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Putative Mechanisms of Cortical Spreading Depression-Induced Neuroprotection

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"Imagination is more important than knowledge. Knowledge is limited.

Imagination encircles the world." -Albert Einstein

In loving memory of my grandparents.

ABSTRACT

Cortical spreading depression (CSD) has been shown to infer protection against ischemia, though the mechanism by which it acts has yet to be determined. It is hypothesized that the reversal of the glial glutamate transporters are primarily responsible for the majority of glutamate release in an ischemic situation and that the down regulation of these glutamate transporters induced by CSDs would lead to a neuroprotective effect.

To determine whether or not the metabotropic glutamate receptors (mGluR) play a role in the CSD-induced down regulation of the glutamate transporters, we have pharmacologically antagonized the specific groups of mGluRs prior to the elicitation of CSD. Neither group I antagonism with AIDA, group I and II antagonism with MCPG nor group III antagonism with MAP4 prior to CSD induction revealed any effect on the plasma membrane content of GLT-1. These studies have also revealed that no down regulation of the GLT-1 transporter was seen three days post-CSD, as was previously reported (Douen et al., 2000). Though a number of factors may have contributed to these differing results, our examination of our techniques could find no conclusive evidence to either support or refute the CSD-induced down regulation of GLT-1.

An examination of the molecular mechanisms that may underlie the neuroprotective effect of CSD has revealed that immediately after 2 hours of CSD, there is a significant increase in ERK phosphorylation while at the same time, no MEK, c-raf or SAPK phosphorylation was observed. These findings may lead to greater insight into the pathway by which CSD may mediate its neuroprotective effect, especially in light of the fact that ERK phosphorylation has been seen to be neuroprotective in both *in vivo* (Shamloo et al., 1999) and *in vitro* (Parrizas et al., 1997) models of neuroprotection.

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List of Abbreviations

ACPD- (1S, 3R)-1-aminocyclopentane-1,3-dicarboxylic acid

AIDA- (R,S)-1-aminodian-1,5-dicarboxylic acid

AMPA- α-amino-3-hydroxy-5-methylisoxazole-4-proprionic acid

BDNF- brain derived neurotrophic factor

bFGF- basic fibroblast growth factor

CSD- cortical spreading depression

EAAT- excitatory amino acid transporter

ERK/MAPK- extracellular signal related protein kinase/ mitogen activated protein kinase

DHPG- dihydroxyphenylglycine

GFAP- glial fibrillary acidic protein

MAP4- (S)-2-amino-2-methyl-4-phosphobutaoic acid

MCA- middle cerebral artery

MCAO- middle cerebral artery occlusion

MCPG- (R,S)-α-methyl-4-carboxyphenylglycine

MEK- mitogen activated protein kinase kinase

mGluR- metabotropic glutamate receptor

NBQX- 6-nitro-7-sulfamoylbenzo(f)quinoxaline-2,3-dione

NMDA- N-methyl-D-aspartate

PKC- protein kinase C

PMSF- phenylmethanesulfonyl fluoride

SDS- sodium dodecyl sulfate

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I would also like to thank my parents and friends for understanding my prolonged absences and neglect of social duties. And lastly, thank you to poor Kittums who had to put up with several years journal articles piled everywhere, including on top of him.

INTRODUCTION

Stroke is one of the leading causes of death in North America and the leading cause of adult neurological disability. Coupled with the brain's susceptibility to ischemic insult, the impetus to find effective neuroprotective strategies and effective treatment to restore function post-stroke is increasing dramatically.

Two *in vivo* animal models are commonly used to emulate stroke in humans. The global ischemia model mimics the human situation of a heart attack where blood flow to the entire brain is compromised (Pulsinelli and Brierley, 1979). The focal ischemia model is accomplished in animals by using a variety of methods, including photothrombic occlusion (Bidmon et al., 1998; Kim et al., 1998), and two middle cerebral artery occlusion (MCAO) models. The thread model of MCAO involves the insertion of a coated thread through the carotid to the middle cerebral artery where the thread blocks blood flow to brain areas served by the distal middle cerebral artery (Longa et al., 1989). The distal clip model of MCAO on the other hand involves exposing the distal middle cerebral artery at the level of the brain and the application of a microaneuryism clip to occlude blood flow distal to those areas (Tamura et al., 1981).

A number of different strategies have been devised to try and protect the brain against an ischemic insult, or to reduce the damage of the brain following a stroke. Numerous pharmacological agents have been tested in the laboratory setting and have been shown to be neuroprotective in animals, though many still remain controversial. The spectrum of agents that have been found to be neuroprotective in animal studies reveals that the molecular mechanisms underlying ischemic damage are not clearly

defined. Efforts to prevent or reduce ischemic damage are contingent upon an understanding of pathophysiology of this type of injury, as well as an appreciation of neuroprotective strategies.

Glutamate and Neurotoxicity

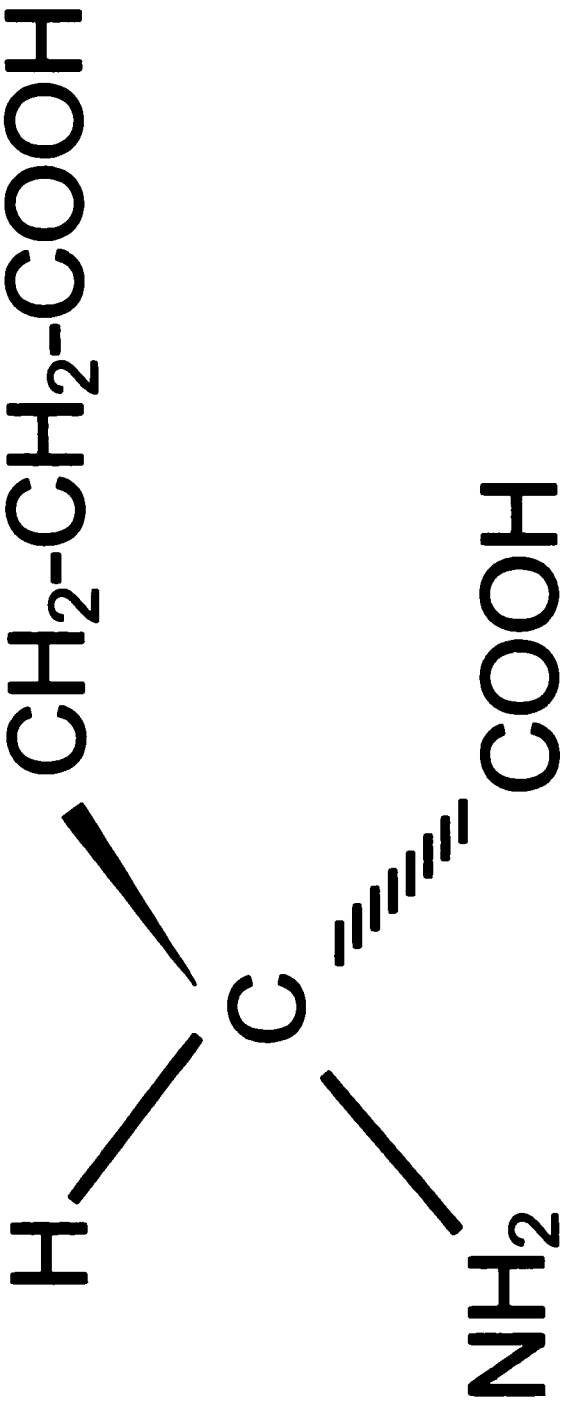
Glutamate (Figure 1-1) is one of the primary neurotransmitters in the brain and it is widely believed that the large amount of glutamate released in an ischemic situation is responsible for much of the damage incurred as a result of reduced blood flow, though this remains controversial. One common hypothesis of the underlying cause of excess glutamate release suggests that the decreased blood flow to an area of the brain causes that particular area to be energetically compromised. This would cause degradation of the ionic gradients established by the ATP-dependent Na^+/K^+ exchanger, leading to a dissipation of the membrane potential and subsequently, depolarization. This depolarization would cause release of glutamate into the synapse. Empirical evidence shows that ischemia causes a dramatic increase in extracellular glutamate levels from micromolar levels to several hundred micromolar (Bouvier et al., 1992; Hagberg et al., 1985, Benveniste et al., 1984). Hagberg and colleagues (1985) found that after a period of ischemia, extracellular glutamate increased to 160 times that of the normal resting level, while Benveniste et al. (1984) showed that there was an eight-fold increase in extracellular glutamate levels during the ischemic period, as measured by *in vivo* microdialysis.

Several mechanisms by which glutamate may be released during ischemia have been proposed. One group (Drejer et al., 1985) has proposed that this release of glutamate is dependent on calcium, suggesting that ischemic glutamate release may be vesicular. They found that cultured cerebellar granule cells release large amounts of glutamate when exposed to anoxic conditions and concluded that the glutamate was released from neurons and not astrocytes. Choi's (Choi, 1985) discovery that cortical cultures, briefly exposed to glutamate, undergo neuronal death and that removal of extracellular calcium attenuates the neuronal loss, provides further support for the hypothesis that calcium plays an important role in glutamate neurotoxicity.

Others have suggested that the large amount of extracellular glutamate during ischemia may be the result of the reversal of the glutamate transporters which are normally responsible for sequestering glutamate from the synapse (Szatkowski et al., 1990; Attwell et al., 1993). Though the former mechanism may play a role in the initial minutes of ischemia, a prolonged effect is unlikely since vesicular release is inhibited in the absence of ATP (Kauppinen et al., 1988). Evidence that the latter proposed mechanism may be the one in operation is accumulating and is discussed below.

Despite the evidence mentioned above, however, the role of glutamate in neurotoxicity still remains controversial. Agents believed to protect against glutamate mediated neurotoxic injury have been shown to have alternative neuroprotective effects that are unrelated to the blockade of glutamate action. For example, once the hypothermic effect of the NMDA receptor antagonist MK-801 is controlled for, its neuroprotective effect is eliminated (Li and Buchan, 1993).

Figure 1-1. The structure of glutamate. Glutamate is an amino acid that is one of the primary excitatory neurotransmitters in the central nervous system.


CH2-CH2-COOH
COOH
C
H
NH2

Glutamate Transporters

Termination of the post-synaptic action of glutamate is primarily the responsibility of the high affinity excitatory amino acid transporters (EAATs) (Rothstein et al., 1995). Four EAATs have been identified in the central nervous system. EAAT1 (GLAST) and EAAT2 (GLT-1) being mainly astrocytic while EAAT3 (EAAC1) is found in neurons of the cortex and EAAT4 in the neurons of the cerebellum (Chaudhry et al., 1995). Knockout studies have revealed that the astrocytic glutamate transporters are responsible for the majority of glutamate clearance in the rat cortex (Rothstein et al., 1996). Selective blocking of GLAST, GLT-1 and EAAT3 synthesis using antisense oligonucleotides has revealed that GLAST and GLT-1 are more significant than EAAT3 in the clearance of glutamate from the extracellular space (Rothstein et al., 1996). The same group also showed that elevated levels of glutamate in the extracellular space are linked to neuronal degeneration. In addition, Mennerick and colleagues (1998) have shown that in hippocampal microcultures, GLT-1 is also localized on the dendrites of excitatory neurons and that GLT-1 inhibition with dihydrokainate causes prolongation of excitatory currents after elimination of glia, suggesting that the neuronal GLT-1 is at least partially responsible for glutamate clearance.

The glutamate transporters operate mainly as a result of the sodium gradient that exists between the intracellular and extracellular compartments (Szatkowski and Attwell, 1994). The glutamate transporter normally transports two sodium ions down its electrochemical gradient into the cell along with a glutamate anion, while one K^+ and one

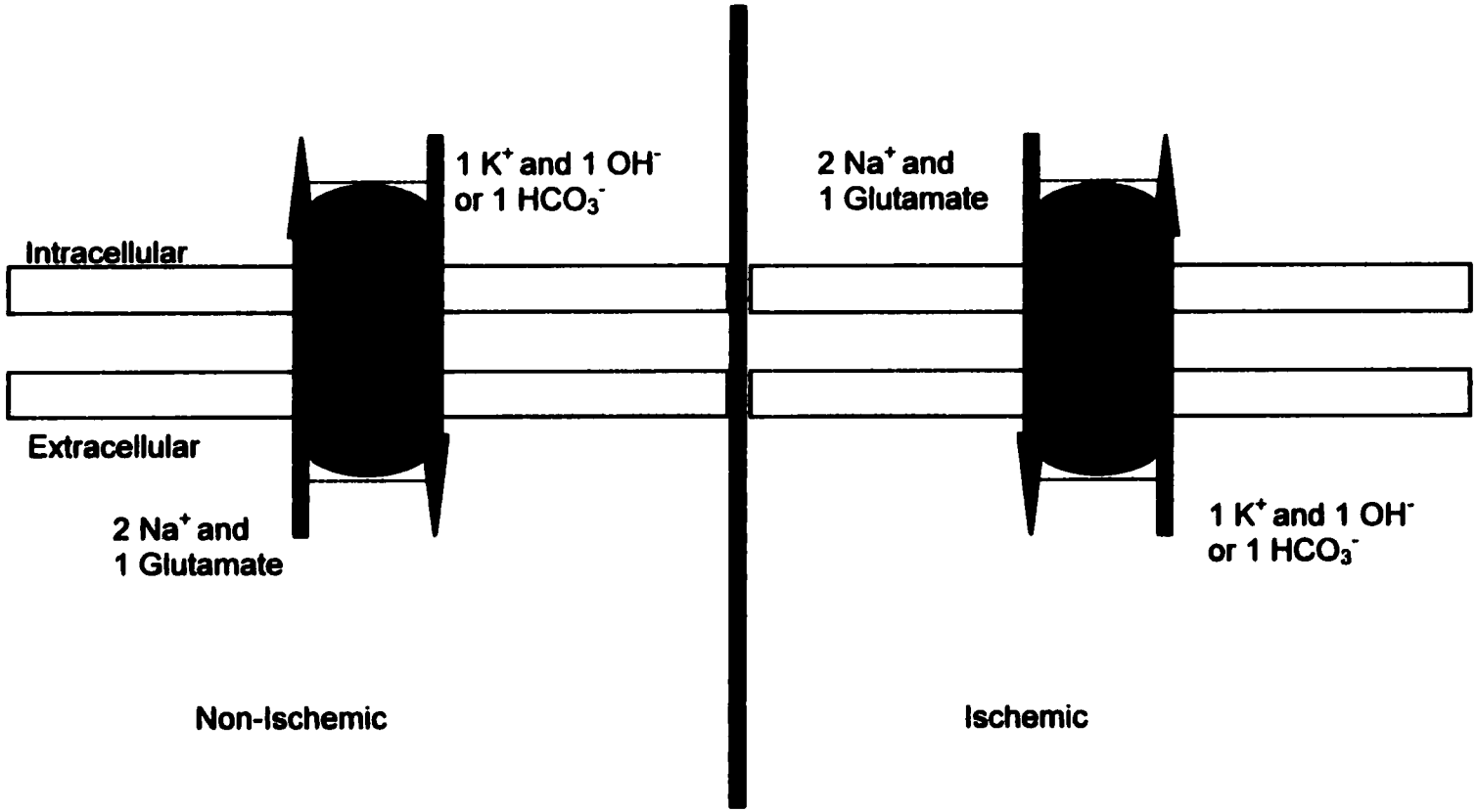
OH^- or one HCO_3^- ion are transported out of the cell (Bouvier et al., 1992). In the ischemic situation, the sodium electrochemical gradient is degraded as a result of the inhibition of the ATP-dependent Na^+/K^+ pump. This would lead to a rise in the intracellular sodium concentration, effectively reversing sodium's electrochemical gradient, thus causing the glutamate transporter to work in reverse, in effect ejecting any glutamate that has been sequestered back into the synapse (Figure 1-2).

Neuroprotection From Cerebral Ischemia

A number of different neuroprotective strategies have been devised in animal models in an attempt to minimize and/or prevent ischemic damage, as well as to promote recovery following a stroke. Many of these strategies have centered around the role of glutamate in neurotoxic injury.

Antagonists to the ionotropic glutamate receptors have been shown to impart acute neuroprotection, though the mechanism through which they act remains controversial. Numerous pharmacological antagonists of the N-methyl-D-aspartate (NMDA) receptors have been shown to impart neuroprotection in a number of different models. In particular, the non-competitive NMDA receptor antagonist MK-801 has been shown to attenuate the severity of ischemic damage (Grabb and Choi, 1999; Bond et al., 1999; Gill et al., 1988) in both *in vivo* and *in vitro* models. Several groups (Dietrich et al., 1995; Hayward et al., 1993), however contend that the neuroprotective effect of NMDA antagonists such as MK-801 is related to its hypothermic action (Li and Buchan, 1993) which has been shown to be neuroprotective (Young et al., 1983; Busto et al.,

Figure 1-2. Glutamate transporters in the normal and ischemic configurations. In the normal brain, the glutamate transporter is responsible for sequestering glutamate from the synapse to end its excitatory action. The glutamate transporter derives its energy from the existing sodium and potassium gradients between the intracellular and extracellular space. It normally removes one glutamate and two sodium ions from the extracellular space in exchange for one potassium and one hydroxide or one bicarbonate ion. In the ischemic situation, energy is compromised and the ATP-dependent Na^+/K^+ pump normally responsible for maintaining the ionic gradient fails. As a result, the glutamate transporter begins to work in reverse, extruding glutamate into the extracellular space.


1 K+ and 1 OH-
or 1 HCO3-
2 Na+ and
1 Glutamate
Intracellular
Extracellular
2 Na+ and
1 Glutamate
1 K+ and 1 OH-
or 1 HCO3-
Non-Ischemic
Ischemic

1989; Nishio et al., 1999; reviewed in Kataoka and Yanase, 1998) rather than the direct reduction of glutamate neurotoxicity. In addition, NMDA antagonists have been ineffective in clinical trials and may indeed have severe neurological consequences if administered (reviewed in Small and Buchan, 1997). MK-801 has been shown to have amnesic effects on rodent memory (Hamm et all, 1994) and administration in songbirds prevents song learning and adaptation (Aamodt et al., 1996). In humans, administration of MK-801 can cause white matter vacuolation (Olney et al., 1989). In addition, focal ischemia appears to intensify the side effects of NMDA antagonists. Clinical trials have shown that stroke patients experience more adverse effects, such as hallucinations and agitation, than normal volunteers given equivalent doses (Grotta et al., 1995). Likewise, MK-801 and CGS19755 psychomimetic side effects were enhanced in rodents following focal ischemia (Loscher et al., 1998).

Administration of antagonists to α-amino-3-hydroxy-5-methylisoxazole-4-proprionic acid (AMPA) receptors may prove to be a more effective neuroprotective strategy. 6-nitro-7-sulfamoylbenzo(f)quinoxaline-2,3-dione (NBQX), a selective AMPA/kainate receptor antagonist has been shown to be neuroprotective in culture (McDonald et al., 1998) and *in vivo* (Hu et al., 1997). Pre-administration of NBQX has also been shown to dramatically reduce the peak concentration of glutamate during ischemia and protect neurons from necrosis in the hippocampus (Gaspary et al., 1994). Administration of NBQX 90 minutes post-ischemic insult has also been shown to be neuroprotective in focal ischemia (Graham et al., 1996). Issues of toxicity, particularly nephrotoxicity (Li and Buchan, 1993) has limited NBQX's use to animal paradigms. YM872, another

AMPA antagonist, may be more promising. Like NBQX, YM872 is capable of reducing brain injury in the rodent after focal ischemia (Shimizu-Sasamata et al., 1998), however, unlike NBQX, no drug precipitation in the kidneys is seen in treated mammals (Takahashi et al., 1998).

Calcium

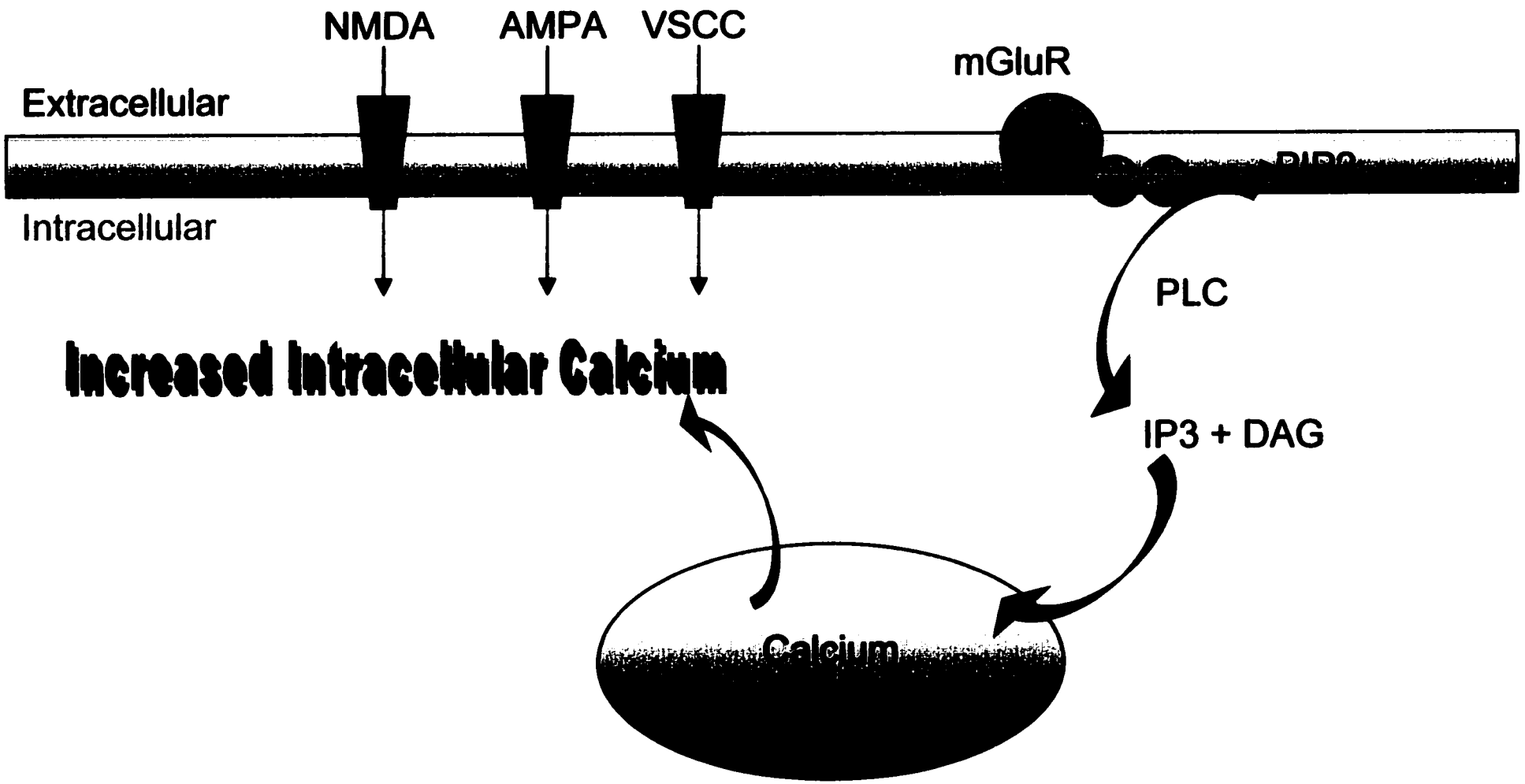
Glutamate's neurotoxic effects are believed to be mediated at least in part by calcium (Choi, 1994). The release of glutamate activates both the ionotropic receptors; the NMDA and AMPA/kainate channels, and the metabotropic glutamate receptors (mGluRs), both of which can alter the calcium levels in a cell.

Intracellular calcium may be increased by the release of calcium that is sequestered in both the endoplasmic reticulum as well as the mitochondria. In the non-ischemic brain, the mitochondria are responsible for lowering cytosolic calcium levels by sequestration into the mitochondria in an energy dependent manner. In situations where cell activity leads to an extensive rise in cytosolic calcium levels, the mitochondria may accumulate large amounts of calcium as a result of the activity of the calcium uniporter (Nicholls, 1985). During an ischemic situation, where there may be massive accumulation of calcium in the mitochondria, there may be the formation of the MPT pore (Gunter et al., 1990, Duchen, 1993). The formation of this MPT pore on the inner mitochondrial membrane allows the release of the accumulated calcium into the cytosolic space, which then leads to the cessation of ATP production which is dependent on a mitochondrial calcium gradient (Mitchell, 1966).

During ischemia, as a result of the degradation of the Na^+ gradient established by the Na^+/K^+ ATPase, intracellular calcium levels are increased in several different ways. The resulting depolarization of the cell causes the opening of the voltage sensitive calcium channel, allowing calcium entry. Cell depolarization would also cause the release of glutamate, as described above and glutamate induced activation of the NMDA and AMPA channels further contribute to the rise in intracellular calcium (Siesjo, 1989). Activation of the mGluRs by glutamate may also contribute to the rise in intracellular calcium levels. Rather than increasing the influx of calcium from extracellular sources however, activation of the mGluRs may cause release of intracellular calcium stores sequestered in the endoplasmic reticulum via a phospholipase C dependent mechanism (Sugiyama et al., 1987, Murphy and Miller, 1988) (summarized in Fig.1-3).

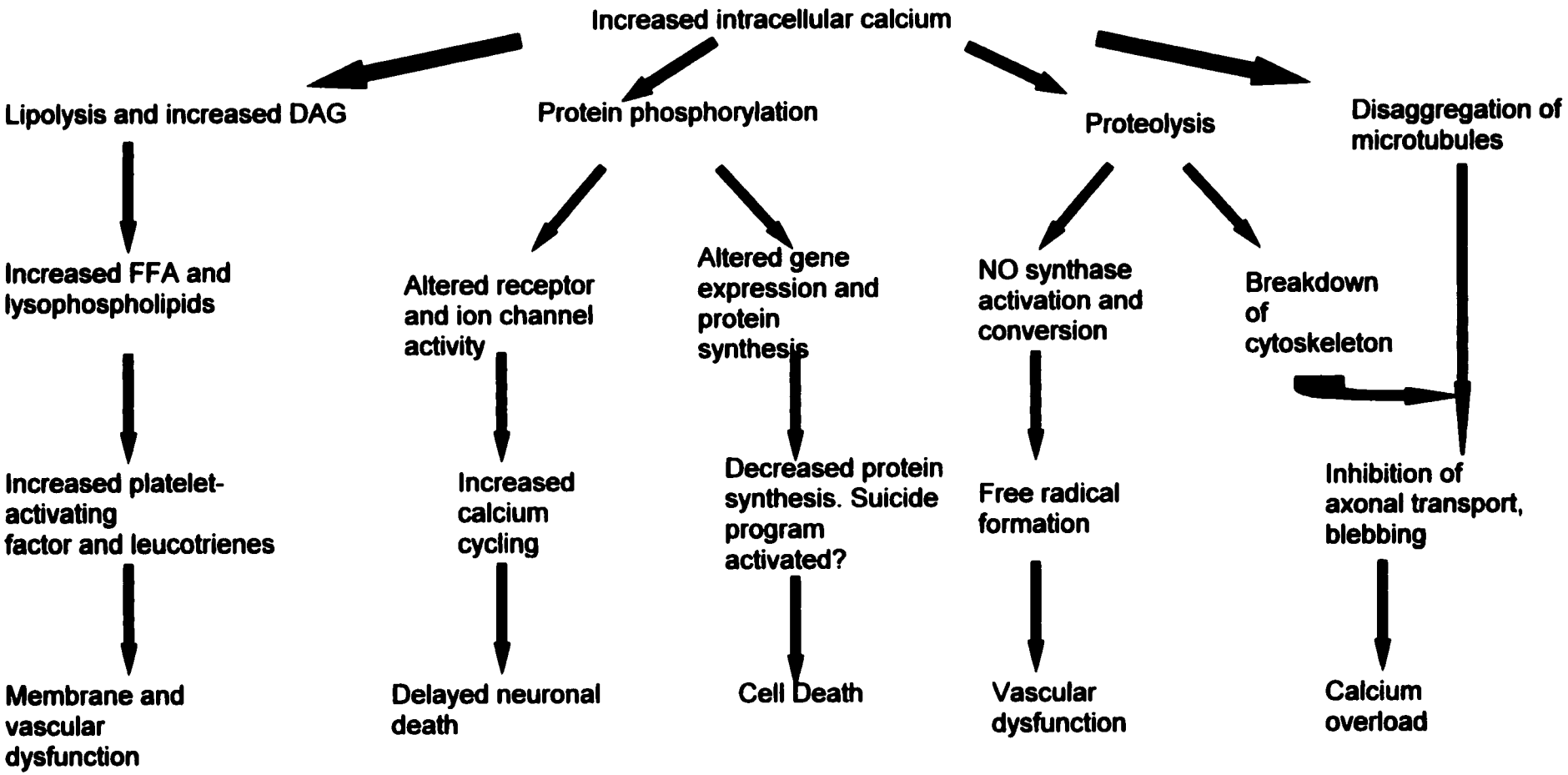
Increased intracellular calcium levels may be detrimental to the cell for a number of different reasons, summarized by Siesjo (1994) (Figure 1-4). In brief, increased calcium levels are capable of activating a number of enzymes such as calpains, which can lead to cleavage and damage of the cellular cytoskeleton, causing altered membrane permeability (Orrenius et al., 1988). Rising intracellular calcium levels can also activate caspases (Takadera and Ohyashiki, 1997), an integral group of enzymes that are believed to play an important role in the initiation of programmed cell death/ apoptosis (Du et al., 1997). In addition, as briefly mentioned above, the depletion of calcium from mitochondrial stores is also detrimental to a cell because ATP production is dependent on a mitochondrial calcium gradient (Mitchell, 1966).

Figure 1-3. The rise in intracellular calcium. Intracellular calcium may be increased from a number of sources. Activation of the NMDA and AMPA receptors by glutamate encourages influx of extracellular calcium. In addition, glutamate may also activate the mGluR which act through a phospholipase C mechanism to increase the release of calcium sequestered in the mitochondria and endoplasmic reticulum. Depolarization also activates the voltage sensitive calcium channels (VSCC) that also allow the influx of extracellular calcium.



Endoplasmic Reticulum and
Mitochondria

Figure 1-4. Summary of the role of calcium in cell death. Calcium may act through a number of different mechanisms to promote cell death. Increased intracellular calclium may act to increase lipolysis, protein phosphorylation, proteolysis and encourage disaggregation of microtubules, all of which may eventually lead to cell death. Adapted from Siesjo, BK., 1994.



Adapted from Siesjo, BK, 1994, Annals of the New York Academy of Sciences

Metabotropic Glutamate Receptors

Extracellular glutamate also acts on the metabotropic glutamate receptors (mGluRs), which are found on both presynaptic and postsynaptic membranes of neurons and astrocytes. The mGluRs are coupled to GTP and contain seven transmembrane spanning units. mGluR classification is based on amino acid sequence homology, second messenger coupling, as well as pharmacological profiles (Camon et al., 1998). The group I mGluRs act through a phosphoinositide hydrolysis and calcium second messenger system. Group I mGluRs include mGluR1 and mGluR5. mGluR 2 and 3 are in the group II mGluRs and are negatively coupled to adenylate cyclase and inhibit the increase of cyclic adenosine monophosphate (cAMP) (Schoepp and Conn, 1993). Group III mGluRs, which includes mGluR4, 6, 7, and 8 are also negatively coupled to adenylate cyclase, but in contrast to the group II mGluRs, are not responsive to ACPD, but rather to L-AP4 (Conn and Pin, 1997).

Pharmacological profiling for group I mGluRs has identified quisqualate as a potent agonist, followed by 3,5-dihydroxyphenylglycine (3,5-DHPG) and 1S,3R-1-amino-1,3-cyclopentanedicarboxylate (ACPD). α-methyl-4-carboxyphenylglucine (MCPG) was the first mGluR group I antagonist to be described and is still widely used due to its high specificity to the group I mGluRs.

(2S, 1'R, 2'R, 3'R)-2-(2,3-dicarboxycyclopropyl)glycine (DCG-IV), L-CCG-I, and 2R,4R-4-aminopyrrolidine-2,4-dicarboxylate (APDC) are the most potent group II agonists in rank order from most to least potent, however only APDC is a specific group II agonist. α-methyl-4-phosphonophenylclycine (MPPG) is the most potent

phenylglycine derivative mGluR group II antagonist known to date, though (2S,1'S, 2'S, 3'R)-2-(2'-carboxy-3'-phenylcyclopropyl) glycine (PCCG-IV) is the most potent antagonist on mGluR2.

L-amino-4-phosphonobutyrate (L-AP4) is a very specific agonist of group III mGluRs. Though the pharmacology of group III mGluRs is poorly characterized, MAP4 appears to be a more selective antagonist than most others described, though it is also a low potency antagonist on mGluR2. (for a more complete mGluR pharmacology review, see Conn and Pin, 1997).

The effect of mGluR agonists and antagonists on ischemia has been equivocal. While pharmacological activation of the group I mgluRs with DHPG has been shown to be detrimental to CA1 hippocampal neurons (Camon et al., 1998), activation of the group II mGluRs using LY354740 has been shown to be neuroprotective in a bilateral artery occlusion paradigm in the gerbil (Bond et al., 1998). . The neurotoxic effects of glutamate are believed to be derived from its effect on elevating intracellular calcium levels (Choi, 1994). The mGluRs may also function in a neuroprotective or neurotoxic fashion by regulating the levels of intracellular calcium.

Preconditioning

Neurons and glia can be preconditioned to better withstand subsequent ischemic episodes both *in vivo* and *in vitro*. A hallmark of preconditioning is that there is a delayed neuroprotective effect that may not occur until several days have passed, in contrast to the very acute effects of glutamate receptor antagonists.

Though many of the mechanisms underlying neuronal preconditioning remain unknown, CNS preconditioning may have mechanisms that are similar to those found in cardiac preconditioning. Both NO (Giddat et al. 1999, Gonzalez-Zulueta et al., 2000) and the adenosine A_1 receptors, along with the K_{ATP} channels (Heurteaux et al., 1995) appear to play roles in both cardiac and neuronal preconditioning. Tauskela et al., (1999) however, have revealed in a cell culture model of neuronal preconditioning, that oxygen glucose deprivation (OGD) does not increase MAPK activity in contrast to what is seen in the heart.

There is increasing evidence that indicates neuronal preconditioning is mediated largely via NMDA receptor activation, the subsequent increase in intracellular calcium and new protein synthesis (Grabb and Choi, 1999). *In vitro* work has also recently revealed a role for raf/MEK/ERK pathway. Gonzalez-Zulueta et al., (2000), have shown that in OGD preconditioning in culture, ras is necessary for preconditioning and that ras alone is sufficient to infer protection against a subsequent OGD insult.

Many of the underlying molecular mechanisms of preconditioning are still poorly understood, however, there is increasing evidence that new gene expression is responsible for mediating the neuroprotective effect. For example a delayed neuroprotective effect is typical of preconditioning stimuli and administration of protein synthesis inhibitors is sufficient to inhibit preconditioning (Barone et al., 1998).

In vitro work has revealed that rodent hippocampi that are exposed to sublethal levels of oxygen-glucose deprivation one to two days prior to lethal levels of oxygen-glucose deprivation are 40-60% more resistant to degeneration when compared to

cultures that were not preconditioned (Khaspekov et al., 1998). Bruer et al. (1997), found similar results in cultured rat cortical neurons preconditioned with oxygen-glucose deprivation.

A number of *in vivo* preconditioning models have also been developed and have been shown to impart a delayed neuroprotection. Matsushima and Hakim (1995) have shown that preconditioning with a sublethal global ischemic episode, but not a sublethal focal ischemic insult, afforded protection against a subsequent focal ischemic period. Glazier et al. (1994), have also demonstrated that focal preconditioning is able to protect the area of the cortex supplied by the middle cerebral artery (MCA) from subsequent global ischemia while areas that were not preconditioned (i.e. areas outside of MCA perfusion) were not protected.

Cortical spreading depression (CSD), first characterized by Leao in 1944, has been shown to play a dual role in ischemia. CSDs are characterized by depolarizations of neuronal and glial cells that propagate from the site of initiation outwards at a rate of approximately 3 mm/min (Leao, 1944). Though CSDs themselves are not associated with neuronal injury in the normal brain (Nedergaard and Hansen, 1988), peri-infarct depolarizations following ischemia have been shown to be detrimental in energetically compromised tissue (Mies et al., 1993).

Administered in advance of ischemia in the normal brain, however, CSDs have been shown to protect the brain from subsequent ischemic episodes (Kobayashi et al., 1995; Matsushima et al., 1996; Matsushima et al., 1998). The mechanism underlying the protective effect of CSDs has yet to be determined, though CSDs have been shown to

cause a transient rise in the extracellular levels of glutamate (Fabricius et al., 1993). Preconditioning with CSDs has also been shown to cause an attenuation of glutamate release in subsequent ischemic episodes (Douen et al., 2000). A number of trophic factors have also been shown to regulated by CSDs, including brain derived neurotrophic factor (BDNF), basic fibroblast growth factor (bFGF) and glial fibrillary acidic protein (GFAP) (Matsushima et al., 1998), though the role these factors may play in neuroprotection remains unknown and controversial, particularly in light of evidence that suggests the timing of the expression of these factors are not well correlated with neuroprotection.

Hypothesis and Rationale

Douen et al. (2000) have demonstrated that CSDs cause a down regulation of the GLT-1 and GLAST glutamate transporters from the plasma membrane. In accordance with the neurotoxic hypothesis, this would allow less glutamate to enter the synaptic cleft during ischemia, thus explaining the neuroprotective effects of CSD.

Though the role of the mGluR in CSD induced neuroprotection has not yet been explored, we believe that they may play a significant role in the down regulation of the glutamate transporters. There is increasing evidence that suggests that activation of the mGluRs is an important step in inducing neuroprotection, though this remains controversial. In a variety of different models, both *in vivo* and *in vitro*, several groups have demonstrated that the mGluRs play essential roles in neuroprotection. Chiamulera et al. (1992) have demonstrated that mGluR activation is neuroprotective in focal

ischemia, while Koh et al. (1991) showed that mGluR stimulation is neuroprotective in culture. Mount et al. (1993) and Siliprandi et al. (1992) have demonstrated similar findings in various cell culture models. In addition, activation of the mGluRs has been shown to phosphorylate ERK1/2 via MEK (Peavy and Conn, 1998), which has recently been revealed to play an essential role in CSD-induced neuroprotection (Kawaguchi et al., 1999). Thus, we wished to examine the role of the mGluRs, if any, on the down regulation of the GLT-1 transporter in the plasma membrane fraction and also investigate the role the mGluRs may have on preconditioning.

In order to determine the underlying molecular events of CSD preconditioning, we also wish to investigate the role the mitogen activated protein (MAP) kinases may play in mediating intracellular events. The role the MAP kinases may play in neuroprotection has been uncertain. In cell culture models, activation of MAP kinases stimulated survival of cells that were serum deprived, normally a signal for cells to undergo apoptosis (Parrizas et al., 1997). Shamloo et al. (1999) found that ischemic preconditioning increases phosphorylation of the ERK proteins and that increased MEK activation was also seen, particularly in the nuclei of the neuronal cell bodies. Alessandrini et al. (1999) however discovered that the inhibition of MEK was neuroprotective in a model of focal ischemia. Investigation of the role of the ERK proteins in a preconditioning paradigm may therefore lead to a greater understanding of the subcellular events that underlie this phenomenon.

Thus, we hypothesize that CSD preconditioning acts through stimulation of the mGluRs to cause intracellular physiological changes via MAP kinase, which results in

GLT-1 down regulation from the plasma membrane and neuroprotection, in accordance with the theory that reduced glutamate efflux from glutamate transporters, leading to delayed neuroprotection.

Our objectives are to:

1) Establish the effect of the mGluRs on CSD waveforms.

2) Determine the role of the mGluRs on CSD-induced plasma membrane down regulation of the GLT-1 transporter.

3) Further elucidate the pathway by which CSD activates ERK1/2.

In our first study, we attempted to determine the role of the mGluRs on CSD-induced down regulation of the glutamate transporters. A number of mGluR antagonists were administered to animals prior to the elicitation of CSDs in order to determine their effects on GLT-1 down regulation. We also investigated the effect the mGluR antagonists may have on the CSD waveforms themselves.

Our second study is an attempt to further elucidate the role MAP kinases may play in a CSD preconditioning paradigm.

The Role of Metabotropic Glutamate Receptors in Cortical Spreading Depression-Induced Down Regulation of GLT-1 From Rat Cerebral Cortex Plasma Membrane

Ava K. Chow, Fuhu Wang, Li Dong, Andre G. Douen and Matthew J. Hogan

Introduction

Though glutamate is one of the primary neurotransmitters in the central nervous system, the accumulation of this amino acid in the synapse has been implicated as an neurotoxin in a variety of neurological diseases, including epileptic brain damage (Collins and Olney, 1982), Huntington's disease and several other neurodegenerative disorders (Plaitkis et al., 1984). Studies have also shown glutamate to be at least partially responsible for mediating the mechanisms underlying ischemic brain damage (Simon et al., 1984; Rothman and Olney, 1986; Choi and Rothman, 1990; Kaku et al., 1993; Rothman and Olney, 1995).

Cortical spreading depression (CSD), first described by Leao in 1944, has been shown to impart neuroprotection in models of ischemia if administered prior to the ischemic insult (Kobayashi et al., 1995; Matsushima and Hakim, 1995; Matsushima et al., 1996; Matsushima et al., 1998). The mechanism by which CSD induces neuroprotection however, remains unclear.

The metabotropic glutamate receptors (mGluRs) are one group of glutamate receptors that may be activated as a result of glutamate release into the synapse. The role of the mGluRs in neuroprotection has so far proven to be ambiguous. Activation of group II mGluRs protects cultured neurons against neurodegeneration induced by excitotoxic concentrations of NMDA (Bruno et al., 1994; Bruno et al., 1995; Buisson and Choi, 1995; Buisson et al, 1996) and attenuates oxygen glucose deprivation-induced neuronal degeneration (Buisson and Choi, 1995). The role of the group I mGluRs in neuroprotection, however, is more uncertain. In cultured neurons, group I mGluR

agonists have been shown to aggravate neuronal damage induced by NMDA, and oxygen glucose deprivation (Buisson and Choi, 1995). On the other hand, conditions such as glutamate and kainate induced neurotoxicity in primary cultures of rat cerebellar granule neurons have shown that activation of the group I mGluRs protects against excitotoxic cell damage (Pizzi et al., 1993). Adamchik and Baskys (2000) suggest that glutamate-induced preconditioning reduces NMDA toxicity by acting through mGluR5, which belongs to the group I mGluR family, possibly through a protein kinase C (PKC)-mediated mechanism. A number of other groups have also found that activation of the mGluRs play an important neuroprotective role in both *in vivo* and *in vitro* models of ischemia (Chiamulera et al., 1992; Koh et al., 1991; Siliprandi et al., 1992).

Though no direct connection has yet been made relating CSDs and the mGluRs, CSDs have been shown to increase glutamate levels during elicitation (Iijima et al., 1998) while decreasing the levels of extracellular glutamate released three days later during ischemia (Douen et al., 2000). The mechanism of CSD induced neuroprotection is also unknown, however, CSDs are known to cause up regulation of a number of biological markers, including neuronal nitric oxide synthase (nNOS) (Shen and Gunlach, 1999), calcium-independent protein kinase C (Koponen et al., 1999), brain derived neurotrophic factor (BDNF), glial fibrillary acidic protein (GFAP) (Matsushima et al., 1998), c-fos, junB, c-jun, and (mitogen-activated protein kinase phosphatase) MKP-1 mRNA (Hermann et al., 1999). It is possible that activation of these intracellular markers may be regulated by the mGluRs particularly in light of the fact that activation of specific mGluRs can also activate many of the same biological markers. For instance,

administration of (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid (ACPD), a metabotropic glutamate receptor agonist, in the rat brain increased the mRNA of BDNF, nerve growth factor, as well as c-fos (Murray et al., 1996). Staton and Bristow (1998) also revealed that ACPD was capable of inducing cell death in cerebellar granule neurons in a concentration dependent manner, while mGluR antagonists reduced glutamate induced cell death.

This study was undertaken to determine the role of the mGluRs in CSD induced GLT-1 down regulation, as well as to determine the effect the mGluRs may have on CSDs. Using the mGluR antagonists, (RS)-1-aminoindan–1,5-dicarboxylic acid (AIDA), a specific group I antagonist, (RS)-α-methyl-4-carboxyphenylglycine (MCPG) a group II and III antagonist and (S)–2-amino–2-methyl–4-phosphonobutaoic acid (MAP4) a group III antagonist, we attempt to elucidate the role of each type of mGluR in CSD. GFAP and BDNF immunoblots were used to determine the efficacy of our CSDs since previous studies have indicated that both may be regulated by CSD stimulation (Matsushima et al, 1998, Kawahara et al., 1999, Kawahara et al., 1997).

Materials and Methods

Reagents

AIDA, MCPG and MAP4 were obtained from Tocris Cookson (Ballwin, MO, USA). Phenylmethanesulfonyl fluoride (PMSF), Tris-HCl, sodium dodecyl sulfate (SDS), bromophenol blue and $NaHCO_3$ were purchased from Sigma (St. Louis, MO, USA) and were all of analytical grade. Methanol, glycerol, NaH_2PO_4, Na_2HPO_4 and

NaCl, all of analytical grade, were obtained from BDH (Toronto, ON, Canada). 2-mercaptoethanol, TEMED, tween 20, acrylamide, bisacrylamide, ethylene diamine tetramine (EDTA), glycine, ammonium persulfate, nitrocellulose paper and the Bradford protein assay kit were obtained from Biorad (Mississauga, ON, Canada). Both the monoclonal Na^+/K^+ ATPase $\alpha 1$ subunit (110 KDa) antibody and the polyclonal GLT-1 antibody (75 KDa) antibody were obtained from Affinity Bioreagents (Golden, CO, USA). Secondary anti-mouse and anti-rabbit horseradish peroxidase conjugated antibodies were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Enhanced chemiluminescence (ECL) supplies were obtained from Amersham (Piscataway, NJ, USA).

All drugs were dissolved in 1N NaOH and then diluted with saline and 1N HCl was used to ensure the pH was between 7.2 and 7.4. AIDA was administered to the animals in an intraperitoneal (I.P.) dose of 1 mg/kg (Moroni et al., 1997). MCPG was given at a dose of 20 μg/rat in 5μL intracerebral ventricularly (I.C.V) (Bordi et al., 1996). 5μL of an 80mM solution of MAP4 was also given I.C.V. (Holscher et al., 1996). Doses, volume and injection rates were selected based on literature that indicated these parameters were capable of eliciting behavioral effects.

Surgery

Male Sprague Dawley rats were obtained from Charles River (Montreal, Canada) weighing between 250-275 grams. They were allowed to habituate to their surroundings for five days before surgical manipulation. At the time of the surgery, rats weighed

between 290-330 grams. All surgical procedures and decapitations were performed under inhalation halothane anaesthesia (4% induction, 0.75% maintenance). All procedures conformed to the guidelines outlined by the Canadian Council of Animal Care. Body temperature of the animals was monitored at all times and maintained between 36.5 and 37.5°C, using a homeothermic feedback blanket system (Harvard Apparatus, South Natick, MA, USA).

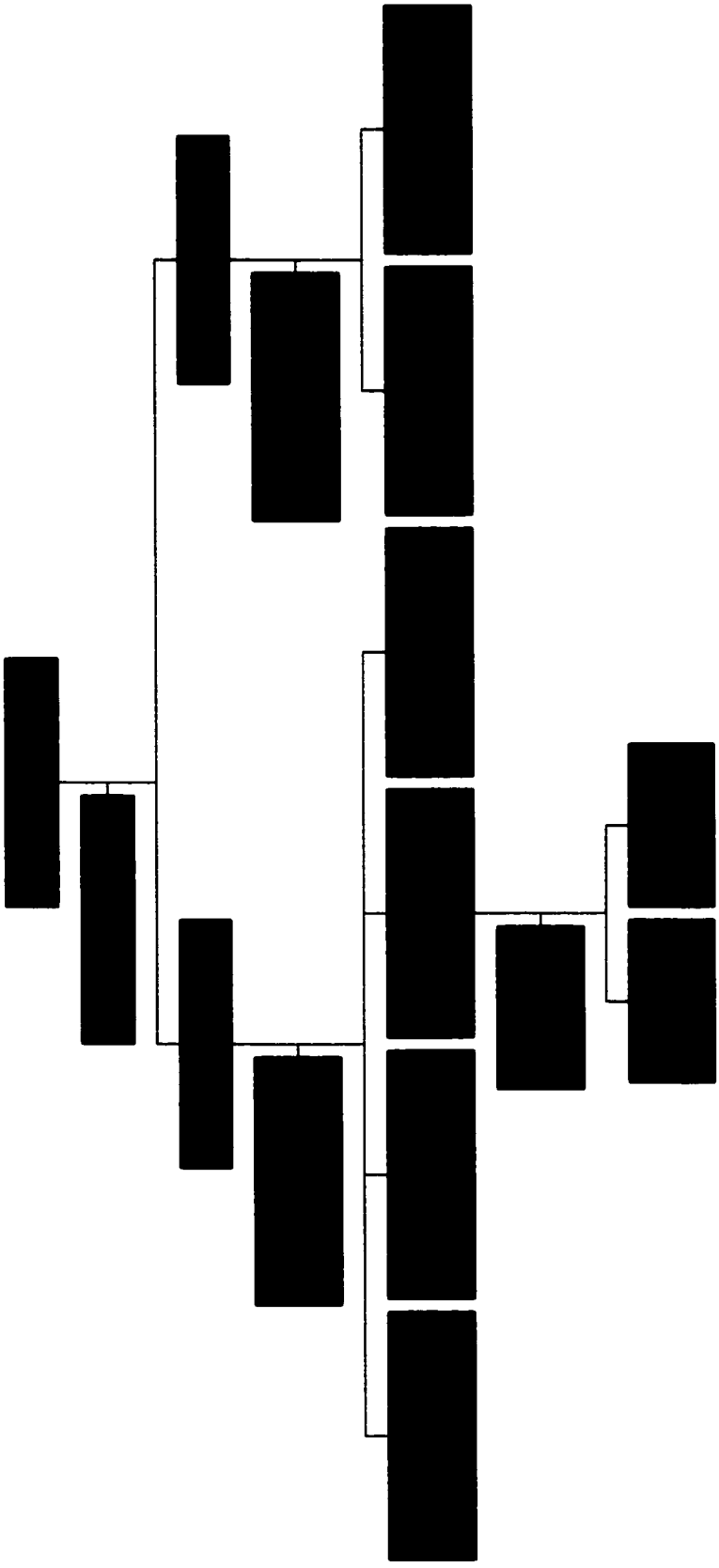
After anesthetization, the tail artery of every animal was catheterized using polyethylene tubing to monitor mean arterial blood pressure (MAP), blood gases, and blood glucose during the experiment. The animals were then mounted in a stereotaxic apparatus (Stoelting Co., Wood Dale IL, USA). A 2 mm burr hole was drilled in the skull over the left occipital cortex (Bregma AP –6.8 mm, ML 5.6 mm) without damaging the dura mater for pledget placement. A 0.5 mm diameter burr hole was placed in the left frontal cortex (Bregma AP –2.0 mm, ML 5.0 mm) without damaging the dura for platinum electrode placement. The platinum electrode was inserted 2 mm into the frontal cortex and then secured to the skull with dental cement. This electrode was used to record the CSD waves. A copper reference electrode was threaded through the skin on the back of the animal's neck. Our follow up studies used silver/silver chloride electrodes as both the recording and reference electrodes. An additional 0.5 mm burr hole was drilled in the skull (Bregma AP –0.4 mm, ML 1.3 mm) for placement of the injection cannula (Alza Corporation, Palo Alto, CA, USA) in those animals where it was required. After filling the cannula with the drug or the vehicle to be infused and elimination of any dead space, the cannula was placed at VD –3.8 mm using a stereotaxic injection arm.

The cannula was secured to the skull by dental cement. 5 μL of the drug or vehicle were infused into the animal's third ventricle over a period of 5 minutes using an injection pump (Harvard Apparatus, South Natick, MA, USA). Animals receiving I.P. injections received 0.5 mL of drug or vehicle as a bolus injection. CSDs in the left hemisphere were induced one hour after I.P. or I.C.V. injection by application of a cotton pledget soaked in 0.5 M KCl. 0.5 M NaCl was placed on the intact dura of the pledget hole for animals receiving sham treatment. The cotton pledgets were replaced every 15 minutes for two hours. In addition, blood pressure and rectal temperature was monitored and recorded every 15 minutes and blood gases and blood glucose measurements obtained every 60 minutes. Two hours after the first pledget placement, the last pledget is removed, the dura rinsed with normal saline, and the skin over the skull sutured. The tail cannula was also removed and the tail sutured. Rectal temperature was monitored and maintained between 36.5 and 37.5°C until animals recovered from anaesthesia, at which point they were returned to their cages. Several animals that were not part of the experimental groups were injected with Evans Blue immediately post-CSD through the implanted cannula to ensure correct cannula placement. All five animals revealed correct cannula placement (data not shown). Because Evans blue is an inhibitor of vesicular glutamate release (Fykse and Fonnum, 1996, Schurmann et al., 1997), and would interfere with our colorimetric protein assay, cannula placement could not be verified in experimental animals. Three days post-CSD, experimental animals were deeply anaesthetized with 4% halothane, decapitated and the brain rapidly removed for plasma membrane extraction.

Subcellular Fractionation

A subcellular fractionation technique, as previously described (Douen et al., 2000) was used to purify plasma membrane. Rats were sacrificed 3 days post-CSD and the brains removed and placed in ice cold saline. Solutions and the brain sample were maintained at 4 °C for all subsequent steps. The pial vessels were removed from the cortex and the left and right cerebral cortices dissected and processed individually. The cortices were weighed and placed in 7 mL of a solution containing 10 mM $NaHCO_3$ – 0.1 mM PMSF, pH 7.0 (buffer A). The cortices were homogenized with a teflon pestle and Wheaton glass homogenizer (VWR, Mississauga, ON, Canada) for 10 strokes at 500 rpm. The glass homogenizer and pestle were then rinsed with an additional 7 mL of buffer A and the rinse added to the homogenized sample. 1 mL of this crude homogenate was removed and stored at –80 °C until subsequent analysis. The remainder of the crude homogenate was centrifuged for 30 min. at 30 000 g. This spin yielded a pellet (P_1) and a supernatant (S_1). S_1 was decanted and centrifuged at 200 000$g_{(max)}$ for 1 hour. The pellet from this spin was re-suspended in 1mL of buffer A and both this pellet as well as the supernatant were then stored at –80 °C. P_1 was re-suspended in 5 mL of buffer A, which was subsequently layered on 5.5 mL of 35% w/v of sucrose made up in buffer A. This preparation was then centrifuged in a titanium Beckman ultracentrifuge rotor for 2 h at 150 000 $g_{(avg)}$. The fraction at the interface between the sucrose and the buffer was carefully removed, washed and re-suspended in 7 mL of buffer A. This fraction was precipitated by centrifugation at 200 000$g_{(max)}$ for 1 hour. The pellet is re-suspended in

Figure 2-1. Flowchart of the subcellular fractionation technique. The corticies are individually homogenized in buffer and then centrifuged at 30 000g for 30 minutes. The supernatant is extracted and then centrifuged at 200 000g for 1 hour to yield a pellet containing the internal membranes of the cell and a supernatant containing the cytosol. The pellet of the first centrifugation is resuspended and placed on a 35% sucrose cushion and then centrifuged for 150 000g for 2 hours to yield a pellet containing the nuclear material, a supernatant containing sucrose, the interface containing the plasma membrane and a top layer of supernatant containing buffer. The interface is carefully removed and resuspended in buffer and centrifuged at 200 000g for 30 minutes to yield a pellet of purified plasma membrane and a supernatant containing sucrose and buffer.



1.2 mL of buffer A, aliquotted and stored at –80 °C until used. A summary of the cell fractionation technique is shown in Figure 2-1. Protein content was determined through the Bradford protein assay using the Biorad protein assay kit.

Western Blotting

Equal amounts of protein, as determined by the protein assay, were applied to a 4% polyacrylamide stacking gel, followed by 10% polyacrylamide separating gel. Proteins were then electrophoretically transferred to nitrocellulose membranes for 2 hours. The nitrocellulose membranes were then rinsed for 30 minutes at room temperature with phosphate buffered saline (PBS) containing 0.1% tween 20 (TPBS) pH 7.2. Membranes were then blocked with 5% w/v powdered skim milk mixed in TPBS for 3 hours at room temperature. After washing for 10 minutes with TPBS, the nitrocellulose membranes were incubated overnight at 4°C with the appropriate primary antibody dilution (Na^+/K^+ ATPase at 1:250, GLT-1 at 1:8000) in 5% w/v powdered skim milk in TPBS. The membranes are then rinsed for 10 minutes with TPBS before incubation at room temperature with 1:5000 dilution of secondary antibody in 1% w/v powdered skim milk in TPBS. After incubation with the horseradish peroxidase conjugated secondary antibody for 3 hours, the membranes are rinsed twice with PBS. The membranes are then exposed to the ECL substrate and either exposed to Amersham Hyperfilm or to the Kodak Digital Science Imaging Station 440 (VWR, Mississauga, ON, Canada). Immunoblotting of each sample was conducted in triplicate.

Verification of the plasma membrane fraction was obtained by performing a

Western Blot using the Na^+/K^+ ATPase antibody as described by Marette et al. (1992).

Immunofluorescence

Sham and CSD animals were anaesthetized three days post-CSD using an I.P. injection of sodium pentobarbital and then transcardially perfused with a 4% paraformaldehyde solution buffered at pH 7.4 with PBS and fixed with 10% sucrose before freezing. Brains were sectioned in 12 μm thick sections and mounted on glass slides. The sections were washed for three times, 10 minutes each, in PBS and then incubated with 5% normal goat serum in PBS in a humidified box at room temperature for 60 minutes. The slides were again washed three times, 10 minutes each in PBS. The primary antibody was appropriately diluted (GFAP 1:500, BDNF 1:200) in 0.1% Triton X-100 in PBS and placed on the sections and incubated overnight in a humidified box at 4C. The slides were again washed and then incubated with the secondary antibody that is conjugated with a florescent marker at a 1:1000 dilution at room temperature for 1 hour. After rinsing once again, the slides were coverslipped with antifade and kept at –80C until viewed.

Statistical Analysis

All data are expressed as mean ± SD. Two way ANOVAs with repeated measures were used to determine differences between CSD, sham, vehicle and drug treated groups for CSD waveform data, plasma membrane GLT-1 levels and physiological variables. Independent t-tests were used to determine if differences existed

between CSD and sham-treated animals for GFAP and Na^+/K^+ ATPase levels. Western data is expressed as a ratio of left treated side compared to contralateral control (right side).

Experimental Design

Ten different experimental groups were used with an n=3 in each group:

1) Animals given an I.P. injection of AIDA (1mg/kg) were subjected to CSDs for 2 hours.
2) Animals given an I.P injection of the AIDA vehicle were subjected to CSDs for 2 hours.
3) Animals given an I.P. injection of AIDA (1mg/kg) were given sham CSD surgeries (NaCl pledget rather than KCl pledget).
4) Animals given an I.P. injection of the AIDA vehicle were subjected to sham surgery.
5) Animals given an I.C.V injection of MAP4 (5μL of 80mM) were subjected to CSD.
6) Animals given an I.C.V injection of MCPG (20μg/rat in 5μL) were subjected to CSD.
7) Animals given an I.C.V injection of the MCPG/MAP4 vehicle were subjected to CSD.
8) Animals given an I.C.V. injection of MAP4 (5μL of 80mM) were subjected to sham surgery.

9) Animals given an I.C.V. injection of MCPG (20μg/rat in 5μL) were subjected to sham surgery.
10) Animals given an I.C.V. injection of the MCPG/MAP4 vehicle were subjected to sham surgery.

All animals were sacrificed three days after the surgery date, since this time point was when Douen et al., 2000, observed maximal down regulation of GLT-1. The corticies rapidly extracted and then differentially centrifuged. The samples were then subjected to Western Blot.

For the immunohistochemistry, the animals were grouped into three experimental groups with n=3 in each group:

1) Animals receiving an I.C.V. injection of MCPG (20μg/rat in 5μL) and subjected to CSD.
2) Animals receiving an I.C.V. injection of the MCPG vehicle and subjected to CSD.
3) Animals receiving an I.C.V. injection of the MCPG vehicle and subjected to sham surgery.

Results

The physiological variables of MAP, arterial blood pH, pCO_2, pO_2, blood glucose and rectal temperature did not vary significantly between the groups or treatments (Table 2-1). The duration of the CSD waves between AIDA and the I.P. vehicle injected treatment group did not vary significantly, nor did the duration of the waves between the

MAP4 and I.C.V. injected groups. CSD wave duration was on average, approximately 75 seconds in duration. There was, however, a significant difference in the duration of the CSD waves between the MCPG treated group and the I.C.V. vehicle controls ($p<0.05$), with the MCPG treated animals having shortened CSD wave duration (vehicle: 1.24±0.007, MCPG: 1.07±0.11). The amplitude of the CSD waves was also considerably reduced in the MCPG animals with a significant difference between the treated group and control animals ($p<0.01$) (vehicle: 4.08±0.17, MCPG: 1.66±0.50) (Table 2-2). Representative waveforms recorded in an animal receiving CSD and vehicle, as well as those in an animal receiving CSD and MCPG are shown in Figure 2-2. To further investigate this phenomenon, silver/silver chloride (Ag/AgCl) recording and reference electrodes were used to measure CSD waves instead of the platinum (Pt) recording and copper reference electrodes. In an ideal situation, a standard hydrogen electrode (SHE) would be used since it has a potential difference of 0V. In biological systems, however, the hydrogen of the electrode can alter the pH of cells and the platinum may act as a catalyst to the many substances in the cell. At least one group has also discovered that long term measurements using platinum electrodes may not be accurate for measuring amplitudes. Carter and Kehr (1997) found that while using platinum electrodes to measure acetyl choline levels, that over time, there is a decrease in peak amplitude, similar to what we observe with CSDs. The accumulation of an unknown brown substance on the platinum electrode and that removal of this brown substance returned the amplitude to its original parameters. In light of these findings, we have elected to use

Table 2-1. Physiological Data. All data shown are mean ± SD with n=3 per group. Using ANOVA for statistical analysis, no significant differences were observed. Mean arterial pressure (MAP) and rectal temperature (Temp.) were recorded every 15 minutes during the surgery period while blood glucose (Blood Glu.), pH, pCO2, pO2 and hematocrit (Hct) were recorded every 60 minutes.

Condition	CSD and AIDA	CSD and no AIDA	sham and AIDA	sham and no AIDA	CSD and MAP4	CSD and MCPG	CSD and vehicle	sham and MAP4	sham and MCPG	sham and vehicle
MAP	79.7±4.7	76.3±5.9	75.4±2.1	83.6±8.6	85.1±3.6	76.6±0.7	77.1±3.0	79.4±1.4	80.7±3.4	85.7±15.3
Temp.	36.5±0.1	37.1±0.1	37.2±0.1	36.6±0.2	37.0±0.1	36.9±0.1	37.0±0.03	37.1±0.1	37.0±0.2	36.8±0.1
Blood Glu.	117.3±9.0	118.5±6.7	109.2±13.6	118.5±15.3	115.2±16.3	112.3±2.8	119.0±8.1	117.2±18.5	132.7±26.3	115.5±10.1
pH	7.4±0.1	7.4±0.01	7.4±0.2	7.4±0.02	7.4±0.02	7.4±0.02	7.4±0.03	7.4±0.02	7.4±0.03	7.4±0.03
pCO2	57.5±4.3	53.9±3.6	57.5±6.4	54.0±31	51.6±4.2	48.8±2.7	52.2±2.2	54.0±3.0	59.8±7.8	56.2±12.9
pO2	220.9±42.7	224.2±34.8	197.9±13.9	246.2±31.5	221.9±6.4	171.1±37.8	176.6±9.1	198.7±2.8	194.0±28.9	163.7±31.2
Hct	39.4±1.0	41.2±3.3	41.1±1.9	39.0±0.5	38.2±3.8	39.4±1.2	40.3±0.9	40.0±2.6	41.1±0.3	42.1±1.7

Table 2-2. CSD waveform data measured using platinum electrodes. Data shown are mean ± SD with n=3 in each group. Data are analyzed using ANOVA. Latency is the period of time between the placement of the 0.5M KCl pledget on the dura and the first CSD measured in minutes. Amplitude is the average amplitude of the CSDs observed over the 2 hour period of CSD elicitation measured in mV. Duration indicates the average length of time it takes for one CSD wave to be completed, measured in minutes. Significant differences are observed for both the amplitude and duration of the MCPG CSDs.

	# CSD	Latency	Amplitude	Duration
AIDA	11±1†	6.13±6.38	4.86±1.90	1.25±0.14
MCPG	15±1.73	13.13±6.60	1.66±0.50*	1.07±0.11*
MAP4	13±1	4.1±1.39	2.22±1.3‡	1.22±0.30
i.p. vehicle	14±1.73	2.27±0.21	6.53±1.91	1.71±0.48
i.c.v. vehicle	14.67±2.31	4.13±3.35	4.08±0.17	1.24±0.007

All values are mean ±SD

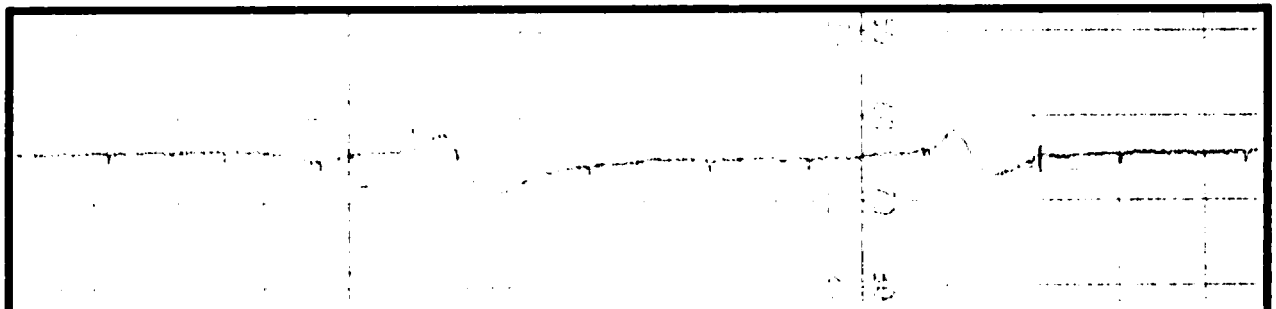
* $p \leq 0.05$ † $p=0.06$ ‡ $p=0.07$

Table 2-3. CSD waveform data using Ag/AgCl electrodes. Data shown are mean ± SD with n=3 in each group. Stsatistics are calculated using ANOVA. Latency is the period of time between the initial placement of the 0.5M KCl pledget onto the dura and the first CSD wave measured in minutes. Amplitude is the average amplitude of all of the waves observed in the 2 hour period when CSDs are elicited, measured in mV. Duration is the average length of the CSD wave measured in minutes. No significant differences are observed.

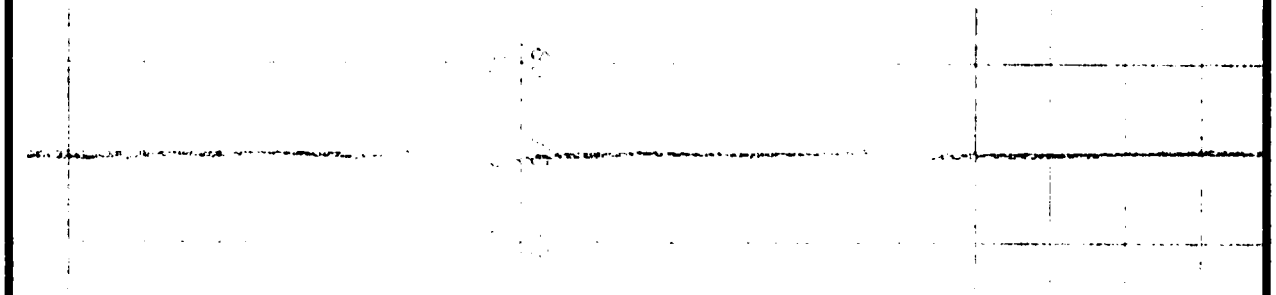
	# CSD	Latency	Amplitude	Duration
Vehicle	12±1.3	11.13±8.48	11.98±3.4	1.25±0.41
MCPG	13±1.73	12.13±6.46	10.31±2.34	1.19±0.27

All values are mean ±SD

Figure 2-2. CSD waveforms measured using platinum electrodes. Amplitude is measured on the Y-axis with each light line indicating 1mV. Time is measured on the X-axis with each dark line indicating one minute. Note the significant reduction in the amplitude of CSDs in animals treated with MCPG one hour before CSD elicitation when compared to animals who were given vehicle.

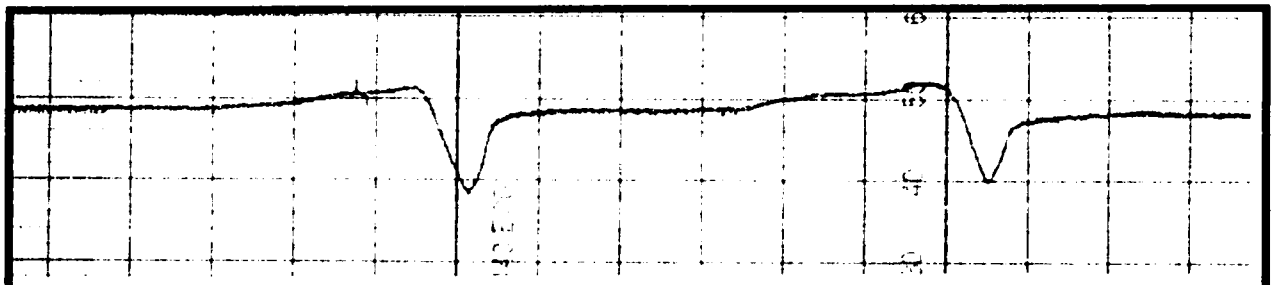


CSDs on a vehicle treated animal measured with platinum electrode

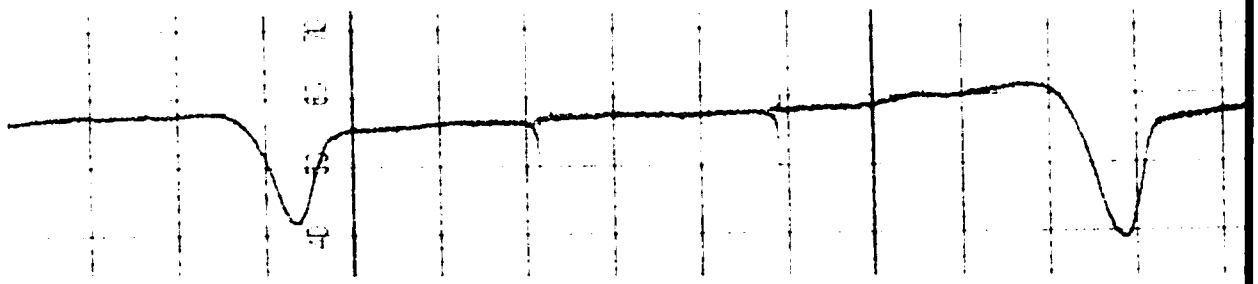


CSDs on an MCPG treated animal measured with platinum electrode

Figure 2-3. CSD waveforms measured using Ag/AgCl electrodes. . Amplitude is measured on the Y-axis with each light line indicating 1mV. Time is measured on the X-axis with each dark line indicating one minute. There is no difference between the animals given vehicle and MCPG one hour prior to elicitation of CSDs, in contrast to what is observed with the platinum electrodes.



CSDs on a vehicle treated animal measured with Ag/AgCl electrode



CSDs on an MCPG treated animal measured with Ag/AgCl electrode

a silver/silver chloride electrode which is relatively inert in biological systems, and despite the fact that this electrode carries a potential difference of 0.222V, its relationship with the SHE is well characterized. Representative CSD waveforms are shown in Figure 2-3. No significant differences in the waveforms were observed between the MCPG-treated group and the vehicle-treated group (Table 2-3).

Western blot analysis of homogenate and plasma membrane fractions with Na^+/K^+ ATPase plasma membrane protein, confirmed that the fraction dubbed the plasma membrane fraction did indeed show enrichment of this protein. There is approximately a ten-fold increase in concentration of the Na^+/K^+ ATPase in the plasma membrane fraction when compared to the homogenate. No significant differences were seen in the concentration of Na^+/K^+ ATPase between CSD and sham-treated animals (Figure 2-4). Efficacy of CSDs in effecting molecular changes was evaluated by Western blot analysis of the homogenate with GFAP, as well as immunohistochemical analysis. CSD-treated hemispheres show a significant up regulation of the GFAP protein, an indication of reactive astrogliosis, typical of CSD (1.97 ± 0.56), when compared to the contralateral hemisphere and to sham animals (1.12 ± 0.28) ($p=0.013$) (Figure 2-5).

Immunodetection for the GLT-1 protein was performed on the plasma membrane fraction extracted from the rat cerebral corticies as previously described (Douen et al., 2000). No significant difference was found between the GLT-1 levels in sham and CSD animals. Repeated measure ANOVA revealed no significant differences between the GLT-1 levels in the AIDA-treated animals and their controls (CSD and AIDA: 1.30 ± 0.34; CSD and vehicle: 0.96 ± 0.11; sham and AIDA: 1.28 ± 0.94; sham and vehicle:

Figure 2-4. Comparison of plasma membrane enrichment in CSD and sham treated animals. Data shown are mean ± SD with n=3 in each group. Semi-quantitative Western Blot analysis reveals that there is approximately a 10 fold increase in the Na+/K+ ATPase levels in the plasma membrane fraction when compared to homogenate ($p<0.01$). No significant differences, based on ANOVA analysis, are observed in enrichment of the Na+/K+ ATPase between CSD-treated animals and sham controls, or between the left and right corticies. A representative Western blot specifically probing for the 110kDa Na+/K+ ATPase is shown.

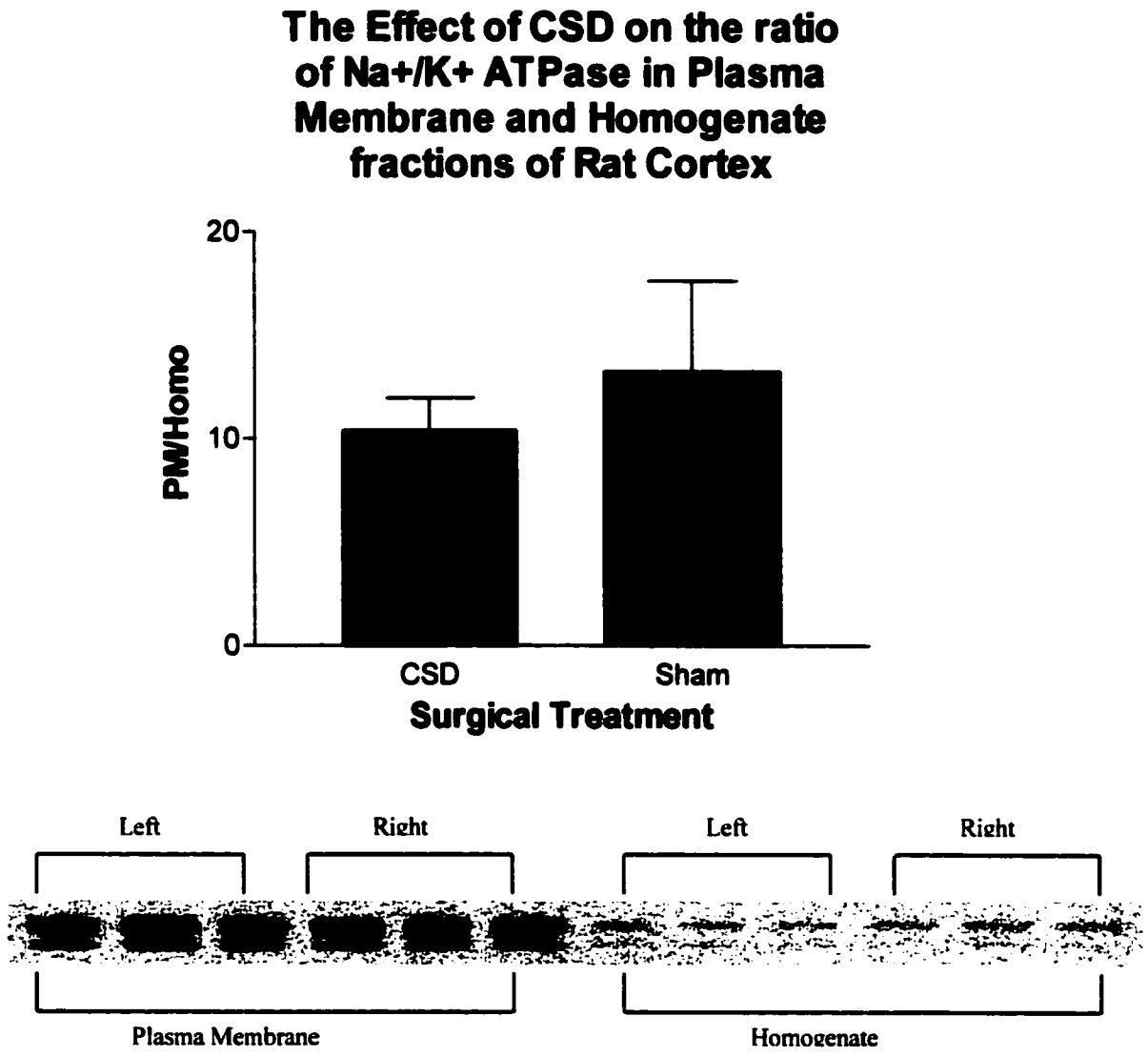

The Effect of CSD on the ratio of Na+/K+ ATPase in Plasma Membrane and Homogenate fractions of Rat Cortex
20
10
0
PM/Homo
CSD
Sham
Surgical Treatment
Left
Right
Left
Right
Plasma Membrane
Homogenate

Figure 2-5. Comparison of GFAP levels as detected by Western blot in CSD and sham treated animals. Data shown are mean ± SD with n=3 in each group. Semi-quantitative Western Blot analysis reveals that there is a significantly higher level of GFAP on the CSD treated cortex (left side) when compared to the contralateral control, as well as with sham-operated animals ($p=0.013$) as determined by ANOVA. Values are plotted as a ratio of the left side compared to the right side. A representative Western Blot is shown.

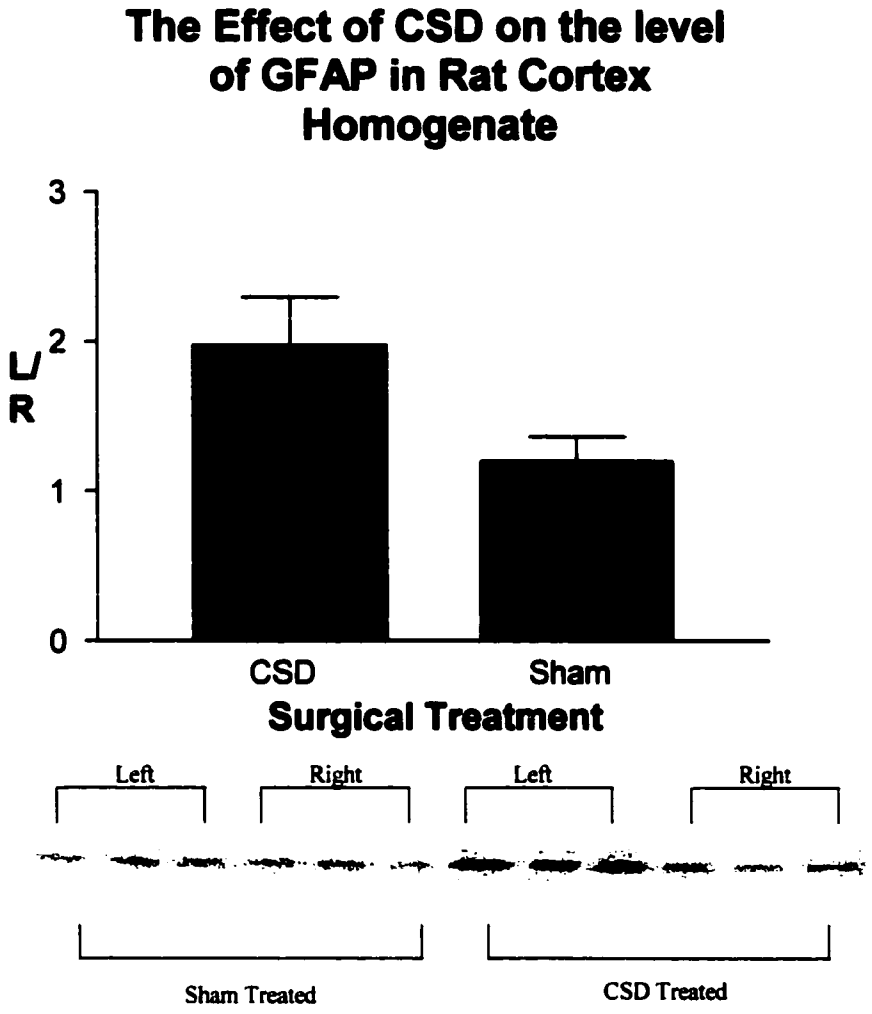
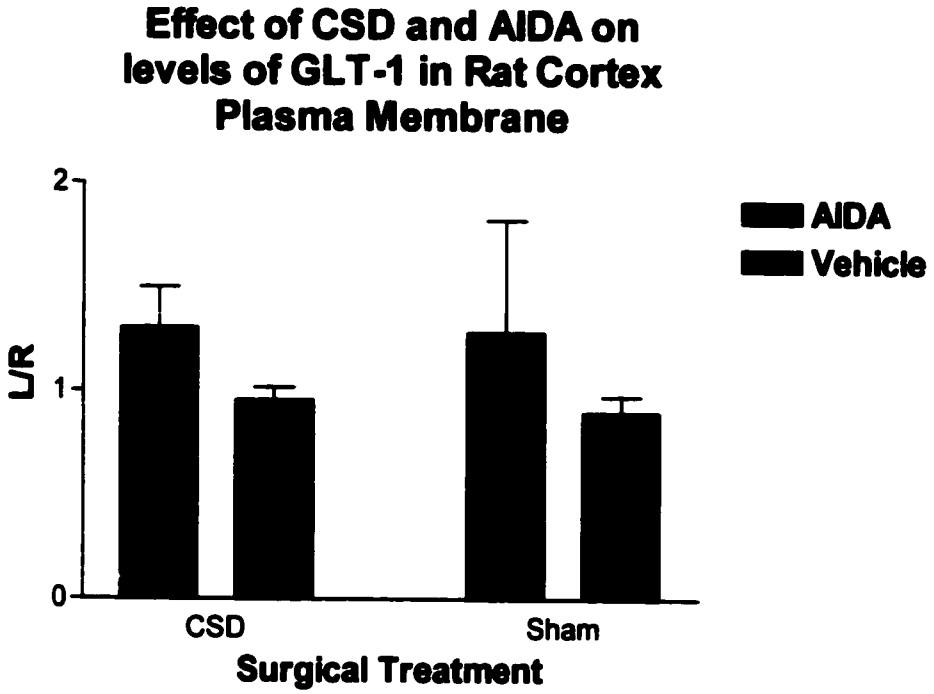

The Effect of CSD on the level of GFAP in Rat Cortex Homogenate
3
2
1
0
L/R
CSD
Sham
Surgical Treatment
Left
Right
Left
Right
Sham Treated
CSD Treated

Figure 2-6. GLT-1 Western blot measurements for sham or CSD surgically treated and AIDA or vehicle treated animals. Data shown are mean ± SD with n=3 in each group. Semi-quantitative Western blot analysis was performed on the plasma membrane fractions of animals treated with either AIDA or vehicle one hour prior to elicitation of CSDs. In each case, the left cortex is the side that is exposed to either 0.5M KCl in the case of CSD animals or to 0.5M NaCl in the case of sham animals. A left to right ratio comparison revealed that there are no significant differences between the CSD-treated animal and sham controls, or between the CSD-treated cortex and the contralateral control as evaluated by ANOVA. In addition, no significant drug effect was seen. Representative blots of each group are shown.


Effect of CSD and AIDA on
levels of GLT-1 in Rat Cortex
Plasma Membrane
2
1
0
L/R
AIDA
Vehicle
CSD
Sham
Surgical Treatment

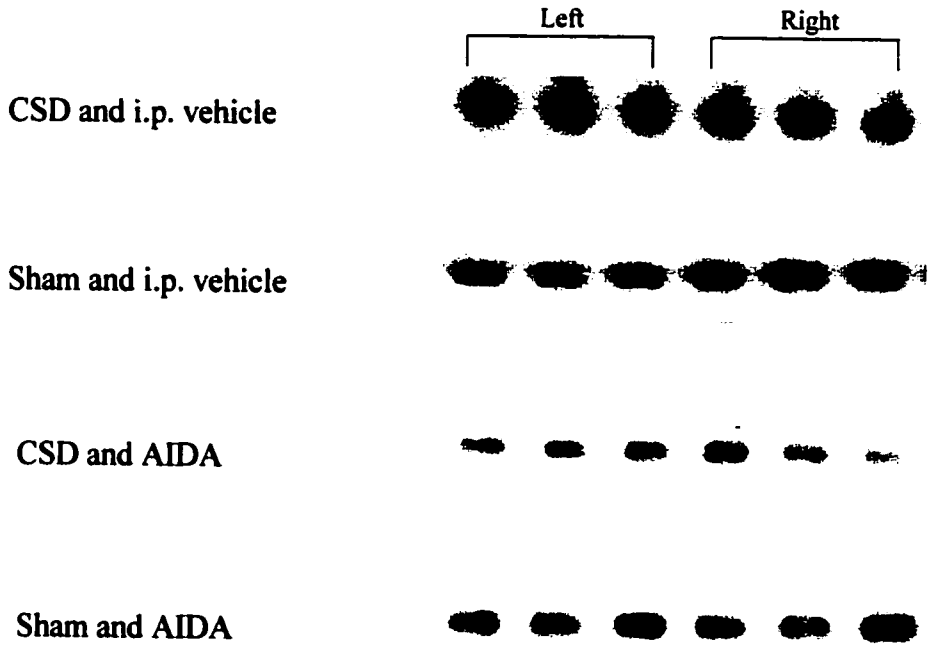

Left
Right
CSD and i.p. vehicle
Sham and i.p. vehicle
CSD and AIDA
Sham and AIDA

Figure 2-7. GLT-1 Western blot measurements for sham and CSD surgically treated and MAP4, MCPG or vehicle treated animals. Data shown are mean ± SD with n=3 in each group. No significant differences. Semi-quantitative Western blot analysis was performed on the plasma membrane fractions of animals treated with MCPG, MAP4 or vehicle one hour prior to elicitation of CSDs. In each case, the left cortex is the side that is exposed to either 0.5M KCl in the case of CSD animals or to 0.5M NaCl in the case of sham animals. A left to right ratio comparison revealed that there are no significant differences between the CSD-treated animal and sham controls, or between the CSD-treated cortex and the contralateral control as evaluated by ANOVA. No drug treatment effects were seen. Representative blots of each group are shown.

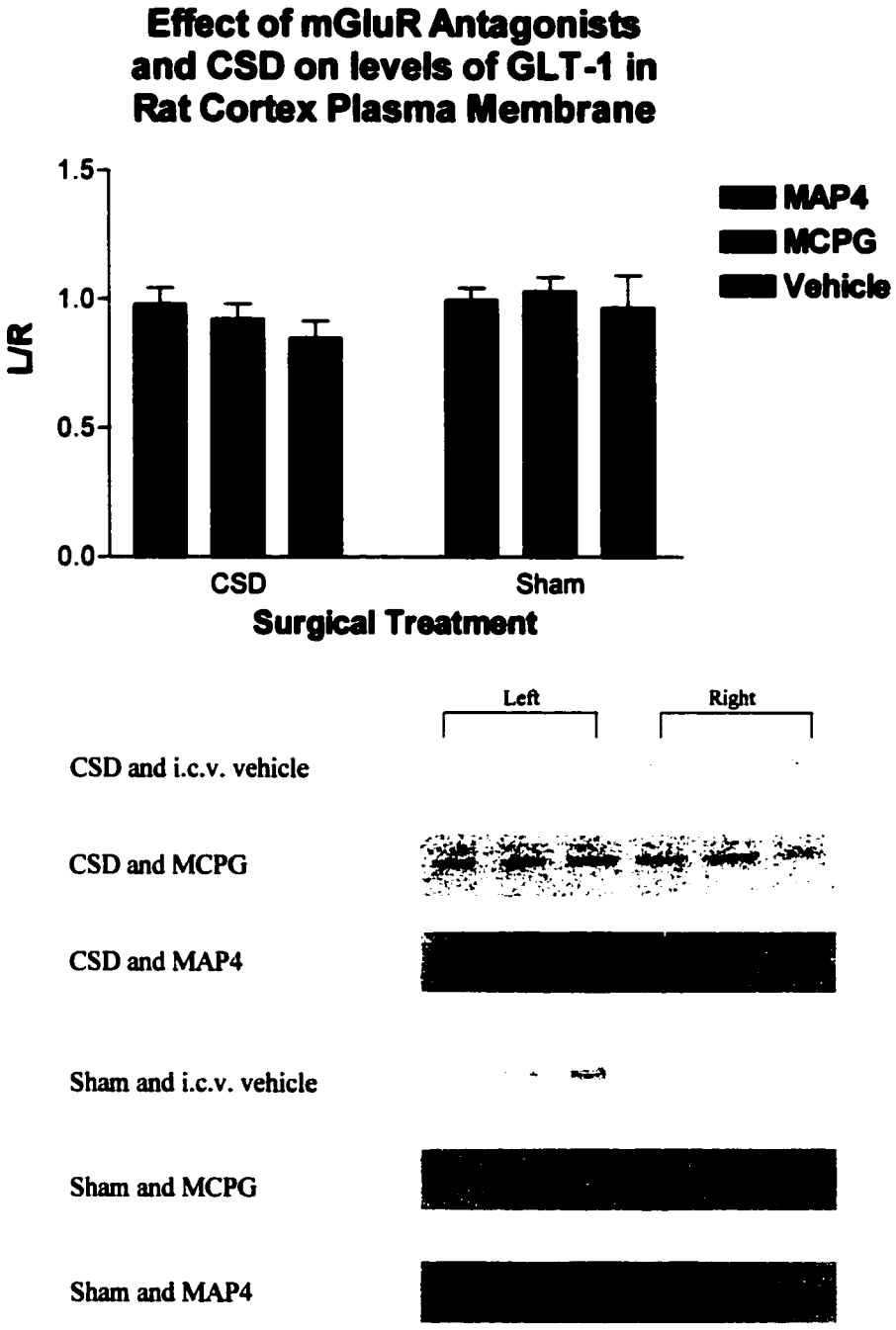

Effect of mGluR Antagonists and CSD on levels of GLT-1 in Rat Cortex Plasma Membrane
1.5
1.0
0.5
0.0
L/R
MAP4
MCPG
Vehicle
CSD
Sham
Surgical Treatment
Left
Right
CSD and i.c.v. vehicle
CSD and MCPG
CSD and MAP4
Sham and i.c.v. vehicle
Sham and MCPG
Sham and MAP4

Figure 2-8. GFAP immunohistochemistry sections on CSD treated animals. Animals subjected to a vehicle injection one hour prior to the elicitation of 2 hours of CSD on the left cortex were perfused and their brains sectioned. Antibodies specific to GFAP were used to probe these sections. Animal #6280 and 6365 show increased immunoreactivity on the CSD treated cortex when compared to the contralateral control. Few conclusions can be made about animal # 6279 since there is high background on the contralateral control.

Animal #	Left	Right
6279	[redacted]	[redacted]
6280	[redacted]	[redacted]
6365	[redacted]	[redacted]

Figure 2-9. GFAP immunohistochemistry sections on CSD and MCPG treated animals. Animals subjected to an MCPG injection one hour prior to the elicitation of 2 hours of CSD on the left cortex were perfused and their brains sectioned. Antibodies specific to GFAP were used to probe these sections. Animal #6278 and 6290 show increased immunoreactivity on the CSD treated cortex when compared to the contralateral control. Few conclusions can be made about animal # 6277 since there is no apparent immunoreactivity on either the CSD-treated side or the contralateral cortex.

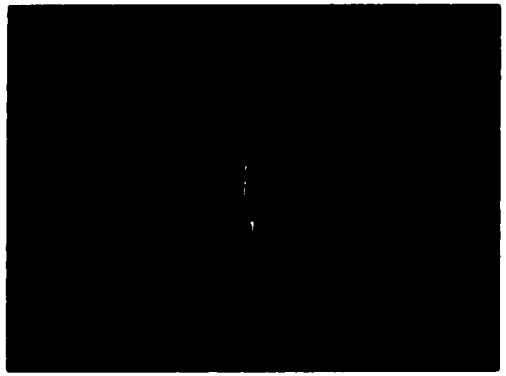
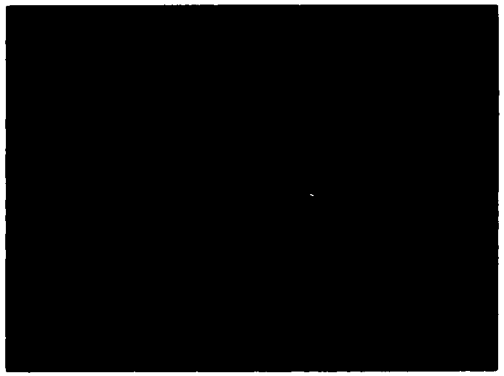
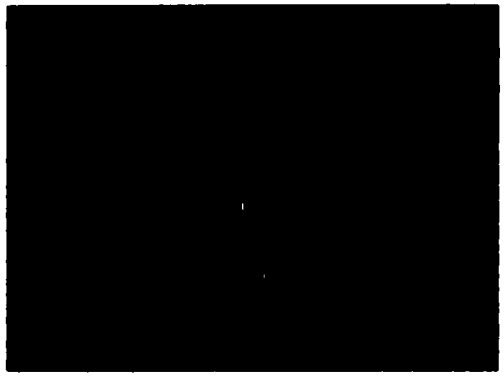
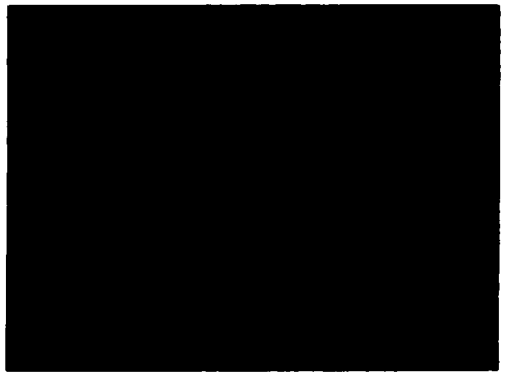
Animal #	Left	Right
6277		
6278		
6290		

Figure 2-10. GFAP immunohistochemistry sections on sham treated animals. Animals subjected to a vehicle injection one hour prior to the placement of a cotton pledget with 0.5M NaCl for 2 hours on the left cortex were perfused and their brains sectioned. Antibodies specific to GFAP were used to probe these sections. No differences in immunoreactivity are observed between the left and right corticies in any of the animals.

Animal #	Left	Right
6273	[redacted]	[redacted]
6274	[redacted]	[redacted]
6281	[redacted]	[redacted]

Figure 2-11. BDNF immunohistochemistry sections on CSD treated animals. Animals subjected to a vehicle injection one hour prior to the elicitation of 2 hours of CSD on the left cortex were perfused and their brains sectioned. Antibodies specific to BDNF were used to probe these sections. No obvious left versus right differences were seen in any of the animals.

Animal #	Left	Right
6279	[redacted]	[redacted]
6280	[redacted]	[redacted]
6365	[redacted]	[redacted]

Figure 2-12. BDNF immunohistochemistry sections on CSD and MCPG treated animals. Animals subjected to an MCPG injection one hour prior to the elicitation of 2 hours of CSD on the left cortex were perfused and their brains sectioned. Antibodies specific to BDNF were used to probe these sections. No obvious left versus right differences were seen in any of the animals.

Animal #	Left	Right
6277	[redacted]	[redacted]
6278	[redacted]	[redacted]
6290	[redacted]	[redacted]

Figure 2-13. BDNF immunohistochemistry sections on sham treated animals. Animals subjected to a vehicle injection one hour prior to the placement of a 0.5M pledget of NaCl on the left cortex were perfused and their brains sectioned. Antibodies specific to BDNF were used to probe these sections. No obvious left versus right differences were seen in any of the animals.

Animal #	Left	Right
6273	[redacted]	[redacted]
6274	[redacted]	[redacted]
6281	[redacted]	[redacted]

0.90± 0.14) (Figure2-6), as well as in the MAP4 or MCPG-treated animals and their controls (CSD and MAP4: 0.98 ± 0.11; CSD and MCPG: 0.92 ± 0.10; CSD and vehicle: 0.85 ± 0.12; sham and MAP4: 1.00 ± 0.08; sham and MCPG: 1.03 ± 0.09; sham and vehicle: 0.97 ± 0.22) (Figure 2-7). No significant changes were seen in GLT-1 protein expression levels in the plasma membrane fractions between the treated cortex and contralateral controls.

Qualitative examination of the immunohistochemistry results showed that there was increased GFAP reactivity in the CSD treated cortex compared to the contralateral control as well as to the sham treated controls. There is no apparent effect of the MCPG on GFAP reactivity (Figures 2-8, 2-9, 2-10). There is no apparent difference between the CSD treated cortex and the contralateral control, as well as between the CSD and sham treated animals in terms of BDNF reactivity (Figures 2-11, 2-12, 2-13).

Discussion

The purpose of these experiments was to determine the role of the mGluRs in the down regulation of the GLT-1 transporters in the rat cortical plasma membranes three days post-CSD, as well as attempt to find a pharmacological intervention that could eliminate CSD-induced neuroprotection without affecting the CSDs themselves. Maki et al. (1994) provided a possible link between the glutamate transporters and the mGluRs. In their experiments, they found that L-glutamate mimicked many of the effects of L-trans-PDC, a glutamate transporter inhibitor and that MCPG blocked the decrease in both the NMDA and non-NMDA mediated excitatory postsynaptic currents induced by L-

trans-PDC. Since regulation of the mGluRs has also been shown to regulate glutaminergic synaptic transmission (Dube and Marshall, 1997), it was feasible that we examine the role of the mGluRs in the down regulation of the GLT-1 transporters in the plasma membrane fraction, as previously reported (Douen et al., 2000), and investigate the role the mGluRs may have on CSD preconditioning.

The results obtained here indicate that KCl-elicited CSD in the rat does not affect the plasma membrane levels of GLT-1 found in the rat cortex three days post-CSD. This is in contrast to the studies previously conducted by Douen et al., (2000) where the time course of GLT-1 down regulation in the CSD-treated hemisphere showed maximal down regulation three days post-CSD.

In attempts to replicate the results obtained previously, we have carefully examined our surgical and molecular techniques. Due to refinement of the surgical methodology, no injury to the brain was seen where the cotton pledget was placed, though CSDs were observed, as recorded by the platinum electrode. This is in contrast to the animals with GLT-1 down regulation, where a small area of damage was seen on the brain directly where the pledget was placed. This damage may have led to initiation of CSDs during the three-day recovery period, which may have been a factor in down regulating the GLT-1 protein. In order to replicate this damage, 3M KCl was placed on the dura for two hours, rather than 0.5M KCl. Damage was seen after removal of the brain three days later and was photographed. Areas anterior to the area of damage, but not including the damaged area, were removed and fractionated according to the same subcellular fractionation protocol as used previously. No down regulation of the GLT-1

was seen in the CSD-treated cortex (data not shown). In one study, the area damaged with 3M KCl was included in the subcellular fractionation but again, no change in the levels of GLT-1 plasma membrane protein was observed (data not shown).

To investigate the effectiveness of our subcellular fractionation technique, the fraction that was dubbed the plasma membrane was probed with Na^+/K^+ ATPase to ensure that enrichment of the plasma membrane proteins had occurred. Results indicate that there was significantly more Na^+/K^+ ATPase found in the plasma membrane fraction when compared to the crude homogenate, suggesting that plasma membrane protein enrichment had indeed occurred.

In order to further purify the plasma membrane fraction and eliminate any possible contaminants, several of the samples which demonstrated no GLT-1 down regulation on the CSD treated cortex were selected and re-homogenized and then centrifuged again according to the subcellular fractionation technique previously established (Douen et al., 2000). Western blot analysis following re-spinning showed no differences in GLT-1 levels between the CSD treated cortex and the internal contralateral control.

The up regulation of glial acidic fibrillary protein (GFAP) is a hallmark of CSD (Kawahara et al., 1999; Matsushima et al., 1998; Bonthius et al., 1994). To ensure the efficacy of the CSDs, the crude homogenate in several samples was subjected to Western blots with the GFAP antibody. Results indicate that up regulation of GFAP did occur. Immunohistochemical results with other samples supported this observation. It is interesting to note, however, that the increase of GFAP in these samples does not appear to be as dramatic as those previously observed. The number, duration and amplitude of

the CSDs in these studies were not different from those previously obtained (Douen et al., 2000, Matsushima et al., 1996).

The immunohistochemical results illustrating a lack of BDNF reactivity is also puzzling, especially since several groups have reported that CSDs are capable of up regulating both BDNF mRNA (Kawahara et al., 1997, Kariko et al., 1998, Matsushima et al., 1998) and BDNF protein (Kawahara et al. 1997, Yanamoto et al., 2000), though we may not have detected an effect due to the time dependent nature of BDNF protein expression.

The lack of GLT-1 down regulation is particularly bewildering, especially in light of evidence that suggests glutamate transporters are indeed regulatable proteins. For example, Ginsberg et al., (1995) showed that GLT-1 and GLAST, both glial transporters, can be down regulated *in vivo* following regional deafferentation, while the neuronal transporter, EAAC1 was not affected, mirroring the results obtained by Douen et al., (2000). Likewise, Rao et al. (1998) demonstrated in a model of percussion injury, which is known to cause spreading depression like depolarizations (Sunami et al., 1989; Rogatsky et al., 1996; Kubota et al., 1989), that GLT-1 and GLAST are down regulated in the cortex exposed to percussion injury.

The effect of the mGluR antagonist MCPG on the amplitude of the CSDs, as recorded by the platinum electrode can be explained by the results obtained by Contractor et al., (1998). They found that commercially available preparations of mGluR agonists and antagonists, including MCPG, had direct effects on native and recombinant NMDA receptors. MCPG, as well as AIDA was found to significantly inhibit NMDA/glycine

induced currents in HEK cells (Contractor et al., 1998). The effect of MCPG on synaptic NMDA receptors however, is highly dependent on the concentrations of NMDA and glycine applied. At saturating concentrations of glycine and NMDA, native NMDA receptors were not affected by MCPG. In addition, Pisani et al. (1997) found that MCPG is capable of preventing NMDA induced membrane depolarization in striatal neurons. Likewise, a number of other groups have discovered that NMDA potentiation is greatly reduced by mGluR antagonists (O'Connor et al., 1994; Colwell and Levine, 1994). This may explain the variability found between animals in our study.

Our sample size of three animals per group was initially selected based on the robust findings of Douen et al., (2000) which demonstrated an 80% down regulation of the GLT-1 transporter three days post CSD. In our current study, however, this GLT-1 down regulation was not seen, though there is a suggestions that AIDA may have an effect on GLT-1 levels. The power of our analysis, however, was not high enough to conclusively determine if this is the case and our small sample size may have led to a type II error. Coupled with Carter and Kehr's (1997) discovery that platinum electrodes may not accurately measure amplitude over prolonged periods of time, no conclusive evidence was found to either prove or disprove the role of mGluR in CSD induced GLT-1 down regulation.

Acknowledgements

Thanks go out to Fuhu Wang for conducting some of the CSD surgeries, Li Dong for the sectioning and immunohistochemistry of the majority of the brain sections and Andre Douen for demonstrating the subcellular fractionation technique and for academic advice.

Cortical Spreading Depression Induced Phosphorylation of ERK 1 and ERK 2 in Rat Cerebral Cortex

Ava K. Chow and Matthew J. Hogan

Introduction

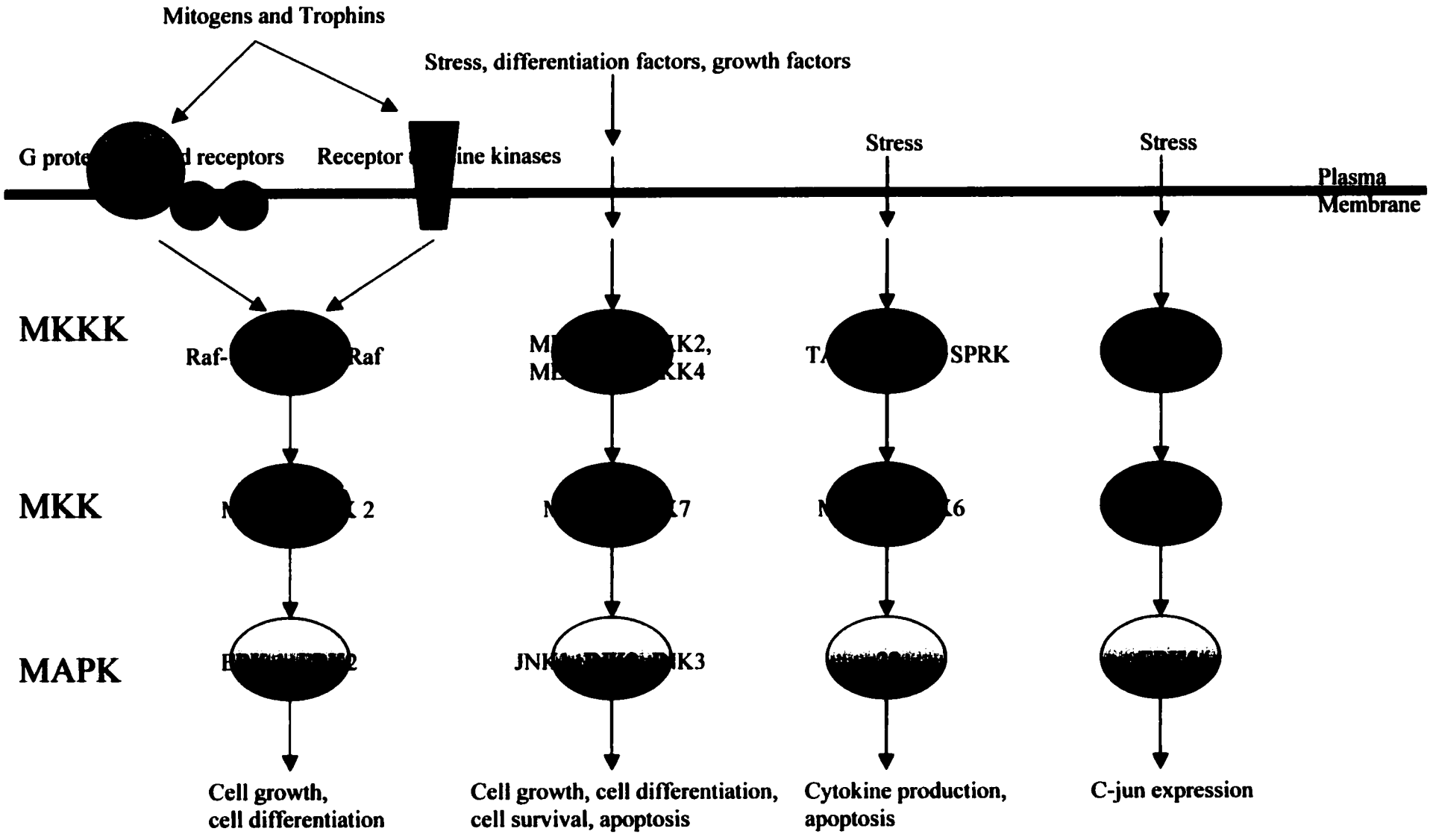
CSD induced preconditioning has been shown to affect the levels of glutamate in the brain both during CSD as well as during subsequent ischemia. Fabricius et al. (1993) have demonstrated that CSD causes a transient rise in extracellular glutamate levels, while Douen et al. (2000) have demonstrated that preconditioning with CSD three days prior to ischemia lowers the extracellular glutamate levels during ischemia, as determined by microdialysis. This released glutamate can have a number of different targets, including NMDA and AMPA channels; the ionotropic glutamate receptors, and the metabotropic glutamate receptors.

Though it is not yet known which of these receptors is responsible for mediating neuroprotection, up regulation of a number of biological markers by CSD is seen, including neuronal nitric oxide synthase (nNOS) (Shen and Gundlach, 1999), calcium-independent protein kinase C (Koponen et al., 1999), brain derived neurotrophic factor (BDNF), glial fibrillary acidic protein (GFAP) (Matsushima et al., 1998), c-fos, junB, c-jun, and MKP-1 mRNA (Hermann et al., 1999).

Several studies have also indicated that mitogen-activated protein (MAP) kinases may play a significant role in the induction of neuroprotection. The MAP kinase cascades are composed of three kinases that are sequentially phosphorylated for activation (Widmann et al., 1999). Four types of MAP kinase pathways have been identified in mammalian cells to date, with extracellular signal regulated kinase (ERK) pathway being the most described (Figure 3-1).

The ERK1 and ERK2 (p44 and p42, respectively) are directly activated by MEK1

Figure 3-1. The MAP kinase cascades. Four main pathways have been identified in mammalian cells to date with the ERK 1/2 pathway being the most described. Each MAP kinase pathway is characterized by a series of three kinases that are sequentially phosphorylated for activation. The MAP kinase cascades can be activated by a variety of factors including G protein coupled receptor activation, receptor tyrosine kinase activation, stress and various growth factors.


Mitogens and Trophins
Stress, differentiation factors, growth factors
Stress
Stress
Plasma Membrane
MKKK
MKK
MAPK
Cell growth, cell differentiation
Cell growth, cell differentiation, cell survival, apoptosis
Cytokine production, apoptosis
C-jun expression

and MEK2, which are in turn activated by a variety of MAP kinase kinase kinases, including c-raf. MEK1 phosphorylates ERK1 and ERK2 on threonine and tyrosine residues, thus activating these MAP kinases (Seger and Krebs, 1995). The main physiological stimuli for activation of the ERK pathway include activation of G-protein coupled receptors, such as the metabotropic glutamate receptors (mGluRs) (Peavy and Conn, 1998) and receptor tyrosine kinases (RTK) (Grosse et al., 2000) by growth factors (Egea et al., 2000) and various cellular stresses (Korneyev, 1998).

Calcium influx through the NMDA channels following stimulation with glutamate has been shown to induce immediate early gene expression that is dependent on the activation of the ERK proteins (Xia et al., 1996). The propagation of CSD has been shown to be dependent on the NMDA channels (Obrenovitch and Zilkha, 1996) and blocking these channels eliminates CSD neuroprotection (Busch et al., 1996).

The role of the MAP kinases in neuroprotection, however, has been equivocal. Activation of the MAP kinases have been shown to promote the survival of PC12 cells that were stimulated to undergo apoptosis by serum withdrawal (Parrizas et al., 1997), though the pathways by which it acts is not yet known. MAP kinases are hypothesized to mediate their neuroprotective effect by mediating down stream signals. MAP kinases have been shown to enhance c-fos expression (Wang and Prywes, 2000) and increased c-fos expression has been linked with cell survival in models of focal and global ischemia (Uemura et al., 1991; Uemura et al., 1991a). Shamloo et al. (1999) found that ischemic preconditioning increases phosphorylation of the ERK proteins and that increased MEK activation was also seen, particularly in the nuclei of the neuronal cell bodies. A number

of neuroprotective agents have also been shown to stimulate ERK phosphorylation, including brain derived neurotrophic factor (BDNF) (Hetman et al., 1999) and estrogen (Singer et al., 1999). In contrast, Alessandrini et al., (1999) have discovered that the prevention of activation of the ERK proteins is neuroprotective in an *in vivo* model of ischemia. Inhibition of the p44/42 MAP kinase pathway has also been shown to be neuroprotective in hippocampal cell culture models of seizure (Murray et al., 1998). This may indicate that the timing of ERK phosphorylation with relation to the noxious stimuli may play a significant role in whether it is neuroprotective or neurotoxic. Kawaguchi et al. (1999) have described the interaction between CSD and the p44/42 MAP kinase pathways. They discovered that CSD is capable of causing phosphorylation of p44/42 MAP kinases and that blockage of this phosphorylation using the MEK inhibitor PD98059 eliminates the neuroprotective effect of CSD, suggesting that ERK phosphorylation is an essential component in CSD induced neuroprotection.

These preliminary experiments were designed to explore the role of the MAP kinases as down stream regulators in CSD in an attempt to elucidate possible mechanisms of neuroprotection.

Materials and Methods

Reagents

Monoclonal antibodies to the phosphorylated form of ERK1/ERK2 were obtained from New England Biolabs (Beverly, MA, USA). Secondary antibodies conjugated with horseradish peroxidase were purchased from Santa Cruz Biotechnology (Santa Cruz, CA,

USA). Enhanced chemiluminescence reagents were obtained from Amersham (Piscataway, NJ, USA). Phenylmethanesulfonyl fluoride (PMSF), Tris-HCl, sodium dodecyl sulfate (SDS), bromophenol blue and $NaHCO_3$ were purchased from Sigma (St. Louis, MO, USA) and were all of analytical grade. Methanol, glycerol, NaH_2PO_4, Na_2HPO_4, KCl and NaCl, all of analytical grade, were obtained from BDH (Toronto, ON, Canada). 2-mercaptoethanol, TEMED, tween 20, acrylamide, bisacrylamide, ethylene diamine tetramine (EDTA), glycine, ammonium persulfate, nitrocellulose paper and the Biorad DC protein assay kit (based on the method of Lowry et al., (1951)) were obtained from Biorad (Mississauga, ON, Canada).

Surgery

CSD were performed as previously described. Sham-operated animals were treated as CSD animals but received 0.5M NaCl in lieu of 0.5M KCl.

Homogenization

The homogenization protocol was adapted from Berman et al., 1998. Rats were decapitated and the brains rapidly removed and placed immediately in ice cold saline. All of the remaining steps were conducted at 4°C. The visible pial vessels were removed from the surface of the brain. The cortex of the brain was carefully removed from the underlying structures and the left and right cortices separated and processed individually. The cortices were then weighed just prior to homogenization with a Teflon pestle and Wheaton glass homogenizer (VWR, Mississauga, ON, Canada) for 10 strokes at 500 rpm

in 7 mL of SDS sample buffer containing 10% glycerol, 5% 2-mercaptoethanol, 2.3 % SDS and 62.5mM Tris-HCl, pH 6.8. The samples were boiled for 5 minutes and immediately frozen in -80°C until used.

Western Blot Analysis

Protein content was determined using the Biorad DC protein assay kit that is based on Lowry's method (Lowry et al., 1951). Equal amounts of protein (50 μg) applied to a 10% polyacrylamide gel according to the method of Laemmli (1970) and underwent electrophoresis for 2 hours. The proteins were then electrophoretically transferred to a nitrocellulose membrane for an additional 2 hours. Membranes were rinsed for 10 minutes with phosphate buffered saline (PBS) with 0.1% tween-20 pH 7.2(TPBS) prior to blocking. Non-specific proteins were blocked with 5% w/v powdered skim milk in TPBS for 3 hours. The nitrocellulose membrane is then rinsed with TPBS for 10 minutes. The phospho-specific antibody to ERK1/ERK2 was diluted 1:1000 in 1% w/v milk TPBS and incubated with the membranes overnight at 4°C. The membranes were again rinsed with TPBS for 10 minutes prior to a 3 hour incubation with the horseradish peroxidase conjugated secondary antibody. After rinsing twice with PBS, membranes were exposed to the ECL substrate and quantified using the Kodak Digital Science Imaging Station 440 (VWR, Mississauga, ON, Canada). All samples were run in triplicate to ensure accurate and even loading.

Results

Animals with a NaCl soaked pledget applied to the dura over the occipital lobe showed no CSD waves while those animals with KCl pledgets showed recurrent CSD waves, with an average of 12±2 waves over the two hour period. CSD duration averaged 68±7 seconds, which is consistent with previous reports of CSD (Douen et al., 2000).

Western blot analysis of the homogenized cortex using an antibody specific for the phosphorylated form of ERK showed significantly higher levels in the hemisphere receiving CSDs when compared to animals receiving sham treatment ($p<0.01$), as well as the contralateral hemisphere ($p<0.01$). No significant differences were seen between the hemispheres in animals receiving sham treatment (Figure 3-2). To determine the upstream activators of the p42/p44 MAP kinase system, we then probed specifically for the phosphorylated form of c-Raf, a classical upstream regulator of ERK phosphorylation. Western blot comparisons of the band at ~77 kDa showed that there are no significant differences between sham-operated and CSD-treated animals, as well as no differences between the CSD-treated hemisphere and the contralateral hemisphere (Figure 3-3). We then investigated the phosphorylation state of MEK1/2, the classical immediate upstream activator of ERK1 and ERK2. Western blots for the phosphorylated form of MEK1/2 showed no difference between the hemisphere receiving CSDs and the contralateral hemisphere. There were also no differences between sham and CSD treated animals (Figure 3-4). No differences were seen between CSD and sham treated animals in Western blots probing for the phosphorylated form of SAPK/JNK (Figure 3-5).

Figure 3-2. Phosphorylated ERK1/2 Western blot measurements for CSD and sham treated animals. Data shown are mean ± SD with n=3 in each group. After 2 hours of KCl elicited CSD on the left cortex, or 2 hours of exposure to 0.5M NaCl, in the case of sham animals, the corticies were rapidly removed, homogenized and were run in Western Blots probing specifically for the phosphorylated form of ERK1/2. Using semi-quantitative Western Blot analysis, left verses right comparisons were plotted, showing that CSD treated animals showed significantly higher levels of phosphorylated ERK when compared to the contralateral cortex, as well as to sham animals ($p<0.01$).

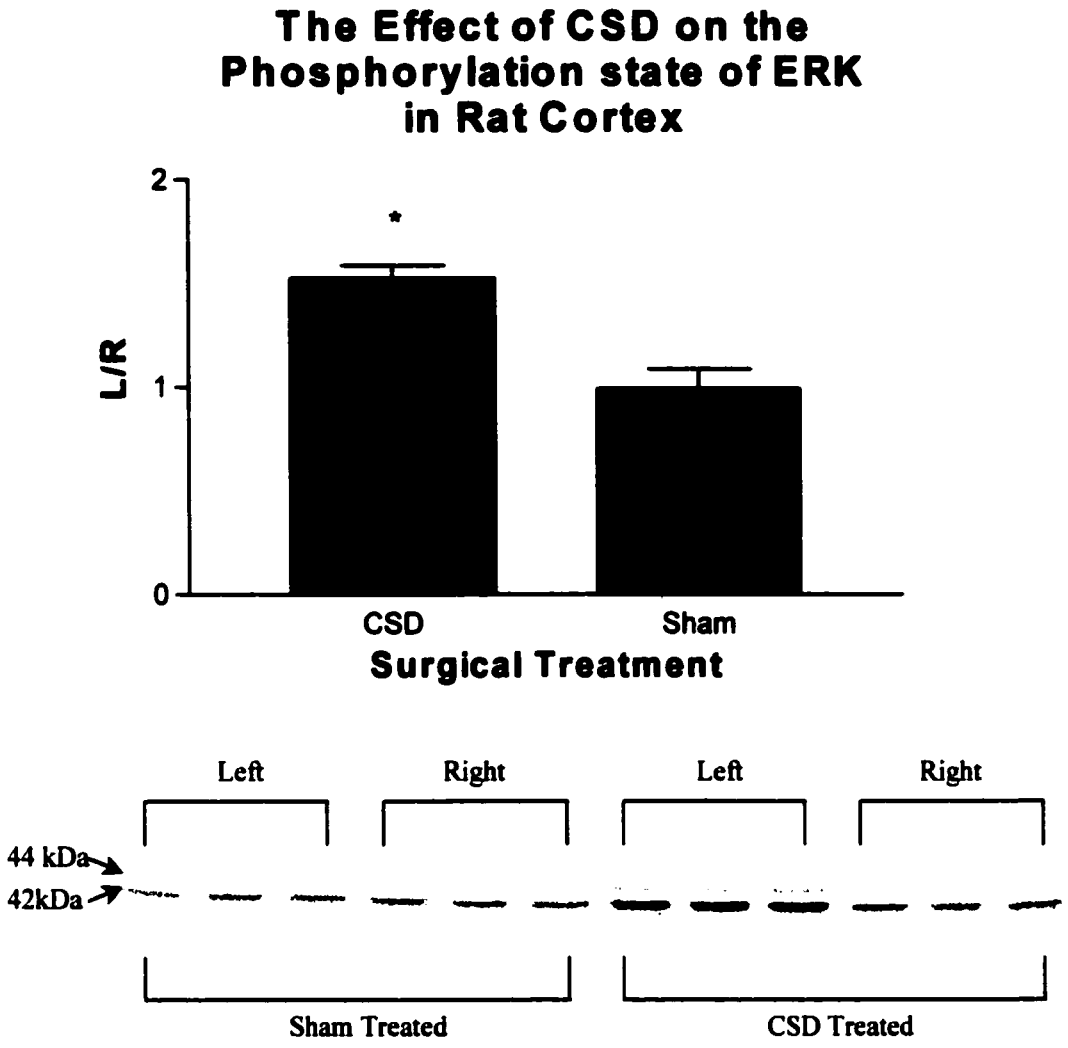

The Effect of CSD on the Phosphorylation state of ERK in Rat Cortex
L/R
2
1
0
*
CSD
Sham
Surgical Treatment
Left
Right
Left
Right
44 kDa
42kDa
Sham Treated
CSD Treated

Figure 3-3. Phosphorylated c-Raf Western blot measurements for CSD and sham animals. Data shown are mean ± SD with n=3 in each group. After 2 hours of KCl elicited CSD on the left cortex, or 2 hours of exposure to 0.5M NaCl, in the case of sham animals, the corticies were rapidly removed, homogenized and were run in Western Blots probing specifically for the phosphorylated form of c-Raf. Using semi-quantitative Western Blot analysis, left verses right comparisons were plotted, showing that no significant differences are seen in the amount of phosphorylated c-Raf between the CSD-treated cortex and the contralateral control, as well as between the CSD-treated animals and sham controls..

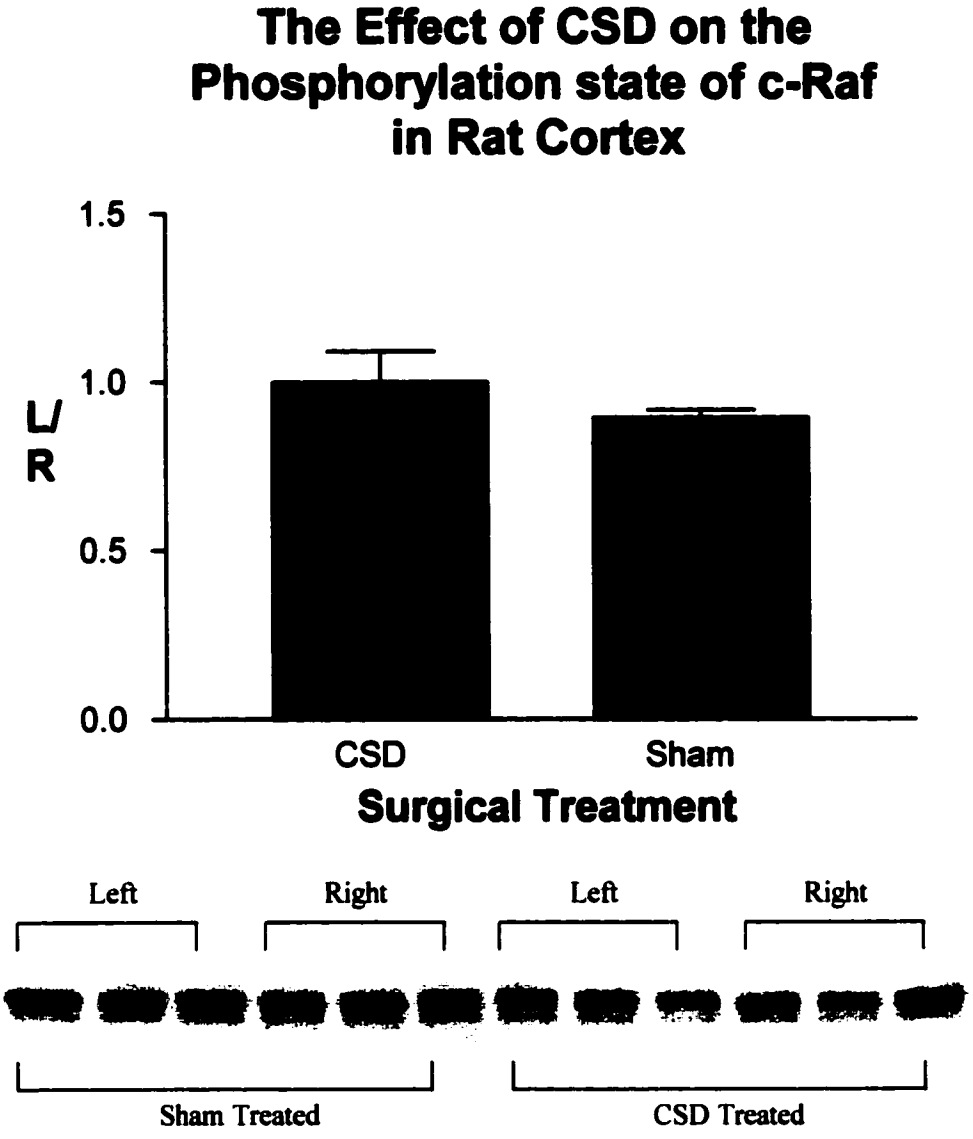

The Effect of CSD on the Phosphorylation state of c-Raf in Rat Cortex
1.5
1.0
0.5
0.0
L/R
CSD
Sham
Surgical Treatment
Left
Right
Left
Right
Sham Treated
CSD Treated

Figure 3-4. Phosphorylated MEK1/2 Western blot measurements for CSD and sham treated animals. Data shown are mean ± SD with n=3 in each group. After 2 hours of KCl elicited CSD on the left cortex, or 2 hours of exposure to 0.5M NaCl, in the case of sham animals, the corticies were rapidly removed, homogenized and were run in Western Blots probing specifically for the phosphorylated form of MEK1/2. Using semi-quantitative Western Blot analysis, left verses right comparisons were plotted, showing that no significant differences are seen in the amount of phosphorylated MEK1/2 between the CSD-treated cortex and the contralateral control, as well as between the CSD-treated animals and sham controls..

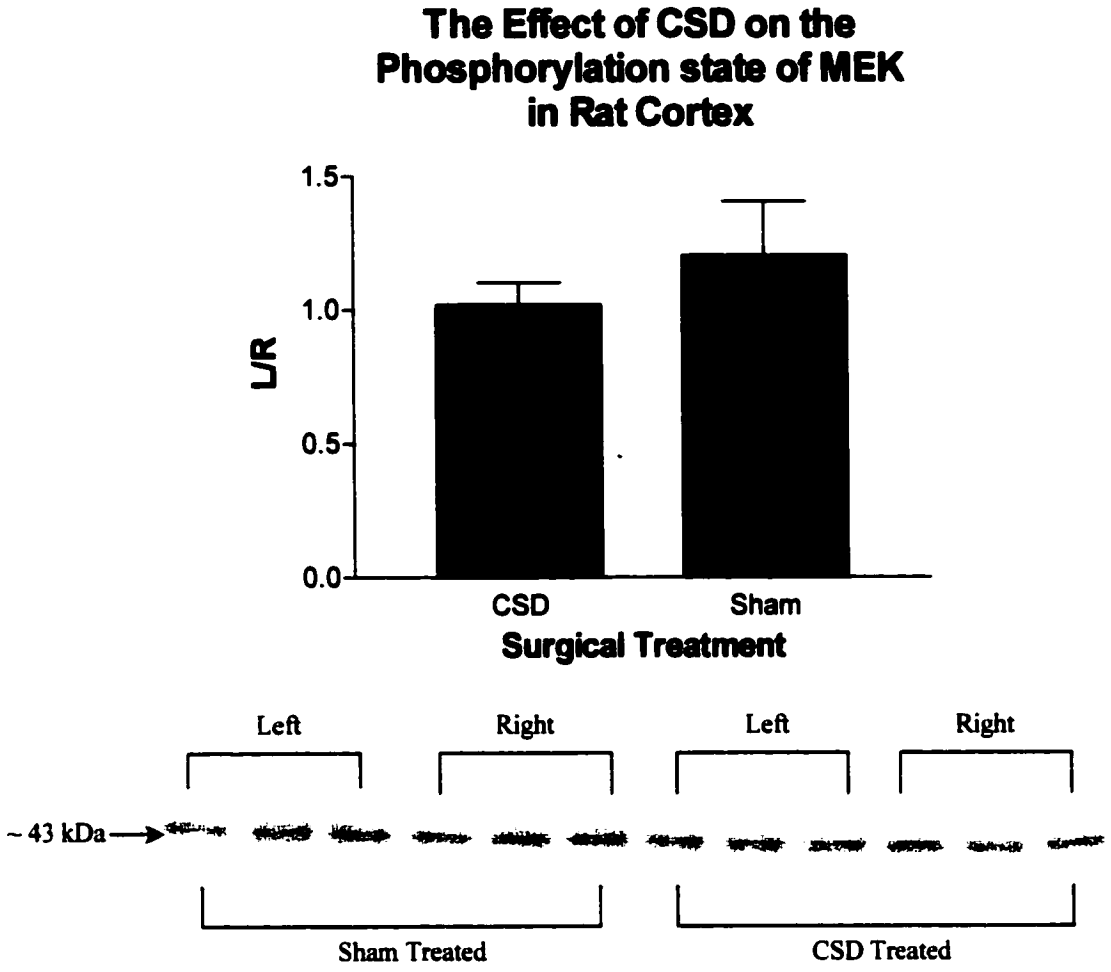
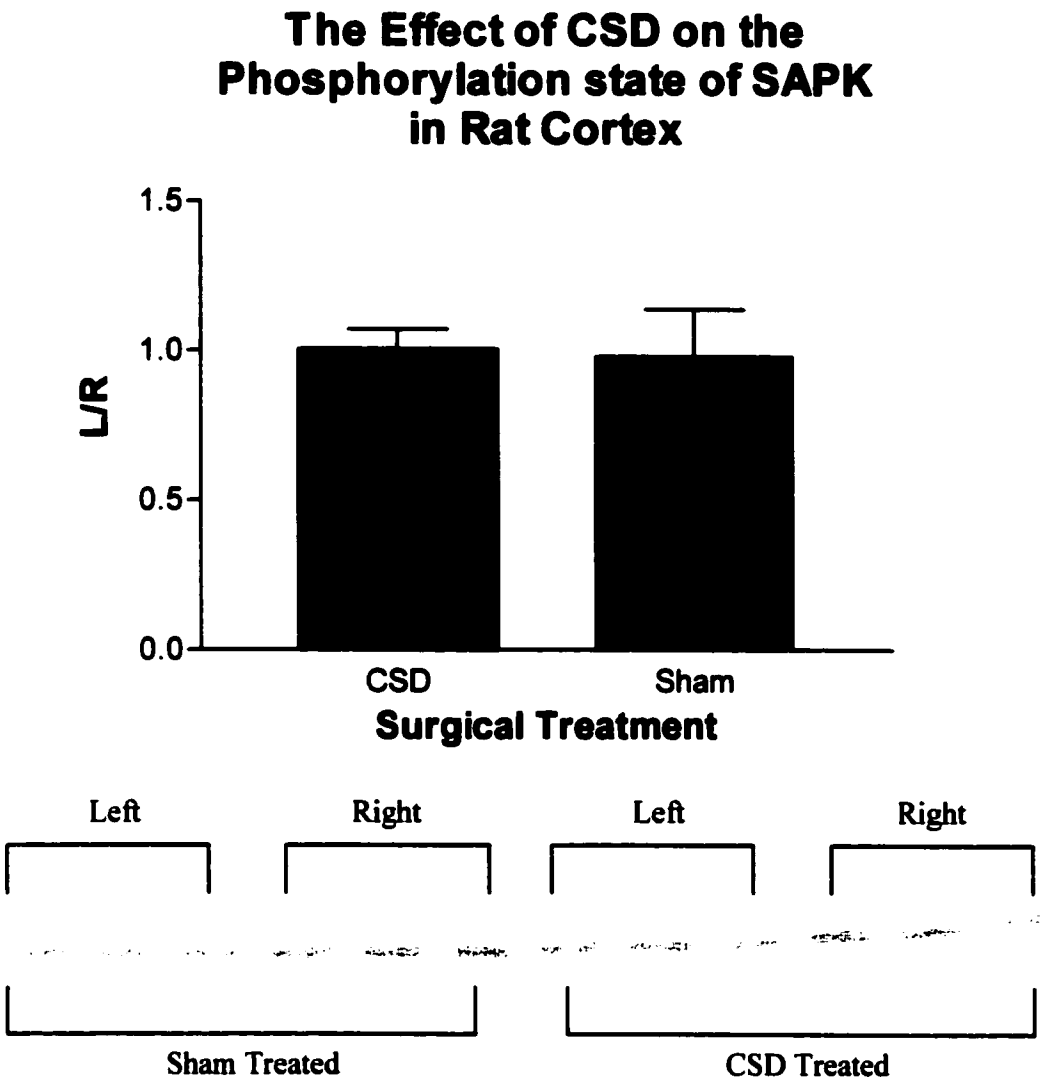

The Effect of CSD on the Phosphorylation state of MEK in Rat Cortex
1.5
1.0
0.5
0.0
L/R
CSD
Sham
Surgical Treatment
Left
Right
Left
Right
~ 43 kDa
Sham Treated
CSD Treated

Figure 3-5. Phosphorylated SAPK Western Blot measurements for CSD and sham treated animals. Data shown are mean ± SD with n=3 in each group. . After 2 hours of KCl elicited CSD on the left cortex, or 2 hours of exposure to 0.5M NaCl, in the case of sham animals, the corticies were rapidly removed, homogenized and were run in Western Blots probing specifically for the phosphorylated form of SAPK. Using semi-quantitative Western Blot analysis, left verses right comparisons were plotted, showing that no significant differences are seen in the amount of phosphorylated SAPK between the CSD-treated cortex and the contralateral control, as well as between the CSD-treated animals and sham controls.


The Effect of CSD on the Phosphorylation state of SAPK in Rat Cortex
1.5
1.0
0.5
0.0
L/R
CSD
Sham
Surgical Treatment
Left
Right
Left
Right
Sham Treated
CSD Treated

Discussion

The role of MAP kinases has been explored in a number of different systems and the ERK pathway has been associated with neuroprotection (Alessandrini et al., 1999; Xia et al, 1995). In an *in vivo* system, Alessandrini et al., (1999) have previously shown that inhibition of MEK1 using PD98059 protected the brain from a subsequent episode of focal ischemia. Though, little is known about the role of the ERK pathway in ischemic preconditioning *in vivo*, recent *in vitro* studies have demonstrated that activation of the p21(ras)/ERK system is required for oxygen-glucose deprivation-induced preconditioning. In their study, Gonzalez-Zulueta et al., (2000) used a constitutively activated ras which conferred neuroprotection while inhibiting the intracellular functions of ras in a dominant negative fashion. Supporting a central role of ras in preconditioning in this model, a dominant negative ras mutant was shown to eliminate the neuroprotective effects of preconditioning induced by oxygen-glucose deprivation (Gonzalez-Zulueta et al., 2000).

Interestingly, an investigation into the differences between cardiac and neuronal ischemic preconditioning revealed that activation of MAPK is an important component of cardiac neuroprotection (Maulik et al., 1996), MAPK activity does not influence neuronal ischemic preconditioning in culture (Tauskela et al., 1999). In contrast, Kawaguchi et al. (1999) have demonstrated that inhibition of MEK1 during CSD using PD98059 is sufficient to eliminate CSD-induced neuroprotection from MCAO-induced ischemic damage.

Our results show that CSDs are capable of up regulating the phosphorylated form of ERK1 and ERK2 on the CSD-exposed cortex when compared to the contralateral control and to sham operated rats. Rosen et al. (1994) have demonstrated that PC12 cells that undergo membrane depolarization show an activation of MEK and ERK via a ras mediated pathway. Several other groups have subsequently demonstrated that activation of ERK promotes survival in PC12 cells (Parrizas et al., 1997; Yan et al., 1998; Xia et al., 1995).

Little else is known, however, about the molecular mechanisms that underlie preconditioning. ERK has been shown to phosphorylate a synaptic vesicle protein, synapsin I that is responsible for synaptic vesicular contact with actin filaments (Jovanovic et al., 1996). Phosphorylation of this protein leads to vesicular dissociation from actin filaments, leading to increased amino acid release (Greengard et al., 1993), thus possibly explaining the increase of glutamate levels seen during CSD preconditioning (Fabricius et al., 1993). This would also explain why inhibition of MEK and therefore ERK activation during ischemia, would be neuroprotective (Alessandrini et al., 1999).

The neuroprotective properties of CSD phosphorylation of ERK may also be related to ERK dependent inhibition of pro-apoptotic proteins. Bergmann et al. (1998) have shown that ERK phosphorylation of the *Drosophola* gene *hid* is critical for inhibiting *hid*'s pro-apoptotic abilities. ERK may also mediate CSD preconditioning by directly translocating to the nucleus (Brunet et al., 1999) and affecting transcription factors such as CREB (Xing et al., 1996), thereby regulating gene expression of various

genes, which may be neuroprotective. CSDs have been known to trigger the expression of a number of immediate early genes such as c-fos (Herrera and Robertson, 1990; Kobayashi et al., 1995). Both *in vivo* and *in vitro*, c-fos expression is closely related to induction of tolerance. Otsuki et al. (1994) have demonstrated that in cultured hippocampal neurons, induction of hypoxic tolerance was correlated with the expression of c-fos proteins. Likewise, in an *in vivo* rat model, ischemic preconditioning stimulated expression of c-fos mRNA, which is correlated with neuroprotection (Belayev et al., 1996). Sgambato et al. (1998) have demonstrated that electrical stimulation of the glutamatergic corticostriatal pathway leads to induction of several immediate early genes, including c-fos and that this induction was dependent on ERK activation.

Another substrate of activated ERK is phospholipase A2 (PLA2). Lin et al. (1993) have demonstrated that ERK is capable of phosphorylating PLA2 on Ser-505 and increasing its enzymatic activity, while mutations at this site rendered cPLA2 an inadequate substrate of ERK. Estevez and Phillis (1997) have demonstrated that quinacrine, a cPLA2 inhibitor, when given upon reperfusion after MCAO, infers neuroprotection on both histological and functional levels.

Our results however, do not indicate a change in the phosphorylation state of MEK, the traditional up stream activator of ERK. In addition, inhibition of MEK1 with PD98059 yielded ambiguous results with CSD inducing phosphorylation of ERK1 and ERK2 in some animals, while not in others. Our results also suggest that the activation of ERK1 and ERK2 by CSD is not induced by raf1. Though the closely related homolog of raf1, B-raf, is highly localized to neuronal and neuroendocrine cells, antibodies to the

activated form were not available. The putative pathway of this protein, however, nevertheless involves the downstream involvement of the MEK protein, which was not observed in this study.

Investigation into whether cross talk between the different MAPK pathways may have phosphorylated the ERK proteins revealed that SAPK/JNK is not phosphorylated by CSD. Interestingly, Sugino et al. (2000) demonstrated that chemical preconditioning was dependent on JNK activation and not on p38 or ERK activation. In contrast, Tsuji et al., (2000) demonstrate that the neuroprotective effect of alpha-phenyl-N-tert-butylnitrone in the gerbil hippocampus acts by suppressing SAPK/JNK and p38 while enhancing the activation of ERK.

It is now obvious that different neuroprotective agents may act through several different routes to mediate their neuroprotective effects. The activation of the ERK pathway we have observed in our *in vivo* model of neuroprotection appears to be consistent with a number of reports that indicate that ERK activation is neuroprotective as a preconditioning paradigm (Kawaguchi et al., 1999), while inhibition of ERK during ischemia reduces infarct volume (Alessandrini et al., 1999). Our results, however, do not demonstrate MEK activation, which is a common element in all of the studies cited here. To the best of our knowledge, MEK is the only known activator immediately upstream of ERK.

GENERAL DISCUSSION

The objectives of these studies were to define the contributions of the mGluRs and the MAP kinases in CSD-induced preconditioning, and to attempt to discover a surrogate marker for neuroprotection.

Preconditioning with CSDs has been shown to be maximally neuroprotective if given three days prior to an ischemic insult (Matsushima et al., 1996). Though no direct connection has yet been made relating CSDs and the mGluRs, CSDs have been shown to increase glutamate levels during elicitation (Iijima et al., 1998) while decreasing the levels of extracellular glutamate released three days later during ischemia (Douen et al., 2000). The mechanism of CSD-induced neuroprotection is also unknown, however, CSDs are known to cause up regulation of a number of biological markers, including neuronal nitric oxide synthase (nNOS) (Shen and Gundlach, 1999), calcium-independent protein kinase C (Koponen et al., 1999), brain derived neurotrophic factor (BDNF), glial fibrillary acidic protein (GFAP) (Matsushima et al., 1998), c-fos, junB, c-jun, and MKP-1 mRNA (Hermann et al., 1999). It is possible that activation of these intracellular markers may be regulated by the mGluRs particularly in light of the fact that activation of specific mGluRs can also activate many of the same biological markers. For instance, administration of (1S,3R)-1-amino-cyclopentane-1,3-dicarboxylic acid in the rat brain increased the mRNA of BDNF, nerve growth factor, as well as c-fos (Murray et al., 1996).

Stimulation of mGluRs may also be an up stream regulator of CSD induced ERK phosphorylation. Peavy and Conn (1998) found that stimulation of the mGluRs in

cortical glia was able to phosphorylate a number of cellular proteins, including ERK2. Blocking mGluRs pharmacologically blocked the phosphorylation of ERK.

We have demonstrated that induction of CSD *in vivo* was capable of phosphorylating the ERK proteins. In recent *in vitro* studies, activation of the p21 (ras)/ERK system is essential for oxygen glucose deprivation-induced preconditioning and that constitutively activated ras infers neuroprotection while inhibition of ras or dominant negative mutant ras eliminates neuroprotection (Gonzalez-Zulueta et al., 2000), suggesting that preconditioning may be dependent on the activation of the ERK system. In further support of this hypothesis, Kawaguchi et al. (1999) have demonstrated that inhibition of MEK1 during CSD using PD98059 is sufficient to eliminate CSD induced neuroprotection from MCAO induced ischemic damage.

Future Experiments

Though these studies have revealed a possible role for the ERK system in neuroprotection, a number of other experiments must be performed in order to determine its role in preconditioning. One such study would be to examine the role of the mGluRs in CSD induced phosphorylation of the ERK proteins. Administration of mGluR antagonists prior to the elicitation of CSDs and then examining the phosphorylation state of the MAP kinases would provide insight into the role of the mGluRs in preconditioning. Using the same paradigm, animals could then be subjected to focal ischemia to examine the role of both the mGluR and the MAP kinases in neuroprotection.

Examination into our discovery that the ERK proteins were phosphorylated

without change in the phosphorylation state of MEK is also necessary. Inhibition of MEK using specific inhibitors, and then examining the phosphorylation state of ERK in animals subjected to CSD may also aid in the elucidation of the preconditioning pathway. If our observation holds true, MEK inhibition should have no effect on the phosphorylation state of ERK, indicating that another pathway may be involved. Our preliminary results were ambiguous, showing that PD98059 dramatically affected CSD-induced ERK phosphorylation in some animals while not affecting it at all in others. The indefinite results may be a consequence of the extreme toxicity of the drug, which caused respiratory and cardiac arrest in a number of animals within seconds of intravenous administration.

REFERENCES

Aamodt, SM., EJ. Nordeen, and KW. Nordeen. 1996. Blockade of NMDA receptors during song model exposure impairs song development in juvenile zebra finches. *Neurobiology of Learning and Memory.* 65: 91-8.

Adamchik, Y., and Y. Baskys. 2000. Glutamate mediated neuroprotection against N-methyl-D-aspartate toxicity: a role for metabotropic glutamate receptors. *Neuroscience.* 99: 731-6.

Alessandrini A., S. Namura, MA. Moskowitz, and JV. Bonventre. 1999. MEK1 protein kinase inhibition protects against damage resulting from focal cerebral ischemia. *Proceedings of the National Academy of Sciences.* 96: 12866-12869.

Attwell, D., B. Barbour, and M. Szatkowski. 1993. Non vesicular release of neurotransmitter. *Neuron.* 11: 401-7.

Barone, FC., White, RF., Spera, PA., Ellison, J., Currie, RW., Wang, X., and GZ. Feurerstein. 1998. Ischemic preconditioning and brain tolerance: temporal histological and functional outcomes, protein synthesis requirement, and interleukin-1 receptor antagonist and early gene expression. *Stroke.* 29:1937-50.

Belayev, L., MD. Ginsberg, OF. Alonso, JT. Singer, W. Zhao, and R. Busto. 1996. Bilateral ischemic tolerance of rat hippocampus induced by prior unilateral transient focal ischemia: relationship to c-fos mRNA expression. *Neuroreport.* 8:55-9.

Benveniste, H., J. Drejer, A. Schousboe and NH. Diemer. 1984. Elevation of the extracellular concentrations of glutamate and aspartate in rat hippocampus during transient cerebral ischemia monitored by intracerebral microdialysis. *Journal of Neurochemistry.* 43: 1369-74.

Bergmann, A., J. Agapite, K. McCall, and H. Steller. 1998 The Drosophila gene hid is a direct molecular target of Ras-dependent survival signaling. *Cell.* 95(3):331-41.

Berman, DE., S. Hazui, K. Rosenblum, R. Seger, and Y. Dudai. 1998. Specific and differential activation of mitogen activated protein kinase cascades by unfamiliar taste in the insular cortex of the behaving rat. *Journal of Neuroscience.* 18: 10037-44.

Bidmon, HJ., J. Wu, I. Buchkremer-Ratzmann, B. Mayer, OW. Witte, and K. Zilles. 1998 Transient changes in the presence of nitric oxide synthases and nitrotyrosine immunoreactivity after focal cortical lesions. *Neuroscience.* 82:377-95.

Bond, A., MJ. O'Neill, CA. Hicks, JA. Monn, and D. Lodge. 1998. Neuroprotective effects of a systemically active group II metabotropic glutamate receptor

agonist LY354740 in a gerbil model of global ischaemia. *Neuroreport* 9:1191-1193.

Bond, A., D. Lodge, CA. Hicks, MA. Ward. and MJ. O'Neill. 1999. NMDA receptor antagonism, but not AMPA receptor antagonism attenuates induced ischaemic tolerance in the gerbil hippocampus. *European Journal of Pharmacology*. 380:91-9.

Bonthius, DJ., JL. Stringer, EW. Lothman, and O. Steward. 1994. Spreading depression and reverberatory seizures induce the upregulation of mRNA for glial fibrillary acidic protein. *Brain Research.* 645(1-2):215-24

Bordi, F., Marcon, C., Chiamulera, C and A. Reggiani. 1996. Effects of the Metabotropic glutamate receptor antagonist MCPG on spatial and context-specific learning. *Neuropharmacology*. 35:1557-65.

Bouvier, M, M. Szatkowski, A. Amato, and D. Attwell. 1992. The glial cell glutamate uptake carrier countertransports pH –changing ions. *Nature*. 360: 471-474.

Bruer, U., MK. Weih, NK. Isaev, A. Meisel, K. Ruscher, A. Bergk, G. Trendelenburg, F. Wiegand, IV. Victorov, and U. Dirnagl. 1997. Induction of tolerance in rat cortical neurons: hypoxic preconditioning. *FEBS Letters*. 414: 117-21.

Brunet, A., D. Roux, P. Lenormand. S. Dowd, S. Keyse, and J. Pouyssegur. 1999. Nuclear translocation of p42/p44 mitogen-activated protein kinase is required for growth factor-induced gene expression and cell cycle entry. *EMBO Journal.* 18: 664-74.

Bruno, V., A. Copani, G. Battaglia, R. Raffaele, H. Shinozaki, and F. Nicoletti. 1994. Protective effect of the metabotropic glutamate receptor agonist, DCG-IV, against excitotoxic neuronal death. *European Journal of Pharmacology.* 256:109-12.

Bruno, V., G. Battaglia, A. Copani, RG. Giffard, G. Raciti, R. Raffaele, H. Shinozaki, and F. Nicoletti. 1995 Activation of class II or III metabotropic glutamate receptors protects cultured cortical neurons against excitotoxic degeneration. *European Journal of Neuroscience.* 7:1906-13.

Buisson, A., and DW. Choi. 1995. The inhibitory mGluR agonist, S-4-carboxy-3-hydroxy-phenylglycine selectively attenuates NMDA neurotoxicity and oxygen-glucose deprivation-induced neuronal death. *Neuropharmacology.* 34:1081-87.

Buisson, A., SP. Yu, and DW. Choi. 1996. DCG-IV selectively attenuates rapidly triggered NMDA-induced neurotoxicity in cortical neurons. *European Journal of Neuroscience.* 8: 138-43.

Busch, E., ML. Gyngell, M. Eis, M. Hoehn-Berlage and KA. Hossmann. 1996.

Potassium-induced cortical spreading depressions during focal cerebral ischemia in rats: contribution to lesion growth assessed by diffusion-weighted NMR and biochemical imaging. *Journal of Cerebral Blood Flow & Metabolism.* 16:1090-9.

Busto, R., MY. Globus, WD Dietrich, E. Martinez, I. Valdes, and MD. Ginsberg. 1989. Effect of mild hypothermia on ischemia induced release of neurotransmitters and free fatty acids in rat brain. *Stroke.* 20: 904-10.

Camon, L., P. Vives, N. De Vera and E. Martinez. 1998. Seizures and neuronal damaged induced in the rat by activation of group I metabotropic glutamate receptors with their selective agonist 3,5-dihydroxyphenylglycine. *Journal of Neuroscience Research* 51: 339-348.

Carter, AJ., and J. Kehr. 1997. Microbore high-performance liquid chromatographic method for measuring acetylcholine in microdialysis samples: optimizing performance of platinum electrodes. *Journal of Chromatography B.* 692: 207-12.

Chaudhry, FA., KP. Lehre, MV. Campagne, OP. Ottersen, NC. Danbolt, and J. Storm-Mathisen. 1995. Glutamate transporters in glial plasma membranes: highly differentiated localizations revealed by quantitative ultrastructural immunocytochemistry. *Neuron.* 15: 711-20.

Chiamulera, C., P. Albertini, E. Valeria, and A. Reggiani. 1992. Activation of metabotropic receptors has a neuroprotective effect in a rodent model of focal ischemia. *European Journal of Pharmacology*. 216: 335-46.

Choi, DW. 1985. Glutamate neurotoxicity in cortical cell culture is calcium dependent. *Neuroscience Letters*. 58: 293-7.

Choi, DW. 1994 Calcium and excitotoxic neuronal injury. *Annals of the New York Academy of Sciences* 747:162-74.

Choi, DW., and SM. Rothman. 1990. The role of glutamate neurotoxicity in hypoxic-ischemic neuronal death. *Annual Reviews of Neuroscience*. 13: 171-82.

Collins, RC., and JW. Olney. 1982 Focal cortical seizures cause distant thalamic lesions. *Science* 218: 177-179.

Colwell, CS., and MS. Levine. 1994. Metabotropic glutamate receptors modulate N-methyl-D-aspartate receptor function in neostriatal neurons. *Neuroscience*. 61: 497-507.

Conn, PJ., and JP. Pin. 1997. Pharmacology and functions of metabotropic

glutamate receptors. *Annual Review of Pharmacology and Toxicology*. 37: 205-37.

Contractor, A., RW. Gereau IV, T. Green, and SF. Heinemann. 1998. Direct effects of metabotropic glutamate receptor compounds on native and recombinant N-methyl-D-aspartate receptors. *Proceedings of the National Academy of Sciences*. 95: 8969-74.

Dietrich, WD., B. Lin, MY. Globus, EJ. Green, MD. Ginsberg and R. Busto. 1995. Effect of delayed MK-801 (dizocilpine) treatment with or without postischemic hypothermia on chroic neuronal survival after global forebrain ischemia in rats. *Journal of Cerebral Blood Flow and Metabolism*. 15: 960-8.

Douen, AG., K. Akiyama, MJ. Hogan, F. Wang, L. Dong, AK. Chow, and AM. Hakim. 2000. Preconditioning with cortical spreading depression decreases intra-ischemic cerebral glutamate levels and down regulates EAAT1 and EAAT from rat cerebral cortex plasma membranes. *Journal of Neurochemistry*. In press.

Drejer, J., H. Benveniste, NH. Diemer, and A. Schousbe. 1985. Cellular origin of ischemia-induced glutamate release from brain tissue in vivo and in vitro. *Journal of Neurochemistry*. 45: 145-51.

Du, Y., KR. Bales, RC. Dodel, E. Hamilton-Byrd, JW Horn, DL. Czilli, LK.

Simmons, B. Ni and SM. Paul. 1997. Activation of a caspase 3-related cysteine protease is required for glutamate-mediated apoptosis of cultured cerebellar granule neurons. *Proceedings of the National Academy of Sciences.* 94: 11657-62.

Dube, GR., and KC. Marshall. 1997. Modulation of excitatory symaptic transmission in locus coeruleus by multiple presynaptic metabotropic glutamate receptors. *Neuroscience* 80: 511-21.

Duchen, MR., McGuinness, O., Brown, LA., and M. Crompton.. 1993. On the involvement of cyclosporin A sensitive mitochondrial pore in myocardial reperfusion injury. *Cardiovascular Research.* 27:1790-4.

Egea, J., C. Espinet, RM. Soler, S. Peiro, N. Rocamora, and JX. Comella. 2000. Nerve growth factor activation of the extracellular signal-regulated kinase pathway is modulated by Ca(2+) and calmodulin. *Molecular & Cellular Biology.* 20:1931-46.

Estevez, AY., and JW. Phillis. 1997. The phospholipase A2 inhibitor, quinacrine, reduces infarct size in rats after transient middle cerebral artery occlusion. *Brain Research.* 752: 203-8.

Fabricius, M., LH. Jensen, and M. Lauritzen. 1993 Microdialysis of interstitial amino acids during spreading depression and anoxic depolarization in rat neocortex. *Brain Research* 612: 61-69.

Finch, EA., and GJ. Augustine. 1998. Local calcium signalling by inositol-1,4,5-trisphosphate in Purkinje cell dendrites. *Nature.* 396: 753-56.

Fykse, EM and F. Fonnum. 1996. Amino acid neurotransmission: dynamics of vesicular uptake. *Neurochemical Research.* 21:1053-60.

Gaspary, H., RP. Simon, and SH. Graham. 1994. BW1003C87 and NBQX but not CGS19755 reduce glutamate release and cerebral ischemic necrosis. *European Journal of Pharmacology.* 262: 197-203.

Gidday, JM., Shah, AR., Maceren, RG., Wang, Q., Pelligrino, DA., Holtzman, DM., and TS. Park. 1999. Nitric oxide mediates cerebral ischemic tolerance in a neonatal rat model of hypoxic preconditioning. *Journal of Cerebral Blood Flow and Metabolism.* 19:331-40.

Gill, R., AC. Foster, and GN. Woodruff. 1988. MK-801 is neuroprotective in gerbils when administered during the post-ischaemic period. *Neuroscience.* 25: 847-55.

Ginsberg, SD., LJ. Martin, and JD. Rothstein. 1995. Regional deafferentation down-regulates subtypes of glutamate transporter proteins. *Journal of Neurochemistry.* 65:2800-2803.

Glazier, SS., DM. O'Rourke, DI. Graham, and FA. Welsh. 1994. Induction of ischemic tolerance following brief focal ischemia in rat brain. *Journal of Cerebral Blood Flow and Metabolism.* 14: 545-53.

Gonzalez-Zulueta., M., AB. Feldman, LJ. Klesse, RG. Kalb, JF. Dillman, LF. Parada, TM Dawson, and VL. Dawson. 2000. Requirement for nitric oxide activation of p21(ras)/extracellular regulated kinase in neuronal ischemic preconditioning. *Proceedings of the National Academy of Sciences of the United States of America.* 97(1):436-41.

Grabb, MC., and DW. Choi. 1999. Ischemic tolerance in murine cortical cell culture: critical role for NMDA receptors. *Journal of Neuroscience.* 19:1657-62.

Graham, SH., J. Chen, JQ. Lan, and RP. Simon. 1996. A dose response study of neuroprotection using the AMPA antagonist NBQX in rat focal cerebral ischemia. *Journal of Pharmacology and Experimental Therapeutics.* 276: 1-4.

Greengard, P., F. Valtorta, AJ. Czernik, and F. Benfenati. 1993. Synaptic vesicle phosphoproteins and regulation of synaptic function. *Science.* 259: 780-5.

Grosse, R., S. Roelle, A. Herrlich, J. Hohn, and T. Gudermann. 2000 Epidermal growth factor receptor tyrosine kinase mediates Ras activation by gonadotropin-releasing hormone. *Journal of Biological Chemistry.* 275:12251-60.

Grotta, J. W. Clark, B. Coull, LC. Pettigrew, B. Mackay, LB. Goldstein, I. Meissner, D. Murphy, and L. Larue. 1995. Safety and tolerability of the glutamate antagonist CGS19755 (Selfotel) in patients with acute ischemic stroke: results of a phase IIa randomized trial. *Stroke.* 26: 602-05.

Gunter, TE., and DR. Pfeiffer. 1990. Mechanisms by which mitochondria transport calcium. *American Journal of Physiology.* 258: C755-86.

Hamm, RJ. BR. Pike, DM. O'Dell, and BG. Lyeth. 1994. Traumatic brain injury enhances the amnesic effect of an NMDA antagonist in rats. *Journal of Neurosurgery.* 81: 267-71.

Hagberg H., A. Lehmann, M. Sandberg,B. Nystrom, I. Jacobson, and A. Hamberger. 1985. Ischemia-induced shift of inhibitory and excitatory amino acids from intra to extracellular compartments. *Journal of Cerebral Blood Flow and Metabolism.* 5: 413-19.

Hayward, NJ, AT. McKnight, and GN. Woodruff. 1993. Brain temperature and the neuroprotective action of enadoline and dizocilpine in the gerbil model of global ischemia. *European Journal of Pharmacology*. 236: 247-53.

Hermann, DM., G. Mies, KA. Hossmann. 1999. Expression of c-fos, junB, c-jun, MKP-1 and hsp 72 following traumatic neocortical lesions in rats—relation to spreading depression. *Neuroscience* 88: 599-608.

Herrera, DG., and HA. Robertson. 1990. Application of potassium chloride to the brain surface induces the c-fos proto-oncogene: reversal by MK-801. *Brain Research*. 510: 166-70.

Hetman, M., K. Kanning, JE. Cavanaugh, and Z. Xia. 1999. Neuroprotection by brain-derived neurotrophic factor is mediated by extracellular signal-regulated kinase and phosphatidylinositol 3-kinase. *Journal of Biological Chemistry*. 274: 22569-80.

Heurteaux, C., Lauritzen, I., Widman, C., and M. Lazdunski. 1995. Essential role of adenosine, adenosine A1 receptors and ATP sensitive K+ channels in cerebral ischemic preconditioning. *Proceedings of the National Academy of Sciences*. 92:4666-70.

Holscher, C., McGlinchey, L., and MJ Rowan. 1996. L-AP4 (L-(+)-2-amino-4-

phosphonobutyric acid) induced impairment of spatial learning in the rat is antagonized by MAP4 ((S)-2-amino-2-methyl-4-phosphonobutanoic acid). *Behavioral Brain Research.* 81:69-79.

Hu, P., NH. Diemer, T. Brahn, and FF. Johansen. 1997. Effects of the AMPA receptor antagonist NBQX on neuron loss in dentate hilus of the hippocampal formation after 8, 10 or 12 minutes of cerebral ischemia in the rat. *Journal of Cerebral Blood Flow and Metabolism.* 17: 147-52.

Iijima, T., C. Shimase, Y. Iwao, and H. Sankawa. 1998. Relationships between glutamate release, blood flow and spreading depression: real-time monitoring using an electroenzymatic dialysis electrode. *Neuroscience Research.* 32:201-7.

Jovanovic, JN., F. Benfenati, YL. Siow, TS. Sihra, JS. Sanghera, SL. Pelech, P. Greengard, and AJ. Czernik. 1996. Neurotrophins stimulate phosphorylation of synapsin I by MAP kinase and regulate synapsin I- actin interactions. *Proceedings of the National Academy of Sciences.* 93:3679-83.

Kaku, DA., RG. Giffard, and DW. Choi. 1993. Neuroprotective effects of glutamate antagonists and extracellular acidity. *Science.* 260: 1516-8.

Kariko K., VA. Harris, Y. Rangel, ME. Duvall, and FA. Welsh. 1998. Effect of

cortical spreading depression on the levels of mRNA coding for putative neuroprotective proteins in rat brain. *Journal of Cerebral Blood Flow and Metabolism.* 18: 1308-15.

Kataoka, K., and H. Yanase. 1998. Mild hypothermia- a revived counter measure against ischemic neuronal damage. *Neuroscience Research.* 32: 103-17.

Kawaguchi, M., JR. Kimbro, JC. Drummond, DJ. Cole, BR. Hu, and PM. Patel. 1999. Effect of MAP kinase inhibition on spreading depression induced acquisition of ischemic tolerance. *Journal of Cerebral Blood Flow and Metabolism.* 19 (suppl.1) S705.

Kawahara, N., SD. Croll, SJ. Wiegard, I. Klatzo. 1997. Cortical spreading depression induces long term alterations of BDNF levels in cortex and hippocampus distinct from lesion effects: implications for ischemic tolerance. *Neuroscience Research.* 29:37-47.

Kawahara N., CA. Ruetzler, G. Mies, and I. Klatzo. 1999 Cortical spreading depression increases protein synthesis and upregulates basic fibroblast growth factor. *Experimental Neurology.* 158:27-36.

Khaspekov, L., M. Shamloo, I. Victorov, and T. Wieloch. 1998. Sublethal in vitro glucose oxygen deprivation protects cultured hippocampal neurons against a subsequent severe insult. *Neuroreport.* 9: 1273-6.

Kim, HY., DK. Vaughan, and S. Ghosh. 1998. Pathways of cerebral calcium accumulation in a model of focal ischemia in rats. *Neurological Research.* 20:169-77.

Kobayashi, S. VA. Harris, and FA. Welsh. 1995. Spreading depression induces tolerance of cortical neurons to ischemia in rat brain. *Journal of Cerebral Blood Flow and Metabolism.* 15: 721-27.

Koh, JY., E. Palmer, and CW. Cotman. 1991. Activation of metabotropic glutamate receptors attenuates N-methyl-D-aspartate neurotoxicity in cortical cultures. *Proceedings of the National Academy of Sciences.* 88: 9431-35.

Koponen, S., R. Keinanen, R. Roivainen, T. Hirvonen, M. Narhi, PH. Chan, and J. Koistinaho. 1999. Spreading depression induces expression of calcium independent protein kinase C subspecies in ischaemia-sensitive cortical layers: regulation by N-methyl-D-aspartate receptors and glucocorticoids. *Neuroscience* 93:985-93.

Korneyev, AY. 1998. Stress-induced tau phosphorylation in mouse strains with different brain Erk 1 & 2 immunoreactivity. *Neurochemical Research.* 23:1539-43.

Kubota, M., T. Nakamura, K. Sunami, Y. Ozawa, H. Namba, A. Yamaura, and H. Makino. 1989. Changes of local cerebral glucose utilization, DC potential and

extracellular potassium concentration in experimental head injury of varying severity. *Neurosurgical Review.* 12 Suppl 1:393-9.

Laemmli, UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-685.

Leao, AAP. 1944 Spreading depression of activity in the cerebral cortex. *Journal of Neurophysiology* 7:199-210.

Li, H., and AM. Buchan. 1993. Treatment with an AMPA antagonist 12 hours following severe normothermic forebrain ischemia prevents CA1 neuronal injury. *Journal of Cerebral Blood Flow and Metabolism.* 13: 933-9.

Lin, LL., M. Wartmann, AY. Lin, JL. Knopf, A. Seth, and RJ. Davis. 1993. cPLA2 is phosphorylated and activated by MAP kinase. *Cell.* 72:269-78.

Longa EZ., PR. Weinstein, S. Carlson, and R. Cummins R. 1989. Reversible middle cerebral artery occlusion without craniectomy in rats. *Stroke.* 20:84-91.

Loscher, W., P. Wlaz, and L. Szabo. 1998. Focal ischemia enhances the adverse effect potential of N-methyl-D-aspartate receptor antagonists in rats. *Neuroscience Letters.* 240: 33-6.

Lowry OH, NJ. Rosebrough, AL. Farr and RJ. Randall. 1951. Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry.* 193:265-275.

Maki, R., MB Robinson, and MA. Dichter. 1994. The glutamate uptake inhibitor L-trans-pyrrolidine-2,4-dicarboxylate depresses excitatory synaptic transmission via a presynaptic mechanism in cultured hippocampal neurons. *Journal of Neuroscience* 14: 6754-6762.

Marette, A., Burdett, E., Douen, A., Vranic, M, and A. Klip. 1992. Insulin induces the translocation of GLUT4 from a unique intracellular organelle to transverse tubules in rat skeletal muscle. *Diabetes.* 41: 1562-9.

Matsushima, K., and AM. Hakim. 1995 Transient forebrain ischemia protects against subsequent focal cerebral ischemia without changing cerebral perfusion. *Stroke* 26:1047-1052.

Matsushima, K., MJ. Hogan, and AM. Hakim. 1996. Cortical spreading depression protects against subsequent focal ischemia in rats. *Journal of Cerebral Blood Flow and Metabolism.* 16: 221-226.

Matsushima, K., R. Schmid-Kastner, MJ. Hogan, and AM. Hakim. 1998. Cortical spreading depression activates trophic factor expression in neurons and astrocytes and protects against subsequent focal brain ischemia. *Brain Research.* 807: 47-60.

Maulik, M., M. Watanabe, YL. Zu, CK Huang, GA. Cordis, JA Schley, and DK Das. 1996. Ischemic preconditioning triggers the activation of MAP kinases and MAPKAP kinase 2 in rat hearts. *FEBS Letters.* 396: 233-237.

McDonald, JW, SP. Althomsons, KL. Hyrc, DW. Choi, and MP. Goldberg. 1998. Oligodendrocytes from forebrain are highly vulnerable to AMPA/kainate receptor mediated excitotoxicity. *Nature Medicine.* 4: 291-7.

Mennerick, S., RP. Dhond, A. Benz, W. Xu, JD. Rothstein, NC. Danbolt, KE. Isenberg, and CF. Zorumski. 1998. Neuronal expression of the glutamate transporter GLT-1 in hippocampal microcultures. *Journal of Neuroscience.* 18: 2290-9.

Mies, G., T. Iijima, and KA. Hossmann. 1993. Correlation between peri-infarct DC shifts and ischemic neuronal damage in rat. *Neuroreport.* 4: 709-11.

Mitchell, P., 1966. Chemiosmotic coupling in oxidative and photosynthetic phosphorylation. *Biological Reviews.* 41:445-502.

Moroni, F., Lombardi, G., Thomsen, C., Leonardi, P., Attucci, S., Peruginelli, F., Torregrossa, SA., Pelligrini-Giampietro, DE., Lunia, R., and R. Pellicciari. 1997. Pharmacological characterization of 1-amino-1,5-dicarboxylic acid, a potent mGluR1 antagonist. *Journal of Pharmacology and Experimental Therapeutics.* 281:721-9.

Mount, HT. CF. Dreyfus and IB. Balck. 1993. Purkinje cell survival is differently regulated by metabotropic and ionotropic amino acid receptors. *Journal of Neuroscience.* 13: 3173-79.

Murphy, SN., and RJ. Miller. 1988. A glutamate receptor regulates Ca^{2+} mobilization in hippocampal neurons. *Proceedings of the National Academy of Sciences.* 85: 8737-41.

Murray, KD., PL. Wood, C. Rosasco, and PJ Isackson. 1996. A metabotropic glutamate receptor agonist regulates neurotrophin messenger RNA in rat forbrain. *Neuroscience* 70: 617-30.

Murray, B., A. Alessandrini, AJ. Cole, AG. Yee, and EJ Furshpan. 1998. Inhibition of the p44/42 MAP kinase pathway protects hippocampal neurons in a cell culture model of seizure activity. *Proceedings of the National Academy of Sciences.* 95: 11975-80.

Nedergaard, M., and AJ. Hansen. 1988. Spreading depression is not associated with neuronal injury in the normal brain. *Brain Research.* 449: 395-398.

Nicholls, DG. 1985. A role for the mitochondrion in the protection of cells against calcium overload? *Progress in Brain Research.* 63:97-106.

Nishio, S., SF, Chen, M. Yunoki, T. Toyoda, M. Anzivino, and KS. Lee. 1999. Hypothermia induced ischemic tolerance. *Annals of the New York Academy of Sciences.* 890: 26-41.

Obrenovitch, TP., and E. Zilkha. 1996. Inhibition of cortical spreading depression by L-701,324, a novel antagonist at the glycine site of the N-methyl-D-aspartate receptor complex. *British Journal of Pharmacology.* 117:931-7.

O'Connor, JJ., MJ. Rowan, and R. Anwyl. 1994. Long lasting enhancement of NMDA receptor mediated synaptic transmission by metabotropic glutamate receptor activation. *Nature.* 367: 557-9.

Olney, JW., J. Labruyere and MT. Price. 1989. Pathological changes induced in cerebrocortical neurons by phencyclidine and related drugs. *Science.* 244: 1360-62.

Orrenius, S., D. McConkey, D. Jones, and P. Nicotera. 1988. Ca2+ activated mechanisms in toxicity and programmed cell death. *Pharmacology.* 2: 319-24.

Otsuki, H., Yamada, K., Yuguchi, T., Tanada, M., and T. Hayakawa. 1994. Prostaglandin E1 induces c-fos and myc proteins and protects rat hippocampal cells against hypoxic injury. *Journal of Cerebral Blood Flow and Metabolism.* 14:150-5.

Parrizas, M., AR. Saltiel, and D. LeRoith. 1997. Insulin-like growth factor 1 inhibits apoptosis using the phosphatidylinositol 3'-kinase and mitogen-activated protein kinase pathways. *Journal of Biological Chemistry* 272:154-161.

Peavy, RD. and PJ. Conn. 1998. Phosphorylation of mitogen activated protein kinase in cultured rat cortical glia by stimulation of metabotropic glutamate receptors. *Journal of Neurochemistry.* 71: 603-612.

Pisani, A., P. Calabresi, D. Centonze, and G. Bernardi. 1997. Enhancement of NMDA responses by group I metabotropic glutamate receptor activation in striatal neurons. *British Journal of Pharmacology.* 120: 1007-14.

Pizzi, M., C. Fallacara, V. Arrighi, M. Memo, and PF Spano. 1993. Attenuation of excitatory amino acid toxicity by metabotropic glutamate receptor agonists and aniracetam in primary cultures of cerebellar granule cells. *Journal of Neurochemistry.*

61: 683-89.

Plaitkis, A., S. Berl, and MD. Yahr. 1984. Neurological disorders associated with deficiency of glutamate dehydrogenase. *Annals of Neurology* 15: 144-153.

Pulsinelli, WA., and JB. Brierly. 1979. A new model of bliateral hemispheric ischemia in the unanesthetized rat. *Stroke*. 10: 267-72.

Rao, VLR. MK. Baskaya, A. Dogan, JD. Rothstein, and RJ. Dempsey. 1998. Traumatic brain injury down-regulates glial glutamate transporter (GLT-1 and GLAST) proteins in rat brain. *Journal of Neurochemistry* 70: 2020-27.

Rogatsky., G., A. Mayevsky, N. Zarchin, and A. Doron. 1996. Continuous multiparametric monitoring of brain activities following fluid-percussion injury in rats: preliminary results. *Journal of Basic & Clinical Physiology & Pharmacology*. 7:23-43.

Rosen, LB., DD. Ginty, MJ. Weber, and ME. Greenberg. 1994. Membrane depolarization and calcium influx stimulate MEK and MAP kinase via activation of ras. *Neuron*. 12: 1207-21.

Rothman, SM and JW. Olney. 1986. Glutamate and the pathophysiology of hypoxic-ischemic brain damage. *Annals of Neurology*. 19: 105-11.

Rothman, SM, and JW. Olney. 1987. Excitotoxicity and the NMDA receptor. *Trends in Neuroscience.* 10: 299-302.

Rothman, SM, and JW Olney. 1995. Excitotoxicity and the NMDA receptor—still lethal after eight years. *Trends in Neuroscience.* 18: 57-8

Rothstein, JD., M. Van Kammen, AI. Levey, LJ. Martin, and RW. Kuncl. 1995. Selective loss of glial glutamate transporter GLT-1 in amyotrophic lateral sclerosis. *Annals of Neurology.* 39: 73-84.

Rothstein, JD. M. Dykes-Hoberg, CA. Pardo, LA. Bristol, L. Jun, RW. Kuncl, Y. Kanai, MA. Hediger, Y. Wang, JP. Schielke, and DF. Welty. 1996. Knockout of glutamate transporters reveals an major role for astroglial transport in excitotoxicity and clearance of glutamate. *Neuron.* 16: 675-86.

Schoepp, DD, and PJ. Conn. 1993. Metabotropic glutamate receptors in brain function and pathology. *Trends in Pharmacological Sciences.* 14: 13-20.

Seger, R., and EG. Krebs. 1995. The MAPK signaling cascade. *FASEB Journal.* 9:726-35

Sgambato, V., C. Pages, M. Rogard, MJ. Besson, and J. Caboche. 1998. Extracellular signal-regulated kinase (ERK) controls immediate early gene induction on corticostriatal stimulation. *Journal of Neuroscience.* 18: 8814-8825.

Shamloo, M., A. Rytter, and T. Wieloch. 1999. Activation of the extracellular signal-regulated protein kinase cascade in the hippocampal CA1 region in a rat model of global cerebral ischemic preconditioning. *Neuroscience.* 93: 81-8.

Shen, PJ., and AL. Gundlach. 1999 Prolonged induction of neuronal NOS expression and activity following cortical spreading depression: (SD): implications for SD- and NO-mediated neuroprotection. *Experimental Neurology* 160: 317-32.

Schurmann, B., Wu, X., Diezel, ID., and V. Lessmann. 1997. Differential modulation of AMPA receptor mediated currents by evans blue in postnatal rat hippocampal neurons. *British Journal of Pharmacology.* 121:237-47.

Shimizu-Sasamata, M., T. Kano, J. Rogowska, GL. Wolf, MA. Moskowitz and EH. Lo. 1998. YM872, a highly water-soluble AMPA receptor antagonist, preserves the hemodynamic penumbra and reduces brain injury after permanent focal ischemia in rats. *Stroke.* 29: 2141-8.

Siesjo, BK. 1994. Calcium mediated processes in neuronal degeneration. *Annals*

of the New York Academy of Sciences. 747:140-61.

Siesjo, BK, F. Bengtsson, W. Grampp, and S. Theander. 1989. Calcium, excitotoxins and neuronal death in the brain. *Annals of the New York Academy of Sciences.* 568: 234-51.

Siliprandi, R., M. Lipartiti, E. Fadda, J. Sautter and H. Maney. 1992. Activation of the glutamate metabotropic receptor protects retina against N-methyl-D-aspartate toxicity. *European Journal of Pharmacology.* 219: 173-4.

Simon, RP., JH. Swan, T. Griffiths, and BS. Meldrum. 1984. Blockade of N-methyl-D-aspartate receptors may protect against ischemic damage in the brain. *Science.* 226:850-52.

Singer, CA. XA. Figueroa-Masot, RH. Batchelor, and DM. Dorsa. 1999. The mitogen-activated protein kinase pathway mediates estrogen neuroprotection after glutamate toxicity in primary cortical neurons. *Journal of Neuroscience.* 19:2455-63.

Small, DL., and AM. Buchan. 1997. NMDA antagonists: their role in neuroprotection. *International Review of Neurobiology.* 40: 137-71.

Staton, PC., and DR. Bristow. 1998. Role of group III metabotropic glutamate receptors in excitotoxin-induced cerebellar granule cell death. *Journal of Neurochemistry*. 71: 1280-8.

Sugino, T., K. Nozaki, and N. Hashimoto. 2000. Activation of mitogen-activated protein kinases in gerbil hippocampus with ischemic tolerance induced by 3-nitropropionic acid. *Neuroscience Letters*. 278(1-2):101-4.

Sugiyama, H., I. Ito, and C. Hirono. 1987. A new type of glutamate receptor linked to inositol phospholipid metabolism. *Nature*. 325: 531-33.

Sunami., K., T. Nakamura, M. Kubota, Y. Ozawa, H. Namba, A. Yamaura, and H. Makino. 1989. Spreading depression following experimental head injury in the rat. *Neurologia Medico-Chirurgica*. 29(11):975-80.

Szatkowski, M., B. Barbour and D. Attwell. 1990. Non-vesicular release of glutamate from glial cells by reversed electrogenic glutamate uptake. *Nature*. 348: 443-46.

Szatkowski, M., and D. Attwell. 1994. Triggering and excitation of neuronal death in brain ischemia: two phases of glutamate release by different mechanisms. *Trends in Neuroscience*. 17: 359-65.

Takahashi, M., JW. Ni, S. Kawasaki-Yatsugi, T. Tyoya, SI. Yatsugi, M. Shimizu-Sasamata, K. Koshiya, SI. Shishikura, S. Sakamoto and T. Yamaguchi. 1998. YM872, a novel selective altha-amino-3-hydroxy-5-methylisoxazole-4-proprionic acid receptor antagonist reduces brain damage after permanent focal ischemia in cats. *Journal of Pharmacology and Experimental Therapeutics.* 284: 467-73.

Takadera, T., and T. Ohyashiki. 1997. Apoptotic cell death and caspase 3 (CPP32) activation induced by calcium ionophore at low concentrations and their prevention by nerve growth factor in PC12 cells. *European Journal of Biochemistry.* 249: 8-12.

Tamura, A., DI. Graham, J. McCulloch, and GM. Teasdale. 1981. Focal cerebral ischemia in the rat: 1. Description of technique and early neuropathological consequences following middle cerebral artery occlusion. *Journal of Cerebral Blood Flow and Metabolism.* 1:53-60.

Tauskela, JS., BR. Chakravarthy, CL. Murray, Y. Wang, T. Comas, M. Hogan, A. Hakim, P. Morley. 1999. Evidence from cultured rat cortical neurons of differences in the mechanism of ischemic preconditioning of brain and heart. *Brain Research.* 827: 143-151.

Tsuji, M., O. Inanami, and M. Kuwabara. 2000 Neuroprotective effect of alpha-phenyl-N-tert-butylnitrone in gerbil hippocampus is mediated by the mitogen-activated protein kinase pathway and heat shock proteins. *Neuroscience Letters.* 282:41-4.

Uemura, Y., NW. Kowall, and MA. Moskowitz. 1991a. Focal ischemia in rats causes time-dependent expression of c-fos protein immunoreactivity in widespread regions of ipsilateral cortex. *Brain Research.* 552:99-105.

Uemura, Y., NW. Kowall, and MF. Beal. 1991. Global ischemia induces NMDA receptor-mediated c-fos expression in neurons resistant to injury in gerbil hippocampus. *Brain Research.* 542:343-7.

Wang, Y., and R. Prywes. 2000. Activation of the c-fos enhancer by the erk MAP kinase pathway through two sequence elements: the c-fos AP-1 and p62TCF sites. *Oncogene.* 19:):1379-85.

Widmann, C. S. Gibson, MB. Jarpe, and GL. Johnson. 1999. Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. *Physiological Reviews.* 79: 143-80.

Xia, Z., H. Dudek, CK. Miranti, and ME. Greenberg. 1996. Calcium influx via the NMDA receptor induces immediate early gene transcription by a MAP kinase/ERK-

dependent mechanism. *Journal of Neuroscience.* 16:5425-36.

Xia, Z., M. Dickens, J., Raingeaud, RJ. Davis, and ME. Greenberg. 1995. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science.* 270:1326-1331.

Xing, J., DD. Ginty, and ME Greenberg. 1996. Coupling of the RAS-MAPK pathway to gene activation by RSK2, a growth-factor regulated CREB kinase. *Science.* 273: 959-963.

Yan, CYI., and LA. Greene. 1998. Prevention of PC12 cell death by N-acetylcysteine requires activation of the Ras pathway. *Journal of Neuroscience* 18: 4042-49.

Yanamoto, H., I. Mizuta, I. Nagata, J. Xue, Z. Zhang, and H. Kikuchi. 2000. Infarct tolerance accompanied enhanced BDNF-like immunoreactivity in neuronal nuclei. *Brain Research.* 877: 331-44.

Young, RS. TP. Olenginski, SK. Yagel and J. Towfighi. 1983. The effect of graded hypothermia on hypoxic-ischemic brain damage: a neuropathologic study in the neonatal rat. *Stroke.* 14: 929-34.