the extreme, this view imagines that the ATD of NR1 can bind multiple modulators, convert the
conformational changes from each binding event into a domain-specific conformational change, and then communicate the sum total of this conformational change to the rest of the subunit, thereby allosterically affecting the function of the channel. This assumption is in opposition to the idea that binding of spermine to a site in the ATD of NR1, or the breaking and formation of a cysteine bond contained on NR1, would change the conformation of the entire NR1/NR2A molecule in a concerted manner.

With this assumption, we expected that mutation of NR1(c79) in NR1a(c79a,c765a,c819a)/NR2A receptors (section 3.4.2.4) would abolish the effect of spermine on DTT potentiation. Because we hypothesized that the putative spermine binding site and NR1(c79) were in the same domain, the modular view would assume that most of the redox sensitivity affected by spermine was that of the NR1(c79)-containing redox sensitivity. Our results show that mutation of NR1(c79) did indeed diminish spermine block of redox sensitivity, but it did not eliminate it completely. This implies that spermine had an effect on the remaining redox sensitivity in the NR2 subunit. This does not support the underlying assumption that a spermine-redox interaction would be predominantly intradomain.

Our finding that mutation of cysteines in the ligand-binding domain of NR1 converted a spermine insensitive receptor (NR1/NR2A) into a spermine sensitive receptor (NR1a(c744a,c798a)/NR2A) also supports the macromolecular concept. The binding site of spermine in potentiation of NR1a(c744a,c798a)/NR2A receptors is unknown. While it is possible that the NR1a(c744a,c798a)/NR2A
mutation causes a local conformational change that creates a spermine binding site (as discussed in section 3.5.2.1), it is also possible that the NR1a(c744a,c798a)/NR2A receptor is permissive for ATD spermine binding sites that are masked in NR1/NR2A receptors. This would support the idea that a global conformational change occurs upon mutation of NR1a(c744) and NR1a(c798). Indeed, NR1a(c744a,c798a)/NR2A receptors are less sensitive to high affinity zinc inhibition (Choi et al., 2001), and NR1a(c744a,c798a)/NR2B receptors are insensitive to spermine potentiation and proton inhibition (Sullivan et al., 1994). The sites of action of these modulatory agents all appear to be discrete from the location of NR1a(c744) and NR1(c798a) and imply that a global conformational change occurs when these residues are altered. This could also explain the significant change in time course of redox sensitivity observed between NR1a(c744a,c798a)/NR2A and NR1/NR2A receptors.

It is of note that NMDA receptors appear to be far more complex than other ionotropic glutamate receptors. While studies revealing previously unknown allosteric modulators of AMPA and Kainate receptors are emerging e.g. (Mott et al., 2003), it appears that AMPA/Kainate receptors are less highly modulated than NMDA receptors. The recent crystal structure of the ligand binding domain of NR1 reveals a considerable difference in "loop 1", a cysteine-rich element, between the ligand binding domains of NR1 and the AMPA receptor subunit GluR2 (Furukawa and Gouaux, 2003). Interestingly, Regalado et al. (2001) suggested that this loop may be the site of interaction between glutamate and glycine binding domains in NR2 and NR1, respectively, and could
explain the physical basis of the allosteric interaction between glutamate and glycine. This leads to the postulation that the structure of NMDA receptors may afford a higher degree of mechanical coupling that AMPA and Kainate receptors, thereby giving NMDA receptors a more "macromolecular" nature than the relatively "modular" AMPA and Kainate receptors.

4.4. The usefulness of site directed mutagenesis as tool to uncover structure-function relationships of ion channels

The central dogma of structural biology is that the three dimensional structure of a protein determines its function. The technique of site-directed mutagenesis has been used extensively to estimate protein structure; specifically to determine the importance of a certain residue, to probe for binding sites, to determine the topology of the protein, and even to explore tertiary and quaternary structure. Determination of structure-function relationships of ion channels by site-directed mutagenesis has been widely used, with varying results. For example, over 100 mutants have been tested to determine the structure of the ligand binding domain in ionotropic glutamate receptors (Dingledine et al., 1999). Mapping these residues onto the crystal structure of the GluR2 ligand binding domain (Armstrong and Gouaux, 2000) reveals that all but one of the residues that contact the ligand are predicted by site directed mutagenesis. Thus site directed mutagenesis can be a useful tool. However, sometimes site directed mutagenesis results can be misleading. For example, it was predicted by site directed mutagenesis that the selectivity filter of potassium channels was formed by π orbitals of aromatic residues (Kavanaugh et al., 1991). When the crystal
structure for KscA was solved, this view proved to be inaccurate (Doyle et al., 1998).

While site-directed mutagenesis may accurately predict ligand binding as measured by channel function, mutation of modulatory sites may involve more complex allosteric changes within the structure of the protein. Residues that alter modulation of NMDA receptors may be involved in forming a binding site for the modulatory agent, but alternatively, could be important in transduction of conformational changes elicited by binding. In addition, these residues may instead (or in addition to) be critical for oligomerization. In this case, mutation may disrupt the quaternary structure of the protein, and have non-specific effects on modulation of channel function.

We have observed that the quadruple mutation in NR1a(e181q,e185q,c765a,c819a)/NR2A generated functional receptors, but that these receptors had unusual properties of modulation. The NR1a(e181q,e185q,c765a,c819a)/NR2A mutant abolished sensitivity to the reducing agent DTT (Figure 16). This would imply that NR1 glutamates 181 and 185 are important for redox sensitivity of NR1a(c744a,c798a)/NR2A receptors. However, this sensitivity is possibly restored in the presence of spermine (Figure 16). It is possible that glutamates 181 and 185 are important for the transduction of the conformational change elicited by redox agents. Alternatively, this mutation could concomitantly abolish redox sensitivity and increase the sensitivity of this receptor to spermine. It is also possible that these negatively charged residues are important for the localized charge of residues permissive of
redox modulation, but this wouldn't explain why the presence of a positively charged molecule (spermine) could restore sensitivity. The results of the NR1a(e181q,e185q,c765a,c819a)/NR2A mutant studies are uninterpretable and an example of why caution should be used in interpreting site-directed mutagenesis studies.

4.5. Summary

In summary, modulation of NMDA receptor function is the result of complex interactions between modulatory agents that are highly interdependent. This study focused on modulation of NMDA receptors by reducing and oxidizing agents and an interaction of redox modulation with spermine modulation. Although the structural bases for this interaction require higher resolution structural data to fully be understood, this study adds to the knowledge base with regards to sites of action and important structural determinants of modulatory agents.
Bibliography


of a pharmacophore and a description of the interaction mode. Journal of Molecular Modeling 8, 65-72.


receptor redox modulatory site in the pathophysiology of seizures. J Neurosci 20, 2409-2417.


required for redox modulation of the NMDA subtype of glutamate receptor. Neuron 13, 929-936.


