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Glutamate Excitotoxicty Activates a Novel Calcium Permeable Ion Channel in Cultured Hippocampal Neurons

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GLUTAMATE EXCITOTOXICITY ACTIVATES A NOVEL CALCIUM PERMEABLE ION CHANNEL IN CULTURED HIPPOCAMPAL NEURONS

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

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DEDICATION

To my family, friends and teachers for their love, support and guidance through the years.

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2APB	2-amino-ethoxy diphenylborate
AM	acetoxymethylester
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	analysis of variance
AP-1	activator protein-1
APV	2-amino-5-phosphonovalerate
ASIC	acid sensing ion channel
ADP	adenosine diphosphate
ATP	adenosine triphosphate
AMILO	amiloride
Ba^{2+}	barium ion
BAPTA	[1,2-bis(2)-aminophenoxy]ethane-*N,N-N',N'*-tetraacetic acid
BRS	basal recording solution
Ca^{2+}	calcium ion
Ca^{2+}-ATPase	ATP-driven Ca^{2+} pump
$[Ca^{2+}]_e$	free extracellular calcium concentration
$[Ca^{2+}]_i$	free intracellular calcium concentration
CaMKII	calcium/calmodulin kinase II
CAN	neuronal calcium activated non-selective channel
CEI	calcium entry inhibitors
Cl^-	chloride ion
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CNS	central nervous system
CsCl	cesium chloride
CTRL	control
DIV	day(s) *in vitro*
DNA	deoxyribonucleic acid
EAAC	excitatory amino acid carrier
EGTA	ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
END	extended neuronal depolarization
EPSP	excitatory post-synaptic potential
ER	endoplasmic reticulum
E_{rev}	reversal potential
GABA	γ-aminobutyric acid
GET	glutamate excitotoxicity

Gd^{3+}	gadolinium ion
GHK	Goldman-Hodgkin-Katz
GLAST	glutamate-aspartate transporter
GLT-1	glutamate transporter-1
GLU	L-glutamate
G proteins	guanosine triphosphate binding proteins
HEPES	*N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid
I	Current
I_{crac}	calcium release activated current
I_{CIC}	calcium injury current
I_{END}	current underlying extended neuronal depolarization
I_{OGD}	current underlying oxygen glucose deprivation
I_{pe}	post-exposure current
IP_3	inositol 1,4,5-tris-phosphate
IP_3R	inositol 1,4,5-tris-phosphate receptor
IPSP	inhibitory post-synaptic potential
IRRC	inability to restore resting free intracellular calcium concentration
I-V relationship	current-voltage relationship
K^+	potassium ion
KA	kainate
Mg^{2+}	magnesium ion
MK-801	5-methyl-10,11-dihydro-5-*H*-dibenzocyclohepten-5,10-imine maleate
MΩ	megaohm
MCPG	(S)-α-methyl-4-carboxyphenylglycine
MCAO	middle cerebral artery occlusion
MEM	minimal essential media
mGluR	metabotropic glutamate receptor
min	minute(s)
ml	milliliter
mM	millimolar
mV	millivolt
MΩ	mega-ohm
n	sample size
Na^+	sodium ion
nm	nanometer
nA	nanoamperes
nM	nanomolar
NMDA	*N*-methyl-$_D$-aspartate
NMDG	*N*-methyl-$_D$-glucamine

PLC	phospholipase C
pS	picosiemens
$P_x:P_y$	permeability ratio
P_{open}	probability of channel opening
RMP	resting membrane potential
RNA	ribonucleic acid
RyR	ryanodine receptor
SAC	stretch-activated channels
SE	status epilepticus
SEM	standard error of the mean
SRF	serum response factor
SOC	store-operated calcium channel
TTX	tetrodotoxin
TRP	transient receptor potential
TRPM-2/7	transient receptor potential melastatin type 2/7
V	voltage
VGCC	voltage-gated calcium channels
VNGC	voltage-gated sodium channels
Zn^{2+}	zinc ion

ABSTRACT

GLUTAMATE EXCITOTOXICTY ACTIVATES A NOVEL CALCIUM PERMEABLE ION CHANNEL IN CULTURED HIPPOCAMPAL NEURONS

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

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Glutamate excitotoxicity is the predominant mechanism implicated in neuronal cell death associated with neurological disorders such as stroke, epilepsy, traumatic brain injury and ALS. Excessive stimulation of NMDA subtypes of glutamate receptors leads to protracted intracellular calcium elevations triggering calcium mediated neurotoxic mechanisms culminating in delayed neuronal cell death. In addition, glutamate excitotoxicity induces a NMDA dependent extended neuronal depolarization mediated by continuous calcium influx that correlates with delayed neuronal death. Attempts to prevent neuronal death by blocking calcium entry into the neurons using calcium channel blockers or NMDA receptor antagonists have failed to provide any beneficial effects in

clinical trials. Thus, calcium continues to enter the neurons despite the presence of calcium entry blockers. This phenomenon is known as the "calcium paradox of stroke" and represents a major problem in developing effective therapies for treatment of stroke. Here employing a combination of patch clamp recordings, fluorescent calcium imaging and neuronal cell death assays in well-characterized *in vivo* and *in vitro* models of glutamate excitotoxicity, we report the discovery of a novel calcium permeable ion channel that is activated by excitotoxic glutamate injury and mediates a calcium current that is an early initiating step in causing neuronal death. Blocking this calcium permeable channel with high concentrations of Zn^{2+} or Gd^{3+} or by removing extracellular calcium for a significant time period after the initial injury is effective in preventing calcium entry, apoptosis and neuronal death, thus accounting for the calcium paradox. This injury induced-calcium permeable channel provides a unique mechanism for calcium entry following stroke and offers a new target for extending the therapeutic window for preventing neuronal death after the initial excitotoxic (stroke) injury.

INTRODUCTION

A. Stroke and Ischemic Brain Injury

Stroke refers to the brain injury that occurs following cerebral ischemia (Sharp et al., 1998). According to the National Institute of Neurological Disorders and Stroke, "stroke" includes the clinical sequelae of cerebral infarction, intracerebral hemorrhage, and subarachnoid hemorrhage (Vaughan and Bullock, 1999). It is the 3rd leading cause of death in USA and a major cause for premature disability amongst adults (Wolf and D'Agostino, 1998). At least 600,000 people suffer from stroke annually with 160,000 resulting in death. Globally, stroke is the third most common cause of death after ischemic heart disease and all types of cancer combined. Two-thirds of stroke deaths occur in less developed countries (Lopez and Murray, 1998). Stroke also caused 3% of the world's disability burden in 1990. Many stroke survivors are permanently disabled and require long-term care, contributing to the cost of related care estimated at $51 billion annually. By 2015, over 50 million healthy life-years will be lost from stroke, with 90% of this burden in low-income and middle-income countries. Stroke patients are also vulnerable to psychiatric diseases. Depression in survivors of stroke is both common and clinically relevant. It is associated with excess suffering, handicap, suicidal ideation and mortality and hampers rehabilitation (Beekman et al., 1998). By 2020, stroke mortality

will have almost doubled, mainly as a result of an increase in the proportion of older people in the population and the future effects of current smoking patterns in less developed countries. Without urgent action, deaths from stroke will increase over the next decade by 12% globally-and by 20% in low-income countries. Clearly stroke is a major neurological disorder with severe economic and public health implications (Lopez and Murray, 1998; Warlow, 1998; Warlow et al., 2003).

Stroke as defined by World Health Organization is a clinical syndrome of rapid onset of focal (or global) cerebral deficits, lasting more than 24 h or leading to death, with no apparent cause other than a vascular etiology. There are three pathological types of stroke: ischemic stroke, embolic stroke and hemorrhagic stroke. Stroke thus results from either ischemia or hemorrhage (Vaughan and Bullock, 1999). Global or focal ischemia is the single most prominent cause of stroke and accounts for 85% of strokes. Global ischemia results from either cardiac arrest or severe hypotension, both of which result in disruption of cerebral perfusion. Focal ischemia results from occlusion of a cerebral vessel. In many cases narrowing of cerebral vessel results from thrombosis or emboli such as blood clots or fat particles lodging in the lumen of a cerebral vessel (thromboembolic stroke). Hemorrhagic stroke accounts for 15% of all the strokes and results from either intracranial hemorrhage or subarachnoid hemorrhage. Generally, intracranial hemorrhage is caused by bleeding from a cerebral vessel secondary to the rupture of an aneurysm or prolonged hypertension. On the other hand, subarachnoid hemorrhage results from either spontaneous or trauma induced rupture of vessels on the surface of the brain (Vaughan and Bullock, 1999). Current treatment options for stroke

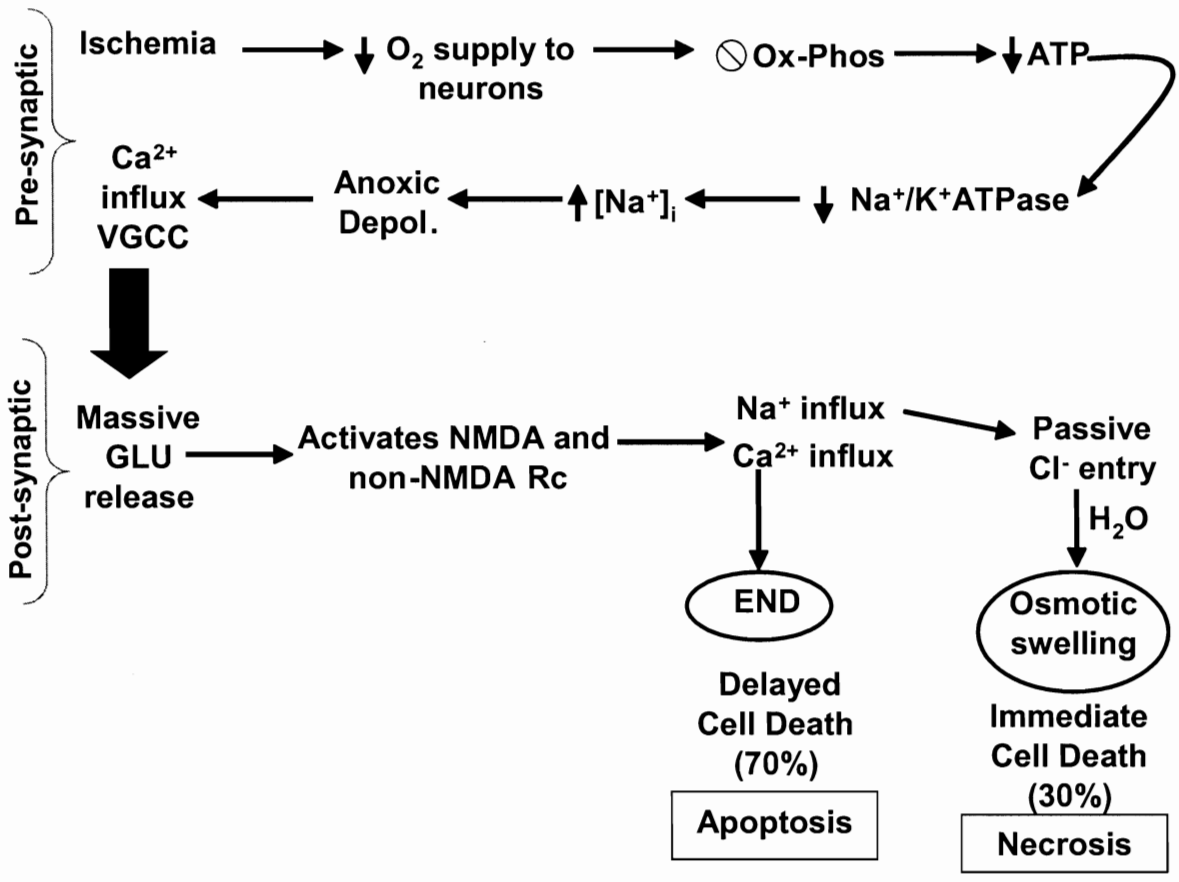
are limited to thrombolytic agents that dissolve the clots and restore the blood supply to the affected brain area (Andrews, 2004). However, these thrombolytic drugs need to be administered immediately upon stroke since they lose their efficacy to prevent brain damage with time.

B. Pathophysiology Of Ischemic Brain Injury

The mechanisms underlying ischemic brain injury are highly complex and diverse. Immediately following ischemia, there is decreased oxygen supply to neurons. This inhibits oxidative phosphorylation by mitochondrial electron chain transport and depletes the cellular stores of energy rich ATP and phosphocretanine (Lipton, 1999). Loss of cellular ATP causes a compensatory switch to anaerobic metabolism resulting in the accumulation of lactate and hydrogen ions precipitating acidosis (Katsura et al., 1993). One of the major effects of this compromised energy state is the decreased activity of the Na^+/K^+ ATPase that is required for maintaining resting membrane potential in neurons. Inhibition of the Na^+/K^+ ATPase also results in increased intracellular Na^+ accumulation producing the so-called "anoxic depolarization"(Katsura et al., 1994; Szatkowski and Attwell, 1994). In response to this depolarization, voltage gated calcium channels open and calcium (Ca^{2+}) enters neuron and causes a massive release of various neurotransmitters including glutamate, γ-aminobutyric acid (GABA), and acetylcholine (Siesjo and Bengtsson, 1989; Lipton, 1999). Extracellular levels of glutamate increase from micromolar to millimolar levels from the ischemic core to the surrounding penumbra (area around the core of the stroke). The excess glutamate acts on postsynaptic

glutamate receptors and floods the postsynaptic neurons with Na^+ and Ca^{2+} ions. Sodium influx is followed by passive chloride entry that results in osmotic swelling responsible for immediate cell death. Prolonged increases in Ca^{2+} signals activate various degradative cascades leading to delayed neuronal cell death. As the severity of ischemia increases, more and more neurons die and release glutamate resulting in larger areas of neuronal death.

Figure 1. Flowchart depicting events underlying neuronal cell death upon ischemia/ stroke. O_2 oxygen; Ox-Phos oxidative phosphorylation, ATP adenosine triphosphate; Na^+/K^+ -ATPase energy dependent sodium potassium pump; VGCC voltage gated calcium channels; GLU glutamate; END extended neuronal depolarization.


Pre-synaptic
Ischemia
↓ O2 supply to neurons
⊘ Ox-Phos
↓ ATP
↓ Na+/K+ATPase
↑ [Na+]i
Anoxic Depol.
Ca2+ influx VGCC
Post-synaptic
Massive GLU release
Activates NMDA and non-NMDA Rc
Na+ influx
Ca2+ influx
Passive Cl- entry
H2O
END
Osmotic swelling
Delayed Cell Death (70%)
Apoptosis
Immediate Cell Death (30%)
Necrosis

C. The Role of Glutamate in Stroke and Excitotoxic Brain Injury

Microdialysis studies in animal models of stroke have revealed that the extracellular levels of excitatory amino acid glutamate are significantly increased from a physiological range of 1-5 μM (Wahl et al., 1994) to 100 μM- 10 mM in the ischemic core (Graham et al., 1990; Wahl et al., 1994; Rusakov and Kullmann, 1998). In addition, extracellular levels of glutamate are elevated several fold within two minutes of injury and increase further with longer periods of ischemia (Benveniste et al., 1989; Mitani and Kataoka, 1991).

Early experiments by Lucas and Newhouse (Lucas and Newhouse, 1957), showed that L-glutamate injections or diet rich in mono-sodium glutamate could destroy the inner layers of the mouse retina, thus suggesting that glutamate could be a neurotoxin. John Olney (Olney, 1969) confirmed glutamate retinotoxicity and further indicated that the structurally related compound kainate also produces brain lesions in immature animals that do not possess a fully developed blood–brain barrier. He also reported that the glutamate-induced retinotoxicity is accompanied by rapid cellular swelling which is most pronounced near dendrosomal components that are currently known to express excitatory amino acid (EAA) receptors. In 1969, Olney coined the term "excitotoxicity", to indicate neurodegeneration presumably mediated by EAAs with prolonged excitation and energy depletion (Olney, 1969). Over the years evidence has accrued in support of the excitotoxic theory. Experimental lesions of glutamergic pathways, for example CA3 input to the CA1 region of the hippocampus, prevented neuronal damage following ischemia (Benveniste et al., 1984; Johansen et al., 1986; Jorgensen et al., 1987; Lipton,

1999). Furthermore, lesions in the area tempesta, a region of the prepyriform cortex that activates hippocampal circuitry, prevented local release of glutamate and was neuroprotective in CA1 region (Kawaguchi et al., 1997). Findings from these and other studies were further supported by experiments in which glutamate was blocked pharmacologically. In the 1980's two research groups Kass and Lipton and Rothman reported that attenuating synaptic transmission by magnesium leads to a reduction in hypoxic/anoxic neuronal death(Kass and Lipton, 1982; Rothman, 1983; Rothman, 1984). Inhibition of glutamate release using the sodium channel blockers tetrodotoxin (Lysko et al., 1994), lidocaine (Fujitani et al., 1994), phenytoin (Taft et al., 1989) and lamotrigine (Rataud et al., 1994) was neuroprotective in several models of ischemia. Likewise, following ischemia, prevention of glutamate release via Ca^{2+} channel blockade (Takizawa et al., 1995) or experimental hypothermia (Mitani and Kataoka, 1991) greatly reduced infarct volume. In addition, experiments with glutamate receptor antagonists, such as γ-D-glutamylglycine, 2-amino-7-phosphnohepatonic acid, and MK-801, showed that blocking excitotoxicity was neuroprotective *in vitro* (Rothman, 1984) and *in vivo* (Simon et al., 1984; Ozyurt et al., 1988; McCulloch et al., 1993).

These findings and others have provided convincing evidence that excessive release and extracellular accumulation of glutamate is responsible for neuronal damage following ischemia. While other pathophysiological mechanisms are likely to be involved for causing neuronal damage, manipulations of glutamate and its receptors have shown the greatest promise in prevention of ischemic brain damage.

D. Glutamate and Glutamate Receptors

Glutamate

L-Glutamate is the most widespread amino acid in the brain and serves a number of functions in the CNS (Nicholls and Attwell, 1990). For example, this dicarboxylic amino acid is a precursor for the inhibitory amino acid neurotransmitter GABA, for the Krebs cycle intermediate α-ketoglutarate and for the amino acid glutamine. Glutamate also acts as a detoxification agent for ammonia products in the brain. In addition to the many metabolic functions of glutamate, the most significant role of glutamate in the brain is its function as the primary excitatory neurotransmitter (Mayer and Westbrook, 1987).

As a neurotransmitter, extracellular glutamate levels must be maintained at controlled levels. Under physiological conditions, extracellular glutamate has been measured in the range of 1–5 μM (Wahl et al., 1994). Although transporters exist to move glutamate into the brain across the blood-brain barrier, the vast majority of glutamate is synthesized de novo from glucose, glutamine, or aspartate (Laterra et al., 1999). Glutamate is stored in synaptic vesicles at concentrations in excess of 20 mM via a magnesium (Mg^{2+})/ATP-dependent transporter (Dingledine and McBain, 1999). The primary mechanism for uptake of extracellular glutamate is a class of high affinity, Na^{+}-dependent glutamate transporters found on neurons and astrocytes. To date, 5 transporters have been characterized: glutamate-aspartate transporter (GLAST), glutamate transporter-1 (GLT-1), and excitatory amino acid carrier-1 (EAAC1), EAAC4, and EAAC5 (Arriza et al., 1997; Gegelashvili and Schousboe, 1997; Vandenberg, 1998). Astrocytes are primarily responsible for the uptake of glutamate at the synapse (Bergles

and Jahr, 1998). Once inside the astrocyte, glutamate is converted to the nonpolar glutamine, which can pass freely from the astrocyte to the neuron. Within the neuron, glutamine is converted back to glutamate to replenish the transmitter pool (Pfrieger and Barres, 1996).

Glutamate receptors

The signaling actions of glutamate are mediated at the neuronal membrane through specialized receptor macromolecules. The binding of glutamate to specific sites on its receptor molecule causes a conformational change that initiates signal transduction cascades in the neuron. Glutamate receptors are broadly categorized based upon the signaling cascade that they trigger. Ionotropic glutamate receptors are coupled to ion permeant channels and under physiological conditions depolarize neurons. In contrast, metabotropic receptors are coupled to guanosine triphosphate-binding proteins (G proteins) and second messenger systems that modulate synaptic transmission (Dingledine et al., 1999). A table summarizing class, properties and functional characteristics of glutamate receptor subtypes is given in Table 1.

Ionotropic glutamate receptors

The ionotropic glutamate receptors are postsynaptic, ligand-gated ion channels (Dingledine et al., 1999). Three types of ionotropic glutamate receptors have been categorized and named according to selective ability of NMDA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), or kainate (KA) to activate them. These

pharmacologically distinct glutamate receptor subtypes have been cloned and have distinct gene families (Dingledine et al., 1999). The AMPA receptor subtype of ionotropic glutamate receptors is comprised of various heteromeric configurations of the GluR1-GluR4 subunits (also known as GluRA-GluRD) (Dingledine et al., 1999). Each of these protein subunits can exist as a "flip" splice variant or a "flop" splice variant, adding to the diversity of the AMPA receptor composition (Dingledine et al., 1999). The KA receptor subtype of ionotropic glutamate receptors contains combinations of subunits derived from 2 distinct gene families, the GluR5–GluR7 family and the KA1–KA2 family (Dingledine et al., 1999). The third subtype of ionotropic glutamate receptors; the NMDA receptor is comprised of at least 1 subunit from the NR1 gene family and varied combinations of NR2A-NR2D subunits (Sucher et al., 1996). An NR3A subunit has also been described which inhibits channel activity (Das et al., 1998). Because of the diversity of subunit composition of the ionotropic glutamate receptor subtypes, the AMPA receptor, the KA receptor, and the NMDA receptor all contribute differently to the excitatory effects of glutamate in the CNS.

α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors

The AMPA receptor contributes to the early, fast component of the excitatory postsynaptic potential (EPSP). The AMPA receptor is a low-affinity glutamate receptor that is typically permeable to the monovalent cations sodium and potassium and impermeable to the divalent cation, Ca^{2+} (Wisden and Seeburg, 1993). This ligand-gated channel demonstrates little voltage dependence, as evidenced by its linear current-voltage

(I-V) relationship (Boulter et al., 1990). AMPA receptor currents are brief (a few milliseconds) due to the low-glutamate affinity and a high rate of desensitization (Dingledine et al., 1999).

The GluR2 subunit has special importance in AMPA receptor composition. GluR2, the most widely expressed subunit in AMPA receptors (Jonas and Burnashev, 1995), confers the Ca^{2+} impermeability of the great majority of AMPA receptors via a specific posttranscriptional modification of this subunit (Sommer et al., 1991). The modification arises when a specific adenosine of the ribonucleic acid (RNA) strand is changed to an inosine by adenine deaminase within the nucleus (Rueter et al., 1995). This RNA edit, which occurs with an approximate efficiency of 90% and high selectivity (Melcher et al., 1996), leads to the substitution of a glutamine with an arginine at the Q/R site (Michaelis, 1998). Since the majority of AMPA receptors contain at least 1 GluR2 subunit (Jonas and Burnashev, 1995), the vast majority of AMPAR are Ca^{2+}-impermeable.

Kainate (KA) receptors

KA receptors are similar in function to AMPA receptors. Like AMPA receptors, KA receptors are voltage-independent, monovalent cation-permeable channels with low affinity and fast kinetics (Michaelis, 1998). KA receptor-mediated EPSPs have smaller peak amplitudes and slower decay kinetics than those derived from AMPA receptors (Frerking and Nicoll, 2000). Extensive RNA editing in these receptors also has

implications on Ca^{2+} permeability (Dingledine et al., 1999) as well as anion permeability (Burnashev et al., 1996).

N-methyl-d-aspartic acid (NMDA) receptors

The NMDA receptor is quite different from the AMPA and KA subtypes of glutamate receptor. First, in addition to permeability to Na^+ and K^+, NMDA receptors have high permeability to Ca^{2+} (Dingledine et al., 1999). Also, NMDA receptors have slower kinetics attributed to a much higher affinity for glutamate (Conti and Weinberg, 1999). The conductance through NMDA receptors can last several hundred milliseconds and constitutes a slower, late phase of the EPSP (Conti and Weinberg, 1999).

Activation of the NMDA receptor is a more complicated process than activation of AMPA and KA receptors. The NMDA receptor requires the binding of a co-agonist, glycine at a strychnine-insensitive glycine site. Originally thought to be a potentiator (Johnson and Ascher, 1987), glycine is actually required for the NMDA receptor to enter the open state (Ozawa, 1993). Another unique characteristic of the NMDA receptor is the voltage-dependent Mg^{2+} blockade, which inhibits NMDA receptor conductance even when both glutamate and glycine are bound to the channel (Nowak et al., 1984).

The binding of Mg^{2+} within the pore is highly voltage-dependent and occurs at a site near or past the middle of the electric field (Ascher and Nowak, 1988). As such, large depolarizations, presumably mediated by activated AMPA and KA receptors, are required to expel Mg^{2+} from the pore. In fact, NMDA receptor inward currents are maximal when the neuron is depolarized to −20 to −30 mV despite the decrease in

driving force (Mayer et al., 1984; Nowak et al., 1984). Thus, the NMDA receptors have a nonlinear I-V relationship, owing to this voltage-dependent Mg^{2+} blockade (Mayer et al., 1984).

Endogenous allosteric modulators finely regulate NMDA receptor function (Dingledine et al., 1999). Zinc, which is concentrated in the synaptic vesicles of some neurons, can inhibit NMDA receptor currents by both voltage-dependent and voltage-independent means (Christine and Choi, 1990). Extracellular cysteine residues on the NMDA receptor act as reduction/oxidation sites. Reduction of these residues enhances NMDA receptor currents, while oxidation inhibits them (McBain and Mayer, 1994). Extracellular pH also functions to modulate the NMDA receptor. NMDA receptors are inhibited by physiologically relevant concentrations of extracellular protons via a reduction in the single-channel opening frequency (Traynelis et al., 1995). Finally, endogenous polyamines such as spermidine and spermine modulate NMDA receptors. Polyamines can cause voltage-dependent inhibition, glycine-dependent potentiation, as well as voltage- and glycine-independent inhibitions (Rock and Macdonald, 1995).

Metabotropic glutamate receptors

G-protein-coupled metabotropic receptors are the other major category of glutamate receptors. There are 8 types of metabotropic glutamate receptors (mGluR1-mGluR8) (Conn and Pin, 1997). mGluRs are further classified on the basis of second messenger systems to which they are linked. Class I mGluRs consist of mGluR1 and 5. Class I mGluRs are activated by quisqualate and are coupled to phospholipase C (PLC).

Class II mGluRs, activated by 2R,4R-4-aminopyrrolidine-2-4-dicarboxylate (APDC), consist of mGluR2 and 3, which inhibit adenylate cyclase activity. The Class III mGluRs (mGluR4 and mGluR6–mGluR8) also inhibit adenylate cyclase activity, but to a lesser extent, and are activated by l-amino-4-phosphonobutyrate (l-AP4) (Conn and Pin, 1997). mGluRs are found both on the presynaptic and postsynaptic membranes. Presynaptic mGluRs decrease neurotransmitter release (Conn and Pin, 1997; Fagni et al., 2000). mGluRs on the postsynaptic membrane regulate the function of ligand-gated ion channels, including all 3 subtypes of ionotropic glutamate receptors (Anwyl, 1999), as well as inhibit the function of voltage-gated Ca^{2+} channels and some potassium channels (Dingledine and McBain, 1999).

Table 1. Excitatory Amino Acid Receptor Subtypes. A table summarizing class, properties and functional characteristics of glutamate receptor subtypes. Table from Doble., 1999.

	NMDA	**AMPA**	**KA**	**mGLUR**
Family	Ion channel	Ion channel	Ion channel	G-protein linked
Structure	Oligomeric	Oligomeric	Oligomeric	Monomeric (7-TM)
Subunits/ Subtypes	1 NR1 subunit 4 NR2 subunit (A-D)	4 GluR subunits (1-4) Flip & Flop splice variant	3 GluR subunits (5-7) 2 KA subunits (1,2)	Class-I: mGluR 1,5 Class-II: mGluR 2,3 Class III: mGluR 4, 6-8
Unitary Conductance	40-50 pS	10-20 pS	<10 pS	
Ionic Selectivity	Na^+, K^+, Ca^{2+}	Na^+, K^+	Na^+, K^+	
Desensitization	Slow	Rapid (AMPA) Slow (kainate)	Rapid (kainite)	
Selective agonist	NMDA Quinolinic acid Ibotenic acid	Quisqualic acid AMPA Kainic acid	Kainic acid Domoic acid	*trans*-ACPD Ibotenic acid Quisqualic acid
Selective antagonists	2-APV MK-801	CNQX	CNQX	Phenylglycines
Regulatory Sites	Glycine Polyamine	Thiazide		

E. Experimental Models of Glutamate Excitotoxicity

Several *in vivo* and *in vitro* models of ischemia have been developed over the years to understand the molecular and pathophysiological basis of ischemia. These models are discussed below.

In vivo models of Ischemia

The most popular models of ischemia include the middle cerebral artery occlusion (transient, permanent or thrombotic), and the complete forebrain ischemia (2-vessel occlusion, 4-vessel occlusion). Hypoxia combined with carotid occlusion forms the basis of these models. Distinct neuronal populations demonstrate different vulnerabilities to ischemia. Thus, a 5-min global ischemia caused delayed neuronal death in all CA1 pyramidal neurons with no effect on other population, while a 20-min ischemia caused neuronal death in hippocampal CA3, cerebellar purkinje neurons, stritial neurons in addition to CA1 region. ATP reductions and other biochemical changes mirror the human ischemia [Reviewed in (Lipton, 1999)]. Ischemia induced by rapid decapitation followed by holding the non-perfused head for varying time intervals preferably at 37^{o}C is also one of the commonly used models to study ischemia. This technique was used many years ago (Lowry et al., 1964) in small animals to study biochemical mechanisms and pathways in global ischemia. The ischemic brain can be freeze-trapped or homogenized for biochemical analysis for metabolic studies.

***In vitro* models of Ischemia**

The *in vivo* models discussed above have been of great value in understanding the pathophysiology of cerebral ischemia. The *in vivo* models have great advantage in correctly depicting blood flow changes and time course of pathological events after ischemia. However, confounding variables such as inflammation, breakdown of blood brain barrier, leukocyte infiltration preclude careful scientific evaluation of any one factor (Lipton, 1999). Thus, *in vitro* models allow one to evaluate effect of discrete variables on the ischemia-induced damage sometimes even at a single neuron level.

Hippocampal brain slices are widely used for studying anoxic or ischemic damage. In this insult the bathing solution is rapidly changed from O_2/CO_2 to N_2/CO_2. When glucose is maintained in the anoxic buffer, the insult is termed anoxia or hypoxia, and when glucose is omitted, the insult is termed *in vitro* ischemia or oxygen/glucose deprivation. ATP levels fall less completely during *in vitro* ischemia than during global ischemia and fall more slowly in the presence of glucose. Generally 5-7 min of *in-vitro* ischemia at 36-37°C, or a period of ischemia extending 2-3 min beyond the anoxic depolarization, leads to rapid damage of CA1 pyramidal cells that lasts for the life of the slice. In addition, primary neuronal/glial cultures from cortex, hippocampus, cerebellum, and hypothalamus of embryo or perinatal rats and mice have been used extensively to study anoxic or ischemic damage. Chemical hypoxia using sodium cyanide or other mitochondrial respiration uncoupler is also one of the commonly used *in vitro* models for ischemia (Lipton, 1999).

F. The Primary Neuronal Culture Model of Glutamate Excitotoxicity

Two independent groups developed the technique of developing primary neuronal cell cultures in 1970's (Bray, 1970; Yamada et al., 1970). Neuronal culture offers several unique advantages over *in vivo* models and also over brain slices. Neurons in culture can easily be subjected to experimental manipulations and observations. They could be made homogenous restricted to neurons from a region of interest in the nervous system- central or peripheral. They are free from blood vessels and other cell types and thus an investigator can study the effect on changing extracellular environment on a neuron. Importantly, primary neuronal cultures are metabolically stable and unlike slices can survive for weeks together. Neurons in culture are stable, develop synaptic connections and exhibit physiological responses, enzymatic activities and genetic expression representative of their *in vivo* counterparts.

Banker and Cowan were the first to develop the technique for culturing hippocampal neurons (Banker and Cowan, 1977). Since then many scientists and research groups across the world have been using their technique with minor variations such as the source of tissue-embryonic or post natal and alterations in the feeding media and frequency. The primary hippocampal neuronal culture model has been successfully adapted to both normal physiology and pathophysiology.

Excitotoxic glutamate exposure in primary neuronal cultures is an established *in-vitro* model of stroke (Choi, 1987; Michaels and Rothman, 1990; Tymianski and Tator, 1996). To produce the excitotoxic insult, a combination of glutamate (50-500μM) and glycine (10μM) is applied to the culture for 5-10min (Sombati et al., 1991; Coulter et al.,

1992; Churn et al., 1995; Limbrick et al., 1995; Limbrick et al., 2001; Limbrick et al., 2003). Using this model, it has been shown that glutamate excitotoxicity results in two distinct types of neuronal death (Choi, 1987). Approximately 30% of the neuronal death observed following glutamate excitotoxicity occurs acutely, within an hour of the glutamatergic insult (Rothman, 1985). Depolarization of the neuronal membrane, disruption of ionic balance and osmotic lysis of the cell mediate this acute component of neuronal cell death. Replacing extracellular sodium or chloride ions with impermeant ions can prevent neuronal death in this phase (Rothman, 1985; Olney et al., 1986). In contrast, delayed neuronal cell death accounts for approximately 70% of the neuronal loss following glutamate excitotoxicity and occurs over the course of hours to days (Rothman et al., 1987). Delayed neuronal cell death is dependent on the presence of extracellular Ca^{2+} ($[Ca^{2+}]_e$) and activation of the NMDA subtype of glutamate receptors (Choi, 1987). Evidence for the distinct pathological processes contributing to acute and delayed neuronal cell death *in-vitro* has been corroborated by research conducted *in-vivo* demonstrating the validity of the primary neuronal culture model (Siesjo and Bengtsson, 1989; Tymianski et al., 1993; Lipton, 1999).

G. The Calcium Hypothesis of Glutamate Excitotoxicity

The finding that glutamate induced delayed neuronal cell death is dependent upon Ca^{2+} permeable NMDA receptor activation and presence of $[Ca^{2+}]_e$ initiated research in determining role of Ca^{2+} in glutamate excitotoxicity. Choi and colleagues using ion substitution experiments found in cortical cell cultures exposed to glutamate that the

removal of $[Na^+]_e$ eliminates acute neuronal swelling but failed to prevent delayed neuronal death. However, replacing $[Ca^{2+}]_e$ fully attenuated the excitotoxicity (Choi, 1985, 1987). Several research groups, using fluorescent Ca^{2+} dyes and indicators have observed a rapid increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$) following the glutamate stimulus. The $[Ca^{2+}]_i$ levels remain elevated as long as the neurons are exposed to glutamate. The Ca^{2+} levels return to the basal levels once the stimulus is removed. However, when neurons are exposed to glutamate for an extended period of time, similar to what occurs during a stroke, the $[Ca^{2+}]_i$ stays elevated even beyond the glutamate removal. Several research groups have observed this phenomenon and alterations in both Ca^{2+} influx and efflux mechanisms have been implicated in glutamate excitotoxicity. The inability to restore resting $[Ca^{2+}]_i$ and a persistent Ca^{2+} influx responsible for an extended neuronal depolarization (END) following excitotoxic glutamate exposure remarkably correlate with delayed neuronal cell death, confirming a strong relationship between excessive Ca^{2+} influx and glutamate-triggered neuronal injury. Further studies with cell permeable Ca^{2+} chelators have been found to afford neuroprotection upon glutamate injury.

Interestingly all Ca^{2+} loads are not always toxic. For example increases in $[Ca^{2+}]_i$ by potassium chloride produced $[Ca^{2+}]_i$ levels similar to the glutamate induced load but were ineffective in producing cell death. Similarly Ca^{2+} loading through L-type voltage sensitive channels was non-toxic. However, similar Ca^{2+} loads produced via NMDA receptors were highly toxic. This led to the development of the "source-specificity" hypothesis that simply states cellular toxicity is contingent upon both the locations of Ca^{2+} entry and increases in Ca^{2+} levels and not upon generalized increases in $[Ca^{2+}]_i$

levels. The strong correlation between excitotoxic neuronal cell death and an inability to restore resting intracellular Ca^{2+} (IRRC) forms the basis for the calcium hypothesis of glutamate excitotoxicity. Indeed recent observations that IRRC occurs in animal models of ischemia *in vivo* and pre-treatment with NMDA receptor antagonist is neuroprotective adds credence to the excitotoxic Ca^{2+} mediated delayed neuronal cell death.

H. Mechanisms for Calcium Induced Neuronal Cell Death

Early observations by pathologists found that Ca^{2+} was deposited in areas of tissue necrosis. These were the first indications that disturbances in Ca^{2+} metabolism can lead to cell death. McLean et al. in 1965 (McLean et al., 1965) reported that livers damaged by toxins had high Ca^{2+} levels, suggesting that Ca^{2+} entry is responsible for tissue damage. Zimmerman and Hulsmann (Zimmerman and Hulsmann, 1966) observed that perfusing isolated heart preparations with Ca^{2+}-free solutions, followed by reperfusion with Ca^{2+} containing solutions, resulted in rapid cessation of contractility followed by massive widespread cell death. Subsequent experiments by Schanne et al. (Schanne et al., 1979) revealed that hepatocytes in cell cultures died in the presence, but not the absence, of $[Ca^{2+}]_e$ when exposed to various membrane-active toxins.

Several lines of evidences have indicated a close relationship between excessive Ca^{2+} influx and neuronal injury in the adult mammalian nervous system. Schlaepfer and Bunge in 1973 (Schlaepfer and Bunge, 1973) observed that amputated axons degenerated only in the presence of $[Ca^{2+}]_e$ ions. Since then several studies have corroborated the essential role of Ca^{2+} ions in various forms of cell death. Toxicity of excitatory amino

acids such as glutamate in cultured neurons and brain slices have confirmed an association between cell death and the requirement of $[Ca^{2+}]_e$ (Choi, 1985; Garthwaite et al., 1986). These observations have given rise to the "calcium hypothesis" of neurotoxicity, which states "neuronal Ca^{2+} overload leads to subsequent neurodegeneration."

A number of pathological processes involving excessive NMDA receptor activation and $[Ca^{2+}]_i$ overloads have been proposed as mediators of delayed excitotoxic neuronal death in ischemia. The formation of free radical species has been implicated in excitotoxicity. The excitotoxic stimulation of the NMDA receptor leads to over-activation of phospholipase A_2 (Lafon-Cazal et al., 1993; Dennis, 1994)and nitric oxide synthase (Dawson et al., 1991; Lipton et al., 1996), resulting in excess arachidonic acid and nitric oxide production, respectively. Arachidonic acid via its subsequent metabolism by cyclooxygenase and lipoxygenase leads to the production of highly reactive oxygen molecules like superoxide ($\cdot O_2^-$) and hydroxyl radicals ($\cdot OH$) (Lafon-Cazal et al., 1993; Dennis, 1994). The reaction of nitric oxide with superoxide forms peroxynitrite ($ONOO^-$), a highly reactive nitrogen species (Dawson et al., 1991; Lipton et al., 1996). Recently, cell death due to changes in the redox state of the cell were found to be mediated by activation of Ca^{2+} permeable ion channels that were gated by the free radicals (Hara et al., 2002; Aarts et al., 2003). Together, these free radicals destroy protein components of the cytoskeleton, nucleic acids, and membrane lipids (Hall et al., 1999).

Mitochondria play a role in the sequestration of the Ca^{2+} influx during glutamate excitotoxicity (White and Reynolds, 1997). However, as NMDA receptor-mediated Ca^{2+} accumulates in mitochondria, superoxide is produced due to the inhibition of the electron transport chain (Dugan et al., 1995). Superoxides, in conjunction with pathological Ca^{2+} accumulation, initiates the opening of the mitochondrial permeability transition pore (Nicholls and Budd, 1998; Bernardi, 1999), and have been associated with the collapse of the mitochondrial proton gradient and subsequent loss of ATP generation. Opening of the mitochondrial permeability transition pores is also associated with the release of cytochrome C, a messenger that triggers apoptosis (Kluck et al., 1997).

A number of enzyme systems have also been associated with excitotoxic NMDA receptor-mediated Ca^{2+} influx. Transient, physiological elevations in $[Ca^{2+}]_i$ are known to regulate activity of a host of protein kinases (Soderling et al., 1994; Braun and Schulman, 1995; Soderling, 1996; Mosior and Epand, 1997). One such enzyme is the Ca^{2+} calmodulin dependent protein kinase II (CaM Kinase II). This enzyme plays an important role in variety of cellular functions ranging from cytoskeletal remodeling, regulation of ion channel activity to affecting release of neurotransmitter (Braun and Schulman, 1995; Churn et al., 1995). Pathological fluctuations in $[Ca^{2+}]_i$ such as during stroke and epilepsy alter the regulation of this enzyme resulting in improper phosphorylations of its substrates (Churn, 1995). A rapid and severe attenuation of CaM Kinase II activity has been observed upon glutamate excitotoxicity *in vitro* and upon transient global ischemia in animals (Hiestand et al., 1992; Churn et al., 1995). The activity of other kinases such as protein kinase C and other regulatory tyrosine kinases is

severely decreased or abolished under the setting of excitotoxicity, a notable exception being the protein kinase A (Aronowski et al., 1993; Hu and Wieloch, 1993a, b; Durkin et al., 1997). These alterations in protein kinase activity could lead to changes in ion channel activity, mitochondrial alterations, and initiation of apoptosis (Lipton, 1999).

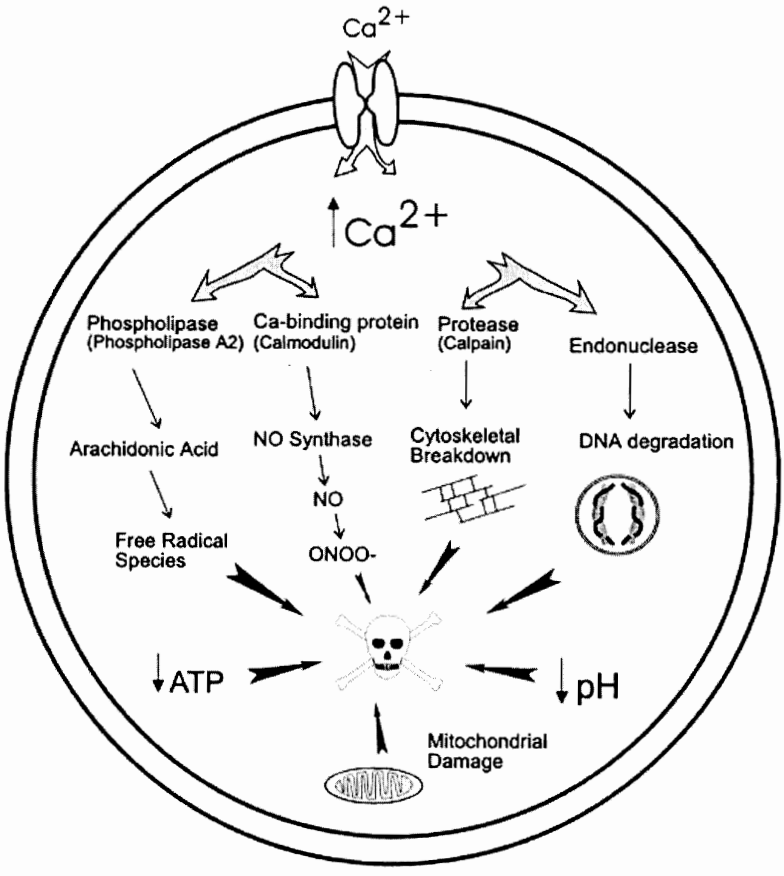
The signaling pathways that mediate neurodegeneration are complex and involve a balance between phosphorylation and dephosphorylation of signaling and structural proteins. Similar to the kinase activity explained above, the activity of Ca^{2+}-dependent phosphates such as calcineurin is also decreased following excitotoxic injury (Morioka et al., 1992; Morioka et al., 1999; Herzig and Neumann, 2000). The phosphorylation states of proteins have immense regulatory capacity, and changes in the normal phosphatase activity could alter the normal activity of these proteins.

Caspases, a family of Ca^{2+}-dependent cystine proteases endonucleases trigger apoptosis during excitotoxicity (Du et al., 1997; Zipfel et al., 2000). Recently, caspase-3 was shown to cleave and inactivate glutamate transporter EAAT2 thus contributing to the excitotoxicity caused by down-regulation of EAAT2 responsible for motoneuron cell death in ALS (Boston-Howes et al., 2006). Caspase inhibitors successfully inhibit apoptotic neuronal death after glutamate exposure (Du et al., 1997).

Calcium overload also activates a family of Ca^{2+}-dependent proteases, the calpains that degrade cytoskeletal components, receptors proteins, G proteins, and Ca^{2+} binding proteins (Emerich and Bartus, 1999). Activity of calpain is increased following excitotoxic injury and the NMDA receptor antagonist, MK-801, blocks this process (Roberts-Lewis et al., 1994; Minger et al., 1998). Calpains dissolve the neuronal

cytoskeleton by degrading essential cytoskeletal proteins such as spectrin, ankyrin and tubulin (Roberts-Lewis et al., 1994; Pettigrew et al., 1996; Emerich and Bartus, 1999) thus, leading to both necrosis and apoptosis. Indeed, calpain inhibition was found to prevent apoptosis upon excitotoxic injury in hippocampal neuronal cultures (Bartus et al., 1994; Hong et al., 1994). Calpain also degrades various receptor proteins including glutamate and ryanodine receptors (Emerich and Bartus, 1999). Recently, that calpain-mediated cleavage of the NR2B subunit was found to occur in neurons and that gave rise to active NMDA receptor forms present on the cell surface after excitotoxic glutamatergic stimulation. Such forms are speculated to contribute to excitotoxicity and synaptic remodeling (Guttmann et al., 2001; Guttmann et al., 2002; Simpkins et al., 2003). Thus, it is clear that calpains have a direct, destructive effect on membrane and cellular proteins and is an important factor in mediating Ca^{2+} induced excitotoxic neuronal cell death. A schematic presentation of mechanisms by which $[Ca^{2+}]_i$ elevations that may trigger secondary Ca^{2+}- dependent phenomenon discussed above are shown in Figure 2.

Figure 2. Schematic and descriptive presentation of proposed mechanisms by which $[Ca^{2+}]_i$ elevations may trigger secondary Ca^{2+}-dependent phenomena, which result in neurotoxicity. NO nitric oxide; ONOO peroxynitrite; ATP adenosine triphosphate; ADP adenosine diphosphate; DNA deoxyribonucleic acid. Figure from Sattler and Tymianski., 2000.


Ca2+
Ca2+
Phospholipase
(Phospholipase A2)
Ca-binding protein
(Calmodulin)
Protease
(Calpain)
Endonuclease
Arachidonic Acid
NO Synthase
Cytoskeletal
Breakdown
DNA degradation
NO
Free Radical
Species
ONOO-
ATP
pH
Mitochondrial
Damage

I. Neuronal Calcium Homeostasis

Calcium ions play a vital role in the regulation number of cellular processes, ranging from cell growth, differentiation to death. Calcium is also essential for triggering neurotransmitter release, modulation of membrane excitability, regulation of synaptic plasticity and gene expression. Thus, maintaining Ca^{2+} homeostasis is crucial for normal neuronal function. Free $[Ca^{2+}]_i$ is maintained around 100 nM compared to approximately 1 mM in the extracellular space. As such, there is a considerable concentration gradient, driving Ca^{2+} into the cell, an effect augmented by the membrane potential. Regulation of resting $[Ca^{2+}]_i$ is maintained through the regulation of the influx, buffering, storage and extrusion of Ca^{2+}. Under normal physiological conditions, these processes are tightly controlled through a combination of restricted Ca^{2+} entry, efficient efflux and restricted intracellular mobility to ensure that dysregulation of Ca^{2+} homeostasis does not occur. A schematic representation of Ca^{2+} homeostasis in neurons is shown in Figure 3.

Influx of extracellular Ca^{2+} across the plasma membrane

The neuronal plasma membrane is relatively impermeable to Ca^{2+} with the exception of three fundamental mechanisms of Ca^{2+} entry: ligand-gated cation channels, voltage-gated Ca^{2+} channels (VGCCs), and store-operated Ca^{2+} channels (SOCs). The NMDA receptor, a ligand-gated cation channel, mediates the vast majority of Ca^{2+} influx during excitatory neurotransmission (Ozawa, 1993). In addition, AMPA and KA receptors of certain subunit compositions, as described previously, are permeable to Ca^{2+} (Jonas and Burnashev, 1995).

Multiple forms of VGCCs have been characterized and cloned. These receptor channels, designated as L-, N-, P-, Q-, and T-types (Tsien et al., 1995; Catterall, 1996), are categorized according to their voltage sensitivities, voltage-dependent and intracellular Ca^{2+}-dependent inactivation rates, and selective sensitivity to inhibiting drugs and toxins (Adams and Olivera, 1994). SOCs represent a third route of Ca^{2+} entry across the plasma membrane. These channels are activated upon depletion of $[Ca^{2+}]_i$ stores (Petersen et al., 1999). Termed "capacitative Ca^{2+} entry" (Putney et al., 2001), SOCs serves to replenish $[Ca^{2+}]_i$ pools such as the ER.

Calcium extrusion across the plasma membrane

Two transport systems exist to pump free $[Ca^{2+}]_i$ out of the neuron into the extracellular space. Because Ca^{2+} extrusion acts against a large Ca^{2+} concentration gradient, these systems are energy-dependent and are, therefore, highly susceptible to ischemic injury (Tymianski and Tator, 1996). The ATP-driven Ca^{2+} pump (Ca^{2+}-ATPase) expends one ATP for each Ca^{2+} ion extruded and is modulated by calmodulin, fatty acids and protein kinases (Carafoli, 1992). The second transport system, the Na^+-Ca^{2+} exchanger, is indirectly coupled to ATP utilization in that it utilizes the Na^+ gradient maintained by the ATP driven Na^+-K^+ exchanger. This electrogenic exchange is triggered by $[Ca^{2+}]_i$ increases and extrudes 1 Ca^{2+} for every 2 or 3 Na^+ ion entering the neuron. Ischemia or persistent depolarization mediated fall in ATP level compromises the Na^+-K^+ exchanger activity that results in increased Na^+ loads in neurons. This could result in reversal of Na^+-Ca^{2+} exchanger activity causing Ca^{2+} influx.

Calcium buffering, sequestration and storage

Ca^{2+} buffering and sequestration can also reduce free $[Ca^{2+}]_i$ levels. Ca^{2+} binding proteins in the cytoplasm, such as calbindin, calmodulin and parvalbumin buffer the vast majority of $[Ca^{2+}]_i$ under physiological conditions (Baimbridge et al., 1992). Mitochondria sequester Ca^{2+} by way of a uniporter driven by the mitochondrial membrane potential (Bernardi, 1999). However, this mitochondrial accumulation only occurs when $[Ca^{2+}]_i$ elevations are prolonged and high (Putney, 1999). Further, mitochondrial sequestration is a temporary buffering system, releasing Ca^{2+} back to the cytoplasm via mitochondrial Na^+-Ca^{2+} and H^+-Ca^{2+} exchangers (Bernardi, 1999). The endoplasmic reticulum (ER) also functions as a Ca^{2+} store. The ER accumulates Ca^{2+} via the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA). This enzyme is similar to that Ca^{2+}-ATPase of the plasma membrane in that it requires ATP. Unlike the plasma membrane Ca^{2+}-ATPase, SERCA function is independent of calmodulin and moves two Ca^{2+} ions into the ER for each ATP molecule utilized (Tymianski and Tator, 1996). Further, the activity of SERCA can be selectively inhibited by thapsigargin (Treiman et al., 1998).

Intracellular Ca^{2+} release

In addition to acting as a sink for $[Ca^{2+}]_i$, the ER also serves as a dynamic Ca^{2+} source (Ogden and Khodakhah, 1996). Two classes of receptors on the ER membrane serve to release stored Ca^{2+} from the ER lumen to the cytoplasm. The ryanodine receptor (RyR) is activated by Ca^{2+} and results in Ca^{2+}-induced Ca^{2+} release (Berridge, 1998).

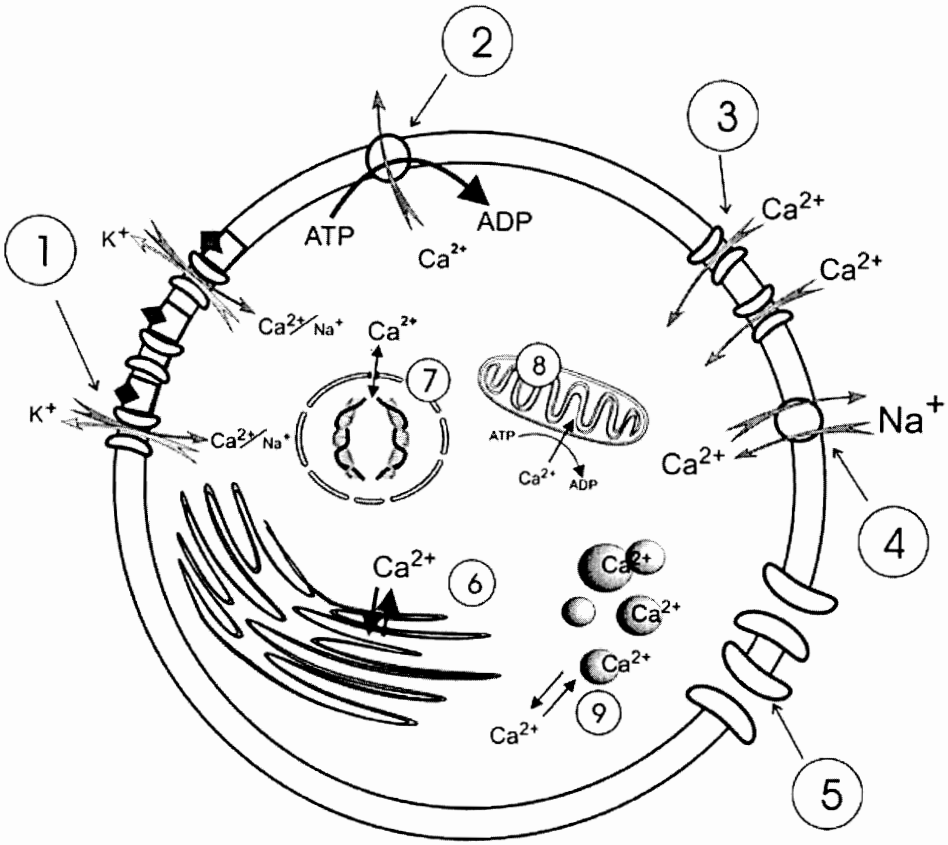
RYRs act to amplify Ca^{2+} signals in a regenerative, positive feedback loop (Putney Jr., 1999). The inositol 1,4,5-tris-phosphate receptor (IP_3R), activated by the second messenger IP_3, is the other class of ER Ca^{2+} release receptors (Ogden and Khodakhah, 1996; Berridge, 1998). IP_3 is produced by the cleavage of phosphatidyl 4,5-bis-phosphate by phospholipase C. IP_3R-mediated Ca^{2+} release can be augmented by the simultaneous presence of Ca^{2+} and IP_3 (Ehrlich et al., 1994). Together, Ca^{2+}-induced Ca^{2+} release by RYRs and IP_3Rs act to produce regenerative Ca^{2+} waves along the ER membrane analogous to sodium action potentials along the plasma membrane (Berridge, 1998; Rose and Konnerth, 2001). The ER is a continuous membrane system that makes close contact with the plasma membrane at specialized regions (Berridge, 1998). Since the ER membrane is continuous with the nuclear envelope, Ca^{2+} waves initiated by Ca^{2+} influx at the plasma membrane can travel to the nucleus, thus, tranducing external signals to the level of gene regulation (Jaffe and Brown, 1994).

Nuclear Ca^{2+} signaling

The nuclear envelope is a continuous extension of the ER and contains RyRs, IP_3Rs and SERCA (Santella and Carafoli, 1997). SERCA has been located on the outer leaflet of the nuclear envelope (Humbert et al., 1996). Thus, the lumen of the nuclear envelope may be able to sequester Ca^{2+} (Carafoli et al., 1997). RyRs and IP_3Rs have been identified on the both the outer and inner leaflets of the nuclear membrane (Payrastre et al., 1992; Santella and Carafoli, 1997). Thus, these two receptor systems allow the nuclear envelope to release Ca^{2+} into the cytoplasm or into the nucleus (Santella

and Carafoli, 1997). Therefore, $[Ca^{2+}]_i$ release initiated by $[Ca^{2+}]_e$ influx at the ER can propagate to the nucleus and continue directly into the nucleoplasm (Berridge, 1998). As a second messenger, Ca^{2+} regulates gene transcription by modulating transcription factors directly or through Ca^{2+}-dependent kinases and phosphatases (Santella and Carafoli, 1997; Mellstrom and Naranjo, 2001).

Figure 3. Schematic representation of Ca^{2+} homeostasis in neurons. 1. Ca^{2+} and Na^{+} influx along with K^{+} efflux in receptor-gated ion channels such as glutamate receptors; 2. Ca^{2+} efflux an ATP requiring ionic pump; 3. Ca^{2+} influx via voltage-gated Ca^{2+} channels; 4. Ca^{2+} efflux via Na^{+}/Ca^{2+} exchanger; 5. additional ionic channels contributing to membrane repolarization and ionic homeostasis; 6. Ca^{2+} sequestration (and release) by endoplasmic reticulum; 7. Ca^{2+} fluxes through the nuclear membrane with potential effects on nucleic acid transcription; 8. Ca^{2+} sequestration by mitochondria; 9. intracellular Ca^{2+} buffering by Ca^{2+} -binding proteins. ATP Adenosine triphosphate; ADP Adenosine diphosphate. Figure from Sattler and Tymianski, 2000.


1
2
3
4
5
6
7
8
9
ATP
ADP
Ca^{2+}
K^+
Na^+
Ca^{2+}/Na^+

J. Excitotoxic Glutamate Exposure Induces An Extended Neuronal Depolarization In Neurons

Cultured hippocampal neurons exposed to excitotoxic levels of glutamate manifest a neuronal depolarization lasting hours even after termination of the glutamate treatment (Sombati et al., 1991). This Extended Neuronal Depolarization (END) is a persistent depolarizing shift of the resting membrane potential of more than 20 mV (Sombati et al., 1991; Coulter et al., 1992). Neurons in END remain viable, respond to glutamate and exclude vital dyes like trypan blue (Coulter et al., 1992). END requires NMDA receptor activation and $[Ca^{2+}]_e$. END is a harbinger of delayed neuronal death, and the percentage of neurons in END correlates with extent of excitotoxic neuronal death in the cultures (Coulter et al., 1992). Several other groups have demonstrated the occurrence of END using different neuronal preparations and confirmed that END depends on NMDA receptor activation and $[Ca^{2+}]_e$ (Calabresi et al., 1995; Chen et al., 1997; Tanaka et al., 1997). Recent studies have demonstrated that a selective Ca^{2+} conductance, distinct from all other conventional routes of Ca^{2+} entry, is activated during excitotoxic glutamate exposure and maintains END (Limbrick et al., 2001).

K. Inability To Restore Basal Intracellular Ca^{2+} Following Glutamate Excitotoxicity

Cultured neurons exposed to excitotoxic glutamate concentration also manifest changes in Ca^{2+} homeostasis. Excitotoxic glutamate injured cultured neurons demonstrate an Inability to Restore Resting $[Ca^{2+}]_i$ (IRRC) (Limbrick et al., 1995). In these cultured

neurons, Ca^{2+} levels do not return to normal levels even after glutamate removal (Connor et al., 1988; Glaum et al., 1990; Limbrick et al., 1995). Like END, IRRC is dependent on NMDA receptor activation and $[Ca^{2+}]_e$, and a strong correlation exists between IRRC and excitotoxic neuronal death (Limbrick et al., 1995).

L. Calcium Influx Constitutes The Ionic Basis Of END

Excessive activation of neuronal glutamate receptors has been implicated in the pathophysiology of stroke, epilepsy, and traumatic brain injury. As mentioned above, it has been demonstrated that excitotoxic glutamate exposure results in the induction of an extended neuronal depolarization (END), as well as protracted elevations in free $[Ca^{2+}]_i$. Both END and the prolonged $[Ca^{2+}]_i$ elevations were shown to correlate with subsequent neuronal death. Recently it was reported that removal of $[Ca^{2+}]_e$ but not Na^+ in the post-glutamate period resulted in complete reversal of END, allowing neurons to rapidly repolarize to their initial resting membrane potential (Limbrick et al., 2003). In addition, removal of $[Ca^{2+}]_e$ was sufficient to eliminate the protracted $[Ca^{2+}]_i$ elevations induced by excitotoxic glutamate exposure. It was further observed that END induced by excitotoxic glutamate exposure was caused by an influx of $[Ca^{2+}]_e$ and removing $[Ca^{2+}]_e$ could reverse the previously irreversible condition of END.

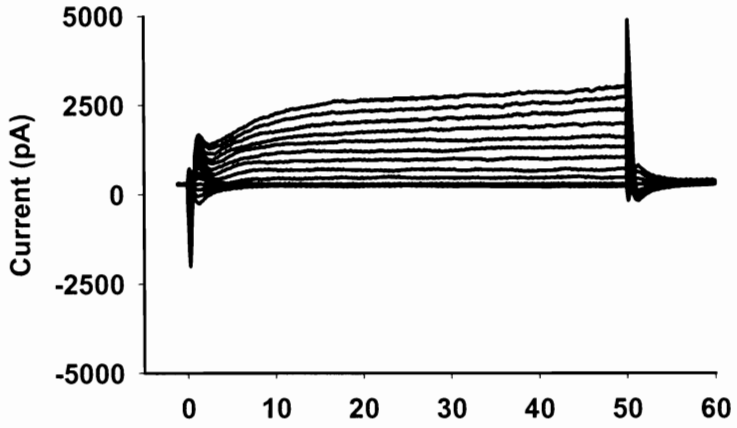
In order to investigate the ionic basis of END, Limbrick (Limbrick, 2000) patch-clamped both control and END neurons at a holding potential of –60 mV and then subjected them to a series of voltage steps in 10 mV increments from –90 mV to +60 mV. No steady state inward current was observed in control neurons, whereas END

neurons demonstrated induction of inward current (I_{END}) at membrane potential <-10 mV. They concluded that glutamate excitotoxicity was associated with the induction of a novel secondary Ca^{2+} conductance that was responsible for IRRC and the maintenance of END (Figure 4). It was suggested that this novel Ca^{2+} current that is responsible for indiscriminant Ca^{2+} entry during END determines the fate of the cell after an excitotoxic insult. It is therefore of pathophysiological importance during stroke, TBI and other forms of neuronal injury (Limbrick, 2000).

Taken together, END, IRRC and the extensive neuronal death induced by excitotoxic glutamate exposure parallel the observed anoxic depolarization, irreversible Ca^{2+} overload and infarction of the ischemic core. The dependence of these phenomena on NMDA receptor activation and $[Ca^{2+}]_e$ has led to the Ca^{2+} hypothesis of glutamate excitotoxicity wherein NMDA receptor mediated Ca^{2+} influx causes the disruption of Ca^{2+} homeostatic mechanisms and Ca^{2+} overload which initiate pathways leading to neuronal death (Choi, 1987; Rothman et al., 1987; Siesjo and Bengtsson, 1989; Dubinsky and Rothman, 1991; Randall and Thayer, 1992; Hartley et al., 1993; Tymianski et al., 1993; Limbrick et al., 1995; Sattler and Tymianski, 2001).

Figure 4. Induction of an inward current, I_{END}, following excitotoxic glutamate exposure. Current traces a representative control (A) or END (B) neuron in response to 50 ms voltage steps in 10 mV increments from –90 mV to +60 mV; holding potential, -60 mV. Current-Voltage (I-V) relationships for control and END conditions. (C) Note the large inward current, I_{END}, observed in END neurons (Limbrick, 2000).

A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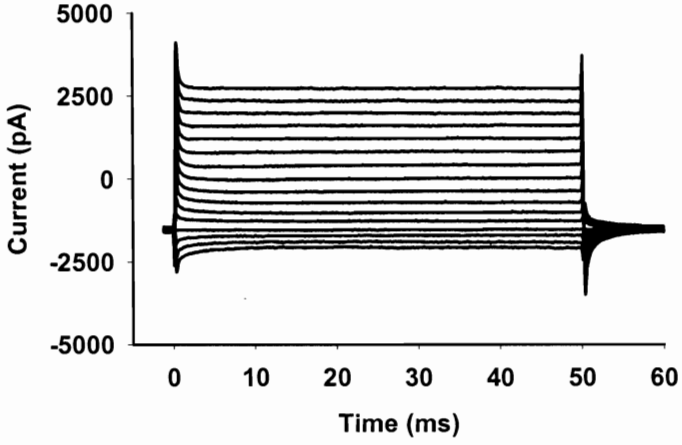
0
-2500
-5000
Current (pA)
0
10
20
30
40
50
60

B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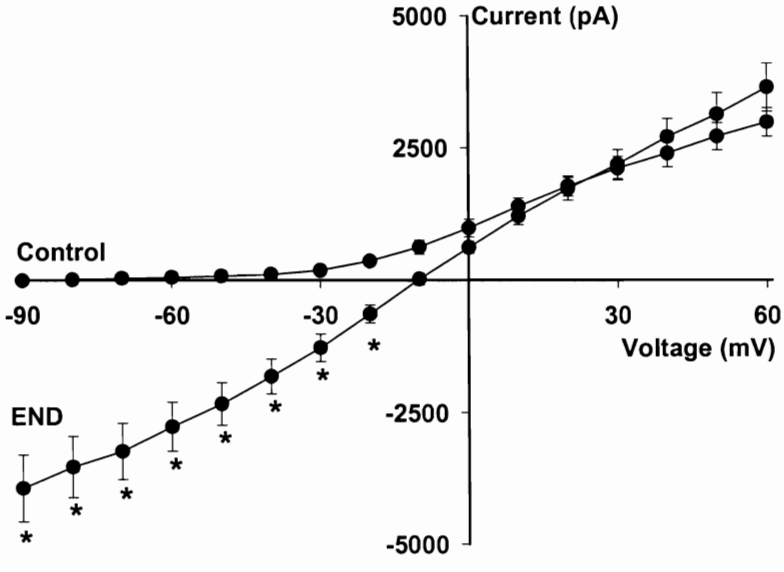
2500
0
-2500
-5000
Current (pA)
0
10
20
30
40
50
60
Time (ms)

C


5000
Current (pA)
2500
Control
-90
-60
-30
30
60
Voltage (mV)
END
-2500
-5000

M. Calcium Paradox Of Glutamate Excitotoxicity and Stroke

Glutamate excitotoxicity is the predominant mechanism mediating the neuronal injury and death associated with stroke, epilepsy and traumatic brain injury, yet the underlying molecular mechanism causing glutamate excitotoxicity remains to be elucidated. There is now conclusive evidence that Ca^{2+} entry via the activation of NMDA subtype of glutamate receptor mediates glutamate excitotoxicity. Blocking NMDA receptors permitted survival of neurons otherwise destined to die from anoxia (Goldberg and Choi, 1990). Further, animal research suggested that blockade of NMDA receptors may protect against ischemic damage in the brain (Simon et al., 1984). Thus, limiting Ca^{2+} entry into the neurons upon glutamate excitotoxicity should prevent neuronal cell death. However, to date all the clinical trials with Ca^{2+} entry antagonists have not been successful. Developing neuroprotective agents by antagonizing glutamate receptors, thus blocking excessive Ca^{2+} rise, has also met limited success. Glutamate receptors antagonists impinge on normal brain function producing adverse side effects and clinical trials have generally failed to benefit the patients (Davis et al., 1997; Morris et al., 1999; Lees et al., 2000). Multicenter clinical trials of glutamate antagonists (Lee et al., 1999; Wahlgren and Ahmed, 2004) have been a failure (See: http://www.stroketrials.org and Tables 2,3 and 4 for updated list of ongoing clinical trials for stroke). Conventional Ca^{2+} entry antagonists prevent neuronal death and END when administered before and during the injury phase of glutamate excitotoxicity (Coulter et al., 1992; Limbrick et al., 2001). However, once the glutamate excitotoxic injury is initiated, attempting to block this Ca^{2+} entry and reduce the increased $[Ca^{2+}]_i$ (the Ca^{2+} plateau) with conventional Ca^{2+} entry

antagonists does not prevent END (Limbrick et al., 2003) or neuronal death (Ikonomidou and Turski, 2002) or improve the outcome after stroke (Horn and Limburg, 2000). This apparent contradiction, where conventional Ca^{2+} entry antagonists do not prevent Ca^{2+} entry or END after glutamate excitotoxicity, and Ca^{2+} continues to enter the neurons, has been referred to as the Ca^{2+} paradox of neuronal death in stroke (Lee et al., 1999; Horn and Limburg, 2001; Ikonomidou and Turski, 2002) and represents a major problem in developing effective stroke therapies (Wahlgren and Ahmed, 2004).

Tables 2, 3 & 4. Tables summarizing drug class, target of action and status in the clinical trials for treatment of stroke. Table generated from information available from online stroke database http://www.stroketrials.org.

DRUGS TO IMPROVE BLOOD FLOW

CLASS	DRUG	ACTION	STATUS
Anti -thrombotic	Heparin	IV anticoagulant	Widely used in acute stroke, but no randomized clinical trials support this use
	Dalteparin	Low molecular weight Heparin	Phase III trial (HAEST) reported no difference from aspirin, 4/00.
	Enoxaparin		Status of stroke trial development unknown
	Nadroparin		No benefit in death or Barthel index at either dose; more intracranial hemorrhage at bid dose.
	Tinzaparin		P-III trial ongoing for acute ischemic stroke.
	Danaparoid		No favorable outcome at 3-mos
Anti-platelet	Asprin	Antiplatelet agent; inhibits thromboxane A2	2 large trials (IST, CAST) showed small (~ 1 %) but significant effect of early aspirin in acute stroke.
	Abciximab	Intravenous platelet aggregation inhibitor,	Phase III trial (AbBEST-II) stopped in Oct'05 due to a high rate of intracranial hemorrhage.
Fibrinogen-depleting	Ancrod	Fibrinogenolytic agent.	Phase III trial on a different dose regimen will begin in the summer of 2005.
Improve Capillary flow	Pentoxifylline	Vasodilator. may improve erythrocyte flexibility.	No known development for stroke or TIA.
Thrombolytics	Pro-Urokinase	Thrombolytic agent	Phase-II showed significant benefit than controls.
	Streptokinase		iv streptokinase increased stroke mortality or morbidity in several studies. No further development in stroke.
	Tenecteplase		Clinical outcome at 3 months was similar to control data from other thrombolytic trials.
	Tissue Plasminogen activator		Approved in acute ischemic stroke within 3 h of onset, June 1996. Study of tPA with stroke onset 3-5 h (ATLANTIS) halted 7/98, lack of efficacy
	Urokinase		Trials for intra-arterial stroke therapy ongoing. Manuf. deficiencies (99) not available in the US.

DRUGS TO PROTECT BRAIN TISSUE (NEUROPROTECTIVE AGENTS)

CLASS	DRUG	ACTION	STATUS
Calcium channel blocker	Nimodipine	L-type Calcium channel blocker	US FDA approved for subarachnoid hemorrhage. Published ischemic stroke trials have reported mixed results.
	Flunarizine	Calcium channel blocker	Phase III trial (FIST) results reported 1996. Drug didn't improve neurologic and functional outcome in patients with acute ischemic stroke.
Calcium chelator	DP-BAPTA	Membrane activated metal ion chelator.	Improvements in the NIHSS 2, 7 and 30 days after the stroke in drug-treated patients.
Free-radical scavengers (Anti-oxidants)	Ebselen	Antioxidant	No difference in incidence of delayed ischemia, Phase-III on-going
	Tirilazad	Lipid peroxidation inhibitor	Excessive Mortality: Trial halted in 1994
GABA agonist	Clomethiazole	GABA agonist	CLASS-I trial: No effect in ischemic stroke
Growth factors	Fibroblast GF		Phase-III trials halted in '98
Leucocyte adhesion inhibitor	Enlimomab	Anti-ICAM antibody	Outcome in Enlimomab group worse than placebo group. Final results published 10/2001.
	Hu23F2G	Antibody against CD11/18	Phase-II success, Phase-III failed
NO inhibitor	Lubeluzole	Nitric oxide inhibitor	No beneficial effect observed in phase-III
Opioid antagonist	Nalmefene	Kappa-antagonist	No significant effect at six month
Phosphatidylcholine precursor	Citicoline	membrane stabilizer	no benefit in primary endpoint. withdrawn 4/98.

Serotonin agonist	Bay x 3702	5-HT1A agonist	Phase-III ongoing
K channel opener	BMS-204352	Activates K channels	Phase-III failed to show efficacy
Sodium channel blocker	Fosphenytoin	Na+ channel blocker, glutamate release blocker	no significant differences between placebo and fosphenytoin in 1^0or 2^0 endpoints at 3 months

GLUTAMATE ANGATONISTS (NEUROPROTECTIVE AGENTS)

CLASS	DRUG	ACTION	STATUS
AMPA antagonists	YM872		ARTIST, Phase-II ongoing
	ZK200775	Competitive AMPA blocker	Halted due to excessive sedation in 1998
KA antagonist	SYM2081		
NMDA antagonist	CGS19755	Competitive blocker	Phase-III showed no benefit in 1997
	Aptiganel	Non- Competitive blocker	Phase-III halted in 1997
	CP-101,606	NR2B antagonist	Phase-III ongoing
	Dextrorphan	Non- Competitive blocker	Unknown
	Magnesium	Ion channel blocker	Multicenter IMAGES trial on going
	MK-801	Non- Competitive blocker	Clinical development for stroke abandoned.
	NPS-1506	NMDA channel blocker	Phase-II trial put on-hold
	Remacemide	Low-affinity antagonist	CNS-related events reported
	ACEA 1021	Glycine-site antagonist	Phase-I safety trials failed, crystals in urine
	GV150526	Glycine-site antagonist	No therapeutic effect in Phase-III
	Eliprodil	Poly-amine site antagonist	Phase III stroke trials abandoned 1997

Unknown/Novel action	Piracetam	Increases CBF; inhibits platelet aggregation.	Piracetam did not influence outcome when given within 12 hours of acute stroke
Neuronal stem cells	619C89	Transplanted Stem-cells	Mixed results, on-going

http://www.stroketrials.org

N. Summary and Rationale

Stroke is one of the leading causes of disability and death in the world, yet the successful treatment of stroke is extremely limited (Bonita et al., 2004). Excessive Ca^{2+} entry and prolonged increases in $[Ca^{2+}]_i$ caused by activation of the NMDA subtype of glutamate receptors are the main causes of glutamate excitotoxic (GET) injury in stroke (Choi, 1994; Choi, 1995; Lipton, 1999). In addition, GET induces an NMDA-dependent extended neuronal depolarization (END) (Sombati et al., 1991; Coulter et al., 1992) mediated by continuous Ca^{2+} influx that correlates with delayed neuronal death (Coulter et al., 1992). Conventional Ca^{2+} entry antagonists prevent neuronal death and END when administered before and during the injury phase of glutamate excitotoxicity (Coulter et al., 1992; Limbrick et al., 2001). However, once the glutamate excitotoxic injury is initiated, attempting to block this Ca^{2+} entry and reduce the increased $[Ca^{2+}]_i$ (the Ca^{2+} plateau) with conventional Ca^{2+} entry antagonists does not prevent END (Limbrick et al., 2003), block neuronal death (Ikonomidou and Turski, 2002) or improve the outcome after stroke (Horn and Limburg, 2000). This apparent contradiction, where conventional Ca^{2+} entry antagonists do not prevent Ca^{2+} entry or END after glutamate excitotoxicity, has been referred to as the Ca^{2+} paradox of neuronal death in stroke (Lee et al., 1999; Horn and Limburg, 2001; Ikonomidou and Turski, 2002) and represents a major problem in developing effective stroke therapies (Wahlgren and Ahmed, 2004). Recently, our laboratory has demonstrated that an influx of $[Ca^{2+}]_e$ is responsible for END and conventional Ca^{2+} entry antagonists did not inhibit this Ca^{2+} influx (Limbrick et al., 2003). In an attempt to solve the Ca^{2+} paradox, this study was initiated to determine

whether glutamate excitotoxicity induces the activation of an injury induced Ca^{2+} permeable ion channel responsible for producing the Ca^{2+} current that underlies END and leads to the initiation of neuronal death pathways ultimately leading to cell death.

O. Central Hypothesis

Stroke is one of the major causes of death and disability around the world. Great advances have been made in understanding the pathophysiology of ischemic brain injury. Excessive glutamate release leading to abnormal Ca^{2+} levels in the cells is thought to be responsible for delayed neuronal death (excitotoxicity). However, upon stroke/ ischemia Ca^{2+} continues to enter the neurons despite the presence of Ca^{2+} entry antagonists (Calcium Paradox). Thus, the route of this Ca^{2+} entry remains unknown.

Recently, our laboratory demonstrated that influx of $[Ca^{2+}]_e$ was responsible for extended neuronal depolarization, a hallmark of excitotoxic neuronal cell death. In an attempt to solve the Ca^{2+} paradox, the specific aims in this study will test the **Central Hypothesis** that "glutamate excitotoxicity induces the activation of an injury induced Ca^{2+} permeable ion channel responsible for producing the Ca^{2+} current that underlies END and leads to the initiation of neuronal death pathways ultimately leading to cell death". The following specific aims are designed to test this central hypothesis.

Aim 1: Determine whether excitotoxic glutamate results in the activation of a glutamate injury induced ion channel

Aim 2: Determine if the glutamate injury induced ion channel is responsible for the END current.

Aim 3: Determine the primary permeant ion for the glutamate injury induced ion channel.

Aim 4: Determine if this glutamate injury induced ion channel accounts for the calcium paradox of excitotoxic neuronal cell death.

To determine if a unique Ca^{2+} permeable ion channel is activated by GET and is responsible for the development of the END, injury induced Ca^{2+} current and neuronal death, we used a combination of patch clamp recordings, fluorescent Ca^{2+} imaging and neuronal cell death assays in both *in vitro* hippocampal neuronal culture model of glutamate excitotoxicity and neurons acutely dissociated from rats subjected to decapitation ischemia. Characterizing these injury-induced alterations may provide important new targets for therapeutic intervention to treat brain injury and offer novel insights into the pathophysiology of stroke.

This study demonstrates that glutamate excitotoxicity activates a previously undetected Ca^{2+} permeable ion channel that causes the Ca^{2+} current responsible for a persistent Ca^{2+} influx that maintains END and over time initiates the irreversible pathways leading to cell death following glutamate excitotoxicity. Inhibiting the Ca^{2+} permeable channel and associated current within the window of opportunity after GET reverses END, blocks Ca^{2+}entry and prevents delayed neuronal cell death. Elucidation of the glutamate excitotoxicity induced Ca^{2+} permeable channel provides an explanation for the apparent Ca^{2+} paradox, where Ca^{2+} continues to enter the neuron despite the presence of conventional Ca^{2+} entry blockers. The development of the injury induced Ca^{2+} permeable channel explains why many of the therapeutic trials employing conventional

strategies to inhibit Ca^{2+} entry have not been effective in treating stroke. The elucidation of the glutamate excitotoxicity induced Ca^{2+} permeable channel provides a new target for stroke treatment that may provide a significant extension of the therapeutic window to prevent neuronal death in stroke.

MATERIALS AND METHODS

A. Primary Hippocampal Cell Culture

Primary mixed hippocampal cultures were prepared by a modified method of Banker and Cowan (Banker and Cowan, 1977), routinely used in our laboratory (DeLorenzo et al., 1998; Limbrick et al., 2001; Sun et al., 2004). Hippocampal tissue was grossly dissected from the brains of 2 day post-natal Sprague-Dawley rats (Harlan, Frederick, MD) at room temperature in a Ca^{2+} and Mg^{2+} -free phosphate-buffered dissection saline that contained glucose/sucrose, N-[2-Hydroethyl]piperazine-N'[2-ethanesulfonic acid] (HEPES) and was supplemented with penicillin (10,000 units/ml) and streptomycin (10,000 μg/ml). After the meninges and vasculature were stripped from the tissue, hippocampi were treated with 0.25% trypsin at 37°C for 30 minutes. Enzymatic treatment was terminated by three washes in dissection saline. Next, mechanical trituration of the tissue was performed through a fire-polished Pasteur pipette in three sets of 20 passes. The concentration of the resultant single cell suspension was estimated using a cell counting chamber (Scientific Apparatus, Philadelphia, PA). The cell suspension was diluted to a final concentration of 1×10^5 cells/ml in Glial Feed (minimal essential media (MEM) with Earle's Salts, 25 mM HEPES, 2 mM L-glutamine, 3 mM glucose, and 10% fetal bovine serum) for the glial preparation or diluted to a final

concentration of 1 x 10^5 cells/ml in Neuronal Feed (MEM with Earle's Salts, 25 mM HEPES, 2 mM L-glutamine, 3 mM glucose, 100 μg/ml transferrin, 5 μg/ml insulin, 100 μM putrescine, 3 nM sodium selenite, 200 nM progesterone, 1 mM sodium pyruvate, 0.1% ovalbumin, 0.2 ng/ml triiodothyroxine, and 0.4 ng/ml corticosterone) supplemented with 5% horse serum for the neuronal preparation.

Cell suspensions in Glial Feed were plated into 35 mm plastic culture dishes or Lab-Tek coverglass chambers (Nunc, Naperville, IL) previously coated with 0.05 mg/ml poly-L-lysine and maintained at 37°C in a 5% CO_2/95% air atmosphere. The media were fully replaced three times per week with Glial Feed. When confluent, glial beds were treated with 5 mM cytosine arabinoside for two days to curtail cell division. On the 13th day in vitro (DIV), the media were fully replaced with a 5% horse serum supplemented Neuronal Feed in preparation for neuronal plating on the following day. At this time, these cultures predominantly consisted of glial cells with few, if any, neurons.

Cell suspensions diluted in Neuronal Feed were plated upon these confluent glial beds. Twenty-four hours after plating, cultures were treated with 5 μM cytosine arabinoside to inhibit non-neuronal growth. Cultures were maintained at 37°C in a 5% CO_2/95% air atmosphere and fed twice weekly with Neuronal Feed (described above). For some cultures the feeding media was Neurobasal-A medium supplemented with B-27 (Invitrogen Corp., San Diego, CA) containing 0.5 mM L-glutamine. These mixed cultures were used for experiments from 13 DIV through the life of the cultures (approximately 21 DIV).

B. Excitotoxic glutamate exposure:

Excitotoxic glutamate injury was induced as described previously (Choi et al., 1987; Rothman et al., 1987; Sombati et al., 1991; Coulter et al., 1992; Limbrick et al., 2003). The recording solution consisted of (in mM): 145 NaCl, 2.5 KCl, 10 HEPES, 10 glucose, 2 $CaCl_2$, and 1 $MgCl_2$ (pH 7.3, 325 mosM adjusted with sucrose). Glutamate (500 μM) was dissolved in recording solution containing $MgCl_2$ (1 mM) and applied with 10μM glycine for 10 minutes. For combined injury experiments, glutamate was dissolved in recording solution containing no added $MgCl_2$. Depending upon the experiment; washout of the glutamate was performed with control recording solution (2 mM $CaCl_2$), Ca^{2+}-free recording solution (0 mM $CaCl_2$), or Na^+-free recording solution. Ca^{2+}-free recording solution was made by omitting $CaCl_2$ but contained no Ca^{2+} chelator. For combined injury experiments control recording solution (2 mM $CaCl_2$) containing no added $MgCl_2$ was used. In some cases, $MgCl_2$ (2 mM) was substituted for $CaCl_2$. High- Ca^{2+} recording solution (10 mM $CaCl_2$) was made by equimolar replacement of NaCl. Na^+-free recording solution was made by equimolar substitution of *N*-methyl-D-glucamine chloride for NaCl. For experiments with Gd^{3+} or Zn^{2+}, the cation salts were included in the pipette or wash solutions (See Table 5 for different recording solutions and their compositions).

C. Patch clamp experiments

Single channel data and membrane potentials were recorded using standard procedures as explained below.

Cell-attached/ On-cell Recordings

Cell-attached single channel recordings were performed on phase bright pyramidal shaped neurons utilizing methods previously described (Hamill et al., 1981). Cell culture media were replaced with recording solution. Cultures were then transferred to a heated stage (Brook Industries, Lake Villa, IL) on a Nikon Diaphot inverted microscope (Garden City, NY) or an Olympus IX 70 inverted microscope for electrophysiological recordings. Patch microelectrodes were pulled from borosilicate glass capillaries (World Precision Instruments, Inc., Sarasota, FL) using a Brown-Flaming P-80C electrode puller (Sutter Instruments, Novato, CA) in 2 or 3 heating cycles. The electrodes were fire-polished on a microforge (Narshige, Tokyo, Japan) and coated with Sylgard # 184 (Dow Corning Corp, MI) or Sigmacote (Sigma Chemicals, St. Louis, MO). The electrodes had a resistance of 5-7 mΩ when filled with solution containing (in mM) 145 NaCl, 2.5 KCl, 10 HEPES, 10 glucose, 2 $CaCl_2$, 1 $MgCl_2$, 10 CsCl, 1 μM MK-801, 10 μM CNQX, 5 μM nifedipine, 10 μM $GdCl_3$ and 1 μM TTX (pH 7.3, 325 mosM adjusted with sucrose). Neurons were bath-perfused with a "high-K^+" solution in which the extracellular KCl concentration was raised from 2.5 to 40 mM to clamp the resting membrane potential near 0 mV. The bath solution consisted of (in mM) 105 NaCl, 40 KCl, 10 HEPES, 10 glucose, 2 $CaCl_2$, 1 $MgCl_2$ and 1 μM TTX (pH 7.3, 325 mosM adjusted with sucrose)

Recordings were obtained in the on-cell configuration (Hamill et al., 1981) using an Axopatch 200A amplifier (Axon Instruments, Foster City, CA) in voltage clamp mode. Briefly, the filled patch microelectrode was mounted on the amplifier headstage

and positioned near the neuron of interest using a course-adjustment manipulator (Narashige, Inc., Tokyo, Japan). Once maneuvered directly above the neuron with a fine-adjustment manipulator (Narashige, Inc., Tokyo, Japan), offset potentials (junction potentials) were nulled. Then, a test square pulse was generated through the amplifier and monitored with an oscilloscope. The patch microelectrode was slowly lowered until making gentle contact with the surface of the neuron, as indicated by a slight change in shape of the test pulse. At this point, negative pressure was slowly applied through the patch microelectrode, increasing the resistance of the seal between microelectrode and neuronal membrane. Formation of a giga-ohm seal was verified and the patch was voltage clamped at –60mV by applying a voltage of opposite sign to the patch pipette solution and the recording was started. Current amplification was accomplished with an Axopatch 200A patch clamp amplifier (Axon Instruments, CA) and recorded on a VCR tape via a Neurocorder (Neurodata, NewYork) using PClamp software (Ver. 9, Axon Instruments Co.) via a Digidata 1322A signal acquisition system. Data were filtered at 2 kHz (3 dB, 4-pole Bessel) and digitized at 10 kHz sampling rate.

Whole-Cell Current Clamp Recordings

Whole-cell current clamp recordings were performed on pyramidal shaped neurons utilizing methods previously described in our laboratory (Sombati et al., 1991; Coulter et al., 1992; Limbrick et al., 2003). Cell culture media was replaced with recording solution. Cultures were then transferred to a heated stage (Brook Industries, Lake Villa, IL) on a Nikon Diaphot inverted microscope (Garden City, NY) or Olympus

IX-70 for electrophysiological recordings. Patch microelectrodes of 3-7 MΩ resistance were pulled from borosilicate glass capillaries (World Precision Instruments, Inc., Sarasota, FL) using a Brown-Flaming P-80C electrode puller (Sutter Instruments, Novato, CA) and filled with an internal solution of 140 mM K^+ gluconate, 1 mM $MgCl_2$, 10 mM HEPES, 1.1 mM EGTA, 4 mM Na_2 ATP, 15 mM Tris phosphocreatine, pH 7.2, osmolarity adjusted to 310 mOsm with sucrose.

Recordings were obtained in the whole-cell current clamp configuration (Hamill et al., 1981) using an Axopatch 200A amplifier or an Axoclamp-2A amplifier (Axon Instruments, Foster City, CA) in current clamp mode. Formation of a giga-ohm seal was accomplished as described above. At this stage the membrane within the opening of the microelectrode tip was ruptured by sharp application of negative pressure giving low resistance access to the cell interior. Capacitance compensation was performed and recording was started.

Data were digitized and stored on videotape using a Neuro-corder DR-890 (Neurodata Instruments Corp, New York, NY) and Sony VCR. Data were later played back for analysis to a Dash IV chart recorder (Astro-Med Inc., Warwick RI) or to a computer via a Digidata 1322A (Axon Instruments, Foster City, CA).

Bath application of glutamate was performed by gravity perfusion at a rate of 1 ml/min and solution changes were controlled through a six-valve perfusion system (Warner Instrument Corp., Hamden, CT). END was defined as a persistent membrane depolarization to around –10 to –20 mV (Sombati et al., 1991; Coulter et al., 1992).

D. Measurement of Neuronal $[Ca^{2+}]_i$

Changes in neuronal $[Ca^{2+}]_i$ were measured using the ratiometric, high affinity ($K_d \approx 224$ nM) fluorescent Ca^{2+} indicator, Fura-2 (Grynkiewicz et al., 1985). Fura-2 is a dual excitation, single emission indicator whose excitation maximum of 380 nm shifts to a lower wavelength upon binding of Ca^{2+} (340 nm) with minimal change in the emission maximum (510 nm). The relative signal intensities measured at 510 nm by alternating excitation at 340 nm and 380 nm can be used to quantify the relative concentrations of Ca^{2+}-bound Fura-2 and Ca^{2+}-free Fura-2, respectively. Therefore, the ratio of measured signal intensity of alternating excitation wavelengths (340/380 nm) is directly related to the total concentration of Ca^{2+} and can be used to measure $[Ca^{2+}]_i$ in neurons.

Cell loading with Fura-2

As described previously by our laboratory (Pal et al., 2001), neurons were loaded with 1 μM acetoxymethylester Fura-2 (Fura-2 AM) (Molecular Probes, Eugene, OR) dissolved in recording solution (0.1% DMSO) for one hour at 37°C in a 5% CO_2/95% air atmosphere. As a membrane permeant compound, Fura-2 AM crosses the neuronal membrane where intracellular esterases cleave the AM moiety trapping the free acid Fura-2 molecule inside. Dye loading was terminated with 3 washes with recording solution, and cells were incubated for 15 min to allow for complete cleavage of Fura-2 AM.

Microfluorometry

Cultures grown on glass coverslip chambers were visualized on an inverted microscope (Olympus IX 70, Olympus America) using a 20X, 0.7 numerical aperture fluorite water immersion objective (Olympus America) maintained at 37°C with a heated stage (Harvard Apparatus Inc.). Fura-2 was excited with a 75 W xenon arc lamp (Olympus Optical Co.) with alternating wavelengths of 340 nm and 380 nm filtered through a Sutter Filter Wheel (Sutter Instruments Co., Novato, CA). Fluorescent emission at 510 nm was captured through a Fura filter cube (Olympus America, Melville, NY) with a dichroic at 400 nm emissions using a cooled digital CCD camera (LSR AstroCam Limited). Image acquisition and processing were controlled by a computer using the Temporal Module of the Perkin Elmer Life Sciences Imaging Suite (Version 4.0). To calculate ratio values, image pairs at each wavelength were captured and digitized every 15 s or 1 min, and the images at each wavelength were averaged over four frames. The background fluorescence was obtained by imaging a field lacking fluorescent indicators. The significance of data was tested by Student's paired or unpaired t test wherever appropriate.

E. Neuronal Cell death assay

Cell viability assays were performed as described previously (Raza et al., 2001). Neurons were analyzed for presence of apoptosis using the Vybrant Apoptosis Assay Kit #3 (Molecular Probes, Eugene, OR). In normal live cells, phosphatidylserine (PS) is located on the cytoplasmic surface of the cell membrane. However, in apoptotic cells, PS

is translocated from the inner to the outer leaflet of the plasma membrane, thus exposing PS to the external cellular environment. The human anticoagulant, annexin V, is a 35–36 kD Ca^{2+}-dependent phospholipid-binding protein that has a high affinity for PS. Annexin V labeled with a fluorophore or biotin can identify apoptotic cells by binding to PS exposed on the outer leaflet. The Vybrant® Apoptosis Assay Kit #3 contains recombinant annexin V conjugated to fluorescein (FITC annexin V) and the red-fluorescent propidium iodide (PI) nucleic acid binding dye. PI is impermeant to live cells and apoptotic cells, but stains dead cells with red fluorescence, binding tightly to the nucleic acids in the cell. Cells were treated with FITC annexin-V 5 µl /100 µl of total volume and 1 µl of 100 µg/ml of PI solution. After 15 min, 400 µl of 1× annexin-binding buffer was added and cells were visualized. Neurons were classified as either apoptotic (green fluorescence), necrotic (red fluorescence) or viable (phase-bright) using an Olympus CK2 inverted microscope (Olympus America, Melville, NY, USA) outfitted with 20x objective and fluorescent phase filters. Images were captured with Q-Capture ver 2.55 (Quantitative Imaging, Burnaby, British Columbia, Canada) using an Olympus Q-fire digital camera mounted on the Olympus CK2 inverted microscope. Fluorescent images were compared to phase bright images to confirm that only pyramidal shaped neurons were counted. Three to five randomly selected fields were counted and averaged per culture dish. The fraction of apoptotic cells in each cultures were calculated as:

$$\text{Fraction apoptotic} = (\text{apoptotic}_{\text{treat}} - \text{apoptotic}_{\text{control}})/ \text{apoptotic}_{\text{glutamate}}.$$

F. Decapitation Ischemia

Decapitation ischemic damage was created using a decapitation ischemia model as described previously (Abe, Yoshida et al. 1983; Wasterlain and Powell 1986; Parsons, Churn et al. 1999; Gerasimov, Artemenko et al. 2004). All animal use procedures were in strict accordance with the National Institutes of Health guidelines for animal use and approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University. Male Sprague-Dawley rats weighing 140-160 g and ~3-6 weeks of age were used in all experiments. Rats were anesthetized with 2.5% halothane prior to rapid decapitation. Ischemia was induced in the rat forebrains post decapitation and prior to dissociation as described (Wasterlain and Powell, 1986). In brief, rats were decapitated with a guillotine model of ischemia. The isolated rat head with intact non-perfused brain was placed immediately in a conventional oven pre-equilibrated to 37°C for 30 min. The time between decapitation and oven placement was kept below 5 s to prevent the neuroprotective effects of hypothermia. Oven temperature was monitored to ensure steady maintenance of 37°C. Immediately after that, brain was rapidly dissected and hippocampal slices were cut using a vibratome and subsequently dissociated to obtain neuronal suspension as below.

G. Isolation of Hippocampal Pyramidal CA1 Neurons

Hippocampal CA1 neurons were acutely isolated by a modification of the methods described previously. Briefly, 15 min prior to acute isolation, the animals were injected with 1 mg/kg MK-801 i.p. to block NMDA receptor activation during the

isolation procedure and to increase viability. The brain was rapidly dissected and placed in a 4°C chilled oxygenated (95% O_2–5% CO_2) artificial cerebrospinal fluid solution (aCSF) composed of (in mM) 201 sucrose, 3 KCl, 1.25 $NaHPO_4$, 6 $MgCl_2$, 0.2 $CaCl_2$, 26 $NaHCO_3$, and 10 glucose (solution A). MK-801 (1 μM) was added to all solutions during the isolation procedure to increase viability and removed 15 min prior to the patch clamp experiments. Hippocampal slices of 450 μm were cut on a 12° agar ramp with a vibratome sectioning system (Vibrotome Series 3000, Pelco 100 Vibrotome sectioning system, Ted Pella Inc, Redding, CA) equipped with a bath refrigeration system (Ted Pella Inc, Redding, CA) and incubated for 10 min in an oxygenated medium at 34°C containing (in mM) 120 NaCl, 5 KCl, 6 $MgCl_2$, 0.2 $CaCl_2$, 25 glucose, and 20 piperazine-N,N′-bis[2-ethanesulfonic acid] (PIPES), pH adjusted to 7.2 with NaOH (solution B). Slices were then treated with 8 mg/ml of Protease XXIII (Sigma Chemical Co.) in solution B for 6–8 min and then thoroughly rinsed with solution B. The CA1 region was visualized in a dark background with the help of a dissecting microscope and 1 mm^2 thick chunks were excised. These tissue preparations were then triturated with a series of pasteur pipettes of decreasing diameter at 4°C in the same medium. The resulting cell suspension was then placed in the center of 35 mm culture dishes and immediately placed in a humidified oxygenated dark chamber at 37°C for 1 h. Polystyrene culture dishes (35 mm diameter) were previously treated overnight with 0.05 mg/ml poly-L-lysine (Sigma) followed by multiple rinses with distilled water. These Poly-L-lysine coated dishes were then treated with Cell-Tak™ (BD-Biosciences) biocompatible cellular adhesive (3.5 $\mu g/cm^2$) for 1hr, rinsed and air-dried. Neuronal suspension placed in the center of

adhesive coated dishes when settled firmly adheres to the bottom. This technique simplifies further manipulations on the dissociated neurons.

H. Data Analysis

To test for statistical differences in the comparison of post-glutamate membrane potentials or neuronal cell-death assay, a one way Analysis of Variance (ANOVA) was run, followed by a *Tukey* or *Dunnett* post-hoc test. In all cases, the α value was set at $p < 0.05$. The data is represented as mean ± SEM. The identifications of single channel opening and closing transitions were accomplished using ClampFit 9.0 (pClamp software suite, Axon Instruments). Statistical tests were run using SigmaStat 2.0 (SPSS, Chicago, IL) and graphs were generated using SigmaPlot 8.0 (SPSS, Chicago, IL).

I. Materials

Reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. MEM, L-glutamine, trypsin, phosphate buffered saline, penicillin-streptomycin, fetal bovine serum, and horse serum used in the tissue culture preparation were obtained from Gibco-BRL (Gaithersburg, MD).

Table 5. Table summarizing different recording solutions and their compositions.

Recording Solutions	Composition (in mM)
bath recording solution (BRS)	145 NaCl, 2.5 KCl, 10 HEPES, 10 glucose, 2 $CaCl_2$, and 1 $MgCl_2$
zero calcium solution	BRS without added $CaCl_2$
zero sodium solution	BRS where NaCl replaced with equimolar NMDG
low magnesium solution	BRS without added $MgCl_2$
Pipette solution (whole-cell)	140 mM K^+ gluconate, 1 mM $MgCl_2$, 10 mM HEPES, 1.1 mM EGTA, 4 mM Na_2 ATP, 15 mM Tris phosphocreatine
Pipette solution (cell-attached)	BRS with 10 CsCl, 1 μM MK-801, 10 μM CNQX, 5 μM nifedipine, 10 μM $GdCl_3$ and 1 μM TTX
High-K^+ solution	105 NaCl, 40 KCl, 10 HEPES, 10 glucose, 2 $CaCl_2$, 1 $MgCl_2$

All the chemicals were dissolved in de-ionized water. The pH was solution was 7.3 and osmolarity adjusted to 325 mOsm using sucrose. The antagonists/ blocker cations were incorporated in pipette solution for on-channel recordings and in the bath solution for whole-cell recordings.

RESULTS

I. Glutamate Excitotoxicity Induces Activation Of A Novel Calcium Permeable Ion Channel In Cultured Hippocampal Neurons

To determine if a unique Ca^{2+} permeable ion channel is activated by glutamate excitotoxicity and is responsible for the development of the END, injury induced Ca^{2+} current and neuronal cell death, we used a combination of patch clamp recordings, fluorescent Ca^{2+} imaging and neuronal cell death assays in an *in vitro* hippocampal neuronal culture model of glutamate excitotoxicity.

Excitotoxic Glutamate exposure produces an Extended Neuronal Depolarization (END) In Neurons

In agreement with previous studies published by our laboratory, excitotoxic glutamate exposure (500 μM glutamate + 10 μM glycine, 10-mins) produced an extended neuronal depolarization (END). Figure 5 shows hippocampal neurons in culture. All the experiments were performed on pyramidal shaped neurons indicated by black arrows in the picture. As shown in Fig. 6, glutamate application produced a stereotypical response in neurons. A short exposure (≤ 5-min) produced rapid initial depolarization followed by a sustained depolarization around -5 to +5 mV. After glutamate washout, the neurons

rapidly repolarized to resting membrane potential and sometimes even exhibited post-glutamate hyperpolarization commonly observed in hippocampal neurons due to activation of Na^+/K^+ exchanger in response to Na^+ entry upon glutamate mediated depolarization (Fig. 6A).

In contrast, following washout after a prolonged 10-min glutamate exposure (excitotoxic insult), neurons only repolarized partially and stayed depolarized at around –15 to –20 mV throughout the duration of the recording (Fig. 6B). This is the extended neuronal depolarization, or END, first described by Sombati et al. (Sombati et al., 1991; Coulter et al., 1992). All the experiments described in this dissertation were performed in this post-glutamate END period.

Excitotoxic glutamate exposure activates an injury induced ion channel

Excitotoxic glutamate exposure produces END and is associated with the induction of a persistent inward current responsible for maintaining END (Limbrick et al., 2003). In order to investigate if this injury-induced current is mediated through activation of an ion channel, we recorded single channel currents in the post-glutamate END period using the cell-attached/ on-cell configuration.

Control neurons exhibited a membrane potential of -55.6 ± 2 mV. Stepping the membrane potential from –90 to +60 mV from a holding potential of –60mV produced no inward or even outward current in control neurons. Thus, recordings from control neurons were essentially silent and demonstrated no channel activity. A total of 10 neurons were recorded under control conditions.

In contrast, as shown in Fig.7, recordings from excitotoxic glutamate injured neurons demonstrated the presence of channel activity. Distinct openings and closings of an ion channel were observed in the post-glutamate END period corresponding to the appearance of the injury-induced current. Thus, the observed channel activity could represent elementary unit conductances of the glutamate injury-induced inward current observed by Limbrick et al (Limbrick, 2000).

Downward deflections from the baseline are indicative of single channel openings and a silent period between two openings is indicative of the channel being closed. A total of 10 neurons were recorded for END conditions and 7-8 times out of 10 patches channel activity was noted upon excitotoxic glutamate exposure. END neurons had a membrane potential of -15.6 ± 1.8 mV. Neurons were then bathed in a Hi-K^+ solution to clamp the resting membrane potential near 0 mV. As shown in Fig.8A, stepping the membrane potential from a holding potential of –60 mV down to more negative hyperpolarizing potentials increased the amplitude of current, whereas the amplitude of the current decreased at more depolarizing potentials.

Distribution of the amplitudes of channel openings across various holding potentials was measured using the 50% threshold method in Clampfit® and the mean amplitude was obtained by fitting the graph using a Gaussian distribution function. Plotting the mean current at the respective voltages generated the current-voltage relationship (I-V curve). The single channel I-V relationship for 10 END neurons is shown in Fig. 8B. The glutamate injury induced channel exhibited an almost linear I-V relationship with a unitary conductance of 60.1 ± 2 pS.

Glutamate injury induced ion channel is responsible for the END current

After establishing that an injury-induced ion channel is activated following glutamate excitotoxicity, we then wanted to investigate if the post injury current as reported by Limbrick (Limbrick, 2000) is in fact mediated by the ion channel observed in this study. This post-injury current was blocked by high concentrations of zinc or gadolinium. Thus, blockade of the single channel by Zn^{2+} and Gd^{3+} in the post-glutamate period would further confirm that it had the same pharmacological profile as Limbrick et al.'s post injury Ca^{2+} current, I_{CIC}.

Cell-attached recordings following excitotoxic glutamate exposure from neurons patched with pipettes containing low concentrations of Zn^{2+} (5 μM) or Gd^{3+} (10 μM) along with normal concentrations of Na^{+} and Ca^{2+} had no effect on the injury-induced channel activity. As shown in Fig. 9A, the amplitude of the channel openings essentially stayed the same in the presence of low concentrations of Gd^{3+} (10 μM). The Current-Voltage relationship (Fig. 9B) showed that the conductance of the channel was slightly reduced to 58.4 ± 1.1 pS in the presence of low concentrations of Gd^{3+}, however this reduction in conductance was not significantly different from the injury induced ion channel conductance ($p = 0.309$). Similarly, incorporation of low concentrations of Zn^{2+} (5 μM) in the extracellular pipette solution did not significantly decrease the amplitude of channel openings (Fig. 11A) or the channel conductance (Fig. 11B). Channel conductance in the presence of Zn^{2+} (5 μM) was 60.5 ± 1.0 and was not significantly different from the injury induced channel conductance alone ($p = 0.715$).

However, excitotoxic glutamate injured neurons when patched with pipette solution containing high concentrations of Zn^{2+} (500 μM) or Gd^{3+} (100 μM) along with normal concentrations of Na^{+} and Ca^{2+} completely abolished the injury induced channel activity. A total of 10 neurons were recorded for each blocker cation concentration. Figs. 10 and 12 shows representative single channel traces from a neuron showing no channel activity when patched with Gd^{3+} (100 μM) or Zn^{2+} (500 μM) respectively at various holding potentials. Inclusion of high concentrations of $ZnCl_2$ or $GdCl_3$ in the extracellular pipette solution completely abolished the injury induced channel activity and the traces resembled to that of a control neuron. Thus, this inhibitory data provides additional evidence that the new glutamate excitotoxicity activated injury-induced channel is responsible for the post injury current (Limbrick, 2001) that mediates END.

Calcium ions are the primary permeant ions for the injury induced ion channel

In order to evaluate the involvement of extracellular ionic species in the maintenance of END and determine which ion is the primary permeant ion for the glutamate injury induced ion channel, we conducted a series of experiments where individual ions were systematically excluded from the extracellular medium.

The mean resting membrane potential for neurons in this study was –60.8 ± 1.5 mV (n = 5). Excitotoxic glutamate exposure produced END exhibiting membrane potentials around –20 mV. Removing extracellular Na^{+} by replacement of NaCl with impermeant N-methyl-D-gluconate (NMDG) had little effect on post-glutamate membrane potentials. Neurons exhibited membrane potential of around –25 ± 2.3 mV for

absence of $[Na^+]_e$ which was not significantly different from END alone. On the other hand removing extracellular Ca^{2+} abolished END. Neurons regained their pre-glutamate membrane potential upon omission of $[Ca^{2+}]_e$. Current clamp recordings demonstrating END (n=5), recovery from END upon removal of $[Ca^{2+}]_e$ (n=5) and no recovery from END upon removal of $[Na^+]_e$ (n=5) in the post-glutamate period are shown in Figure 13 A-C.

Cell-attached single channel recordings in the presence and absence of Na^+ or Ca^{2+} ions were performed to investigate which ion was permeating through the injury induced ion channel. Single channel recordings in the post-glutamate END period revealed the presence of the injury-induced ion channel. Cations were then sequentially replaced from the pipette solution. Sodium ions were replaced by equimolor substitution of sodium chloride (NaCl) with impermeant N-methyl-D-glucamine (NMDG) chloride. Sodium replacement from the extracellular pipette solution had no significant effect on channel activity. The channel activity was essentially maintained despite Na^+ replacement (Fig. 14A). Removal of Na^+ did not produce a significant decrease in conductance. Unitary conductance was 59.3 ± 0.5 pS after removing Na^+, a value that was not significantly different than in the presence of Na^+ ($p = 0.486$) (Fig. 14B). A total of 7 neurons were recorded for removal of Na^+. Thus, Na^+ appears to be a poor charge carrier and contributes little to injury induced channel conductance.

In contrast, removing extracellular Ca^{2+} by omitting $CaCl_2$ from the pipette solution completely abolished the injury induced channel activity. Figure. 15 shows representative single channel recordings from a neuron patched with no added Ca^{2+} in the

pipette solution. Similar results were obtained in 7 patches. As shown in Figs. 10 and 12 for high Zn^{2+} or Gd^{3+}, upon omission of $[Ca^{2+}]_e$, no channel openings were observed at any of the holding potentials and the single channel traces resembled to that of a control patch. Although Na^+ may carry a trace amount of current under Ca^{2+} free conditions, this current is small and could not be resolved in our preparation and patch clamp configuration. These observations suggest that Ca^{2+} is the primary ion permeating through the injury induced ion channel.

Direct measurements of $[Ca^{2+}]_i$ were employed to further establish that Ca^{2+} influx was responsible for END and that Ca^{2+} was the major ion permeating the injury induced channel. A total of 5 sets of fluorescent Ca^{2+} imaging were performed for conditions described above. Fig. 16 shows the average $[Ca^{2+}]_i$ traces of population of neurons subjected to excitotoxic glutamatergic insult and then washed with control recording solution or Na^+ free recording solution or a Ca^{2+} free recording solution. Under all the treatment conditions, exposure to 500 μM glutamate + 10 μM glycine caused a rapid and large increase in $[Ca^{2+}]_i$ as evidenced by increase in Fura-2 340/380 ratio around 1.5-1.6. The ratio stayed constant in this range for as long as glutamate was present, in this case an excitotoxic exposure lasting 10-mins. Washout of glutamate after this 10-min period with control recording solution produced a slight decrease in the 340/380 ratios to around 1.4-1.5, where it remained till the termination of experiment 80-mins later.

Omission of $[Na^+]_e$ by replacement of NaCl with NMDG-Cl in the wash solution produced no changes in Fura-2 348/380 ratios with the average ratio of 1.4-1.5, very similar to that of control wash solution. Thus, perfusion with Na^+-free wash solution did

not accelerate $[Ca^{2+}]_i$ recovery and in fact resulted in a continuance of the levels obtained during the glutamate-treatment itself (Fig. 16).

In contrast, omission of $[Ca^{2+}]_e$ in the wash solution had a drastic effect on the excitotoxic glutamate induced protracted $[Ca^{2+}]_i$ elevations, resulting in the restoration to the basal $[Ca^{2+}]_i$ Fura-2 340/380 ratio. As shown in Fig. 16, upon glutamate exposure, a similar peak Fura-2 340/380 ratio of 1.6 was obtained. Washout of glutamate with a recording solution containing no added $CaCl_2$ produced a complete restoration of elevated 340/380 ratios to the pre-treatment levels in about 45-50 minutes.

Taken together, these results indicate that Ca^{2+} is the primary permeant ion for the injury induced channel and the persistent elevated $[Ca^{2+}]_i$ levels post injury are mediated by extracellular Ca^{2+} entry through the injury-induced channel. Further, these studies also indicate that the protracted $[Ca^{2+}]_i$ levels are of an extracellular origin and not from intracellular Ca^{2+}- release or from intracellular stores.

Conventional glutamate receptor-gated or voltage-gated ion channels do not mediate the injury induced ion channel activity

In order to investigate if the conventional glutamate receptor-gated or voltage-gated ion channels were the molecular basis of the injury induced ion channel, we conducted a series of experiments using pharmacological inhibitors of well-characterized cation channels known to exist in the hippocampal neurons (Chen et al., 1997; Sensi et al., 1997; Dingledine et al., 1999). Single channel cell-attached recordings were conducted on control and glutamate injured hippocampal neurons in the presence of Ca^{2+}

entry inhibitors (CEI), consisting of MK-801 (10 μM), CNQX (10 μM), $GdCl_3$ (10 μM) and nifedipine (5 μM) in the extracellular pipette solution. The CEI mixture has been shown to inhibit voltage gated calcium channels (nifedipine), the NMDA channel (MK-801), AMPA/ KA channels (CNQX), and the TRP channel family ($GdCl_3$) (Sattler et al., 1998; Sattler et al., 1999; Aarts et al., 2002; Aarts et al., 2003). Thus, recording in the presence of the CEI mixture should inhibit the major mechanisms of Ca^{2+} entry following GET (Aarts et al., 2003). To block voltage-gated sodium channels, tetrodoxin (TTX, 1 μM) was also incorporated in the wash solution. Incorporating Cs^+ ions (CsCl, 10 mM) in the pipette solution blocked the potassium channels.

Cell-attached single channel recordings from control neurons demonstrated no channel activity. Conversely, glutamate-injured neurons manifested distinct openings and closings of an ion channel that existed in the presence of CEI corresponding to the appearance of the post injury current in the post-glutamate END period (Fig. 17A). As shown Fig. 17B, in the presence of CEI's the channel had a unitary conductance of 59.6 ± 1.1 pS which was not significantly different from the injury-induced ion channel conductance ($p = 0.679$). A total of 10 neurons were studied for the presence of CEI's. These results suggest that the injury-induced Ca^{2+} permeable ion channel is largely independent of the activation of glutamate-gated, voltage-gated Ca^{2+} or TTX-sensitive Na^+ channels.

Taken together, these results further confirm that a novel channel activity is responsible for END and I_{CIC} and is present in glutamate-injured neurons in the presence

of CEI's. The fact that Ca^{2+} entry is observed despite presence of CEI's points towards to the possible role of this injury-induced ion channel in explaining Ca^{2+} paradox.

Neuroprotection with Gadolinium: Injury-Induced Ca^{2+} permeable ion channel accounts for the "Ca^{2+} paradox"

Our earlier observations showed that during post glutamate-injury, Ca^{2+} still continues to enter the neuron despite the presence of Ca^{2+} entry inhibitors (CEI). The CEI's failed to block the injury induced Ca^{2+} permeable ion channel suggesting that the current mediated by this channel could account for the Ca^{2+} paradox, since Ca^{2+} ions were still continuing to enter the neuron even in the presence of conventional Ca^{2+} entry antagonists. Thus, to test whether activation of this channel could explain the Ca^{2+} paradox, we investigated if blocking this channel activity could prevent neuronal death after glutamate excitotoxicity. We measured the fraction of the cells undergoing apoptosis upon excitotoxic glutamate exposure in the presence and absence of various agents in a post-glutamate injury intervention paradigm.

Apoptosis was measured using a fluorescent Annexin-V and Propidium Iodide (PI) assay that identifies cells by color: apoptotic cells (green), necrotic cells (red) and viable cells (phase bright). A series of hippocampal neuronal culture plates were subjected to control or excitotoxic glutamate exposure. Ten minutes later they were washed with control solution or solutions containing: CEI mixture or nitric oxide synthase inhibitor, L-NAME (300 μM) or acid sensing ion channel inhibitor, Amiloride (100 μM) or a solution containing high Gd^{3+} (100 μM) or low Gd^{3+} (10 μM) followed by

incubation with the fluorescent dyes. Upon washout of dyes with respective solutions, the fraction of apoptotic cells were measured as described in the methods section.

Each column in the bar graph shown in Fig. 18 represents an average fraction or the apoptotic cells measured from (N = 6) culture plates calculated according to a formula described in methods section. A 10-min exposure to control solution produced a small insignificant amount of apoptotic cells (< 5%). However, excitotoxic glutamate exposure produced a robust increase in the fraction of cells undergoing apoptosis (>90%). Treatment with CEI mixture or L-NAME or Amiloride or low Gd^{3+} produced only a small decrease in fraction of cells undergoing apoptosis. This decrease was not statistically significant when compared with glutamate-alone control ($p = 0.297$).

In contrast, treatment with 100 μM Gd^{3+} had a dramatic effect on the fraction of apoptotic cells. High Gd^{3+} treatment reduced the number of cells undergoing apoptosis to approximately 20%. Gadolinium intervention produced a statistically significant decrease in the fraction apoptotic cells when compared with glutamate-alone control ($p<0.05$). Thus, high Gd^{3+} treatment afforded neuroprotection to the cells otherwise destined to die upon glutamate excitotoxicity.

These results demonstrate that inhibiting the injury-induced I_{ClC}-Ca^{2+}-permeable channel blocks both END and delayed neuronal cell death following glutamate excitotoxicity before the irreversible neuronal death pathways are activated. The data also provide an explanation for the Ca^{2+} paradox demonstrating that inhibiting the previously unknown injury-induced Ca^{2+}-permeable channel was able to prevent Ca^{2+} entry, reverse END and thus resolve the Ca^{2+} paradox.

Blockade of the injury induced Ca^{2+} permeable channel: Extension of the therapeutic window

After demonstrating the neuroprotective action of Gd^{3+} mediated injury induced channel blockade, we next investigated if there is a window of opportunity to block this injury induced Ca^{2+} permeable ion channel after the injury and still observe a neuroprotective effect. To elucidate the therapeutic window, we either removed $[Ca^{2+}]_e$ at various time points after the injury and measured the Fura-2 $[Ca^{2+}]_i$ 340/380 ratio or in parallel cultures intervened with Gd^{3+} (100 μM) at the same time-points after injury and measured the fraction of apoptotic cells.

A series of hippocampal neuronal culture plates were subjected to excitotoxic glutamate injury. After 10 mins, glutamate was washed out with control solution and returned to the incubator. At every 1hour time point starting at 0hour (immediately after 10-min glutamate insult) and up to 4hour, one plate was removed from the incubator and washed with Ca^{2+} free solution or 100 μM Gd^{3+} containing solution. The fraction of apoptotic neurons or the Fura-2 ratio was then measured independently at each time point. A total of 6 plates were measured for the fraction of apoptotic neurons or $[Ca^{2+}]_i$ Fura-2 ratio for each of the time points.

Inhibition of the injury-induced Ca^{2+}-permeable channel with 100μM Gd^{3+} produced a significant reduction in the number of apoptotic cells and conferred protection to the neurons when administered for up to 1hr after GET. In addition, up to 50% neuroprotection was also observed even out to 2 hrs after GET. The neuroprotective effect of Gd^{3+} was significant up to 3 hrs after the injury compared to glutamate alone.

Beyond this time point the fraction of neurons undergoing apoptosis started to increase despite the presence of Gd^{3+} (Fig. 19).

Ratiometric Fura-2 experiments employing Ca^{2+} free wash solution after excitotoxic glutamate exposure demonstrated that in injured neurons elevated $[Ca^{2+}]_i$ recovered to basal levels for up to 1hour after removal of $[Ca^{2+}]_e$. The percentage of neurons buffering elevated $[Ca^{2+}]_i$ upon $[Ca^{2+}]_e$ removal were significantly different up to 3 hrs. Beyond this time point the percentage of neurons that could restore basal $[Ca^{2+}]_i$ levels started to decrease despite removal of $[Ca^{2+}]_e$ (Fig. 20).

These experiments indicate that there is a window of opportunity of up to 2 hour where it is possible to intervene and restore basal $[Ca^{2+}]_i$ levels by inhibiting the novel injury-induced Ca^{2+}-permeable channel before the irreversible effects of injury are initiated.

Status Epilepticus along with glutamate excitotoxicity produces a severe injury compared to excitotoxic glutamate injury alone

As shown above (Fig. 16), our laboratory and others have demonstrated that removal of $[Ca^{2+}]_e$ restored basal $[Ca^{2+}]_i$ in injured neurons in the well established neuronal culture model of glutamate excitotoixity and stroke (Hartley and Choi, 1989; Manev et al., 1989; Randall and Thayer, 1992; Limbrick et al., 2003; Chinopoulos et al., 2004). However, producing excitotoxic injury in the absence of extracellular Mg^{2+}, causes the levels of $[Ca^{2+}]_i$ to remain elevated despite the removal of $[Ca^{2+}]_e$ (Khodorov, 2004). Incubating neurons in culture in 0 Mg^{2+} is known to induce continuous

epileptiform activity, status epilepticus (Churn and DeLorenzo, 1998). To further distinguish these injuries, we compared the effects of glutamate excitotoxicity alone and with 0 Mg^{2+} on the ability of removal of $[Ca^{2+}]_e$ to restore basal $[Ca^{2+}]_i$ or END potentials after the injury.

Figure 21 shows current clamp recordings for excitotoxic glutamate injury in the presence and absence of extracellular Mg^{2+}. A total of 6 neurons were analyzed for both the conditions. A rapid depolarization of membrane potential was observed under both the conditions. As shown previously and in Fig. 9 above, wash out of glutamate with control recording solution produced END. Omission of $[Ca^{2+}]_e$ from the wash solution abolished END and restored resting membrane potentials only in neurons injured in presence of Mg^{2+}. Removal of $[Ca^{2+}]_e$ failed to restore resting membrane potentials in neurons receiving excitotoxic injury in the absence of Mg^{2+} and END persisted in these neurons (Fig. 21B).

Figure 22 show the Fura-2 340/380 ratios for excitotoxic glutamate injury in the presence and absence of extracellular Mg^{2+}. A total of 5 experiments were performed for both the conditions. Rapid increases in Fura-2 ratios were observed under both conditions. However, neurons injured in the absence of $[Mg^{2+}]_e$ had higher 340/380 peak ratios compared to neurons injured in the presence of Mg^{2+}. Further, as shown in Fig. 12, removal of $[Ca^{2+}]_e$ restored basal $[Ca^{2+}]_i$ in neurons injured in presence of Mg^{2+}. In contrast, following excitotoxic injury in the absence of Mg^{2+}, neurons failed to recover basal $[Ca^{2+}]_i$ 340/380 ratios despite the removal of $[Ca^{2+}]_e$.

These experiments indicate that the combined status epilepticus and glutamate excitotoxicity produces much more severe injury than glutamate excitotoxicity alone.

Traditional routes of Ca^{2+} entry are not probable candidates for the injury induced ion channel

To rigorously establish that the injury-induced I_{CIC}-Ca^{2+} permeable channel represents a new route of Ca^{2+} entry, we conducted extensive pharmacological studies using established inhibitors of other Ca^{2+} channels, ionic mechanisms, intracellular Ca^{2+} systems and injury induced cation currents to determine if inhibiting these other Ca^{2+} systems could inhibit the injury-induced channel mediating the Ca^{2+} conductance activated following glutamate excitotoxicity. We utilized these well-characterized inhibitors under conditions that were shown to block these Ca^{2+} systems in this model of glutamate excitotoxicity. We intervened with various inhibitors by incorporating them in the wash solution during the post-glutamate END phase and observed recovery from END by measuring the membrane potential in a current-clamp configuration. Each column in the bar graph (Figs. 23 and 24) represents the mean membrane potentials ± SEM from N = 5 neurons.

Blockade with effective concentrations of inhibitors of voltage gated Ca^{2+} channels (nifedipine, 5 μM; $GdCl_3$, 10 μM), the NMDA channel (MK-801, 1 μM), AMPA/ KA channels (CNQX, 10 μM; NBQX, 10 μM), the metabotropic glutamate receptor (mCPG, 250 μM) and Na^+ conductances (TTX, 1 μM) had no effect on decreasing END. Inhibiting the forward and reverse mode of Na^+/ Ca^{2+} exchanger

(bepridil, 50 μM and $(Na)_o$ removal), chloride channels (DIDS, 100 μM), and stretch receptors channels ($GdCl_3$, 10 μM) had no effect on diminishing the END potentials after glutamate excitotoxicity. Blocking store operated Ca^{2+} channels (SKF-96365, 10 μM) or compensating for mitochondrial injury by addition of an ATP regenerating system (4 mM ATP and 22 mM phosphocreatinine) had no effect on reducing END. Blocking the newly discovered TRPM-7 channel (L-NAME, 300 μM) or the acid sensing ion channel (amiloride, 100 μM) activated upon anoxic/ hypoxic injury also had no effect on restoring END potentials (Fig. 23).

In contrast, as shown earlier with the single channel data, END was blocked by high concentrations of Gd^{3+} (100 μM) or Zn^{2+} (500 μM). Low concentrations of these ions were ineffective. Similarly, END was abolished by removal of $[Ca^{2+}]_e$ but not $[Na^+]_e$ from the wash solution (Fig. 24).

Thus, these results indicate that traditional routes of Ca^{2+} entry do not mediate END and that this injury induced Ca^{2+} permeable channel represents a novel route of Ca^{2+} entry into the neurons and offers a novel therapeutic target for preventing neuronal death following excitotoxicity.

II. Decapitation Ischemia Induces Activation Of A Novel Calcium Permeable Ion Channel In Hippocampal Neurons

After establishing the activation of a calcium permeable ion channel upon glutamate excitotoxicity *in vitro*, it was important to investigate whether a similar channel was activated upon glutamate excitotoxicity *in vivo*. Excitotoxic brain injury was induced in 2-week old Sprague-Dawley rats by subjecting them to decapitation ischemia (Abe, Yoshida et al. 1983; Wasterlain and Powell 1986; Parsons, Churn et al. 1999; Gerasimov, Artemenko et al. 2004). Hippocampal slices were obtained from control and injured brains and were then dissociated to yield neuronal suspension as previously described (Gibbs et al., 1996; Chen et al., 1997; Chen et al., 1998; Raza et al., 2001; Raza et al., 2004). Patch clamp experiments were performed on these acutely dissociated neurons to investigate if the *in vitro* glutamate excitotoxicity triggered Ca^{2+} permeable ion channel had an *in vivo* counterpart.

Ischemia by rapid decapitation followed by 30-mins of hold time at 37^0C in a non-perfused state is a well-established model of ischemia. Excitatory amino acid toxicity plays a major role in causing neuronal injury and death in the decapitation model of brain injury (Abe et al., 1983; Gerasimov et al., 2004). Our laboratory and others (Gibbs et al., 1996; Chen et al., 1997; Chen et al., 1998; Raza et al., 2001; Raza et al., 2004) have demonstrated that acutely isolated hippocampal neurons can be studied *in vitro* (Fig. 25) and show minimal signs of necrosis or apoptosis (Raza et al., 2001; Raza et al., 2004).

Decapitation ischemia activates an ion channel activity in acutely dissociated neurons

To investigate if a unique ion channel activity is induced by glutamate excitotoxicity *in vivo*, acutely dissociated hippocampal neurons from control hippocampi and from hippocampi subjected to decapitation ischemia were patch-clamped in a cell-attached configuration.

Fig. 26A shows recordings from control and ischemic neurons. A total of 10 neurons were recorded for each condition. Control neurons showed no channel activity at any holding potentials. In contrast, immediately upon seal formation, distinct single channel currents were observed in neurons acutely isolated from ischemia-injured hippocampi. Similar to the *in vitro* neuronal culture experiments described earlier, the holding potential was varied. Channel activity was noted across all the holding potentials and the amplitude of the openings changed with respect to the voltage. Plotting the amplitude of openings at various holding potentials generated the current-voltage relationship or the I-V curve as shown in Fig. 26B. The I-V relationship was linear and slope of this line when measured gave the single-channel conductance of 52.3 ± 0.8 pS for this channel. The conductance of this channel was close to the conductance of injury induced Ca^{2+} permeable channel activated in excitotoxic glutamate-injured cultures.

Traditional glutamate receptor-gated or voltage-gated ion channels are not the probable candidates for the decapitation ischemia activated ion channel

After observing an ion-channel activity in decapitation ischemia injured neurons, we next investigated if conventional glutamate-receptor gated or voltage-gated ion

channels were responsible for mediating this channel activity. Similar to the *in vitro* culture experiments we used a pharmacological approach. Inhibitors to the glutamate-receptor gated or voltage gated ion channels were incorporated in the extracellular pipette solution. Despite the presence of effective concentrations of inhibitors of voltage gated Ca^{2+} channels (nifedipine, 5 μM; $GdCl_3$, 10 μM), the NMDA channel (MK-801, 1 μM), AMPA/ KA channels (CNQX, 10 μM; NBQX, 10 μM), Na^+ conductance (TTX, 1 μM) and K^+ conductance (CsCl, 10 mM) the single-channel activity continued. As shown in Fig.27A, despite the presence of these antagonists, ischemia-injured acutely dissociated neurons demonstrated persistent openings and closings of an ion-channel at various holding potentials. A total of 10 neurons were studied. The I-V relationship for the presence of blocker was linear and the single channel conductance was 51.2 ± 2.6 pS, which was not significantly different for the channel conductance in the absence of antagonists ($p = 0.254$). These experiments suggest that voltage-gated Na^+ or K^+ channels or glutamate-receptor gated ion channels are not mediating the ischemia induced single channel activity.

Calcium ions are the primary permeant ions for the ischemia-induced channel

Our next aim was to investigate which ions are permeating through this ischemia-induced ion channel. To resolve this we followed the standard ion substitution approach as described earlier. Sodium and calcium ions were sequentially omitted from the extracellular pipette solution and the channel activity was recorded in the presence or absence of these ions.

Omission of Na^+ ions by equimolar replacement of NaCl with impermeant NMDG-Cl in the pipette solution did not affect the channel activity. As shown in Fig. 28A, replacement of Na^+ did not change the channel activity across various holding potentials. However, the amplitude of channel openings was significantly reduced at all the holding potentials. A total of 6 neurons were studied for the absence of Na^+. The I-V relationship for Na^+ omission is shown in Fig 28B. The conductance of the ischemia-induced ion channel was slightly reduced in the absence of $[Na^+]_e$ to 49.3 ± 0.5 pS. However, these changes were significantly different from values obtained in the presence of Na^+ ($p = 0.02$). Thus, although Na^+ ions appear to be slightly contributing to the ischemic injury induced current, it is a poor charge carrier for this channel.

Calcium substitution was achieved by omitting $CaCl_2$ from pipette solution. Omission of $[Ca^{2+}]_e$ completely abolished the ischemia-induced ion channel activity. Similar results were obtained in 6 different neurons under these conditions. No inward current was observed at any holding potentials and the traces resembled that of a control neuron (Fig. 29). Sodium ions are known to permeate through ion channels when the Ca^{2+} mediated divalent block is removed. However, we didn't observe any channel activity indicative of Na^+ entry in absence of Ca^{2+}. Although this could be occurring to some extent in our preparation, the resolution of our recordings and limitations of the patch-clamp configuration might be a limiting factor. Nonetheless, these observations strongly indicate that Ca^{2+} is the primary ion permeating through the decapitation ischemia induced ion channel.

High concentrations of Zn^{2+} and Gd^{3+} block the ischemia induced ion channel

After demonstrating that Ca^{2+} is the primary permeant ion for the ischemia-induced ion channel our next aim was identify blockers for this channel. Based on our previous experience in vitro with the effect of Zn^{2+} and Gd^{3+} on the glutamate excitotoxicity activated ion channel *in vitro*, we decided to investigate the effects of these blocker cations on ischemia induced ion channel activity in acutely dissociated neurons. These divalent or triavalent ions were incorporated in the extracellular pipette solution.

Cell-attached recordings in the presence of low concentrations of Zn^{2+} (5 μM) or Gd^{3+} (10 μM) had no effect on the channel activity. As shown in Fig 30A, channel activity was maintained despite presence of Gd^{3+} (10 μM). The amplitude of channel opening was not affected. The slope of I-V relationship revealed a single channel conductance of 51.1 ± 0.2 pS (Fig. 30B) which was not statistically different from the conductance measured in the absence of 10 μM Gd^{3+} ($p = 0.115$).

Similarly, incorporation of Zn^{2+} (5 μM) did not inhibit the ischemic injury induced channel activity (Fig. 31A). Although the amplitude of channel opening was significantly reduced in the presence of Zn^{2+} (5 μM), the conductance of channel opening (51.1 ± 1.3 pS) as obtained from I-V plot (Fig. 31B) was not statistically different from the conductance measured in the absence of 5 μM Zn^{2+} ($p = 0.435$).

In contrast, incorporating high concentrations of the divalent or the trivalent blocker cation, Zn^{2+} (500 μM) or Gd^{3+} (100 μM) along with normal concentrations of Na^{+} and Ca^{2+} completely abolished the ischemia induced single channel activity. Figures 32 and 33 show representative single channel traces from neurons showing no channel

activity when patched with Gd^{3+} (100 μM) or Zn^{2+} (500 μM) at various holding potentials. As shown in Fig. 29 for $[Ca^{2+}]_e$ removal, inclusion of high concentrations of $ZnCl_2$ or $GdCl_3$ in the extracellular pipette solution completely abolished the injury induced channel activity and the traces resembled to that of a control neuron. A total of 6 neurons were recorded for each blocker cation concentration.

Taken together, all these experiments strongly indicate that the ion channel activated by excitotoxic glutamate injury in cultured hippocampal neurons *in vitro* is also activated upon decapitation ischemia induced glutamate excitotoxicity *in vivo*. The close range of conductance and kinetic characteristics for these two channels, Ca^{2+} selectivity, similar insensitivity to Ca^{2+} entry inhibitors or low concentrations to blocker cations and similar blockade by high concentrations of Zn^{2+} or Gd^{3+} all strongly indicate that the excitotoxic glutamate activated ion channels observed in these two systems are the same.

Figure 5. Digital Photograph of hippocampal neurons in culture. The neurons were grown on confluent glial beds *in vitro* for 13-21 days. Electrophysiological or imaging experiments were performed on pyramidal shaped neurons indicated by arrows.

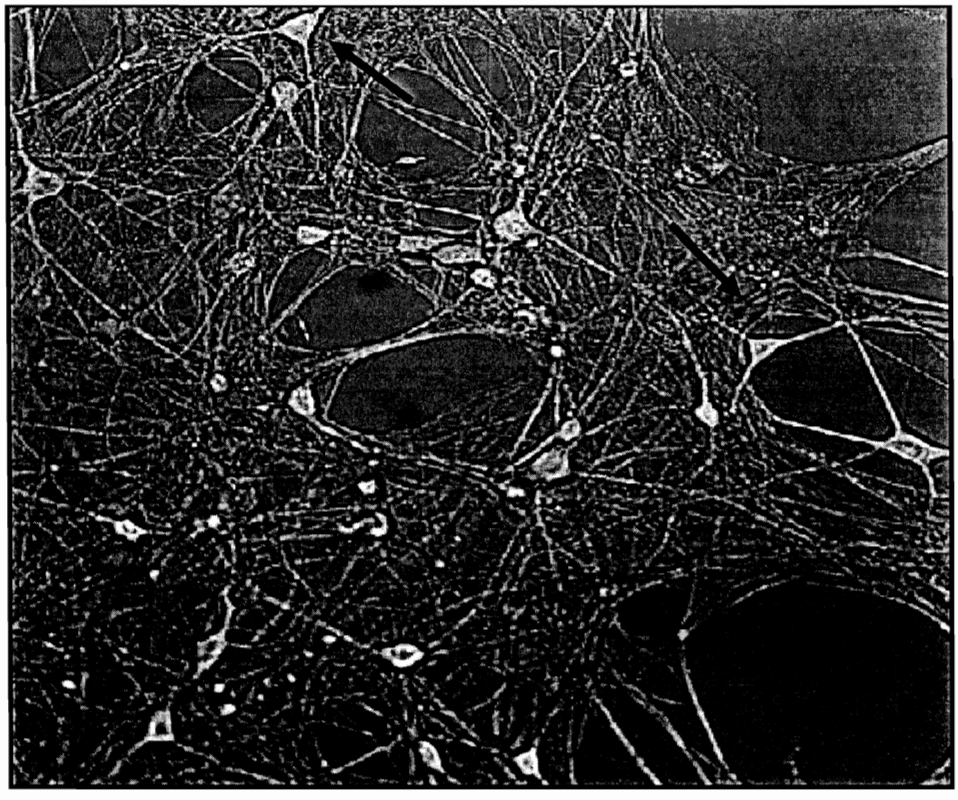


Figure 6. Excitotoxic glutamate (GLU) exposure induces an extended neuronal depolarization (END) in cultured hippocampal neurons. (A) Current clamp recording from a neuron subjected to glutamate (500 μM + 10 μM glycine) for a short duration (5-min). The neuron shows rapid depolarization when exposed to glutamate, stays depolarized as long as glutamate is present in the bath solution and after 5-mins when washed with control-recording solution regains its resting membrane potential. (B) Current clamp recording from a neuron subjected to glutamate (500 μM + 10 μM glycine) for a long duration (10-min). This constitutes the excitotoxic insult. Despite removal of glutamate after the 10-min period, the neuron stays depolarized for as long as recording is continued. This is the END phase. All the experiments in this dissertation were performed in this post-glutamate END phase.

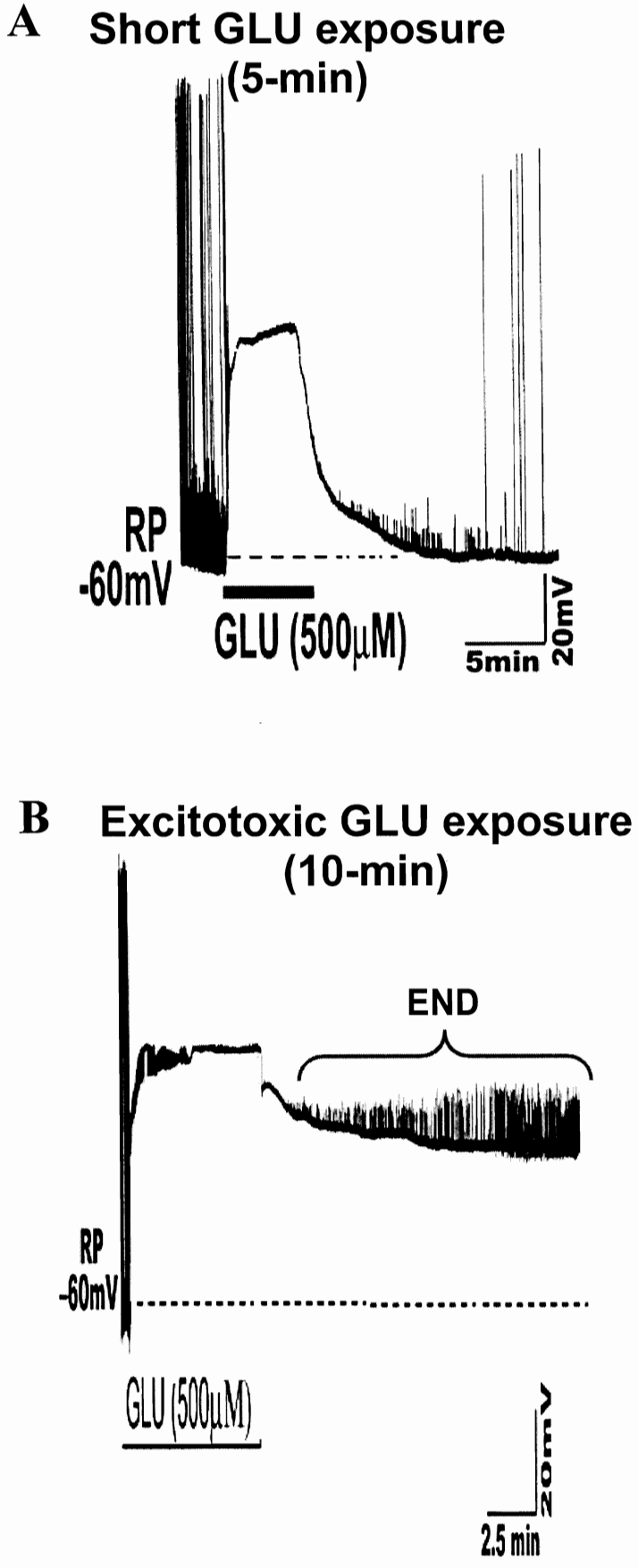

A
Short GLU exposure
(5-min)
RP
-60mV
GLU (500μM)
5min
20mV
B
Excitotoxic GLU exposure
(10-min)
END
RP
-60mV
GLU (500μM)
20mV
2.5 min

Figure 7. Excitotoxic glutamate exposure activates an injury induced ion channel in hippocampal neurons. Recording of channel activity from cultured hippocampal neurons in the post glutamate "END" phase. Downward deflections from baseline indicate single channel openings from hippocampal neurons subjected to excitotoxic glutamate insult. The trace is a 4-sec continuous recording at a holding potential of –60mV in a cell-attached patch clamp mode. Records were filtered at 2kHz. A total of 10 neurons were recoded.

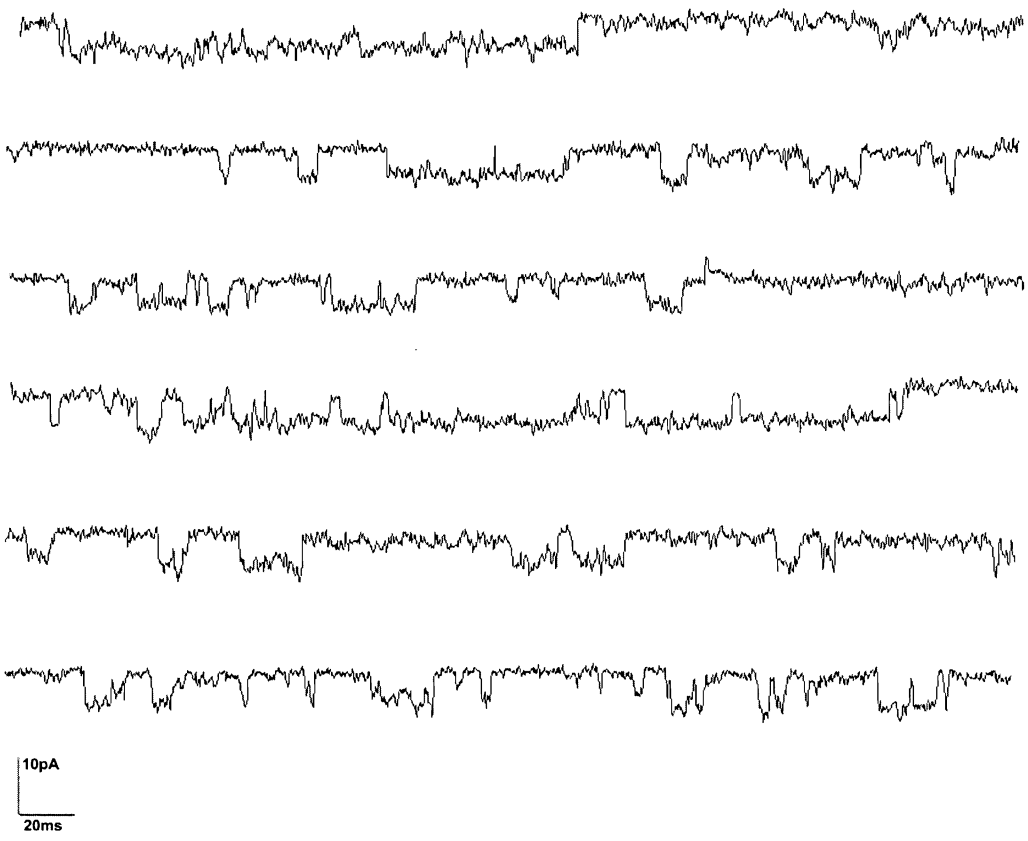
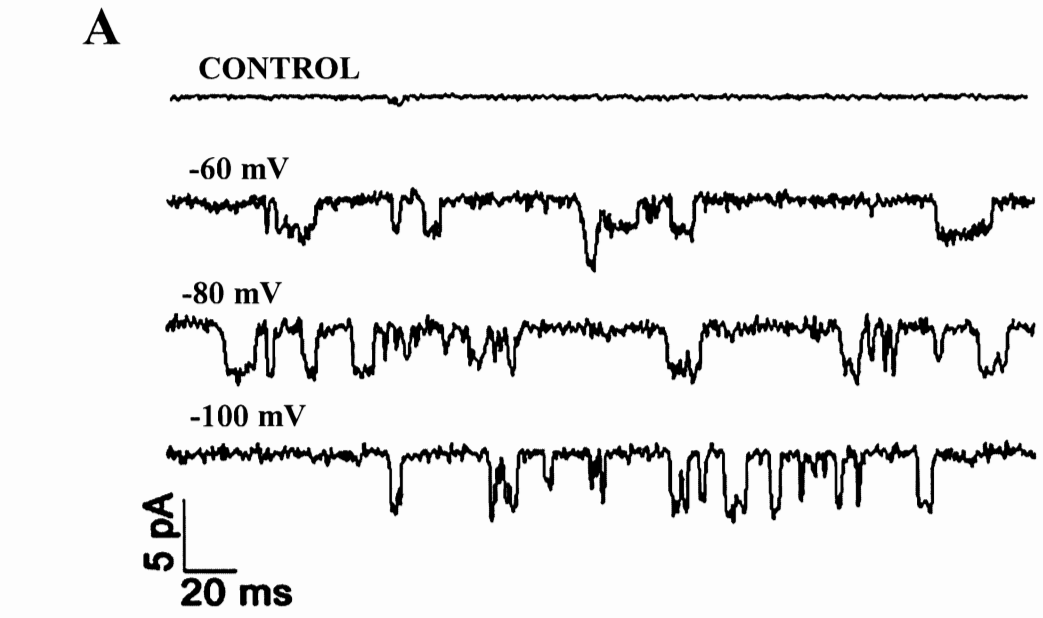

10pA
20ms

Figure 8. Induction of injury induced channel in hippocampal neurons. *A*. Representative trace showing induction of single-channel activity in cell-attached patches from excitotoxic glutamate injured neurons (n=10) at holding potentials ranging from –60mV to –100 mV. No single-channel events were observed in control neurons (n=10). *B*. I-V relationship for channel activity from GET injured neurons. Channel had a slope conductance of 59.5 pS.


A
CONTROL
-60 mV
-80 mV
-100 mV
5 pA
20 ms

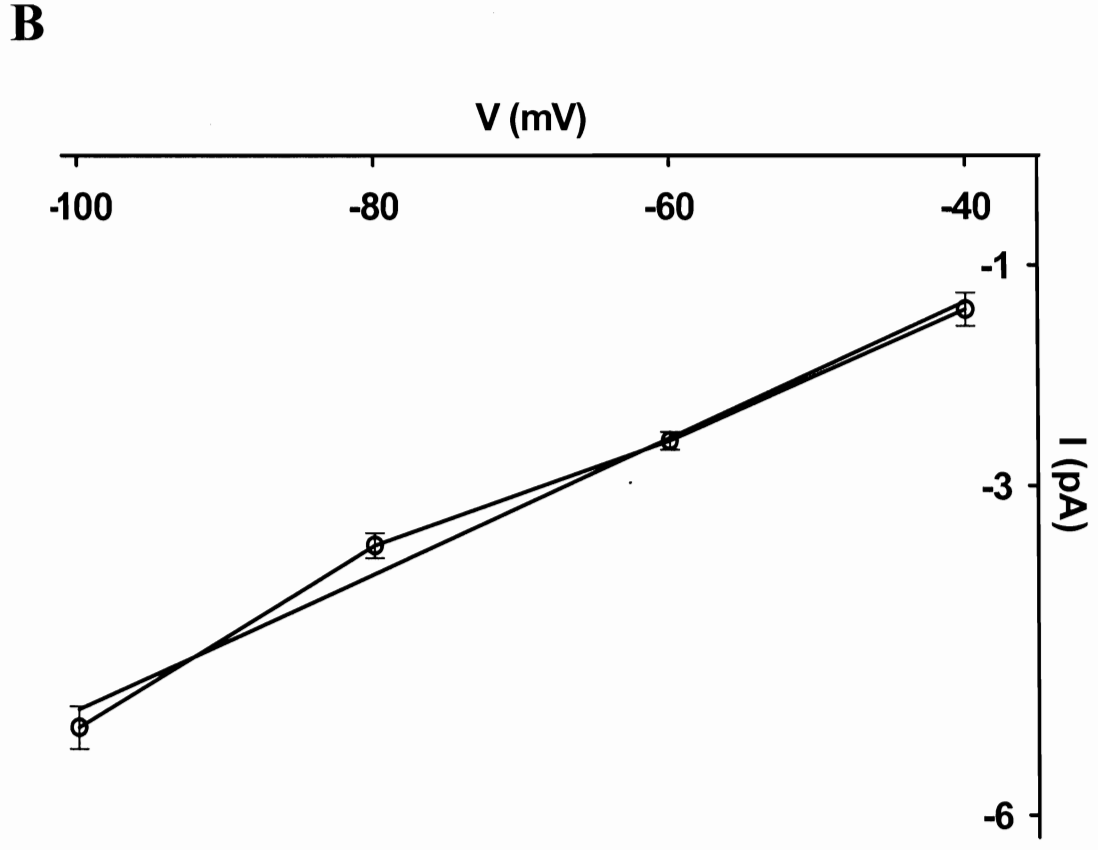
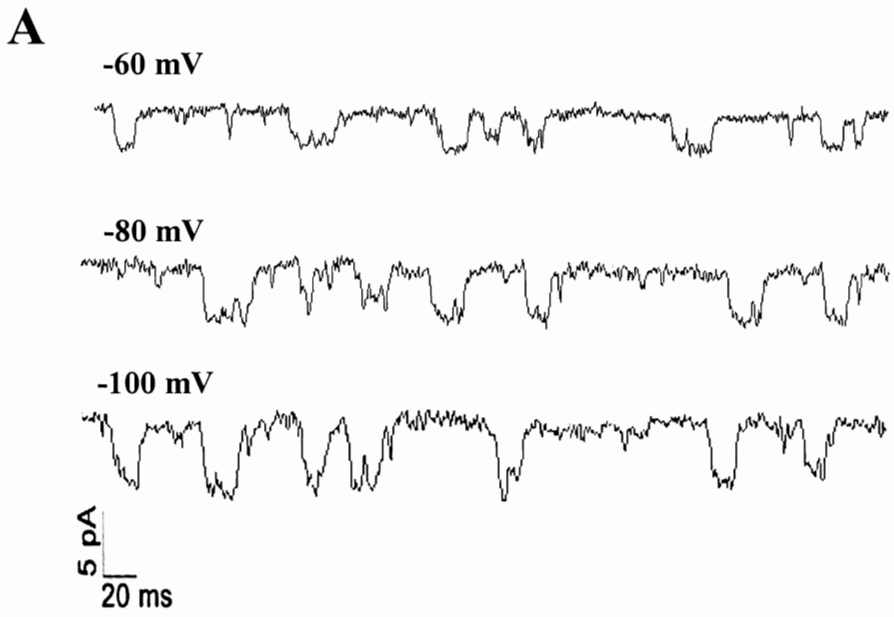

B
V (mV)
-100
-80
-60
-40
-1
-3
-6
I (pA)

Figure 9. Low concentrations of Gd^{3+} (10 μM) do not block the glutamate injury induced channel activity. *A*, Representative single channel recordings from glutamate injured neurons patched with pipette solutions containing 10 μM Gd^{3+} in the post-glutamate END period. Distinct channel activity was observed at all the holding potentials ranging from –60 mV to –100 mV. *B*, Current-Voltage (I-V) relationship for the presence and absence of Gd^{3+} (10 μM). A total of 10 neurons were recorded for the presence of 10 μM Gd^{3+}.


A
-60 mV
-80 mV
-100 mV
5 pA
20 ms

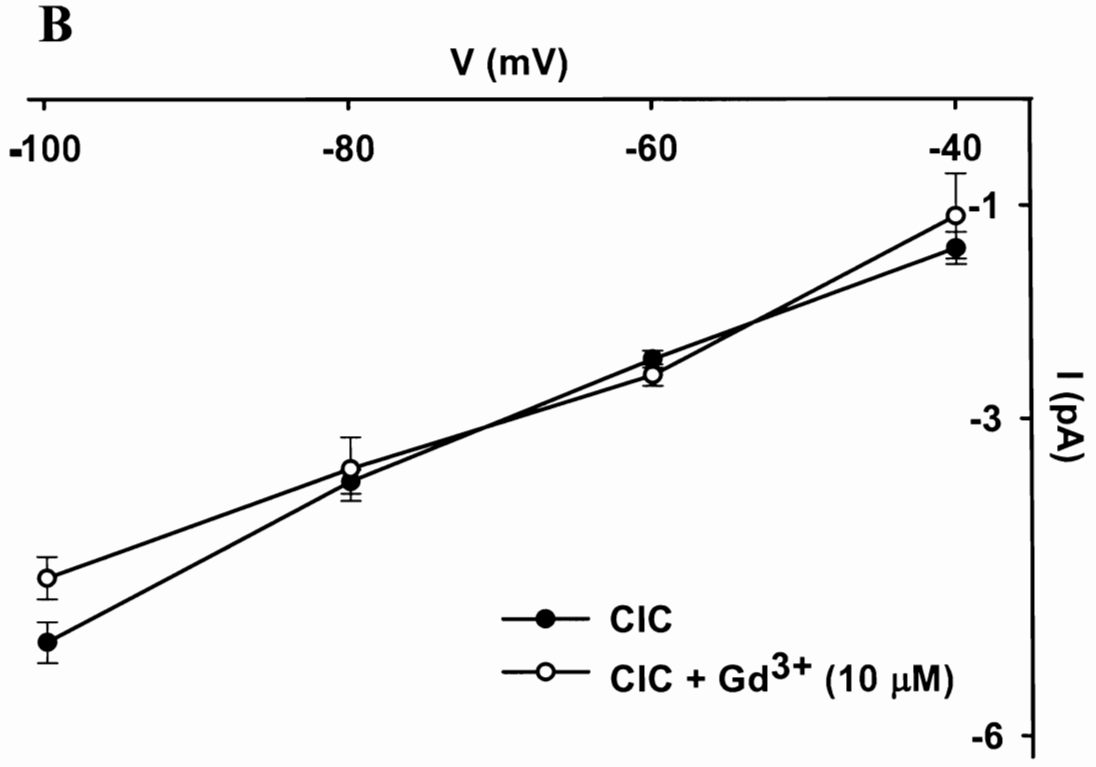

B
V (mV)
-100
-80
-60
-40
-1
-3
-6
I (pA)
ClC
ClC + Gd3+ (10 μM)

Figure 10. High concentrations of Gd^{3+} (100 μM) completely abolish the glutamate injury induced channel activity. Representative single channel recordings from glutamate injured neurons patched with pipette solutions containing 100 μM Gd^{3+} in the post-glutamate END period. Note the absence of channel activity at all the holding potentials ranging from –60 mV to –100 mV. A total of 10 neurons were recorded for the presence of 100 uM Gd^{3+}.

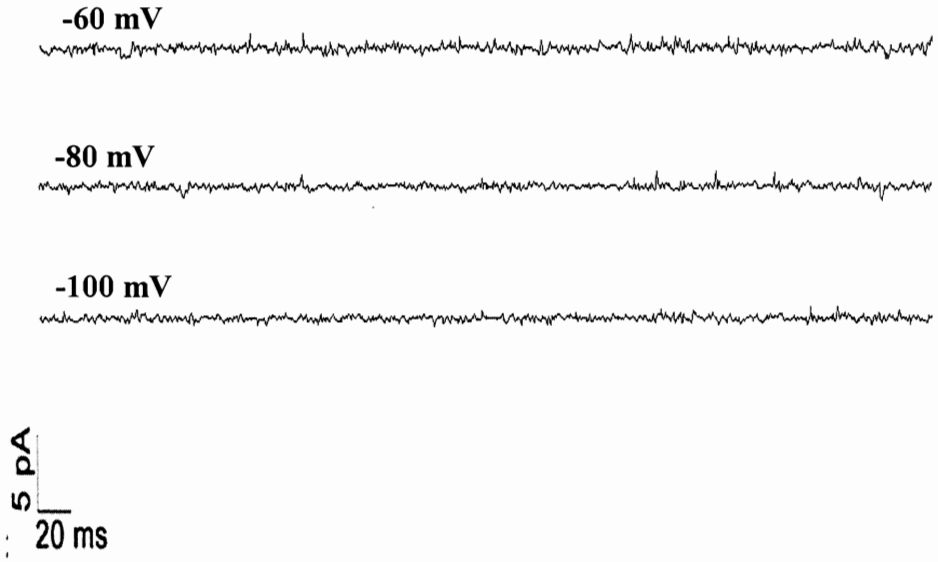

-60 mV
-80 mV
-100 mV
5 pA
20 ms

Figure 11. Low concentrations of Zn^{2+} (5 μM) do not block the glutamate injury induced channel activity. *A*, Representative single channel recordings from glutamate injured neurons patched with pipette solutions containing 5 μM Zn^{2+} in the post-glutamate END period. Distinct channel activity was observed at all the holding potentials ranging from –60 mV to –100 mV. *B*, Current-Voltage (I-V) relationship for the presence and absence of Zn^{2+} (5 μM). A total of 10 neurons were recorded for the presence of 5 μM Zn^{2+}.

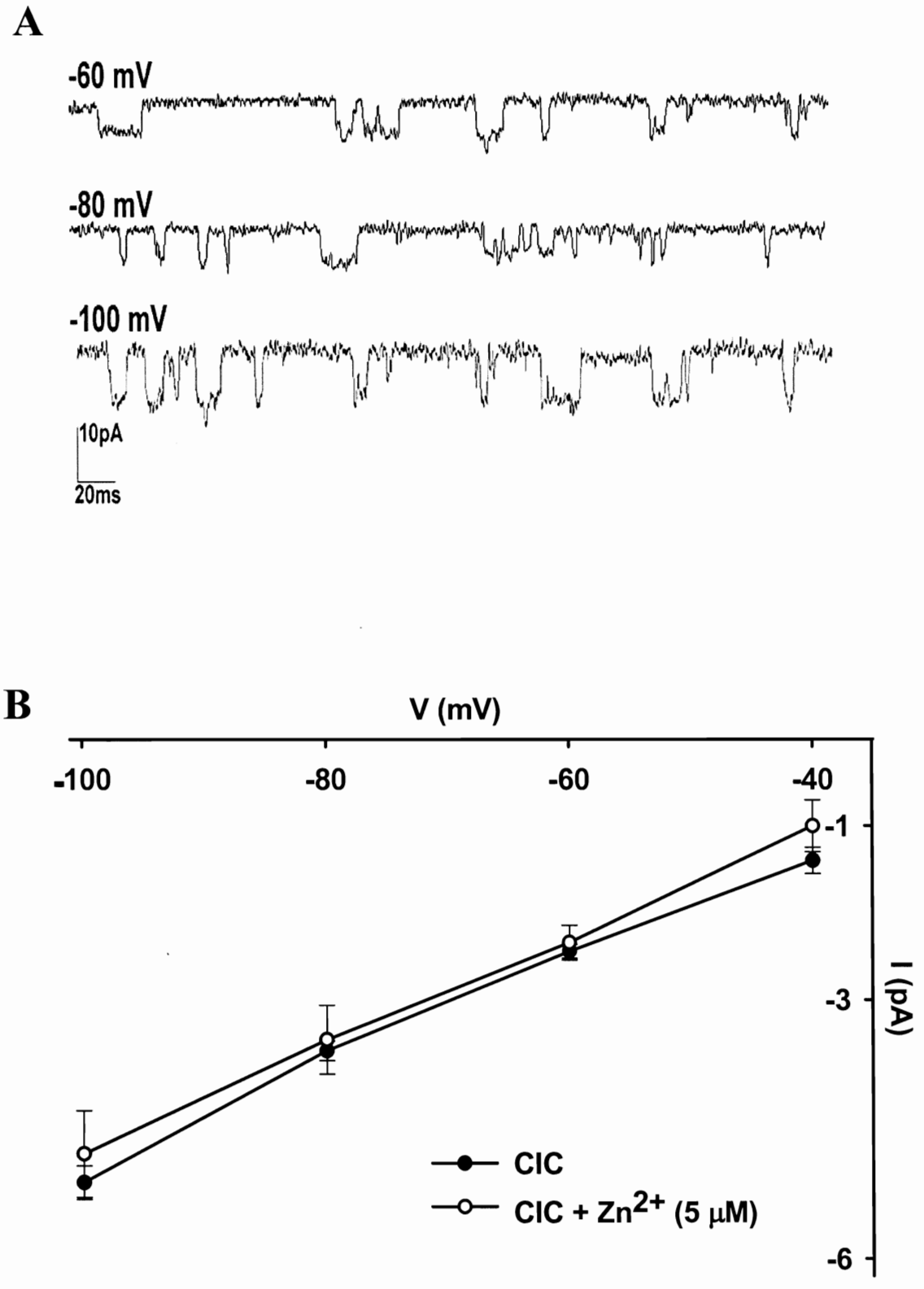

A
-60 mV
-80 mV
-100 mV
10pA
20ms
B
V (mV)
-100
-80
-60
-40
-1
-3
-6
I (pA)
CIC
CIC + Zn2+ (5 μM)

Figure 12. High concentrations of Zn^{2+} (500 μM) completely abolish the glutamate injury induced channel activity. Representative single channel recordings from glutamate injured neurons patched with pipette solutions containing 500 μM Zn^{2+} in the post-glutamate END period. Note the absence of channel activity at all the holding potentials ranging from –60 mV to –100 mV. A total of 10 neurons were recorded for the presence of 500 μM Zn^{2+}.

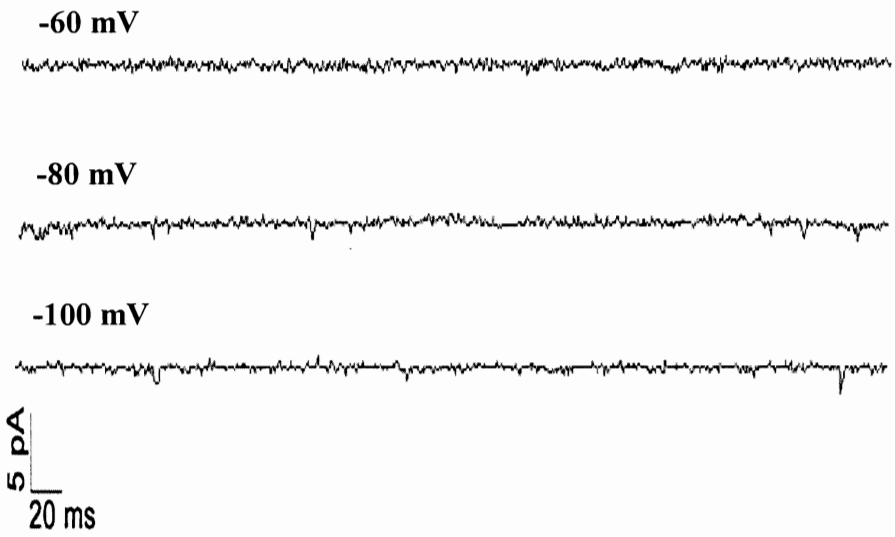

-60 mV
-80 mV
-100 mV
5 pA
20 ms

Figure 13. Extracellular Ca^{2+} but not Na^{+} was required for the maintenance of extended neuronal depolarization (END). In order to induce END, neurons were first treated with 500 μM glutamate and 10 μM glycine for 10-min. *A-C*: Representative membrane potential recordings from neurons in which glutamate was terminated by wash with the following solutions: *A*, control extracellular recording solution containing normal concentrations of Na^{+} and Ca^{2+}; *B*, Na^{+} - free extracellular solution (Na^{+} was substituted with NMDG); *C*, Ca^{2+} -free extracellular solution (containing normal Na^{+} but no added Ca^{2+}). Bars indicate periods of glutamate treatment (below each trace) and the post-glutamate wash (above each trace), and initial resting membrane potential for each neuron is shown to the left of each trace. A total of 5 neurons were recorded for each wash condition.

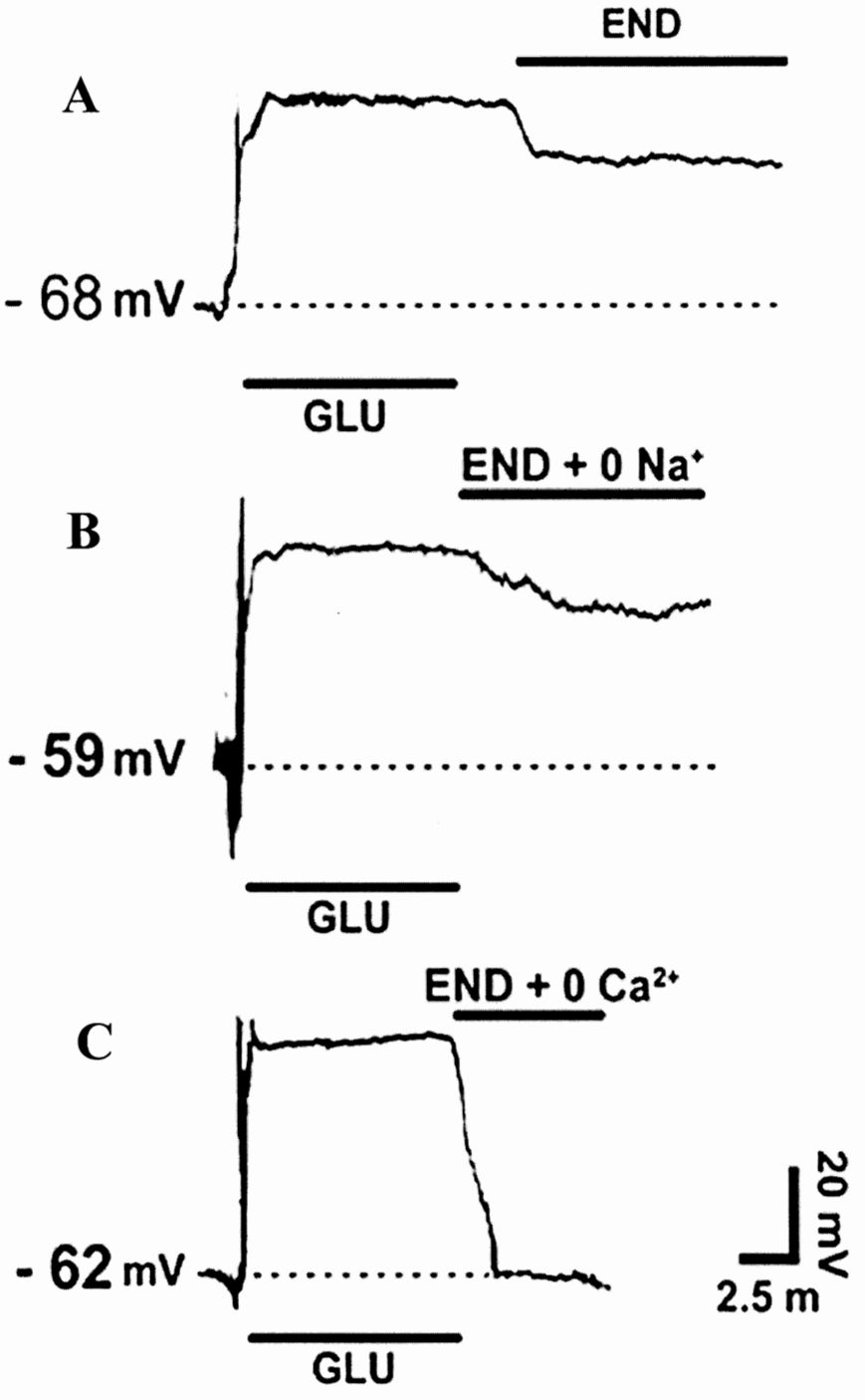
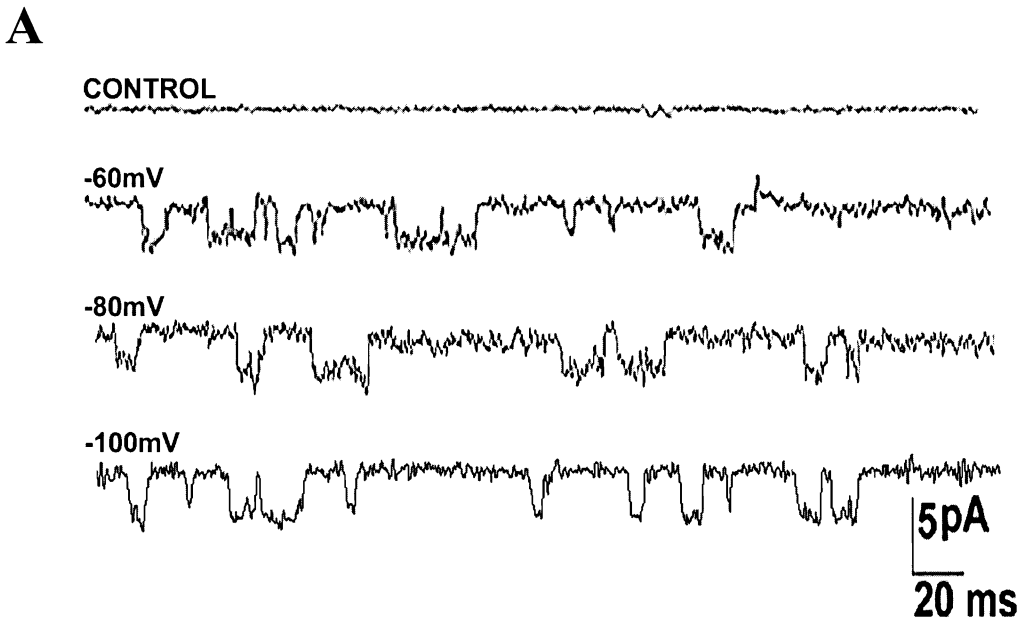

END
A
- 68 mV
GLU
END + 0 Na+
B
- 59 mV
GLU
END + 0 Ca2+
C
- 62 mV
GLU
20 mV
2.5 m

Figure 14. Extracellular Na^+ removal maintains the excitotoxic glutamate induced channel activity. Substituting NaCl with NMDG in the pipette solution constituted the Na^+ removal. *A*, Representative cell-attached recordings showing presence of channel activity at various holding potentials in the post-glutamate END period despite the absence of Na^+ in the extracellular solution. *B*, Current-Voltage (I-V) relationship for the presence and absence of Na^+. The slight reduction in the conductance of channel in the absence of Na^+ that was not significantly different. A total of 7 neurons were recorded for the absence of Na^+.


A
CONTROL
-60mV
-80mV
-100mV
5pA
20 ms

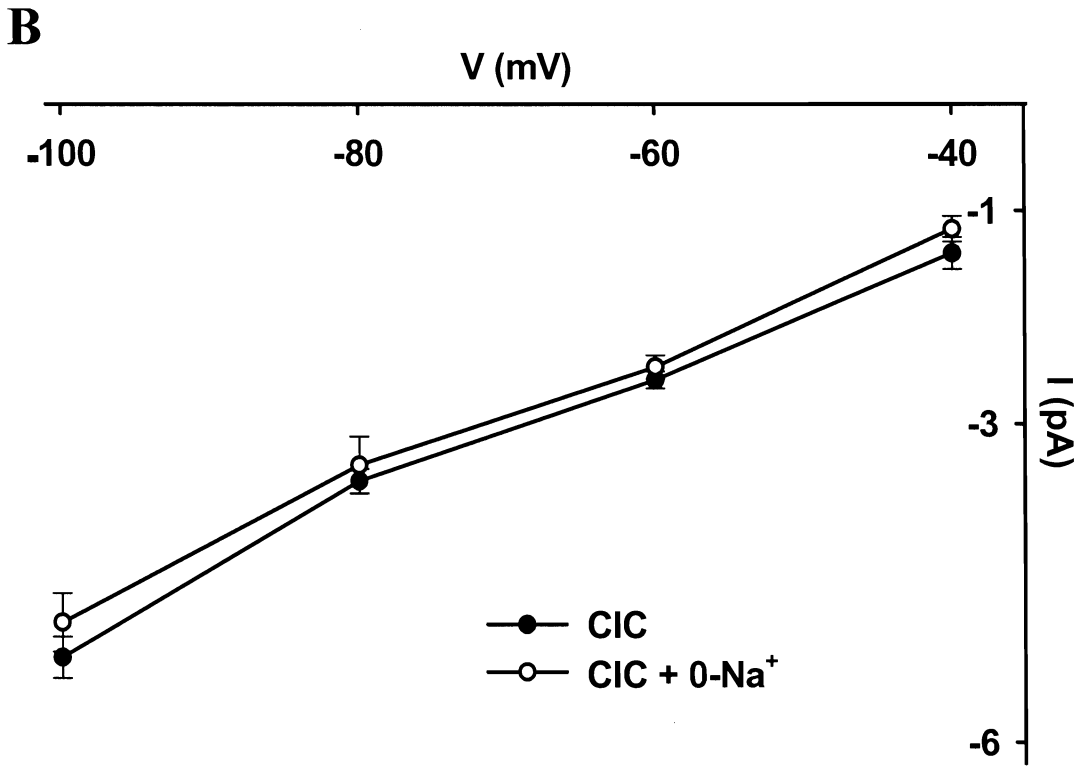

B
V (mV)
-100
-80
-60
-40
-1
-3
-6
I (pA)
ClC
ClC + 0-Na+

Figure 15. Removing extracellular Ca^{2+} abolishes the glutamate injury induced channel activity. Omission of $CaCl_2$ from the pipette solution constituted the Ca^{2+} removal. Representative single channel traces showing no channel activity across all the holding potentials in the absence of extracellular Ca^{2+}. A total of 7 neurons were recorded for the absence of Ca^{2+}.

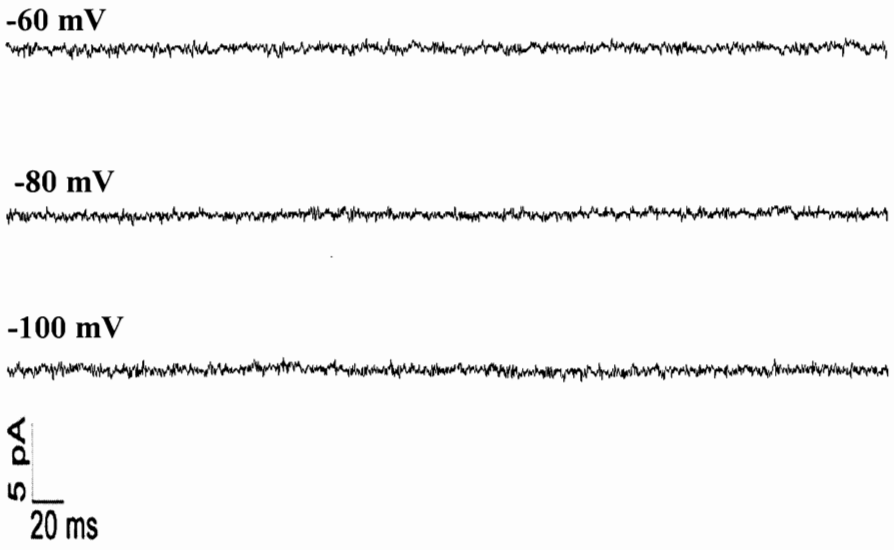

-60 mV
-80 mV
-100 mV
5 pA
20 ms

Figure 16. $[Ca^{2+}]_i$ expressed as fura-2 340/380 ratio values demonstrate that removal of $[Ca^{2+}]_e$ (n=5), but not $[Na^+]_e$ (n=5) leads to recovery of glutamate induced sustained elevated $[Ca^{2+}]_i$ (n=5) elevations. Fura-2 ratio values were monitored as neurons were exposed to excitotoxic glutamate and then washed with: extracellular solution containing normal concentrations of Na^+ and Ca^{2+}; Na^+ -free extracellular solution (Na^+ replaced with NMDG); or Ca^{2+} -free extracellular solution (no added $CaCl_2$). The glutamate exposure period is indicated by solid black bar at the top.

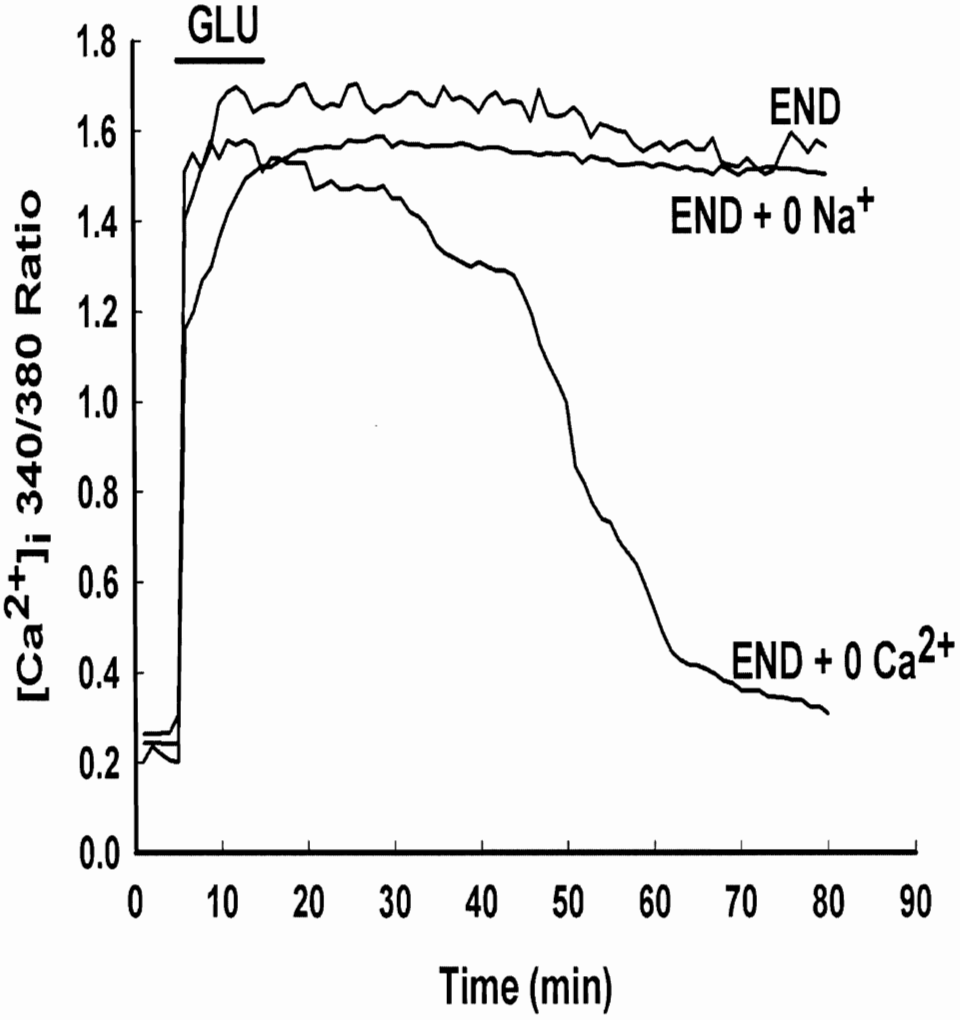

GLU
END
END + 0 Na+
END + 0 Ca2+
[Ca2+]i 340/380 Ratio
Time (min)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
0
10
20
30
40
50
60
70
80
90

Figure 17. Traditional Ca^{2+} entry antagonists do not block glutamate injury induced single channel activity. *A*, Representative single channel recordings from four neurons at a holding potential of –60 mV in a cell-attached mode. The neurons were patched with pipette solutions containing MK-801 (1μM), CNQX (10 μM), Nifedipine (5 μM), Gd^{3+} (10 μM), Cs (10 mM) and TTX (1 μM). *B*, Single channel Current-Voltage (I-V) relationship in the presence and absence of Ca^{2+} entry inhibitors (CEI). Note the identical I-V curves and single channel conductances for the two conditions. A total of 10 neurons were recorded for the presence of CEI's.

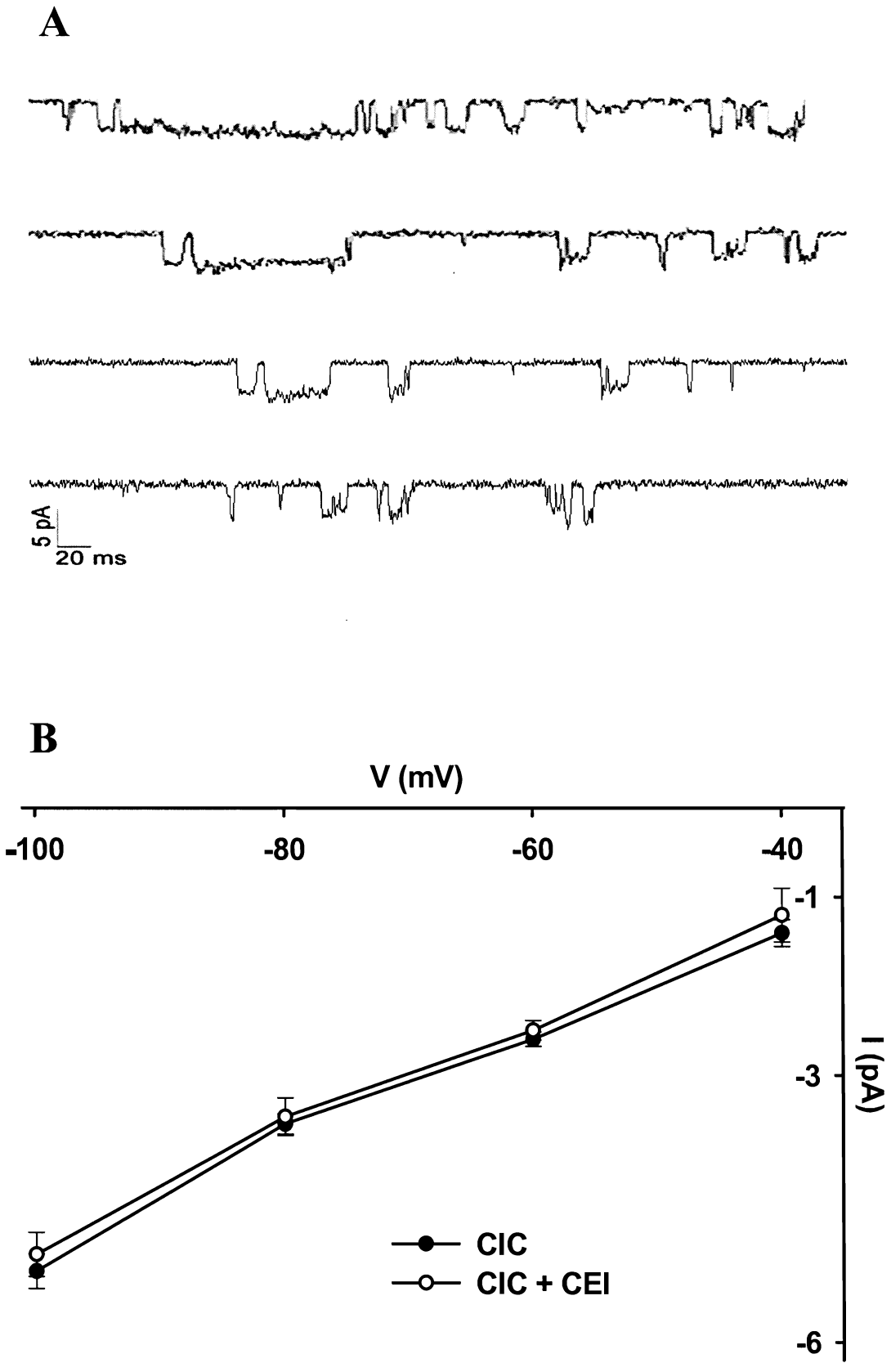

A
5 pA
20 ms
B
V (mV)
-100
-80
-60
-40
-1
-3
-6
I (pA)
CIC
CIC + CEI

Figure 18. Neuroprotection with Gadolinium. Hippocampal neurons in cultures were subjected to excitotoxic glutamate insult and then treated with wash solutions containing normal basal recording solution (BRS), BRS containing 10 μM Gd^{3+} or L-NAME (300 μM) or Amiloride (100 μM) or Gd^{3+} (100 μM). Fraction apoptotic neurons were calculated as outlined in materials and methods using the Annexin-V and PI stain. Blocking the injury induced ion channel with Gd^{3+} (100 μM) was neuroprotective while inhibitors of I_{OGD}/TRPM-7 (L-NAME and 10 μM Gd^{3+}) or ASIC's (AMILO) had no effect on apoptotic cell count when compared to glutamate alone. Asterisks denote differences from GLU (*$p < 0.05$). A total of 6 experiments were conducted for each treatment condition. (AMILO= Amiloride, ASIC= Acid Sensing Ion Channels, I_{OGD}= Oxygen Glucose Deprivation Current, TRPM7= Transient Receptor Potential Channel-subtype 7).

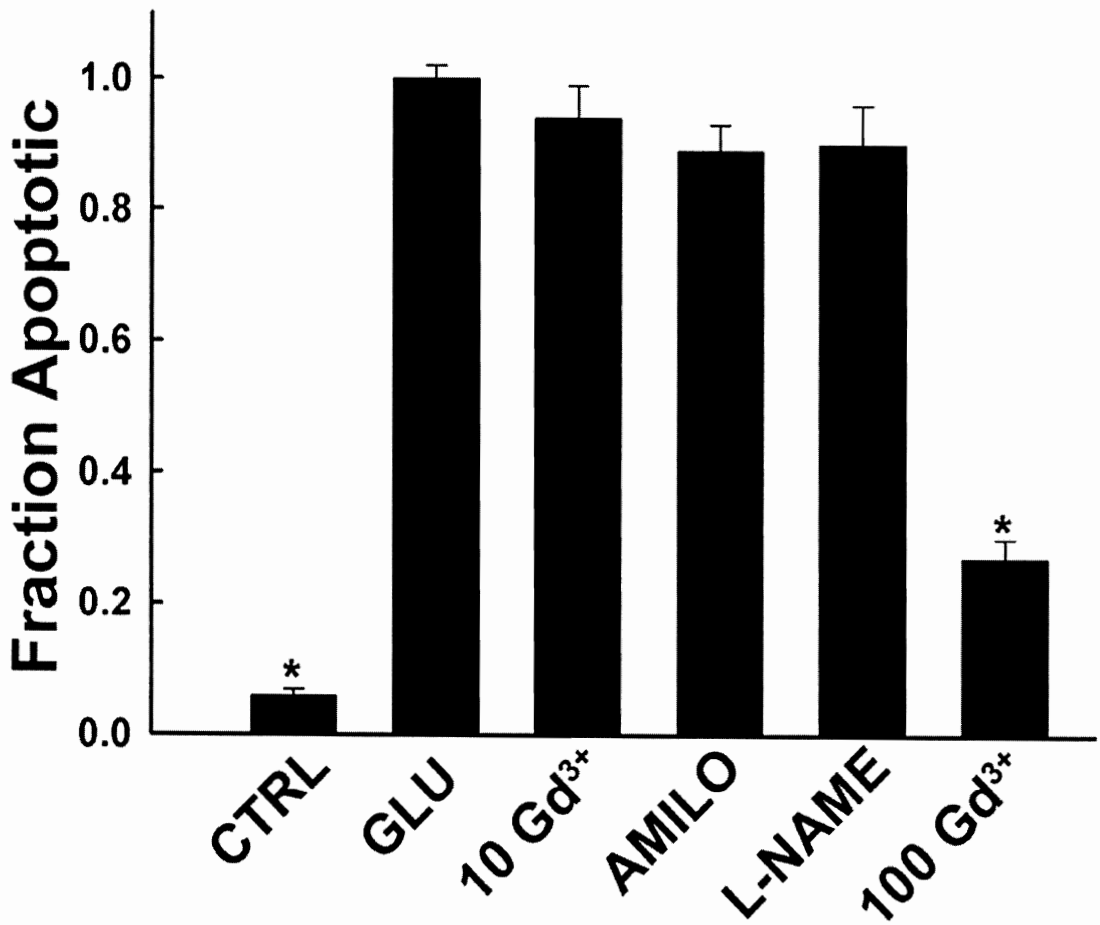

Fraction Apoptotic
1.0
0.8
0.6
0.4
0.2
0.0
*
*
CTRL
GLU
10 Gd3+
AMILO
L-NAME
100 Gd3+

Figure 19. Blockade of the injury induced Ca^{2+} permeable channel: Extension of the therapeutic window. Neurons in cultures were subjected to excitotoxic glutamate insult. Glutamate was then washed off using control solution. At various time points starting at 0-hr and up to 4-hr, the neurons were treated with 100 μM Gd3+ containing solution. The percentage of neurons undergoing cell death (both apoptotic and necrotic) was measured using the Annexin-V and PI stain. A total of N=6 plates were measured for each time point and data is expressed as mean ±SEM. Asterisks denote differences from GLU (*$p < 0.05$)

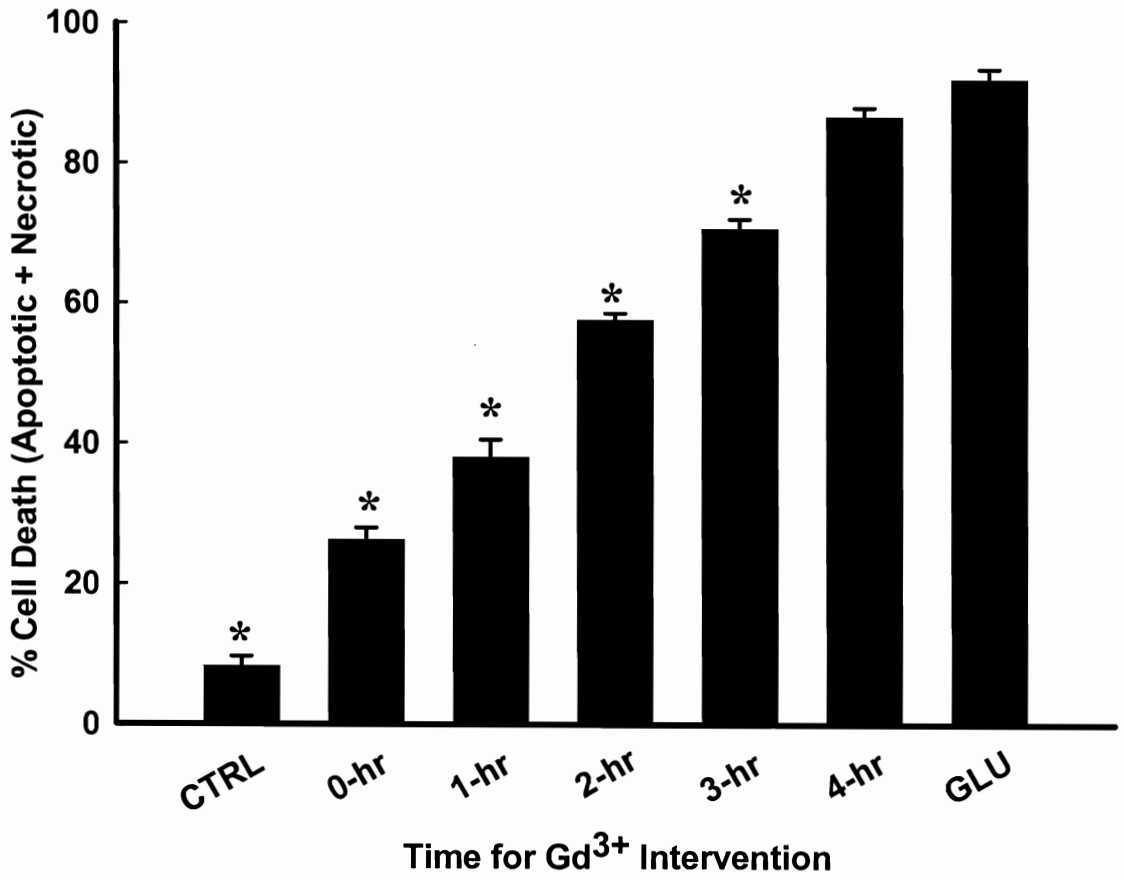

100
80
60
40
20
0
% Cell Death (Apoptotic + Necrotic)
*
*
*
*
*
CTRL
0-hr
1-hr
2-hr
3-hr
4-hr
GLU
Time for Gd3+ Intervention

Figure 20. Blockade of the injury induced Ca^{2+} permeable channel: Extension of the therapeutic window. Neurons in cultures were subjected to excitotoxic glutamate insult. Glutamate was then washed off using control solution. At various time points starting at 0-hr and up to 4-hr, the neurons were treated with solution containing no added Ca^{2+}. The percentage of neurons restoring the elevated Fura-2 $[Ca^{2+}]_i$ ratio were measured using the standard fluorescent Ca^{2+} imaging technique. A total of N=6 plates were measured for each time point and data is expressed as mean ±SEM. Asterisks denote differences from GLU (*$p < 0.05$)

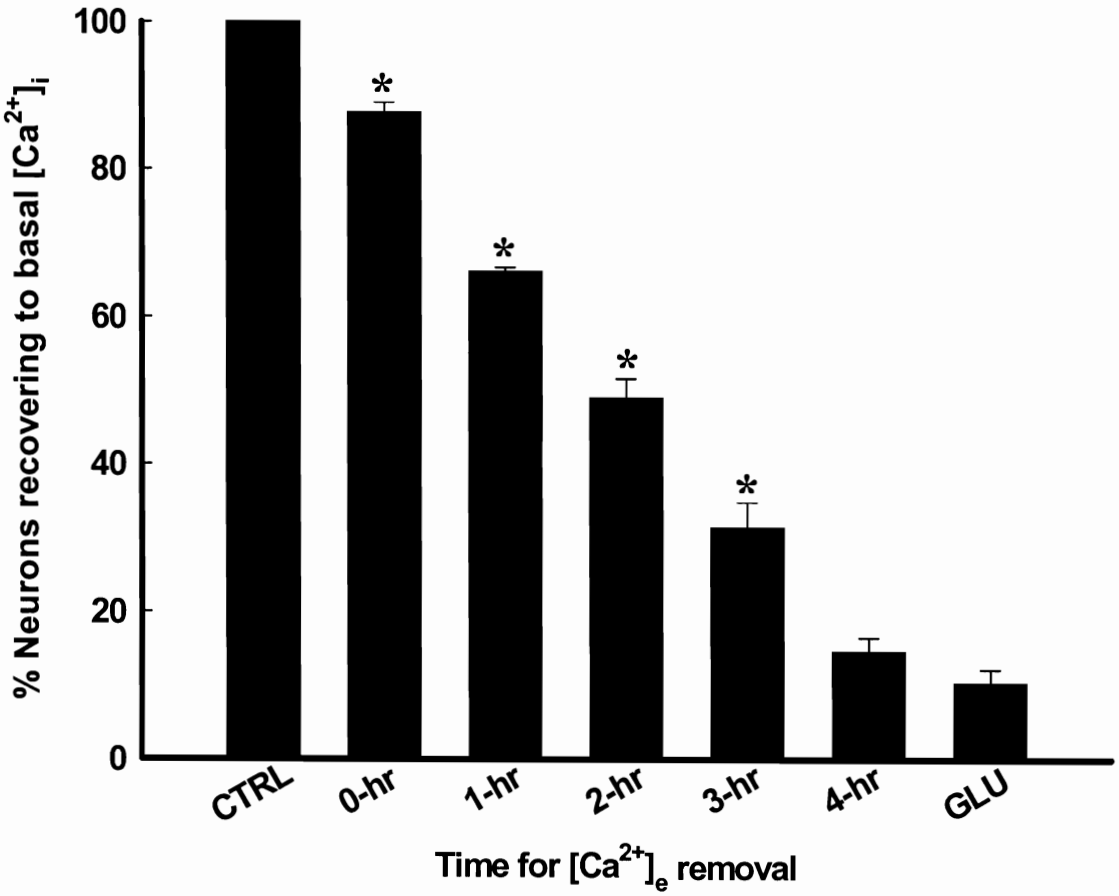

100
80
60
40
20
0
% Neurons recovering to basal [Ca2+]i
*
*
*
*
CTRL
0-hr
1-hr
2-hr
3-hr
4-hr
GLU
Time for [Ca2+]e removal

Figure 21. Representative membrane potential recording from neurons responding to Ca^{2+} removal. The neuron in top panel, *A*, was subjected to excitotoxic glutamate and was treated with normal recording solution. This neuron demonstrates an END. Neuron in middle panel, *B*, received a double injury in the form of excitotoxic glutamate exposure along with zero-Mg^{2+}. Washout of glutamate with zero-Ca^{2+} solution doesn't restore membrane potential and neuron goes into END. Neuron in bottom panel, *C*, is subjected to excitotoxic glutamate in the presence of Mg^{2+} as in A. It recovers resting membrane potential upon washout with zero-Ca^{2+} solution. A total of 6 neurons were recorded for each conditions.

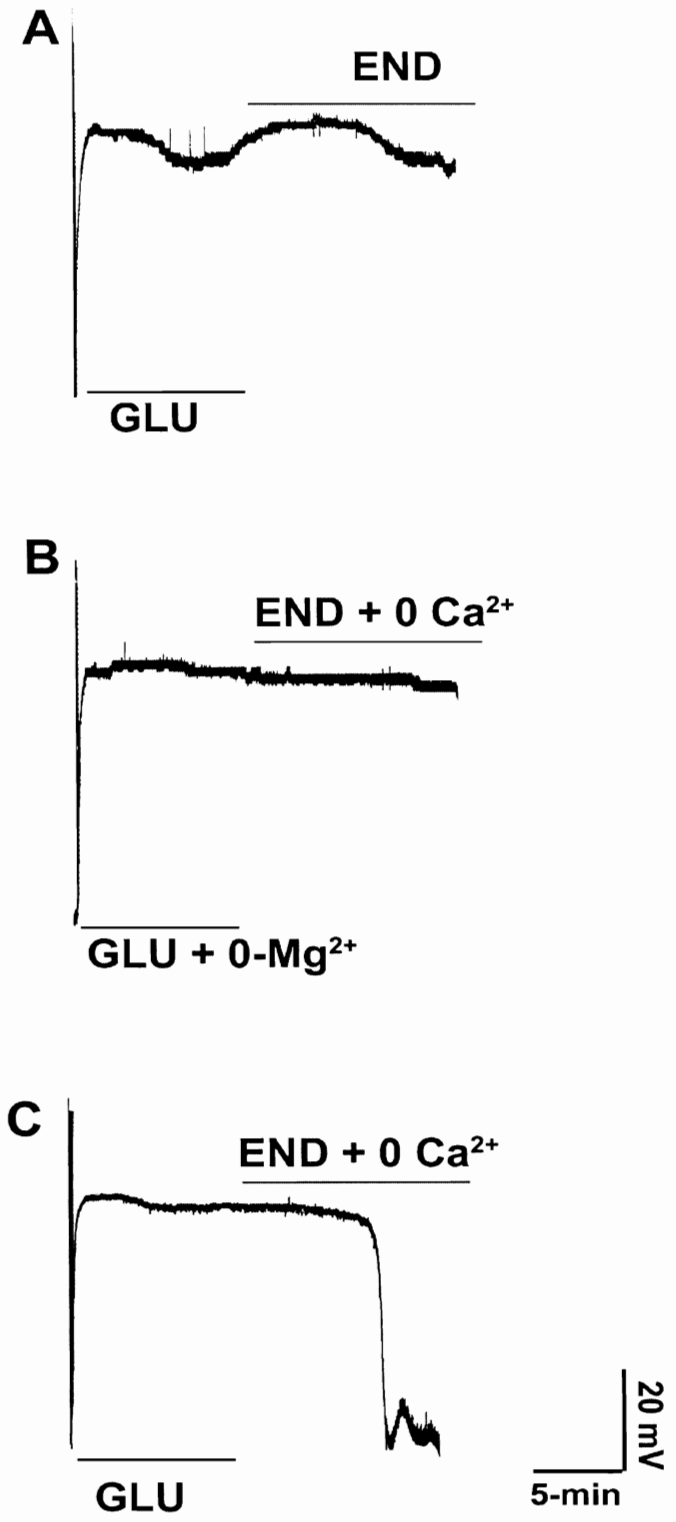

A
END
GLU
B
END + 0 Ca2+
GLU + 0-Mg2+
C
END + 0 Ca2+
GLU
20 mV
5-min

Figure 22. $[Ca^{2+}]_i$ expressed as fura-2 340/380 ratio values demonstrate that removal of $[Ca^{2+}]_e$ leads to recovery of glutamate induced sustained elevated $[Ca^{2+}]_i$ elevations only when the excitotoxic insult is produced in the presence of Mg^{2+}. Fura-2 ratio values were monitored as neurons were exposed to excitotoxic glutamate in the presence or absence of Mg^{2+} and then washed with either extracellular solution containing normal concentrations of Na^+ and Ca^{2+} or Ca^{2+} -free extracellular solution (no added $CaCl_2$). The glutamate exposure period is indicated by solid black bar at the top. Experiments were repeated 5 times for each tratement condition.

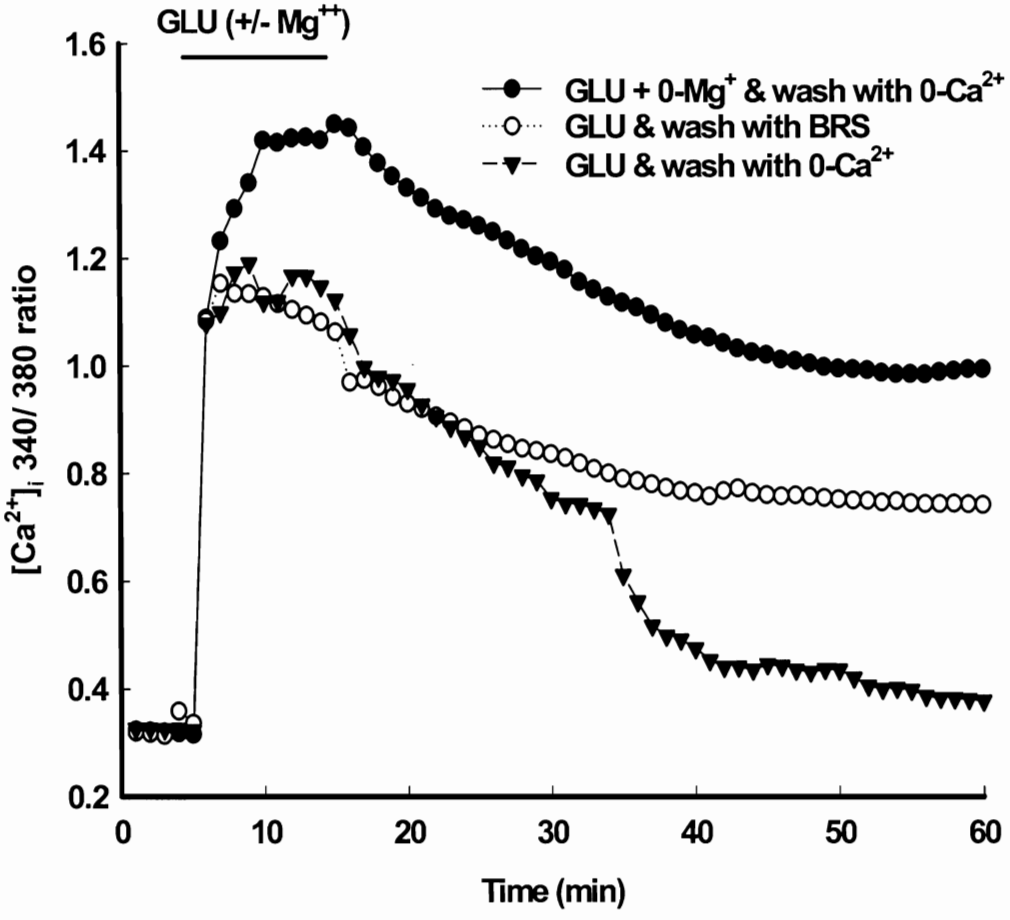

GLU (+/- Mg++)
GLU + 0-Mg+ & wash with 0-Ca2+
GLU & wash with BRS
GLU & wash with 0-Ca2+
[Ca2+]i 340/ 380 ratio
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
10
20
30
40
50
60
Time (min)

Figure 23. Excitotoxic glutamate exposure induces a novel Ca^{2+} permeable channel that accounts for the Ca^{2+} paradox of delayed neuronal death. Mean membrane potentials ± SEM for control (CTRL), END, and END + various inhibitors/ conditions: CEI, Ca^{2+} entry inhibitors; mCPG, á-methyl-4-carboxyphenyl glycine; TTX, tetrodotoxin; BEP, bepridil; DIDS, 4, 4'- diisothiocyanatostilbene-2, 2'- disulfonic acid; SKF, SKF-96365; ATP; L-NAME; AMILO, amiloride; 10 Gd^{3+} and 5 Zn^{2+} (N=5). Asterisks denote statistical difference from pre-glutamate control condition ($p<0.05$; One Way ANOVA, post-hoc Tukey test).

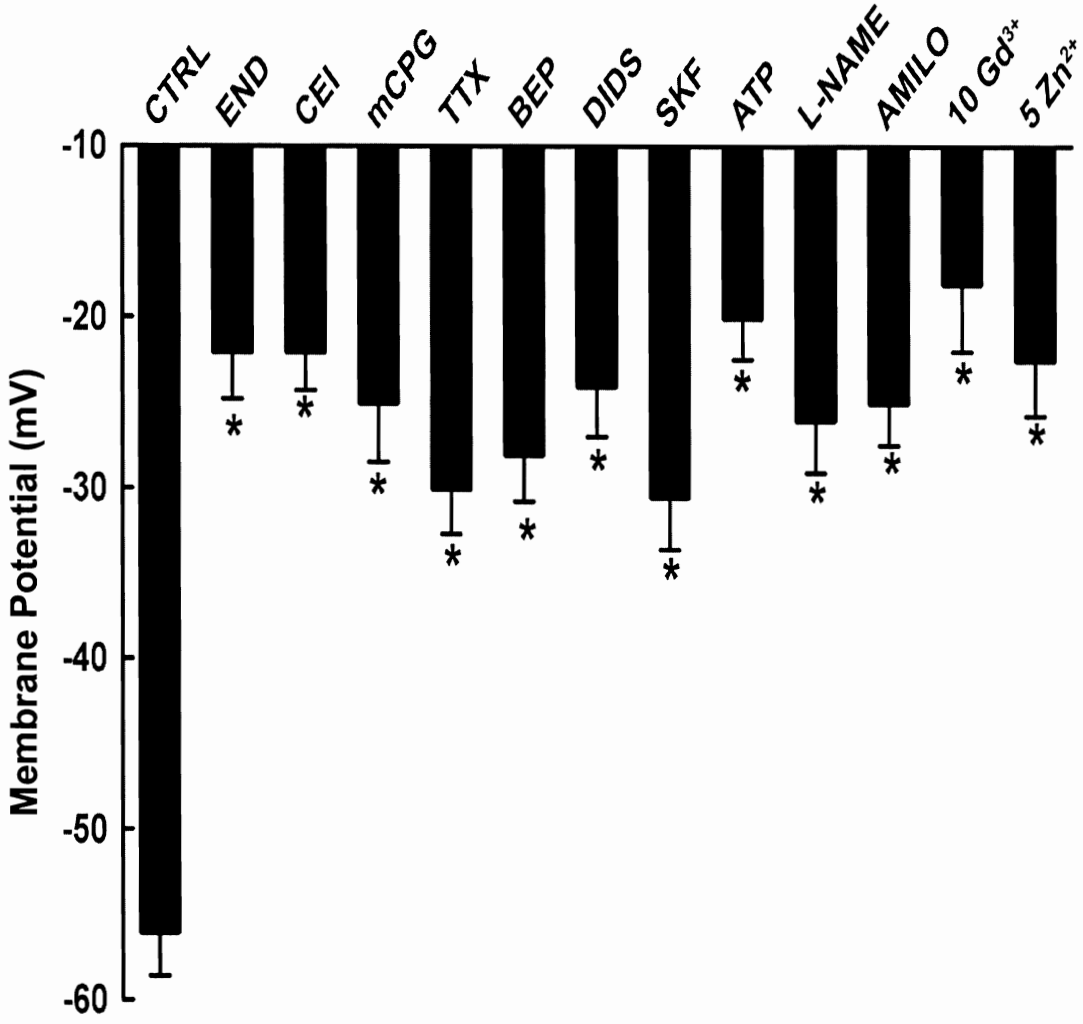

CTRL
END
CEI
mCPG
TTX
BEP
DIDS
SKF
ATP
L-NAME
AMILO
10 Gd3+
5 Zn2+
Membrane Potential (mV)
-10
-20
-30
-40
-50
-60
*

Figure 24. Effect of removing extracellular Na^+ or Ca^{2+} or treatment with high concentrations of Zn^{2+} or Gd^{3+} on neuronal membrane potentials following excitotoxic glutamate exposure. Bars represent mean membrane potential ± SEM for neurons in END or treated in the post-glutamate period with 0 Na^+ or 0 Ca^{2+} containing solution or normal extracellular solutions containing Zn^{2+} (500 μM) or Gd^{3+} (100 μM). The first bar CTRL depicts the mean membrane potential ± SEM for neurons before treatment with glutamate (N=5). Asterisks denote statistical difference from pre-glutamate control condition ($p<0.05$; One Way ANOVA, post-hoc Tukey test).

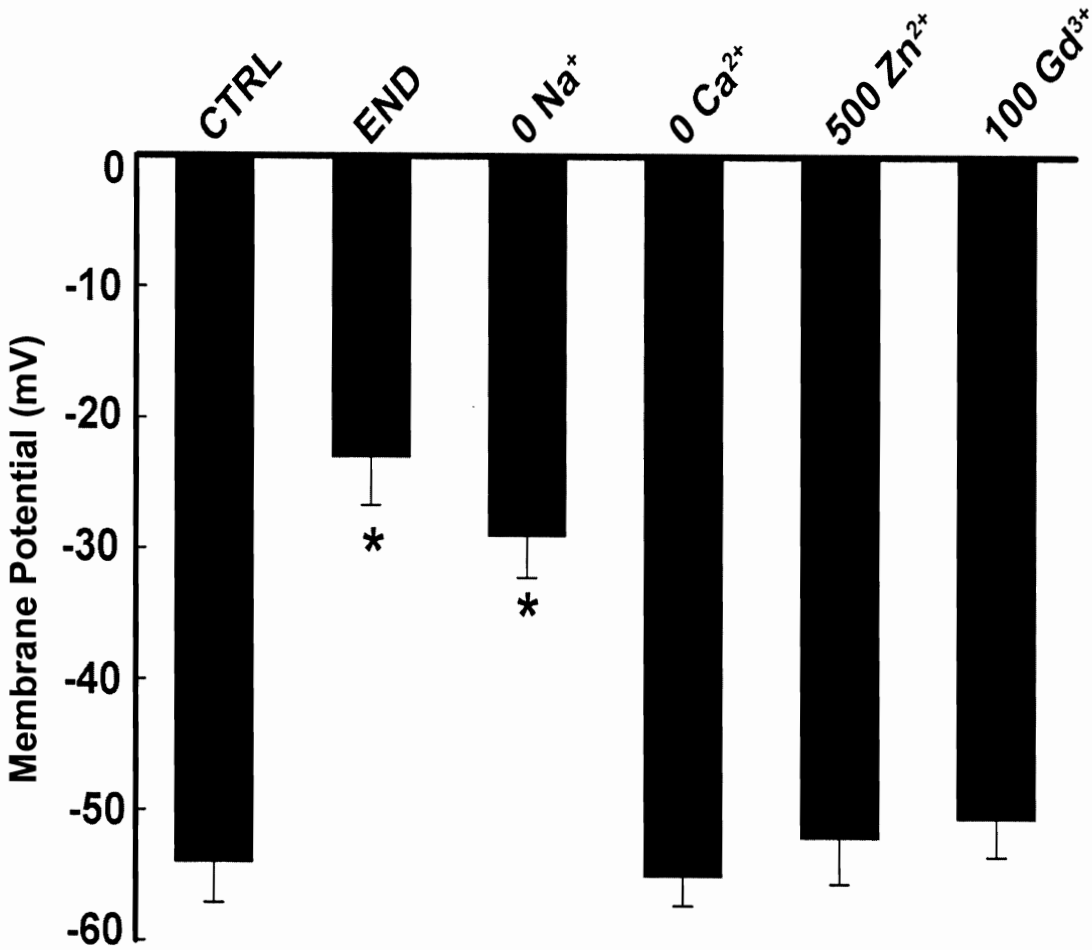
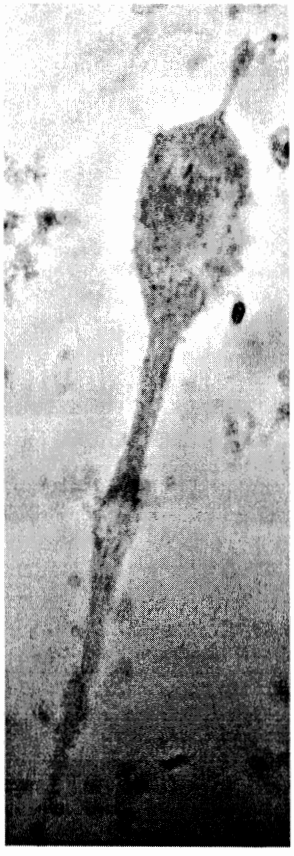
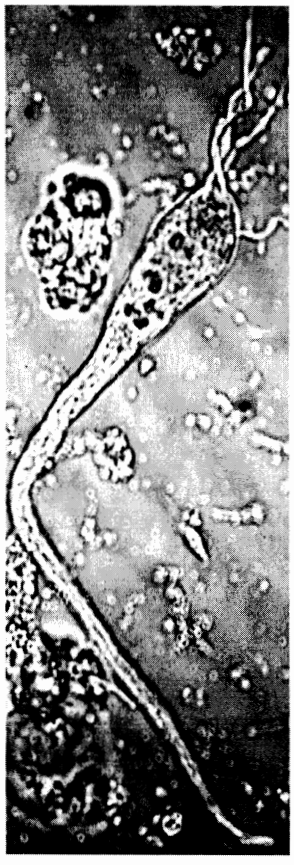

CTRL
END
0 Na+
0 Ca2+
500 Zn2+
100 Gd3+
0
-10
-20
-30
-40
-50
-60
Membrane Potential (mV)
*
*

Figure 25. High-resolution images of acutely dissociated hippocampal neurons from three different preparations.





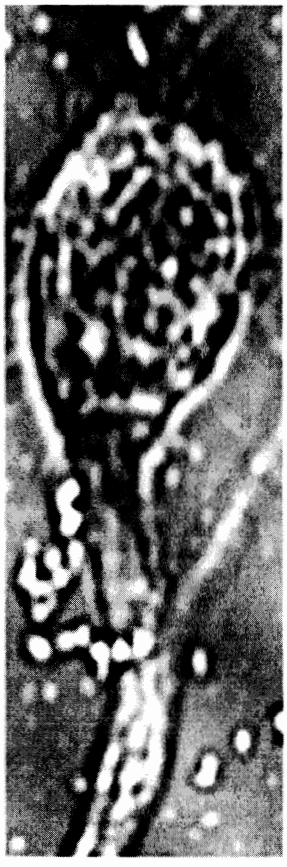
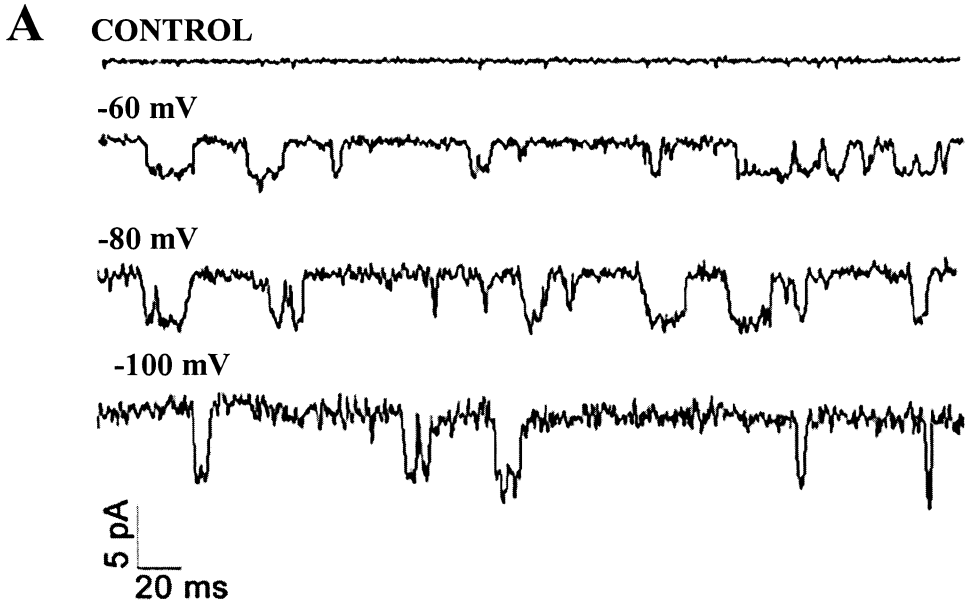


Figure 26. Decapitation Ischemic injury activates an ion channel in acutely dissociated hippocampal neurons. *A*, Representative trace showing induction of single-channel activity in cell-attached patches from decapitation ischemia injured neurons (n=10) at holding potentials ranging from –60mV to –100 mV. No single-channel events were observed in acutely dissociated non-injured control neurons (n=10). *B*, Current-Voltage (IV) relationship for channel activity from ischemia- injured neurons. The channel had a slope conductance of 49.5 pS.


A
CONTROL
-60 mV
-80 mV
-100 mV
5 pA
20 ms

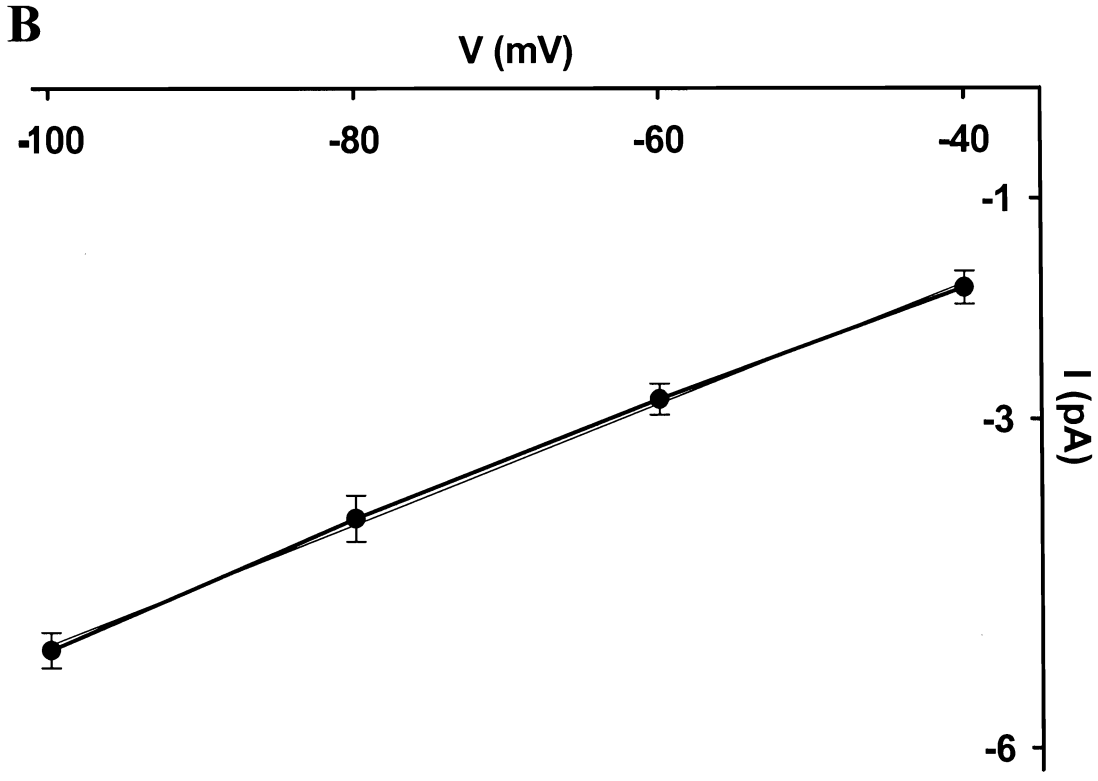
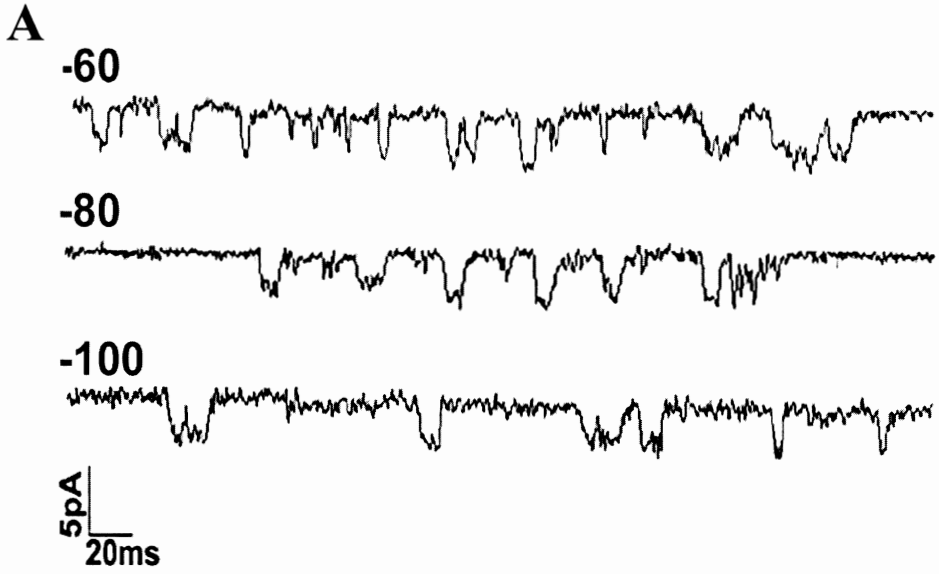

B
V (mV)
-100
-80
-60
-40
-1
-3
-6
I (pA)

Figure 27. Traditional glutamate gated or voltage gated ion channels do not mediate the decapiation ischemia induced single channel activity. *A*, Representative single channel traces from acutely dissociated neurons patched with pipette solutions containing MK-801 (1μM), CNQX (10 μM), Nifedipine (5 μM), Gd^{3+} (10 μM), Cs (10 mM) and TTX (1 μM). Note the presence of distinct single channel activity across all the holding potentials ranging from –60 to –100 mV. *B*, Current-Voltage (I-V) relationship for the presence and absence of glutamate gated or voltage gated ion channel blockers. A total of 10 neurons were recorded for the presence of inhibitor combination.


A
-60
-80
-100
5pA
20ms

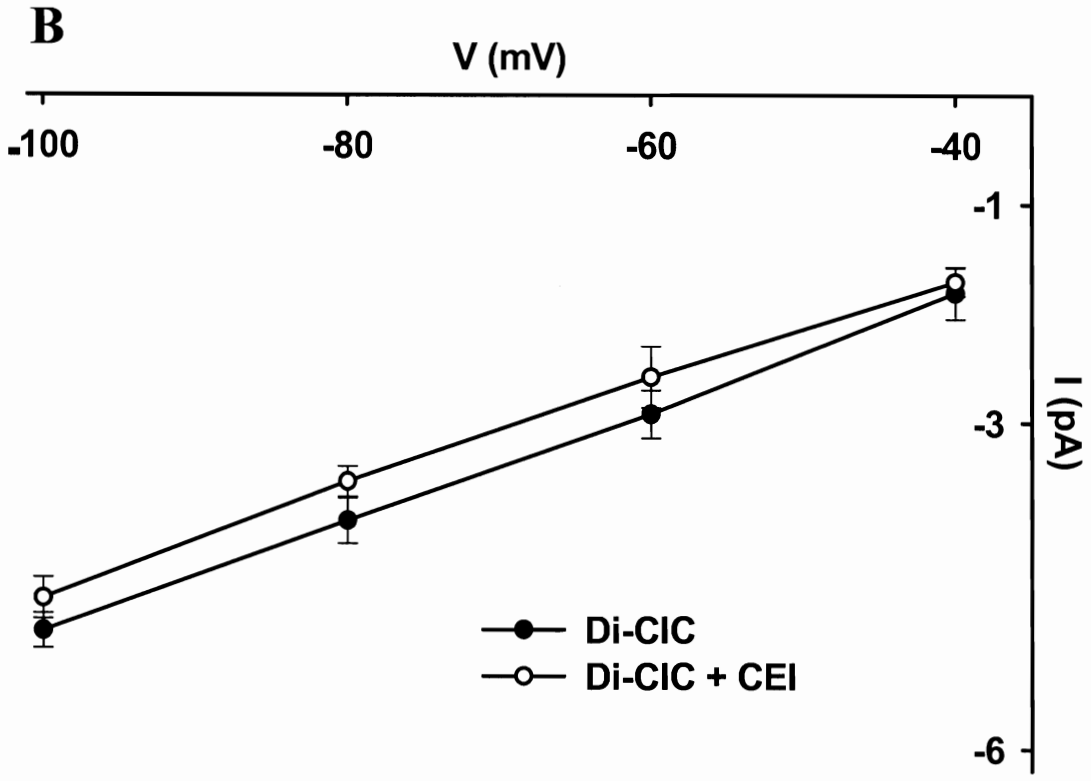
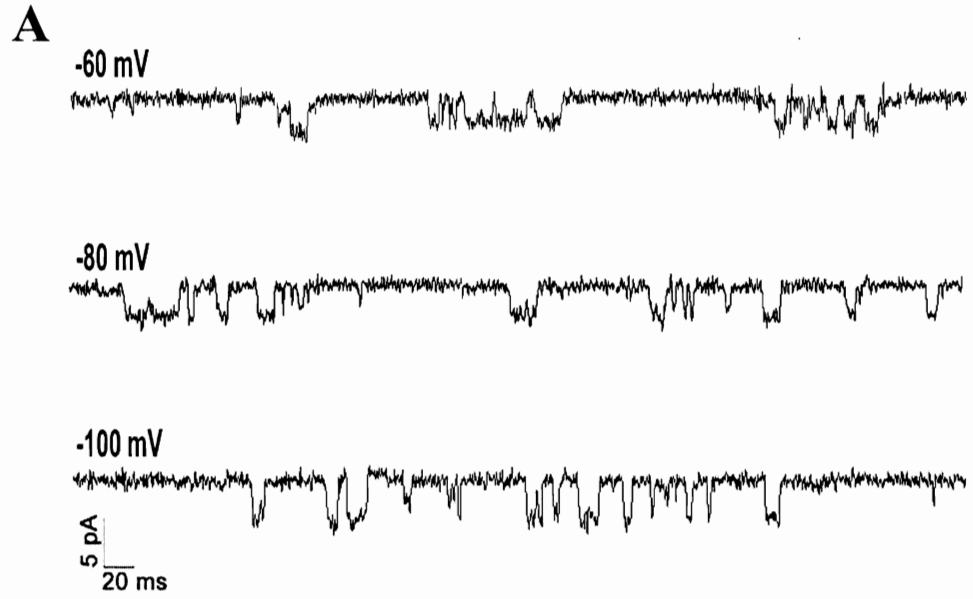

B
V (mV)
-100
-80
-60
-40
-1
-3
-6
I (pA)
Di-ClC
Di-ClC + CEI

Figure 28. Extracellular Na^+ removal maintains the decapitation ischemia injury induced channel activity. Substituting NaCl with NMDG in the pipette solution constituted the Na^+ removal. *A*, Representative cell-attached recordings showing presence of channel activity at various holding potentials despite the absence of Na^+ in the extracellular solution. *B*, Current-Voltage (I-V) relationship for the presence and absence of Na^+. A total of 6 neurons were recorded for the absence of Na^+.


A
-60 mV
-80 mV
-100 mV
5 pA
20 ms

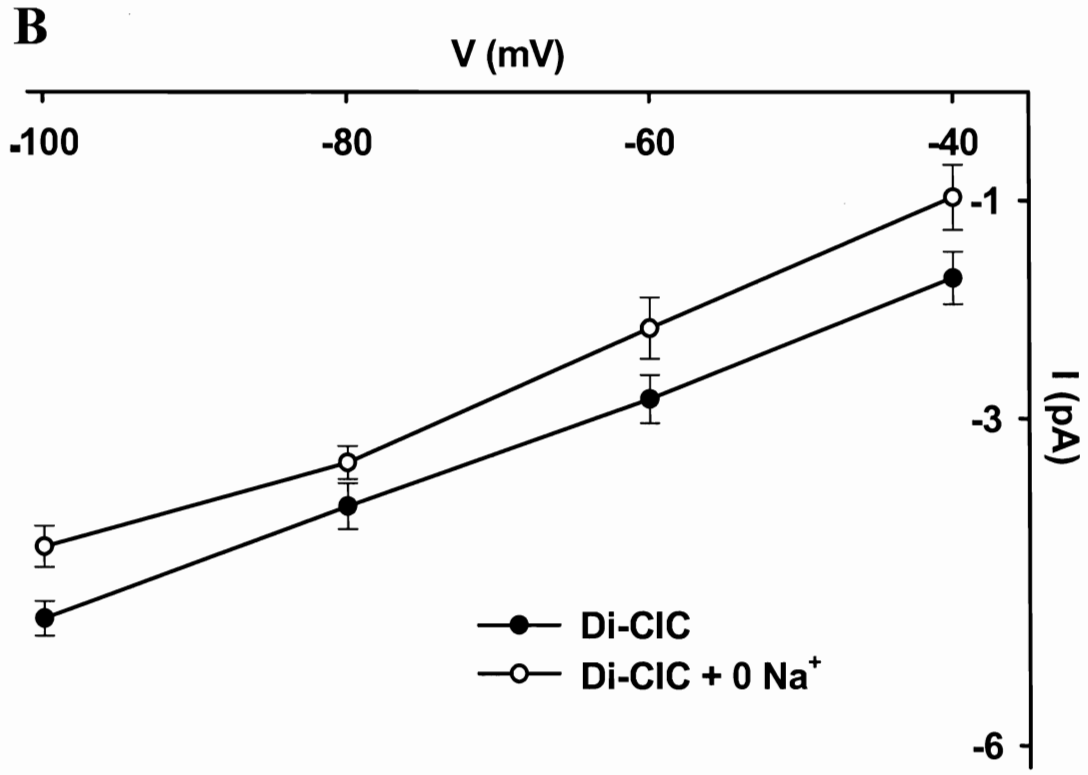

B
V (mV)
-100
-80
-60
-40
-1
-3
-6
I (pA)
Di-ClC
Di-ClC + 0 Na+

Figure 29. Removing extracellular Ca^{2+} abolishes the decapitation ischemia injury induced channel activity. Omission of $CaCl_2$ from the pipette solution constituted the Ca^{2+} removal. Representative single channel traces showing no channel activity across all the holding potentials in the absence of extracellular Ca^{2+}. A total of 6 neurons were recorded for removal of extracellular Ca^{2+}.

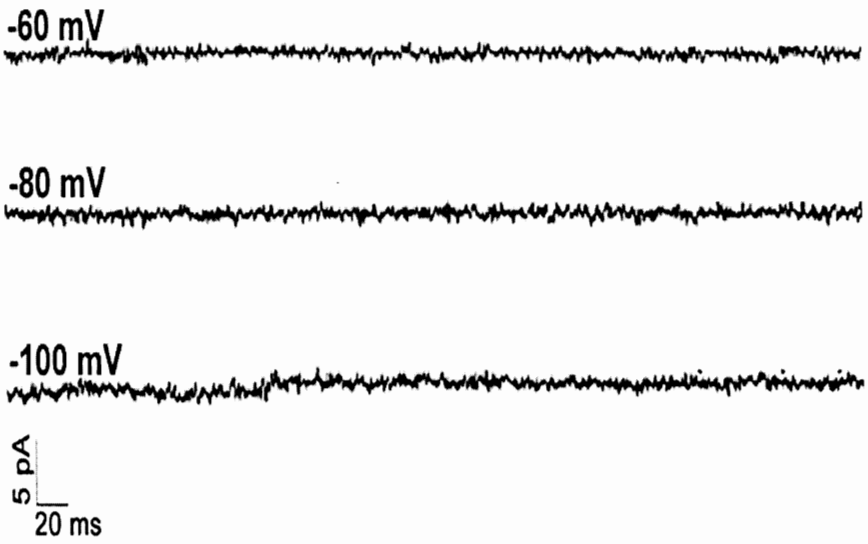

-60 mV
-80 mV
-100 mV
5 pA
20 ms

Figure 30. Low concentrations of Gd^{3+} (10 μM) do not block the decapitation ischemia injury induced channel activity. *A*, Representative single channel recordings from acutely dissociated decapitation ischemia injured neurons patched with pipette solutions containing 10 μM Gd^{3+}. Distinct channel activity was observed at all the holding potentials ranging from –60 mV to –100 mV. *B*, Current-Voltage (I-V) relationship for the presence and absence of Gd^{3+} (10 μM) [N=6].

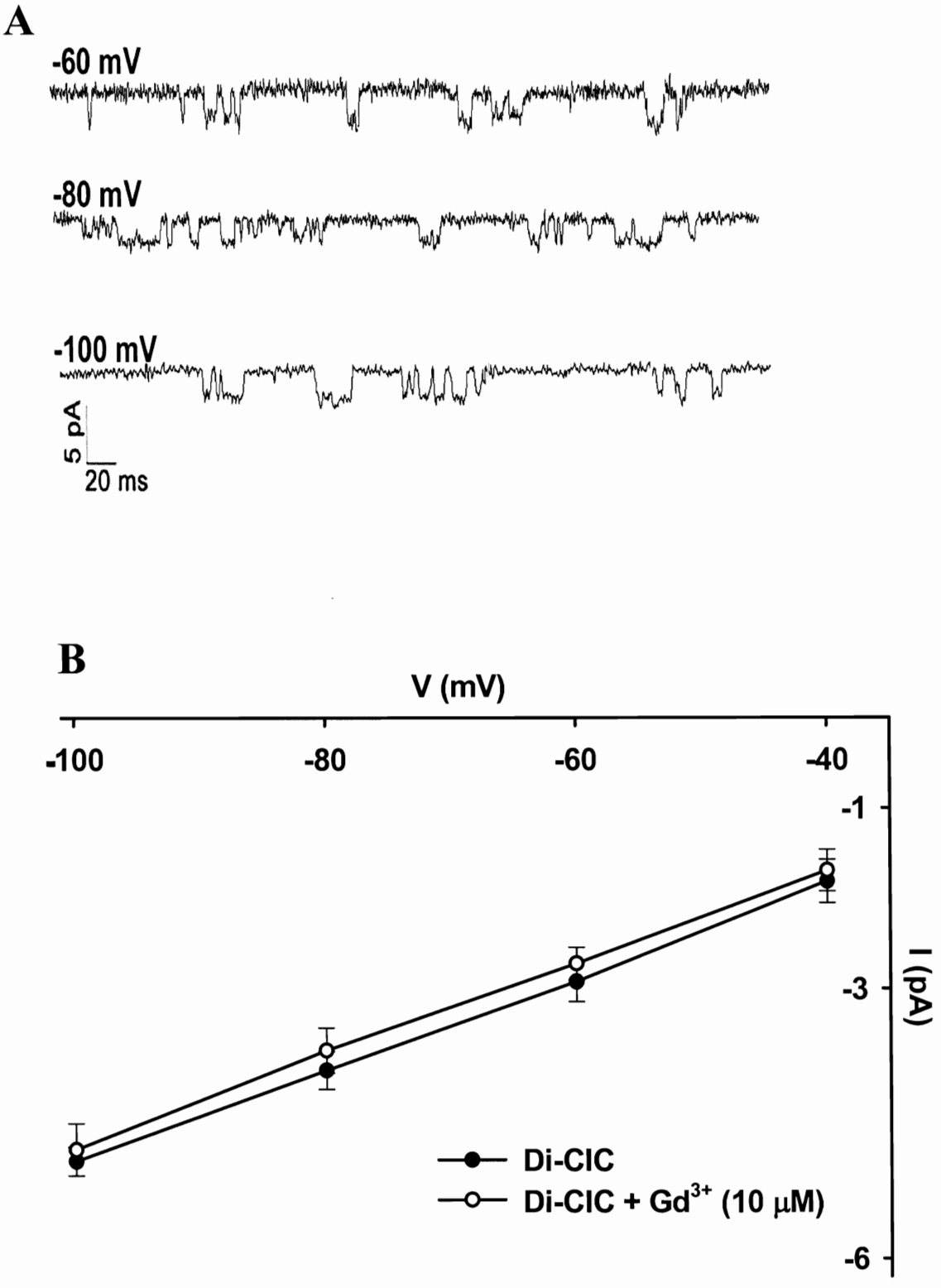

A
-60 mV
-80 mV
-100 mV
5 pA
20 ms
B
V (mV)
-100
-80
-60
-40
-1
-3
-6
I (pA)
Di-ClC
Di-ClC + Gd3+ (10 μM)

Figure 31. Low concentrations of Zn^{2+} (5 μM) do not block the decapitation ischemia injury induced channel activity. *A*, Representative single channel recordings from acutely dissociated decapitation ischemia injured neurons patched with pipette solutions containing 5 μM Zn^{2+}. Distinct channel activity was observed at all the holding potentials ranging from –60 mV to –100 mV. *B*, Current-Voltage (I-V) relationship for the presence and absence of Zn^{2+} (5 μM). Six neurons were recorded for the presence of 5 μM Zn^{2+}.

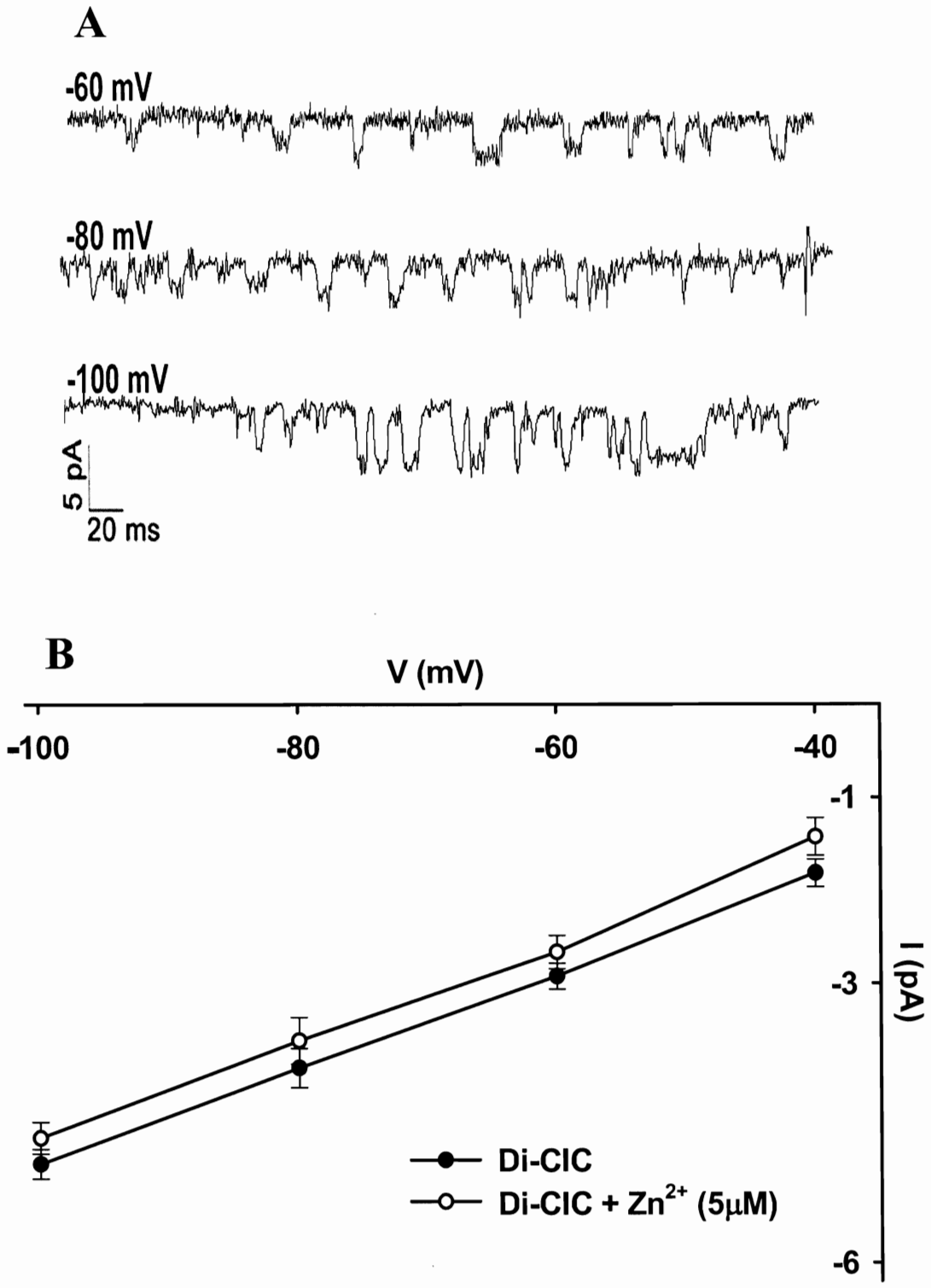

A
-60 mV
-80 mV
-100 mV
5 pA
20 ms
B
V (mV)
-100
-80
-60
-40
-1
-3
-6
I (pA)
Di-CIC
Di-CIC + Zn2+ (5μM)

Figure 32. High concentrations of Gd^{3+} (100 μM) completely abolish the decapitation ischemia injury induced channel activity. Representative single channel recordings from acutely dissociated decapitation ischemia injured neurons patched with pipette solutions containing 100 μM Gd^{3+}. Note the absence of channel activity at all the holding potentials ranging from –60 mV to –100 mV [N=6].

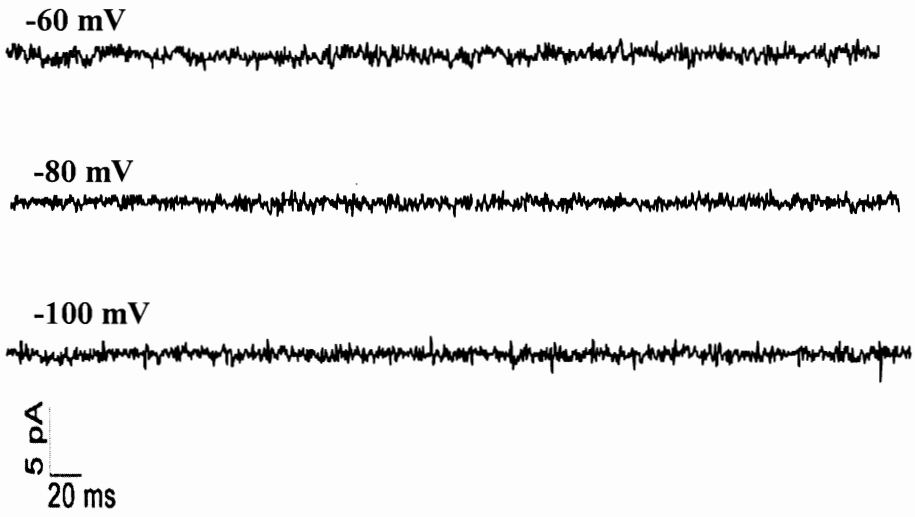
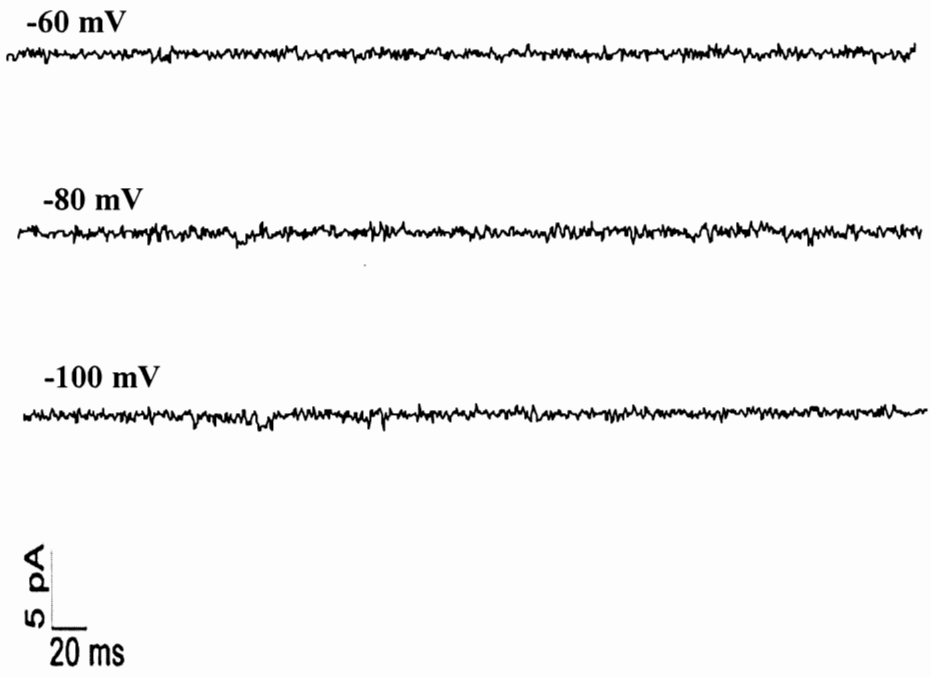

-60 mV
-80 mV
-100 mV
5 pA
20 ms

Figure 33. High concentrations of Zn^{2+} (500 μM) completely abolish the decapitation ischemia injury induced channel activity. Representative single channel recordings from acutely dissociated decapitation ischemia injured neurons patched with pipette solutions containing 500 μM Zn^{2+}. Note the absence of channel activity at all the holding potentials ranging from –60 mV to –100 mV. [N=6]


-60 mV
-80 mV
-100 mV
5 pA
20 ms

DISCUSSION

This study demonstrates that glutamate excitotoxicity activates a previously undetected Ca^{2+} permeable ion channel in both hippocampal neuronal cultures and decapitation ischemia injured dissociated neurons. The data indicate that this novel channel causes the Ca^{2+} injury current (I_{CIC}) responsible for a persistent Ca^{2+} influx that maintains END and initiates over time the irreversible pathways leading to cell death. The channel conducted an inward current and exhibited a conductance of approximately 60 pS. This channel was not voltage dependent over the range of voltages studied. It was more selective towards Ca^{2+} than Na^{+}. Inhibiting this Ca^{2+} permeable channel and the associated current, I_{CIC}, with high concentrations of Zn^{2+} or Gd^{3+} within the window of opportunity after glutamate excitotoxicity reverses END, blocks Ca^{2+}entry and prevents delayed neuronal cell death. Elucidation of the I_{CIC}- Ca^{2+} permeable channel provides an explanation for the apparent Ca^{2+} paradox, where Ca^{2+} continues to enter the neuron despite the presence of conventional Ca^{2+} entry blockers. The development of the I_{CIC}-Ca^{2+} permeable channel explains why many of the therapeutic trials employing conventional strategies to inhibit Ca^{2+} entry have not been effective in treating stroke (Ikonomidou and Turski, 2002; Muir and Lees, 2003; Wahlgren and Ahmed, 2004). Stroke is associated with massive release of glutamate in the brain. Glutamate acts on NMDA receptor that lets Ca^{2+} into the neurons. Persistent activation of NMDA receptors

leads to further elevations in $[Ca^{2+}]_i$ triggering neurotoxic mechanisms leading to cell-death, a phenomenon called as excitotoxicity (reviewed in Sattler and Tymianski, 2001). Calcium entry blockers developed in an effort to limit neuronal death after stroke have failed to show any beneficial effect after stroke and thus have failed in the clinical trials (Horn and Limburg, 2001; Ikonomidou and Turski, 2002; Muir and Lees, 2003; Wahlgren and Ahmed, 2004). After excitotoxicity, Ca^{2+} still continues to enter the neuron despite the presence of Ca^{2+} blockers. This is known as the Ca^{2+} paradox of stroke. In this dissertation an attempt was made to resolve the Ca^{2+} paradox. It was hypothesized that glutamate excitotoxicity activates a novel Ca^{2+} permeable ion channel which cannot be blocked by conventional Ca^{2+} entry antagonists. This channel is responsible for mediating a Ca^{2+} conductance responsible for maintaining END and ultimately leading to delayed neuronal cell death.

Previous studies from our laboratory have shown that glutamate excitotoxicity induces an extended neuronal depolarization (END) (Sombati et al., 1991; Coulter et al., 1992) in neurons and a Ca^{2+} conductance was responsible for maintaining neurons in END (Limbrick et al., 2003). However, the molecular basis of this injury induced Ca^{2+} current was not known. The data provided in this dissertation provide evidences that glutamate excitotoxicity activates a novel Ca^{2+} permeable ion channel that mediates the Ca^{2+} current responsible for producing END. The following lines of evidence support our conclusion: a) activation of the ion channel in the post-glutamate END period coinciding with the appearance of the injury induced Ca^{2+} current, b) similar ionic selectivity while conducting no current in the absence of $[Ca^{2+}]_e$, c) similar insensitivity to blockade by

Ca^{2+} entry inhibitors and d) analogous sensitivity to blocker cations like Zn^{2+} and Gd^{3+}. All these evidences indicate that the observed ion channel activity represents elementary unit conductances of the injury-induced current I_{ClC} activated by glutamate excitotoxicity and responsible for END.

Persistent Ca^{2+} entry into neurons even in the absence of glutamate triggers neurodegenerative cascades responsible for neuronal cell death upon stroke (Aarts and Tymianski, 2003a, b). The majority of Ca^{2+} increase in neurons occurs either via influx from Ca^{2+} permeable NMDA or AMPA receptors, voltage gated Ca^{2+} channels, release from intracellular Ca^{2+} stores or some defects in Ca^{2+} homeostatic mechanisms (Sattler and Tymianski, 2000; Delorenzo et al., 2005). However, development of neuroprotective drugs targeted at limiting this Ca^{2+} entry into the neurons after a stroke have been largely unsuccessful in clinical trials (Lees et al., 2000; Muir and Lees, 2003). Thus, Ca^{2+} continues to enter the neuron despite the presence of Ca^{2+} entry inhibitor giving rise to the Ca^{2+} paradox. Our observation that an ion channel activated upon excitotoxic glutamate exposure that is not blocked by conventional Ca^{2+} entry inhibitors raises the possibility that this ion-channel could account for the Ca^{2+} paradox. Indeed, blockade of this channel with high concentrations of Gd^{3+} or $[Ca^{2+}]_e$ removal not only prevented the $[Ca^{2+}]_i$ increase and END but it also afforded neuroprotection to the neurons otherwise destined to die after glutamate excitotoxicity thus supporting our conclusions.

Numerous studies have demonstrated a causal relationship between ischemia induced neuronal cell death and $[Ca^{2+}]_i$ accumulation (Kristian and Siesjo, 1996, 1998; Lo et al., 2003; Lo et al., 2005; Singhal et al., 2005). Thus, a critical question relates to

the source of this Ca^{2+}. Various research groups have confirmed our observation that it is an influx of an $[Ca^{2+}]_e$ responsible for glutamate excitotoxic damage (Choi, 1985, 1987; Glaum et al., 1990; Randall and Thayer, 1992). This massive Ca^{2+} influx ultimately leads to delayed Ca^{2+} dysregulation culminating in END (Limbrick et al., 1995; Limbrick et al., 2001). The genesis of delayed Ca^{2+} dysregulation is thought to be in the influx of $[Ca^{2+}]_e$ since removing $[Ca^{2+}]_e$ completely abolishes END, neurons regain their resting membrane potential (Limbrick et al., 2003) and $[Ca^{2+}]_i$ returns to basal pre-glutamate levels (Hartley and Choi, 1989; Manev et al., 1989; Randall and Thayer, 1992; Limbrick et al., 2003; Chinopoulos et al., 2004). However, many others believe the contrary. For example, Khodorov et al has repeatedly shown that the delayed Ca^{2+} dysregulation finds its origin in mitochondrial Ca^{2+} release (Khodorov et al., 2002; Khodorov, 2004). In order to shed further light on this issue and also to determine relative contribution of intracellular and extracelluar Ca^{2+} in mediating the post-glutamate excitotoxic injury a series of experiments were performed in the presence and absence of Mg^{2+} on the ability of removal of $[Ca^{2+}]_e$ to restore basal $[Ca^{2+}]_i$ in the same hippocampal neuronal model used in this study. This is the major difference between our method of producing glutamate injury and some other research groups. Producing injury employing both excitotoxic glutamate and the removal of extracellular Mg^{2+} causes the levels of $[Ca^{2+}]_i$ to remain elevated despite the removal of $[Ca^{2+}]_e$ (Khodorov et al., 2002; Khodorov, 2004).

Our laboratory has shown that incubating neurons in culture in 0 Mg^{2+} induces continuous epileptiform activity, status epilepticus (DeLorenzo et al., 1998; Blair et al., 2006). In addition, the voltage-dependent Mg^{2+} block of NMDA receptor is lifted during

metabolic inhibition such that in absence of Mg^{2+} minimal concentration of glutamate produces excitotoxicity (Zeevalk and Nicklas, 1992). The combination of status epilepticus and glutamate excitotoxicity produces a much more severe injury than glutamate excitotoxicity alone (Waterhouse et al., 1998) with the inclusion of early mitochondrial injury, where release of Ca^{2+} from the mitochondria plays a major role in contributing to the elevated $[Ca^{2+}]_i$ such that is not reversed even by the removal of $[Ca^{2+}]_e$ (Khodorov, 2004). However, this combined injury is not characteristic of the ischemic injury produced by stroke or glutamate excitotoxicity alone. We found that neurons receiving double injury had higher $[Ca^{2+}]_i$ peaks compared to neurons receiving only excitotoxic glutamate injury. Further, removal of $[Ca^{2+}]_e$ restored basal $[Ca^{2+}]_i$ only in neurons injured by glutamate excitotoxicity and not following combined glutamate excitotoxicity and 0 Mg^{2+} injury. The presence or absence of Mg^{2+} during glutamate excitotoxicity is responsible for the ability of the neurons to respond to removal of $[Ca^{2+}]_e$ following injury. The combined injury not only activates the I_{ClC} channel, but also immediately causes mitochondrial injury and activates the irreversible cascade leading to neuronal death. Further recently it was reported that removal of the endogenous blockade by Mg^{2+} of the NMDA receptor in cultured hippocampal neurons triggers a self-perpetuating cycle of excitotoxicity (Skaper et al., 2001).

In addition, ratiometric Fura-2 experiments employing Ca^{2+} free wash solution after glutamate excitotoxicity demonstrated that glutamate-injured neurons recovered from elevated $[Ca^{2+}]_i$ back to basal levels for up to 1hour after removal of $[Ca^{2+}]_e$. Beyond this time point the percentage of neurons that could restore basal $[Ca^{2+}]_i$ levels

started to decrease despite removal of $[Ca^{2+}]_e$. This indicates there is a window of opportunity of up to 1 h where it is possible to intervene and restore basal $[Ca^{2+}]_i$ levels by inhibiting the novel injury induced Ca^{2+}-permeable channel before the irreversible effects of injury are initiated, resulting in loss of Ca^{2+} buffering capacity and release of mitochondrial Ca^{2+}. This is in line with recent findings that mitochondria maintain their resting level for Ca^{2+} for about 45-mins after glutamate excitotoxicity, even in the face of rising cytosolic Ca^{2+} levels (Bano et al., 2005). There is also evidence that mitochondrial respiration is retained for a relatively long time in cerebellar neurons undergoing excitotoxicity (Jekabsons and Nicholls, 2004). Moreover, it was recently reported that mitochondria in the acutely dissociated CA1 neurons maintained their membrane potential ($\Delta\psi_m$) despite increases in $[Ca^{2+}]_i$ upon toxic glutamate exposure (Larsen et al., 2006). These results clearly distinguish the combined injury from the glutamate excitotoxic alone injury and demonstrate that it is possible to reverse the increased $[Ca^{2+}]_i$ following glutamate excitotoxicity in the early phase during the critical time window of opportunity while the I_{ClC} channel is the major source of increased $[Ca^{2+}]_i$.

Thus, while intracellular Ca^{2+} stores (Verkhratsky, 2005) and other cation conductances (Chen et al., 1998; Aarts et al., 2003; Xiong et al., 2004) may play some role in ischemia induced Ca^{2+} elevations, our data demonstrate that the activation of the I_{ClC}- Ca^{2+} permeable channel during glutamate excitotoxicity is responsible for the majority of the massive Ca^{2+} influx during the first hour after glutamate excitotoxicity. During this 1 h time window, intervention with Gd^{3+} or removal of $[Ca^{2+}]_o$ can restore elevated $[Ca^{2+}]_i$. However, beyond this time point, in addition to I_{ClC} other irreversible

ionic mechanisms are activated, including mitochondrial breakdown, Ca^{2+} release from intracellular stores and alteration of Ca^{2+} homeostatic mechanisms. Once the irreversible Ca^{2+} deregulating mechanisms are initiated, it is no longer possible to lower the elevated $[Ca^{2+}]_i$ by removing $[Ca^{2+}]_e$. The discovery that the activation of the I_{ClC}- Ca^{2+} channel causes the majority of Ca^{2+} influx during the first hour after glutamate excitotoxicity in the cascade leading to neuronal death offers new hope for therapeutic intervention to prevent neuronal injury and brain damage in stroke by inhibiting this novel Ca^{2+} permeable channel.

After demonstrating that glutamate excitotoxicity activates a novel Ca^{2+} permeable ion channel in cultured hippocampal neurons, we then investigated if a similar phenomenon was occurring upon brain injury *in vivo*. We used the well-established decapitation ischemia model to produce brain injury (Abe et al., 1983; Wasterlain and Powell, 1986; Parsons et al., 1999; Gerasimov et al., 2004). Glutamate excitotoxicity plays a major role in producing neuronal injury and cell death in this model of brain injury. Neurons were acutely dissociated from injured and sham hippocampal slices. Our laboratory and many others (Gibbs et al., 1996; Chen et al., 1997; Chen et al., 1998; Raza et al., 2001; Raza et al., 2004) have demonstrated that acutely isolated hippocampal neurons can be studied in vitro using patch clamp or fluorescent Ca^{2+} imaging and they show minimal signs of necrosis or apoptosis (Raza et al., 2001; Raza et al., 2004). Immediately upon seal formation, in the presence of Ca^{2+} entry inhibitors, distinct single channel currents were observed in neurons acutely isolated from ischemia-injured hippocampi, but not in neurons acutely isolated from control hippocampi. Ischemia

injured acutely isolated neurons had a linear I-V curve with a slope conductance of 52.23 ± 1.61 pS that was close to the I_{ClC}-Ca^{2+}-permeable channel's conductance activated by glutamate excitotoxicity. The *in vivo* current carried by this I_{ClC}-Ca^{2+}-channel also demonstrated a strong Ca^{2+} selectivity, passing no current in absence of Ca^{2+} and was blocked by high, but not low concentrations of Zn^{2+} and Gd^{3+}. These results indicate that I_{ClC}-Ca^{2+}-channel activated by glutamate excitotoxicity *in vitro* was identical to the I_{ClC}-Ca^{2+}-channel activated *in vivo* by ischemia.

Preliminary fluorescent Ca^{2+} imaging results using neurons acutely dissociated from middle cerebral artery occluded (MCAO) rats, another model for stroke, showed increased Fura-2 $[Ca^{2+}]_i$ 340/380 ratio in neurons dissociated from the occluded, injured hemisphere but not from the contra-lateral, uninjured hemisphere. Further, the increased $[Ca^{2+}]_i$ ratio in MCAO injured neurons could be restored when $[Ca^{2+}]_e$ was removed from the bath solution suggesting that an influx of extracellular Ca^{2+} was playing an important role in maintaining the elevated $[Ca^{2+}]_i$ ratio in MCAO injured neurons. However, presence of conventional Ca^{2+} entry inhibitors failed to reduce the elevated $[Ca^{2+}]_i$ ratio indicating that traditional Ca^{2+} influx pathways are not mediating the Ca^{2+} influx. This also demonstrated the presence of a possible Ca^{2+} paradox in this MCAO model of ischemia/ stroke/ glutamate excitotoxicity. Interestingly, the elevated $[Ca^{2+}]_i$ ratio in MCAO injured neurons was restored only upon early removal of $[Ca^{2+}]_e$ immediately upon acute dissociation after the injury. However, neurons isolated from animals allowed to recover for 24-hrs after the injury, while still demonstrating significantly higher $[Ca^{2+}]_i$ ratio than its non-injured contra lateral hemisphere, failed to restore basal $[Ca^{2+}]_i$ ratio

despite the removal of $[Ca^{2+}]_e$. This suggests that similar to the excitotoxic glutamate injured neurons both *in vivo* and *in vitro*, whatever mechanism that is responsible for maintaining the elevated $[Ca^{2+}]_i$ ratio in MCAO injured neurons is activated very early in the injury cascade and could potentially be inhibited early thereby limiting the neuronal loss with certain therapeutic window of opportunity. However, beyond this therapeutic window, once the irreversible Ca^{2+} deregulating mechanisms are initiated, it is no longer possible to lower the elevated $[Ca^{2+}]_i$ despite removing $[Ca^{2+}]_e$. It will be interesting to further confirm these preliminary observations. It will also be insightful to perform patch clamp analysis on these MCAO injured dissociated neurons and look for the presence of an injury activated current and compare it's properties to the glutamate excitotoxicity activated current expressed in hippocampal cultures and decapitation ischemia injured neurons.

Since the activation of the injury-induced Ca^{2+} permeable channel and the associated current could be observed immediately after excitotoxic glutamate exposure, it is plausible that the injury-induced current does not result from the synthesis of new channel proteins. Thus, it is interesting to elucidate the molecular basis of the excitotoxic glutamate activated ion channel. Given the high Ca^{2+} permeability of this channel and a requirement of NMDA for channel activation one probable candidate is the NMDA receptor gated channel. We used NMDA-receptor antagonists MK-801 and APV either alone or in combination. MK-801 is a voltage-dependent blocker of NMDA-receptor ion channel. Thus its ability to block is limited under depolarized (END) condition. APV is a competitive antagonist and its ability to block NMDA-receptor depends upon the period

of glutamate exposure. Our observations that both the competitive (APV) and non-competitive (MK-801) pharmacological inhibitors to NMDA channel do not block the post-glutamate injury induced channel activity provide strong evidence that NMDA receptor does not mediate the injury-induced current. Similarly the inability of CNQX, an AMPA/KA receptor antagonist, to block the injury induced channel activity suggests that Ca^{2+} permeable AMPA receptors do not represent the molecular basis for excitotoxic glutamate activated channel.

END neurons are characterized by membrane potentials of approximately –15 to –20 mV. Activation of voltage-gated Ca^{2+} channels is expected to occur at these potentials. Thus, the observed injury mediated channel activity could be due to the activation of the voltage-gated Ca^{2+} channels. However, our experiments with Ca^{2+} channel blockers, nifedipine and 10 μM Gd^{3+} suggest that the Ca^{2+} channels are not mediating the injury induced ion channel activity. In addition, voltage-gated Ca^{2+} channels undergo inactivation rapidly after the onset of depolarization. But we observed a persistent channel activity for prolonged period in the depolarized END phase. Taken together, we can conclude that voltage-gated Ca^{2+} channels are not the mediators of the glutamate injury induced Ca^{2+} permeable channel.

Under conditions of persistent depolarization, the normal Na^{+} electrochemical gradient is also disrupted resulting in increased intracellular Na^{+}. As a consequence, the normal role of Na^{+}/Ca^{2+} exchanger can actually reverse and the exchanger starts contributing to Ca^{2+} entry (Hoyt et al., 1998; Blaustein and Lederer, 1999). However, inhibition of reverse mode of Na^{+}/Ca^{2+} exchanger with amiloride derivative bepridil or

removal of $[Na^+]_e$ could not block the channel activity or prevent END. In addition, the rate of Ca^{2+} entry upon glutamate injury appears to be too fast to be mediated by an exchanger protein. These evidences indicate that reverse mode of the Na^+/Ca^{2+} exchanger is not contributing to END and do not represent the cellular basis for the glutamate injury induced ion channel.

In the recent literature the TRP family of cation permeable channels have emerged as potential candidates for delayed Ca^{2+} dysregulation following anoxia or changes in the redox status of the cell (Aarts and Tymianski, 2005). Within the TRP family, TRPM-2 and TRPM-7, have been found to confer susceptibility to cell death following H_2O_2 insult and anoxia (Hara et al., 2002; Aarts et al., 2003). The transient receptor potential (TRP) cation channels are a family of about 20 mammalian proteins united by a common primary structure and permeability to monovalents and Ca^{2+} (Pedersen et al., 2005; Ramsey et al., 2006). Of particular interest is TRPM7, a cation channel containing a functional C-terminal kinase domain (Nadler et al., 2001; Runnels et al., 2001). Heterologously over-expressed TRPM7 channels in HEK-293 cells exhibit a high Ca^{2+} permeability, an outwardly rectifying I-V curve, enhancement by low $[Ca^{2+}]_e$, and block of monovalent currents by Gd^{3+}. Intracellular Mg^{2+} and Zn^{2+} also block the TRPM7 currents (Kozak and Cahalan, 2003). These divalent cations both permeate TRPM7 channels and block the monovalent cation flow through them (Kerschbaum et al., 2003). Moreover, their over-expression is lethal to HEK-293 cells (Nadler et al., 2001; Monteilh-Zoller et al., 2003) but can be prevented simply by elevating extracellular Mg^{2+} to restore Mg^{2+} homeostasis (Schmitz et al., 2003). Indeed recently it was shown

that oxygen-glucose deprivation or chemical anoxia using sodium cyanide in cortical neurons results in the induction of a lethal cation current mediated by TRPM7. Blocking either the cation current with 10 μM Gd^{3+} or inhibiting the expression of TRPM7 channels using RNAi prevented the anoxic neuronal death (Aarts et al., 2003; Aarts and Tymianski, 2005).

In general the TRP currents are smaller and demonstrate greater monovalent permeability. The anoxia activated outwardly rectifying TRPM-7 currents are: 1) at ~100 pA at –80 mV (Aarts et al., 2003), 2) demonstrate limited Ca^{2+} permeability (P_{Ca}:P_{Na} = 3:1) (Clapham et al., 2005), 3) are blocked by low concentrations (10 μM) of $GdCl_3$ (Aarts et al., 2003; Clapham et al., 2005). The H_2O_2 activated TRPM-2 currents are at ~1000 pA at –80 mV and are abolished by replacement of Na^+ with NMDG or by omission of $[Ca^{2+}]_e$ (Hara et al., 2002; Clapham et al., 2005). In contrast, glutamate excitotoxicity activated currents carried by the injury induced Ca^{2+} permeable channel are: 1) large, ~3500 pA at –80 mV, 2) are highly Ca^{2+} permeable (P_{Ca}:P_{Na}= 12.5:0.25) and 3) are not abolished by low $GdCl_3$ (10 μM) or by replacement of Na^+ with NMDG (Limbrick, 2000). The I_{ClC}- channel currents were blocked only by high concentrations of $GdCl_3$ (100 μM) and by omission of $[Ca^{2+}]_e$. Based on the kinetic characteristics and pharmacological comparisons it appears that the TRPM-2/7channels are not mediating the I_{ClC} observed here. However, the large number of TRP channels along with their diverse co-assembling abilities and lack of specific blockers makes it difficult to rule out a novel form of these channels.

Ischemia or excess glutamate release is known to cause a fall in extracellular pH. Under these circumstances Acid Sensing Ion Channels (ASIC) are activated that mediate a cation influx suggested to produce ischemic neuronal death (Allen and Attwell, 2002). ASIC channels are potentiated by membrane stretch and arachidonic acid (Allen and Attwell, 2002) produced as a consequence of increased phosphilipase A_2 activity in response to increased intracellular Ca^{2+} (Rehncrona et al., 1982). During ischemia, although the pH falls by up to one unit as a result of metabolism becoming anaerobic (Kraig et al., 1983; Silver and Erecinska, 1992), it takes several minutes to do so, which is too slow to evoke the large transient component of ASIC currents. Further channel desensitization also occurs as the pH falls preventing a large current from developing (Allen and Attwell, 2002). Recently, Xiong and co-authors (Xiong et al., 2004) reported that acidosis activates ASICs in mouse cortical neuronal cultures and MCAO rats. They showed that acidosis activated an inward depolarizing current mediated by cations. This amiloride sensitive current had high Na^+ and low Ca^{2+} permeability. This current required activation of ASIC*1a* since cortical cultures prepared from ASIC*1a*$^{-/-}$ mice didn't exhibit the activation of the current. However, our observations that amiloride (100 μM) or its derivative, bepridil (50 μM) had no significant effect on END, failed to block Ca^{2+} influx and did not prevent cell death after glutamate excitotoxicity strongly indicates that ASICs were not responsible for mediating the excitotoxic glutamate injury activated Ca^{2+} currents.

Yet another candidate for this injury-induced channel could be the stretch activated channel. Membrane stretch is produced when the extra-cellular fluid becomes

hypotonic, or during ischemia when the extra-cellular [K^+] rises to 60 mM a few minutes after the start of a stroke (Hansen, 1985; Walz et al., 1993). Glutamate is known to cause profuse neuronal swelling. Swelling acts as a stimulus for opening of swelling/ stretch activated mechanosensitive channels that result in the influx of cations including Ca^{2+} (Viana et al., 2001). Stretch activated ion channels are reported in various tissues including the central nervous system. Studies indicate that the stretch activated ion channels have a single channel conductance of approximately 32pS (Kanzaki et al., 1999). These stretch activated channels are blocked by low concentrations of Gd^{3+}. However, at these concentrations [Gd^{3+} (10 μM)] where the stretch activated channels would have been blocked, Gd^{3+} failed to block the glutamate injury induced ion channel activity, Ca^{2+} influx and failed to prevent neuronal cell death. Moreover the I_{ClC} Ca^{2+} permeable channel had conductance two times higher in magnitude than the stretch activated channels. All these evidences suggest that the stretch activated ion channels are not mediating the injury induced ion channel activity.

Another candidate for this putative END channel could be the neuronal Ca^{2+}-activated non-selective channel. Presence of this channel has been reported in hippocampal neurons amongst other tissues. Single channel conductance for these CAN channels is in the range of 17-40 pS (Partridge and Valenzuela, 2000). Blockers are available for these classes of ion channels such as Gadolinium and Flufenamic acid. However at the concentrations known to block the neuronal Ca^{2+}-activated non-selective channels, Gd^{3+} or flufenamic acid were not able to block the glutamate injury induced ion

channel activity ruling out the neuronal Ca^{2+}-activated non-selective channels as probable candidates for excitotoxic glutamate activated ion channel.

Chen et al in 1997 reported that excitotoxic neuronal injury with NMDA can also induce a non-selective post exposure cation current (I_{pe}) in acutely dissociated hippocampal neurons (Chen et al., 1997; Chen et al., 1998). This current also required NMDA receptor activation and/or increase in $[Ca^{2+}]_i$ for activation. However, I_{pe} was not highly selective for Ca^{2+} (P_{Ca}:P_{Na}=7:1). In addition, I_{pe} was blocked by reducing both $(Na^+)_e$ and $(Ca^{2+})_e$, but not by removing $(Ca^{2+})_e$ alone. In contrast, the glutamate injury induced current is highly Ca^{2+} selective and as reported by Limbrick et al has permeability ratios of (P_{Ca}:P_{Na}=50:1) and is completely abolished by removal of $(Ca^{2+})_e$ alone (Limbrick, 2000). These evidences indicate that I_{pe} and the excitotoxic glutamate injury activated Ca^{2+} current are two different species and I_{pe} doesn't appear to be Ca^{2+} selective enough for being able to mediate END.

Taken together, our findings demonstrate that the excitotoxic glutamate injury activated I_{ClC}-Ca^{2+} channel appears to be different from TRPM-7, ASIC or I_{pe} and these conductances are not significantly contributing to the initial Ca^{2+} entry and END during the first hour of the post-glutamate treatment paradigm. These results indicate that the I_{ClC}-Ca^{2+}-channel activated by glutamate excitotoxicity is a unique mechanism for Ca^{2+}entry and offers a novel therapeutic target for preventing neuronal death following glutamate excitotoxicity.

The activation, but not the maintenance of END and the I_{ClC}- Ca^{2+} permeable channel is dependent upon NMDA receptor activation during glutamate excitotoxicity

(Limbrick et al., 2003), since the presence of the NMDA channel inhibitor, MK-801, during glutamate injury prevents the ability of glutamate to induce cell death and END. Moreover, the results demonstrate that MK-801 administered after glutamate excitotoxicity did not inhibit Ca^{2+} entry during END or blocked the channel activity. In addition, depolarization alone didn't induce END. Treatments with high concentrations of potassium chloride or substitution of $(Ca^{2+})_e$ with $(Ba^{2+})_e$ during glutamate excitotoxicity both caused neuronal depolarization of similar magnitude upon glutamate excitotoxicity in the presence of $(Ca^{2+})_e$ but did not cause the induction of END (Coulter et al., 1992). These findings indicate that the I_{ClC}- Ca^{2+} permeable channel is activated by an NMDA/Ca^{2+} mechanism. It is possible that NMDA dependent induction of the I_{ClC}-channel may occur through activation of a dormant channel or modification of an existing channel or protein in the membrane. Since I_{ClC} mediating channel activity could be observed immediately following the standard 10-min glutamate stimulation, it is plausible that I_{ClC} doesn't occur from the synthesis and delivery of new channel proteins to the plasma membrane, since this may be too rapid a time frame for *de novo* protein synthesis and insertion into the membrane. Excitotoxic stimulation would activate second messenger cascades leading to post-translational modifications of existing membrane proteins. Indeed NMDA dependent changes in protein phosphorylation (Churn et al., 1995; Durkin et al., 1997), protease activity such as the calpains (Minger et al., 1998; Simpkins et al., 2003) and numerous other second messenger effects have been reported during glutamate excitotoxicity. Such alterations in key proteins or enzyme activities could result in the activation of the I_{ClC}- Ca^{2+} permeable channel. Indeed, NMDA

mediated Ca^{2+} influx has been demonstrated to cause depolymerization of cytoskeletal elements resulting in changes in receptor density, function and localization (Allison et al., 1998; Sattler et al., 2000). Post-translational events such as proteolytic cleavage of a preexisting membrane protein or protein phosphorylation represent rapid changes that could underlie activation of the I_{ClC}- Ca^{2+} permeable channel during the 10 minutes of glutamate excitotoxicity. Our preliminary studies with cycloheximide- a compound that blocks synthesis of newly synthesized proteins did not block development of END, suggesting that glutamate excitotoxicity mediated synthesis and delivery of new channel proteins to cell membrane is unlikely to cause the novel channel activity. Additional, preliminary experiments with generalized protease inhibitor PMSF failed to block END indicating that preoteolytic cleavage of existing membrane proteins might not underlie the observed channel activity. Interestingly preliminary experiments with generalized kinase inhibitor K-252a blocked the development of END suggesting that a kinase phosphorylation based event of an existing membrane protein could be the likely mechanism for the activation of the injury induced Ca^{2+} permeable ion channel. It will be extremely insightful to further investigate these possibilities with more specific inhibitors and elucidate the molecular basis of the activation of the I_{ClC}- Ca^{2+} permeable channel and thus determine the mechanisms activating this injury induced Ca^{2+} entry.

Glutamate excitotoxicity is accompanied by protracted increases in neuronal $[Ca^{2+}]_i$ that persist well beyond the period of glutamatergic injury (Dubinsky, 1993; Limbrick et al., 1995). These sustained elevations in $[Ca^{2+}]_i$ represent a prolonged imbalance in neuronal Ca^{2+} homeostasis and correlate with subsequent excitotoxic

neuronal cell death (Limbrick et al., 1995). In general, the inability of neurons to restore resting $[Ca^{2+}]_i$ following glutamate excitotoxicity could result from either a persistent influx of $[Ca^{2+}]_e$ and/ or from sustained impairment of neuronal Ca^{2+} sequestration/ extrusion mechanisms. Indeed recent discoveries that TRPM-7 (Aarts et al., 2003) and ASIC's (Xiong et al., 2004) allow for Ca^{2+} entry upon hypoxia-ischemia and that the plasma membrane Na^+/Ca^{2+} exchanger undergoes cleavage upon excitotoxicity (Bano et al., 2005) suggests that both these possibilities exist. Protracted Ca^{2+} increases upon glutamate excitotoxicity causes diverse pathophysiological changes including generation of free radicals, neuronal acidity, activation of proteases, to name a few, all of which trigger neurodegenerative processes (Lipton, 1999; Lo et al., 2005). However, our findings that I_{CIC}-Ca^{2+} permeable channel is activated immediately upon glutamate excitotoxicity and blocking its activity after glutamate excitotoxicity for sometime prevents $[Ca^{2+}]_i$ accumulation suggests that activation of I_{CIC}-Ca^{2+} permeable ion channel could represent the first step in the genesis of the injury induced $[Ca^{2+}]_i$ plateau upon glutamate excitotoxicity.

Our results indicate that blocking the I_{CIC}- Ca^{2+} permeable channel up to 1 h after glutamate excitotoxicity can still reverse END and prevent neuronal death. This indicates that there is a significant time period after the initial stroke injury to prevent cell death by blocking Ca^{2+} entry through the I_{CIC} channel prior to the irreversible induction of apoptosis. In addition, these findings have potentially broader implications, since the I_{CIC}-Ca^{2+} permeable channel may be activated under sub-lethal glutamate exposure and play a role in neuronal plasticity. The possible identification of a novel molecular target

compliant to pharmacological manipulations opens exciting avenues for the treatment of acute and chronic neurological disorders associated with glutamate excitotoxicity. The elucidation of the Ca^{2+} injury induced ion channel provides a new target for stroke treatment that may provide a significant extension of the therapeutic window to prevent neuronal death in stroke.

SUMMARY AND CONCLUSIONS

Excitotoxicity is the process by which L-glutamate, the primary excitatory neurotransmitter in the central nervous systems, damages neurons. Glutamate excitotoxicity is the predominant established mechanism responsible for neuronal death in acute neurological disorders such as stroke, epilepsy and traumatic brain injury (TBI) and is also implicated for neuronal death in amyotrophic lateral sclerosis (ALS). Persistent stimulation of NMDA receptors because of excessive release of glutamate causes indiscriminant Ca^{2+} entry into the neurons. Small changes in Ca^{2+} levels are required for normal brain functioning such as learning, memory, long-term potentiation (LTP) etc. However, prolonged elevations in $[Ca^{2+}]_i$ under pathological conditions such as stroke, epilepsy or TBI activates a plethora of neurotoxic mechanisms, triggers neurodegeradative pathways ultimately leading to neuronal cell death. One of the consequences of glutamate excitotoxicity is an NMDA-receptor dependent extended neuronal depolarization that has been demonstrated to remarkably correlate with impending neuronal death. A Ca^{2+} selective current was found to be the electrophysiological basis of END. Blocking the NMDA receptor or Ca^{2+} entry into the neurons was shown to prevent ischemia induced delayed cell death both in animal models and in vitro models only when administered before the injury. However, all the clinical trials with conventional Ca^{2+} entry inhibitors have failed to demonstrate any clinical

while NMDA activation is required for the initiation of the excitotoxicity cascade, other long-lasting non-NMDA dependent changes in neuronal physiology may endure following glutamate removal. The studies in this dissertation try to resolve the Ca^{2+} paradox by providing evidence that glutamate excitotoxicity activates a novel Ca^{2+} permeable ion channel that is responsible for the indiscriminant Ca^{2+} entry ultimately leading to neuronal cell death.

Using single channel patch clamp analyses, fluorescent Ca^{2+} imaging and cell death assays we demonstrated that an ion channel is activated by glutamate excitotoxicity both in the *in vivo* and *in vitro* models of ischemia/ stroke. Conventional Ca^{2+} entry blockers did not block this channel and Ca^{2+} continued to enter despite the presence of these antagonists suggesting that this channel could underlie the Ca^{2+} paradox of stroke. This channel is selective for Ca^{2+} allowing no current in the absence of $[Ca^{2+}]_e$. Traditional Ca^{2+} entry antagonists did not block this channel and it was only upon $[Ca^{2+}]_e$ removal or in the presence of high concentrations of Zn^{2+} or Gd^{3+} that this channel was blocked, a property shared by glutamate injury induced Ca^{2+} selective current observed by Limbrick et al. These results suggest that observed channel activity is in fact the elementary unit conductance of this same injury induced current. Blockade of this channel with either $[Ca^{2+}]_e$ removal or intervention with high concentrations of Gd^{3+} also rescued the neurons from undergoing apoptosis even up to 1-hr after the injury. This indicates that there is a therapeutic window of opportunity within which blockade of this channel prevents neuronal death. Beyond this time point irreversible Ca^{2+} deregulating mechanism are initiated ultimately leading to neuronal cell death.

The findings in this dissertation may represent an important new direction for elucidating the pathophysiological mechanisms underlying excitotoxic brain injuries. It provides a potential pharmacological target for the treatment of stroke and related brain insults with a significant therapeutic window.

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VITA

Laxmikant Sudhir Deshpande was born on August 31,1976 in the Amravati district of Maharashtra state in India and is an Indian citizen. He obtained a Bachelor's degree in Pharmaceutical Sciences (B.Pharm) in 1998 and a Master's degree in Pharmacology and Toxicology (M.Pharm) in 2000 from Nagpur University Department of Pharmaceutical Sciences, Nagpur, India. Mr. Deshpande joined the PhD program in the Department of Pharmacology and Toxicology at Virginia Commonwealth University in Fall-2001 and subsequently joined Dr. Robert DeLorenzo's lab in Summer-2002 to begin doctoral work on pathophysiology of stroke and other brain injuries.

Mr. Deshpande has been publishing actively, has presented his work at University, National and International meetings. He has received awards for his work such as the Best Paper Award by Virginia Academy of Sciences in 2004 and 2005, the John C. Forbes Research Honor Colloquium Seminar in 2004 and a travel grant from VCU Graduate School in 2005. His work was also selected for a press release by Society for Neuroscience during its 35th Annual Meeting in Washington, DC in 2005. He was also awarded the Anthony Ambrose Award for the Best Graduate Student in the Department of Pharmacology and Toxicology in 2004.

Mr. Deshpande has been active in various student bodies and committees. He was Representative of MCV Honor Council from 2003-2006 and served as the Secretary for the MCV Honor Society in 2004-05. He also served as the President of Pharm-Tox Student Organization (PTSO) in 2004-05. He also served as the Editor of the Medical Sciences section of the VA Academy of Sciences in 2004-05. He was also the graduate student representative to the Central VA Chapter of the Society for Neuroscience in 2005-06 and a group leader in Quester's program from 2001-2005.

A list of Mr. Deshpande's publications and abstracts is provided below:

Deshpande LS, Limbrick DD, Sombati S, DeLorenzo RJ. Activation of an injury induced calcium current initiates neuronal death in stroke. *(In preparation)*

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Deshpande L.S., Raza, M. Blair, R.E., Sombati S.S., DeLorenzo R.J. Aging Causes Elevated Intracellular Calcium Levels And Altered Calcium Homeostatic Mechanisms In Hippocampal Neurons. 82nd Virginia Academy of Sciences Meeting, May 26-28 2004, Richmond, VA.

Deshpande L.S., et al. Poster presentations at 19, 20, 21 & 22nd Daniel T. Watts Research Poster Symposium, Graduate Student Association's Annual Research Symposium, Annual Pharm-Tox Department Research retreat and Central VA Chapter of Society for Neuroscience annual research symposium. Oral presentations in Pharmtox graduate student seminar series. (2002-05)

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