CELLULAR AND MOLECULAR MECHANISMS OF N-METHYL-D-ASPARTATE RECEPTOR MEDIATED CALCIUM-DEPENDENT NEUROTOXICITY IN CORTICAL CELL CULTURES

by

Rita Sattler

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy Graduate Department of Physiology University of Toronto

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CELLULAR AND MOLECULAR MECHANISMS OF N-METHYL-D-ASPARTATE RECEPTOR MEDIATED CALCIUM-DEPENDENT NEUROTOXICITY IN CORTICAL CELL CULTURES

Rita Sattler, Ph.D. Thesis Abstract, 1999
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The N-methyl-D-aspartate receptors (NMDARs) have been ascribed a key role in excitotoxicity owing to their ubiquitous distribution in central neurons, and to their high permeability to Calcium (Ca^{2+}) ions. NMDAR overactivation was thought to trigger excitotoxicity by excessive intracellular Ca^{2+} accumulation that exceeds the cell's regulatory capacity. Here, we established that the chief determinant of Ca^{2+} neurotoxicity is the route of Ca^{2+} entry, and not the total Ca^{2+} load. Whereas neurons were rapidly damaged when loaded with Ca^{2+} ions through NMDARs, a similar Ca^{2+} load incurred through alternative pathways, such as voltage sensitive calcium channels (VSCCs), was innocuous. First, we investigated whether it is the synaptic localization of NMDARs that is responsible for the toxic potential of NMDAR-mediated Ca^{2+} loading. Perturbing F-actin, a cytoskeletal protein that participates in targeting synaptic NMDARs in dendritic spines, reduced the number of dendritic NMDAR clusters and the NMDAR-mediated component of spontaneous miniature EPSCs, while the AMPA receptor-mediated component was unchanged. This selective perturbation of synaptic NMDARs had no effect on neuronal death or the accumulation of ^{45}Ca^{2+} evoked by applying exogenous
NMDA, which reaches both synaptic and extrasynaptic receptors. However, it increased survival and decreased $^{45}\text{Ca}^{2+}$ accumulation in neurons exposed to oxygen-glucose deprivation, which causes excitotoxicity by synaptic glutamate release, and causes toxicity mainly by activation of synaptic receptors. Thus, it is not the synaptic localization of the NMDARs that determines their toxic potential. We next examined whether the association of NMDARs with distinct membrane-associated macromolecular complexes initiate and/or propagate neurotoxic Ca$^{2+}$ signaling. We studied excitotoxic NMDAR signaling by suppressing the expression of the NMDAR scaffolding protein PSD-95, which selectively attenuated excitotoxicity triggered via NMDARs, but not by other glutamate or Ca$^{2+}$ channels. NMDAR function was unaffected, as receptor expression, NMDA-currents and $^{45}\text{Ca}^{2+}$ loading via NMDARs were unchanged. Suppressing PSD-95 selectively blocked Ca$^{2+}$-activated nitric oxide production by NMDARs, but not by other pathways, without affecting neuronal nitric oxide synthase (nNOS) expression or function. Thus, PSD-95 is required for the efficient coupling of NMDAR activity to nitric oxide toxicity, and imparts specificity to NMDAR-mediated excitotoxic Ca$^{2+}$ signaling.
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Who did what:
All excitotoxicity experiments as well as biochemistry/molecular biology assays described in this thesis were planned, performed, and analyzed by myself, with constructive critical input from my supervisors Dr. Milton Charlton and Dr. Michael Tymianski as well as from the program committee. All cortical tissue culture preparations were done by myself, while all hippocampal tissue culture preparations were done by Ella Czerwinska under my supervision.

All electrophysiological experiments were designed by myself. However, the recordings were performed, in my presence, by Drs. Zhigang Xiong and Wei-Yang Lu of the Department of Physiology, University of Toronto, Toronto, Canada. Dr Xiong and myself analyzed these data together.
Major Publications arising from this work


Sattler R, Xiong Z, Lu WY, MacDonald JF, Tymianski M. Distinct roles of synaptic and extra-synaptic NMDA receptors in excitotoxicity. Submitted to J Neurosci.


Tymianski M, **Sattler R**, Bernstein G, Jones OT (1997). Preparation, characterization and utility of a novel antibody for resolving the spatial and temporal dynamics of the calcium chelator BAPTA. Cell Calcium 22(1), 111-120.


**Book Chapters**


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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate</td>
</tr>
<tr>
<td>AMPAR</td>
<td>AMPA receptor</td>
</tr>
<tr>
<td>AS</td>
<td>Antisense</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine-triphosphate</td>
</tr>
<tr>
<td>APV</td>
<td>DL-2-amino-5-phosphonovaleric-acid</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>Ca(^{2+})</td>
<td>Calcium</td>
</tr>
<tr>
<td>&quot;Ca(^{45})&quot;</td>
<td>Radioactive labeled Ca(^{45})</td>
</tr>
<tr>
<td>[Ca(^{2+})](_i)</td>
<td>Free intracellular calcium concentration</td>
</tr>
<tr>
<td>Cl(^-)</td>
<td>Chloride</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CNQX</td>
<td>6-cyano-7-nitroquinoxaline</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>EAA</td>
<td>Excitatory amino acid</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>L-VSCC</td>
<td>L-type Voltage Sensitive Calcium Channels</td>
</tr>
<tr>
<td>MAGUK</td>
<td>Membrane-associated guanylate kinases</td>
</tr>
<tr>
<td>Min</td>
<td>Minute</td>
</tr>
<tr>
<td>MS</td>
<td>Missense</td>
</tr>
<tr>
<td>Na(^+)</td>
<td>Sodium</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>NMDAR</td>
<td>NMDA receptor</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>nNOS</td>
<td>Neuronal nitric oxide synthase</td>
</tr>
<tr>
<td>ODN</td>
<td>Oligodeoxynucleotide</td>
</tr>
<tr>
<td>OGD</td>
<td>Oxygen-glucose deprivation</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PSD</td>
<td>Postsynaptic density</td>
</tr>
<tr>
<td>PSD-95</td>
<td>Postsynaptic density protein 95</td>
</tr>
<tr>
<td>VSCC</td>
<td>Voltage sensitive Calcium Channels</td>
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INTRODUCTION

1. GENERAL

Calcium (Ca$^{2+}$) ions are ubiquitous intracellular messengers governing a large number of cellular functions such as the control of cell growth and differentiation, membrane excitability, exocytosis and synaptic activity. Because of this, neurons must tightly regulate the cytosolic Ca$^{2+}$ concentration ([Ca$^{2+}$]) to achieve a sufficiently high signal-to-noise ratio for efficient Ca$^{2+}$-signaling to occur. The resting free [Ca$^{2+}$], must remain at very low levels (around 100nM, or $10^3$ times lower than extracellular [Ca$^{2+}$]), so that relatively small or localized increases in [Ca$^{2+}$] can be used to trigger physiological events such as the activation of an enzyme or an ion channel. Neurons have therefore evolved complex homeostatic mechanisms to control both [Ca$^{2+}$] and the intracellular location of Ca$^{2+}$ ions (for a general review of Ca$^{2+}$ homeostasis in neurons see refs Graham et al., 1990; Rahn et al., 1991; Zhou and Neher, 1993; Bindonkas and Miller, 1995). These mechanisms consist of complex interactions between four general categories of events: Ca$^{2+}$ influx, Ca$^{2+}$ buffering, internal Ca$^{2+}$ storage and Ca$^{2+}$ efflux (Fig.1).

Under physiological conditions, a delicate interplay between these processes allows multiple Ca$^{2+}$ dependent signaling cascades to be regulated independently within the same cell. However, it is believed that excessive Ca$^{2+}$ loading, exceeding the capacity of Ca$^{2+}$-regulatory mechanisms, may inappropriately activate Ca$^{2+}$-dependent processes which either lie dormant or normally operate at low levels. When overactivated, such
Figure 1: A schematic representation of Ca\(^{2+}\) homeostasis in neurons. 1, Ca\(^{2+}\) and Na\(^{+}\) influx along with K\(^{+}\) efflux in receptor-gated ion channels, such as glutamate receptors. 2, Ca\(^{2+}\) efflux via an ATP-requiring ionic pump. 3, Ca\(^{2+}\) influx via voltage-gated Ca\(^{2+}\) channels. 4, Ca\(^{2+}\) efflux via Na\(^{+}\)/Ca\(^{2+}\) exchanger. 5, additional ionic channels contributing to membrane repolarization and ionic homeostasis. 6, Ca\(^{2+}\) sequestration (and release) by endoplasmic reticulum. 7, Ca\(^{2+}\) fluxes through the nuclear membrane with potential effects on nucleic acid transcription. 8, Ca\(^{2+}\) sequestration by mitochondria. 9, intracellular Ca\(^{2+}\) buffering by Ca\(^{2+}\)-binding proteins. ATP, adenosine triphosphate; ADP, adenosine diphosphate.
-Figure 1-
processes including enzymes (e.g., proteases, lipases, endonucleases) and other metabolic machinery directly damage neurons or lead to the formation of toxic reaction products which ultimately cause cell death (Fig. 2). In spite of two decades of research supporting the association between Ca\(^{2+}\) excess and neurotoxicity, the precise molecular mechanisms by which Ca\(^{2+}\) neurotoxicity occurs remain poorly understood.

1.1 HISTORICAL BACKGROUND OF CALCIUM AND CYTOTOXICITY

A significant step toward an understanding of cytodestructive mechanisms was initiated with the observation that disturbances in Ca\(^{2+}\) metabolism can lead to cell death. In early studies, pathologists noted that calcium was deposited in areas of tissue necrosis. McLean et al. (1965) observed that livers that had been damaged by toxins accumulated calcium, and suggested that calcium entry may be responsible for tissue damage. In isolated heart preparations, Zimmerman and colleagues (1966) observed that perfusion with calcium deficient solutions, followed by reperfusion with solutions containing calcium, resulted in rapid cessation of contractility followed by massive widespread cell death (the "Calcium paradox"). Subsequent experiments by Schanne and colleagues (1979) revealed that primary cell cultures of adult hepatocytes were killed when exposed to various membrane-active toxins in the presence, but not the absence, of extracellular Ca\(^{2+}\). The authors arrived at the conclusion that Ca\(^{2+}\) influx into cells with disrupted plasma membranes was a requirement for the observed toxicity, and termed Ca\(^{2+}\) influx the "final common pathway of cell death".
Figure 2: A schematic and descriptive presentation of proposed mechanisms by which intracellular Ca$^{2+}$ elevation ($\uparrow$Ca) may trigger secondary Ca$^{2+}$-dependent phenomena, which result in neurotoxicity. NO, nitric oxide; ONOO$^-$, peroxynitrite; ATP, adenosine triphosphate, ADP, adenosine diphosphate, DNA, deoxyribonucleic acid.
Figure 2
However, the idea of Ca\textsuperscript{2+} overload as an absolute requirement for cell death was challenged by other investigators. For example, reports subsequent to that of Schanne and colleagues indicated that cytotoxicity in hepatocytes can be produced in the absence of calcium (e.g. Smith et al., 1981; Fariss et al., 1985). More recently, it has been shown that a rise in cytosolic free Ca\textsuperscript{2+} occurred after, not before critical injury to hepatocytes exposed to chemical hypoxia (Lemasters et al., 1987). Similarly, cell death in the heart under some conditions can be triggered independently of variations in extracellular Ca\textsuperscript{2+} (Chizzonite and Zak, 1981), suggesting that in cardiac muscle, mechanisms other than those responsible for the “calcium paradox” may be operative. These works confirmed that mechanisms other than those triggered by Ca\textsuperscript{2+} excess can also be cytotoxic.

Although cellular Ca\textsuperscript{2+} overload is unlikely to be a common pathway mediating all forms of cell death, several lines of observations support a close relationship between excessive Ca\textsuperscript{2+} influx and neuronal injury in the adult mammalian nervous system. As early as 1973, Schlaepfer and Bunge showed that amputated axons degenerated only if Ca\textsuperscript{2+} ions were present in the extracellular medium. Many studies of the toxicity of excitatory amino acids in cultured neurons and brain slices confirmed an association between the observed toxicity and the presence of extracellular Ca\textsuperscript{2+} (Choi, 1985; Garthwaite et al., 1986). The repeated observations of the requirement of Ca\textsuperscript{2+} in neurodegeneration has given rise to the ‘Calcium hypothesis’ of neurotoxicity which simply states that ‘neuronal Ca\textsuperscript{2+} overload leads to subsequent neurodegeneration’.

However, the association between Ca\textsuperscript{2+} excess and neuronal cell death was not absolute, as other investigators produced evidence which was seemingly contradictory to
the Ca\(^{2+}\) hypothesis. For example, Collins and colleagues showed that in ciliary ganglion neurons depolarizing concentrations of extracellular potassium promote neuronal survival by causing a sustained increase in intracellular calcium (Collins et al., 1991; see also Price et al., 1985). Such contradictions illustrated that the rules governing the relationship between Ca\(^{2+}\) overload and neurotoxicity were poorly understood. Also, despite numerous subsequent studies that supported a link between Ca\(^{2+}\) overload and neurotoxicity, the detailed mechanisms by which Ca\(^{2+}\) ions may trigger neuronal cell death remained to be defined. One major reason for the former is that, to date, virtually every major Ca\(^{2+}\)-dependent intracellular process has been implicated, at some time, as the causative toxic mechanism. For example, neurotoxic actions of Ca\(^{2+}\) overload have been ascribed to the overstimulation of enzymes such as calpains and other proteases, protein kinases, nitric oxide synthetase, calcineurins and endonucleases. Presumably, this leads to an overproduction of toxic reaction products such as free radicals, lethal alterations in cytoskeletal organization, or activation of genetic signals leading to cell death. This large array of potential neurodegenerative mechanisms has made it difficult to propose a focused means of treating Ca\(^{2+}\)-dependent neurodegeneration, and has led to the conclusion that once Ca\(^{2+}\)-toxicity is triggered, therapeutic strategies may have to address several processes simultaneously, rather than a single neurotoxic mechanism.

1.2 CALCIUM AND THE “EXCITOTOXICITY HYPOTHESIS”

Glutamate is the major excitatory neurotransmitter in the mammalian nervous system. It is present at millimolar concentrations in mammalian central gray matter and
has a ubiquitous excitatory effect on central neurons. The postsynaptic effects of this endogenous excitatory amino acid (EAA) are mediated by a number of pharmacologically and functionally distinct cell-membrane receptors, i.e. the ionotropic N-methyl-D-aspartate (NMDA), kainate, and 2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl) propionate (AMPA) receptors as well as metabotropic receptors (Hollmann and Heinemann, 1994). Activation of ionotropic receptors leads to the opening of their associated ion channel which is permeable to sodium, potassium and calcium ions, depending on the receptor type. Metabotropic receptors are not necessarily associated with an ion channel pore, but mediate their actions through GTP-binding protein-dependent mechanisms leading to a mobilization of $\text{Ca}^{2+}$ ions from internal stores such as the endoplasmic reticulum by inositol triphosphate formation.

Even before the realization that cellular $\text{Ca}^{2+}$ accumulation may play a critical role in neurodegeneration, early observations by Lucas and Newhouse (1957) revealed that glutamate may be a potent neurotoxin. They found that systemic injections of L-glutamate into immature mice destroyed the inner neural layers of the retina. These observations remained in relative obscurity until they were reproduced by Olney (1969; 1978), who confirmed the retinotoxicity of glutamate and showed further that the structurally related compound kainate produced brain lesions in immature animals who did not possess a fully developed blood-brain barrier. Olney also found in his studies that this glutamate-induced toxicity was accompanied by rapid cellular swelling which was most marked near dendrosomal components of neurons that are now known to contain EAA receptors. He coined the term “excitotoxicity”, whereby excitatory amino acids
produced neurodegeneration (Olney, 1969). Since its inception, excitotoxicity has been considered as a major mechanism in many human disease states such as cerebral ischemia (stroke), nervous system trauma, epilepsy, and chronic neurodegenerative disorders.

Excitotoxicity is thought to occur due to an excessive synaptic release of glutamate. Its role in hypoxic neuronal damage was established by studies in which blocking either synaptic transmission or postsynaptic glutamate receptors diminished the sensitivity of central neurons to hypoxia. For example, Kass and Lipton (1982) and Rothman (1983) reported that attenuating synaptic transmission by magnesium reduced hypoxic/anoxic neuronal death. Soon thereafter, the neuroprotective activity of glutamate receptors antagonists such as γ-D-glutamylglycine, 2-amino-7-phosphonohepatonic acid and MK-801 was shown in vitro (Rothman, 1984) and in vivo (Simon et al., 1984; Ozyrt et al., 1988).

To date virtually every glutamate receptor subtype has been implicated in mediating neurotoxicity. Although the molecular basis of glutamate toxicity is uncertain, there is general agreement that it is largely Ca²⁺-dependent (Choi, 1985, 1987, 1995). Also, it is generally accepted that the NMDA subtype of glutamate receptors plays a key role in mediating at least certain aspects of glutamate neurotoxicity, possibly owing to their high Ca²⁺ permeability (Choi, 1988; Tymianski, 1996). Several studies of neurotoxicity in cultured neurons have established important pathological roles for the ionic changes caused by glutamate and other EAAs, especially for extracellular Ca²⁺ and extracellular Na⁺. Glutamate-evoked Na⁺ influx has been proposed to contribute to toxicity in some instances. For example, hippocampal cultures (Rothman, 1985) and retina (Olney et al.,
1986) exhibited irreversible, Na\(^+\)-mediated, toxic swelling even in the absence of extracellular Ca\(^{2+}\). However, as initially proposed by Berdichevsky et al. (1983), Choi and colleagues emphasized the role of Ca\(^{2+}\) influx during glutamate neurotoxicity (Choi et al., 1987; Choi, 1987). They showed that although the replacement of extracellular Na\(^+\) abolished the acute neuronal swelling in cortical cell cultures in response to a brief glutamate challenge, most neurons still developed delayed degeneration over the ensuing day. However, if extracellular Ca\(^{2+}\) was absent during the glutamate challenge, the cells swelled transiently but neuronal loss was markedly reduced. Choi's observations suggested two possible mechanisms of excitotoxic neuronal death, distinguished on the basis of differences in time course and ionic dependence. First, an acute, Na\(^+\)- and Cl\(^-\)-dependent component marked by immediate cell swelling, and second a delayed, Ca\(^{2+}\)-dependent component marked by delayed cell degeneration, that could be mimicked by the Ca\(^{2+}\) ionophore A23187. Choi concluded that at lower glutamate exposures the Ca\(^{2+}\) component was the more significant cause of neuronal cell death.

1.3 RELATIONSHIP BETWEEN CALCIUM LOAD AND NEUROTOXICITY

A significant body of evidence now implicates Ca\(^{2+}\) ions in the toxicity process (see above). However, despite the availability of various methods to measure intracellular calcium ions, there have been difficulties in actually determining "how much Ca\(^{2+}\) is too much" for a cell. The inability to pin-point the degree of Ca\(^{2+}\) loading which produces toxicity, or the molecular mechanisms of Ca\(^{2+}\) neurotoxicity, has been ascribed by some
to the ubiquitous involvement of Ca\(^{2+}\) ions in so many different processes, to complexities of cellular Ca\(^{2+}\) regulation, or to methodological limitations of past studies.

Several techniques have been applied to determine the quantity of Ca\(^{2+}\) required to trigger toxicity. In cultured neurons, [Ca\(^{2+}\)], can be measured using fluorescent Ca\(^{2+}\) indicators such as fura-2 (Gryniewicz et al., 1985; Morre et al., 1990). When exposed to glutamate, fura-2 loaded neurons exhibit a transient rise in [Ca\(^{2+}\)], which declines even in the continued presence of the neurotransmitter. This decline is presumably due to the recruitment of [Ca\(^{2+}\)], lowering mechanisms, such as increased Ca\(^{2+}\) buffering and sequestration, and increased Ca\(^{2+}\) extrusion. Those cells that cannot adapt to the Ca\(^{2+}\) load then exhibit a large secondary, irreversible Ca\(^{2+}\) increase, which denotes an irreversible deregulation of Ca\(^{2+}\) homeostasis indicative of impending cell death (Glaum et al., 1990; DeCoster et al., 1992; Randall and Thayer, 1992; Tymianski et al., 1993). However, this delayed [Ca\(^{2+}\)], rise is likely the consequence, rather than the cause of neurodegeneration as removing extracellular Ca\(^{2+}\) after the initial [Ca\(^{2+}\)], increase abolishes the secondary Ca\(^{2+}\) rise, but not neuronal cell death (Tymianski et al., 1993 a, b). Thus, [Ca\(^{2+}\)], measurements with fluorescent Ca\(^{2+}\) indicators showing a delayed loss of Ca\(^{2+}\) homeostasis conflict with the hypothesis that Ca\(^{2+}\) toxicity is a consequence of this homeostatic disturbance. Instead, they suggest that neurotoxic processes can be triggered early on, at a time where Ca\(^{2+}\) regulatory mechanisms are still operative.

Efforts have been made to measure the magnitude of the early (initial) Ca\(^{2+}\) increase needed to trigger neurodegeneration, but with controversial results. The use of fluorescent free Ca\(^{2+}\) indicators for this purpose has been criticized, as this method does
not reflect the total Ca\textsuperscript{2+} load incurred by the cell, but only the small fraction of free Ca\textsuperscript{2+} ions, while Ca\textsuperscript{2+} ions that are buffered or sequestered will not be detected. Critics argue that it is this larger bound, or sequestered, fraction of Ca\textsuperscript{2+} ions, which may trigger toxicity. Also, early investigations using high affinity Ca\textsuperscript{2+} indicators such as fura-2 (K\textsubscript{d} for Ca\textsuperscript{2+} = 200-250 nM) have been criticised because of the possibility that if [Ca\textsuperscript{2+}]\textsubscript{i} increases to micromolar levels (presumed toxic), it may approach the saturation limits of the indicator and fluorescence measurements may no longer reflect true [Ca\textsuperscript{2+}]\textsubscript{i}.

Nevertheless, these problems have been addressed by various means in an effort to determine the degree to which [Ca\textsuperscript{2+}]\textsubscript{i} must rise to cause toxicity. One approach has been to restrict the degree to which [Ca\textsuperscript{2+}]\textsubscript{i} can rise following a toxic insult to determine what range of [Ca\textsuperscript{2+}]\textsubscript{i} must be toxic. This was achieved by pretreating the cells with a range of (non-fluorescent) Ca\textsuperscript{2+} buffers having diverse Ca\textsuperscript{2+} affinities. The buffer’s affinity puts an upper limit on the [Ca\textsuperscript{2+}]\textsubscript{i} rise produced by the toxic insult. This approach revealed that [Ca\textsuperscript{2+}]\textsubscript{i} must exceed several micromolar for toxicity to occur, and concluded that fura-2 must underestimate this value (Tymianski et al., 1994). More recently, the advent of low Ca\textsuperscript{2+} affinity, fluorescent Ca\textsuperscript{2+} indicators, has facilitated the more direct measurement of [Ca\textsuperscript{2+}]\textsubscript{i} changes produced by neurotoxic stimuli and confirmed the findings of the previous approach (Hyrc et al., 1997).

Many questions could not be answered conclusively with the free Ca\textsuperscript{2+} indicator approach. Beyond the original topic (“How much Ca\textsuperscript{2+} is too much?”), the questions of the actual relationship between Ca\textsuperscript{2+} loading and cell death (is there a threshold?),...
compartment in which Ca^{2+} toxicity is triggered, and of the fraction of Ca^{2+} (free vs. bound) responsible for the toxicity remain open.

Due to the limitations inherent in free Ca^{2+} measurements, some investigators turned to measurements of total Ca^{2+} fluxes using radiolabeled ^{45}Ca^{2+} in an attempt to better establish the relationship between Ca^{2+} loading and neurodegeneration. These studies, as well as studies using fluorescent Ca^{2+} indicators, have contributed some conflicting interpretations of what the relationship between Ca^{2+} overload and toxicity is. However, two main hypotheses describing this relationship have arisen: 1) Ca^{2+}-induced neurotoxicity is simply a linear function of the quantity of Ca^{2+} which enters the cell (hereafter termed the 'Ca^{2+} load hypothesis'; Hartley et al., 1993; Eimerl and Schramm, 1994; Lu et al., 1996) and 2) Ca^{2+}-induced neurotoxicity is, like many other Ca^{2+}-regulated physiological processes, regulated through distinct Ca^{2+}-signaling pathways linked to specific routes of Ca^{2+} influx (the 'source-specificity hypothesis'; Tymianski et al., 1993b).

1.3.1 Calcium load hypothesis

Based on the original 'Ca^{2+}-hypothesis', this view suggests that neurodegeneration is simply a function of the quantity of Ca^{2+} which enters the cell. This hypothesis has been best corroborated by studies using radiolabeled ^{45}Ca^{2+}. For example, Manev et al. (1989) and Marcoux et al. (1990) reported that cultured neurons exposed to glutamate or anoxia experienced delayed ^{45}Ca^{2+} accumulations, likely representing the secondary Ca^{2+} deregulation phenomenon described above. In the same year, abstracts from Choi and
colleagues reported that in cortical neurons exposed to glutamate (Kurth et al., 1989) or anoxia (Goldberg et al., 1989), $^{45}\text{Ca}^{2+}$ measurements correlated precisely with cell death. Additional quantitative data on glutamate neurotoxicity were recently published by the same laboratory (Hartley et al., 1993). These demonstrated a very strong linear correlation between $^{45}\text{Ca}^{2+}$ accumulation and cell death when neurons were exposed to differing concentrations of glutamate, or to 500μM glutamate for different exposure durations. Furthermore, agents known to attenuate acute glutamate neurotoxicity such as the NMDA antagonist D-APV and dextrorphan reduced neuronal $^{45}\text{Ca}^{2+}$ accumulation in a manner proportional to their protective effect. The tight linear correlation between $^{45}\text{Ca}^{2+}$ accumulation and glutamate-induced cell death has been reproduced. Schramm and Eimerl (1994) reported a linear correlation coefficient of 0.85 (p<0.001) when plotting $^{45}\text{Ca}^{2+}$ uptake against cell death due to NMDA and glutamate. This linear phenomenon applies not only to tissue cultures, but to acute hippocampal brain slice preparations, in which anoxia-induced electrophysiological cell damage correlated linearly with $^{45}\text{Ca}^{2+}$ accumulation ($r = 0.927$; Lobner and Lipton, 1993). More recently, further results suggesting a simple linear relationship between neuronal $\text{Ca}^{2+}$ accumulation and neurodegeneration have appeared in similar experiments (Lu et al., 1996).

It is difficult to comment about the actual quantity of $\text{Ca}^{2+}$ which produced damage in the above experiments. Lobner and Lipton showed that 50% damage after 5 min of anoxia in hippocampal slices was due to about 4 nmoles of $\text{Ca}^{2+}$ per mg dry weight. Schramm and Eimerl report the figure of about 10 fmol $^{45}\text{Ca}^{2+}$ per cell, about three times the endogenous $\text{Ca}^{2+}$ content, as producing 60% toxicity in their paradigms. This translates to
millimolar Ca\(^{2+}\) concentrations within each cell (about 2.5 mM in a spherical, 20\(\mu\)m diameter neuron).

In paradigms where glutamate, NMDA, or anoxia were used to produce toxicity, \(^{45}\)Ca\(^{2+}\) accumulation measurements appear presently to have resolved methodological difficulties in correlating Ca\(^{2+}\) quantity with neurotoxicity. However, certain inconsistencies remain. For example, if a linear 1:1 relationship between Ca\(^{2+}\) content and cell death is assumed, then Ca\(^{2+}\) influx evoked by pathways other than through NMDA receptor channels should evoke cell death exactly in proportion to the degree of Ca\(^{2+}\) accumulation. However, this is not always the case: Hartley et al. (1993) showed that when neurons were depolarized with high potassium, or challenged with non-NMDA receptor agonists such as kainate, neurotoxicity was less predicted from the amount of \(^{45}\)Ca\(^{2+}\) accumulation. A similar dissociation between \(^{45}\)Ca\(^{2+}\) accumulation and cortical neuronal death was observed in the laboratory of Marcoux: they showed that while NMDA dependent Ca\(^{2+}\) influx and neurotoxicity were well correlated, anoxia-induced \(^{45}\)Ca\(^{2+}\) accumulation- but not neurotoxicity- could be blocked by Ca\(^{2+}\) channel blockers, tetrodotoxin, and phenytoin (Weber et al., 1988; Marcoux et al., 1989). These results are reminiscent of those described by Rothman using free Ca\(^{2+}\) measurements, which suggested that a general elevation in cytoplasmic calcium does not necessarily predict neurodegeneration (Dubinsky and Rothman, 1991). It is apparent from the above that while linear relationships between Ca\(^{2+}\) accumulation and neurotoxicity may exist, additional factors may influence the slope of such relationships.
1.3.2 Source specificity hypothesis

Modern knowledge of physiological Ca$^{2+}$ signaling indicates that despite the ubiquitous presence of Ca$^{2+}$ ions in cells, different Ca$^{2+}$-dependent processes, including synaptic plasticity and gene expression, are separately regulated through distinct signaling pathways linked to specific routes of Ca$^{2+}$ influx (Bading et al., 1993; Lerea and McNamara, 1993; Ghosh, Greenberg, 1995; Friedlander et al., 1997). Taken at face value, the Ca$^{2+}$-load hypothesis implies a departure from this principle, and suggests that Ca$^{2+}$-induced neurodegeneration is triggered by a general overactivation of the many Ca$^{2+}$-dependent processes within the cell. If so, then ‘Ca$^{2+}$ overload’ might simply indicate the point at which excessive crosstalk occurs between the many Ca$^{2+}$-regulated signaling pathways which are normally kept separated by functioning homeostatic mechanisms.

An alternative to the Ca$^{2+}$ load hypothesis is that, like the many other Ca$^{2+}$-regulated physiological processes in the cell, Ca$^{2+}$ toxicity occurs through analogous, distinct Ca$^{2+}$-signaling pathways linked to specific routes of Ca$^{2+}$ influx (the ‘source-specificity hypothesis’). If so, then the search for the mechanisms of Ca$^{2+}$-induced neurotoxicity might be focused on accurately identifiable molecular targets, rather than against all or most of the many Ca$^{2+}$-dependent phenomena already identified in cells.

The source-specificity hypothesis was originally based on experiments performed with free Ca$^{2+}$ indicators. The aim was to determine whether equivalent increases in [Ca$^{2+}$], produced through different Ca$^{2+}$ influx pathways would be equally toxic. These experiments revealed that, in contrast with the Ca$^{2+}$ loading hypothesis, free Ca$^{2+}$ loads produced through L-type voltage-sensitive Ca$^{2+}$ channels (L-VSCCs) were not harmful,
whereas similar [Ca\textsuperscript{2+}], increases produced via NMDA receptors were toxic (Tymianski et al., 1993b). These findings focused the search for Ca\textsuperscript{2+}-neurotoxic mechanisms, at least in such model systems, to Ca\textsuperscript{2+} -activated processes that are preferentially associated with NMDA receptors. The data suggested that a prospect exists to identify specific molecular targets whose manipulation may block Ca\textsuperscript{2+} influx-mediated neurotoxicity. The source-specificity hypothesis proposed that such targets, consisting of rate-limiting enzymes or substrates, might be physically co-localized with NMDA receptors (Tymianski et al., 1993b).

1.4 THE MOLECULAR FRAMEWORK AT THE POSTSYNAPTIC DENSITY

Evidence for the existence of signaling molecules with the potential to act as neurotoxic triggers has arisen from recent knowledge of the molecular composition of the postsynaptic side of the synapse. This molecular framework was thought to enable neurons to target ion fluxes in very distinct patterns to achieve specific physiological effects such as triggering of one particular enzyme.

The specialization of the postsynaptic side of the synapse was first discovered in the 1950s using electron microscopic techniques (Palade and Palay, 1954; DeRobertis and Bennett, 1954). The electron dense and highly differentiated structure on the postsynaptic side was called the postsynaptic density (PSD) and consists of numerous cytoskeletal and regulatory proteins, some of which contact the cytoplasmic domain of ion channels in the postsynaptic membrane (for review see Ziff, 1997).
Electrophysiological and immunocytochemical studies revealed that membrane receptors and ion channels are often localized and clustered at selected subcellular sites on the postsynaptic side of the synapse (Froehner, 1993; Craig et al., 1994). This spatially localized aggregation of receptors ensures a rapid and robust response to released neurotransmitters and allows the neuron to cope with the diversity of synaptic inputs, including excitatory (glutamate-mediated) and inhibitory (GABA-mediated) signals. The purpose of membrane receptor/ion channel clustering at the PSD may be to provide a mechanism for linking the transmembrane ion flux to the molecular machinery responsible for second messenger pathways specifically associated with that ion channel class. Thus, specific molecular components associated with postsynaptic membrane receptor clusters likely also constitute the above mentioned Ca\(^{2+}\)-dependent trigger molecules that regulate pathological signal transduction during excitotoxicity.

1.4.1 Postsynaptic density proteins

Glutamate receptors were originally divided into separate classes based on pharmacological and molecular criteria (see above, see also Hollmann and Heinemann, 1994). Since then, it has become apparent that they differ not only in their function during synaptic transmission and plasticity, but also in the mechanisms and molecules that target and cluster these receptors at the plasma membrane (for review see O'Brien et al., 1998). Recent work that has focused on the molecular composition of the PSD may help to understand the distinctiveness of receptor-specific signal transduction machineries of physiological as well as pathological events.
1.4.1.1 Membrane-associated guanylate kinases

One group of sub-membrane proteins, which is involved in membrane receptor clustering, is known as the MAGUK family for membrane-associated guanylate kinases. Several mammalian homologous families of MAGUKs have been identified: PSD-95/synapse associated protein 90 (SAP90) (Cho et al., 1992; Kistner et al., 1993), chapsyn-110/PSD-93 (Kim and Sheng, 1996; Brenman et al., 1996), SAP102 (Muller et al., 1996) and SAP97/hdlg (Muller et al., 1995), all of which are found in brain synapses. MAGUKs share a common domain organization: multiple N-terminal PDZ domains, a src homology domain 3 (SH3 domain), and a carboxy-terminal yeast guanylate kinase (GuK) homology domain.

Each of those domains can function as a site for protein-protein interaction. The PDZ domains are named after three of the homologous proteins that contain them: PSD-95/SAP90, discs large (Dlg-A, a Drosophila protein found at septate junctions) and zonula occludentes-1 (ZO-1, a vertebrate protein found at epithelial cell tight junctions) (Kennedy, 1995, see also Fanning and Anderson, 1996; Ponting et al., 1997). SH3 domains are also frequent sites of protein-protein interactions (for review see Pawson, 1995). The specific function of the yeast GuK homology domain in these proteins is unclear.

Recent studies using the yeast two-hybrid system demonstrated direct interactions between NMDA receptor subunits and members of the PSD-95/SAP90 family (Ehlers et al., 1996; Gomperts, 1996; Kornau et al., 1997). This interaction occurs through the carboxy-terminal cytoplasmic tail of the NMDAR subunits 2 (NR2), and certain splice
**Figure 3**: Domain organization of PSD-95. SH3, src homology domain 3; GuK, guanylate kinase homology domain
variants of the NMDAR subunit 1 (NR1) (Kornau et al., 1995), with the PDZ domains of PSD-95/SAP 90. The last three amino acids of the carboxyl termini of the NMDAR subunits have defined a consensus motif threonine/serine X valine (T/SXV, where ‘X’ is any amino acid) that has been found to be critical for binding with the PDZ domains (Kornau et al., 1995; Kim et al., 1995; Doyle et al., 1996; Cabral et al., 1996). The functional significance of the interaction between NMDARs and PSD proteins is still unclear. However, it has been suggested that the interaction between NMDARs and PSD-95 may be important for the synaptic targeting and clustering of NMDARs (for review see O'Brien et al., 1998; see also Niethammer et al., 1996).

In addition to receptor clustering and targeting it has been suggested that PSD-95 family proteins play a role in synaptic organization by linking NMDARs to downstream signal-transduction enzymes. This may not only occur through PDZ domain interactions but also via protein-protein interactions at the other conserved domains of the MAGUKs, the SH3 and GuK-domain. GuK domains usually catalyze the conversion of GMP to GDP using ATP, suggesting a signaling function. GuKs in MAGUKs do not show any kinase activity, but a family of proteins has been identified that binds to the guanylate kinase homology domain of PSD-95: GKAP for GK-associated protein (Kim et al., 1997) or SAPAPs for SAP90/PSD-95-associated proteins (Takeuchi et al., 1997). Their function remains unknown, although it is hypothesized that they are involved in intracellular signaling pathways by acting as an adapter molecule between PSD-95 and yet unidentified intracellular signaling molecules. Although SH3 domain motifs are found in
a variety of signaling molecules (for review see Pawson and Scott, 1997), a binding partner for the MAGUK SH3 domain has yet to be found.

PSD-95 family members are also capable of binding to cytoplasmic signaling proteins via PDZ-PDZ domain interactions, thereby linking the NMDAR to signal transduction cascades. The PDZ domain of neuronal nitric oxide synthase (nNOS) binds to the second PDZ domain of both PSD-95 and PSD-93 (Brenman et al., 1996). Although NR2 and nNOS compete in vitro for binding at the PDZ 2 domain of PSD-95/SAP90, NR2 can independently bind PSD-95/SAP90’s first PDZ domain (Niethammer et al., 1996). Thus, PSD-95 may allow for the formation of a ternary complex between NMDAR, PSD-95 and nNOS. This molecular scaffold, which brings NMDARs into a close apposition to nNOS, may explain the preferential activation of nNOS by Ca2+ coming through the NMDARs over Ca2+ entry through other channels (Craven and Bredt, 1998).

Recently a novel Ras-GTPase activating protein has been isolated, SynGAP, that interacts with the PDZ domains of PSD-95 and SAP102 via its C-terminal amino acids (Kim et al., 1998). The GTPase activating domain shares significant homology with other RasGAPs and has RasGAP activity. The Ras-GTPase activating activity of SynGAP is inhibited by phosphorylation by CaMKII located in the PSD protein complex (Chen et al., 1998). Thus, the authors suggest that the PSD-95 family, through SynGAP, may play a role in the regulation of synaptic Ras signal transduction cascades.

In addition, a recent study showed that PSD-95 is linked to Fyn, a Src family protein tyrosine kinase (PTK) as well as other Src family PTKs such as Src, Yes and Lyn.
(Tezuka T. et al., 1999). The interaction occurs via a region, which includes the third PDZ domain of PSD-95 and the SH2 domain of Fyn, thus Fyn and NMDARs bind to different domains of PSD-95. Hence, this finding represents another NMDA receptor-associated PSD-95-based complex, which may play a role in regulation of synaptic transmission and plasticity.

PSD-95 associated complex formations may also be important during neuronal morphogenesis, dendritic outgrowth, synapse formation, and activity dependent reorganization of the postsynaptic signaling machinery. This has been suggested from recently published data, which describe the interaction of PSD-95 with Citron, a Rho-effector in the brain (Furuyashiki et al., 1999; Zhang et al., 1999). Rho family proteins are small GTPases that act as molecular switches in various cellular processes critical for the regulation of cell morphology and cell polarity.

1.4.1.2 Non-NMDAR binding proteins

Unlike NMDARs, AMPA receptor subunits do not exhibit T/SXV motifs, suggesting that they do not interact with the PSD-95 family of proteins. However, two of the AMPA receptor subunits (GluR2 and GluR3) terminate in a very similar sequence (ESVKI) and bind to a protein called GRIP (glutamate receptor interacting protein) (Dong et al., 1997). GRIP contains seven PDZ domains and no other recognizable protein-binding domain. The interaction with the AMPA receptor occurs through PDZ domains four and five, whereas none of the PDZ domains bind to the T/SXV motif, hence GRIP does not bind to NMDARs. GRIP is selectively expressed in neurons and is
colocalized at excitatory synapses with AMPA receptors. However, GRIP does not induce AMPA receptor clustering when they are coexpressed in heterologous expression systems, indicating that other proteins may be required for AMPA receptor synaptic clustering.

Recent studies on glutamate receptors in C. elegans have indicated that complexes of PDZ-containing proteins may be required for the appropriate synaptic targeting of AMPA receptors (Rongo et al., 1998). A recent study showed that the C termini of the GluR2 and GluR3 subunits also interact with the PDZ domain-containing protein PICK1 (protein interacting with C kinase). PICK1 was originally isolated due to its interaction with protein kinase C (PKC) (Staudinger et al., 1995; Staudinger et al., 1997). It is colocalized with AMPA receptors at excitatory synapses and is associated with AMPA receptors in the brain. Moreover, PICK1 was shown to induce the clustering of AMPA receptors in heterologous expression systems. These results indicated that PICK1 and GRIP as well as other proteins that interact with the C termini of AMPA receptors, such as the n-ethylmaleimide-sensitive factor (NSF) (Nishimune et al., 1998; Song et al., 1998; Osten et al., 1998), may cooperate to regulate the targeting and clustering of AMPA receptors at excitatory synapses.

The scheme of glutamate receptor clustering by PDZ-containing proteins was further extended by the identification of Homer, a small protein containing only one PDZ domain, which binds to a subset of metabotropic glutamate receptors (mGlur1α and mGlur5) (Brakeman et al., 1997). Homer was first identified as a neuronal immediate early gene (IEG) based on its rapid induction in rat hippocampal granule cell neurons
following excitatory synaptic activity. The same group of investigators recently presented a comprehensive picture of the family of Homer-related proteins and defined their functional properties (Xiao et al., 1998). Like Homer, all new family members were shown to bind to group 1 mGluRs, but they were all constitutively expressed. The authors suggest a mechanism in which the Homer IEG acts as a natural "dominant negative," in dynamic competition with constitutively expressed Homer family members, to regulate synaptic metabotropic function.

1.4.2 Cytoskeletal elements

Apart from the interaction of intracellular synaptic proteins with membrane-bound proteins a physical bond and functional coupling of synaptic membrane proteins to a specific set of cytoskeletal proteins has emerged at the PSD. The filamentous network of dendritic spines is comprised mainly of actin and actin-regulating proteins and is distinguished from the dendritic cytoskeleton by the near absence of microtubules (for review see Harris and Kater, 1994). One link in the interaction of NMDARs with cytoskeletal elements has been the discovery of α-actinin-2, a protein that binds to actin filaments and to the C-terminal domain of all NMDAR1 exonic splice variants as well as to the C-terminal domain of NMDAR2B, but not NR2A (Wyszynski et al., 1997, 1998; see also Figure 4). The binding of α-actinin-2 to the NMDAR is directly antagonized by Ca²⁺/calmodulin, a protein that binds to NR1 and spectrin (Ehlers et al., 1996), hence Ca²⁺ influx through the NMDAR may induce release of the receptor from the actin cytoskeleton.
Figure 4: Schematic model of the molecular composition at the postsynaptic density. NMDAR, NMDA receptor; AMPAR, AMPA receptor; mGluR, metabotropic glutamate receptor; PSD-95, postsynaptic density protein – 95; nNOS, neuronal nitric oxide synthase; SH3, src homology domain 3; GuK, guanylate kinase (GK) homology domain; NSF, N-ethylmaleimide-sensitive fusion protein; GRIP, Glutamate receptor interacting protein; GKAP, GK associated protein; SynGAP, Ras-GTPase activating protein
A recent report suggests that, unlike PSD-95/SAP90, the actin cytoskeleton does not participate in receptor clustering, but rather serves to tether NMDAR clusters to synaptic sites in dendritic spines (Allison et al., 1998). Depolymerization of F-actin causes a redistribution of NMDAR clusters to non-synaptic sites, but does not alter the binding between NMDARs and PSD-95/SAP90 (Allison et al., 1998). Also, it has been shown that Ca\(^{2+}\) influx through NMDAR directly causes actin cytoskeletal depolymerization (Shorte, 1997), which protects against excitotoxicity (Furukawa et al., 1995). Whether those processes may be related to the phenomenon of Ca\(^{2+}\) induced rundown of NMDAR currents (Rosenmund and Westbrook, 1993) has yet to be determined. The authors proposed that rundown of NMDAR currents was enhanced by cytochalasin-D, an actin cytoskeletal depolymerizing agent, and prevented by phalloidin or ATP, which stabilizes the actin cytoskeleton (Rosenmund and Westbrook, 1993). Thus, rundown may result from disassembly of the actin cytoskeleton from the NMDAR and could reflect a compartmentalization of a channel regulatory protein by actin filaments.

Much less characterized are potential interactions of NMDAR with other synaptic cytoskeletal elements. Ehlers and colleagues (1998) discovered an interaction of NR1 with the 68kDa neurofilament subunit NF-L in a manner regulated by alternative splicing of NR1. NF-L and NR1 were found to colocalize in neuronal dendrites and growth cones, but were much less abundant at the PSD. The authors propose that this interaction could anchor NMDAR to non-synaptic plasma membrane sites, from where they may be recruited to the synaptic sites upon specific signaling. Lin et al (1998) identified and
characterized another novel putative cytoskeletal protein that interacts with the C-terminal tail of NR1 dependent on the alternatively spliced C1 exon cassette: Yotiao. It was found to be specifically concentrated in the neuromuscular junction (NMJ) as well as in neuronal synapses, suggesting that it may have a general function in organizing postsynaptic specialization. Niethammer et al (1998) were successful in the search for a protein, CRIPT, that binds to the 3rd PDZ domain of PSD-95/SAP90. CRIPT colocalizes in the brain with PSD-95/SAP90 in the PSD and coimmunoprecipitates with PSD-95 and tubulin. Despite the fact that PSDs are known to lack microtubules (Harris and Kater, 1994), the authors suggest that CRIPT may support an interaction of PSD-95/SAP90 with a tubulin-based cytoskeleton at the PSD (Niethammer et al., 1998).

1.5 GOALS OF THE PRESENT WORK

Despite the availability of various methods to measure intracellular calcium ions, the precise relationship between "Ca\(^{2+}\) excess" and neurotoxicity has remained controversial (Michaels and Rothman, 1990; Randall and Thayer, 1992; Tymianski et al., 1993a; Hartley et al., 1993). Because strong evidence demonstrates that many forms of neurodegeneration are Ca\(^{2+}\) dependent (Schlaepfer and Bunge, 1973; Choi, 1985, 1987, 1988; Goldberg et al., 1989), some have suggested that toxicity is simply a function of the quantity of Ca\(^{2+}\) which enters the cell (see above: Ca\(^{2+}\) load hypothesis; also Hartley et al., 1993; Eimerl and Schramm, 1994; Lu et al., 1996). The inability to pin-point the degree of Ca\(^{2+}\) loading which produces toxicity, or the molecular mechanisms of Ca\(^{2+}\) -dependent neurotoxicity, has been ascribed to the ubiquitous involvement of Ca\(^{2+}\) ions in
so many different processes, to complexities of cellular Ca\textsuperscript{2+} regulation, or to methodological limitations of past studies (see above).

Recent studies have shown that different physiological Ca\textsuperscript{2+}-dependent processes, including synaptic plasticity and gene expression, are separately regulated through distinct signaling pathways linked to specific routes of Ca\textsuperscript{2+} influx (Bading et al., 1993; Lerea and McNamara, 1993; Ghosh, Greenberg, 1995; Friedlander et al., 1997). If similar specificity also links the source of Ca\textsuperscript{2+} influx to pathways causing cell death, then the treatment of neurodegeneration might be targeted against accurately identifiable molecular targets, rather than against all or most of the many Ca\textsuperscript{2+}-dependent phenomena already identified in cells.

The purpose of this thesis was to first extend the studies by Tymianski and colleagues (1993b) by correlating neuronal cell death with total \textsuperscript{45}Ca\textsuperscript{2+} loading, rather than free intracellular Ca\textsuperscript{2+} rises and to thereby confirm their hypothesis that the specificity of physiological Ca\textsuperscript{2+} signaling is applicable to Ca\textsuperscript{2+} neurotoxicity. We further sought to determine the molecular mechanisms underlying the observation that NMDAR mediated Ca\textsuperscript{2+} loading appears to be more toxic than Ca\textsuperscript{2+} loading through alternative Ca\textsuperscript{2+} influx pathways.

We therefore sought to test the following hypotheses:

**MAIN HYPOTHESIS 1**

Distinct influx pathways, not Ca\textsuperscript{2+} load, determine neuronal vulnerability to Ca\textsuperscript{2+} neurotoxicity
Sub-Hypotheses 1:

I) \([Ca^{2+}]\), elevations triggered by various agonists reflect \(Ca^{2+}\) entry through multiple pathways.

II) The route of \(Ca^{2+}\) influx determines the relationship between total \(Ca^{2+}\) loading and the free intracellular \(Ca^{2+}\) concentration ([\(Ca^{2+}\)]).

III) Calcium influx through NMDA receptors is more toxic than equivalent \(Ca^{2+}\) influx through alternative \(Ca^{2+}\) influx pathways, such as \(Ca^{2+}\) channels and/or AMPA/Kainate receptors.

IV) The route of \(Ca^{2+}\) influx determines the relationship between total \(Ca^{2+}\) loading and \(Ca^{2+}\) induced morphological alterations.

The initial experiments to test the above mentioned hypotheses were designed to confirm the observation that NMDAR mediated neuronal \(Ca^{2+}\) loading is more toxic than equal \(Ca^{2+}\) loading through non-NMDA receptors or \(Ca^{2+}\) channels (Tymianski et al., 1993). To address previous criticisms the experiments were done in primary cortical neuronal cultures, rather than spinal cord neurons. Also, instead of correlating neuronal cell death with free intracellular \(Ca^{2+}\) rises as measured using fluorescent \(Ca^{2+}\) indicators, we chose to measure total \(Ca^{2+}\) loading using radioactive labeled \(^{45}Ca^{2+}\) as described by others (Hartley et al., 1993; Eimerl and Schramm, 1994).

The results obtained from this set of experiments strengthened our as yet unproved theory that \(Ca^{2+}\) influx through NMDAR channels is selectively coupled to neurotoxic signaling pathways, while \(Ca^{2+}\) influx through non-NMDARs or \(Ca^{2+}\)
channels is much less effective in triggering neuronal cell death. To explain this phenomenon, we further sought to test the following hypotheses:

**MAIN HYPOTHESIS 2:**
Lethal NMDAR-mediated Ca^{2+} signals are linked to neurotoxic signaling pathways by the interaction of NMDARs with a multimolecular complex, or a unique compartmentalization of NMDARs to subcellular sites that are essential to cell survival.

An important feature arising from studies of the role of glutamate receptor targeting and clustering was that the accumulation of synaptic glutamate receptor clusters in spines is governed by excitatory synaptic activity, and increases when activity is suppressed (Rao and Craig, 1997; O'Brien et al., 1998). Conversely, excitotoxicity produces a rapid and profound loss of dendritic spines in cultured neurons (Halpain et al., 1998), mimicking the loss in dendritic spine synapses observed in several neurological conditions including epilepsy, schizophrenia, aging and prion protein-related diseases (Jeffrey et al., 1997; Jiang et al., 1998; Garey et al., 1998). However, NMDARs are also found at extrasynaptic sites (Rao and Craig, 1997; Clark et al., 1997), raising the possibility that the synaptic and extrasynaptic subsets of NMDA receptors play different physiological and pathological roles in the cell. Thus the experiments aimed to test the above mentioned hypotheses were based on these studies that suggested that receptor
localization at dendritic spine synapses might be critical to excitotoxicity, and that dendritic spines constitute the subcellular sites that govern neuronal vulnerability to excitotoxicity.

F-actin, a cytoskeletal protein which is concentrated in dendritic spines (Matus et al., 1992; Kaech et al., 1997) may be responsible for targeting NMDARs to synaptic sites, as treatment with actin depolymerizing agents selectively reduces the numbers of synaptic NMDAR clusters without affecting non-synaptic clusters (Allison et al., 1998). We therefore sought to use F-actin depolymerizing agents as a tool to study the excitotoxic potential of synaptic versus extrasynaptic NMDARs and to test whether the synaptic localization of the NMDAR is crucial for its neurotoxic potential.

**Main-Hypothesis 2.A:**

Synaptic localization of NMDARs does not determine the toxic potential of NMDAR-mediated Ca\(^{2+}\) loading.

**Sub-Hypotheses 2.A:**

I) Perturbing the F-actin cytoskeleton does not affect NMDAR function.

II) Perturbing the F-actin cytoskeleton does selectively affect synaptic NMDAR localization and synaptic activation of NMDARs.

III) Depolymerization of F-actin does not affect NMDAR-mediated Ca\(^{2+}\) loading or neurotoxicity produced by exogenously applied NMDA.
IV) Depolymerization of F-actin does protect against NMDAR-mediated Ca\(^{2+}\) loading and neurotoxicity evoked by oxygen-glucose deprivation (OGD), a neurotoxic challenge that is anticipated to injure neurons by activating synaptic NMDARs preferentially.

This selective perturbation of synaptic NMDARs had no effect on neuronal death or the accumulation of \(^{45}\text{Ca}^{2+}\) evoked by applying exogenous NMDA, which reaches both synaptic and extrasynaptic receptors. However, it increased survival and decreased \(^{44}\text{Ca}^{2+}\) accumulation in neurons exposed to oxygen-glucose deprivation, which causes excitotoxicity by synaptic glutamate release. Thus, synaptic and extrasynaptic NMDARs are equally capable of triggering excitotoxicity and it is not the synaptic localization of the NMDAR that determines the neurotoxic potential of NMDAR-mediated Ca\(^{2+}\) neurotoxicity.

Extrasynaptic NMDARs may therefore still be toxic because they remain in association with the intracellular molecules that are responsible for triggering neurotoxicity. Based on this idea was the following hypothesis:

**Main Hypothesis 2.B:**

The interaction of NMDARs with PSD-95 and its interaction with intracellular enzymes such as nNOS form a multimolecular complex, which is responsible for NMDAR-mediated Ca\(^{2+}\) neurotoxicity. Perturbing the interaction between
NMDARs and PSD-95 by reducing PSD-95 protein expression levels will reduce the toxic potential of Ca$^{2+}$ influx through NMDARs.

**Sub-Hypotheses 2.2:**

I) Use of antisense oligodeoxynucleotides to PSD-95 will selectively reduce PSD-95 protein expression levels.

II) Reducing PSD-95 expression levels in cultured neurons and thereby disrupting the NMDAR-specific neurotoxic signaling pathway will protect against NMDA-induced neurotoxicity.

III) Reducing PSD-95 expression levels and thereby disrupting the NMDAR-specific neurotoxic signaling pathway will not alter NMDAR-mediated Ca$^{2+}$ loading.

IV) Reducing PSD-95 expression levels will not alter NMDAR function.

V) Reducing PSD-95 expression levels will affect NMDAR-mediated nitric oxide (NO) signaling and toxicity, due to the disruption of the protein-protein interaction of nNOS with PSD-95 and the thereby reduced ability of NMDAR-mediated Ca$^{2+}$ influx to activate nNOS (see above).

The results obtained from these experiments indicated that PSD-95 is required for efficient coupling of NMDAR activity to nitric oxide toxicity, and therefore provide a molecular model accounting for the specificity of Ca$^{2+}$ neurotoxicity to Ca$^{2+}$ influx via NMDARs.
MATERIALS AND METHODS

Tissue Culture

*High density mixed cortical cultures:* Mixed cortical cell cultures containing both neurons and glia were prepared from embryonic Swiss mice at 15 days of gestation as previously described (Sattler et al., 1997), with minor modifications from Choi (1987). Briefly, cerebral cortices from 10 to 12 embryos were incubated for 10-12 min in 0.05% trypsin in EDTA, dissociated by trituration, and plated on poly-L-ornithine-coated 24-well plates (Corning) or glass coverslips at a density of 0.43 x 10^6 cells/well or 0.9 x 10^6 cells/cover slip. Plating medium consisted of Eagle’s minimum essential media (MEM, Earle’s salt) supplemented with 10% heat-inactivated horse serum (ICN), and (in mM) 2 glutamine, 25 glucose, and 26 bicarbonate. The cultures were maintained at 37 °C in a humidified 5% CO₂ atmosphere. After 3-5 days in vitro, growth of non-neuronal cells was halted by a 24-48h exposure to 10μM FDU-solution (5μM Uridine, 5μM (+)-5-Fluor-2'-Deoxyuridine). This method produces cultures in which >85% of the cells were neurons, based on immunohistochemical staining for glial fibrillary associated protein (exclusive to astrocytes), and for the NMDAR1 subunit (data not shown). The cultures were used for experiments after 12-16 days in vitro.

*Oligodeoxynucleotide-treated cortical cultures:* Cortical cultures were prepared as described above and switched to serum-free media at 24h [Neurobasal with B27 supplement (Gibco)]. Filtersterilized phosphodiester antisense (AS) (5'-GAATGGGTCACCTCC-3'), sense (SE), and missense (MS) (5'-
CCGCTCTATCGAGGA-3') ODNs (5μM) were added in culture medium during feedings at 4, 6, 8 and 10 days after plating. Cultures were used for all experiments on day 12. ODN sequences exhibited no similarity to any other known mammalian genes (BLAST search, Altschul et al., 1997).

*Low density hippocampal cultures:* Mouse hippocampal cultures were prepared using previously described methods (Banker and Cowan 1977, Goslin and Banker, 1991). Briefly, hippocampi were dissected from 18 d mouse embryos and dissociated using trypsin and trituration through a Pasteur pipette. The neurons were plated on coverslips coated with poly-L-lysine in minimal essential medium (MEM) with 10% horse serum at an approximate density of 2400 cells/cm². After the neurons had attached to the substrate, they were transferred to a dish containing a glial monolayer and maintained for up to 3 weeks in serum-free MEM with N2 supplements.

**Drugs and solutions**

The control solution contained (in mM): 121 NaCl, 5 KCl, 20 d-glucose, 10 HEPES acid, 7 HEPES-Na salt, 3 NaHCO₃, 1 Na-pyruvate, 1.8 CaCl₂, and 0.01 glycine, adjusted to pH 7.4 with NaOH. High-K⁺ solution was made by substituting Na⁺ with K⁺ to a total of 50mM K⁺. The high Ca²⁺ solution was prepared by increasing, the concentration of CaCl₂ to 10 mM. Zero sodium experiments were performed in control solution in which NaCl was exchanged with N-methyl-glucamine, NaHCO₃ exchanged with KHCO₃, Na-pyruvate omitted, and HEPES-Na salt exchanged with HEPES acid. Oxygen glucose deprivation (OGD) was performed in a glucose-free bicarbonate-
buffered solution containing (in mM): 121 NaCl, 5 KCl, 1 Na-pyruvate, 1.8 CaCl₂, 25 NaHCO₃, 0.01 glycine, adjusted to pH 7.4 with HCl.

Stock solutions of nimodipine (Miles Pharmaceuticals Inc.), 6-cyano-7-nitroquinoxaline (CNQX; Research Biochemicals Inc), S(-)-Bay K 8644 (Research Biochemicals Inc), 18α-glycyrrhetonic acid, Cytochalasin-D and Latrunculin-A (Molecular Probes Inc.) were prepared in DMSO and kept at -20°C until used. DL-2-amino-5-phosphonovaleric acid (APV) stocks, MK-801 stocks and Tetrodotoxin (TTX) stocks were prepared in distilled water and also stored at -20°C until used. (RS)-1-Aminoindan-1,5-dicarboxylic acid (Tocris Cookson), (RS)α-Methylserine O-phosphate mono phenyl ester (MSOPPE, Tocris Cookson), (S)-4-Carboxyphenylglycine (4-CPG, Tocris Cookson), L-trans-Pyrrolidine-2,4-dicarboxylic acid (L-trans-PDC, Tocris Cookson) and (±)-1-Aminocyclopentane-trans-1,3-dicarboxylic acid (trans-ACPD, Tocris Cookson) were prepared before each use in control solution. Tetanus Toxin (Tntx, List Biochemical Laboratories Inc.) was dissolved as described by the supplier (25 μg/100 ul sterile water) and further diluted in control solution. Solutions of sodium nitroprosside (SNP, dissolved in control solution) were prepared in the dark immediately before application to the cultures to minimize photolysis. A 100mM stock of S-nitrosocysteine (SNOC) was produced immediately before each use from a mixture of 100mM L-cysteine and 100mM NaNO₂ by acidification with 5% (v/v) 10N HCl. SNOC was added to control solution at room temperature and the pH was brought back to 7.4 with 1N NaOH. It was applied within minutes of its synthesis.
Stock solutions of glutamate, NMDA, AMPA and kainate were prepared before each use in control solution. Propidium iodide (PI; 1 mg/ml stock; Molecular Probes Inc.) was prepared in distilled water and dissolved to a final concentration of 50 µg/ml. This concentration of PI produced no observable effects on cell morphology or survival, as demonstrated by the low cell mortality in all control groups. All compounds were always diluted to their final concentrations in the experimental solution. All solutions were sterile-filtered prior to use. Unless otherwise noted above, all chemicals were obtained from Sigma (St. Louis, MO, USA).

**Determination of cell death**

Cell death was determined by serial quantitative measurements of Propidium Iodide (PI) fluorescence using a multiwell plate fluorescence scanner (Cytofluor II, PerSeptive Biosystems, Framingham, MA, USA) as described previously (Sattler et al., 1997). Briefly, the culture medium in each tissue culture well was replaced with control solution containing 50 µg/ml PI, and a baseline fluorescence reading was taken. Sequential readings were then taken at appropriate intervals over the 24 hours following the experimental manipulations. The fraction of dead cells in each culture at a given time was calculated as: Fraction dead = (F_t - F_o)/ F_{NMDA} Where F_t = PI fluorescence at time t, F_o = initial PI fluorescence at time zero and F_{NMDA} = background subtracted PI fluorescence of identical cultures from the same dissection and plating, 24 hours after a 60 min exposure to 1 mM NMDA at 37 °C. Based on manual observations at the time of validation of this technique, this NMDA exposure routinely produced near complete
neuronal death in each culture but had no effect on surrounding glia (also see Bruno et al., 1994; David et al., 1996; Sattler et al., 1997). Adding Triton X-100 (0.1%) to cultures treated in this manner produced an additional 10-15% increase in PI fluorescence due to permeabilization of non-neuronal cell membranes, consistent with a 10-15% glial component in the cultures.

Measurements of $[Ca^{2+}]_i$ and $[Na^+]_i$.

Cultures, grown on glass coverslips and loaded with fura-2/AM (2 μM) or SBFI/AM (10 μM; both from Molecular Probes Inc.), were mounted in a temperature-controlled microscope-stage incubator (Medical Systems Corp. model TC-202). They were then viewed with an inverted microscope (Nikon Diaphot, equipped with Xenon epifluorescence optics) through a fluorite oil-immersion lens (Nikon CF UV-F x40, NA = 1.3) in contact with the coverslip bottom. A second-generation microchannel-plate intensified CCD-array camera (Quantex Corp. Model QX-100) was used to record the fluorescence emissions of the indicator (>510 nm for both fura-2 and SBFI) evoked by excitation through narrow band-pass filters (340 ± 5 nm / 380 ± 6.5 nm) housed in a computer-controlled filter wheel. Fluorescence images were gathered on a PC-based personal computer. The system allowed for a maximal time-resolution of 2 seconds between successive $[Ca^{2+}]_i$ or $[Na^+]_i$ measurements. 4-8 images were averaged at each excitation wavelength. Each average was corrected for background fluorescence and camera dark current by subtracting a frame taken at the beginning of each experiment at each excitation wavelength from an area of the coverslip devoid of cells. Changes in
[Ca\textsuperscript{2+}], or [Na\textsuperscript{+}], in individual neurons were determined by averaging the fluorescence values for all pixels within a boundary traced around the perimeter of the cell soma prior to starting the experiment. Measurements were obtained simultaneously from 10-30 neurons per randomly selected field. Results were expressed as the background subtracted 340/380 nm fura-2 or SBFI fluorescence ratio. The cultures were perfused at 1-2 ml/min. Changes in [Ca\textsuperscript{2+}]\textsubscript{i} were evoked by brief (20 s) applications of a given agonist in the presence of appropriate antagonists (see Fig. 5). [Na\textsuperscript{+}], changes were measured in cultures exposed to 60 min agonist applications (see Fig. 13).

**Measurements of total Ca\textsuperscript{2+} load**

Ca\textsuperscript{2+} loading was determined using measurements of \textsuperscript{45}Ca\textsuperscript{2+} accumulation in the cells. Cultures were washed x1 with control solution and then challenged for a given duration (usually 60 min) with the agonist and/or antagonists in solution containing \textsuperscript{45}CaCl\textsubscript{2} (0.85 μCi/well, 0.5-0.6 mM). In some experiments the cultures were pretreated with \textsuperscript{45}CaCl\textsubscript{2} containing control solution for 14 hrs at room temperature (RT). Thereafter, the cells were rinsed x4 in cold control solution, lysed with 0.2% sodium dodecyl sulfate (SDS) and counted in a scintillation counter. The counts were normalized to the \textsuperscript{45}Ca\textsuperscript{2+} counts obtained from sister cultures exposed for 1 hr to 1mM NMDA in the presence of nimodipine (2 μM) and CNQX (10 μM) at RT, which selectively destroyed all neurons in the culture. The result of this normalization is thus the fraction of the maximal \textsuperscript{45}Ca\textsuperscript{2+} load obtainable in the neurons under study. This normalization method is similar to that used in previous reports (e.g., Hartley et al., 1993), and was selected over normalizing the
"Ca²⁺ reading to the total protein content in the cultures as the latter measurement reflects both the neuronal and glial compartments.

**Simultaneous measurements of [Ca²⁺]ᵢ and total Ca²⁺ load**

In some experiments we measured changes in both free and total Ca²⁺ in the same cells. Of each 24 well culture plate, 12 cultures were loaded with 5 µM fura-2/AM and the other 12 with 5 µM fluo-3/AM. All cultures were then identically exposed to a 60 min insult using either high-K⁻ or NMDA in ⁴⁵CaCl₂ containing solution as above. [Ca²⁺]ᵢ was measured in the multiwell plate fluorescence scanner adjusted for fluo-3 recordings (excitation 485±20, emission 530±30 nm), and for exciting fura-2 at 360±40 nm which provides a [Ca²⁺]-insensitive emission (at 530±30 nm). The Ca²⁺ -insensitivity of the fura-2 signal was confirmed in multiple pilot experiments using high-K⁻ and NMDA (data not shown). Relative changes in [Ca²⁺]ᵢ were calculated using the following formula: $[\text{Ca}^{2+}]_i = \frac{(F_\text{f} - F_\text{f-back})}{(F_\text{fu} - F_\text{fu-back})}$ where $F_\text{f}$ and $F_\text{fu}$ are the fluorescence intensities of fluo-3 and fura-2, respectively, and $F_\text{f-back}$ and $F_\text{fu-back}$ are the respective backgrounds. This background-subtracted fluo-3/fura-2 ratio, taken from fluorescence emissions from appropriate wells in the same culture plate, compensates for any loss of indicator due to dye extrusion, dye loss due to cell death, and differences in cell density between different plates. After the measurement period (usually 60 min), the cells were washed and lysed and ⁴⁵Ca²⁺ accumulation was determined as above.

**Standard experimental protocols**
Excitotoxicity Assay: In most experiments, the cultures were washed x1 in control solution, and then subjected to a challenge (usually 60 min) with a range of concentrations of glutamate, NMDA, AMPA, kainate, high-K−, or the Ca2+ ionophore A23187. Appropriate antagonists (one or more of 2 μM nimodipine, 10 μM CNQX and 10 μM MK-801) were added to isolate the route of Ca2+ loading to a specific influx pathway. After the insult, the cultures were washed x2 and maintained in control solution containing all three antagonists to ensure that toxicity recorded at later times was triggered by this initial insult rather than by delayed depolarization and/or EAA release. Cell survival was measured as above at different times up to 24 hours. Unless otherwise stated, all experiments were performed at 24°C.

45Ca2+ accumulation Assay: The cultures were treated equally to those during the toxicity assay (see above) in the presence of 45Ca2+. After the insult (usually 60 min), the cells were washed with 45Ca2+-free control solution, lysed and the cell-content counted in a scintillation counter.

Oxygen-glucose deprivation (OGD): After taking a baseline PI fluorescence reading (see above) the cultures were transferred to an anaerobic chamber containing a 5% CO2, 10% H2 and 85% N2 (<0.2%O2) atmosphere (Goldberg, Choi, 1993). They were washed x3 with 500 μl of deoxygenated glucose-free bicarbonate solution in the presence of the appropriate antagonists (mostly 2 μM nimodipine and 10 μM CNQX in order to isolate NMDAR activation, see above excitotoxicity-assay) and kept in the anoxic environment for varying time intervals (37°C). OGD was terminated by washing the cultures with oxygenated glucose-containing (20mM) bicarbonate solution containing all
antagonists (nimodipine, CNQX and MK-801). The cultures were maintained for further 22 hrs at 37°C in a humidified 5% CO₂ atmosphere. PI fluorescence readings were taken at appropriate time intervals (mostly 6hrs, 12hrs, 24hrs).

**Determination of ATP content**

ATP was extracted from cultures grown in 24 well plates and measured with a luciferin/luciferase-based bioluminescent somatic cell assay kit (Sigma, St. Louis, MO). The emitted light was detected using the multi-well plate fluorescence scanner from which we removed the excitation light and the emission filters. The utility of this apparatus for ATP content determinations was validated using standard ATP solutions. The luminescence readings, in arbitrary units, were linearly correlated with ATP concentration within the ranges of ATP contents obtained from the cultures.

**Kainate activated Cobalt uptake**

Staining for Co²⁺ uptake was performed as per Pruss et al. (1991) and Turetsky et al. (1994). Briefly, cells were exposed to 100 μM kainate (or 100μM NMDA for a control) plus 5 mM CoCl₂ in uptake buffer (in mM: 139 sucrose, 57.7 NaCl, 5 KCl, 2 MgCl₂, 1 CaCl₂, 12 glucose, 10 HEPES; pH 7.6) for 30 min at RT. The cultures were then washed in uptake buffer containing 5 mM EDTA to chelate any extracellular cobalt. After a following 5 min incubation in 0.12% (NH₄)₂S, the cells were washed three times in uptake buffer, and finally fixed in 4% paraformaldehyde for 30min at RT. Enhancement of the CoS precipitation was performed by washing the fixed cells 3 times in
development buffer (in mM: 292 sucrose, 15.5 hydroquinone, 42 citric acid) and then incubating them in 0.1% AgNO₃ in development buffer at 50°C. This solution was changed every 15 min until the silver enhancement was complete (usually 4 changes). The reaction was terminated by washing the cultures 3 times with development buffer.

**DiI staining/assessment of cell morphology**

To examine axonal, somatic and dendritic morphology after Ca²⁺ loading, cultures grown on glass coverslips were exposed to a specific agonist/antagonist regime and then labeled with the membrane tracer DiI as described by Park et al. (1996) and Faddis et al. (1997). Briefly, they were fixed in 4% paraformaldehyde with 0.025% glutaraldehyde in PBS for 30 min at RT. DiI stock solution (0.5 mg/ml) was prepared in absolute ethanol and kept at -20°C. Prior to use, the solution was diluted 1:100 in PBS, vortexed for 2 min and sonicated for another 2 min. After the fixation step, the cultures were washed twice with PBS and incubated in DiI suspension for 70 min at RT. Cultures were then washed again with PBS and viewed through a x40 oil-immersion lens (Nikon CF UV-F x40, NA = 1.3) using an inverted laser-scanning confocal microscope equipped with an argon/krypton laser (MRC 1000, Bio-Rad, Hertfordshire, England). The following confocal parameters were used: 514 nm excitation, 560 nm emission wavelengths, pin-hole size set at 0.8 mm, laser intensity at 3%, gain at 1320, black level at zero. Due to the three-dimensional nature of the neurons and their neurites, sequential confocal planes were acquired at 0.5 μm intervals, and stacked to produce a two-dimensional image of the cell.
Immunoblotting

To determine the expression level of PSD-95/SAP90 in the oligodeoxynucleotide-treated cultures, tissue was harvested and washed with Phosphate-buffered saline (PBS) containing Phenylmethylsulfonyl fluoride (PMFS) to inhibit intracellular proteases. The harvest was then resuspended in Laemmli’s sample buffer (Laemmli, 1970) containing: Sodium dodecyl sulfate (SDS), Tris-HCl, glycerol, mercaptoethanol, bromphenol blue. The samples were boiled for 5 min and DNA was removed by several passages through a 18 gauge needle, following centrifugation. 1-10µg protein in sample buffer was resolved by electrophoresis on a 7.5% SDS-polyacrylamide gel. The proteins were transferred electrophoretically to a nitrocellulose membrane (0.45µm, Bio Rad) and blocked overnight in Tris-buffered saline (TBS, pH 7.5) containing 5% non-fat dried milk at 4°C. The blots were then incubated for 2 hr at RT with primary antibody diluted in blocking solution. Primary antibodies used: mouse monoclonal anti-PSD-95 IgGl, Transduction Laboratories, diluted 1:250; rabbit polyclonal anti-PSD-93, Synaptic Biosystems GmbH, diluted 1:1000; rabbit polyclonal anti-SAP102, Synaptic Biosystems GmbH, diluted 1:2000; mouse monoclonal anti-NR1 IgG2a, PharMingen, Canada, diluted 1:1000; mouse monoclonal anti-nNOS (NOS type I) IgG2a, Transduction Laboratories, diluted 1:2500. After repeated washing with TTBS (TBS containing 0.05% Tween-20) and TBS, the blots were treated with sheep anti-mouse Ig or donkey anti-rabbit Ig antibodies conjugated to horseradish peroxidase (Amersham Life Science, diluted 1:2000 in blocking solution). After 2 hrs at room temperature, the blots were re-washed and immunoreactive proteins were detected by
enhanced chemi-luminescence (ECL, Amersham Life Science). Densitometric analysis allowed quantification of the protein levels using an imaging densitometer (Bio-Rad GS-670). Molecular weights were determined using Kaleidoscope pre-stained markers (Bio-Rad).

**Immunostaining**

*F-actin*: F-actin was labeled with rhodamine phalloidin. Cultures were fixed in warm 4% paraformaldehyde and 4% sucrose in PBS for 15 min and were permeabilized with 0.25% Triton X-100 for 5 min. The cultures were incubated with 10% bovine serum albumin (BSA) for 30 min at 37°C to block nonspecific staining and were incubated with rhodamine phalloidin (Molecular Probes, 1:4000) in 3% BSA for 2 hr at 37°C.

*NMDA receptors*: Neurons were simultaneously fixed and permeabilized with icecold methanol for 20 min. The cultures were repeatedly washed and then blocked with 10% goat serum in PBS followed by incubation with rabbit polyclonal anti-NR1 IgG (Upstate Biotechnology) at a concentration of 3µg/ml in 2% goat serum/0.2% Triton X-100. The cultures were then washed again, and incubated with secondary antibody: Goat anti-rabbit IgG conjugated to Cy™5 (Jackson ImmunoResearch Laboratories, Inc). Immunostaining was visualized with the 647nm laser line of a confocal microscope (BioRad MRC 1000) through a x40 oil immersion lens (Nikon, NA 1.3).
NADPH-Diaphorase staining

Neurons were stained for NADPH-diaphorase as described before (Dawson et al., 1993). Briefly, cells were washed three times with control solution and fixed for 30 min at 4°C in a 4% paraformaldehyde, 0.1 M phosphate buffer. The fixative was washed away with TBS (50 mM Tris-HCl, 1.5% NaCl, pH 7.4). The reaction solution containing 1 mM NADPH, 0.2 mM nitroblue tetrazolium, 0.2% Triton X-100, 1.2 mM sodium azide, 0.1 M Tris HCl, pH 7.2, was applied to the fixed cell cultures for 1 hr at 37°C. The reaction was terminated by washing with TBS.

Treatment with Actin-perturbing agents

Cultures were treated with varying concentrations of cytochalasin-D or latrunculin-A for 12 hrs by directly applying the drugs from concentrated DMSO stocks into the media. In all experiments in which actin depolymerizing agents were applied, the cultures were treated chronically with 100 μM APV from day 2 until the cultures were used. This chronic APV treatment causes the number of synaptic NMDAR clusters to increase (Rao, Craig, 1997).

Electrophysiology

NMDA currents: Whole cell patch-clamp recordings in the cultured neurons were performed and analyzed as described in Xiong et al., (1997). During each experiment a voltage step of -10 mV was applied from holding potential and the cell capacitance was calculated by integrating the capacitative transient. The extracellular solution contained

50
(in mM): 140 NaCl, 5.4 KCl, 1.3 CaCl₂, 25 HEPES, 33 glucose, 0.01 glycine, and 0.001 tetrodotoxin (pH = 7.3-7.4, 320 - 335 mOsm). A multi-barrel perfusion system was employed to rapidly exchange NMDA containing solutions. The pipette solution contained (in mM): 140 CsF, 35 CsOH, 10 HEPES, 11 EGTA, 2 tetraethylammonium chloride (TEA), 1 CaCl₂, 4 MgATP, pH 7.3 at 300 mOsm.

Miniature EPSCs: Spontaneous miniature excitatory post-synaptic currents (mEPSCs) were recorded as described previously (Lu et al., 1999). The extracellular solution contained 0.5 μM tetrodotoxin, 1 μM strychnine, 10 μM bicucullin methiodide and 1 μM glycine. mEPSCs were filtered at 2 kHz and stored on tape prior to off-line acquisition and analysis with an event detection program (SCAN, Strathclyde software; courtesy of Dr. J. Dempster). For event detection, the trigger level was set at approximately three times of the baseline noise. False events were eliminated by subsequent inspection of the raw data. In general, more than 80 events were acquired for averaging.

After establishing the whole-cell configuration and stable baseline recordings, mEPSCs with both AMPA and NMDA components (without APV and Mg²⁺ added to extracellular solution) were recorded for about 5 min to acquire sufficient numbers of events. The perfusion solution was then changed to one containing 20 mM APV plus 2 μM Mg²⁺ to record AMPA-only mEPSCs. The NMDA-only mEPSCs were obtained by subtracting AMPA-only mEPSCs from the total mEPSCs.
**Determination of cGMP formation**

Determination of cGMP formation was performed 10 min after challenging the cultures with NMDA, kainate, or high-K⁺ with the Biotrak cGMP enzymeimmunoassay system according to the kit manufacturer’s instructions (Amersham). The assay is based on the competition between unlabeled cGMP and a fixed quantity of peroxidase labeled cGMP for a limited amount of cGMP specific antibodies. The cultures were prepared as follows: after the EAA insult, cells were treated with 65% (v/v) ice-cold ethanol in control solution. The supernatant was transferred in a centrifuge tube and combined with a second wash of ethanol. The extracts were centrifuged at 2000xg for 15 min at 4°C and the supernatant transferred into a fresh tube. The extracts were dried under vacuum and dissolved in a suitable volume of assay buffer prior to the analysis.
RESULTS

PART 1: Relationship between neuronal cell death and $^{45}\text{Ca}^{2+}$ accumulation for distinct, pharmacologically isolated $\text{Ca}^{2+}$ influx pathways.

$[\text{Ca}^{2+}]_i$ elevations triggered by various agonists reflect $\text{Ca}^{2+}$ entry through multiple pathways.

We first established the conditions required for restricting neuronal $\text{Ca}^{2+}$ entry to distinct influx pathways using the $\text{Ca}^{2+}$ indicator fura-2. The chief $\text{Ca}^{2+}$ influx pathways were defined by measuring the effects of their specific agonists and antagonists on $[\text{Ca}^{2+}]_i$. Antagonist concentrations were: nimodipine 2 $\mu$M, MK-801 10 $\mu$M, CNQX 10 $\mu$M. No agonist evoked a measurable $[\text{Ca}^{2+}]_i$ rise in the absence of extracellular $\text{Ca}^{2+}$ (not shown).

Repeated, reproducible $[\text{Ca}^{2+}]_i$ transients caused by NMDA (30 $\mu$M; Fig. 5A1) were partly attenuated by the dihydropyridine $\text{Ca}^{2+}$ channel blocker nimodipine, and completely blocked by adding the NMDA receptor antagonist MK-801 (Fig. 5A2). MK-801 alone also completely blocked the $[\text{Ca}^{2+}]_i$ transient (not shown), indicating that NMDA receptor (NMDAR) activation was the initial trigger of the $[\text{Ca}^{2+}]_i$ rise. Blocking L-type $\text{Ca}^{2+}$ channels with nimodipine and non-NMDA ionotropic glutamate receptors with CNQX significantly attenuated the NMDA-evoked $[\text{Ca}^{2+}]_i$ transient (Fig. 5A3, 6A). The surprisingly small $[\text{Ca}^{2+}]_i$ rise remaining after blocking $\text{Ca}^{2+}$ channels and non-NMDARs (leaving that which actually enters through NMDAR channels) was unlikely to be caused by effects of CNQX on NMDARs because the concentration used (10 $\mu$M) was an order of magnitude below that which significantly affects NMDA currents (Randle et
also, increasing CNQX to 25 and 50 μM had no attenuating effect on the NMDA evoked [Ca²⁺]i rise (not shown). Thus, a large portion of the [Ca²⁺]i rise in the absence of antagonists was produced by Ca²⁺ influx through pathways other than NMDARs. NMDA at > 30μM in the presence of CNQX and nimodipine evoked larger [Ca²⁺]i elevations (not shown). However, reproducible [Ca²⁺]i transients as shown in figure 5A1 could not be achieved, possibly due to cell damage by toxic NMDA exposure.

[Ca²⁺]i transients evoked by AMPA (30 μM) were significantly attenuated by nimodipine and almost completely blocked by adding CNQX, confirming that much of the Ca²⁺ influx occurred by secondary activation of L-VSCCs (Fig 5B2, 6A). Blocking NMDARs (MK-801) did not further attenuate the AMPA-evoked [Ca²⁺]i rise (Fig 5B3, 6A). Similar results were obtained with kainic acid as the agonist (Fig 5D1, 5D2, 6A).

Cell depolarization with 50 mM high-K⁺ also produced reproducible [Ca²⁺]i transients (Fig. 5C1). These were only partly attenuated by nimodipine (Figs. 5C2, 6A), but almost completely blocked by combining nimodipine, CNQX and MK-801 (Figs. 5C2, 6A). Depolarizing the cells in only MK-801 and CNQX therefore isolated that portion of the [Ca²⁺]i transient produced by Ca²⁺ influx through L-VSCCs (Figs. 5C3, 6A). The small remaining [Ca²⁺]i transient in the presence of all three antagonists (Figs. 5C2, C3) was not attenuated further by the N-type VSCC blocker ω-conotoxin GⅡA (1 μM; not shown). The possible contribution of P-type VSCCs (McDonough et al., 1996) to this small residual [Ca²⁺]i rise was not excluded since distinguishing between VSCC types was immaterial to this study.
Figure 5: $[\text{Ca}^{2+}]_i$ transients triggered by a given agonist reflect $\text{Ca}^{2+}$ entry through multiple pathways. Cortical neurons loaded with fura-2/AM were subjected to 20s exposures to EAAs, high-K, or $\text{Ca}^{2+}$ ionophore in the presence or absence of different antagonists as indicated. $[\text{Ca}^{2+}]_i$ is presented as the background-subtracted 340/380nm fura-2 fluorescence ratio. Agonist concentrations were chosen so as to produce easily reproducible $[\text{Ca}^{2+}]_i$ transients upon repetitive agonist applications (panels A1-D1). The applied antagonists (black bars) were nimodipine (NIM, 2µM), CNQX (10µM) and MK-801 (10µM). CTRL: antagonist-free control solution, N: NMDA (30µM), A: AMPA (30µM), K+: 50 mM high-K solution, KA: kainate (30µM), A-23187: 4-bromo-A23187 (5µM). Each tracing represents the time-course of $[\text{Ca}^{2+}]_i$ averaged from several neurons (n) in the same field. Standard errors (S.E.) are shown where they exceed symbol size. A: NMDA applications produces reproducible $[\text{Ca}^{2+}]_i$ transients (A1) reflecting a small contribution by $\text{Ca}^{2+}$ influx through VSCCs (A2) and a large contribution via AMPA/Kainate receptors (A3). B: AMPA-induced $[\text{Ca}^{2+}]_i$ transients (B1) reflect a significant $\text{Ca}^{2+}$ influx via VSCCs (B2), but not via NMDA receptors (B3). C: Depolarization with high-K+ produces $[\text{Ca}^{2+}]_i$ transients (C1) which are only partly mediated by VSCCs, having a significant contribution from $\text{Ca}^{2+}$ influx via secondary glutamate receptor activation (C2,3). D: Similar to AMPA, kainate-evoked $[\text{Ca}^{2+}]_i$ transients (D1) reflect $\text{Ca}^{2+}$ influx via VSCCs and glutamate receptor channels (D2). E: 4-Bromo-A23187 can also evoke $[\text{Ca}^{2+}]_i$ transients both in the presence (not shown) and absence of antagonists. Note that the $[\text{Ca}^{2+}]_i$ transients evoked by glutamate (G) and the other agonists (panels A-D) produced $[\text{Ca}^{2+}]_i$ increases far below those seen with the ionophore, and thus, far below the saturation level of fura-2.
Figure 6: Diverse combinations of nimodipine (NIM; 2μM), CNQX (10μM) and MK-801 (10μM) are sufficient to isolate Ca$^{2+}$ loading to distinct Ca$^{2+}$ influx pathways. A: Summary of the representative experiments illustrated in figure 1. Each bar represents mean (+ S.E.) peak [Ca$^{2+}$]i measurements taken from the number of neurons indicated (n), pooled from several experiments (N). Each peak [Ca$^{2+}$]i measurement was normalized to its own control (transient evoked in the absence of antagonists). Arrows indicate the peak [Ca$^{2+}$]i value obtained through the isolated influx pathway. Inset: Diagrammatic representation of influx pathway isolation strategy. “Other” represents potential Ca$^{2+}$ influx pathways not covered by the three antagonists. B: The three antagonists are sufficient to block completely Ca$^{2+}$ loading evoked through glutamate receptors and VSCCs. Cultures were exposed for 60 min to EAAs or high-K$^+$ in media containing $^{45}$CaCl$_2$ and all three antagonists (nimodipine, 2μM, CNQX, 10μM and MK-801, 10μM), and $^{45}$Ca$^{2+}$ accumulation measured as described (Methods). Sister cultures were identically treated and cell death measured at 24 hours. EAAs were applied in media containing 1.8mM Ca$^{2+}$ at the following concentrations (in μM): NMDA and glutamate at 0, 20, 40, 60, 80 and 100, Kainate at 0, 10, 30, 100, 300, 1000. The high-K$^+$ solution contained 10mM Ca$^{2+}$ and increasing concentrations of Bay-K 8644 (0, 100, 200, 300, 400 and 500nM). Note that the three antagonists effectively abrogate all measurable Ca$^{2+}$ accumulation and toxicity (compare with fig. 5). Each symbol represents measurements averaged from 2-4 cultures each for $^{45}$Ca$^{2+}$ and cell death. Standard errors are shown where they exceed symbol size.
Figure 6
The fura-2 fluorescence ratios produced by the various agonists were well below those produced by adding the Ca^{2+} ionophore 4-bromo-A23187 (5 μM; not shown) and thus, below the apparent saturation limits of the Ca^{2+} indicator.

To explore for additional routes of Ca^{2+} influx which are unaffected by the above antagonists, cultures in ^45Ca^{2+} containing media were exposed for 60 min to a range of concentrations of NMDA, L-glutamate, kainate, or high-K^- (50 mM) in the presence of all three antagonists (nimodipine, CNQX and MK-801). ^45Ca^{2+} loading was evaluated at the end of the exposure (see Methods). Sister cultures were similarly treated, and cell death evaluated at 24 hrs. Fig 6B shows that the three antagonists virtually eliminated all ^45Ca^{2+} accumulation and cell death, indicating that alternative routes of Ca^{2+} influx which are unaffected by the three antagonists would contribute little (if any) to Ca^{2+} loading or to cell survival in our studies. The agonist/antagonist combinations defined in Fig 6A (insert) can thus be used to pharmacologically isolate Ca^{2+} entry to distinct pathways.

Temporal and quantitative characterization of neurotoxicity and calcium loading

Neurotoxicity and Ca^{2+} accumulation are both time-dependent. Optimal times for evaluating cell survival and Ca^{2+} loading were determined by exposing cultures to L-glutamate (0-1000 μM) for 60 min in nimodipine and CNQX. They were then returned to a solution containing the above antagonists + MK-801. For each glutamate concentration, this treatment caused cell death and ^45Ca^{2+} accumulation to peak at 24 hrs and 60 min, respectively (Figs. 7A, B). These peak times were thus used for cell death and ^45Ca^{2+} determinations in this study.
Figure 7: Characterization of cell death and of ⁴⁵Ca²⁺ accumulation in cultures exposed to NMDA or L-glutamate. A: Cultures were exposed to a 60 min insult with L-glutamate (0, 10, 30, 100, 300 or 1000 μM) and cell death was measured in the multiwell-plate fluorescence scanner (see Methods) at hourly intervals for 24 hours thereafter. Each symbol represents an average reading from 4 cultures. Standard errors are shown where they exceed symbol size. B: Cultures were similarly exposed to L-glutamate at the indicated concentrations in ⁴⁵Ca²⁺ containing media. Ca²⁺ accumulation was measured at 10, 30, 60 min and 5 hours after the challenge onset. ⁴⁵Ca²⁺ accumulation increased throughout the 60 min insult, and decreased from its peak by 5 hours. Each bar represents the mean (+S.E.) ⁴⁵Ca²⁺ accumulation from four cultures. C: Less than 10% of the measured ⁴⁵Ca²⁺ accumulation occurs in non-neuronal cells. Bars indicate the mean (+S.E.) ⁴⁵Ca²⁺ accumulation in cultures exposed to 1mM NMDA for 60 min at 37°C 24 hours prior to exposure to the indicated agonist. The NMDA pretreatment kills >95% of neurons in the plate, leaving only non-neuronal cells [1239]. D,E: Concentration-response relationships between 60 min NMDA () and glutamate (■) applications and the resultant ⁴⁵Ca²⁺ accumulation at 60 min and cell death at 24 hours. Experiments were performed in the presence of nimodipine (2μM) and CNQX (10μM). Each symbol represents the mean of 4 cultures. Different symbols shapes indicate experiments in cultures from different dissections. Standard errors are shown where they exceed symbol size.
Figure 7
Figure 8: Simultaneous measurements of free and total calcium accumulation in the same cells are highly correlated when the \( \text{Ca}^{2+} \) influx occurs through pharmacologically distinct influx pathways. Using \(^{45}\text{Ca}^{2+}\)-containing media, cultures were challenged for 60 min with NMDA (in the presence of nimodipine, 2\( \mu \)M and CNQX, 20\( \mu \)M), or with high-K (50mM, with 10\( \mu \)M MK-801, 10\( \mu \)M CNQX, 10mM \( \text{Ca}^{2+} \) and 0, 100, 200 or 300nM Bay-K 8644). Changes in [\( \text{Ca}^{2+} \)]\(_i\) were measured in the multiwell plate fluorescence scanner as described using the fluo-3/fura-2 ratio or the calcium-green 5N/fura-2 ratio (see Methods). \(^{45}\text{Ca}^{2+}\) accumulation was then measured in the same cultures at the end of the 60 min challenge. A: Challenges with 30\( \mu \)M NMDA and 50mM high-K\(^-\) (without Bay-K 8644), which evoke similar changes in [\( \text{Ca}^{2+} \)]\(_i\), also produce similar \(^{45}\text{Ca}^{2+}\) accumulations in the same cells (inset). Note the differences in peak [\( \text{Ca}^{2+} \)]\(_i\) despite similar average changes in [\( \text{Ca}^{2+} \)]\(_i\) and total \( \text{Ca}^{2+} \). B: Measurements performed in different cultures with the low \( \text{Ca}^{2+} \) affinity indicator calcium green 5N produce identical results to those seen with fluo-3. C: Time-course of [\( \text{Ca}^{2+} \)]\(_i\) with increasing concentrations of Bay-K 8644 in cultures challenged with high-K. D: Time-course of [\( \text{Ca}^{2+} \)]\(_i\) with increasing concentrations of NMDA. E: High correlation between \(^{45}\text{Ca}^{2+}\) accumulation and the fluo-3/fura-2 ratio averaged over 60 min in cells challenged with high-K\(^-\) (from 2 experiments similar to C), or with NMDA (from 3 experiments similar to D). Solid lines: least-squares best fit line. Each symbol represents the mean fluo-3/fura-2 ratio and/or \(^{45}\text{Ca}^{2+}\) measurement from 4 cultures. \(^{45}\text{Ca}^{2+}\) accumulation in C was measured and presented as the fraction of the maximal achievable \(^{45}\text{Ca}^{2+}\) load in the cultures (Methods). Standard errors are shown where they exceed symbol size.
Optimal agonist concentrations for titrating neuronal Ca\(^{2+}\) loading were obtained from concentration-response curves derived for each agonists/antagonist combination in this study. For L-glutamate and NMDA (with nimodipine and CNQX), cell death and Ca\(^{2+}\) accumulation peaked at 100 μM (Figs. 7D, E). Ca\(^{2+}\) accumulation produced by 50 mM high-K\(^{+}\) (in MK-801 and CNQX) only reached levels equal to those produced by NMDA or L-glutamate in increased [Ca\(^{2+}\)]\(_c\) (10 mM) and 300-500 nM S(-)-Bay K 8644, an L-type VSCC agonist (see figs. 8C,9B).

We have previously established that, in the protocol described (Fig 7A), glutamate and NMDA toxicity was restricted to neurons, leaving glia unaffected (Sattler et al., 1997, also Koh, Choi, 1987; Bruno et al., 1994; David et al., 1996). To determine the cell type undergoing \(^{45}\)Ca\(^{2+}\) accumulation under the same circumstances, neurons were selectively destroyed by a 60 min pretreatment with NMDA (1mM, 37°C; Sattler et al., 1997). After 24hrs, the same cultures were exposed for 60 min to L-glutamate (0-300 μM), NMDA (0-1000 μM) or high-K\(^{+}\) (50 mM, in 10 mM Ca\(^{2+}\) + 500 nM S(-)-Bay K 8644). No \(^{45}\)Ca\(^{2+}\) accumulation above control values was observed in the absence of neurons (Fig. 7C). Also, no free [Ca\(^{2+}\)]\(_i\) changes, as evaluated with fura-2, were produced in glial cells by any of the agonist/antagonist combinations used (not shown). Thus, Ca\(^{2+}\) loading occurred primarily in neurons.
The relationship between $[\text{Ca}^{2+}]_i$ and total Ca$^{2+}$ loading depends on the route of Ca$^{2+}$ entry.

NMDA in the presence of non-NMDAR and L-VSCC antagonists produced surprisingly small $[\text{Ca}^{2+}]_i$ elevations as compared with the other agonists (Fig 5A3, 6A). Given the central role of NMDARs in Ca$^{2+}$-mediated neurotoxicity (Tymianski et al., 1993b), we examined this issue in greater detail by studying both $[\text{Ca}^{2+}]_i$ and $^{45}\text{Ca}^{2+}$ accumulation in the same neurons.

Free $[\text{Ca}^{2+}]_i$ recordings were made in cultures grown in 24 well plates using $^{45}\text{Ca}^{2+}$-containing solutions so that after the $[\text{Ca}^{2+}]_i$ recordings, the cells were lysed, and $^{45}\text{Ca}^{2+}$ accumulation was measured in the same cells (Methods). $[\text{Ca}^{2+}]_i$ indicator extrusion and photobleaching were minimal (<3%) and identical for both indicators (fura-2, fluo-3; not shown). Ca$^{2+}$ loads were evoked by 60 min exposures to NMDA (with nimodipine and CNQX) or to high-K$^+$ (50 mM, in 10 mM Ca$^{2+}$, CNQX, MK-801 and 0-300 nM S(-)-Bay K 8644). These insults caused minimal or no cell death over 60 min (Fig 8A, D), so both $[\text{Ca}^{2+}]_i$ and $^{45}\text{Ca}^{2+}$ measurements were obtained from living cells. Agonist concentrations were chosen to produce a range of $^{45}\text{Ca}^{2+}$ accumulation from baseline to the maximum achievable $^{45}\text{Ca}^{2+}$ content.

The time-course of free $[\text{Ca}^{2+}]_i$ changes evoked by high-K$^+$ (Fig. 8A) and by NMDA (Fig. 8B) differed markedly. Cultures challenged with Ca$^{2+}$ through L-VSCCs exhibited a rapid rise in $[\text{Ca}^{2+}]_i$, followed by a decline to a lower, stabler plateau. In contrast, Ca$^{2+}$ entry through NMDAR channels caused a lower initial $[\text{Ca}^{2+}]_i$ rise, followed by a gradual increase which never stabilized, particularly at NMDA
concentrations exceeding 20 μM. Free [Ca$^{2+}$]$_i$ changes were highly correlated with the $^{45}$Ca$^{2+}$ accumulation measured in the same cells (Fig 8E). However, the slope of the relationship between [Ca$^{2+}$]$_i$ and $^{45}$Ca$^{2+}$ load depended on the agonist. Specifically, NMDA produced a higher average [Ca$^{2+}$]$_i$ increase for a given total $^{45}$Ca$^{2+}$ load as compared with high-K$^+$ (Fig 8E), suggesting that differences exist in the cell’s ability to handle Ca$^{2+}$ loading through different pathways.

Previous studies using fluorescent [Ca$^{2+}$]$_i$ indicators could not consistently demonstrate a causal relationship between [Ca$^{2+}$]$_i$ and neurotoxicity (Michaels and Rothman, 1990; Randall and Thayer, 1992; Tymianski et al., 1993a). This has recently been attributed to the saturation of high-Ca$^{2+}$ affinity indicators such as fura-2 by high [Ca$^{2+}$]$_i$, causing them to underestimate neurotoxic Ca$^{2+}$ levels (Tymianski et al., 1994; Hyrc et al., 1997). For this reason, we did not report absolute (calibrated) [Ca$^{2+}$]$_i$ values in this study. However, our [Ca$^{2+}$]$_i$ measurements were in complete agreement with $^{45}$Ca$^{2+}$ loading throughout the entire range of feasible $^{45}$Ca$^{2+}$ loads (Fig. 8E). Therefore, high-affinity indicators retained their utility for demonstrating the differences in neurons’ handling of Ca$^{2+}$ through different influx pathways, even if calibrated [Ca$^{2+}$]$_i$ values would have been underestimated. Similar results to Fig. 8E were obtained using the low affinity indicator calcium green-5N instead of fluo-3 (Fig. 8A vs 8B). Thus, the critical impact of the route of Ca$^{2+}$ loading on [Ca$^{2+}$]$_i$ homeostasis (Figs. 8A,E), not indicator saturation, may have contributed to previous failures of conventional [Ca$^{2+}$]$_i$ measurements to predict neurotoxicity. Also, in the present study, the variability of our measurements was significantly reduced by relating [Ca$^{2+}$]$_i$ and total Ca$^{2+}$ in the same
cells, and by obtaining averaged [Ca\(^{2+}\)], measurements from the entire cell sample rather than from a select few neurons in each culture.

Source-specificity of Ca\(^{2+}\) neurotoxicity in cultured cortical neurons.

We now examined whether Ca\(^{2+}\) neurotoxicity is a function of total Ca\(^{2+}\) load (Hartley et al., 1993; Eimerl and Schramm, 1994; Lu et al., 1996), or of the source of Ca\(^{2+}\) influx (Tymianski et al., 1993b). If neurodegeneration is simply a function of Ca\(^{2+}\) loading, then it should be independent of the route of Ca\(^{2+}\) influx. As in all experiments, \(^{45}\)Ca\(^{2+}\) accumulation was expressed as the fraction of the maximum obtainable Ca\(^{2+}\) loading in the neurons (Methods).

Ca\(^{2+}\) entry was isolated to NMDARs by treating the cultures for 60 min with 0-100 µM NMDA in nimodipine and CNQX (Fig 6A, insert), causing concentration-dependent increases in \(^{45}\)Ca\(^{2+}\) accumulation. However, neurotoxicity at 24 hrs remained low until the Ca\(^{2+}\) load exceeded 80% of maximum (Fig. 9A). Thus, a threshold must be exceeded before Ca\(^{2+}\) ions become toxic when Ca\(^{2+}\) loading is achieved by this method.

NMDA toxicity (Fig 9A) required Ca\(^{2+}\) influx, as it could not be produced in Ca\(^{2+}\) free media (0 Ca\(^{2+}\), 100 µM EGTA, not shown), and showed a clear (albeit non-linear) relationship with Ca\(^{2+}\) loading. This is in accordance with the established Ca\(^{2+}\)-influx requirement for EAA toxicity in this and other models as reported in many previous studies (Choi, 1985, 1987; Garthwaite and Garthwaite, 1986; Ellren and Lehmann, 1989; Michaels and Rothman, 1990; Randall and Thayer, 1992; Tymianski et al., 1993a; Hartley et al., 1993). This requirement for Ca\(^{2+}\) influx excludes the possibility that
**Figure 9:** Source-specificity of Ca\(^{2+}\) neurotoxicity in cultured cortical neurons. Cultures were challenged for 60 min using different agonists/antagonist combinations shown to isolate Ca\(^{2+}\) influx to a specific route of entry (see Fig. 2A). A range of agonist concentrations was used to evoke different degrees of cell death and of Ca\(^{2+}\) loading (see Fig 3D,E). Normalized \(^{45}\)Ca\(^{2+}\) accumulation (see Methods) was evaluated after 60 min (the peak Ca\(^{2+}\) load; Fig. 3B) of agonist/antagonist exposure. Sister cultures were identically exposed, but maintained for a further 23 hours (in the presence of all 3 antagonists- nimodipine, CNQX , and MK-801), and cell death was measured at 24 hours. 

**A:** NMDA (0-100mM) applied in the presence of nimodipine and CNQX.  

**B:** 50mM K\(^+\) solution applied in 10mM Ca\(^{2+}\), S(-)-Bay K 8644 (0-500nM), CNQX and MK-801.  

**C:** 4-Bromo A23187 (0-30mM) applied in the presence of all three antagonists.

Symbols in A-C represent the average measurement from 4 cultures each for \(^{45}\)Ca\(^{2+}\) and cell death determinations. \(^{45}\)Ca\(^{2+}\) accumulation was measured and presented as the fraction of the maximal achievable \(^{45}\)Ca\(^{2+}\) load in the cultures (Methods). Different symbol shapes indicate experiments from different culture dissections. Error bars are shown where they exceed symbol size. Solid lines indicate hand-drawn “best-fit” curves (mathematical fitting was not used, as the biological significance of the curve shape [e.g., linear vs. exponential] is not known).
NMDA toxicity was mediated by a coincident, Ca\(^{2+}\)-independent, event also triggered by NMDAR activation.

Neurons challenged for 60 min with high-K\(^{-}\) were not injured. In contrast to Ca\(^{2+}\) loading via NMDARs (Fig. 9A), Ca\(^{2+}\) loading through L-VSCCs was not toxic, even at maximal Ca\(^{2+}\) accumulation identical to that obtained with NMDA (Fig. 9B). Similar results were obtained in cultures pre-incubated (37\(^{\circ}\)C) in \(^{45}\)Ca\(^{2+}\)-containing media for 14 hrs prior to experiments (not shown), ensuring that the measured \(^{45}\)Ca\(^{2+}\) accumulation in Figs. 9A,B reflected a net Ca\(^{2+}\) influx, not bidirectional Ca\(^{2+}\) cycling through the cell membrane in which native Ca\(^{2+}\) is exchanged for \(^{45}\)Ca\(^{2+}\).

In contrast with high-K\(^{-}\), the divalent cation ionophore 4-bromo-A23187 (0-30 \(\mu\)M), which permeabilizes both internal and external cell membranes causing non-specific Ca\(^{2+}\) entry (Kristal and Dubinsky, 1997), was highly toxic even in the absence of any significant \(^{45}\)Ca\(^{2+}\) accumulation (Fig 9C). At 24 hrs, no neurons had survived treatment with 20-30 \(\mu\)M. Although this ionophore may have also permeabilized glia to Ca\(^{2+}\), most appeared morphologically intact, and only a few (<10%) stained PI (not shown). Similar results were obtained with A23187 and digitonin, another membrane permeabilizing agent (not shown).

Neurons undergo cell swelling and exhibit dendritic varicosities within minutes to hours following exposure to EAAs or to anoxia (Choi et al., 1987; Choi, 1987; Bindonkas and Miller, 1995; Park et al., 1996; Faddis et al., 1997). Cell swelling is likely an osmotic consequence of Na\(^{+}\) influx through glutamate receptors (Choi, 1987), whereas the ionic dependence of dendritic varicosities is less clear, but may involve Ca\(^{2+}\) ions for their
initiation or maintenance (Bindonkas and Miller, 1995; Faddis et al., 1997). These morphological changes have been associated with neuronal injury both in-vitro and in-vivo (Olney, 1971; Emery, Lucas, 1995 and refs. above). Therefore, we examined the relationship of these changes to neuronal Ca\(^{2+}\) loading through distinct pathways. Cultures were exposed to agonists under conditions identical to those in figure 9, using agonist concentrations which produced maximal (100\%) \(^{45}\)Ca\(^{2+}\) accumulation. After the 60 min exposure, the cultures were either fixed immediately (Fig 10A,C,E,G) or returned to solution containing all antagonists and fixed after 5 hrs (Fig 10B,D,F,H) or 23 hrs (not shown). These time-points reflect the height of Ca\(^{2+}\) loading (60 min; Fig 7B), and later times (5 and 23 hrs) in which some recovery from the Ca\(^{2+}\) load occurs. The fixed neurons were then stained with the fluorescent membrane dye DiI, revealing the cell soma, extensive dendritic arborization, and dendritic spines.

Neurons challenged with high-K\(^{+}\) (Fig. 10C,D) were morphologically similar to controls (Fig. 10A,B) at 1, 6, and 24 hrs, even though they were maximally Ca\(^{2+}\) -loaded to an extent which was clearly lethal when loading occurred through NMDARs (Figs 9A, 12A,B). Ca\(^{2+}\) loading alone is therefore insufficient to produce cell death, cell swelling or dendritic varicosities. In contrast, similar Ca\(^{2+}\) loading via NMDARs using L-glutamate (Fig 10E,F) or NMDA (not shown) produced the anticipated cell swelling and dendritic beading with complete cytodestruction at 24 hrs (not shown). These morphological changes were probably independent of the Ca\(^{2+}\) load, because they were also produced by the Ca\(^{2+}\) ionophore (Fig 10G,H), which caused neurodegeneration with much less Ca\(^{2+}\)
Figure 10: The route of Ca\textsuperscript{2+} influx, not the quantity of Ca\textsuperscript{2+} loading, dictates morphological alterations. Cultures were exposed for 60 min to the indicated agonists under conditions producing maximal Ca\textsuperscript{2+} loading through distinct pathways. After 1 hr (A,C,E,G) or 6 hr (B,D,F,H), they were fixed, stained with DiI, and imaged as described (Methods). A,B: control (unchallenged) cultures. C,D: Cultures challenged with 50mM K\textsuperscript{+} in the presence of 10mM Ca\textsuperscript{2+}, 500nM Bay-K 8644, 10μM CNQX and 10μM MK-801. E,F: Cultures challenged with 100μM L-glutamate in 1.8mM Ca\textsuperscript{2+}, 2μM nimodipine and 10μM CNQX. G,H: Cultures challenged with 10μM 4-bromo A23187 in 1.8mM Ca\textsuperscript{2+}, 2μM nimodipine, 10μM CNQX, 10μM MK-801. Challenges with A23187 produced smaller Ca\textsuperscript{2+} accumulations than glutamate and high-K\textsuperscript{+} (Fig 5D) but were highly lethal. Lethal challenges with glutamate and A23187 produced cell swelling and dendritic beading (arrows), whereas equal Ca\textsuperscript{2+} loading through voltage-gated Ca\textsuperscript{2+} channels produced no changes in cell morphology (compare A,B with C,D) even though the conditions for high-K\textsuperscript{+} and glutamate produced identical Ca\textsuperscript{2+} loads in the neurons (see Fig. 5B2,C).
Figure 10
Figure 11: Neurons challenged with kainic acid undergo little $^{45}$Ca$^{2+}$ accumulation and cell death. A: Representative phase image of cortical neurons challenged with 100 mM kainate and stained for Co$^{2+}$ positivity. B: Transmitted light image of the same field, showing Co$^{2+}$ positive neurons. These constituted only 8.4% of the total number of neurons (based on counts from 13 fields in 3 cultures). C: Kainic acid (0, 10, 30, 100, 300 and 1000 mM) was applied for 60 min in solution containing 10 mM Ca$^{2+}$. The resultant $^{45}$Ca$^{2+}$ accumulation and cell death were measured at 60 min and 24 hours, respectively. Symbols in C represent the average measurement from 4 cultures each for $^{45}$Ca$^{2+}$ and cell death determinations. $^{45}$Ca$^{2+}$ accumulation in C was measured and presented as the fraction of the maximal achievable $^{45}$Ca$^{2+}$ load in the cultures (Methods). Different symbol shapes indicate experiments from different culture dissections. Error bars are shown where they exceed symbol size.
accumulation (Fig 9C). Thus the source, not the degree of Ca\textsuperscript{2+} loading, was the likely determinant of the observed morphological changes.

Some have proposed that Ca\textsuperscript{2+} neurotoxicity following AMPA/kainate receptor activation is related to the degree of Ca\textsuperscript{2+} loading (Lu et al., 1996). However, only a fraction of AMPA receptors exhibit Ca\textsuperscript{2+} permeability (Hollman et al., 1991; Gasic and Heinemann, 1992; Jonas et al., 1994; Bochet et al., 1994; Ruano et al., 1995). Neurons bearing Ca\textsuperscript{2+}-permeable channels can be identified immunohistochernically using kainate-activated cobalt uptake, and would be vulnerable to Ca\textsuperscript{2+} neurotoxicity through AMPA receptors (Pruss et al., 1991; Turetsky et al., 1994). These comprised only 8.4% of neurons in our cultures (Fig.11A,B). Under conditions which isolate Ca\textsuperscript{2+} influx to non-NMDA ionotropic glutamate receptors (using nimodipine and MK-801, Fig. 6A), a 60 min kainate application (0-1000 μM) produced little \textsuperscript{45}Ca\textsuperscript{2+} accumulation and cell death even in 10mM [Ca\textsuperscript{2+}]e (Fig.11C). This, coupled with our finding that most AMPA/kainate-evoked Ca\textsuperscript{2+} loading is actually due to cell depolarization and Ca\textsuperscript{2+} influx through L-VSCCs (Fig. 6A), made it difficult in this study to comment on the relationship between Ca\textsuperscript{2+} influx and neurodegeneration through this route.

A component of glutamate neurotoxicity additive to that produced by Ca\textsuperscript{2+} ions.

Others have described essentially linear relationships between neuronal Ca\textsuperscript{2+} loading and glutamate neurotoxicity (Hartley et al., 1993; Eimerl and Schramm, 1994). This was reproduced in the present study using L-glutamate (0-100 μM) with or without antagonists (Figs 12A,B). The observed linear relationship between glutamate-evoked
Ca\(^{2+}\) loading and cell death was unlike that observed with NMDA (compare with Fig 9A). Thus, isolating Ca\(^{2+}\) accumulation to NMDAR channels (nimodipine, CNQX, Fig. 6A, insert) unmasked a component of glutamate toxicity exceeding that of NMDA at equal intermediate Ca\(^{2+}\) loads (compare with Fig 9A). This component was not reduced by raising the CNQX concentration from 10 to 50 \(\mu\)M, excluding a contribution of unblocked AMPA/kainate receptors, which might be activated by glutamate (not shown). We therefore investigated other possible causes of this phenomenon.

Glutamate, the endogenous neurotransmitter, activates both ionotropic (iGluR) and metabotropic (mGluR) glutamate receptors. Both may mediate the release of intracellular Ca\(^{2+}\) stores (Simpson et al., 1995) which, if contributing significantly to neurotoxicity, could confound the present findings and account for the excess toxicity of glutamate over NMDA. To examine the role of mGluRs, experiments were repeated in the presence of a range of concentrations of diverse mGluR agonists and antagonists (Table 1). However, none of these affected the degree of \(^{45}\)Ca\(^{2+}\) accumulation, cell death or the relationship between them, making it unlikely that mGluRs contribute significantly to the excess toxicity of glutamate over NMDA.

Glutamate is released at excitatory synapses and is recovered by reuptake mechanisms, whereas NMDA is not. The contribution of endogenous glutamate release to our glutamate toxicity paradigm was tested by blocking vesicular transmitter release
**Figure 12:** Glutamate-evoked Ca\(^{2+}\) loads produce greater toxicity and ATP depletion as compared with NMDA. Experiments in A,B, exploring the relationship between \(^{45}\)Ca\(^{2+}\) accumulation and toxicity, were performed and plotted identically to those in Fig 9. A: Effect of L-glutamate (0-100\(\mu\)M) applied without antagonists. B: L-glutamate (0-100\(\mu\)M) applied with nimodipine and CNQX. Compare with Fig. 4A. The lines indicate hand-drawn “best-fit” curves (mathematical fitting was not used, as the biological significance of the curve shape [e.g., linear vs. exponential] is not known). C: Glutamate application produces greater ATP depletion than NMDA. Cultures were exposed for 60 min to the indicated concentrations of either L-glutamate or NMDA in nimodipine and CNQX. After 3 hrs, the cells were lysed and the ATP content measured (Methods). Each bar represents the average (+ S.E) of 4 cultures. Data were normalized to ATP measurements from similarly treated controls (sham washes, no agonist exposure). Asterisks: significant difference between glutamate and NMDA groups (unpaired t-test, p<0.05). D: Same data as in (C), normalized to the \(^{45}\)Ca\(^{2+}\) accumulation produced by the different EAA concentrations, illustrating the profound ATP depletion by glutamate at intermediate \(^{45}\)Ca\(^{2+}\) loads. Symbols in A,B,D represent averages from 4 cultures each for \(^{45}\)Ca\(^{2+}\) and cell death (A,B) or ATP (D) determinations. Different symbol shapes in A,B indicate experiments from different culture dissections. Error bars are shown where they exceed symbol size.
Figure 12
using combinations of TTX (500 nM - 1 μM), a blocker of voltage gated sodium channels and/or tetanus toxin (TnTX; 10-50 nM), which blocks neurotransmitter release by cleaving synaptobrevin (Ahnert-Hilger and Bigalke, 1995; Poulain et al., 1996; Ahnert-Hilger et al., 1996). Neither compound affected glutamate-evoked ⁴⁴Ca²⁺ accumulation or neurotoxicity (Table 1), indicating that the toxicity observed in Fig. 12A,B was due to exogenous (applied), not endogenous glutamate. Glutamate re-uptake, on the other hand, was important to cell survival because blocking glutamate reuptake using L-trans-PDC (Bridges et al., 1991) increased both the ⁴⁴Ca²⁺ accumulation and neurotoxicity produced by any given glutamate concentration (not shown). However, neither blocking glutamate reuptake nor transmitter release altered the fact that for any given intermediate ⁴⁴Ca²⁺ accumulation, glutamate remained more lethal than NMDA (Table 1).

Additional hypotheses were tested to account for the increased toxicity of L-glutamate over NMDA. Glutamate also activates Ca²⁺ changes in astrocytes (Finkbeiner, 1992), as characterized by regenerative waves produced by internal Ca²⁺ release. These Ca²⁺ changes may propagate to neurons via gap junctions (Nedergaard, 1994), possibly causing toxicity. However, 18α-glycyrrrethonic acid, an astrocyte gap junction blocker (Davidson and Baumgarten, 1988), failed to reduce the toxicity of L-glutamate for a given ⁴⁴Ca²⁺ load, having no effect on the relationship between glutamate-induced ⁴⁴Ca²⁺ accumulation and neurotoxicity (Table 1). Thus, it is unlikely that Ca²⁺ ion diffusion from astrocytes, or the activation of Ca²⁺ release from neuronal stores by astrocytes, accounts for the increased toxicity of glutamate. Finally, since most experiments were performed at room temperature (24°C), the NMDA and glutamate experiments were repeated at 37°C.
to exclude a temperature-dependent artifact. However, the higher temperature did not affect the disparity between glutamate NMDA toxicity (not shown).

ATP depletion has been associated with neurodegeneration. To determine whether the excess toxicity of L-glutamate over NMDA at intermediate Ca$^{2+}$ loads was paralleled by differences in ATP levels, cultures were challenged as before with a range of concentrations of NMDA or glutamate (both 0-100 μM) in the presence of nimodipine and CNQX. The highest and lowest NMDA and glutamate concentrations produced similar degrees of $^{45}$Ca$^{2+}$ accumulation and cell death (Figs 9A, 12B). Also, they produced similar ATP depletion (Fig 12C). However, cultures challenged with intermediate glutamate concentrations (20-80 μM), sustaining intermediate $^{45}$Ca$^{2+}$ loads, exhibited a significantly greater ATP depletion as compared with NMDA (Fig. 12C,D).

NMDA toxicity is due to Ca$^{2+}$, not Na$^{+}$.

NMDAR channels are permeable to Na$^{+}$, whereas VSCCs generally are not. We therefore examined whether Na$^{+}$, rather than Ca$^{2+}$ ion entry, could explain NMDAR toxicity over VSCCs.

Cultures were challenged either with NMDA or with high-K$^{-}$ under conditions shown in figure 5 to produce identical, maximal Ca$^{2+}$ loading. The relative change in intracellular Na$^{+}$ concentration ([Na$^{+}$]) was measured with the ratiometric Na$^{+}$ indicator SBFI for 60 min (Methods). Cultures challenged with high-K$^{-}$ exhibited no significant [Na$^{+}$] increase over this period (Fig. 13A,C), suggesting that either voltage-gated Na$^{+}$ channels inactivated, that Na$^{+}$ extrusion mechanisms compensated for the Na$^{+}$ load, or
both. In contrast, [Na\(^+\)]\(_i\) in cultures challenged with NMDA rose to levels similar to those produced by the Na\(^+\) ionophore gramicidin which, presumably, equalizes [Na\(^+\)]\(_i\) and extracellular Na\(^+\) concentration ([Na\(^+\)]\(_e\)) (Fig 13B,C).

Nevertheless, cell death following NMDAR activation was produced by Ca\(^{2+}\) loading, not by Na\(^+\), because cultures exposed to 100 µM NMDA (with 2 µM nimodipine, 10 µM CNQX) in zero [Na\(^+\)]\(_e\) exhibited identical "Ca\(^{2+}\) accumulation and cell death to cultures challenged in normal [Na\(^+\)]\(_e\). Cell death in zero [Na\(^+\)]\(_e\) media was reduced, in parallel with "Ca\(^{2+}\) accumulation, by MK-801 (Fig 13D). Similar results were obtained with low (10 mM) [Na\(^+\)]\(_e\) (not shown). Thus, Ca\(^{2+}\), not Na\(^+\) loading, was primarily responsible for NMDA neurotoxicity.
**Figure 13:** Calcium, not sodium, is responsible for the observed neurotoxicity of NMDA receptors. Relative changes in $[\text{Na}^+]_i$ were measured in cultures loaded with the ratiometric Na$^+$ indicator SBFI/AM (Methods) and challenged for 60 min with high-K$^-$ or with NMDA. 5 μM gramicidin, a Na$^+$ ionophore, was added at the end of each experiment to illustrate the maximum obtainable SBFI ratio. A: Effect of 50mM high-K$^-$ applied under conditions known to produce a maximal Ca$^{2+}$ influx, which was not toxic (Fig 9B). $[\text{Na}^+]_i$. B: Effect of 100 μM NMDA applied under conditions known to produce a maximal Ca$^{2+}$ load equivalent to that produced by the high-K$^-$ insult. n = number of neurons from which tracings were derived. Symbols indicate means ± S.E. C: Average maximum change from baseline in the SBFI ratio in cultures subjected to the indicated agonists as in A,B. Numbers in brackets indicate number of experiments. Asterisk (*) indicates differences from all other groups (ANOVA with post-hoc t-tests, p<0.0001). D: Effects of blocking NMDA receptors (10 μM MK-801) on $^{45}$Ca$^{2+}$ accumulation and on neurotoxicity in Na$^+$ free medium, which excludes the role of Na$^+$ influx on the observed toxicity. Each bar represents the mean (+ S.E.) of 4 cultures. Data were normalized to cultures exposed for 60 min to 1mM NMDA at 37°C in normal [Na$^+$]o.
Figure 13
### Table 1

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<th>Concentration</th>
<th>Agonist</th>
<th>Effect</th>
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<td>(RS)-1-Aminoisindan-1,5-dicarboxylic acid</td>
<td>Group I mGlu-R antagonist</td>
<td>500μM</td>
<td>Glutamate</td>
<td>None</td>
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<tr>
<td>MSOPPE</td>
<td>Group II/III mGlu-R antagonist</td>
<td>500μM</td>
<td>Glutamate</td>
<td>None</td>
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<tr>
<td>(RS)-1-Aminoisindan-1,5-dicarboxylic acid + MSOPPE</td>
<td>Group I + Group II/III antagonists</td>
<td>500μM each</td>
<td>Glutamate</td>
<td>None</td>
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<td>4-CPG</td>
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<td>500μM, 1000μM</td>
<td>Glutamate</td>
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<td>trans-ACPD</td>
<td>Group I and II mGluR antagonist</td>
<td>500μM</td>
<td>NMDA</td>
<td>None</td>
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**and L-trans-Pyrolidine-2,4-dicarboxylic acid (L-trans-2,4-PDC)**

<table>
<thead>
<tr>
<th>Compound:</th>
<th>Action:</th>
<th>Concentration</th>
<th>Agonist</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamate uptake carrier inhibitor</td>
<td>10μM, 30μM, 100μM</td>
<td>Glutamate (endo- genous)</td>
<td>None</td>
<td></td>
</tr>
</tbody>
</table>

- TTX | Na⁺ channel Blocker | 500nM | Glutamate | None |
- TTX | Na⁺ channel Blocker | 500nM | NMDA | None |
- TTX + tetanus toxin | Na⁺ channel blocker and release blocker | 500nM + 10nM | Glutamate | None |
- TTX + tetanus toxin | Na⁺ channel blocker and release blocker | 500nM + 10nM | NMDA | None |
- Tetanus toxin | Transmitter release blocker | 50nM | Glutamate | None |
- 18α-glycyrrhetonic acid | gap-junction blocker | 10, 30, 60μM | Glutamate | None |

**Legend:**

Metabotropic glutamate receptors, vesicular neurotransmitter release and gap junctions do not contribute to the Ca²⁺-influx-independent component of glutamate neurotoxicity. Summary of additional experiments not shown in the figures. Each experiment was replicated at least x2. Cultures were exposed to glutamate or NMDA at 0,
20, 40, 60, 80 and 100 μM concentrations in the presence of 2μM nimodipine, 10 μM CNQX, and one of the compounds above. After 60 min they were washed in control solution and either used for 45Ca²⁺ determinations or left in control solution containing 2 μM nimodipine, 10 μM CNQX and 10 μM MK-801 for another 23 hours. In experiments involving tetrodotoxin (TTX), the blocker was applied for the full 24 hours. In experiments involving tetanus toxin, the cells were pre-incubated with the agent at 37°C for two hours. Cell death was monitored at 24hrs as described (see Methods). At least four cultures were subjected to each agent at each agonist concentration. None of the compounds altered the relationship between ⁴⁵Ca²⁺ loading and neurotoxicity from that illustrated in figure 9A (for NMDA) and in 12B (for glutamate).
PART 2: Molecular mechanisms of NMDAR-mediated Ca\(^{2+}\) - dependent neurotoxicity.

The results in part 1 show that NMDAR-mediated Ca\(^{2+}\)-dependent neurotoxicity cannot be reproduced when neurons are loaded with equivalent quantities of Ca\(^{2+}\) entering the cell through alternative Ca\(^{2+}\) influx pathways, such as voltage sensitive calcium channels. Unfortunately, the present culture system did not allow us to investigate the neurotoxic effects of Ca\(^{2+}\) loading through AMPA receptors (see Fig. 11). However, a recent study performed in our laboratory investigated the Ca\(^{2+}\)-dependence of AMPAR-toxicity using neuronal cultures from GluR2-deficient mice (Iihara et al. manuscript in submission). This work showed that despite the increased Ca\(^{2+}\) permeability in the GluR2-deficient neurons, the vulnerability to kainate toxicity was unchanged. Consequently, NMDARs must possess as yet uncharacterized properties that permit them to trigger Ca\(^{2+}\)-mediated neuronal damage more effectively than other Ca\(^{2+}\) sources. We hypothesized that such properties may include a unique association of NMDARs with rate limiting substrates or enzymes that trigger neurotoxicity, or a unique compartmentalization of NMDARs with subcellular sites essential to cell survival (Tymianski et al., 1993b, 1996; Sattler et al., 1998).
A. Functional consequences of synaptic localization of NMDARs on NMDAR-mediated Ca\(^{2+}\) - dependent neurotoxicity.

Glutamate receptors (NMDA and non-NMDA) accumulate in the form of clusters at synaptic spines (Craig et al., 1994; Kornau et al., 1995; Rao and Craig, 1997; O’Brien et al., 1998), while also present at non-synaptic sites (Rao and Craig, 1997; Clark et al., 1997). The synaptic clustering is regulated by excitatory synaptic activity (Rao and Craig, 1997; O’Brien et al., 1998), which raises the possibility that the synaptic localization of NMDARs at the spines may play a role in neuronal vulnerability to excitotoxicity. NMDARs are localized to synaptic sites through interaction with cytoskeletal and/or scaffolding proteins, forming a large macromolecular complex at the postsynaptic density (Gomperts, 1996; Wyszynski et al., 1997; Ponting et al., 1997; Ehlers et al., 1998; Allison et al., 1998). The cytoskeletal element F-actin may participate in targeting synaptic NMDARs in dendritic spines (Matus et al., 1992; Kaech et al., 1997; Allison et al., 1998), as treatment with actin depolymerizing agents selectively reduces the number of synaptic NMDAR clusters without affecting extrasynaptic clustering (Allison et al., 1998). We used this tool to test the hypothesis whether it is the synaptic localization of the NMDAR that is responsible for the selective vulnerability of NMDAR activation, or whether it is the direct association with a macromolecular complex that triggers NMDAR-mediated Ca\(^{2+}\) - dependent neurotoxicity.
Effects of actin depolymerizing agents on neuronal F-actin.

We studied neuronal actin using rhodamine phalloidin, a fluorescent actin stabilizing compound used to stain, visualize and quantify F-actin (Cooper, 1987; Shorte, 1997; Allison et al., 1998). Rhodamine phalloidin labels F-actin in cell bodies and processes, and is highly fluorescent in dendritic spines where F-actin is concentrated. The cultures were treated for 12h with the actin depolymerizing agents, stained, and viewed with a confocal microscope.

Treating cortical neurons with cytochalasin-D (1-10µM) caused actin to agglomerate (Fig. 14A), without attenuating the overall rhodamine phalloidin fluorescence (Fig 14B). This is consistent with the anticipated action of the cytochalasins, which, while causing F-actin to break down into shorter filaments, still bind phalloidin (Cooper, 1987). Hence, the rhodamine fluorescence intensity was unchanged (Fig. 14B). Treating cortical neurons with latrunculin-A (0.1-5µM) dramatically reduced the visualization of rhodamine phalloidin in the cells (Fig. 14A), and reduced its fluorescence by 70% (Fig. 14B). This suggests a more potent actin depolymerization by this agent.

Owing to the high density of neurons and their dendritic arbors in the cortical neuronal cultures, we could not reliably visualize the fate of dendritic spines following treatment with actin depolymerizing agents. As these were the compartments of greatest interest, we repeated the experiments in hippocampal neurons grown at low density (see Methods). F-actin staining, which appeared homogeneous in dendritic shafts and spines of sham treated neurons (Fig. 15A) was changed by the cytochalasin-D treatment (10 µM) to a punctate pattern similar to that seen in the cell soma in cortical cells (Fig 14A).
Figure 14: Differential effects of depolymerizing agents on actin in cultured cortical neurons. A. Rhodamine phalloidin fluorescence images of neurons treated with 0.1% DMSO (sham), cytochalasin-D (10μm) or latrunculin-A (1μm), obtained using identical confocal excitation, emission and gain settings. Treatment with cytochalasin-D caused actin to agglomerate into clumps (arrow), whereas latrunculin-A reduced actin staining throughout the cells. Scale bar, 30μm. B. Quantification of rhodamine-phalloidin fluorescence in cultured cortical neurons treated with actin depolymerizing agents. The cultures were grown in 24 well plates, treated with the agents at the indicated concentrations for 12h, and imaged with the confocal microscope using identical settings for each well. An averaged, background subtracted fluorescence intensity was derived from 15-20 randomly selected fields taken from 6 separate cultures per condition (shown as mean ± SE averaged pixel values). 1 μm latrunculin-A induces a 70% decrease in rhodamine phalloidin fluorescence. Asterisk: statistically different from sham (t_{38} = 24.6, p <0.0001).
A

Sham  

Cytochalasin  

Latrunculin

B

- Bar chart showing averaged pixel values for Sham, Cytochalasin, and Latrunculin treatments.

Figure -14-
Figure 15: Effects of actin depolymerizing agents on actin filaments in dendrites and spines of cultured hippocampal neurons. The cultures were treated with 10 μM cytochalasin-D or 1 μM latrunculin-A for 12 h, stained with rhodamine phalloidin, and imaged with the confocal microscope using identical settings. A, Homogeneous actin staining in control neuron treated with sham (0.1% DMSO) solution. *Inset:* Homogeneous staining of dendritic shafts and spines. B, Cytochalasin-D induces agglomeration of F-actin throughout the entire neuron. *Inset:* maintenance of actin clumps in dendritic spines. C, Latrunculin-A dissolves F-actin throughout the cell. *Inset:* uniform loss of rhodamine phalloidin staining in dendritic shafts and spines. Scale bar in C, 20 μm. Insets of (A), (B) and (C) show magnified views of the indicated dendrites (numbers). All images are representative of neurons in N > 6 cultures per condition.
As described by Allison et al. (1998), actin in the spines appeared resistant to destruction by this agent (Fig. 15B, insert).

Treatment for 12h with latrunculin-A completely dissolved F-actin in the cell, including actin in dendritic shafts and spines (Fig. 15C). This was consistent with results obtained by visualizing cortical neurons (Fig. 14) and those obtained by others (Allison et al., 1998). Therefore, based on imaging criteria, this agent might be more suitable for perturbing actin at synaptic sites.

We also examined the effects of concentration and treatment duration with the actin depolymerizing agents in both cortical and hippocampal cultures. The effects of cytochalasin-D (concentrations tested: 0.1, 3, 10 and 30 µM) on producing a punctate distribution of actin was maximal at 10 µM. The effects of latrunculin-A (concentrations tested: 0.1, 0.3, 1, 3 and 5µM) on reducing actin staining reached a peak at 1µM (data not shown). There were no differences between 12 and 24h treatment periods for either agent. Also, cell morphology (by phase-contrast optics) and 24h survival were unchanged by the drugs (see sections below).

Disruption of F-actin does not affect NMDA-evoked ionic currents.

Previous studies have established an intimate structural and functional relationship between F-actin and NMDARs. F-actin is bound to NMDARs via α-actinin (Wyszynski et al., 1997), and its state of polymerization may play an important role in regulating NMDA channel activity (Rosenmund and Westbrook, 1993). Therefore, to determine the functional consequences of depolymerizing actin in our cortical neurons,
we recorded whole cell NMDA-evoked currents from cultures treated with the depolymerizing compounds. The concentrations selected were based on their effects on F-actin in the imaging studies (Figs. 14,15).

Cortical neuronal cultures were treated for 12 h with either cytochalasin-D (10 μM), latrunculin-A (1 μM) or DMSO (0.1%). F-actin depolymerization had no effect on passive membrane properties, including input resistance and membrane capacitance [Capacitance: DMSO: $51.4 \pm 2.5$ pF (n=23); cytochalasin-D: $56.1 \pm 3.0$ pF (n=20), latrunculin-A: $48.8 \pm 2.4$ pF (n=27), one-way ANOVA, $F = 1.98, p = 0.15$]. Fig. 16A shows representative traces of whole-cell recordings of currents elicited by brief applications of 3-300 μM NMDA. Peak NMDA currents were not significantly different in cultures treated with cytochalasin-D or latrunculin-A: DMSO: $1969.7 \pm 141.5$ pA (n=23); cytochalasin-D: $2231.3 \pm 223.6$ pA (n=20), latrunculin-A: $2020.4 \pm 125.8$ pA (n=27) (Fig. 16A, one-way ANOVA, $F = 0.68, p = 0.51$). NMDA concentration-response relationships were also unaffected (Fig. 16B; EC$_{50}$ DMSO: $23.2 \pm 2.5$ μM (n=8); cytochalasin-D: $22.4 \pm 2.9$ (n=8); latrunculin-A: $18.1 \pm 2.1$ (n=7); one-way ANOVA, $F = 1.26, p = 0.30$). Also, there were no observable differences in NMDA current density (Fig. 16C, ANOVA, $F = 0.23, p = 0.79$) and desensitization (Fig. 16D, ANOVA, $F = 0.12, p = .92$). Identical results were obtained with higher concentrations of latrunculin-A (5μM) in separate studies (data not shown). Thus, in spite of the dramatic alterations produced by the actin depolymerizing agents on rhodamine-phalloidin staining (Figs. 14,15), macroscopic currents evoked by adding exogenous NMDA were unchanged.
Figure 16: NMDA-evoked ionic currents are not affected by the actin perturbing agents. Cultured cortical neurons were treated for 12 h with latrunculin-A (1μM) or cytochalasin-D (10μM) and maintained in solutions containing the agents until recordings were made. A. Representative NMDA-evoked currents obtained with 3-300 μM NMDA in control (sham-treated) cultures and in cultures treated with the depolymerizing agents. B. NMDA concentration-response curves: EC₅₀ for control: 23.2 ± 2.5 μM (n=8); latrunculin-A: 18.1 ± 2.1 μM (n=8); cytochalasin-D: 22.4 ± 2.9 μM (n=7), one-way ANOVA, F=1.26, p=0.30. Symbols represent mean ± SE. Error bars are shown where they exceed symbol size. C, NMDA current density measurements elicited with 300 μM NMDA. In pA/pF: control: 39.1 ± 2.6, (n=23); latrunculin-A: 41.8 ± 2.0, (n=27); cytochalasin-D: 40.9 ± 4.4, (n=20), one-way ANOVA, F=0.23, p=0.79. D, Analysis of NMDA current desensitization. Iₛₛ = steady-state current; Iₚₑᵃᵏ = peak current. Control: n=23; latrunculin-A: n=27, cytochalasin-D: n=20. Columns in (C) and (D) indicate the mean ± SE.
Depolymerizing F-actin in dendritic spines selectively targets synaptic NMDARs.

Next, we used both imaging and electrophysiological approaches to study the effects of perturbing F-actin on the localization and function of synaptic NMDARs. We used latrunculin-A (5μM), as this compound had the most pronounced effects on actin in the cells (Figs 14A, 15C). First, cortical and hippocampal neurons treated with latrunculin-A for 12h were stained for the essential NMDAR subunit NR1. We were unable to reliably resolve the distribution and localization of NR1 clusters in the cortical cells owing to the density of neurons and their processes in the cultures (data not shown). However, both sham and latrunculin-treated hippocampal cultures exhibited punctate NR1 immunostaining as reported by others (Fig 17A; Kornau et al., 1995; Halpain et al., 1998). These could be quantified by counting individual clusters per unit dendrite length as shown in Fig. 17B and described by Rao and Craig (1997). Depolymerizing F-actin with latrunculin-A reduced the total number of NMDAR clusters in dendrites of hippocampal neurons from 8.9 ± 0.6 to 6.1 ± 0.4 clusters per 10 μm of dendrite length (Fig 17C; t_{56} = 4.16, p = 0.0001). Our results are consistent with the experiments of Allison et al., (1998), where depolymerizing F-actin with latrunculin-A reduced the total number of NMDAR clusters in dendrites of hippocampal neurons by a similar degree. Using co-localization studies with the presynaptic marker synaptophysin, these authors showed that this decrease was entirely due to a selective loss of synaptic NMDAR clusters, as clusters that did not co-localize with synaptophysin were unaffected.
Figure 17: Disrupting F-actin reduces the number of NMDAR clusters in dendrites of pyramidal hippocampal neurons. The cells were treated with 1 μM latrunculin-A for 12h and immunostained for the NMDAR subunit NR1. A. NR1 immunostaining of neurons treated with sham (0.1% DMSO) and latrunculin-A-containing solutions illustrating the punctate appearance of NMDAR clusters (representative of 3 separate experiments). B. Method of counting NMDAR clusters per unit dendrite length. 1-2 dendrites were randomly selected in 12 different neurons under each condition, and imaged using identical confocal parameters. The total number of clusters was counted (arrowheads) over 20μm of dendrite length, and expressed as the averaged counts per 10 μm. C. Latrunculin-A reduced the average number of NMDAR clusters from 8.9 ± 0.6 to 6.1 ± 0.4 clusters per 10 μm of dendrite length ($t_{36} = 4.16, p = 0.0001$).
Figure 18: Dissolving F-actin selectively reduces the NMDA component of spontaneously evoked miniature excitatory post-synaptic currents (mEPSCs). Cultured cortical neurons were treated with 5 μM latrunculin-A for 12h. mEPSCs were recorded for 5 min in the whole-cell configuration. N_sham = 13 neurons, N_latrunculin = 11 neurons. A. Representative averaged traces showing mEPSCs recorded from cultured mice cortical neurons without (left) and with latrunculin-A treatment (right). Both AMPA and NMDA containing mEPSCs (1) were recorded without APV and Mg²⁺. AMPA-only mEPSCs (2) were recorded with 20 μM APV and 2 mM Mg²⁺ in the perfusion solution. NMDA-only mEPSCs (3) were obtained by subtracting trace 2 from trace 1. B. Summary data showing the effect of latrunculin on the area (pA x ms) of different components of mEPSCs. The area (A) of mEPSCs was integrated over 50ms. A_total-sham 137.0 ± 12.0, A_total-latrunculin 88.3 ± 7.2 (p=0.002); A_AMPA-sham 60.8 ± 5.8, A_AMPA-latrunculin 61.0 ± 3.5 (p=0.974); A_NMDA-sham 76.0 ± 12.0, A_NMDA-latrunculin 27.3 ± 6.6 (p=0.003). Asterisks: statistical difference as compared with sham (Student’s t-test).
The findings above support the use of latrunculin-A to perturb synaptic NMDA receptors selectively. Since the actin depolymerizing agents had no effect on macroscopic whole cell NMDA currents (Fig. 16), latrunculin treatment might indicate that while NMDAR localization might be rearranged, function is grossly unaffected. It is difficult, based on imaging experiments, to determine whether the NMDAR clusters migrate away from dendritic spines, whether they dissociate, or both. Regardless, if the number of synaptic NMDAR clusters is reduced, then their activity might be affected. Therefore, we next examined miniature spontaneous excitatory post-synaptic currents (mEPSCs) in our cells, as these currents are mediated by synaptic receptors.

Recordings were made in cortical neuronal cultures treated with latrunculin-A (5 μM) for 12h. Using the whole-cell configuration, spontaneous mEPSCs were recorded for about 5 min to acquire sufficient numbers of events. Representative averaged traces from these recordings are shown in Fig. 18A for sham and latrunculin-treated cultures. Both AMPA and NMDA containing mEPSCs (Fig. 18A, trace 1) were recorded without APV and Mg²⁺ in the extracellular solution. The AMPA component of the mEPSCs was then recorded by switching the neurons to an extracellular solution containing 20 μM APV and 2 mM Mg²⁺ to block NMDAR activation (Fig. 18A, trace 2). Subtracting the fast AMPA-component from the total mEPSC revealed the slower NMDA-component of spontaneous mEPSCs (Fig. 18A, trace 3). Integration of the area (A) of the different mEPSC-components revealed that latrunculin-A treated neurons exhibited a significantly reduced total mEPSC (Fig. 18B). This was due entirely to a selective reduction of the NMDA-component, as the AMPA component remained unaffected (Fig. 18B).
Depolymerizing F-actin does not affect NMDAR-mediated Ca\textsuperscript{2+} loading or neurotoxicity produced by exogenously-applied NMDA

We next examined the effects of depolymerizing actin on NMDAR-mediated excitotoxicity using two established *in vitro* models. First, by applying exogenous NMDA to the cultures (Choi et al., 1988; Sattler et al., 1998). Then, by exposing the cultures to oxygen-glucose deprivation (Goldberg et al., 1987; Abdel-Hamid and Tymianski, 1997).

Applying NMDA directly to the bath should result in a uniform concentration of NMDA in the extracellular medium, and affect all NMDA receptors equally, irrespective of their location. Cortical neuronal cultures were treated with either latrunculin-A (1-5 μM) or cytochalasin-D (0.1-30 μM) for 12 hrs. The cells were then exposed to a range of NMDA concentrations (0, 30, 100 μM) for 60 min in the presence of CNQX (10 μM) and nimodipine (2 μM), antagonists of non-NMDARs and voltage-gated Ca\textsuperscript{2+} channels, to isolate both Ca\textsuperscript{2+} entry and neurotoxicity to NMDARs (validated in Sattler et al., 1998).
Figure 19: Disrupting F-actin has no impact on excitotoxicity or neuronal Ca\(^{2+}\) loading evoked by exogenous NMDA. Cultured cortical neurons were pretreated with the indicated concentrations of latrunculin-A (0-5\(\mu\)M) or cytochalasin-D (0-30\(\mu\)M) before undergoing exposure to 0, 30 or 100 \(\mu\)M NMDA for 60 min (in 2\(\mu\)M nimodipine and 10\(\mu\)M CNQX, see Materials and Methods). The cultures were then maintained for a further 23 h to measure neuronal survival (A1, B1) or used for \(^{45}\)Ca\(^{2+}\) accumulation measurements (A2, B2). A: Effects of treatment with latrunculin-A on NMDAR-mediated excitotoxicity (A1) or \(^{45}\)Ca\(^{2+}\) loading (A2). B: Effects of treatment with cytochalasin-D on NMDAR-mediated excitotoxicity (A1) or \(^{45}\)Ca\(^{2+}\) loading (B2). Symbols in A1, B1 represent the mean survival (±SE) of 4-64 cultures per experimental condition, obtained from at least 2 (usually 4-6) different dissections. Error bars are shown where they exceed symbol size. The lines indicate the least-squares linear regression curves obtained for each NMDA concentration. Columns in A2, B2 represent the mean (±SE) \(^{45}\)Ca\(^{2+}\) accumulation averaged from 16-36 cultures obtained from 4-6 different culture dissections.
Figure 1.9

A1

Fraction Dead

Latrunculin-A [μM]

0 μM NMDA
30 μM NMDA
100 μM NMDA

A2

Latrunculin-A

0 μM
1 μM

45Ca²⁺ accumulation

NMDA [μM]

0
30
100

B1

Fraction Dead

Cytochalasin-D [μM]

0 μM NMDA
30 μM NMDA
100 μM NMDA

B2

Cytochalasin-D

0 μM
0.1 μM
10 μM

45Ca²⁺ accumulation

NMDA [μM]

0
30
100
The cells were then washed and observed for a further 23 h in control solution containing CNQX, nimodipine and MK-801 (10 μM). Cell survival was monitored by measuring propidium iodide fluorescence as an index of cell death (Methods; Sattler et al., 1997; Tymianski et al., 1998). Sister cultures were identically treated and used immediately after the insult for determinations of NMDAR-mediated 45Ca²⁺ accumulation (Sattler et al., 1998).

Figure 19 summarizes the effects of latrunculin-A (1-5μM; Fig. 19A) and cytochalasin-D (0.1-30μM; Fig. 19B) on NMDAR-mediated toxicity and Ca²⁺ loading. In the absence of an NMDA challenge, even the highest concentrations of depolymerizing agents were well tolerated by the cells (0 μM NMDA groups in Figs. 19A1,B1). Interestingly, in spite of these compounds’ profound effects on the polymerization state of F-actin (Figs. 14,15), neither one affected excitotoxicity produced over a range of NMDA concentrations (Figs. 19A1, B1). Furthermore, the accumulation of ⁴⁵Ca²⁺ in the cells throughout the NMDA application was also unaffected by the depolymerizing agents (Figs. 19A2, B2). These results are consistent with the lack of effect of both depolymerizing agents on macroscopic NMDA-mediated ionic currents (Fig. 16), and suggest that NMDAR activation can trigger excitotoxicity irrespective of receptor localization.
Depolymerizing F-actin reduces NMDAR-mediated Ca\(^{2+}\) loading and neurotoxicity evoked by oxygen-glucose deprivation (OGD).

Oxygen-glucose deprivation releases synaptic glutamate and causes neurotoxicity that is mediated primarily by NMDA receptors and Ca\(^{2+}\)-dependent mechanisms (Rothman, 1983, 1984; Goldberg et al., 1987; Goldberg and Choi, 1993). In cultured cortical neurons, OGD causes vesicular glutamate release, as both glutamate accumulation and OGD toxicity are blocked by pretreatment with tetanus toxin (Monyer et al., 1992) which prevents synaptic vesicle exocytosis (Bergey et al., 1987; Ahnert-Hilger and Bigalke, 1995). OGD also causes nonvesicular transmitter release via reverse operation of glutamate transporters (Attwell et al., 1993; Szatkowski and Attwell, 1994) which are enriched in neurons at pre- and postsynaptic sites (Rothstein et al., 1994). Thus, OGD may trigger mechanisms that preferentially cause glutamate to accumulate at synaptic sites. Unlike excitotoxicity produced by exogenous NMDA, which targets both synaptic and extrasynaptic receptors (Fig. 19), OGD is anticipated to injure neurons by a mechanism that preferentially activates synaptic NMDARs. Since disrupting actin with latrunculin-A selectively perturbs synaptic NMDARs (Figs 17,18), we asked whether neurons treated with this agent would exhibit altered vulnerability to OGD.

We exposed cultured cortical neurons to combined oxygen-glucose deprivation in the presence of CNQX (10 \(\mu\)M) and nimodipine (2 \(\mu\)M) to block non-NMDARs and Ca\(^{2+}\) channels. After 2 hrs, the cells were washed and kept for further 22 hrs in oxygenated glucose-containing bicarbonate solution containing CNQX, nimodipine and MK-801.
Sister cultures were equally exposed to OGD and used for $^{45}\text{Ca}^{2+}$ accumulation assays to measure Ca$^{2+}$ loading through NMDARs.

Cultures that has been pretreated with latrunculin-A for 12 h were significantly less vulnerable to OGD-induced NMDAR-mediated neurotoxicity than controls (Fig. 20A; N =6 cultures from two different dissections, $t_{10} =4.18$, $p=0.002$). This protective effect was exactly paralleled by reduced $^{45}\text{Ca}^{2+}$ accumulation in the latrunculin-treated neurons (Fig. 20B; N=6 cultures from 2 different dissections, $t_{10} = 4.07$, $p = 0.002$). Thus, neurons in which synaptic NMDAR function was selectively perturbed by depolymerizing actin (Figs. 16,17,18) also exhibited reduced vulnerability to excitotoxic insults that are preferentially mediated by synaptic NMDRs.
Figure 20: Depolymerization of F-actin attenuated NMDR-mediated cell death and neuronal Ca\textsuperscript{2+} loading when excitotoxicity was evoked by oxygen-glucose deprivation (OGD). Cultures were pretreated with 1 \textmu M latrunculin-A for 12 h, after which they were exposed to 2h of OGD in the presence of non-NMDAR and Ca\textsuperscript{2+} channels antagonists (CNQX and nimodipine, respectively) to isolate Ca-influx to NMDARs (Methods). The cells were observed for a further 22 h to measure cell death, whereas sister cultures were used to measure \textsuperscript{45}Ca\textsuperscript{2+} accumulation after the 2h OGD exposure. A, Cell death was significantly reduced in the latrunculin-A treated cultures as compared to sham cultures ($t_{10} = 4.18$, $p=0.002$). Each bar represents the mean $\pm$ SE of 6 cultures obtained from 2 different dissections. B, \textsuperscript{45}Ca\textsuperscript{2+} accumulation was significantly reduced in the latrunculin-A treated cultures ($t_{10} = 4.07$, $p=0.002$). Each bar represents the mean (+ SE) of 6 cultures obtained from 2 different dissections.
B. Functional consequences of the interaction of NMDARs with PSD-95 on
NMDAR-mediated Ca\(^{2+}\) - dependent neurotoxicity.

The data obtained in part 2.0 indicate that the signaling mechanisms that participate in NMDAR-mediated excitotoxicity are not governed by the synaptic localization of NMDARs. Since conditions that preferentially attenuated synaptic NMDAR function had no effect on the toxicity of exogenous NMDA (Fig. 19, part 2.0), extrasynaptic NMDARs must still be linked to the second messenger pathways that trigger neuronal damage. Recent data on the molecular organization of neuronal synapses support the idea of the existence of a macromolecular complex that is associated with the NMDAR. One favorable candidate for joining into this complex is postsynaptic density protein PSD-95/SAP90, which is specifically bound to NMDARs via the second of its three PDZ domains (see introduction and (Kornau et al., 1995; Kim and Sheng, 1996; Niethammer et al., 1996). PSD-95 also interacts with other intracellular signaling molecules, including neuronal nitric oxide synthase (nNOS; Brenman et al., 1996; Brenman et al., 1996; Stricker et al., 1997), an enzyme that participates in NMDAR-mediated nitric oxide signaling pathways (Garthwaite et al., 1988; Bredt and Snyder, 1989, 1990). We therefore sought to test the hypothesis that PSD-95 acts as a scaffolding protein that links Ca\(^{2+}\) ions coming through the NMDAR to intracellular signaling molecules, such as nNOS, and that this link is responsible to the preferential triggering of neurotoxic cascades by NMDAR activation.
Application of antisense oligodeoxynucleotides specifically reduces PSD-95 protein expression

To study the role of PSD-95 as a molecule that might link Ca\(^{2+}\) influx through the NMDAR to intracellular neurotoxic signal transduction pathways, we sought to eliminate PSD-95 from the PSD. A recently developed approach to modulate gene expression in a sequence-specific manner at the translational level in mammalian cells is the use of synthetic antisense oligodeoxynucleotides (for review see Murray and Crocket, 1992; Nicot and Pfaff, 1997; Brussaard, 1997, also Caceres and Kosik, 1990; Wahlstedt et al., 1993). The application of the antisense technique can, in principle, be used for any gene for which sequence information is available and in any system into which antisense oligodeoxynucleotides can be introduced. The result is the creation of a ‘mutant’ in which the level of a single chosen protein, in our case PSD-95, is selectively reduced or abolished. Several phosphodiester antisense ODNs corresponding to various regions of mouse PSD-95/SAP90 messenger RNA (GeneBank Acc. No. D50621) were synthesized. To promote stability of ODNs, serum was omitted from the tissue culture medium after one day in vitro (see Methods). ODNs were added at day 4 in vitro and re-added every other day during feedings. The effects of the treatment on PSD-95 protein expression levels were resolved by conventional Western blot analysis of treated tissue samples with PSD-95 antibodies (see Methods). A 15-mer phosphodiester antisense ODN corresponding to nucleotides 435-449 (5'-GAATGGGTCACCTCC-3') selectively suppressed in a dose-dependent manner PSD-95 expression levels to < 10% of control levels (Fig. 21A,B). Treatment with sham washes (SH), sense oriented (SE) and missense
Figure 21: Selective reduction of PSD-95 expression with antisense oligodeoxynucleotides (ODNs) to PSD-95. A, Immunoblot showing representative effects of sham (SH) washes, and PSD-95 antisense (AS), sense (SE) and missense (MS) oriented ODNs, on PSD-95 expression. PC: positive control tissue from purified rat brain cell membranes. Asterisk: non-specific band produced by the secondary antibody, useful to control for protein loading and blot exposure times. B, Densitometric analysis of PSD-95 expression pooled from N experiments. Asterisk: different from other groups, one-way ANOVA, $F = 102, p<0.0001$. ODNs were used at 5μM except where indicated (AS 2μM). Each bar represents the mean + SE.
A

Anti PSD-95

SH AS MS SE SH PC

-95kD

B

PSD-95 expression Normalized to Sham

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-Figure 21-

115
Figure 22: Representative phase contrast and propidium iodide fluorescence images of PSD-95 deficient (AS) and control (SE) cultures 24 h after a 60 min challenge with 30μM NMDA. Scale bar: 100 μm.
oriented (MS) ODNs had no effect on PSD-95 protein expression levels. The treatment with ODNs had no effect on neuronal survivability and morphology as gauged by viability assays (see below) and phase-contrast microscopy (not shown).

**NMDA toxicity is significantly reduced in neurons deficient in PSD-95 without effecting NMDAR-mediated Ca\(^{2+}\) loading**

To examine the impact of PSD-95-deficiency on NMDAR-triggered excitotoxicity, we challenged ODN-treated cultures with NMDA as described in Part I. Briefly, the cultures were exposed to a range of NMDA concentrations (10-100 μM, 60 min). The insult was preformed in the presence of antagonists of non-NMDARs (CNQX, 10 μM) and Ca\(^{2+}\) channels (nimodipine, 2 μM) to selectively trigger Ca\(^{2+}\) neurotoxicity via NMDAR activation. The cells were washed and kept for further 23 h in control solution containing CNQX, nimodipine and MK-801 (NMDAR antagonist, 10 μM). Cell survival was monitored during that time by measuring propidium iodide fluorescence as an index of cell death (see Methods, also Sattler et al., 1997). In all four groups (sham, antisense, sense and missense ODN) we observed a concentration-dependent effect of NMDA exposure on neuronal survival (Figs. 22 and 23A). However, NMDA toxicity was significantly reduced in neurons deficient in PSD-95 across a range of insult severities (Fig. 23A; EC\(_{50}\): AS: 43.2 ± 4.3; SE: 26.3 ± 3.4, Bonferroni t-test, p <0.005). This protective effect could have been explained by a decreased Ca\(^{2+}\) load through NMDARs in the PSD-95 deficient neurons. To test for this possibility, we measured NMDA-induced Ca\(^{2+}\) loading in sister cultures that were treated identically with NMDA and were
Figure 23: Increased resilience of PSD-95 deficient neurons to NMDA toxicity in spite of Ca^{2+} loading. A, NMDA toxicity was decreased at 24h in PSD-95 deficient neurons following selective NMDAR activation x 60 min (n=16 cultures/bar pooled from N=4 separate experiments). Asterisk: differences from sense (SE), missense (MS) and sham (SH) treated cultures (Bonferroni t-test, p<0.005). Death is expressed as the fraction of dead cells produced by 100μM NMDA in sham-ODN-treated controls (validated in Sattler et al. 1997). B, No effect of PSD-95 deficiency on NMDAR-mediated Ca^{2+} loading (n = 12/bar, N = 3; reported as the fraction of ^{45}Ca^{2+} accumulation achievable over 60 min in the sham controls by 100μM NMDA, which maximally loads the cells with calcium). Each bar represents the mean + SE.
A  NMDA + CNQX + NIM

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B  NMDA + CNQX + NIM

$^{45}$Ca accumulation

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-Figure 23-
Figure 24: PSD-95 deficiency does not affect toxicity and Ca\(^{2+}\) loading produced by activating non-NMDARs and Ca\(^{2+}\) channels. Cultures were treated with SH washes or AS or SE ODNs as in Fig. 21. A, Selective activation of AMPA/kainate receptors with kainate in MK-801 (10\(\mu\)M) and nimodipine (NIM; 2\(\mu\)M) produces toxicity over 24h (A1) irrespective of PSD-95 deficiency, with minimal \(^{45}\)Ca\(^{2+}\) loading (A2). B, Selective activation of VSCCs produces little toxicity (B1), but significant \(^{45}\)Ca\(^{2+}\) loading (B2) that is also insensitive to PSD-95 deficiency. Each bar represents the mean (+SE) from \(n = 4\) cultures in all experiments.
A1
Kainate+ MK-801 + NIM x 24h

Fraction Dead

0 30 60 100 300
Kainate [µM]

A2

0 30 60 100 300
Kainate [µM]

45Ca accumulation

B1
High-K+ + MK-801 + CNQX in 10 mM calcium

Fraction Dead

B2

0 30 60 100 300
Kainate [µM]

45Ca accumulation

- Figure 24 -
used for \(^{45}\text{Ca}^{2+}\) accumulation measurements right after the 60min insult (see Methods). Similar to the toxicity results, we detected a concentration-dependent rise in \(^{45}\text{Ca}^{2+}\) accumulation over a range of NMDA concentrations in all four groups. However, PSD-95 deficiency in the antisense ODN treated group had no effect on NMDAR-mediated \(\text{Ca}^{2+}\) loading (Fig. 23B). Therefore, PSD-95 deficiency induces resilience to NMDA toxicity despite maintained \(\text{Ca}^{2+}\) loading.

**PSD-95 deficiency has no effect on non-NMDA receptor-mediated neurotoxicity or calcium loading**

We next examined whether the increased resilience to \(\text{Ca}^{2+}\) loading in PSD-95 deficient neurons was specific to NMDARs. Non-NMDAR toxicity was produced using kainic acid (30-300 \(\mu\)M), an incompletely desensitizing AMPA/kainate receptor agonist (Burnashev et al., 1995), in the presence of NMDAR and \(\text{Ca}^{2+}\) channel antagonists (see Methods and Sattler et al., 1997). Kainate-exposure caused dose-dependent neuronal cell death, which was unaffected in PSD-95 deficient neurons challenged for either 60 min (not shown) or 24 h (Fig. 24A1). As shown in Part I (Fig.11), more than 90% of neurons in the present culture system express \(\text{Ca}^{2+}\)-impermeable AMPA receptors. Hence, the observed AMPA-receptor mediated toxicity occurred without significant \(^{45}\text{Ca}^{2+}\) loading (Fig. 24A2). Therefore, we were unable in the present culture system, to detect effects of antisense ODN treatment on AMPAR-mediated \(\text{Ca}^{2+}\) loading. However, we were able to induce \(\text{Ca}^{2+}\) loading through L-VSCCs which is equivalent to that induced via NMDAR activation (Fig. 9), but which is non-
toxic (Figs. 9 and 24B1). Exposing ODN treated neurons to high-K⁺ in the presence of MK-801 and CNQX plus Bay-K 8644 (see Methods) showed that PSD-95 deficiency had no effect on Ca²⁺ channel-mediated Ca²⁺ loading (Fig. 24B2). Thus, suppressing PSD-95 expression affects neither toxicity nor Ca²⁺ fluxes triggered through pathways other than NMDARs.

**Protein expression levels of NMDARs and other NMDAR-associated MAGUKs are unchanged with antisense ODN treatment**

One explanation for the protective effect of PSD-95 deficiency could be an altered expression level of either NMDARs or other NMDAR-associated MAGUKs, such as PSD-93/Chapsyn 110 or SAP-102 (Sheng and Kim, 1996). The hypothesis would be that NMDARs are downregulated in PSD-95 deficient cultures and hence NMDAR-mediated toxicity would be reduced. On the other hand, PSD-93/Chapsyn 110 or SAP-102 could be up- or downregulated and thereby alter vulnerability to NMDAR-mediated Ca²⁺ neurotoxicity. We therefore performed immunoblot analyses for NMDAR subunit NR1 and NR2A/B (not shown), PSD-93/Chapsyn 110 and SAP-102 (Fig. 25). None of the proteins examined revealed an altered expression level by treatment with antisense ODNs to PSD-95. Hence, altered expression of NMDARs and their associated proteins was unlikely to explain reduced NMDA toxicity in PSD-95 deficiency (Fig. 23A,B). These data are consistent with data obtained from newly generated mutant mice expressing a truncated form of PSD-95 (Migaud et al., 1998). The authors showed that
Figure 25: No effect of perturbing PSD-95 on expression levels of other postsynaptic density proteins or NMDARs. Immunoblots of PSD-95 ODN-treated cultures probed for PSD-95 (A), NR1 (B), PSD-93 (C), and SAP-102 (D) using specific antibodies. PC: positive control tissue from purified rat brain cell membranes.
the total amount of NR1, NR2A and NR2B proteins as well as PSD-93/chapsyn 110, SAP102, and SAP 97 was the same in wild-type and mutant mice.

Reduction of PSD-95 expression levels does not alter NMDAR function or NMDAR localization

Another explanation for the protective effects of PSD-95 deficiency in NMDAR-mediated Ca\(^{2+}\) neurotoxicity could be a modulatory role of PSD-95 on NMDAR function. Hence, eliminating PSD-95 by antisense ODNs could alter NMDAR properties and concomitantly make receptor activation less efficient and consequently less toxic. We therefore investigated NMDA currents in ODN-treated neuronal cultures using the whole-cell patch clamp technique (Fig. 26, see also Methods). PSD-95 deficiency had no effect on passive membrane properties, including input resistance and membrane capacitance [Capacitance: AS 55.0 \(\pm\) 2.6 pF (n = 18); SE 52.7 \(\pm\) 3.2 pF (n = 19); SH 48.1 \(\pm\) 3.4 pF (n = 17; ANOVA, F = 1.29, p = 0.28)]. Thus, treatment with antisense ODNs did not affect the health of the neurons, nor did the cells show any changes in cell size due to PSD-95 deficiency. This was consistent with light microscopic observations of cell morphology (not shown) and cell viability as shown in Fig. 23. Whole-cell currents were elicited by brief applications of 3-300 \(\mu\)M NMDA/10 \(\mu\)M glycine in magnesium-free medium (see Methods). Peak currents were not significantly different in cultures treated with antisense ODNs: AS: 2340 \(\pm\) 255 pA (n = 18); SE: 2630 \(\pm\) 276 (n = 19); SH: 2370 \(\pm\) 223 (n = 17) (Fig. 26A, inset; one-way ANOVA, F = 0.43, p = 0.65). NMDA dose-response relationships, an electrophysiological measure of the affinity of the receptor to an agonist, also
remained unchanged (Fig. 26A; EC$_{50}$ AS: 16.1 ± 0.8 μM (n=7); SE: 15.5 ± 2.1 (n=6); SH: 15.9 ± 2.9; one-way ANOVA, F= 0.02, p = 0.98). Measurements of NMDA current density, which is an indicative assessment for the number of receptors per unit membrane, did not reveal any difference between ODN-treated groups (Fig. 26C). Nor did the analysis of NMDA current desensitization (Fig. 26D). This suggests, that NMDAR channel properties are unaffected by a lack of PSD-95.

To further examine the effect of PSD-95 binding on NMDAR function, a 9 aa peptide (KLISSIESDV) corresponding to the C-terminal domain of the NR2B subunit characterized by the T/SXV motif (Kornau et al., 1995) was injected into neurons that were not treated with ODNs. At 0.5mM, this peptide was shown to competitively inhibit the binding of PSD-95 to GST-NR2B fusion proteins (Kornau et al., 1995), and was therefore predicted to uncouple NMDARs from PSD-95. Thus, this manipulation represented an alternative approach to interrupt the proposed signaling complex consisting of NMDAR, PSD-95 and intracellular enzymes. Intracellular dialysis of 1mM T/SXV or control peptide (CSKDTMEKSESL) was achieved through patch pipettes (3-5 MΩ). We also added the fluorescent tracer Lucifer Yellow (LY) into the pipette, which enabled us to follow visually the dialysis of the pipette-content. Incubation with either one of the peptides for over 30 min showed no effects on NMDA currents despite extensive dialysis of LY into the cell soma and dendrites (Fig. 27A + insert). Peak current amplitudes were T/SXV: 2660 ± 257 pA (n= 9), control peptide: 2540 ± 281 pA (n= 10; t$_{(17)}$ = 0.31, p = 0.76). This manipulation, where we examined single cells rather than whole cell cultures, also allowed us to study the effects of PSD-95 binding on NMDAR
Figure 26: Perturbing PSD-95 does not effect NMDA receptor function. A, Representative NMDA currents obtained with 3-300μM NMDA in AS, SE and sham treated cortical neuronal cultures. B, C, D, NMDA dose-response curves NMDA current density measurements elicited with 300μM NMDA (AS: n = 18; SE: n =19; SH: n = 17; one-way ANOVA F=1.101, p=0.3403), as well as analysis of NMDA current desensitization. $I_{ss}$ = steady-state current; $I_{peak}$ = peak current. AS: n=15; SE: n = 16; SH: n = 16 (ANOVA, F=0.1440, p=0.8663). Time constants for current decay were AS: 1310 ± 158 ms; SE, 1530 ± 185 ms; SH: 1190 ± 124 ms (ANOVA, F= 1.22, p= 0.31). Each bar in (C) and (D) represents the mean + SE. Error bars in (B) are shown were they exceed symbol size.
Figure 26.

(A) Antisense, Sense, and Sham recordings.

(B) Normalized amplitude vs. NMDA concentration.

(C) Current density (pA/pF) comparison.

(D) Ratio (I_{ss}/I_{peak}) comparison.
Figure 27: Microinjection with T/SXV peptide does not alter NMDAR function or NMDAR localization. Cultured neurons were dialyzed with 1mM T/SXV or control peptide in the presence of Lucifer Yellow (insert). A, NMDA currents were elicited with 300μM NMDA in neurons loaded with control peptide or T/SXV peptide. The treatment with T/SXV peptide had no effect on NMDA currents (normalized to max. current) over 30 min, despite extensive dialysis of LY into the cell soma and dendrites (insert). Peak current amplitudes were T/SXV: 2660 ± 257 pA (n= 9), control: 2540 ± 281 pA (n= 10; t(17) = 0.31, p = 0.76). Error bars are shown where they exceed symbol size. B, After the current measurements, neurons were fixed and immunostained for NMDAR subunit NR1 (see Material and Methods). The asterisk indicates one representative neuron that has been dialyzed with T/SXV peptide. Comparison of these neurons with non-treated ones being in close apposition revealed no apparent difference in NR1 staining patterns.
localization in our culture system. After the electrophysiological experiments, we fixed the cultures and immunostained them with anti-NR1 antibodies (see Methods). The presence of LY in the peptide-treated cells allowed us to identify easily the manipulated cells (Fig. 27A insert). Viewing the immunostained cultures with a confocal microscope revealed no difference in the staining pattern of NR1 between T/SXV peptide and control peptide treated neurons (Fig. 27B). These observations were consistent with the current density measurements obtained above in antisense ODN-treated cultured neurons (Fig. 26C) as well as with the data obtained in the PSD-95 mutant mice as presented by Migaud et al (1998). Their immunogold studies of NR1 in CA1 stratum radiatum detected no difference between the mutant mice and the wild-type mice in the characteristics or density of NR1 immunogold labeling of asymmetric synapses. They also showed that NMDA currents, including synaptic currents, as well as NMDAR current-voltage relation did not change in the PSD-95 mutant neurons.

**PSD-95 deficiency perturbs NMDAR-mediated NO neurotoxicity**

We found no effects of PSD-95 deficiency on NMDAR expression (Fig. 25) and localization (Fig. 27), on expression levels of other NMDAR associated MAGUKs (Fig. 25), nor on NMDA-evoked currents (Fig. 26). In addition, NMDAR function gauged by measuring NMDA-evoked $^{45}$Ca$^{2+}$-accumulation was unaffected (Fig. 23B). In agreement with the results obtained by Migaud et al (1998) in PSD-95 mutant mice, we concluded that PSD-95 does not modify NMDA receptor properties. Thus, the neuroprotective
consequences of PSD-95 deficiency (Fig. 23A) must be due to events downstream from NMDAR activation, rather than to altered NMDAR function.

The second PDZ domain of PSD-95 binds to the C-terminus of NR2 subunits and to other intracellular proteins (see introductory part). Among these is nNOS (Brenman et al., 1996), an enzyme that catalyzes the production of nitric oxide (NO), a short-lived signaling molecule that also mediates Ca^{2+}-dependent NMDA toxicity in cortical neurons (Dawson et al., 1991, 1993; Huang et al., 1994). NO synthesis is triggered by the influx of calcium, which, when complexed with calmodulin, activates the biosynthetic activity of nNOS (Garthwaite et al., 1988; Bredt and Snyder, 1989, 1990). Although never demonstrated experimentally, the NMDAR/PSD-95/nNOS complex was postulated to account for the preferential production of NO by calcium influx through NMDARs over other pathways (Brenman et al., 1996). Hence, the protective effects seen in the PSD-95 deficient neurons may be explained by eliminating the interaction between NMDAR/PSD-95/nNOS and thereby reducing the activation of nNOS by Ca^{2+} influx through the NMDAR.

To first determine whether NO signaling plays a role in NMDA toxicity in our culture system, we treated the cells with N\textsuperscript{6}-nitro-L-arginine methyl ester (L-NAME), a NOS inhibitor (Dawson et al., 1991, 1993). The cultures were exposed to a range of NMDA concentrations (0-100 μM, x 60min) in the presence of L-NAME (0-1000 μM) (Fig. 28). L-NAME protected the neurons against NMDA toxicity in a dose-dependent manner. This result indicated that the protective effects of PSD-95 deficiency could indeed be due to a detachment of the toxic NO signaling pathway from NMDARs.
Figure 28: NOS inhibitors protect against NMDA toxicity in the present culture system. L-NAME protects against NMDA toxicity in a dose-dependent manner. Cultures were exposed to different NMDA concentrations (0-100 μM) in the presence of antagonists of non-NMDARs (CNQX, 10 μM) and Ca channels (nimodipine, 2 μM). Asterisk: difference from 0 μM L-NAME (Bonferroni t-test, p<0.05). Each bar represents the mean (+SE) from n = 8 cultures pooled from N = 2 separate experiments.
Figure 29: Decreased PSD-95 expression selectively reduced NMDAR-mediated cGMP formation. A, In PSD-95 deficient neurons challenged with NMDA under conditions that isolated Ca\(^{2+}\) influx to NMDARs cGMP production was markedly attenuated (>60%; one-way ANOVA, \(p<0.0001\)); Each bar represents the mean (+ SE) of 12 cultures obtained from 3 different dissections. B, Decreased PSD-95 expression levels had no effect on nNOS expression levels. Western blot analysis of sham (SH), missense (MS), and antisense (AS) oligodeoxynucleotide-treated neurons did not reveal any differences between groups. PC = positive control tissue from purified rat brain cell membranes. Also, there was no effect of AS oligos on NADPH diaphorase staining of neurons as compared to SE-treated neurons. Data in (A) are expressed as the fraction of cGMP produced in SE-treated cultures by 100 \(\mu\)M NMDA. Asterisk: differences from both SH and SE controls.
To test for that possibility, we examined the effect of PSD-95 deficiency on NO signaling and toxicity using cGMP formation as a surrogate measure of NO production by Ca$^{2+}$-activated nNOS (Ferrendelli et al., 1974; Garthwaite et al., 1989; Bredt and Snyder, 1989). ODN-treated cultures were challenged with a range of NMDA concentrations (0-100µM, x 60min) in the presence of non-NMDAR and Ca$^{2+}$ channel antagonists to isolate Ca$^{2+}$ influx to NMDARs (see Fig. 6A). The cells were then lysed and cGMP formation was determined using a commercially available cGMP enzymeimmunoassay-kit (see Methods). cGMP was formed in all groups examined (AS, SE and SH-treated cultures), however in neurons lacking PSD-95 cGMP production was markedly attenuated (>60%; Fig. 29A, one-way ANOVA, p<0.0001).

To ensure that this effect was not due to an altered expression level or function of nNOS, we first measured protein expression levels in ODN-treated cultures using conventional Western Blot analysis. The immunoblot for anti-nNOS showed no impact of PSD-95 deficiency on nNOS expression (Fig. 29B). To identify NOS neurons in our cultures, we stained the cells for NADPH diaphorase (Dawson et al., 1993, see also Methods) and examined whether morphology or the total amount of NADPH$^+$ neurons changed in PSD-95 deficient cultures. Figure 29B shows representative transmitted images of NADPH$^+$ neurons in AS and SE-treated cultures, indicating that there is no effect of PSD-95 deficiency on NADPH$^+$ neurons' morphology. Also, we found no difference in the total number of NOS neurons between experimental groups (SH: 361 ± 60, SE: 354 ± 54, AS: 332 ± 42 staining neurons /10mm coverslip, 3 coverslips/group).
Figure 30: PSD-95 deficiency had no effect on VSCC and AMPAR-mediated cGMP formation. A, Neurons were exposed to high K⁺ (50mM) in the presence of Ca channel agonist BayK8644 (0-500nM). cGMP formation was determined for neurons treated with either antisense (AS) or sense (SE) oligodeoxynucleotides and revealed no differences between groups. B, AS, SE and sham (SH) treated neurons were exposed to kainate (0-1000 μM). Measurements of cGMP formation indicated that no cGMP was produced when neurons were treated with kainate. This can be explained by low levels of AMPAR-mediated Ca loading in the present culture system. Each bar represents the mean (+SE) from (A): n=8 cultures and (B): n=4 cultures. Data are expressed as the fraction of cGMP produced in SE-treated cultures by 100 μM NMDA.
A

High-K⁺ + MK-801 + CNQX
in 10 mM calcium

Fraction of Max cGMP formation

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<th>Condition</th>
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<td>* 100 nM</td>
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High-K⁺ (50 mM)

B

Kainate+ MK-801 + NIM

Fraction of Max cGMP formation

<table>
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<tr>
<th>Kainate [μM]</th>
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<th>SE</th>
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-KFigure 30-
Figure 31: Bypassing nNOS activation with NO donors restored toxicity in neurons lacking PSD-95. Antisense (AS), sense (SE) or sham (SH) treated neurons were exposed to nitric oxide (NO) donors nitroprosside (0, 300, 1000 μM). Application of the donor was highly toxic, irrespective of PSD-95 deficiency in the AS-treated groups. Each bar represents the mean (+SE) from n=8 cultures from 2 different experiments.
We then tested for cGMP formation induced by Ca\(^{2+}\) influx through alternative Ca\(^{2+}\) influx pathways. Loading the cells with Ca\(^{2+}\) through L-VSCCs by application of high-K\(^{-}\) in the presence of Bay-K8644, a maneuver that matches Ca\(^{2+}\) loads with those attained by activation of NMDAR (compare Fig. 9), did induce similar amounts of cGMP formation (Fig. 30A). However, cGMP levels were unaffected in neurons lacking PSD-95. This result further confirmed that nNOS activity in PSD-95 deficient neurons was unaltered. AMPA/kainate receptor activation failed to load the cells with Ca\(^{2+}\) (Fig. 11), and thus failed to increase cGMP levels (Fig. 30B).

Our findings indicate that suppressing PSD-95 selectively reduces NO production efficiency by NMDAR-mediated Ca\(^{2+}\) influx, but preserves NO production by Ca\(^{2+}\) influx through other pathways. To test whether the protective effects of this reduced NO production are indeed based on the disconnection of the NMDAR/PSD-95/nNOS complex, we bypassed nNOS activation with NO donors: sodium nitroprosside (SNP, Dawson et al. 1993, Fig. 31) and S-nitrosocysteine (SNOC, Brorson et al., 1999, data not shown). Both NO donors restored toxicity in neurons lacking PSD-95, indicating that reduced NMDA toxicity in PSD-95 deficient cells was unlikely to be caused by altered signaling events downstream from NO formation.
DISCUSSION

Overview

This work describes the cellular and molecular mechanisms of NMDAR-mediated Ca\(^{2+}\)-induced neurotoxicity, a process implicated in many forms of neurodegeneration including stroke, epilepsy and chronic neurodegenerative disorders (Lipton and Rosenberg, 1994). First, we described the relationship between neuronal Ca\(^{2+}\) loading and neurotoxicity for specific Ca\(^{2+}\) influx pathways. By pharmacologically isolating different Ca\(^{2+}\) influx pathways such as glutamate receptors and Ca\(^{2+}\) channels we provided evidence that, contrary to the Ca\(^{2+}\) hypothesis, neuronal survival is not primarily determined by the degree of neuronal Ca\(^{2+}\) loading. We demonstrated instead that the main determinant of Ca\(^{2+}\) toxicity is the route through which Ca\(^{2+}\) ions gain access to the intracellular space (Fig. 9). Specifically, we showed that the toxic potential of NMDAR-mediated Ca\(^{2+}\) loading cannot be reproduced when neurons are loaded with equal amounts of Ca\(^{2+}\) coming through other Ca\(^{2+}\) influx pathways, such as non-NMDA receptors or Ca\(^{2+}\) channels. We also showed that, depending on the route of Ca\(^{2+}\) influx, neurons could handle large Ca\(^{2+}\) loads without alterations in morphology or survivability (Figs. 9, 10). In other instances, there exist apparent “Ca\(^{2+}\) thresholds” above which Ca\(^{2+}\) loading becomes rapidly toxic.

These observations provided us with the basis for postulating that the NMDAR is linked to a macromolecular complex that enables Ca\(^{2+}\) coming through the NMDAR to trigger neurotoxicity. Confirming this hypothesis, we were able to show in subsequent
experiments that there indeed exists a macromolecular complex that is bound to the NMDAR and which is tethered to the postsynaptic density by the interaction with cytoskeletal proteins, such as the F-actin cytoskeleton. Disrupting F-actin with actin depolymerizing agents did not affect NMDA-evoked whole cell currents (Fig. 16). However, the number of synaptic NMDAR clusters was reduced, and the NMDA component of spontaneous miniature excitatory post-synaptic currents was selectively attenuated (Figs. 17, 18). The reduction in synaptic NMDARs did not affect neuronal death nor $^{45}$Ca$^{2+}$ accumulation evoked by exogenous NMDA. However, both were attenuated when NMDAR excitotoxicity was evoked by oxygen-glucose deprivation, which causes synaptic glutamate release (Fig. 20). Thus, it is not the subcellular localization of NMDARs that determines their excitotoxic potency, but the link to a macromolecular complex that is responsible for Ca$^{2+}$-dependent triggering of neurotoxic cascades.

In subsequent studies we sought evidence for the existence of this macromolecular complex that is linked to the NMDAR and that we hypothesized to be responsible for the unique potential of NMDAR-mediated Ca$^{2+}$-dependent neurotoxicity. Based on the recently acquired knowledge of the molecular composition at the postsynaptic density and its protein-protein interactions, we hypothesized that NMDARs are linked to neurotoxic signaling pathways by their direct interaction with postsynaptic density protein PSD-95 (Cho et al., 1992; Kornau et al., 1995, Fig.4). To test this hypothesis we decreased protein expression levels of PSD-95 by treating primary cortical neuronal cultures with antisense oligodeoxynucleotides (Fig. 21). This maneuver
selectively protected neurons from NMDAR-mediated Ca\textsuperscript{2+}-neurotoxicity without effecting Ca\textsuperscript{2+} loading through the NMDAR, NMDAR localization or NMDAR functions (Figs. 23, 26, 27). PSD-95 was shown by others to bind to nNOS, an enzyme that catalyzes the production of nitric oxide (NO), a short-lived signaling molecule that also mediates Ca\textsuperscript{2+}-dependent NMDA toxicity in cortical neurons (Dawson et al., 1991, 1993). We therefore sought to investigate the effect of suppressing PSD-95 expression on NO signaling and toxicity using cGMP formation as a surrogate measure of NO production by Ca\textsuperscript{2+}-activated nNOS (Dawson et al., 1991, 1993; Jaffrey and Snyder, 1995). PSD-95 deficiency had no impact either on nNOS expression (Fig. 29B), or on the morphology (Fig. 29B) or counts of NADPH diaphorase-stained neurons. However, in neurons lacking PSD-95 challenged with NMDA cGMP production was markedly attenuated (Fig. 29A). Like inhibited toxicity (Fig. 23), cGMP formation was unaffected in neurons loaded with Ca\textsuperscript{2+} through alternative Ca\textsuperscript{2+} influx pathways (Fig. 30). Thus, our findings indicated that suppressing PSD-95 selectively reduces NO production efficiency by NMDAR-mediated Ca\textsuperscript{2+} influx, but preserves NO production by Ca\textsuperscript{2+} influx through other pathways. This suggests that PSD-95 is required for the efficient coupling of NMDAR activity to nitric oxide toxicity, and imparts specificity to excitotoxic Ca\textsuperscript{2+} signaling.

The data presented in this thesis indicate that in cultured murine cortical neurons, Ca\textsuperscript{2+} neurotoxicity occurs through specific signaling pathways triggered by Ca\textsuperscript{2+}-dependent molecular substrates linked with NMDARs via PSD-95, which imparts NMDARs with signaling and neurotoxic specificity through the coupling of the receptor
activity to critical second messenger pathways, such as NO formation. Thus we provide for the first time a molecular model accounting for the specificity of Ca²⁺ neurotoxicity to Ca²⁺ influx via NMDARs.

Part 1: Distinct influx pathways, not total Ca²⁺ load, determine neuronal vulnerability to Ca²⁺ neurotoxicity.

Our data suggest that the source of Ca²⁺ entry, not the Ca²⁺ load, is the main determinant of the neurotoxic potential of Ca²⁺ ions. The source of Ca²⁺ also influences injury-associated morphology, and the relationship between free [Ca²⁺]ᵢ and total Ca²⁺ accumulation. In the case of NMDA-mediated toxicity, there exists an apparent threshold below which Ca²⁺ loading is well tolerated by neurons. Additionally, L-glutamate exhibits neurotoxic effects additive to those produced by Ca²⁺ loading via NMDARs.

Source-specificity of Ca²⁺ neurotoxicity implies that acute Ca²⁺ neurotoxicity is triggered by distinct signal transduction pathways rather than by a general overactivation of Ca²⁺-dependent phenomena. A prospect therefore exists to identify specific molecular targets whose manipulation may block Ca²⁺ influx-mediated neurotoxicity. These Ca²⁺-dependent molecules must be preferentially stimulated by NMDAR activity. We previously proposed that such targets, consisting of rate-limiting enzymes or substrates, may be physically co-localized with NMDARs (Tymianski et al., 1993b). Since then, candidates for such molecules have been identified. For example, Ca²⁺ influx through
NMDARs, by interaction with calmodulin, activates neuronal nitric oxide synthase (nNOS) and generates nitric oxide (NO; Garthwaite et al., 1988; Bredt, Snyder, 1990). NO, in excess, causes excitotoxicity (Dawson et al., 1991). nNOS has recently been shown to interact with postsynaptic density proteins where NMDARs abound (Brenman et al., 1996; Brenman et al., 1996). This led to the hypothesis that the ability of NMDAR activation to cause NO formation is due to the physical co-localization of NMDARs with nNOS, coupling the NMDA-mediated Ca\(^{2+}\) influx with this enzyme (Brenman and Bredt, 1997). Other possible targets for triggering NMDAR mediated, Ca\(^{2+}\)-influx dependent, neurotoxicity include a number of Ca\(^{2+}\)-sensitive second messengers such as Ca\(^{2+}\)-calmodulin dependent protein kinases, protein phosphatases, and adenylate cyclases, which have been identified in postsynaptic densities (Kitamura et al., 1993; Ghosh and Greenberg, 1995). Such enzymes have been associated with distinct Ca\(^{2+}\) signaling pathways regulating glutamate receptor function, gene expression, synaptic plasticity and neurotoxicity (Pirollet et al., 1992; Bading et al., 1993; Lerea and McNamara, 1993; Kitamura et al., 1993; McGlade-McCulloh et al., 1993; Hajimohammadreza et al., 1995; Churn et al., 1995).

Additional conceivable targets for neurotoxic Ca\(^{2+}\) influx may include intracellular Ca\(^{2+}\) sensors mediating Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) from intracellular stores. This Ca\(^{2+}\) release would compound the Ca\(^{2+}\) load incurred by influx, resulting in toxicity. If so, then, given the dependence of Ca\(^{2+}\) toxicity on the route of initial influx (Fig. 9), CICR would also have to be preferentially coupled to NMDA-mediated Ca influx over other routes such as VSCCs.
Alternatives exist to the hypothesized physical co-localization of NMDARs and rate limiting molecules governing neurotoxic cascades. For example, it is possible that Ca\(^{2+}\) signals generated by distinct influx pathways may exhibit diverse spatial and temporal characteristics. In the present context, this may be due to a different spatial distribution of NMDARs from VSCCs, or by different kinetics of diverse Ca\(^{2+}\) influx pathways producing different Ca\(^{2+}\) signaling patterns (e.g., see Fig. 8C vs 8D). These dissimilar patterns might provoke distinct homeostatic responses within the cell, including a preferential activation of neurotoxic pathways by one pattern over another. Evidence for such phenomena governing physiological events is mounting in non-neuronal cells (Dolmetsch et al., 1997; Berridge, 1997). However, analogous events leading to neuronal cell death have yet to be studied. It is also conceivable that agonist applications in the present paradigms produced bidirectional Ca\(^{2+}\) fluxes through the cell membrane, but with a net intracellular Ca\(^{2+}\) accumulation. In this light, the excess toxicity of NMDAR channels over L-VSCCs for equal total Ca\(^{2+}\) loads might be explained by greater Ca\(^{2+}\) influx through NMDAR channels which is equally better extruded from the cell as compared with L-VSCCs. This intriguing possibility would require that Ca\(^{2+}\) efflux pathways be preferentially compartmentalized with NMDARs, and merits further study.

A component of glutamate neurotoxicity additional to that produced by Ca\(^{2+}\) loading.
Our study revealed a previously unnoticed process whereby L-glutamate produced an excess toxicity over NMDA in cultures incurring intermediate Ca\(^{2+}\) loads (Fig. 12A,B). This excess toxicity of L-glutamate was not produced by EAA receptor activation including AMPA and mGluRs, or by endogenous glutamate release (Table 1,). Also, it was synergistic with NMDAR activation but not explained by NMDARs activity alone since this was accounted for by the \(^{45}\)Ca\(^{2+}\) measurements. The mechanism by which the excess glutamate toxicity occurs therefore remains open.

Glutamate is taken up by energy-dependent glutamate uptake carriers whereas NMDA, an exogenous compound, is not (Nicholls and Attwell, 1990; Attwell et al., 1993; Asztely et al., 1997). Impaired glutamate uptake contributed to excitotoxicity in this and other studies (Rothstein et al., 1996; Velasco et al., 1996). However, the increased toxicity observed in the presence of the glutamate uptake inhibitor was not due to the additional Ca\(^{2+}\) load incurred by the rise in extracellular glutamate, as that was accounted for in the normalized \(^{45}\)Ca\(^{2+}\) measurements. One possible explanation for this effect is that glutamate, the endogenous compound, imposes a metabolic burden on glutamate reuptake mechanisms which is lacking with NMDA. This hypothesis is compatible with our finding that glutamate challenges depleted cellular ATP levels more effectively than NMDA (Fig. 12C,D). Notably however, blocking glutamate uptake failed to reduce glutamate toxicity to levels seen with NMDA for any given Ca\(^{2+}\) load. Therefore, alternative explanations must be considered. An intriguing possibility, advanced by Murphy et al. (1989), that glutamate toxicity is due to its ability to inhibit cystine uptake, causing a decrease in glutathione levels and an accumulation of
intracellular peroxides. It is also possible that glutamate attenuates cellular ATP levels due to the metabolic burden imposed on intracellular regulatory processes by intracellular glutamate accumulation rather than on ATP requirements for glutamate transport. Finally, despite having tried numerous mGluR antagonists together and in combination (Table 1), we have not conclusively shown that all mGluRs in the cells were blocked. The possibility that mGluRs not responsive to the blockers employed, or that other, “unrecognized” glutamate receptors types might exist in our preparation should be considered.

Given the relative resilience of neurons to sub-maximal Ca\(^{2+}\) loading via NMDARs (Fig. 9A), this previously unidentified, additional process of glutamate toxicity may constitute an important excitotoxic mechanism under conditions producing sub-maximal Ca\(^{2+}\) loading, and merits further study.

**Ca\(^{2+}\) threshold for NMDA neurotoxicity**

As noted, cultures loaded with Ca\(^{2+}\) ions by NMDAR activation exhibited remarkable resilience to Ca\(^{2+}\) loading, until the load exceeded 80% of maximum (Fig. 9A). At that point, small increments in Ca\(^{2+}\) loading were highly neurotoxic. Given that most (>95%) Ca\(^{2+}\) ions which enter a neuron under physiological conditions are buffered (Neher and Augustine, 1992; Zhou and Neher, 1993), one possibility is that the Ca\(^{2+}\) buffering machinery of the cell is overwhelmed when a given Ca\(^{2+}\) load is reached. For example, neuronal mitochondria are currently believed to buffer both physiological and pathological Ca\(^{2+}\) loads (Werth and Thayer, 1994; White and Reynolds, 1995; Wang and
Exposure to EAAs produces mitochondrial dysfunction, and the production of secondary excitotoxic products such as reactive oxygen species (Reynolds and Hastings, 1995; Dugan et al., 1995; Schinder et al., 1996). Mitochondria also exhibit a Ca\textsuperscript{2+} load-dependent phenomenon, the mitochondrial permeability transition (Bernadi et al., 1992; Petronilli et al., 1993; Brutovetsky and Klingenberg, 1996; Kristal and Dubinsky, 1997), which fits the concept of a Ca\textsuperscript{2+} threshold for instigating the failure of ionic homeostasis. Under this hypothesis, Ca\textsuperscript{2+} buffering by mitochondria would fail when Ca\textsuperscript{2+} loading reaches the level required to trigger the permeability transition. In support of this was our finding that challenging cultures with A23187, a Ca\textsuperscript{2+} ionophore which also permeabilizes the mitochondria thereby simulating early permeability transition (Reed and Lardy, 1972; Kristal and Dubinsky, 1997), also evokes a high degree of neurotoxicity for small increments in $^{45}$Ca\textsuperscript{2+} accumulation (Fig. 9C).

The above hypothesis does not explain why Ca\textsuperscript{2+} neurotoxicity was restricted to NMDA-evoked Ca\textsuperscript{2+} entry, and was not seen when equal Ca\textsuperscript{2+} loads were delivered via L-VSCCs (Fig. 9B). The source-specificity hypothesis, however, suggests an alternative explanation (Fig. 32): To trigger neurotoxicity, [Ca\textsuperscript{2+}]i or total Ca\textsuperscript{2+} loads must reach very high levels in the vicinity of NMDARs in order to activate signals leading to neurodegeneration (Tymianski et al., 1993b, 1994). As Ca\textsuperscript{2+} levels in the vicinity of NMDARs increase, more Ca\textsuperscript{2+} ions are channeled into pathways, which, through second messenger systems such as NO production, incapacitate the cell’s Ca\textsuperscript{2+} homeostatic machinery. This does not exclude the possibility that Ca\textsuperscript{2+} ions, having interacted with Ca\textsuperscript{2+}-binding domains near NMDARs, then diffuse further into the cytoplasm.
Figure 32: Schematic depiction of the source-specificity hypothesis. For Ca$^{2+}$ influx to efficiently trigger neurotoxicity, distinct submembrane proteins physically couple NMDARs to neurotoxic second messengers.
At this stage, they would be handled by the cell's Ca\(^{2+}\) buffering and/or extrusion processes like Ca\(^{2+}\) ions which enter the cell through other pathways. Therefore, under the source-specificity hypothesis, phenomena such as Ca\(^{2+}\) -dependent mitochondrial damage occur not due to excessive Ca\(^{2+}\) accumulation within mitochondria, but rather, due to neurotoxic processes triggered by Ca\(^{2+}\) ions prior to their sequestration. In this context, the ability of A23187 to trigger neurodegeneration at lower Ca\(^{2+}\) loads (Fig. 9C) may be due to the fact that the ionophore destroys the cell by directly disrupting intracellular organelles rather than by producing Ca\(^{2+}\) entry. Also, under this hypothesis, the "Ca\(^{2+}\) threshold" of NMDA toxicity is determined by the threshold of Ca\(^{2+}\) required to activate neurotoxic cascades in the vicinity of NMDARs, not by the total Ca\(^{2+}\) entry into the cell.

These notions are supported by the finding that Ca\(^{2+}\) loads produced by activating NMDARs were poorly handled by the cell as compared with Ca\(^{2+}\) entry through L-VSCCs (Fig. 8). The temporal pattern of NMDA-evoked [Ca\(^{2+}\)]\(_i\) increases exhibited a progressive [Ca\(^{2+}\)]\(_i\) rise with time (Fig. 8D), a pattern constituting the secondary Ca\(^{2+}\) overload phenomenon previously characterized in EAA-challenged neurons, reflecting irreversibly impaired Ca\(^{2+}\) homeostasis in cells that are destined to degenerate (Randall and Thayer, 1992; Tymianski et al., 1993a). This Ca\(^{2+}\) homeostatic failure may represent the consequence, not the cause, of early Ca\(^{2+}\) -triggered neurotoxic events. In the present study, deregulation of Ca\(^{2+}\) homeostasis following a toxic NMDA exposure began almost immediately (Fig. 8D), at times when total \(^{45}\)Ca\(^{2+}\) loading was still well below 80% of maximum, the "neurotoxic threshold" for NMDA (Fig. 9A; also see \(^{45}\)Ca\(^{2+}\) accumulation at 10 and 30 min, Fig. 7B). This almost immediate onset of deregulated Ca\(^{2+}\) homeostasis
is consistent with the source specificity hypothesis, but not with the theory that general Ca\(^{2+}\) loading must reach a threshold prior to the onset of neurotoxicity.

**Part 2.A: Synaptic NMDAR localization does not determine the neurotoxic potential of NMDAR-mediated Ca\(^{2+}\)-loading**

Treating cortical and hippocampal neurons with cytochalasin-D and latrunculin-A disrupted neuronal F-actin to different degrees (Figs. 14, 15), with no apparent effect on macroscopic NMDA-evoked whole-cell currents (Fig. 16) or NMDA-evoked \(^{45}\)Ca\(^{2+}\) accumulation (Fig. 19A2, B2). Latrunculin-A, the agent that disrupted actin in dendritic spines most effectively (Fig. 15C), caused a reduction in the total number of NMDAR clusters in the dendrites (Fig. 17) and selectively reduced synaptic NMDAR activity (Fig. 18). When used to probe the role of synaptic and extrasynaptic receptors in excitotoxicity, perturbing F-actin with latrunculin did not affect excitotoxicity evoked by exogenous NMDA (Fig. 19), but reduced excitotoxicity caused by OGD which preferentially activates synaptic NMDARs (Fig. 20).

Our data indicate that signaling mechanisms that participate in NMDAR-mediated excitotoxicity are not governed by the synaptic localization of NMDARs. Since conditions that preferentially attenuated synaptic NMDAR function had no effect on the toxicity of exogenous NMDA (Fig. 19), extrasynaptic NMDARs must still be linked to
the second messenger pathways that trigger neuronal damage. This implies that the actin cytoskeleton is not involved in neurotoxic NMDAR signaling.

Allison et al. (1998) demonstrated that destabilizing actin with latrunculin-A reduced the numbers of synaptic NMDA receptors, but that they remained associated with PSD-95. Halpain et al. (1998) further showed that the association of NMDARs and PSD-95 remained unperturbed by excitotoxic insults with NMDA, which causes F-actin depolymerization (Shorte, 1997). Taken together, these data suggest a model in which F-actin plays a structural role in targeting NMDARs and their associated signaling complexes to synaptic sites (Fig. 33). The optimal positioning of such complexes may maximize the efficiency of activating NMDARs and their associated signal transduction pathways. Depolymerizing F-actin perturbs the efficiency of postsynaptic receptor activation, and reduces the probability of activating neurotoxic signaling molecules (Fig. 33B). However, this occurs without disrupting the association of NMDARs with the molecules responsible for initiating these neurotoxic signaling cascades.

In spite of the above-proposed model (Fig. 33), it remains difficult to determine the precise mechanism by which treatment with latrunculin-A reduced the activation of synaptic NMDARs. Consistent with our results (Fig. 17), Allison et al. (1998) showed in hippocampal neurons that this compound reduced the numbers of NMDAR clusters in dendrites. However, they also showed a similar reduction by latrunculin of the numbers of AMPA receptor clusters using immunostaining for the GluR1 subunit. Since depolymerizing actin in spines selectively attenuated the NMDA component of spontaneous mEPSCs while leaving the AMPA component intact (Fig. 18), the loss of
receptor cluster immunostaining may not translate directly to an effect on receptor function. Rather, the effect may occur at a level of organization that is not easily detectable by conventional optical means. For example, AMPA receptors are readily solubilized from adult rat hippocampal tissue and cultured neurons using Triton X-100 extraction (Wenthold et al., 1996; Allison et al., 1998) whereas NMDA receptors and other core components of the PSD such as PSD-95 are relatively detergent insoluble (Cohen et al., 1977; Kennedy, 1997). This may indicate that AMPA receptors are less tightly anchored to structures in the PSD, and that their submicroscopic localization may be less affected than that of NMDARs upon treatment with actin-perturbing agents. Studies using immunogold histochemistry, suggest that AMPARs are preferentially localized at the periphery, whereas NMDARs are found at the highest concentration in the middle of the synaptic apposition (Bernard et al., 1997; Kharazia and Weinberg, 1997). Thus, the impact of destabilizing the cytoskeleton may have different implications for the function of these two receptor classes.

It has been reported that cytochalasins can protect cultured hippocampal neurons against glutamate and amyloid β-peptide toxicity by stabilizing neuronal calcium homeostasis (Furukawa and Mattson, 1995; Furukawa et al., 1995). The authors achieved this by pretreating the cells with cytochalasin-D for 1h at concentrations of 1-100nM. These findings are consistent with a proposed action of cytochalasins in promoting NMDA channel rundown by mimicking the effects of Ca²⁺-mediated actin depolymerization (Rosenmund and Westbrook, 1993). In the present study, we found no effect of cytochalasin-D on NMDA-evoked ionic currents (Fig. 3), NMDA excitotoxicity
(Fig. 19B1) or NMDA-evoked \(^{45}\text{Ca}^2+\) accumulation (Fig. 19B2). However, our cortical neurons were treated with this depolymerizing agent for a longer duration (12h), and generally at higher concentrations (up to 30\(\mu\)M). In the present experiments, our aim was to achieve a maximal depolymerizing effect on actin based on imaging with rhodamine-phalloidin (Figs. 14, 15). Thus, we cannot exclude the possibility that brief exposure to low concentrations of cytochalasins might affect receptor activity, \(\text{Ca}^{2+}\) homeostasis or survivability by mechanisms that were not addressed in our experiments.

The finding that extrasynaptic NMDARs are fully capable of triggering excitotoxic neuronal damage may imply different mechanisms in different human neurological diseases. Excitotoxic neuronal damage is thought to play a role in traumatic brain and spinal cord injuries (Faden et al., 1989; Tecoma et al., 1989), as CNS trauma may produce a rapid disruption of cellular membranes causing a diffuse rise in extracellular glutamate levels (Brown et al., 1998). By its nature, traumatic injury may result in excitotoxic neuronal damage via both synaptic and non-synaptic receptors. Conversely, transient focal cerebral ischemia, transient global ischemia and epilepsy are disorders of synaptic overactivity that occurs in the absence of overt early neuronal damage (Kirino, 1982; Petito et al., 1987; During and Spencer, 1993). Under these pathological circumstances that do not cause the loss of neuronal membrane integrity the glutamate receptors that trigger excitotoxicity would likely be located in synaptic sites.

Our data reveal a distinct functional role of the actin cytoskeleton. This protein appears to target NMDARs with their associated scaffolding, clustering and signaling macromolecules to synaptic sites, but not to affect the functionality of these complexes.
Figure 33: Schematic depiction of the distinct contributions of synaptic and extrasynaptic NMDA receptors to OGD-mediated excitotoxicity. A, NMDARs are tethered to the synapse by interactions with the F-actin cytoskeleton via α-actinin. During an excitotoxic insult, presynaptically released glutamate binds to the NMDAR and induces Ca$^{2+}$ influx. Ca$^{2+}$ ions entering the postsynaptic cell through NMDAR channels then trigger neurotoxicity by interacting with a macromolecular complex that is linked to the NMDAR. B, Depolymerization of the F-actin cytoskeleton reduces the number of synaptic NMDAR clusters. Glutamate is released into the synaptic cleft, but activates a smaller number of NMDARs resulting in decreased Ca$^{2+}$ influx and a decreased activation of NMDAR-associated neurotoxic signaling molecules. This results in decreased neuronal cell death.
Figure 33

Cell Death

Presynaptic

Latrunculin-A

Postsynaptic

Neuroprotection

Glutamate

NMDA receptor

Calcium

Macromolecular complex

α-actinin

F-actin

Cell Death

Neuroprotection
Recent evidence indicates that this targeting is a dynamic process, as the accumulation of both AMPA and NMDA receptors at synapses is highly responsive to the stage of neuronal development and their level of endogenous excitatory activity (Rao and Craig, 1997; O'Brien et al., 1998; Liao et al., 1999). For example, it is possible to regulate the numbers of synaptic glutamate receptor clusters in cultured neurons by pharmacologically inhibiting or increasing excitatory synaptic activity (Rao and Craig, 1997; O'Brien et al., 1998).

**Part 2.B: Specific coupling of NMDAR-mediated excitotoxicity to nitric oxide signaling by postsynaptic density –95 protein**

This study indicates that suppressing PSD-95 expression uncoupled NO formation from NMDAR activation (Fig. 29), and partially protected neurons against NMDAR toxicity (Fig. 23A,B) without affecting receptor function as determined by measurements of NMDA currents and NMDAR-mediated Ca\(^{2+}\) loading (Figs 26, 27). We further showed that the protective effect from PSD-95 deficiency is due to mechanisms downstream from NMDAR activation, and upstream from NO-mediated toxic events (Fig. 31). Therefore NMDAR signaling and its neurotoxic specificity can be explained by the coupling of NMDA receptor activity to critical second messenger pathways via PSD-95.
Our data therefore point to a structural role of PSD-95 rather than a modulatory one. This is consistent with data recently obtained in a study using mice with mutant PSD-95 (Migaud et al., 1998). The authors showed that in mice carrying a targeted mutation in the PSD-95 gene, synaptic NMDAR currents, subunit expression, localization and synaptic morphology were all unaffected by the mutation. However, the frequency function of NMDA-dependent LTP and LTD was shifted to produce enhanced LTP at different frequencies of synaptic stimulation. Hence their results also point to a modification of downstream events of NMDAR activation due to the loss of PSD-95, rather than an alteration of NMDAR function. This idea was further supported by a study describing the importance of the intracellular C-terminal domain of the NR2 subunits (Sprengel et al., 1998), which is the domain responsible for binding to PSD-95. The authors showed that C-terminal truncation of NR2 subunits in mutant mice does not interfere with the formation of gateable receptor channels that can be synaptically activated, but instead impaired the mice in cellular signal transduction events such as LTP. Tezuka et al. (1999) also suggested a scaffolding role for PSD-95 by binding proteins that mediate tyrosine phosphorylation of NR2A by Src-family protein tyrosine kinases, such as Fyn. The idea of PSD-95 as a solely scaffolding protein that brings intracellular signaling molecules in close vicinity to the membrane receptor is apparently contradictory to one recent study carried out by Yamada et al. (Yamada et al., 1999). By injecting PSD-95 cRNA into *Xenopus* oocytes expressing the NMDAR the authors showed a decreased sensitivity of the receptor channel to glutamate as well as inhibition of protein kinase C-mediated potentiation of the NMDAR channel. They suggest that
PSD-95 functionally modulates the channel activity of the ε2/ζ1 NMDAR and that PSD-95 may play a protective role against neuronal excitotoxicity. The contradiction of these results, in which PSD-95 was designated a NMDAR-modulatory role, to our findings and those of others (Tsunoda et al., 1997; Sprengel et al., 1998; Migaud et al., 1998; Yamada et al., 1999) might be explained by the differences in the culture systems and their distinct underlying cellular and molecular machinery.

In the present study we describe a situation in which we were able to reduce NMDAR-mediated NO formation and neurotoxicity without effecting NMDAR-mediated Ca\(^{2+}\) loading (Fig. 23). Thus, despite the fact that Ca\(^{2+}\) ions are still entering the cell they are no longer capable of triggering certain Ca\(^{2+}\)-dependent cascades. This finding is consistent with a concept first implied as a result of studies describing the mechanisms of Ca\(^{2+}\)-dependent gene expression (Bading et al., 1993; Lerea, McNamara, 1993; Bito et al., 1997). The concept suggests that Ca\(^{2+}\) activates distinct signaling pathways and immediate early gene responses depending on its mode of entry and depending on the availability of the particular mechanisms for signal processing, such as key enzymes, at the specific site of Ca\(^{2+}\) entry (Bading et al., 1993; Lerea, 1997). The present study extends this idea further by showing that also pathological Ca\(^{2+}\)-dependent signaling depends on the route of Ca\(^{2+}\) entry and not the total amount of neuronal Ca\(^{2+}\) loading (see Part 1 and Sattler et al., 1998). In addition, we were now able to demonstrate for the first time the existence of a macromolecular complex that links NMDAR-mediated Ca\(^{2+}\) influx to neurotoxic signaling pathways and most likely also to
physiological signaling events. Perturbing this complex by removing one of the linking molecules clearly prevented Ca\(^{2+}\) ions from activating NMDAR-linked signaling events.

Thus, this study provides for the first time direct evidence for the existence of a macromolecular complex consisting of NMDARs, PSD-95 and nNOS. This complex has been hypothesized before by others to be responsible for NMDAR-mediated NO signaling (Brenman et al., 1996a, b, 1997), but it has never been demonstrated directly. By eliminating PSD-95 we presumably relocated nNOS far enough away from the NMDAR channel pore, to enable Ca\(^{2+}\) ions coming through the receptor from activating nNOS. This idea was supported by the fact that first, nNOS activity was maintained (Figs. 29 and 30) and secondly we were able to restore NO-mediated toxicity in PSD-95 deficient cultures by adding NO-donors (Fig. 31). Thus the loss of PSD-95 did indeed perturb the activation of nNOS rather than effecting downstream events of NO toxicity. NO has been proposed to play a major role in NMDAR-mediated neurotoxicity, as a variety of NOS inhibitors blocks NMDA neurotoxicity in cultures (Dawson et al., 1991; Dawson et al., 1993; Dawson and Snyder, 1994; Dawson and Dawson, 1996) and nNOS-deficient mice show reduced NMDA-induced neurotoxicity, while kainate-induced toxicity was uneffected by nNOS-deficiency (Dawson et al., 1996). The authors also showed that bypassing nNOS activity by application of NO donors regained the nNOS-deficient neurons' sensitivity to NO toxicity. Thus, preventing NMDAR-mediated Ca\(^{2+}\) entry from activating nNOS, either by eliminating nNOS or by perturbing its colocalization with the NMDAR, could be an efficient approach to protect against NO-mediated neurotoxicity.
CONCLUSIONS

We have shown that in cultured murine cortical neurons, Ca\(^{2+}\)-dependent neurotoxicity, like many other physiological Ca\(^{2+}\)-dependent events, occurs through specific signaling pathways triggered by Ca\(^{2+}\)-dependent molecular substrates linked with specific plasma membrane receptors. Thus it is not the quantity of intracellular Ca\(^{2+}\) accumulation that determines the neurons’ vulnerability to cell death but rather the route of Ca\(^{2+}\) entry and its associated molecular scaffold. Thus, cell death by overactivation of NMDARs during an excitotoxic insult is not caused by the receptor’s ability to cause greater Ca\(^{2+}\) rises over other Ca\(^{2+}\)-influx pathways, but rather by activation of NMDAR associated signaling molecules.

We showed that a macromolecular complex consisting of NMDARs, PSD-95 and its associated molecules imparts NMDARs with signaling and neurotoxic specificity through the coupling of the receptor activity to critical second messenger pathways, such as NO formation. This macromolecular complex is tethered to the synapse by cytoskeletal proteins such as F-actin. Therefore, the actin cytoskeleton plays a structural role in targeting NMDARs and their associated signaling complexes to synaptic sites, where maximal efficiency of NMDAR activation and their associated signal transduction pathways is given.

We provided for the first time a molecular model accounting for the specificity of Ca\(^{2+}\)-dependent neurotoxicity to Ca\(^{2+}\) influx via NMDARs over other neuronal Ca\(^{2+}\)-influx pathways (Fig. 34). Our results have broader consequences, as NMDAR activation and NO signaling are also critical to neuronal plasticity, memory and behavior. Thus this
work provides evidence for a potential mechanism by which PSD-95 may govern important physiological and pathological aspects of neuronal functioning.
Figure 34: Model of NMDAR-mediated Ca\(^{2+}\)-dependent neurotoxicity. The NMDAR is linked to neurotoxic signaling molecules, such as nNOS, via scaffolding protein PSD-95. Activation of nNOS by Ca\(^{2+}\) influx through the NMDAR causes formation of nitric oxide (NO) which diffuses to neighboring cells inducing NO-mediated neurotoxicity. Cytoskeletal elements such as F-actin play a structural role by tethering the macromolecular complex at synaptic sites for maximal efficiency of NMDAR activation. nNOS, neuronal nitric oxide synthase; NMDA-R, N-methyl-D-aspartate receptor;
GLUTAMATE
NMDA-R
Ca²⁺
F-actin
nNOS
NO
O₂⁻
NO
PSD-95

-Figure 34-
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