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Involvement of the NMDA Receptor in Moderate Ethanol Preconditioning-Dependent Neuroprotection From Amyloid-Beta in Vitro

Robert Matthew Mitchell

Loyola University Chicago

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LOYOLA UNIVERSITY CHICAGO

IN Volvement of the NMDA RECEPTOR IN MODERATE ETHANOL PRECONDITIONING-DEPENDENT NEUROPROTECTION FROM AMYLOID-BETA IN VITRO

A DISSERTATION SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL IN CANDIDACY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY PROGRAM IN MOLECULAR AND CELLULAR BIOCHEMISTRY

BY

ROBERT MATTHEW MITCHELL

CHICAGO, ILLINOIS

DECEMBER 2010
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<tr>
<td>4-AP</td>
<td>4-aminopyridine</td>
</tr>
<tr>
<td>5-HD</td>
<td>5-hydroxydecanoic acid</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>AG-1478</td>
<td>N-(3-Chlorophenyl)-6,7-dimethoxy-4-quinazolinamine</td>
</tr>
<tr>
<td>ALS</td>
<td>amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine monophosphate</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate</td>
</tr>
<tr>
<td>AP-2</td>
<td>adaptor protein 2</td>
</tr>
<tr>
<td>AP-5</td>
<td>DL-2-amino-5-phosphonopentanoic acid</td>
</tr>
<tr>
<td>ApoE4</td>
<td>apolipoprotein E ε4 allele</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine-5’-triphosphate</td>
</tr>
<tr>
<td>Aβ</td>
<td>amyloid beta</td>
</tr>
<tr>
<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>Cdk5</td>
<td>cyclin-dependent kinase 5</td>
</tr>
<tr>
<td>CIE</td>
<td>chronic intermittent ethanol</td>
</tr>
<tr>
<td>CM-HBSS</td>
<td>calcium and magnesium-free Hanks balanced salt solution</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
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<tr>
<td>CPP</td>
<td>3-[(+/-)-2-carboxypiperazin-4-yl]-propyl-1-phosphonate</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element-binding</td>
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<tr>
<td>Cy2, 3 and 5</td>
<td>cyanine dyes</td>
</tr>
<tr>
<td>Cys</td>
<td>cystine</td>
</tr>
<tr>
<td>DHHC</td>
<td>Asp-His-His-Cys (protein family)</td>
</tr>
<tr>
<td>DIV</td>
<td>day(s) in vitro</td>
</tr>
<tr>
<td>DlgA</td>
<td>drosophila disc large tumor suppressor</td>
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<tr>
<td>DNase</td>
<td>deoxyribonuclease I</td>
</tr>
<tr>
<td>DPCPX</td>
<td>8-Cyclopentyl-1,3-dipropylxanthine</td>
</tr>
<tr>
<td>ECL</td>
<td>enhanced chemiluminescence detection</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>ERK1/2</td>
<td>extracellular signal-regulated kinases 1 and 2</td>
</tr>
<tr>
<td>EtOH</td>
<td>ethanol</td>
</tr>
<tr>
<td>FAK</td>
<td>focal adhesion kinase</td>
</tr>
<tr>
<td>FAT</td>
<td>focal adhesion targeting</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>fESPS</td>
<td>field excitatory postsynaptic potentials</td>
</tr>
<tr>
<td>FREM</td>
<td>band 4.1/ezrin/radixin/moesin homology</td>
</tr>
<tr>
<td>GABA</td>
<td>gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GBSS</td>
<td>Gay's balanced salt solution</td>
</tr>
<tr>
<td>GFAP</td>
<td>glial acidic fibrillary protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>gp120</td>
<td>HIV-1 coat glycoprotein 120kD</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>HS</td>
<td>horse serum</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>LDH</td>
<td>lactate dehydrogenase</td>
</tr>
<tr>
<td>LTD</td>
<td>long term depression</td>
</tr>
<tr>
<td>LTP</td>
<td>long team potentiation</td>
</tr>
<tr>
<td>M 1-4</td>
<td>transmembrane spanning domains 1-4</td>
</tr>
<tr>
<td>MAGUK</td>
<td>membrane associated guanylate kinase</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein (MAP) kinase</td>
</tr>
<tr>
<td>MEK</td>
<td>mitogen-activated protein kinase/extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>MEM</td>
<td>minimum essential media</td>
</tr>
<tr>
<td>MEP</td>
<td>moderate ethanol preconditioning</td>
</tr>
<tr>
<td>mEPSCs</td>
<td>miniature excitatory postsynaptic currents</td>
</tr>
<tr>
<td>mitoK\textsubscript{ATP}</td>
<td>mitochondrial ATP-dependent potassium</td>
</tr>
<tr>
<td>MPP+</td>
<td>1-methyl-4-phenylpyridinium</td>
</tr>
<tr>
<td>MPT</td>
<td>mitochondrial permeability transition</td>
</tr>
<tr>
<td>MPTP</td>
<td>1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine</td>
</tr>
<tr>
<td>NAD</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NaN3</td>
<td>sodium azide</td>
</tr>
<tr>
<td>NBQX</td>
<td>3-dihydroxy-6-nitro-7-sulfamoyl-benzo[g]quinoxaline-2,3-dione</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>NFκB</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NIH</td>
<td>National Institutes of Health</td>
</tr>
<tr>
<td>NMDAR</td>
<td>N-methyl-D-aspartate subclass of glutamate receptor</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NR1</td>
<td>NMDAR subunit 1</td>
</tr>
<tr>
<td>NR2A-D</td>
<td>NMDAR subunits 2A-D</td>
</tr>
<tr>
<td>NR2B-pY1472</td>
<td>NMDAR2B phosphotyrosine 1472</td>
</tr>
<tr>
<td>PATs</td>
<td>palmitoyl acyltransferases</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson's disease</td>
</tr>
<tr>
<td>PDZ domains</td>
<td>post synaptic density protein, drosophila disc large tumor suppressor and zonula occludens-1 protein</td>
</tr>
<tr>
<td>PI</td>
<td>propidium iodide</td>
</tr>
<tr>
<td>PIPES</td>
<td>piperazine-N,N'-bis(2-ethanesulfonic acid)</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PKB/Akt</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PP2</td>
<td>4-Amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine</td>
</tr>
<tr>
<td>PP2B</td>
<td>protein phosphatase 2B (also know as calcineurin)</td>
</tr>
<tr>
<td>Prx</td>
<td>peroxiredoxin</td>
</tr>
<tr>
<td>PSD-95</td>
<td>post synaptic density protein 95kD</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene fluoride</td>
</tr>
</tbody>
</table>
Pyk2  proline-rich tyrosine kinase 2
Rac1  Ras-related C3 botulinum toxin substrate 1
ROS  reactive oxygen species
SAP-102  synapse-associated proteins 102kD
SH1  Src homology domain 1
SH2  Src homology domain 2
SH3  Src homology domain 3
SH4  Src homology domain 4
Src  Rous sarcoma virus
STEP  striatal enriched protein phosphatase
TRPC  transient receptor potential canonical
tSXV  terminal serine, any amino acid, valine motif
Tx-100  Triton-X 100
Zo-1  zonula occludens-1 protein
βARKct  C-terminus of β-adrenergic receptor kinase
Ψm  mitochondrial inner-membrane potential
ABSTRACT

Alzheimer’s disease (AD) is a progressive, mentally crippling, and eventually fatal form of dementia with growing prevalence in aging populations. In 2009 it was estimated that 5.3 million Americans have AD, with 5.1 million older than age 65 and 200,000 under 65. That accounts for 1 in 8 adults over 65. Furthermore, AD costs Americans 148 billion dollars in direct expenses, and is the leading cause of dementia and the 6th leading cause of death. Since improved medical care in general is increasing average life span and age is the primary risk factor for AD, there is need for basic science research into the causes of and potential pharmacological treatments for this debilitating neurodegenerative disease.

Preconditioning refers to endogenous adaptive responses to mild insults that induce a tolerant state, such that preconditioned tissues become resistant to a normally lethal insult. Our laboratories have shown that subchronic moderate ethanol preconditioning (MEP, 20-30mM for ~6d) protects organotypic brain slice cultures from amyloid-beta (Aβ), the neurotoxic peptide most often associated with AD. It is possible that the mechanism of MEP neuroprotection from Aβ toxicity may further our understanding of recent epidemiological results demonstrating a reduced risk of AD dementia in older moderate alcohol consumers.

Whether MEP causes events analogous to those triggered during/by ischemic and other types of neuroprotective preconditioning has been little studied. Therefore, the
objective of this project was to elucidate the possible “sensor” roles of certain cell surface receptors in MEP-mediated neuroprotection from Aβ in vitro. Specifically, Ga \textsubscript{i} protein-coupled receptors and/or glutamatergic N-methyl-D-aspartate receptors (NMDAR) have been shown to be involved in other neuroprotective preconditioning mechanisms. I hypothesize that MEP mediates neuroprotection from Aβ by the sensor activity of one or more of these cell surface receptors. The experiments employed rat cerebellar mixed cultures that show dose-related neurotoxicity in response to Aβ challenge and robust neuroprotection by MEP.

The results of these studies implicate NMDAR activation—but rule against adenosine A1 receptors or other Ga \textsubscript{i/o} protein-coupled receptors—in attaining the neuroprotected phenotype promoted by MEP. Whereas inhibition of adenosine A1 receptors with the selective antagonist, DPCPX, or of Ga \textsubscript{i/o} protein-coupled receptors with pertussis toxin failed to block MEP neuroprotection, inhibition of NMDAR with either the selective antagonist, AP5, or open channel blocker, memantine, effectively blocked it. Support for these findings was further demonstrated by the ability of preconditioning with NMDA itself to neuroprotect from Aβ-insult directly.

MEP was then shown to modulate NMDAR by increasing receptor subunit expression and enhancing synaptic receptor localization, ultimately triggering pathways favoring prosurvival and protection against the neurotoxic Aβ. Specifically, NMDAR subunits NR1, NR2B and NR2C were elevated by day 2 of MEP treatment and remained increased through day 6. MEP also increased post-synaptic scaffolding protein PSD-95 and the phosphorylation of tyrosine 1472 of NR2B—both of which are documented
markers for synaptically-localized NMDAR complex. These findings agree well with a growing literature on the dichotomous role of synaptic and extra-synaptic NMDAR on neuronal survival vs. excitotoxicity, respectively.

Furthermore, two non-receptor tyrosine kinases known to modulate NMDAR synaptic activity and localization, Src and Pyk2, were shown to be phospho-activated in the cerebellar cultures at a timepoint that correlated with the increases in PSD-95 and NR2B-pY1472, implicating them in the targeting of the NMDAR to the synapse. These findings are intriguing, since PSD-95 has been shown to target Pyk2 to the synapse and provide Src with molecular scaffolding. Src can then interact with multiple MAPK pathways.

Seeking further evidence, deconvolution microscopy was employed in order to visualize NMDAR localization in the cultures. The results of these experiments, done in collaboration with Dr. Edward Campbell’s laboratory in the Department of Microbiology and Immunology LUMC, showed an increase in PSD-95 association with NR2 subunits at day 6 of MEP. These results are consistent with the role of synaptic NMDAR in engendering the neuroprotective phenotype, in contrast to extra-synaptic NMDAR.

It has been recently reported that synaptic NMDAR activity enhances resistance of neurons from oxidative stress by upregulating the peroxiredoxin-thioredoxin antioxidant pathway (Papadia et al., 2008). In exploring whether or not peroxiredoxins were increased in association with augmented NMDAR activity in the MEP model, initial evidence indicated that cytosolic peroxiredoxin-2 is increased by day 6, but not earlier—correlating with the appearance of statistically significant MEP neuroprotection. This
result suggests a previously unappreciated “effector” in preconditioning neuroprotection. Since Aβ is known to induce oxidative stress, this finding may partially explain why MEP reduces Aβ neurotoxicity.

Summarizing, the results show that MEP acts through a sensor, NMDAR, to promote neuroprotection. They provide insights into the molecular mechanism by which a receptor once thought responsible for the neurotoxicity of AD pathophysiology actually is modulated in a prosurvival way to induce neuroprotection. Hopefully, future research based on these preconditioning mechanisms might lead to new avenues of therapy that might be useful in AD, age-related cognitive decline, and potentially even vascular dementia (stroke).
CHAPTER ONE
INTRODUCTION AND OVERVIEW

Alzheimer’s disease (AD) is the leading cause of dementia. In 2009 the Alzheimer’s Association annual AD facts and figures publication estimated 5.3 million Americans of all ages have AD with 5.1 million older than age 65 and 200,000 under 65. That accounts for 1 in 8 adults over 65 or 13% and a new case every 70 seconds. Furthermore, AD costs Americans $148 billion in direct expenses and is the 6th leading cause of death. Additionally, during the period between 2000 and 2006 the reported causes of death from heart disease, prostate cancer and stroke were reduced by -11.5, -14.3 and -18.1%, respectively, whereas over the same period, the reported cause of death from AD increased by a striking 47.1%.

The etiology of AD is complex and is confounded by a milieu of contributing risk factors, including cognitive decline associated with normal aging and environmental circumstances such as social engagement, intellectual stimulation, diet and exercise, as well as certain genetic predispositions. The most notable genetic risk factor is the apolipoprotein E ε4 (ApoE4) allele that is observed in nearly 40% of sporadic AD. In fact one report that studied 42 families with late onset AD found that with increasing copy number of the allele, the risk of AD increased from 20% to 90% and the mean age of
onset decreased from 84 to 68 (Corder et al., 1993). Furthermore, microarray studies have identified numerous genetic mutations associated with AD, many of which seem to have completely divergent functions. This perplexing aspect of the disease makes identifying drug targets extremely difficult.

Two pathological hallmarks of AD are insoluble extracellular deposits of amyloid beta (Aβ) peptide, sometimes referred to as senile plaques, and twisted strands of hyper-phosphorylated tau protein within neurons, called neurofibrillary tangles. While there is debate over the exact mechanisms involved in the progression of Alzheimer’s disease, it is clear that Aβ peptide fragments formed from the proteolytic processing of amyloid precursor protein (sequential actions of β- and γ-secretases) are directly neurotoxic in vivo and in vitro. The prevailing theory for the development of AD, the “Amyloid Hypothesis”, posits that Aβ peptide overproduction is the main cause of the disease. Furthermore, it has been suggested that soluble levels of Aβ in the brain, more so than amyloid plaque burden, correlate with cognitive deficits among AD patients (Terry et al., 1991; Naslund et al., 2000). Finally, Aβ peptides target memory processes in vivo (Haass and Selkoe, 2007).

Aside from acetylcholinesterase inhibitors, memantine is the only FDA-approved drug that has shown promise in delaying AD-related cognitive decline. Memantine at physiologically relevant concentrations is believed to act primarily on the N-methyl-D-aspartate subclass of glutamate receptors (NMDAR) at extra-synaptic sites to reduce excitotoxicity (Leveille et al., 2008) while allowing normal function of synaptically
localized NMDARs. However, it has also been shown to have affinity for α7 subunit-containing nicotinic acetylcholine receptors (Aracava et al., 2005) that are also implicated in AD (Snyder et al., 2005). Furthermore, although improving medical care is increasing average life span, and age is the primary risk factor for AD, investigation into mechanisms that prevent or delay it are of the utmost importance.

The beneficial effects of moderate ethanol consumption on cardiovascular health are today well recognized (Agarwal, 2002); however, less appreciated is the growing evidence that alcohol in moderation produces beneficial effects in the brain. Several lines of evidence now suggest that moderate, as opposed to heavy, alcohol consumption is associated with a decreased probability of dementia, including AD, later in life (Zuccala et al., 2001; Mukamal et al., 2003). One recent report from participants in the Ginkgo Evaluation of Memory Study found that, after controlling for demographics, smoking, co-morbidities, depression, social activity, and baseline cognition, moderate alcohol intake (defined as 8-14 drinks/week) was associated with a 40% lower risk of dementia in participants with normal cognition at the start of the study. However, this same study revealed that participants with mild cognitive impairment at the start of the study who drank heavily (>14 drinks/week) had a higher rate of progression to dementia.

Although two other studies found that moderate alcohol consumption decreased the risk of AD (ApoE4 negative), they only reached statistical significance when they analyzed wine intake as opposed to any form of alcohol (Orgogozo et al., 1997; Luchsinger et al., 2004). However, two more recent meta-analyses (a more powerful
statistical analysis) found beneficial effects of moderate drinking on the relative risk for Alzheimer’s disease regardless of the type of alcohol consumed (Peters et al., 2008; Anstey et al., 2009). Thus it appears that there might be some mechanism by which moderate drinking affords neuroprotection from the neurodegenerative effects of AD pathology.

Preconditioning refers to endogenous adaptive responses to mild insults that induce a tolerant state, such that preconditioned tissues become resistant to a normally lethal insult. Examples of preconditioning initiators include brief ischemia, hypoxia and an array of chemicals that induce cellular tolerance via a “sensor-transducer-effector” mechanism (Dirnagl et al., 2003). Our laboratories have shown that subchronic moderate ethanol preconditioning (MEP, 20-30mM for ~6d) protects organotypic brain slice cultures from dementia-associated neurotoxins such as HIV-1 gp120 or amyloid beta (Aβ) (Collins et al., 2000; Belmadani et al., 2004). MEP neuroprotection from Aβ toxicity may relate mechanistically to the aforementioned epidemiological results showing a reduced risk of dementia in older moderate alcohol consumers. Assessing whether MEP causes events analogous to those triggered during/by ischemic and other types of preconditioning has been little studied and was the focus of this project. Gα protein-coupled receptors and or N-methyl-D-aspartate sensitive glutamate receptors (NMDARs) have been implicated in neuroprotective preconditioning mechanisms. Therefore, the objective of this specific project was to elucidate their possible “sensor” roles in MEP-mediated neuroprotection from Aβ in vitro. I hypothesized that MEP
mediates neuroprotection from Aβ by the sensor activity of one or more of these cell surface receptors. Experiments employed a model system of cerebellar mixed cultures that show dose-related neurotoxicity in response to Aβ challenge and robust neuroprotection by MEP. Although the cerebellum is relatively resistant to amyloid peptides in vivo (Kim et al., 2003), cerebellar granule neurons have been shown to be vulnerable to Aβ in vitro (Allen et al., 1999). Additionally, Aβ 25-35 was employed as the toxic insult, which, although not physiological, has a neurotoxic mechanism apparently identical to the most neurotoxic form in vivo, Aβ 1-42 (Frozza et al., 2008).

The results of these studies implicate NMDAR activation—but rule against adenosine A1 or other Gαi/o protein-coupled receptors—in attaining the neuroprotected phenotype due to MEP. In short, whereas inhibition of the adenosine A1 receptor with the selective antagonist DPCPX, or of Gαi/o protein-coupled receptors with pertussis toxin failed to block MEP neuroprotection, inhibition of NMDARs with either the selective antagonist, AP5, or open channel blocker, memantine, effectively blocked it. Support for these findings was further demonstrated by the ability of preconditioning with NMDA itself to neuroprotect from Aβ-insult directly.

MEP was shown to act via at least one novel mechanism, in that it modulates NMDARs to increase subunit expression and enhance synaptic receptor localization, ultimately triggering pathways favoring protection against the neurotoxic Aβ. Specifically, MEP elevates NMDAR subunits NR1, NR2B and NR2C by day 2 of the treatment, elevations that persist through day 6. Importantly, MEP also increases post-
synaptic scaffolding protein PSD-95 and phosphorylation of tyrosine 1472 of the NMDAR2B subunit; both are markers for synaptically localized NMDAR complex. These findings are in harmony with a growing literature on the dichotomous role of synaptic and extra-synaptic NMDARs on neuronal survival vs. excitotoxicity, respectively (Vanhoutte and Bading, 2003).

Two non-receptor tyrosine kinases known to modulate NMDAR synaptic activity and localization, Src and Pyk2, were shown to be phospho-activated at a timepoint that correlated with the increases in PSD-95 and NR2B-pY1472 expression, implicating their role in the targeting of the NMDAR to the synapse. These particular results are extremely intriguing, since PSD-95 has been shown to target Pyk2 to the synapse and provide molecular scaffolding for Src (Bartos et al., 2010). Src can then interact with multiple MAPK pathways, lending support for the hypothesis that MEP enhances postsynaptic responses via formation of a “postsynaptic signalosome” (Collins et al., 2009). The exact nature of this interaction is not yet fully understood and is the focus of additional study.

Deconvolution microscopy was employed in order to visualize NMDAR localization. The results of these experiments showed an increase in the association of PSD-95 with NR2 subunits at day 6 of the preconditioning paradigm. These results are entirely consistent with the requirement of synaptic NMDARs to engender the neuroprotective phenotype in contrast to extra-synaptic NMDARs.

Recently, it was shown that synaptic NMDAR activity enhances resistance of neurons from oxidative stress by upregulating the peroxiredoxin-thioredoxin antioxidant
pathway (Papadia et al., 2008). Therefore I investigated whether the peroxiredoxins were increased by MEP. I found that indeed there is evidence that cytosolic peroxiredoxin-2 is increased by day 6 of MEP, but not earlier—correlating with the appearance of MEP neuroprotection. This identifies a previously unappreciated “effector” in preconditioning neuroprotection. Since Aβ is known to induce oxidative stress, this finding may partially explain why MEP reduces Aβ neurotoxicity.

Summarizing, the results show that MEP acts through the NMDAR to engender neuroprotection and provides clarity on the molecular mechanism by which a receptor once thought responsible for the neurotoxicity of AD pathophysiology actually is modulated in a pro-survival way by MEP to induce neuroprotection. Hopefully, in continuing with this work, research may lead to new avenues of therapy within the spectrum of AD, age-related cognitive decline and potentially vascular dementia. The following background presents a selected discussion of a vast but pertinent literature and relevant data related to this project.
2.1 Alzheimer’s Disease

Alzheimer’s disease (AD) is a progressive, mentally crippling and eventually fatal form of dementia with growing prevalence in aging populations. Early symptoms of AD include difficulty remembering names and recent events, apathy and depression. Over time, symptoms become more severe and include impaired judgment, disorientation, confusion, behavior changes, and trouble speaking, swallowing and walking in later stages. In the final stages of the disease people lose their ability to communicate, and to recognize loved ones and require constant medical attention. Symptoms result from massive synaptic degeneration that leads to neuronal death and shrinking of brain matter. Two pathological hallmarks of AD are insoluble deposits of Aβ peptide referred to as senile plaques and twisted strands of hyper-phosphorylated tau protein found within neurons called neurofibrillary tangles. Although it is currently unknown whether these histological markers accumulate as a result of disease progression or are themselves causative factors, amyloid peptides are directly neurotoxic in vivo and in vitro, and genetic manipulations that enhance accumulation of these pathological hallmarks account for the majority of animal models of the disease. Neurofibrillary tangles appear to be the result of Aβ pathology (Canevari et al., 2004).
AD is the leading cause of dementia. In 2009 it was estimated that 5.3 million Americans of all ages have AD, with 5.1 million older than age 65 and 200,000 under 65 (derived from the Alzheimer’s Association 2009 “Facts and Figures”). That accounts for 1 in 8 adults over 65 or 13%. Furthermore, AD costs Americans 148 billion dollars in direct expenses and is the 6th leading cause of death. During a period between 2000 and 2006 the reported causes of death for heart disease, prostate cancer and stroke were reduced by -11.5, -14.3 and -18.1%, respectively, whereas over the same period the reported cause of death from AD increased by a striking 47.1% (2009).

Although improving medical care in general is increasing average life span, since age is the primary risk factor for AD, clearly there is need for basic science research into the cause of and potential pharmacological treatment of this crippling neurodegenerative disease. This point is underscored by the failure of most pharmacological approaches that target the NMDAR to have beneficial outcomes. Because of the enormous socioeconomic burden and growing prevalence of AD, investigation into its cause and potential therapeutics is of great concern.

2.2 Mechanisms of amyloid neurotoxicity

The prevailing theory of the cause of AD is the “Amyloid Hypothesis” that posits that amyloid peptides and the dysregulation of their production are the cause of AD. The exact mechanism of Aβ neurotoxicity is complex and is thought to involve altered fluctuations in intracellular calcium (Ho et al., 2001) and excessive production of reactive
oxygen species (ROS) (Benzi and Moretti, 1995). While there is debate over the exact mechanisms involved in the progression of AD, it is clear that the peptide fragments formed from the proteolytic processing of amyloid precursor protein (sequential actions of β- and γ-secretases) are directly neurotoxic in vivo and in vitro. Correlations between the brain levels of these peptide products and the degree of cognitive impairment in AD have demonstrated that synaptic loss is the major anatomical correlate of cognitive decline in AD (Naslund et al., 2000).

There is also evidence that in AD patients, living neurons show decreased synaptic content and transcripts of vesicle trafficking machinery, underscoring the fact that AD is a synaptopathy (Coleman and Yao, 2003). As such, postsynaptic density preparations from cingulate cortex and hippocampus of AD brains have an increased density of pro-caspase-3 and active caspase-3 when compared to controls (Louneva et al., 2008). Soluble amyloid oligomers have been shown to target memory processes (Haass and Selkoe, 2007). For example, soluble amyloid oligomers rapidly impair plasticity of excitatory synaptic transmission in hippocampus (Hu et al., 2008) and facilitate NMDAR- and mGluR-dependent long-term depression (LTD) of electrical activity in hippocampal CA1 neurons (Li et al., 2009).

However, there does seem to be an important physiological role for amyloid peptides in normal brain function (Pearson and Peers, 2006). Plant and colleagues found that inhibiting the production of amyloid peptides in primary central neurons with secretase inhibitors or an antibody that binds Aβ resulted in a marked decrease in cell
viability, which was rescued by addition of Aβ 1-40 but not 1-42 or Aβ 25-35 (Plant et al., 2003). Furthermore, picomolar concentrations of a preparation containing monomeric and oligomeric Aβ enhanced hippocampal LTP and enhanced both reference and contextual fear memory in mice (Puzzo et al., 2008). Higher (nanomolar) concentrations had inhibitory effects. These effects were shown to be mediated by α7 nicotinic acetylcholine receptors. Therefore, it must be remembered that AD results from a dysregulation of normal physiological processes in the brain.

2.3 Aβ and NMDAR

The etiology of AD has long been thought to involve the NMDAR, which are important intermediaries in learning and memory, although the exact nature of this interaction is a matter of debate. Early hypotheses predicted an overactivation of NMDAR by Aβ in AD, but the data available demonstrate an inhibitory effect of amyloid peptides on NMDAR-dependent postsynaptic potentials and disruption of the postsynaptic density. Importantly, naturally secreted Aβ oligomers potently inhibit hippocampal LTP in vivo (Walsh et al., 2002). Long term potentiation (LTP) and long term depression (LTD) are cellular forms of memory that are activity-dependent and have opposing effects on postsynaptic strength. LTP, induced by way of high-frequency tetanic stimulus in vitro or by conditioned learning in vivo, enhances postsynaptic signals in hippocampus, whereas LTD induced chemically or otherwise depresses them. These effects on the excitability of neurons in response to different tonic stimuli are believed to
underlie memory consolidation and learning, since they can last for prolonged durations, and impairment of these effects by various stimuli impairs cognition in several animal models including AD. Although multiple forms of this phenomenon exist, NMDAR-facilitated forms are believed to be fundamental to memory and learning (Lacor et al., 2007). In hippocampus, LTP increases the surface expression of NMDARs in a protein kinase C (PKC)- and tyrosine kinase-dependent manner (Grosshans et al., 2002). Furthermore, Aβ impairs NMDAR-dependent LTP but neither non-NMDAR dependent LTP nor the induction of LTD in dentate gyrus slice cultures (Wang et al., 2004b); these effects were attributed to inflammatory microglia-induced production of superoxide and nitric oxide. Interestingly, an elegant study by Shankar and colleagues found that amyloid oligomers induced progressive loss of hippocampal dendritic spines, and when applied acutely, decreased NMDAR-dependent calcium influx through synaptic spines in organotypic hippocampal slice cultures (Shankar et al., 2007). Total antagonism of NMDAR with 20µM 3-[(±)-2-carboxypiperazin-4-yl]-propyl-1-phosphonate (CPP) blocked this effect and partial blockade (200-400nM CPP) mimicked it. The researchers concluded that the partial inhibition of NMDARs by Aβ oligomers or partial pharmacological inhibition, pathways that favor LTD, were responsible for the decrease in spine density and were dependent on activity of the actin disassembling enzyme, coflin, and the protein phosphatase, calcineurin (Shankar et al., 2007).

Roselli and coworkers found that soluble Aβ(1-40) caused dose- and time-dependent decreases in PSD-95 that were NMDAR- and calcium-dependent and were
inhibited by GABA receptor blocker bicuculline or a metabotropic II/III glutamate receptor antagonist, but were not AMPA receptor-dependent since 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX), an AMPA antagonist, failed to have an effect (Roselli et al., 2005). They further showed the decrease in PSD-95 to involve cdk5 and the proteasome pathway. However, an Aβ(1-42) preparation containing monomeric and oligomeric peptides bound to NMDAR-containing synapses and inhibited NMDAR function. The effect was not due to a direct NMDAR inhibition, since acute application of their preparation failed to alter NMDAR currents in hippocampal cultures or xenopus oocytes expressing recombinant NMDAR. Pre-incubation with this preparation decreased NMDAR subunit immunoreactive spines and surface expression of NR2B-containing NMDAR. Employing a transgenic model of AD, Dewachter and coworkers also showed that postsynaptic density preparations from APP[V717I] transgenic mice contained decreased concentrations of NR2B and PSD-95 (Dewachter et al., 2007).

Others have found that application of Aβ cause endocytosis of NMDARs in cortical neurons and decreased NMDAR positive synaptic spines. This study showed a potent inhibitory effect of Aβ peptides on NMDA- evoked currents and, importantly, inhibition of NMDA signaling via CREB. These researchers also provide compelling evidence that the effects of Aβ on NMDAR surface expression were α-7 nicotinic acetylcholine receptor-dependent, protein phosphatase 2B (PP2B, also know as
calcineurin)- and striatal enriched protein phosphatase (STEP)-dependent, and correlated with decreased phosphorylation of NR2B-Y1472 (Snyder et al., 2005).

Although the exact nature of the effect of Aβ on the NMDAR and its role in synaptic strength, architecture, and learning and memory processes remains to be fully elucidated, loss of functional NMDAR synapses is associated with behavioral and cognitive deficits in AD patients (Venkitaramani et al., 2007).

2.4 Moderate ethanol and Alzheimer’s

Although several reports indicate a beneficial effect of moderate-to-light ethanol consumption on the incidence of AD, two recent meta-analyses have helped to clarify this effect in humans. Anstey and colleagues analyzed data from 15 prospective studies including 14,646 participants with AD, vascular dementia (VaD) and any dementia and showed the relative risk (RR) of moderate drinkers to non-drinkers to be 0.72, 0.75 and 0.74, respectively (Anstey et al., 2009). When more generally classified into “drinkers” or “non-drinkers”, RR became 0.66 for AD and 0.53 for any dementia, but no significant difference for any dementia. There was not enough data to determine the RR for VaD under these criteria. Heavy drinkers did not have an increased risk, but the authors’ state that this could be due to sampling bias. “…alcohol drinkers in late life have reduced risk of dementia. It is unclear whether this reflects selection effects in cohort studies commencing in late life, a protective effect of alcohol consumption throughout adulthood, or a specific benefit of alcohol in late life” (Anstey et al., 2009).
Additionally, a meta-analysis by Peters et al. (2008) evaluated the relationship between alcohol consumption and incident cognitive decline or dementia in the elderly. The inclusion criteria were longitudinal studies of subjects 65 or older, with primary outcomes of incident dementia/cognitive decline. They identified 23 studies (20 epidemiological cohort and three retrospective matched case-controls nested in a cohort) and found statistically reduced RR of dementia (0.63) and AD (0.57) among drinkers when compared to abstainers, but not for VaD (0.82) or cognitive decline (0.89). “However, studies varied with differing lengths of follow up, measurement of alcohol intake, inclusion of true abstainers and assessment of potential confounders. Because of the heterogeneity in the data these findings should be interpreted with caution. However, there is some evidence to suggest that limited alcohol in earlier adult life may be protective against incident dementia later” (Peters et al., 2008). Thus, it appears that there is a significant degree of neuroprotection from AD and general dementia observed in moderate-to-light drinking adults. It is possible that this may reflect to some degree the MEP neuroprotection from Aβ observed in vitro.

2.5 Preconditioning

Conserved among organisms is an inherent ability to adapt to stress or environmental change. Preconditioning in general refers these adaptive responses to mild stressful stimuli that induce a tolerant state, such that preconditioned tissues are rendered resistant to a much stronger and normally lethal insult. There are two classical phases of
preconditioning, the acute phase that is short-lived (typically 1-3h) and independent of protein synthesis (not blocked by cycloheximide) (Perez-Pinzon et al., 1997; Perez-Pinzon and Born, 1999), and the delayed phase that is long-lasting (typically from 1-7 days) and dependent on new protein synthesis (Barone et al., 1998). The first description of cardiac preconditioning in mammals described this early phase and was published in 1985 by Murry and colleagues. They demonstrated that brief periods of ischemia induced tolerance to a later, more prolonged normally lethal episode of ischemia in dog heart (Murry et al., 1986). Since then, examples have grown to include not only ischemia, but a number of traumas and a wide array of chemicals that induce tolerance, as well as examples of cross-tolerance, remote preconditioning and postconditioning. Furthermore, preconditioning has been demonstrated in multiple organ systems, including heart, brain (Kitagawa et al., 1990), lung (Luo et al., 1997) and kidney (Islam et al., 1997). However, although a great body of literature has promoted a better understanding of the mechanisms involved, many aspects have yet to be fully elucidated. One recent report that is highly relevant to our work demonstrated that moderate to low (single bolus, 42-46 mg/kg peak blood level) ethanol preconditioning is capable of inducing delayed (24h) cross-tolerance that is protective against global cerebral ischemia in gerbils (Wang et al., 2007) via a mechanism involving NADPH oxidase-derived ROS. Interestingly, many of the underlying mechanisms of preconditioning-mediated protection are homologous throughout various tissue types and stimuli. Dirnagl et al. (2003) provides a conceptual framework that separated the various signaling mechanisms common in brain
preconditioning into “sensors” (events that signal to the cell that there is a stressor present), “transducers” (molecules such as kinases and transcription factors that transduce information from the environment to the nucleus), and “effectors” (that are the physical entities such as heat shock proteins and anti-apoptotic enzymes that account for the protected state) (Dirnagl et al., 2003). However, although this model of brain preconditioning provides a logical paradigm to investigate specific signaling events within preconditioning models, it should be recognized that many of these signaling pathways and intermediaries overlap spatially and temporally. What follows is a review of some of the key research in the area of preconditioning mechanisms of heart and brain.

2.6 NMDA receptors in preconditioning

In the brain, NMDAR activation is commonly associated with excitotoxicity. However, several models of preconditioning have demonstrated the role of NMDARs in mediating neuroprotection (Kato et al., 1992; Miao et al., 2005; Jiang et al., 2005; Wang et al., 2006; Xifro et al., 2006). Exposure of murine cortical cell cultures to preconditioning doses of glutamate or NMDA has been shown to be neuroprotective (Grabb and Choi, 1999). Similarly, exposure of cerebellar granule cell cultures to sub-excitotoxic concentrations of NMDA has been shown to cause neuroprotection attributable to release of brain-derived neurotrophic factor (BDNF) acting on receptor tyrosine kinase B (TrkB) (Marini et al., 1998). Both NMDA and BDNF exposure result in activation of the transcription factor nuclear factor κB (NF-κB), and inhibition of NF-
κB with an oligonucleotide decoy reversed neuroprotection from glutamate-mediated excitotoxicity (Wu et al., 2005). Neuroprotection in this model correlates with expression of anti-apoptotic bcl-2. NMDA receptor activation is also associated with mitogen-activated protein kinase (MAPK) activity. In a rat model of ischemic preconditioning (IPC), inhibition of NMDAR signaling blocked ERK5 activation, an effect that reversed hippocampal CA1 neuroprotection (Wang et al., 2006). That same study showed inhibition of ERK5 with antisense oligonucleotide also prevented neuroprotection, demonstrating dependence on NMDAR-mediated ERK5 activation for IPC. In that regard, transient calcium influx into hippocampal neurons causes calcium/calmodulin kinase (CaMK)-dependent Ras/MAPK activation (Wu et al., 2001). This work exposed a biphasic activation of cyclic AMP response element binding protein (CREB). The early phase was dependent on CaMK and the latter phase on MAPK. In another study, NMDA was capable of protecting cerebellar granule cell cultures from potassium deprivation (Xifro et al., 2006). Protection in this model correlated with inhibition of pro-apoptotic c-jun N-terminal kinase (JNK) and expression of anti-apoptotic bcl-2.

2.7 \( G_{\alpha,i} \), protein-coupled receptors in preconditioning

Much of what is known about the early cell signaling events in preconditioning result from research on ischemic preconditioning of heart. Advances made in the field of ischemic preconditioning have paved the way for insight into several other forms of preconditioning. One such advance was identification of the involvement of G-protein
coupled receptors in preconditioning. Activation of $G_\alpha_i$ protein-coupled receptors is one of the earliest signals in the induction of ischemic preconditioning-mediated protection.

Adenosine is an established mediator of ischemic preconditioning in the heart and in the brain, and the adenosine $A_1$ receptor is expressed in several tissues including brain (Heurteaux et al., 1995). In heart, adenosine (Liu et al., 1991), bradykinin (Goto et al., 1995), and opioids (Bell et al., 2000) signal through $G_\alpha_i$ protein-coupled receptors and are capable of stimulating preconditioning-mediated protection. The 7-transmembrane domain receptors are coupled to heterotrimeric $G$ proteins having $G_\alpha$ and $G_\beta_\gamma$ subunits. Upon ligand binding, the $G_\alpha$ subunit exchanges GDP for GTP and dissociates. Both $G_\alpha$ and $G_\beta_\gamma$ subunits are biologically active, and cessation of signaling occurs by the inherent GTPase activity of $G_\alpha$, which upon hydrolysis of GTP, re-assembles with $G_\beta_\gamma$. $G_\alpha_i$ signaling inhibits adenyl cyclase and may both activate $K^+$ channels and inhibit $Ca^{2+}$ channels. $G_\beta_\gamma$ is coupled to PI3 kinase (PI3K) (Naga Prasad et al., 2000), which is believed to be a critical mediator of protection in several forms of preconditioning.

2.8 Kinase signaling in preconditioning

PI3K phosphorylates membrane phospholipids, leading to recruitment and activation of phosphatidylinositol-specific phospholipase C (PI-PLC) and hydrolysis of membrane phosphatidylinositol 4,5-bisphosphate. This results in release of inositol trisphosphate (IP3) and diacylglycerol (DAG) that in turn activate isoforms of protein kinase C (PKC) (Nishizuka, 1992). Activation of at least one PKC isoform is thought to be a ubiquitous transducer of preconditioning stimuli, and the phosphoinositide products,
DAG and IP3, are common activators of PKC isoforms. In hippocampal slice cultures, εPKC has been shown to be required for both NMDA- and ischemia-mediated preconditioning (Raval et al., 2003). εPKC and δPKC, members of the novel class of PKC isozymes, have been identified as controlling survival/apoptosis signaling in preconditioning. These two enzymes have been shown to have opposing effects, with εPKC favoring protection (Speechly-Dick et al., 1994) and δPKC initiating apoptosis (Bright et al., 2004).

PI3K can also mediate protection by way of protein kinase B (PKB), also known as AKT (Chan et al., 1999). PKB may be a potent modulator of protection, since it is capable of phosphorylating several mediators of preconditioning. In heart, acetylcholine, which induces protection through G protein-coupled muscarinic receptors, has been shown to transactivate the epidermal growth factor (EGF) receptor, leading to activation of PKB (Krieg et al., 2002). This activation was blocked by both the Src inhibitor 4-Amino-5-(4-chlorophenyl)-7-(t-butyl)pyrzolo[3,4-d]pyrimidine (PP2) and the EGF receptor inhibitor N-(3-Chlorophenyl)-6,7-dimethoxy-4-quinazolinamine (AG-1478). The transactivation of EGF by G protein-coupled receptors may be mediated through Gβγ, since sequestration of the subunit with C-terminus of β-adrenergic receptor kinase (βARKct, an inhibitor of the Gβγ subunit of G protein-coupled receptors) inhibited transactivation (Pierce et al., 2001). PKB phosphorylates and activates endothelial nitric oxide synthase (eNOS) and 70-kDa ribosomal protein S6 kinase (p70S6K), both of which are implicated in preconditioning. Also, PKB phospho-inhibits GSK3β and BAD, two
proteins that have apoptotic functions. GSK3β is intimately involved with MPT pore formation, and its inhibition has been hypothesized to be the “master switch” upon which preconditioning signals culminate (Juhaszova et al., 2004). Proapoptotic BAD, which also favors MPT formation, is sequestered by 14-3-3 protein upon phosphorylation (Scheid and Woodgett, 2001).

In the heart, extracellular signal-regulated kinase (ERK) is another postulated transducer of preconditioning-mediated protection (Fryer et al., 2001). It has been demonstrated that ischemic preconditioning results in PKC-dependent phosphorylation of ERK (Baines et al., 2002). Also, delayed preconditioning mediated by activation of mitochondrial $K_{\text{ATP}}$ is abrogated by inhibition of ERK with MEK1/2 inhibitor, PD 98059. Indeed, ERK activation may be due to mito$K_{\text{ATP}}$ dependent release of ROS (Gross et al., 2003). A role of ERK activation in receptor-mediated endosomal trafficking and survival signaling has also been proposed, since blocking endosomal trafficking, inhibition of PI3K and sequestration of G$\beta\gamma$ all inhibit ERK phosphorylation and IPC-mediated protection (Tong et al., 2003).

### 2.9 PKC and redox signaling in preconditioning

The translocation and activation of PKC isozymes is very sensitive to redox signaling, since antioxidants inhibit PKC-mediated responses (Gopalakrishna and Jaken, 2000). Structural data support the direct activation of PKC by redox signaling. The N-terminal domain of PKC contains a cysteine-rich zinc finger motif susceptible to
oxidation by H$_2$O$_2$. When oxidized, the enzyme loses its auto-inhibitory effect and becomes active (Gopalakrishna and Anderson, 1989). Conversely, the C-terminal domain contains cysteines that are targets of antioxidants (Gopalakrishna and Anderson, 1991). Modifications of this type decrease PKC activity. It is therefore possible that through its redox properties, PKC couples mitochondrial ROS generation to gene transcription in the preconditioning pathway.

2.10 ROS in preconditioning

Based on emerging evidence, particularly concerning the role of mitochondria, ROS might function as cellular second messengers in preconditioning signaling. This is supported by the observation that antioxidants such as N-acetylcysteine (a glutathione precursor) (Das and Maulik, 2003) and dimethyl thiourea (a hydroxyl radical scavenger) (Das et al., 1999) abolish the delayed protective effect of ischemic preconditioning in heart. PI3K (Qin and Chock, 2003) and MAPKs (Pain et al., 2000) have established roles in preconditioning and both are activated by ROS. Certain redox-sensitive transcription factors have been shown to be critical in ischemic preconditioning. If nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB) activity is inhibited with SN 50 peptide (an inhibitor of NFkB nuclear translocation), ischemic preconditioning protection of heart is abolished (Das et al., 1999). Furthermore, NFkB is translocated into the nucleus during ischemic preconditioning, and this action is blocked by antioxidants (Das et al., 1999). This activity may be mediated by redox-sensitive tyrosine kinases.
(Gopalakrishna and Anderson, 1989). NFκB is responsible for control of several apoptosis-related genes, including anti-apoptotic bcl-2 and pro-apoptotic genes bax and p53 (Bromme and Holtz, 1996). Thus, redox signaling in this way is capable of regulating apoptosis.

2.11 Mitochondria and preconditioning

The mitochondria represent a central mediator of apoptosis, but they are now being recognized as intermediaries of cytoprotection as well. Although the precise roles of mitochondria in preconditioning-mediated protection are topics of debate, it is generally accepted that preconditioning-initiated protection involves both the mitochondrial ATP-dependent potassium (mitoK\textsubscript{ATP}) channel and inhibition of mitochondrial permeability transition (MPT) pore formation. Many of the known cellular mediators of protection have also been shown to activate the mitoK\textsubscript{ATP} channel, including nitric oxide (NO) (Sasaki et al., 2000), PKC (Sato et al., 1998) and ROS (Zhang et al., 2001). Activation of the mitoK\textsubscript{ATP} channel leads to depolarization of the mitochondrial inner-membrane potential (\(Ψ_m\)), which attenuates mitochondrial Ca\textsuperscript{2+} accumulation during ischemia/reperfusion (Liu et al., 1998). This effect has been correlated with a decrease in MPT pore formation (Murata et al., 2001).

In another study using isolated mitochondria, both diazoxide (mitoK\textsubscript{ATP} channel opener) and myristic acid (PKC activator) were protective from anoxic injury (Korge et
al., 2002), and these effects were abolished by 5-hydroxydecanoic acid (5-HD), an inhibitor of the mitoK\textsubscript{ATP} channel. Protection in this study correlated with depolarization of Ψ\textsubscript{m} and decrease in mitochondrial Ca\textsuperscript{2+} accumulation. Furthermore, there was inhibition of MPT formation upon reoxygenation, indicating a potential correlation between mitoK\textsubscript{ATP} channel activation and inhibition of mitochondrial-driven apoptosis. Activation of the mitoK\textsubscript{ATP} channel and subsequent depolarization of Ψ\textsubscript{m} have also been demonstrated to result in generation of superoxide anion in a preconditioning model of angiotensin II-mediated protection (Kimura et al., 2005). This was demonstrated elsewhere, with the addition of diazoxide (Pain et al. 2000). In this last study, protection afforded by diazoxide was blocked by inhibition of mitochondrial ROS with antioxidants. These findings are compelling when considering the ability of ROS to affect diverse mediators of preconditioning-mediated protection.

2.12 CREB and preconditioning

CREB, a transcription factor affecting multiple genes in response to intracellular calcium, has been shown to mediate both the development of hypoxia/ischemia-induced brain damage, and for mechanisms of brain repair (Walton et al., 1996). In addition, CREB-dependent gene expression is involved in learning and memory, neuronal differentiation and survival (Carlezon et al., 2005). Considering the critical role of CREB in neuronal homeostasis, it is not surprising that it is a central intermediary of brain preconditioning. For example, CREB is required for ischemic preconditioning-induced
ischemic tolerance in gerbil hippocampal CA1 neurons (Hara et al., 2003). Phospho-activated CREB is also increased for days following transient ischemia in rat brain dentate granule cells (Hu et al., 1999).

What is more, CREB signaling has been shown to upregulate anti-apoptotic bcl-2 following oxygen-glucose deprivation preconditioning in vitro and ischemic preconditioning in rats (Meller et al., 2005). Neuroprotection in this study was blocked by CREB oligonucleotide decoy, which also blocked Bcl-2 expression. Also, inhibitors of protein kinase A, CaMK and mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK) inhibited preconditioning protection. In another study, increases in intracellular calcium through NMDA receptors or L-type calcium channels caused enhanced CREB phosphorylation, an effect mediated by mitochondrial calcium-dependent superoxide signaling (Hongpaisan et al., 2003); however, another study found NADPH-oxidase and not mitochondria to be responsible for NMDAR-dependent ROS in hippocampus (Brennan et al., 2009).

2.13 Synaptic vs. extra-synaptic NMDAR and CREB

Interestingly, it is not merely the activity but importantly the localization of the NMDAR that appears important for mediating its neuroprotective vs. excitotoxic effects. This is because of an emerging literature on the opposing roles of synaptic vs. extra-synaptic NMDARs in neuronal survival and death pathways (Vanhoutte and Bading, 2003). Hardingham and coworkers demonstrated that calcium influx through NMDARs
caused CREB activation and expression of neuroprotective BDNF, similar to stimulation of L-type calcium channels. However, activation of extrasynaptic NMDARs triggered by bath application of NMDA or ischemia caused a dominant CREB inhibition that blocked BDNF expression (Hardingham et al., 2002). Furthermore, it has been demonstrated that preconditioning doses of NMDA favor activation of synaptic NMDARs by enhancing action potential firing and that this is required for both early and late phases of preconditioning-neuroprotection (Soriano et al., 2006). This mechanism also involved CREB-dependent activation of BDNF. In addition, pharmacologically enhancing neuronal activity by a combination of the potassium channel blocker 4-aminopyridine (4-AP), and the GABA-A antagonist, bicuculline, for 1-2 days resulted in potent and prolonged (up to 3 days) elevations in tolerance to simulated ischemia (Tauskela et al., 2008). This effect correlated with activation of ERK1/2 and CREB and was blocked by NMDAR and L-type voltage gated calcium channel antagonists, further confirming the role of synaptic neurotransmission in the preconditioning-dependent neuroprotection.

2.14 The NMDA receptor

The NMDAR is a subclass of excitatory ionotropic glutamate receptors that is selectively activated by the synthetic amino acid N-methyl-D-aspartate. These receptors are unique among neurotransmitter receptors, since they require the binding of a co-agonist glycine (or D-serine) along with L-glutamate for activity, have a high degree of voltage dependence, have high permeability to calcium, and display slow
activation/deactivation kinetics. Voltage dependence is achieved by a magnesium blockade of the channel pore at resting membrane potentials that is released upon depolarization, often by simultaneous stimulation of the AMPA subclass of ionotropic glutamate receptor. The functional NMDAR consists of a heteromeric assembly (most likely to be a tetramer, but not yet unequivocally proven) of NR1, NR2 and NR3. Seven genes encode for the NMDAR subunits and include NR1, NR2A-D and NR3A-B. Each subunit is composed of an extracellular regulatory domain and agonist binding domain, four transmembrane spanning domains (M1-4, the M2 domain is actually a membrane re-entrant domain similar to the selectivity filter of potassium channels) that form the channel pore, and an intracellular C-terminal domain. The NR1 subunit contains the binding site for the co-agonist glycine, has a short C-terminal domain, and is obligatory for receptor function. NR1 undergoes extensive splicing resulting in 8 different splice variants NR1-1a,1b to NR1-4a,4b. Of these, NR1-2a,b and NR1-4a,b are missing an ER retention signal encoded in the C1 cassette (Standley et al., 2000) and thus exit independent of NR2 assembly. The NR2 subunit contains the glutamate binding domain and a long C-terminal domain that dictates many channel properties. For example, inhibition of NR2B but not NR2A subunit activity sharply reduces LTP and spontaneous activity-driven potentiation in hippocampal slice cultures; this is due to the affinity of the subunits for CamKII (Barria and Malinow, 2005). Furthermore, both NR1 and NR2 are phosphorylated by different serine/threonine kinases and tyrosine kinases. NR1 is phosphorylated exclusively on serine residues by the activity of PKA and PKC. PKC
phosphorylation of serine 890 of NR1 disrupts NMDAR clustering (Tingley et al., 1997), but phosphorylation of serine 890 together with PKA phosphorylation of serine 897 is required for increased NMDAR surface expression (Scott et al., 2001) in a mechanism involving disruption of an ER retention signal. NR2C is also serine phosphorylated by PKA, PKB/Akt, and PKC. NR2A&B are serine and tyrosine phosphorylated by multiple serine/threonine and tyrosine kinases. NR2 phosphorylation is more complex, since it affects not only localization but also channel properties. For example, PKC phosphorylation of NR2A on serine1291 and 1312 enhance channel conductance (Grant et al., 2001). However, phosphorylation of NR2B at tyrosine 1472 within its C-terminal YKEL sequence, the adaptor protein 2 (AP-2)-interacting motif, disrupts interaction with AP-2 which inhibits clathrin-mediated endocytosis and preserves its membrane localization (Lavezzari et al., 2003).

Additionally, phosphorylation of serine 1480 within the C-terminal PDZ ligand (IESDV) by casein kinase II disrupts interaction of NR2B with the PDZ domains of post synaptic density protein PDS-95 and synapse-associated protein SAP-102, causing dissociation from the post synaptic density (Chung et al., 2004). Thus receptor subunit composition, post-translational modification and interaction with various modulatory and signaling machinery (discussed in more detail later) create a great deal of NMDAR functional diversity within the CNS and are hypothesized to account for the diverse roles of the receptor in brain development, learning, memory and disease.
2.15 Ethanol and the NMDA receptor

The NMDAR is a well recognized target of ethanol within the CNS. The acute effects of ethanol on NMDAR neurotransmission are inhibitory (Chu et al., 1995) and are believed to mediate (at least in part) the sedative effects of intoxication. With continued ethanol exposure adaptive responses are believed to occur in the brain to compensate for the inhibitory effects of ethanol on NMDAR signaling. This compensatory upregulation of NMDAR is thought to underlie withdrawal symptoms associated with alcoholism, such as tremor and seizures as well as accounting for plasticity changes that occur during development of tolerance. For example, in vivo exposure to a 14-day chronic intermittent ethanol (CIE) protocol via ethanol vapor chamber caused a marked increase in field excitatory postsynaptic potentials (fESPS) of pharmacologically isolated NMDAR-currents in the CA1 region of acutely isolated hippocampal slices at 7 days post-withdrawal (Nelson et al., 2005). These electrophysiological changes correlated with increased expression of NR2A and NR2B. Furthermore, CIE (75mM for 14 h followed by 10 h withdrawal, repeated 5 times), upregulated NR1 and NR2B in cultured cortical neurons at days 2 and 5 post-withdrawal (Qiang et al., 2007). The increase was correlated with increased surface expression but not endocytosis. The increase in surface expression was confirmed by immunocytochemistry and shown to be mainly synaptic, correlating with increases in PSD-95 immunoreactive clusters, and was PKA-dependent. Also, chronic ethanol (50mM, 4 days, EtOH vapor container) caused significant increases in the size and density of synapsin-associated clusters (synaptic marker) containing NR1
and NR2B and enhanced synaptic NMDAR mEPSCs in hippocampal neurons via a PKA-dependent mechanism (Carpenter-Hyland et al., 2004). Finally, ethanol causes increases in NR2B phosphorylation in dorsal—but not ventral—striatal slice cultures in a mechanism requiring Fyn kinase activity (Wang et al., 2007). This study also corroborated Fyn-dependent phosphorylation of NR2B with ethanol-induced long term facilitation of NMDAR currents in dorsal but not ventral striatum. In contrast to chronic effects of ethanol, acute ethanol exposure has been shown to have the opposite effect on phosphorylation of NR2 subunits. Alvestad et al. reported that a 10 min application of ethanol (100mM) decreased phospho-NR2B concomitant with decreases in NMDAR field excitatory postsynaptic potential slope and amplitude (Alvestad et al., 2003). These changes were reduced by co-application of a phospho-tyrosine phosphatase inhibitor.

2.16 Post synaptic density protein 95kD (PSD-95)

Fidelity in postsynaptic signal transduction requires spatially and temporally coordinated organization of signaling machinery. At excitatory synapses this is a highly dynamic process that allows for changes in synaptic strength such as LTP and LTD. PSD-95 is a member of the membrane associated guanylate kinase (MAGUK) family of PDZ domain containing proteins. MAGUK family members share a common structural arrangement containing three PDZ (PSD95, Drosophila disc large tumor suppressor and Zonula occludens-1 protein) domains, an SH3 domain and a kinase-dead guanylate kinase (GK) domain. PSD-95 and other MAGUK proteins are found enriched at
postsynaptic densities where they function as scaffolds for transmembrane proteins and anchors to the cytoskeleton. Many NR2 subunits of the NMDAR contain a seven amino acid carboxy terminal tSXV (serine, any amino acid, valine) motif that binds to the second PDZ domain of PSD-95. This interaction facilitates the postsynaptic clustering of NMDAR (Kornau et al., 1995). The SH3 domain of PSD-95 is known to associate with proline rich regions of non-receptor tyrosine kinases. Furthermore, because PSD-95 expression increases the number, activity and size of glutamatergic spines, it is a suspected modulator of synaptic strength and plasticity (El-Husseini et al., 2000). Support for this hypothesis was demonstrated by Delint-Ramirez and colleagues, who found that PSD-95 and NMDARs were recruited to synaptic lipid rafts in the hippocampus and insular cortex 24h following spatial memory training (Morris water maze), and this caused tyrosine phosphorylation of NR2B-Y1472 (Delint-Ramirez et al., 2008). Furthermore, NR2B Tyr-1472 phosphorylation is enhanced after induction of LTP in the hippocampus (Nakazawa et al., 2006) and fear learning in the amygdala. This study suggests that Src-mediated phosphorylation of Tyr-1472 is involved in synaptic plasticity.

PSD-95 interacts with several ion channels, including AMPA, NMDA and potassium channels, and facilitates their clustering and interaction with signaling machinery at excitatory synapses. Therefore, PSD-95 is believed to be an integral part of synaptic development and plasticity. However, mechanisms that govern its ability to coordinate ion channel assembly and clustering are only partially understood. PSD-95 has
two distinct post-translational modifications that govern its clustering, and both appear to
depend on synaptic activity—these are phosphorylation and palmitoylation. Early work
showed that PSD-95 partitions as an integral membrane protein in brain homogenates and
is thioester-linked to palmitic acid, a 16-carbon fatty acid (Topinka and Bredt, 1998).
Multimerization of PSD-95 occurs through a unique N-terminal 13 amino acid motif
which contains two conserved cysteine residues that are the substrates for palmitoylation
(Christopherson et al., 2003).

Palmitoylation of PSD-95 is both necessary and sufficient to cause channel
clustering. Palmitoylation is a reversible reaction catalyzed by protein palmitoyl
acyltransferases (PATs), which are members of the DHHC (Asp-His-His-Cys) protein
family. Recently, Noritake and coworkers demonstrated the activity-dependent
palmitoylation of PSD-95 and determined that DHHC2, a dendritically localized PAT,
but not DHHC3, a Golgi-resident, mediates the reaction. Specifically, inhibition of
synaptic activity causes rapid translocation of DHHC2 to postsynaptic densities where it
acts on PSD-95 to facilitate palmitoylation and subsequent clustering and association
with ion channels (Noritake et al., 2009).

In addition to palmitoylation, PSD-95 can be further modified by phosphorylation
at serine 295. Modification of this type is also activity-dependent and causes
accumulation of PSD-95 at the postsynaptic density and recruitment of AMPA receptors.
Phosphorylation of PSD-95 is mediated by the Rac1-JNK pathway and is enhanced in
response to chronic silencing and inhibited by increases in synaptic activity. Furthermore,
chemically-induced LTD by NMDA treatment causes a rapid decrease in ser-295 phosphorylation, and a phosphomimetic mutant, S295D of PSD-95, inhibited NMDA-induced AMPA receptor internalization and blunted LTD induction (Kim et al., 2007). Thus, the activity-dependent modulation of PSD-95 is dynamic, as is the nature of the synapse itself.

2.17 Pyk2

NMDA receptor activity correlates with protein tyrosine phosphorylation (Rostas et al., 1996). The mechanisms responsible for NMDAR tyrosine phosphorylation and subsequent increase in activity are only partially understood. Two likely non-receptor tyrosine kinases involved in this process are Src-family and Pyk2. Pyk2 is a member of the focal adhesion kinase (FAK) family of protein tyrosine kinases, sharing a high degree of homology with FAK. Pyk2 has 48% identity and 65% similarity to FAK, and like FAK it lacks a transmembrane region, myristoylation sites, and SH2 and SH3 domains (Avraham et al., 1995). Also, the focal adhesion targeting (FAT) domain of Pyk2 is repressed in most instances (Schaller and Sasaki, 1997). However a striking difference between the two is that Pyk2 can be activated by multiple stimuli that increase intracellular calcium by both PKC-dependent and PKC-independent pathways (Soltoff et al., 1998). Pyk2 contains FREM (band 4.1/ezrin/radixin/moesin homology), kinase, proline-rich and focal adhesion targeting (FAT) domains. Furthermore, Pyk2 is
autophosphorylated at Tyr-402 during activation. Pyk2 is necessary and sufficient for the induction of CA1 LTP, and tetanic stimuli cause activation of Pyk2 (Huang et al., 2001).

Although Pyk2 is well known to be activated by calcium, the calcium-dependent mechanism of Pyk2 activation in postsynaptic densities has remained elusive. Very recently, some light has been shed on this matter. Bartos and colleagues report that Pyk2 is activated via a unique mechanism involving calcium/calmodulin and PSD-95 (Bartos et al., 2010). They provide strong evidence that Pyk2 is trans-autophosphorylated in response to calcium entry through NMDAR and that this causes postsynaptic clustering of Pyk2 and physical interaction with PSD-95. Furthermore, this oligomerization is caused by Pyk2 binding to PSD-95, allowing for the close proximity required for trans-autophosphorylation. The interaction is achieved through the interaction of the SH3 domain of PSD-95 interacting with the proline-rich domain of Pyk2.

Bartos et al. (2010) further hypothesize that under basal conditions, the PSD-95 SH3 domain is bound to the guanulate kinase domain obscuring interaction with Pyk2. However, calcium entry through NMDARs causes activation of calmodulin and binding of Ca/Calmodulin to the HOOK domain (disorganized linker region between the GK and SH3 domain) of PSD-95. This causes displacement of the SH3/GK interaction, allowing for binding and clustering of Pyk2 and subsequent trans- autophosphorylation (two Pyk2 monomers phospho-activate each other in trans). Thus, they provide a plausible mechanism accounting for the calcium-induced activation of Pyk2 and provide insight on the role of Pyk2 in the induction of LTP in hippocampal CA1 neurons. Furthermore, they
show that PKC is not required for Pyk2 activation, since inhibitors of PKC fail to block activation. However, activators of PKC do lead to activation of Pyk2 (Siciliano et al., 1996).

Pyk2 is also involved in the recruitment of Src to the NMDAR complex. This is critical for many of Pyk2’s functions, including induction of LTP, coupling to ERK and JNK MAPK pathways, and cerebellar granule cell survival. Specifically, upon activation by phosphorylation of Pyk2-Y402, a modification that alleviates Pyk2 autoinhibition, Src then binds to Y402 via interaction of its SH2 domain and causes further phosphorylation of Pyk2 at Y579 and Y580 of the activation loop, augmenting Pyk2 catalytic activity (Avraham et al., 2000). Subsequently, the Pyk2-activated Src phosphorylates NR2 subunits in the NMDAR complex (Wang and Salter, 1994). Phosphorylation of NMDAR by Src upregulates NMDAR function and thus gates NMDAR-dependent synaptic potentiation (Salter and Kalia, 2004). Pyk2 acting via Src is necessary and sufficient for CA1 LTP. Tetanic stimulation increases the interaction of Pyk2 and Src (Huang et al., 2001) and this pathway is critical for NMDAR-dependent activation of the ERK MAP kinase pathway (Lev et al., 1995). Pyk2 is also critical for the survival of cerebellar granule neurons in response to repolarization-induced apoptosis. Dominant-negative Pyk2 caused 60% reduction in total neuronal number at 24h post-transfection in granule cells cultured in depolarizing conditions (Strappazzon et al., 2007).

As mentioned above, Pyk2 has several unique characteristics that mediate its ability to both instigate cell survival via activation of ERK (Lev, et al., 1995) and
transduce neuronal stress/death pathways through its interaction with JNK pathways (Tokiwa et al., 1996). Its ability to protect neurons is not limited to its role in the NMDAR-ERK pathway. This is because it has also been shown to mediate the neuroprotective effect of the transient receptor potential canonical (TRPC) channel. Interestingly, Yao and colleagues demonstrated that platelet-derived growth factor (PDGF)-mediated neuroprotection was mediated via the TRPC in a Pyk2/ERK/CREB-dependent manner (Yao et al., 2009). Specifically, blocking TRPC5 or TRPC6 with an inhibitor or siRNA induced suppression of PDGF-mediated neuroprotection in primary neurons. Furthermore, PDGF was able to protect dopaminergic neurons in the substantia nigra from HIV Tat-induced neurotoxicity. TRPC3 and 6 activation protect cerebellar granule neurons from serum deprivation-induced neurotoxicity and promote their survival in rat cerebellum (Jia et al., 2007). In vitro inhibition of TRPC3 or 6 suppressed BDNF-dependent neuroprotection and CREB activation. Additionally, overexpression of these channels enhanced neuronal survival in vivo and in vitro in a CREB-dependent manor.

Pyk2’s capacity to become rapidly activated in response to stressful stimuli, and to interact with both ERK and JNK pathways, suggest it may have a role as an intermediary in preconditioning-dependent signal transduction cascades. Nevertheless, it has been little studied in this regard. One study found an increased phosphorylation of NR2A and NR2B after transient (5 min) non-lethal ischemia, concomitant with an increased association of PSD-95, NR2B, Pyk2, FAK and Src that peaked at 3h
reperfusion; with the exception of Pyk2 (which remained elevated in the NMDAR complex), they returned to basal levels 72h later. However, these changes were not observed with lethal 30 min ischemia (Zalewska et al., 2005). Although the authors of this report were not directly investigating ischemic preconditioning, but rather the differences between lethal and non-lethal ischemia, their model of non-lethal 5 min ischemia is consistent with a preconditioning stimulus.

2.18 Src family non-receptor tyrosine kinases

Src was first described by Bishop and Varmus in vertebrate tissues as a protooncogene based on homology to avian Rous carcinoma virus (Spector et al., 1978). Src is the largest family of non-receptor tyrosine kinases with nine members. Of these, Src, Fyn, Lyn, Lck and Yes are expressed in the CNS. Src-family kinases are comprised of a number of conserved domains, some of which appear frequently in heterologous signal-related proteins, including G protein-coupled receptors, receptor tyrosine kinases, cytokine receptors and cell-adhesion receptors. Within the Src family, the catalytic Src homology domain (SH1), the phosphotyrosine binding SH2 domain, the proline rich-binding SH3 and amino-terminal domains share high homology. SH2 and SH3 domains are examples of common protein-protein interaction motifs found in a variety of proteins (Pawson and Scott, 1997). These two domains also bind intramolecularly and are responsible for autoinhibition (Xu et al., 1997). Tyrosine 527 of Src is phosphorylated and interacts intramolecularly with the SH2 domain (Liu et al., 1993), causing inhibition
of kinase activity (Cooper et al., 1986). Inhibitory intramolecular interaction further occurs between the SH3 domain and the linker region connecting SH2 and catalytic domains. These interactions are interrupted by binding to sequences in other proteins causing activation of Src. Tyrosine 416 in the catalytic domain is autophosphorylated (Smart et al., 1981). The SH3 domain binding sequence is a 10 amino acid, proline-rich motif containing the consensus sequence pXXp (where X is any amino acid) (Ren et al., 1993). The N-terminal SH4 domain contains a consensus sequence for lipid modifications, such as myristoylation and/or palmitoylation, critical for interaction with cell membranes (Resh, 1993). Indeed, Src acts as a central mediator and convergence point for a number of critical cellular signaling pathways.

Src-family kinases have long been known to modulate the activity of ion channels. Src was first shown to modulate NMDAR activity in the CNS (Wang and Salter, 1994) and then voltage-gated potassium channels (Fadool et al., 1997), L-type calcium channels (Cataldi et al., 1996), GABAA receptors (Moss et al., 1995) and nicotinic acetylcholine receptors (Wang et al., 2004a). Exogenous Src or a peptide activator of Src enhances NMDAR peak currents (Cheung and Gurd, 2001). Src coimmunoprecipitates with multiple PSD components (Husi et al., 2000). Src activation is necessary and sufficient for inducing LTP and functions by up-regulating NMDAR (Lu et al., 1998). Src phosphorylates multiple components of the NMDAR complex, including NR2A (Kohr and Seeburg, 1996) and NR2B (Cheung and Gurd, 2001). Thus Src-dependent modulation of NMDAR activity is believed to be responsible for many of
the physiological processes of the receptor, including LTP (Soderling and Derkach, 2000), synaptic transmission, and plasticity (Ali and Salter, 2001).

2.19 Peroxisiredoxins in neuroprotection and AD

Peroxiredoxins (prx) are a ubiquitous family of antioxidant proteins that reduce and detoxify H$_2$O$_2$, peroxynitrite, and organic hydroperoxides. They are divided into three subclasses: typical 2-Cys (2-Cys refers to the catalytic site containing 2 cysteine residues, prx1-4), atypical 2-Cys (prx5), and 1-Cys (prx6). All three classes undergo the same initial oxidation reaction whereby an oxidant attacks the peroxidatic (undergoes initial oxidation, see Fig. 1) cysteine, forming S-hydroxycysteine. The second step for the 2-Cys peroxiredoxins involves a resolving cysteine (reduces S-hydroxycysteine to form a disulfide bond, see Fig.1) that is attacked (oxidized) by the sulfenic acid of the peroxidatic cysteine to form a disulfide bond. Typical and atypical 2-Cys peroxiredoxins differ in the location of the resolving cysteine residues. The catalytic cysteine residues for the typical 2-Cys peroxiredoxins are located on two juxtaposed subunits, which form the catalytic site, and when oxidized, result in an intersubunit disulfide bond. This causes multimerization and is thought to be involved, in part, in the role of prxs in mediating ROS signal transduction (Rhee et al., 2005). Conversely, the catalytic residues of the atypical 2-Cys peroxiredoxins residues lie within the same subunit and the oxidation step therefore results in an intrasubunit disulfide bond. Finally, oxidation of the peroxidatic
cysteine of 1-Cys prx6 is not resolved by a neighboring cysteine and must be reduced by some other reducing equivalent (such as glutathione).
Reduction of hydrogen peroxide (or other ROS) by the peroxiredoxins (prxs) all occurs through the same initial reaction. An oxidant attacks the peroxidatic cysteine, forming S-hydroxycysteine (a sulfenic acid). The second step for the 2-Cys prxs involves a resolving cysteine, which is attacked (oxidized) by the sulfenic acid intermediate, producing a disulfide bond. The 2-Cys prxs contain two family members, typical and atypical. They differ by the location of their resolving cysteine residues. For the typical, the resolving cysteine is located on a juxtaposed subunit, and oxidation therefore results in intersubunit disulfide bond formation. The atypical has its resolving cysteine on the same subunit, and thus oxidation results in formation of an intrasubunit disulfide bond. The 1-Cys prx has no resolving cysteine and thus reduction of its S-hydroxycysteine must be by some other reducing equivalent such as glutathione.
Reduction and thus reactivation of the 2-Cys peroxiredoxins occurs mainly through the thiol-reducing thioredoxin/thioredoxin reductase system, which utilizes reducing equivalents from NADPH and is coupled to the pentose phosphate pathway (Wood et al., 2003). However, the disulfide bond formation is slow and thus the sulfenic acid intermediate can be further oxidized to sulfinic and sulfonic acids. This process was thought to be irreversible, but more recently it was shown that it is reversible by the ATP-dependent activity of sulfiredoxin for the typical 2-Cys prxs (Woo et al., 2005).

Peroxiredoxins are prevalent antioxidant enzymes in the CNS and also show distinct subcellular, cellular and anatomical distribution profiles. Prx1, prx2, and prx6 (1-Cys) appear mainly in the cytosol, whereas prx3 is primarily mitochondrial and prx4 is secreted (Wood et al., 2003). Prx5 (atypical 2-Cys) shows a more widespread localization, including cytosol, mitochondria, and peroxisomes (Seo et al., 2000). Furthermore, whereas neurons show moderate staining for prx2-5, oligodendrocytes show robust staining for prx1 and 4 and moderate reactivity of prx6 (Jin et al., 2005). Also, in this study, prx1 was shown to be primarily microglial, whereas prx6 was found in astrocytes. These results contrast with an earlier study that showed prx1 was expressed primarily in astrocytes, while prx2 was expressed exclusively in neurons in cerebral cortex, cerebellum, basal ganglia, substantia nigra, and spinal cord (Sarafian et al., 1999). Nevertheless, the distinct subcellular, cellular and anatomical distribution of these antioxidant enzymes within the CNS implies differential and important roles for CNS function.
Since first being characterized (Chae et al., 1994), this thiol-specific antioxidant family has rapidly gained notoriety for its role in cytoprotection and disease. Prx isoforms have been shown to have aberrant expression in certain diseases such as AD, Parkinson’s disease, Down’s syndrome (Krapfenbauer et al., 2003), Creutzfeldt-Jacob disease (Krapfenbauer et al., 2002), and ALS (Kato et al., 2005). Furthermore, prx2 is upregulated in AD brain and in 2 transgenic mouse models of AD, and transfection with prx2 protects cultured cortical neurons from toxic Aβ (Yao et al., 2007). These authors propose that prx2 is a marker for early AD and provide evidence that the interaction between amyloid-binding alcohol dehydrogenase and Aβ mediates up-regulation of prx2. They propose that blocking this interaction could be a therapeutic target; however, this is not likely the sole pathway for AD-associated ROS, and it remains to be determined if blocking this association will have beneficial outcomes in vivo.

An additional study showed overoxidation of prx2 in AD brain (Cumming et al., 2007). Furthermore, they show that a neuronal-like cell line (PC12) that has been developed for resistance to Aβ has increased expression of prx1, 2 & 6. Prx2 is phosphorylated on threonine 89 in a calpain/p35/Cdk5-dependent mechanism in response to MPP+ and/or MPTP (mitochondrial stress) and this causes inhibition of its peroxidase activity (Qu et al., 2007). Furthermore, a phospho-mimetic mutant was more sensitive to, and a phospho-null mutant protected against, mitochondrial stress in vitro and in vivo. Additionally, prx2 has been shown to mediate, at least in part, the neuroprotective effects of pituitary adenylate cyclase activating polypeptide in cultured cerebellar granule
neurons (Botia et al., 2008). They further show that prx2 expression is induced by both cyclic AMP and PKC stimulators, and also show upregulation of prx2 with exposure to 325 mM EtOH *in vitro* and 2.5 g/kg *in vivo* that peaks at 24h of exposure; it is of note that the *in vitro* concentration is an extremely high level of EtOH. They further speculate on prx2’s therapeutic potential for the treatment of some neurodegenerative diseases. Finally, as mentioned above, thioredoxin (trx) is critical in the maintenance of prxs in the reduced state and thus their ability to function as antioxidant enzymes. In this regard, it has been demonstrated that trx overexpressing mice show resistance to focal ischemia, a known ROS-dependent neurodegenerative insult (Takagi et al., 1999).
CHAPTER THREE

MATERIALS AND GENERAL METHODS

3.1 Chemicals and antibodies

Hoechst 33342, propidium iodide (PI), DL-2-amino-5-phosphonopentanoic acid AP-5, 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX), pertussis toxin, memantine, NMDA, deoxyribonuclease I (DNAse), Tween 20, Triton-X 100, protease and phosphatase inhibitor cocktails, and penicillin/streptomycin were from Sigma Co. (St. Louis MO). Neurobasal-A media, B27, trypsin and fetal bovine serum (FBS) were from Gibco (Carlsbad, CA). American Peptide (Sunnyvale, CA) provided Aβ25-35 and Aβ35-25. Polyacrylamide gels were obtained from GenScript (Piscataway, NJ). Western blot buffers and enhanced chemiluminescence detection (ECL) solutions were from Pierce Chemical Co. (Rockford, IL). The antibodies obtained from Upstate Co. (Temecula, CA) were anti-NR1-CT (1:1000) and anti-NR2B (1:1000); from Chemicon International (Temecula, CA), anti-NR2C (1:1000), PSD-95 (1:1000), NR2B-pY1472 (1:1000), neuronal specific nuclear antigen (NeuN, 1:1000) and glial acidic fibrillary protein (GFAP, 1:1000); and from Cell Signaling (Danvers, MA), actin (1:1000) and horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (1:1000-1:20,000).
3.2 Cerebellar mixed primary cultures

The culture protocol is modified from (Brewer et al., 1993). 7-10 day old Sprague-Dawley rat pups are cryoanesthetized on ice for 2-3 min and rapidly decapitated. The brain is carefully removed and placed into ice-cold Gays balanced salt solution (GBSS) supplemented with 4.5 mg/ml glucose. The cerebellum is then carefully removed and placed into ice-cold minimal essential media (MEM) supplemented with 6.5 mg/ml glucose. Meninges are removed and the cerebella are sectioned manually into approximately 6 pieces. Pieces are then transferred into calcium- and magnesium-free Hanks balanced salt solution (CM-HBSS) containing 0.25% trypsin and 0.5 mg/ml DNAse at 37°C. Tissue is incubated for 10 min, and pieces are transferred into MEM with 10% fetal bovine serum (FBS) and 5% horse serum (HS) to inactivate trypsin. Cells were dispersed by triturating with two fire polished pipettes that have decreasing size. Next, cells are passed through a 70µm cell strainer. Viable cell density is determined by trypan blue exclusion on a hemocytometer. Cells are diluted to a concentration of 800,000 cells/ml in serum-free Neurobasal-A media supplemented with 2% B27 (Gibco), 500µM L-glutamine and 1% penicillin-streptomycin. Cells are then plated on poly-L lysine-coated 12 well plates at 1 ml/well. All of the media is changed after one hour. Half of the media is changed every three days until DIV10, at which point 100% of the media is changed. This technique allows glia to proliferate to near confluence and produces neurons with robust processes and intricate neural networks (see Fig. 2B).
3.3 Ethanol pretreatment and Aβ exposure

Culture media is changed to media containing 30mM ethanol (1.8µl EtOH/1ml media) or media alone. Culture plates containing ethanol are placed in airtight containers that have small open dishes of 90mM ethanol to minimize evaporative ethanol loss from experimental wells. Ethanol levels will be monitored via Sigma NAD-ADH reagent multiple test reagent (product number N 7160). Control plates are treated similarly, but with water alone. The container is allowed to equilibrate with incubator atmosphere for 15 min and then sealed. Culture media and solution dishes are changed at day 3. At day 6, 100% of ethanol media or control media is removed and media with or without 25µM Aβ (25-35) is added. For drug treatments, a parallel set of plates is prepared, differing only by the presence of added drug.

3.4 Lactate dehydrogenase assay

Media were collected from cultures and spun at 5000g for 5 min. The manufacturer’s microplate protocol was used to determine toxicity. Lactate dehydrogenase (LDH) activity correlated linearly ($R^2 = 0.9992$; see Fig. 2) with the number of live cerebellar cells lysed over the activity ranges in these experiments. Neurotoxicity is reported as LDH activity/100µL culture media.
Figure 2: Positive control for LDH assay

Cerebellar cells were trypsinized and manually dissociated via triturating with a fire polished pipette, counted with a hemocytometer and diluted to the indicated cell concentration. Cell suspensions were then lysed by addition of 1% Triton-X100 and assayed for LDH activity. Regression data are represented over LDH activity ranges observed in experiments. Four cell concentrations are shown covering ~300-300,000 cells/ml, and n=3 for each concentration.
LDH activity of lysed (1% Tx-100) cerebellar cells

\[ y = 1E-06x + 0.0041 \]

\[ R^2 = 0.9992 \]
3.5 Analyses of cytotoxicity by Hoechst/PI staining

Cerebellar cell cultures were incubated 15 min at 37°C with 5 µM Hoechst 33342, which labeled nuclei of all cells, and 5 µg/ml propidium iodide (PI), which stained dead/dying neurons. The extent of cytotoxicity defined as percent of dying neurons was obtained by dividing the number of PI-positive neurons by the total number of Hoechst-stained nuclei and multiplying by 100. Approximately 500 nuclei were counted per well and six wells per experimental group. Images were subsequently analyzed with NIH ImageJ 1.34s (NIH, Bethesda, MD, USA). Note: NMDA preconditioning is not neurotoxic as determined by this method (Fig. 3).
Figure 3: NMDA preconditioning is not neurotoxic in cerebellar mixed cultures

Cerebellar cultures 13DIV were treated with the indicated concentrations of NMDA for 24h and assayed for viability via dual fluorescence staining with Hoechst 33342 (staining all nuclei) and PI (staining dead/dying cells). Data shown as means +/- sem, n = 6.
3.6 Western blot analyses

Protein (5-20µg) from cellular extracts was loaded and run on 4-12% polyacrylamide gels. After separation, protein was transferred to polyvinylidene fluoride (PVDF) membrane. Membranes were blocked with 1 or 3% BSA in TBS with 0.1% Tween 20 for 60 min. Primary antibody was diluted based on manufacturer’s recommendation in 1 or 3% BSA TBS with 0.1% Tween 20. Membranes were incubated at rt for 60 min or overnight @ 3°C. HRP-conjugated secondary antibody was added in TBS with 0.1% Tween 20 for 2h. Membranes were then incubated with developing buffer and exposed to X-ray film. Following development, films were scanned and analyzed with NIH ImageJ 1.34s. Unless otherwise stated, actin was used to normalize loading.

3.7 Immunocytochemistry for identification of cell types

Following ethanol or control treatment, cultures were washed 2 times with pre-warmed phosphate-buffered saline (PBS), fixed for 15 min at 37°C with 4% paraformaldehyde, and stored in PBS containing 0.05% NaN₃ for later viewing. Monolayers were permeabilized with PBS containing 0.3% Triton X-100 and blocked with 5% serum of secondary antibody host for 3 h at 27°C. Primary antibodies were incubated overnight at 4°C in blocking buffer, washed, and incubated with corresponding biotinylated secondary antibody. Streptavidin-conjugated cyanine dye (Cy2 or Cy3,
1:200) was then applied for 2 h at 27°C, washed, and stored in PBS containing 0.05% NaN₃. Primary delete experiments showed specificity of cell-type antibodies. Cultures were digitized on an inverted epiluminescent fluorescent microscope equipped with a Nikon digital camera (Nikon Inc., Melville, NY, USA) for image acquisition. Images were subsequently analyzed with NIH ImageJ 1.34s.

3.8 Deconvolution microscopy

Cerebellar cultures were grown on 18mm acid-washed glass coverslips coated with poly-L lysine. At 10DIV, cultures were treated with 30mM ethanol (MEP) or control (C) media for 6 days and then fixed with a cocktail of 100mM PIPES buffer pH 6.8 with 4% formaldehyde. Monolayers were then permeabilized/blocked in PBS with 0.1% Triton-x and 10% normal goat serum for 3 h. Following permeabilization/blocking, the primary antibodies were diluted to the appropriate concentrations in the same perm/block cocktail, with the exception of addition of 5µM Hoechst33342 (used to visualize nuclei), and incubated overnight in the dark at room temperature. Primary antibody dilutions were as follows: chicken α-βIII tubulin, 1:500; mouse α-PSD-95, 1:200; and rabbit α-pan NR2, 1:500. Next, coverslips were rinsed 3 times with PBS containing 0.1% Triton-X100 and stained with the appropriate secondary antibodies (Cy2 conjugated α-rabbit, Cy3 α-chicken, and Cy5 α-mouse; all goat at 1:400) for 1 h and washed times.
After the final rinse, coverslips were mounted with fluoro-gel antifaden (to minimize photo bleaching) and dried for immediate imaging. Image stacks were collected blindly on a Delta Vision deconvolution microscope (60X objective). Deconvolved image stacks were then rendered in 3D and quantified with Imaris 6.3.1. An algorithm was constructed using Imaris by first selecting the PSD-95 channel (that was not visible at the time of data collection, since Cy5 emission spectrum is invisible to the naked eye) and inclusion criteria were set for PSD-95 immunoreactive surfaces. This same algorithm was then used to analyze all subsequent image stacks and retrospectively analyzed for statistical significance using either Systat 11.00.01 or GraphPad Prism 5. At least 20 neurons were counted per group.
CHAPTER FOUR

RESULTS

4.1 Reductions in dose-related Aβ neurotoxicity by moderate ethanol preconditioning (MEP) in mixed cerebellar cultures

4.1.1 Introduction

Our laboratories have shown that MEP protects against Aβ-induced neurotoxicity in hippocampal-entorhinocortical slice cultures (Belmadani et al. 2004). In order to explore in more detail the neuromolecular mechanisms responsible for this apparent neuroprotection, I employed cerebellar mixed cultures as a model system. This in vitro system is advantageous for several reasons when compared to the HEC slice model. One, it allows for relatively high throughput screening of pharmaceuticals that help to identify signaling mechanisms (e.g., cell surface receptors) underlying MEP-neuroprotection, and may mirror other preconditioning paradigms. Second, it allows high resolution immunocytochemical analysis of neurons and glia within the cultures to aid in identifying cellular and molecular differences between groups that may account for the observed neuroprotection. Finally, these cultures respond with robust and highly reproducible MEP-dependent neuroprotection from amyloid neurotoxicity.
4.1.2 Results

Figure 4A, left column, shows phase contrast micrographs of control (top) and MEP-treated (bottom) cerebellar cultures. MEP treatment does not seem to affect the way these cultures appear. Right column, images show neuronal (NeuN+, top) and glial (GFAP+, bottom) cell types present in these cultures. Arrows indicate neuronal cells based on morphology that stain positive for NeuN but negative for GFAP. These results show both neurons and astrocytes are present in these cultures at the time of Aβ exposure. Figure 4B is a schematic representation of our MEP protocol. Mixed cerebellar cultures are grown for 10 days and exposed to 30mM ethanol or control media for an additional 6 days. Following MEP or control treatment, mixed cerebellar cultures contain ~15-20% NeuN-positive neuronal cell types and a mixture of non-neuronal, primarily GFAP-positive cell types (Fig. 4C). MEP treatment affects neither the neuronal viability (Fig. 4C) nor the total cell number of these cultures (not shown). At 16DIV, MEP untreated cultures displayed dose-related Aβ25-35 neurotoxicity (Fig. 4D, 25 µM for 24 hr). The toxicity due to Aβ25-35 is significantly counteracted in ethanol-preconditioned cultures (Fig. 4E) as determined by released LDH. As expected, the reverse peptide Aβ35-25 (25 µM) was not neurotoxic (results not shown). Interestingly, our laboratory frequently observes a neuroprotective effect of MEP even from toxicity associated with general media change in the MEP-treated vs. control groups. A similar effect has been observed in HEC slice cultures (Thomas and Morrisett, 2000).
Figure 4: Cerebellar mixed cultures, dose-related Aβ neurotoxicity and neuroprotection by moderate ethanol preconditioning (MEP)

A. Left column: phase contrast micrographs of control (top) and MEP-treated (bottom) cerebellar cultures (16DIV). Note: upon visual inspection there appears to be little difference between control and MEP-treated cultures. Right column: images show neuronal (NeuN+, top) and glial (GFAP+, bottom) cell types present in these cultures. Arrows indicate neuronal cells based on morphology, that stain positive for NeuN but negative for GFAP. B. MEP protocol: cerebellar cultures (10DIV) were treated with 30mM EtOH or control media for 6 d, rinsed and exposed 24 hr to 25µM Aβ25-35 or vehicle. C. MEP did not alter neuronal composition of cerebellar cultures. Cerebellar cultures were treated as in B and the percent of total (Hoechst 33342-positive) cells that are positive for NeuN were quantified in MEP and control treated cultures (n=3 at least 100 cells counted per well). D. Dose-related neurotoxicity of cerebellar cultures (16DIV) to Aβ25-35 as measured by LDH release; *p<0.05 vs. no Aβ25-35 (n=6). E. Aβ neurotoxicity in cerebellar cultures was prevented by MEP. *p<0.05 vs. control; 1-way ANOVA with Scheffe post hoc test (n = 6).
4.1.3 Discussion

The phase contrast micrographs depicted in Figure 4A indicate that MEP appears by visual inspection not to be neurotoxic. This possibility was confirmed by immunocytochemical analyses (Fig. 4C) showing that MEP does not alter the neuronal composition of the cultures nor the total number of cells (not shown). This is an important control, since the reduction in LDH release in MEP-treated vs. cultures could be due to a decrease in the number of neurons present at the time of amyloid exposure. Next, MEP naive cultures respond with dose-related toxicity in response to amyloid challenge (Fig. 4D) and are robustly resistant to this toxicity following MEP treatment (Fig. 4E), indicating that these cerebellar cultures are a satisfactory model system to study the neuromolecular mechanisms that induce neuroprotection from Aβ by MEP. Furthermore, although the cerebellum is relatively resistant to amyloid peptides in vivo (Kim et al., 2003), cerebellar granule neurons have been shown to be vulnerable to Aβ in vitro (Allen et al., 1999). Aβ 25-35, employed as the amyloid insult throughout these experiments, appears to act via a mechanism identical to the most neurotoxic form in vivo, Aβ 1-42 (Frozza et al., 2008).

LDH activity is commonly used as a measurement of Aβ-induced neurotoxicity both in purified (Koh and Choi, 1987; Khan et al., 2007) and mixed neuronal cultures (Hartley et al., 1999). Furthermore, LDH release correlates with mitochondrial-driven neuronal death (Deshpande et al., 2006). Finally, although both MTT and LDH assays are effective measurements of mitochondrial-driven apoptosis, LDH is the more accurate
assay, as it is independent of mitochondrial function and is therefore a better endpoint measurement of neuronal apoptosis (Lobner, 2000).

4.2 NMDAR-dependency of moderate ethanol preconditioning-induced neuroprotection

4.2.1 Introduction

Common among preconditioning paradigms is the activation of at least one class of cell surface receptor. Soon after its discovery, ischemic preconditioning (IP) of brain was found to be dependent on the activity of the NMDAR (Kato et al., 1992). Since then several models of preconditioning have demonstrated the role of NMDAR in mediating neuroprotection (Jiang et al., 2005). In a rat model of ischemic preconditioning (IP), inhibition of NMDAR signaling blocked ERK5 activation, an effect that reversed hippocampal CA1 neuroprotection (Wang et al., 2006). That same study showed inhibition of ERK5 with antisense oligonucleotide also prevented neuroprotection, demonstrating dependence on NMDAR-mediated ERK5 activation for IP.

Activation of Gαi, protein-coupled receptors is one of the earliest signals in the induction of IP-mediated protection in heart. Adenosine is an established mediator of IP in the heart and in the brain (Heurteaux et al., 1995). In heart, adenosine (Liu et al., 1991), bradykinin (Goto et al., 1995), and opioids (Bell et al., 2000) signal through Gαi, protein-coupled receptors and are capable of stimulating preconditioning-mediated
protection. Therefore I investigated the role of these receptors in MEP’s neuroprotective mechanism.

4.2.2 Results

To ascertain if adenosine/Gαi/o protein-coupled receptors and/or NMDAR, both cell surface receptors implicated in other forms of brain preconditioning neuroprotection, are indeed involved in MEP neuroprotection, this experiment examined whether antagonism of these receptors reduced or prevented Aβ neurotoxicity. Figure 5 shows that, when present in culture media for the first 3 days of preconditioning, neither the specific adenosine A1 receptor antagonist, DPCPX (Fig. 5A), nor the Gαi/o protein-coupled receptor inhibitor, pertussis toxin (Ptx, Fig. 5B), significantly counteracted neuroprotection from Aβ after 6 days of MEP. Pertussis toxin inhibits all Gαi/o protein-coupled receptors, several of whom have been implicated in multiple preconditioning paradigms in brain and heart (see Introduction). It is important to note that, although somewhat toxic to these cultures based on the reduced total LDH values observed, pertussis toxin had no effect on MEP’s neuroprotective mechanism. However, AP-5, a highly specific NMDAR antagonist, completely abolished the neuroprotective effects of MEP (Fig. 5C). Furthermore, memantine, a moderate affinity NMDAR open channel blocker, antagonized most of the MEP neuroprotection (Fig. 5D). These results are consistent with the relatively early “sensor” role of the NMDAR in MEP’s neuroprotective mechanism and further support the importance of this receptor in multiple forms of brain preconditioning.
Figure 5: MEP-neuroprotection abolished by NMDAR antagonists, unaffected by antagonists of adenosine A1 receptors or $G_{i/o}$ protein-coupled receptors

MEP of cerebellar cultures (as in Fig. 4) was performed with the respective receptor antagonists present for the first three days of the 6d MEP protocol, followed by 25 μM Aβ 25-35 or vehicle for 24 h. After the 24 h exposure to Aβ, media LDH activity was assayed. A. DPCPX (100 nM), adenosine A1 receptor antagonist, did not prevent MEP neuroprotection. B. Pertussis toxin (Ptx, 50 ng/ml), $G_{i/o}$ protein-coupled receptor antagonist, also did not alter MEP neuroprotection. C. AP-5 (50 μM), NMDAR antagonist, completely abolished MEP neuroprotection. D. Memantine (50 μM), NMDAR antagonist, significantly inhibited MEP neuroprotection. *p<0.001 vs. control; #p<0.001 vs. MEP; 1-way ANOVA with Scheffe post hoc test (n = 6).
4.2.3 Discussion

The results of these studies implicate NMDAR activation—but rule against adenosine A1 or other $G_{\alpha_{i/o}}$ protein-coupled receptors—in attaining the neuroprotected phenotype due to MEP. Whereas inhibition of the adenosine A1 receptor with the selective antagonist DPCPX or of $G_{\alpha_{i/o}}$ protein-coupled receptors with pertussis toxin failed to block MEP neuroprotection, inhibition of NMDARs with either the selective antagonist AP5 or open channel blocker memantine effectively blocked it. These results are seemingly counterintuitive, since ethanol even at low concentrations is a well-known inhibitor of the NMDAR (Chu et al., 1995). Therefore, I chose to confirm the ability of NMDAR stimulation to induce neuroprotection from Aβ via NMDA preconditioning and to study in more detail the mechanism in which MEP is acting through the NMDAR to engender the neuroprotective phenotype.

4.3 Neuroprotection from Aβ toxicity by NMDA Preconditioning as determined by dual fluorescence staining

4.3.1 Introduction

Data in Figure 5 identify the role of NMDARs early in the mechanism of MEP-dependent neuroprotection. This is consistent with other forms of preconditioning. Furthermore, exposure of murine cortical cell cultures to preconditioning doses of glutamate or NMDA has been shown to be neuroprotective (Grabb and Choi, 1999).
Similarly, exposure of cerebellar granule cell cultures to sub-excitotoxic concentrations of NMDA has been shown to cause neuroprotection attributable to release of BDNF acting on TrkB (Marini et al., 1998). Both NMDA and BDNF exposure result in activation of the transcription factor NF-κB. Further, inhibition of NF-κB with an oligonucleotide decoy reversed neuroprotection in this model (Wu et al., 2005). Neuroprotection in this model correlates with expression of anti-apoptotic bcl-2. NMDAR activation is also associated with MAPK activity. Moreover, it has been demonstrated that preconditioning doses of NMDA favor activation of synaptic NMDARs by enhancing action potential firing and this is required for both early and late phases of preconditioning-neuroprotection (Soriano et al., 2006). This mechanism also involved CREB-dependent activation of BDNF. In addition, pharmacologically enhancing neuronal activity by a combination of the potassium channel blocker 4-aminopyridine and the GABAA antagonist bicuculline for 1-2 days resulted in potent and prolonged (up to 3 days) elevations in tolerance to simulated ischemia (Tauskela et al., 2008). This effect correlated with activation of ERK1/2 and CREB and was blocked by NMDAR and L-type voltage gated calcium channel antagonists, further confirming the role of synaptic neurotransmission in the preconditioning-dependent neuroprotection.

4.3.2 Results

If NMDAR activation is an essential early event in MEP neuroprotection, I hypothesized that direct stimulation of this receptor via NMDA preconditioning should elicit similar protection from Aβ insult in our cultures. As shown in Figure 6A, cultures at
DIV13 were preconditioned with 50µM NMDA for 24h (determined to be subneurotoxic, see Fig. 3) and then exposed for 24h to 12.5µM Aβ 25-35 or media only. The DIV13 time point represents a culture age corresponding to halfway through the MEP exposure and was chosen based on this and our previous work indicating that both subclasses (NR1 and NR2) of NMDAR subunits were expressed at that time. In Figure 6B are representative live-cell photomicrographs of dual fluorescence staining with Hoechst 33342 and propidium iodide (PI), a combination shown to correlate well in cerebellar cultures with other cytotoxic measures (Bachis et al. 2003). Hoechst 33342 labels the nuclei of all cells and PI stains dying/dead neurons in the control (C) and NMDA-preconditioned cultures. PI-stained cell densities between control and NMDA-preconditioned groups did not differ, verifying that NMDA treatment was not neurotoxic. However, cultures treated with Aβ demonstrated increased density of PI-labeled degenerating neurons compared to C or NMDA, whereas the NMDA-preconditioned cultures treated with Aβ (NMDA+Aβ) displayed robust neuroprotection from Aβ toxicity. Figure 6C is a quantification of these results. Thus NMDAR activation by NMDA ligand binding or, by extension, ethanol preconditioning-augmented NMDAR activity, promotes a neuroprotected state in these cerebellar cell cultures, rendering them resistant to Aβ insult.
Figure 6: Neuroprotection from Aβ toxicity by NMDA preconditioning as determined by dual fluorescence staining with Hoescht 33342 and PI

A. Cerebellar cultures (13DIV) were treated for 24h with NMDA (50µM) or control media followed by 24h ± 25µM Aβ 25-35. B. Representative images of neurotoxicity assayed by live cell dual fluorescence staining. C. Quantification of Aβ 25-35 neurotoxicity (% Hoescht stained nuclei that are PI- positive) in NMDA-preconditioned or control cultures. *p<0.001 vs. control; 1-way ANOVA with Scheffe post hoc test (n = 6).
A

13DIV

Measure Toxicity

Control

24h control media

+/- 24h Aβ

NMDA

24h 50 μM NMDA

+/- 24h Aβ

B

C

NMDA Preconditioning

% Neurotoxicity +/- SEM

Control  Aβ  NMDA  NMDA + Aβ

*
4.3.3 Discussion

The fact that preconditioning with subneurotoxic NMDA ligand itself affords neuroprotection from Aβ in our cerebellar cultures lends additional support to the possibility that NMDAR activation mediates moderate ethanol-initiated prosurvival signaling, and is consistent with NMDA preconditioning/neuroprotection results using other primary cultures (Soriano et al. 2006; Smith et al. 2008). NMDAR excitability appears critical as well in preconditioning with glutamate or prolonged elevations in electrical activity, since NMDAR antagonists block the resultant neuroprotection against subsequent ischemia (Lin et al. 2008; Tauskela et al. 2008). However, despite these findings, it should be acknowledged that NMDA’s protective mechanism and ethanol’s mechanism of neuroprotection may differ in other ways.

4.4 Elevation of NMDAR subunits by ethanol preconditioning and abrogation of potentiation of obligatory NR1 subunit by memantine

4.4.1 Introduction

The NMDAR is a well recognized target of ethanol within the CNS. The acute effects of ethanol on NMDAR neurotransmission are inhibitory (Chu et al., 1995). However, chronic ethanol has been shown to increase NMDAR expression and activity. Exposure in vivo to chronic intermittent ethanol (CIE) increases fESPS of pharmacologically isolated NMDAR in the CA1 region of acutely isolated hippocampal
slices (Nelson et al., 2005). These electrophysiological changes correlated with increased expression of NR2A and NR2B. Furthermore, CIE upregulates NR1 and NR2B in cultured cortical neurons (Qiang et al. 2007). The increase was correlated with NMDAR surface expression but not endocytosis. Surface expression was confirmed by immunocytochemistry and was shown to be mainly synaptic, correlating with increases in PSD-95 immunoreactive clusters. Also, chronic ethanol causes significant increases in the size and density of synapsin-associated clusters containing NR1 and NR2B, and enhanced synaptic NMDAR mEPSCs in hippocampal neurons (Carpenter-Hyland et al., 2004). Finally, a recent in vivo study demonstrated improved memory in rats chronically fed moderate, but not high, ethanol-containing diets (Dewachter et al., 2009). This effect was hippocampal NR1-dependent, since hippocampal infusion of siRNA directed against NR1 abolished ethanol’s facilitatory effects on cognition, and hippocampal NR1 over-expression mimicked the effects.

4.4.2 Results

Data in Figure 5 indicate NMDAR involvement early in MEP’s neuroprotective mechanism. Therefore, I examined whether changes in the levels of NMDAR subunits could underlie increases in receptor activity. Data in Figure 7 demonstrate that NR1, the obligatory subunit for NMDAR function, and NR2B and NR2C, which contain glutamate binding domain(s), were each significantly elevated by day 2 of ethanol exposure and remained elevated after 6 days of preconditioning (Fig. 7A and Fig. 7B). The elevation of NMDAR subunits at day 4 in ethanol preconditioning is not shown for simplicity. It was
also apparent that co-treatment with memantine antagonist, which counteracts MEP neuroprotection from Aβ and restores the peptide’s neurotoxic effects (Fig. 5D), completely prevented the 2 day elevations in the obligatory NR1 subunit (Fig. 7A and Fig. 7B).
Figure 7: Significant increases in NMDAR subunits by day 2-6 of ethanol preconditioning and prevention of obligatory NR1 subunit increases with memantine

Cerebellar cultures (10DIV) were treated with 30mM ethanol or control media for 2d or 6d; protein extracts were subjected to immunoblot analysis. A. Representative immunoblots of NMDAR subunits NR1, NR1 with 45µM memantine, NR2B and NR2C after ethanol (E) or control (C) media treatment. B. Quantification of NR immunoblot analyses normalized to actin and expressed as % control. *p<0.05 vs. control by student t-test (n = 3-6).
4.4.3 Discussion

Ethanol is known to acutely inhibit and chronically potentiate NMDAR currents, although exact mechanisms remain a matter of intense investigation (Nagy 2008). I find that persistent increases in subunits NR1, NR2B and NR2C occur by the first 2 days of moderate ethanol exposure. Such increases in receptor subunits could be a principal reason for increased NMDAR activity leading to neuroprotection. This possibility was supported by the fact that memantine blocked the ethanol-dependent increases in NR1, the receptor’s obligatory subunit—thus lessening NMDAR activity—concurrent with abolishing MEP neuroprotection. Of obvious relevance to these in vitro findings are in vivo studies demonstrating improved NR1-dependent memory performance in rats chronically fed moderate ethanol-containing diets (Kalev-Zylinska and During 2007). RNA interference knockdown of hippocampal NR1 abolished ethanol’s facilitatory effects on cognition, while NR1 overexpression mimicked ethanol’s effects. Thus, although NR2 subunits are known ethanol targets (Woodward et al. 2006), changes in NR1 subunits often mirror NMDAR function.

4.5 Increased synaptically-localized NMDAR indicated by NR2B phosphotyrosine-1472 and PSD-95 after 2 days of ethanol preconditioning

4.5.1 Introduction

Synaptic NMDAR activity has been correlated with activation of pro-survival signaling within neuronal populations. Phosphorylation of NR2B at tyrosine 1472 within
its C-terminal AP-2-interacting motif preserves its membrane localization (Lavezzari et al., 2003). Additionally, PSD-95 expression increases the number, activity and size of glutamatergic spines and is a suspected modulator of synaptic strength and plasticity (El-Husseini et al., 2000). Support for this was demonstrated by Delint-Ramirez and colleagues. This group found that PSD-95 and NMDAR were recruited to synaptic lipid rafts in the hippocampus and insular cortex 24h following spatial memory training (Morris water maze) and this caused tyrosine phosphorylation of NR2B-Y1472 (Delint-Ramirez et al., 2008). Furthermore, NR2B Tyr-1472 phosphorylation is enhanced after induction of LTP in the hippocampus and fear learning in the amygdala (Nakazawa et al., 2006). This study suggests that Src-mediated phosphorylation of Tyr-1472 is involved in synaptic plasticity. Therefore, I chose to investigate if there were changes in these markers of synaptic NMDAR complex early in the ethanol preconditioning protocol.

4.5.2 Results

Questioning whether MEP-induced NMDAR activity was primarily synaptic, and considering recent reports on the critical importance of synaptic vs. extrasynaptic localization on NMDAR pro-survival signaling, I examined ethanol’s effects on levels of tyrosine 1472-phosphorylated NR2B (NR2B-pY1472) and PSD-95 relatively early in the 6 day preconditioning protocol. The activity and synaptic localization of NMDAR are believed to require phosphorylation of subunits, especially tyrosine 1472 of NR2B subunit, by Src family tyrosine kinases (Yu et al. 1997; Goebel-Goody et al. 2009). Also, PSD-95 is a cytoskeletal protein involved in the dynamic regulation of synaptic NMDAR
complex formation that links the receptors to downstream signaling moieties (Delint-Ramirez et al. 2008; Kurisaki et al. 2008). As shown in Figure 8A and Figure 8B, after 2 days of moderate ethanol exposure, the levels of both NR2B-pY1472 (2B-pY1472) and PSD-95 were significantly elevated. Furthermore, although ethanol preconditioning potentiated NR2B protein levels at this time (Fig. 7 and Fig. 8A), tyrosine kinase activity acting on NR2B—most likely of the src family—was evidently increased as well, as demonstrated by the significantly increased ratio of NR2B-pY1472/total NR2B (pY/2B in Fig. 8B) in ethanol-treated cultures. The results point to NMDAR subunit upregulation in association with enhanced synaptic NMDAR localization as relatively early events of the ethanol preconditioning mechanism.
Figure 8: Increased synaptically-localized NMDAR Complex indicated by increases in NR2B phosphotyrosine-1472 PSD-95 and NR2B-pY1472/Total NR2B by Day 2 of MEP

Cerebellar cultures (10DIV) were treated with 30mM ethanol (E) or control (C) media for 48h, protein extracted and subjected to immunoblot analyses. A. Representative immunoblots of post-synaptic scaffolding protein PSD-95, NMDAR subunit NR2B phosphorylated tyrosine 1472 (2B-pY1472) and total NR2B. B. Immunoblot quantification of PSD-95, NR2B-pY1472 (normalized to actin) and the ratio of 2B-pY1472 to total NR2B, expressed as % control. *p<0.05 vs. control by student t-test (n = 3-6).
4.5.3 Discussion

An exciting finding of NMDAR dynamics related to our ethanol preconditioning model is our evidence for early MEP-dependent receptor increases that reflect synaptic as opposed to extrasynaptic NMDAR. Specifically, levels of NR2B phospho-tyrosine 1472, which are associated with decreased endocytosis and synaptic enrichment of NMDAR (Yu et al. 1997; Goebel-Goody et al. 2009), and levels of PSD-95, which implicate increased synaptic receptor localization (Delint-Ramirez et al. 2008; Kurisaki et al. 2008), are increased. These findings are in harmony with data showing that increases in synaptic NMDAR signaling are associated with downstream pro-survival events (Soriano et al. 2006; Soriano and Hardingham 2007). Furthermore, the significantly augmented ratio of NR2B-pY1772/total NR2B at an early MEP timepoint of 2 days indicates the possibility of increased activity of Src-family kinase(s) (Goebel-Goody et al. 2009). Src has only recently been demonstrated to be involved in ischemic and NMDA preconditioning of neurons (Head et al., 2008), although it has been reported in heart preconditioning. Therefore I wanted to examine if Src activation could explain the increases in NMDAR2B-Y1472 phosphorylation. Also, Pyk2 was investigated, since it is known to facilitate NMDAR activity.
4.6 Phosphoactivation of Src family and Pyk2 non-receptor tyrosine kinases by MEP

4.6.1 Introduction

MEP appears to be mediating an early recruitment of NMDAR to the PSD as described in Figure 8. Two non-receptor tyrosine kinases associated with the PSD are Pyk2 and Src. Both play an intimate role in the physiological role of excitatory neurotransmission (Lev et al., 1995). Pyk2 associates with and is trans-autophosphorylated/activated directly by PSD-95 and has also been shown to be required for LTP (Bartos et al., 2010). Furthermore, Src (Ali and Salter, 2001) (Lu et al., 1998) and possibly Fyn (Nakazawa et al., 2001) have been shown to phosphorylate the NR2B subunit directly, preventing NMDAR endocytosis and enhancing synaptic localization. I explored the possibility that MEP was activating these synaptic signaling moieties, which may be associated with the early phosphorylation of NR2B phospho-tyrosine 1472 and upregulation of PSD-95.

4.6.2 Results

Figure 9A shows that at 48 h of MEP, there are significant elevations in the phospho-activated forms of pSrc and pPyk2 synaptic non-receptor tyrosine kinases. Figure 9B shows the quantification of these observations. The results provide further biochemical evidence that MEP increases the synaptic localization and the activity of signaling moieties associated with synaptic NMDA receptor complex. The novelty of
these observations may lie in their ability to transduce signals to multiple downstream targets and cross-talk with survival signals of the MAPK family.
Figure 9: Phosphoactivation of Src-family and Pyk2 non-receptor tyrosine kinases by 48 h of MEP, correlating with phosphorylation of NR2B tyrosine 1472

A. Cerebellar cultures (10DIV) were treated with 30mM ethanol (E) or control (C) media. Protein was extracted and subjected to immunoblot analyses for anti-p-Src (Tyr416) and p-Pyk2 (Tyr402) at 48 h. Blots were then stripped and probed with corresponding non-phosphorylated antibodies. B. Quantification of A. Data represents %phospho/total. *p<0.05 (n= 3-4). NOTE: anti-Fyn was used as loading control for p-Src due to recognition of multiple p-Src family members.
A 2 Day MEP

C  E

cpy2c

Fyn

Pyk2

B

pSrc

Control  Ethanol

pSrc/Total Src (%)  *

pPyk2

Control  Ethanol

pPyk2/Total Pyk2 (%)  *
4.6.3 Discussion

The phospho-activated form Src was elevated by 48 h of MEP a timepoint consistent with the observed increase in NR2B-pY1472. There seems to be some debate in the literature as to the exact Src-family member that is responsible for the NR2B-Y1472 phosphorylation. However, the anti phospho Src-family antibody (recognizes Src-family phospho-activation domain) employed in this study identified 3 bands that were elevated when compared to control, all of which are consistent with the hypothetical mol. wt. of at least Src and Fyn; and levels of total Fyn (used as loading control) did not differ. This implies that MEP is acting via Src-family kinases to increase NR2B-Y1472 phosphorylation that is strongly correlated with synaptic receptor localization. Furthermore, this indicates that a degree of synaptic plasticity is occurring in these preconditioned cultures.

Elevations in the phospho-activated form of Pyk2 further support data implicating increased synaptic NMDAR activity early in MEP’s neuroprotective mechanism. Pyk2, like Src, has been implicated in synaptic plasticity. This non-receptor tyrosine kinase is responsive to increases in intracellular calcium and interacts with MAPK pathways. Interestingly, a recent report sheds light on this mechanism, one that may well be involved in MEP. Pyk2 binds to PSD-95 in a calcium/calmodulin-dependent way (Bartos et al., 2010) providing the close proximity required for its trans-autophosphorylation of tyrosine 402. Phosphorylation of this site activates Pyk2 and provides a docking site for Src family kinases (binding via their SH2 domain to Pyk-pY402), which further phosphorylates and augments Pyk2 kinase activity. Src as previously mentioned is well
known to phospho-activate NMDAR and maintain them at the synapse. Thus it seems plausible that MEP enhances the synaptic NMDAR complex and may, speculatively, induce formation of a “postsynaptic signalosome” that acts as a “sensor” complex in MEP-dependent neuroprotection.

**4.7 Increased NMDAR2B and PSD-95 colocalization by MEP**

**4.7.1 Introduction**

Biochemical data are highly indicative of a mechanism by which MEP increases synaptic NMDAR and subsequently the receptor’s ability to engender a neuroprotective phenotype. However, it should follow that there is a quantitative increase in synaptic localization of the NMDAR complex. In order to approach this issue, deconvolution microscopy was employed. This imaging technique in conjunction with Imaris, a program that assembles 3D images from deconvolved image z-planes, allows for the highly accurate quantification of fluorescent images in 3 dimensions. A Delta vision deconvolution microscope in the laboratory of Dr. Edward Campbell, Department of Microbiology and Immunology, LUMC, was utilized for all image acquisition. This microscope is equipped with a multi-filter (narrow band pass, excitation emission) wheel and allows for simultaneous allocation of images from multiple fluorescent channels. Thus, in order to ascertain whether there was an enhancement of NMDAR synaptic content in MEP vs. control cultures, I employed antibodies for βIII tubulin, as a neuronal marker, PSD-95, as a component of the postsynaptic density, and NR2 subunits
(panNR2), for visualization of the NMDAR complex. These antibodies were selected based on their optimal affinity to epitope for fluorescence microscopy.

4.7.2 Results

The experimental approach was to utilize this technique to identify post-synaptic densities via PSD-95 immunoreactivity and then quantify the amount of NMDAR complex (defined by NR2 intensities) within these postsynaptic densities. The logic behind this approach is that if there is an increase in the NMDAR immunofluorescence within the postsynaptic densities in MEP compared to control, than indeed MEP is increasing the synaptic content of the NMDAR complex. Control (Fig. 10, top panel) and MEP-treated neurons (Fig. 10, bottom panel) were identified by βIII tubulin immunoreactivity (column 1, Fig. 10). PSD-95 (column 2, Fig. 10) was blind at the time of image acquisition (the emission spectrum for Cy5, the fluorophore linked to the PSD-95 secondary antibody, is invisible to the naked eye). An antibody that recognizes all NR2 subunits was employed for NMDAR visualization (Fig. 10, column 3). Column 4 of Figure 10 is a merged image of PSD-95 and NR2. Inlays under each representative image are an enlarged view of a neurite from the above channel. Shown in Figure 10, it appears that upon visual inspection that there is an enhancement of the NR2/PSD-95 immunofluorescence; amount of yellow in comparing neurite to neurite.

However, the power of this technique is the highly accurate quantification of images. Figure 11 depicts the quantification of these images. Image stacks were collected for both groups (blindly and in the same sitting) and deconvolved for later quantification.
An algorithm was constructed using Imaris by first selecting the PSD-95 channel (not visible at the time of data collection) and inclusion criteria set for PSD-95 immunoreactive surfaces that were clearly visible to the naked eye. This same algorithm was then used to analyze all subsequent image stacks and retrospectively analyzed for statistical significance using either Systat or Graphpad prism. Figure 11A is a plot of the mean intensities of panNR2 vs. PSD-95 within the areas defined by PSD-95. Red dots in Figure 11A represent the control surfaces (defined by PSD-95 immunoreactivity and plotted as mean intensities of panNR2 vs. PSD-95, see Fig. 11D for clarity) and the blue diamonds represent the MEP-treated surfaces. This plot reveals a general tendency for increased NR2 intensities within the PSDs for the MEP treated vs. control cultures. Figure 11B is a graphical representation of the data in Figure 11A for the NR2 intensities within the PSD. Figure 11C is the mean PSD fluorescence for control and MEP within the PSDs. Figure 11D is a pictorial view of the surfaces identified by PSD-95 (red) and NR2 (green) within these surfaces. It is evident from theses data that MEP increases the synaptic NMDAR complex and increases the PSD-95 intensities after 6 days of MEP.
Figure 10: Increased colocalization of PSD-95 and NR2 subunits by MEP

Representative images of cerebellar cultures (10DIV) treated with control (upper panel) or 30mM ethanol (MEP, lower panel) media for 6 days. Cultures were fixed and stained with the appropriate antibodies (chicken α-βIII Tubulin 1:500, mouse α-PSD-95 1:200 and rabbit α-pan NR2 1:500), rinsed 3X and stained with the appropriate 2’ antibody (Cy2 conjugated α-rabbit, Cy3 α-chicken and Cy5 α-mouse; all goat & 1:400). Images were collected on a Delta Vision deconvolution microscope (60X objective) and a representative z-plain is shown. Inset below each channel is an enlarged view of a neurite from the image above. Image stacks were cropped and channel colors chosen using the MacBiophotonics ImageJ bundle. Intensities for individual channels are constant between groups. Scale bar = 5µm.
Figure 11: Quantification of PSD-95 and NR2 intensities following MEP

Cerebellar cultures (10DIV) were treated with control or 30mM ethanol (MEP) media for 6 days, fixed and stained with the appropriate antibodies. Image stacks were collected with a Delta Vision deconvolution microscope (60X objective) and analyzed with Imaris 6.3.1. 3D surfaces were defined by PSD-95 punctum and analyzed for PSD-95 and pan NR2 mean intensity per surface. A. Plot of Mean NR2 Vs. mean PSD-95 intensities; red square = Control and blue diamond = MEP. B. Mean NR2 intensities for surfaces defined by PSD-95. C. Mean PSD-95 intensities. D. Representative 3D image generated with Imaris 3.1 showing PSD-95 (red) and pan NR2 (green). Gray surfaces represent areas quantified. At least 20 neurons were imaged per group. *p<0.0001.
4.7.3 Discussion

It is interesting that as early as two days there are increases in PSD-95 and NR2BpY1472, which may indicate a strengthening of the NMDAR synaptic content of the cultures early in the preconditioning paradigm. What is potentially more striking is that the co-localization as defined by deconvolution microscopy is also apparent at day 6 of MEP when neuroprotection is evident. Together with the data indicating increases in pPyk2 and pSrc at the 2 day timepoint, the data seem to be consistent with MEP initiating plasticity at an early time that results in a persistent increase in the NMDAR content of postsynaptic densities. This has several possible implications for the mechanism of MEP and quite possibly modalities of brain preconditioning in general.

My biochemical data indicate a strengthening of the synaptic NMDAR complex in MEP’s neuroprotective mechanism, and this agrees well with the growing literature on the neuroprotective role of synaptic NMDAR activity. However, it should follow that there is a physical increase in the synaptic NMDAR content, and in fact, deconvolution microscopy revealed an increase in the colocalization of NR2 (pan-NR2 antibody) and PSD-95 following the 6 day MEP protocol. When PSD-95 puncta were identified using the quantification software Imaris, MEP-treated cultures had a significantly elevated mean NR2 intensity compared to control. Thus it appears that an early activation of the non-receptor tyrosine kinases, Src and Pyk2, and a concomitant increase in the expression of PSD-95 and NMDAR subunits results in an increase in the synaptic NMDAR complex in preconditioned cultures. Furthermore, these changes are consistent with those reported during synaptic plasticity in vivo, since early tyrosine kinase and NR2B phosphorylation
also result in a sustained increase in the synaptic NMDAR complex. Thus at least in vitro, MEP appears to cause plasticity responses that elicit a neuroprotective phenotype in response to amyloid challenge.

4.8 NMDAR-dependent antioxidant system enhanced by moderate ethanol preconditioning

4.8.1 Introduction

Data thus far have been consistent with a mechanism involving recruitment and strengthening of the NMDAR synaptic complex. Recently, Papadia and coworkers reported in an elegant study isolating synaptic NMDAR that synaptic NMDAR activity enhanced a neuron’s intrinsic antioxidant defenses via the peroxiredoxin thioredoxin pathway (Papadia et al., 2008). Given that Aβ toxicity involves excessive ROS and that synaptic NMDAR content is enhanced in MEP-treated compared to control cerebellar mixed cultures, this study provides a novel effector mechanism to help explain the neuroprotected phenotype. If MEP is enhancing the neuron’s pro-survival capacity via the synaptic NMDAR complex, then it may be upregulating the peroxiredoxin thioredoxin pathway, thereby reducing Aβ neurotoxicity. Speculatively, the upregulation of antioxidant enzymes due to NMDAR activity (or other routes of synaptic calcium entry) may predispose a neuron receiving excitatory synaptic input through NMDAR to have resistance to oxidative stress (calcium entry is a known source of ROS), even from pathological insults.
4.8.2 Results

In order to explore this possibility, I investigated the relationship between MEP and the small molecular weight antioxidant peroxiredoxins. Although this work is preliminary at this juncture, the initial results show strong evidence that the 2-Cys peroxiredoxin2, a cytosolic member of this protein family, is increased at day 6 of MEP, consistent with a time point when neuroprotection is evident (Fig. 12). Furthermore, memantine co-treatment blocks this induction, consistent with MEP enhancing NMDAR signaling and causing the upregulation of this pathway. However, more work needs to be done, since the other enzymes in this pathway, mainly thioredoxin and thioredoxin reductase, are seemingly equally as important for antioxidant defenses.
Figure 12: Increased expression of peroxiredoxin II by MEP and reversal by memantine

Cerebellar cultures (10DIV) were treated with 30mM ethanol (E) or control (C) media for 2 and 6 days (w./w.o. memantine), protein extracted and subjected to immunoblot analyses. Extracts were probed with an antibody for pan 2 cys-peroxiredoxin and bands were identified by their theoretical Mol. Wt. Lane 1 is an equal concentration of protein lysate from rat brain cerebella at postnatal day 8. A non-specific (NS) band of ~100kD is shown for loading control.
2 Cys-peroxiredoxins

Prx 2, and 4 in cytosol, Prx3 in mitochondria
4.8.3 Discussion

Figure 12 provides evidence that imply the NMDAR changes observed in response to MEP are initiating the neuroprotective phenotype by increases in prx2. This is a cytosolic antioxidant observed primarily in neurons. Augmentation of this pathway has been linked to increased synaptic NMDAR activity (Papadia et al. 2008). The preliminary experiments showed increases in this enzyme at day 6 of MEP, but not earlier. Importantly, memantine co-treatment, which blocks NR1 expression and MEP neuroprotection, also blocked the increased expression of prx2, indicating that prx2 may be a previously unappreciated NMDAR-dependent “effector” in this preconditioning paradigm.

Another interesting point regarding prx2 is that it is increased in AD brain. This may indicate a natural process during AD progression which upregulates this antioxidant enzyme, presumably in an attempt to protect neurons from accumulating amyloid burden (Yao et al., 2007). Further, this publication shows that transfection with prx2 is sufficient to block Aβ neurotoxicity. An aspect of this paper that was not addressed is that their control transfection was PSD-95, which provided no protection from Aβ. It has been shown that PSD-95 alone is capable of clustering NMDAR complex, but the relatively short post-transfection timeframe (72 hour, additionally these mouse cortical neurons were transfected at plating and only allowed to develop for 72 h prior to Aβ exposure) may be such that it was unable to produce the increase in NMDAR activity and so increase the expression of prx2 (biochemical data showing expression of prx2 were not supplied for transfection experiments). The relationship between NMDAR synaptic
activity and this antioxidant system (Papadia et al. 2008) was, to my knowledge, not known at the time of this publication. It is conceivable that the increase in prx2 observed in vitro with MEP could occur in vivo. Such an early increase in prx2 may occlude the increased expression of prx2 in response to elevated amyloid peptides during AD progression, thereby preserving neuronal function.
CHAPTER FIVE

OVERALL DISCUSSION AND SIGNIFICANCE

One overall conclusion derived from these experiments is that neuroprotection engendered by preconditioning rat cerebellar cultures with moderate ethanol concentrations involves NMDAR activation as a likely neuroprotective sensor.

Pharmacological inhibition of NMDAR early during MEP disallows later neuroprotection. In contrast, antagonism of adenosine A$_1$ receptors or of G$_{o/o}$ protein-coupled receptors is without effect (Fig. 5). The data agree with preconditioning results demonstrating NMDAR involvement in ischemic preconditioning published soon after the discovery of ischemic neuronal tolerance (Kato et al. 1992). More recent reports further confirm a neuroprotective role for NMDAR in ischemic preconditioning (Grabb and Choi 1999; Raval et al. 2003).

The fact that preconditioning cerebellar cultures with subneurotoxic NMDA ligand itself affords neuroprotection from Aβ (Fig. 6) strengthens the idea that NMDAR activation is capable of mediating moderate ethanol-initiated prosurvival signaling. This is consistent with NMDA preconditioning/neuroprotection results using other primary cultures (Soriano et al. 2006; Smith et al. 2008). NMDAR excitability also appears critical in preconditioning with glutamate or prolonged elevations in electrical activity.
since NMDAR antagonists block the resultant neuroprotection against subsequent ischemia (Lin et al. 2008; Tauskela et al. 2008). However, despite these findings, it should be acknowledged that NMDA’s protective mechanism and ethanol’s mechanism of neuroprotection may differ in other ways.

Ethanol is known to acutely inhibit and chronically potentiate NMDAR currents, although exact mechanisms remain a matter of intense investigation (Nagy 2008). Persistent increases in subunits NR1, NR2B and NR2C occur by the first 2 days of moderate ethanol exposure and persist through day 6 (Fig. 7). Such increases in receptor subunits could be a principal reason for increased NMDAR activity leading to neuroprotection, as shown in Figure 13A. This possibility is supported by the finding that memantine blocked the ethanol-dependent increases in NR1, the receptor’s obligatory subunit—thus lessening NMDAR activity—concurrent with abolishing MEP neuroprotection. Of relevance to these in vitro findings are in vivo studies demonstrating improved NR1-dependent memory performance in rats chronically fed moderate ethanol-containing diets (Kalev-Zylinska and During 2007). RNA interference knockdown of hippocampal NR1 abolished ethanol’s facilitatory effects on cognition, while NR1 overexpression mimicked ethanol’s effects. Thus, although NR2 subunits are known ethanol targets (Woodward et al. 2006), changes in NR1 subunits often appear critical for NMDAR function.

A further critical aspect of MEP is our evidence for early NMDAR subunit increases that indicate the involvement of synaptic as opposed to extrasynaptic NMDAR. Specifically, MEP elevates levels of NR2B phospho-tyrosine 1472, which are associated
with synaptic enrichment of NMDAR and decreased NMDAR endocytosis (Yu et al. 1997; Goebel-Goody et al. 2009), and levels of PSD-95, which implicate increased synaptic receptor localization (Delint-Ramirez et al. 2008; Kurisaki et al. 2008), as represented in Figure 13. These findings are in harmony with data showing that increases in synaptic relative to extrasynaptic NMDAR signaling are associated with downstream pro-survival events (Soriano et al. 2006; Soriano and Hardingham 2007).

Furthermore, the significantly augmented ratio of NR2B-pY1772/total NR2B at an early MEP timepoint of 2 days indicates increased activity of Src-family kinase(s) (Goebel-Goody et al. 2009). This possibility was confirmed, since the phospho-activated form of Src (Fig. 9) was elevated by 48 h of MEP, a timepoint consistent with the observed increase in NR2B-pY1472. There is debate in the literature as to the exact Src-family member that is responsible for the NR2B-Y1472 phosphorylation. However, the antiphospho-Src-family antibody (recognizes Src-family phospho-activation domain) employed in this study identified 3 bands that were elevated when compared to control, all of which are consistent with the hypothetical mol. wt. of at least Src and Fyn (Fig. 8); levels of total Fyn (used as loading control) did not differ. This implies that MEP is acting via Src-family kinases to increase NR2B-Y1472 phosphorylation. Since this NR2B-Y1472 phosphorylation correlates with synaptic receptor localization, the finding suggests that a significant amount of synaptic plasticity is occurring in these preconditioned cultures.
Figure 13: Hypothetical model for the involvement of the NMDAR complex in MEP neuroprotection against Aβ neurotoxicity

Ethanol preconditioning requires NMDAR activity early in its neuroprotective mechanism. NMDA preconditioning is capable of inducing neuroprotection from Aβ, lending additional support to the involvement of the NMDAR receptor in MEP-mediated neuroprotection. A. MEP increases expression of NMDAR subunits, phosphorylation of NR2B-Y1472 and postsynaptic scaffolding protein PSD-95 by day 2. Pyk2 and Src are also phospho-activated by day 2 of MEP treatment, a timepoint when elevations in these proteins are evident and NR2B-pY1472 is increased. B. By day 6 of MEP, a timepoint when MEP-dependent neuroprotection is evident, microscopy data show an increase colocalization of NR2 and PSD-95, as well as increased expression of the antioxidant enzyme prx2. Therefore, MEP causes recruitment of the NMDAR complex to the postsynaptic density within the neuron. This mechanism provides two potential explanations for MEP’s ability to protect from Aβ-neurotoxicity: 1) MEP increases NMDAR-dependent prosurvival signaling in general such as CREB activation and antioxidant defenses and 2) MEP shifts the pool of available receptor from the extrasynaptic or excitotoxic receptor to synaptic sites, increasing receptor efficacy in the face of amyloid burden and producing an environment less susceptible to amyloid neurotoxicity.
A Day 2 MEP

- NR2B-Y1472
- pSrc + pPyk2
- NMDAR Subunits
- NR2B-pY1472
- PSD-95

B Day 6 MEP

- PSD-95
- pSrc?
- pPyk2?

NEUROPROTECTION
- Peroxiredoxin 2
- Heat shock proteins (S. Sivaswamy, PhD dissertation)

Synapse
Another finding that correlates well with increased synaptic NMDAR activity early in MEP’s neuroprotective mechanism is the elevations in the phospho-activated form of Pyk2 (Fig. 8). Pyk2, like Src, has been implicated in synaptic plasticity. This non-receptor tyrosine kinase, which is responsive to increases in intracellular calcium, interacts with MAPK pathways. Interestingly, a very recent report found that Pyk2 binds to PSD-95 in a calcium/calmodulin-dependent manner (Bartos et al., 2010). This provides the close proximity required for its trans-autophosphorylation and activation of tyrosine 402. Phosphorylation of this site activates Pyk2 and provides a docking site for Src family kinases (binding via their SH2 domain to Pyk-pY402), which further phosphorylate and augment Pyk2 kinase activity. Src, as previously mentioned, is well known to phospho-activate NMDAR at the synapse. Thus it seems plausible that MEP enhances the synaptic NMDAR complex and may, speculatively, induce formation of a “postsynaptic signalosome” complex in MEP’s neuroprotection (Collins et al., 2009, see Fig. 13).

My biochemical data are strongly indicative of a strengthening of the synaptic NMDAR complex in MEP’s neuroprotective mechanism. This agrees with a growing literature on the neuroprotective role of synaptic NMDAR activity. However, it should follow that there is a physical increase in the synaptic NMDAR content. Indeed, deconvolution microscopy revealed an increase in the colocalization of NR2 (pan-NR2 antibody) and PSD-95 following the 6 day MEP protocol (Fig. 10). When PSD-95 puncta were identified using the quantification software Imaris, MEP-treated cultures had a significantly elevated mean NR2 intensity vs. control (Fig. 11). Therefore, an early
activation of the non-receptor tyrosine kinases Src and Pyk2 and a concomitant increase in the expression of PSD-95 and NMDAR subunits (Fig. 13A) result in an increase in the synaptic NMDAR complex in preconditioned compared with control cultures (Fig. 13B). These changes are consistent with those reported during synaptic plasticity in vivo, since early tyrosine kinase and NR2B phosphorylation results in sustained increases in the synaptic NMDAR complex. Thus at least in vitro, MEP appears to be causing plasticity responses that elicit a neuroprotective phenotype.

A final line of evidence that the NMDAR changes observed in response to MEP are initiating the neuroprotective phenotype is the increases in prx2 (Fig. 12). This cytosolic antioxidant is observed primarily in neurons. The peroxiredoxin-thioredoxin pathway has also been linked to synaptic NMDAR activity (Papadia et al., 2008). I observed increases in this enzyme at day 6 of MEP but not earlier. Importantly, memantine co-treatment, which blocks NR1 expression and MEP neuroprotection, also suppressed the increased expression of prx2 (Fig. 12). As depicted in Figure 13, this implicates prx2 as a previously unrecognized NMDAR-dependent “effector” in MEP. Another interesting point regarding this particular enzyme is that it is increased in AD brain, suggesting that there is a natural process during AD progression by which this antioxidant enzyme is increased, possibly to protect neurons from the accumulating amyloid burden (Yao et al., 2007).

The authors further show that transfection with prx2 is sufficient to block Aβ neurotoxicity. However, their control transfection was PSD-95, which provided no protection from Aβ. It has been shown that PSD-95 alone is capable of clustering
NMDAR complex, but the relatively short post-transfection timeframe (72 hour, additionally these mouse cortical neurons were transfected at plating and only allowed to develop for 72h prior to Aβ exposure) may be such that it was unable to produce the increase in NMDAR activity and so increase the expression of prx2 (biochemical data showing expression of prx2 were not supplied for transfection experiment). It was not known at the time of the Yas publication the relationship between NMDAR synaptic activity and this antioxidant system (Papadia et al., 2008). It is conceivable that the increase in prx2 observed in vitro with MEP occurs in vivo and there is some evidence for this possibility (Botia et al., 2008). Such an early increase in prx2 may supersede the expression in response to elevating amyloid peptides, preserving neuronal function in response to soluble amyloid burden during AD progression.

Although growing evidence suggests a physiological function of amyloid peptide fragments pertaining to regulation of synaptic transmission and homeostasis (Pearson and Peers 2006), aberrant Aβ peptide overproduction is considered central to AD pathophysiology and possibly age-related mild cognitive decline. This concept is evidenced by the facts that synaptic loss correlates well with cognitive decline in vivo (Terry et al. 1991) and that Aβ peptides experimentally induce alterations in synapse composition and density (Lacor et al. 2007). AD is reaching epidemic proportions among aging populations (Wang and Ding 2008) and effective pharmaceutical approaches to slow AD progression are lacking. Investigations into the molecular mechanisms of ethanol preconditioning may help to clarify roles of NMDAR in AD etiology and offer new strategies for halting synaptic degeneration while preserving synaptic connectivity.
A recent report showed that Aβ-containing monomeric and oligomeric peptides bind to postsynaptic NMDAR-containing synapses, inhibit NMDAR, and decrease NMDAR immunoreactive spines and surface expression of NR2B-containing NMDAR (Dewachter et al. 2009). The authors also found decreased concentrations of NR2B and PSD-95 in a transgenic mouse model of AD. Others have reported that the suppressive effects of Aβ on NMDAR surface expression correlate with decreased NR2B-pY1472 (Snyder et al. 2005). Our results indicate that ethanol preconditioning might combat neurodegenerative processes induced by Aβ and other neurotoxic proteins (e.g., HIV-1 gp120) by preserving NMDAR-associated synaptic density and connectivity. MEP-mediated neuroprotection seems to entail the ability of synaptically-localized NMDAR to enhance intrinsic antioxidant defenses—specifically, via peroxiredoxin and thioredoxin pathways. In view of considerable evidence that AD is characterized by excessive oxidative stress (Sultana et al. 2009), such a mechanism seems plausible.
CHAPTER SIX
CONCLUDING REMARKS

Brain preconditioning by multiple stimuli have shown the NMDAR to be an important sensor for preconditioning and as such, the results in this study are consistent with those published results. However, emerging evidence seems to implicate the specificity of synaptic responses as opposed to total activity in attaining NMDAR-dependent neuroprotection. NMDAR synaptic activity has been linked to CREB activation and its downstream prosurvival signaling. Importantly, Aβ inhibits NMDA-stimulated CREB activation (Snyder et al., 2005). Since CREB seems to play a critical role in preconditioning, CREB signaling should be investigated in MEP’s neuroprotective mechanism.

Another interesting point is that amyloid peptides have been postulated to act as gain-of-function molecules for the NMDAR. Ethanol even in moderation can upregulate the NMDAR in vitro and in vivo. Thus, one might expect that Aβ would enhance excitotoxicity in ethanol-treated cultures. My results show MEP-induced upregulation of NMDAR at a timepoint that correlates with neuroprotection from Aβ. Furthermore, there is growing evidence that Aβ acts as an inhibitor of the NMDAR (Dewachter et al., 2007; Puzzo et al., 2008) and mediates excitotoxic calcium entry through α-7 nicotinic acetylcholine receptors (Snyder et al., 2005). This may partially explain why memantine, which has affinity for the α-7 receptor similar to that of the NMDAR
(Aracava et al., 2005), is the only NMDAR inhibitor that has been approved by the FDA for treatment of AD. Nevertheless, there is an obvious discrepancy in the literature on the mechanisms of AD pathology that demands further exploration.

This work has identified the NMDAR as a probable sensor of MEP-neuroprotection, as has been documented for other forms of preconditioning-induced neuroprotection. However, MEP seems to act via a distinct, novel and until now undescribed mechanism. That is, MEP increases the NMDAR content of the postsynaptic density. MEP, potentially by shifting the NMDAR pool from extra-synaptic (not associated with the PSD) to synaptic (associated with the PSD) surfaces, modulates the membrane content of the receptor to a more prosurvival, anti-excitotoxic phenotype, essentially providing less opportunity for extra-synaptic noise while inducing a higher propensity for legitimate synaptic transmission. This phenotype may also prime the neuron to accommodate a greater oxidant stress, as it has been primed to deal with the oxidant load by enhanced endogenous antioxidants.

Furthermore, this same phenotype could be rendering neurons resistant to the elevated glutamate that has been reported in AD brains. Amyloid peptides seem to have important physiological functions in normal neuronal transmission and this point must be considered when designing logical therapeutic approaches to treating AD. With this in mind, it seems that MEP may be inducing an extracellular environment that is resistant to the actions of Aβ, and in so doing, thwart off the progression of AD or possibly induce a more regulated control of the amyloidogenic processes. Designing drugs that encapsulate these properties, but lack the undesirable effects of alcohol (such as the propensity for
abuse), would be a novel approach to treating both the neurodegenerative processes and the cognitive deficits associated with the AD.

It would appear that amyloid peptides have both physiological roles and in excess pathological roles. Memantine is the only FDA-approved drug for the treatment of moderate to severe AD. Memantine has been reported to act by enhancing the efficacy of synaptic transmission, inhibiting the pathological activation of extrasynaptic NMDARs while preserving the physiological function of the receptor in normal synaptic (depolarized, voltage-sensitive) transmission (Leveille et al., 2008). Ethanol preconditioning, or by extension, moderate alcohol consumption, found in some studies to be protective against dementia in older individuals, may be working similar to memantine—but potentially by a different mechanism, by trafficking the receptor from the extrasynaptic to synaptic regions, thereby increasing the efficacy of synaptic (prosurvival) events while decreasing the pathological extrasynaptic excitotoxicity.


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VITA

Robert Matthew Mitchell was born the second of March, 1980 in Elgin, Illinois, the youngest child of Ralph and Julianne Mitchell. Robert attended Larkin High School in Elgin and graduated with the class of 1998. In the summer of 1998, Robert enrolled in classes at Elgin Community College. While attending ECC, Robert was invited to join the Phi Theta Kappa Honors Society and the ECC Honors program. He completed his studies there in December 2000 and graduated with high honors, attaining Associates Degrees in both art and science. In spring of 2001 Robert entered the Biochemistry program at Northern Illinois University. While attending NIU, Robert was accepted into the universities Honors Program and in May of 2003, graduated with High Honors receiving the B.S. degree with a major in biochemistry and a minor in biology. That year Robert was accepted into the Molecular & Cellular Biochemistry doctoral program at Loyola University Medical Center. In 2005, he joined the laboratory of Dr. M. A. Collins to study the molecular mechanisms of neuroprotection by moderate ethanol preconditioning, with Dr. E.J. Neafsey as co-advisor.

In 2007, Robert accepted a position as a part-time faculty member at Loyola’s Lakeshore Campus instructing the undergraduate Neuroscience lab course under Dr. Louis Lucas. He remained as a co-instructor during the fall semesters through 2009.

During his research duration in the Collins-Neafsey laboratories, Robert has been independently funded for four years—two years through the LUMC Alcohol Research
Program, NIH T32 (P.I., Dr. Elizabeth Kovacs) and two years by an Early Researchers grant from the Illinois Department of Public Health Alzheimer’s Disease Research Fund.