EXCITOTOXIC MECHANISMS IN THE HIPPOCAMPUS AND CEREBELLUM DURING BRAIN ISCHEMIA

By

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Abstract

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Brain ischemia, or lack of blood flow to the brain, causes damage to neural tissue, and is a leading cause of death and disability. Brain ischemia triggers a sequence of events, which can be classified in two phases: the first is a small rise in extracellular K^+ with little change in the other principal ion concentrations, the cessation of EEG activity, and an increase in the frequency of miniature excitatory post-synaptic potentials (mEPSCs). The second phase is marked by a severe disregulation of all principal ionic gradients, an associated large and sustained ischemic depolarization, and a massive increase in extracellular glutamate concentration caused by the reversal of glutamate transporters. Because of the severity of the second phase, much ischemia research has centered on elucidating its mechanisms; however, the early phase likely plays a prominent role in delayed selective neuronal death in ischemia sensitive neurons such as the CA1 pyramidal neurons and the Purkinje cells in the cerebellum. Similarly, because of its extreme sensitivity, much ischemia research has focused on the hippocampus; however the mechanisms gleaned from the hippocampus may not extrapolate to other brain regions, especially those with fundamentally different cellular and molecular make-ups like the cerebellum.

The goal of this thesis is to improve our understanding of the early events of the ischemic response generally, and to increase our understanding of ischemic responses across brain regions. I found that, like CA1 neurons in the hippocampus, ischemia causes Purkinje

cells to depolarize to near 0mV. However in contrast to the hippocampus, the current that drives the Purkinje cells ischemic depolarization (ID) is generated mainly by non-desensitizing AMPA receptors. This ID current is triggered by glutamate that is released by the reversal of glutamate transporters, but unlike the hippocampus, it is also largely reduced by the removal of calcium from the extracellular solution.

The mechanisms underlying early responses to ischemia were also investigated. In hippocampal CA1 neurons, ischemia causes an increase in intracellular calcium concentration ($[Ca^{2+}]_c$) and parallel increase in mEPSC frequency prior to the severe ionic disreguation and ischemic depolarization current that characterizes the second phase of the ischemic response. Despite occurring simultaneously, the increase in $[Ca^{2+}]_c$ and in mEPSC frequency are independent of each other. The increase in mEPSC frequency is caused by the depolymerization of actin filaments and the increase in $[Ca^{2+}]_c$ is glutamate-independent. Like the hippocampus, the first phase of the ischemic response in the cerebellum is marked by an increase in $[Ca^{2+}]_c$; however there is no increase in mEPSC frequency during the first five minutes of the response in Purkinje cells. This difference in the ischemic response could be explained by intrinsic differences in the synaptic input to each of these cells. Evoked responses in CA1 neurons induced by stimulation of CA3 axons are reduced by actin filament stabilization, due to a decrease in the probability of release. In contrast, actin filament stabilization has no effect in the parallel fiber and climbing fiber input to Purkinje cells, suggesting that actin plays a limited role in synaptic activity in these terminals. Thus, the early and late ischemic responses vary in different brain regions and this thesis has covered several gaps in the existing knowledge in the ischemia field.

Chapter 1:

Introduction

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1.1 Introduction to Brain Ischemia

1.1.1 Clinical Implications of Brain Ischemia

Brain ischemia, or the lack of blood flow to the brain, is a condition that can arise in a number of situations, such as cardiac arrest, stroke, injuries involving large losses of blood, or for fetuses, in a variety of complications during the perinatal period. Brain ischemia can either be focal, when a confined region of the brain surrounding a blocked artery is affected; or global, when the total blood supply to the brain is lost. Within minutes, global cerebral ischemia is experienced in humans by a loss of consciousness and seizures (Rossen *et al*., 1943). The symptoms and outcome from focal ischemia will depend on the brain region that has been affected. These usually are numbness or weakness of the face, arm or leg, especially on one side of the body; sudden confusion; trouble speaking, understanding, seeing, or walking; dizziness; and loss of balance and coordination. If the total blood supply is interrupted for long enough (Cole and Corday, 1956), or, in focal ischemia, if the affected region is critical for survival, brain ischemia

will result in death. Brain ischemia survivors many times are faced with the consequences of long-term disabilities, memory loss, seizures, epilepsy; and an increased risk for recurrent episodes of ischemia (Leys *et al.*, 2002; Bladin *et al.*, 2000, Reviewed in Silverman *et al*., 2002). The neurological disorders resulting from ischemia are attributed to neuronal damage; however unfortunately, there are no treatments that salvage neural tissue damaged by ischemia.

Time is the most critical factor for determining the severity of the outcome of ischemia. Brain ischemia can be categorized into two groups according to its duration: transient ischemic attacks (TIAs), for symptoms that subside within 24 hours; and stroke, for when symptoms last longer than 24 hours, which may also be interpreted as permanent loss of blood flow. Even though the symptoms of TIAs subside within 24 hours, many times long-term disabilities ensue and brain damage can be detected by diffusion-weighted magnetic resonance imaging (DW-MRI; Kimura *et al*., 1999; Rovira *et al*., 2002; Inatomi *et al*., 2004; Barber *et al*., 1999; Augustin *et al*., 2000). The detrimental outcomes that can result from very short periods of brain ischemia highlight the extreme sensitivity of the brain to blood flow interruption. Strokes are invariably accompanied by severe brain damage, also called an infarct, and can be easily detected by magnetic resonance imaging (MRI). About 795,000 Americans suffer from stroke every year, and about one third of these die as a result, ranking stroke as the third most common cause of death in the

United States (Am Heart Assoc., 2009). Thus brain ischemia is a very serious condition which deserves much attention to understand how it leads to brain damage.

Despite the seriousness of this neurological disorder, currently there are no treatments to salvage neural tissue from ischemic damage. Treatments for brain ischemia are currently limited to the restoration of blood flow. When treatment occurs within a brief span after stroke onset, blood vessel-occluding clots can be solubilized with tissue plasminogen activator (NINDS rt-PA Stroke study group, 1995), urokinase (Furlan *et al*., 1999), heparin (Kay *et al*., 1995), or the more recently developed ancrod (Sherman *et al*., 2000). All attempts at mitigating damage in post-ischemic brain tissue have failed so far (Kidwell *et al*., 2001) and thousands of Americans continue to die and suffer from ischemia-related long-term disabilities every year. There are many speculations for the reasons for failure of clinical trials, but surely one contributing factor is our incomplete understanding of the mechanisms that cause damage.

1.1.2 Transient Ischemia and the Selective Vulnerability of Neurons

Time is critical when determining the outcomes resulting from brain ischemia. From both *in vivo* and *in vitro* ischemia studies, it has been demonstrated that, for very transient

ischemic episodes, not all neurons are equally sensitive to ischemia; rather certain neuronal types are more susceptible to damage than others. A transient bilateral carotid occlusion in the Mongolian gerbil for as little as 5 minutes can produce a lesion in the CA1 area of the hippocampus while sparing all other neuronal population from neuronal death (Kirino, 1982; Kirino and Sano, 1984; Crain *et al*., 1988). Similar durations of two-vessel occlusions or cardiac arrests in the rat produce the same result (Smith *et al*., 1984; Blomqvist and Wieloch, 1985), as well as in monkeys (Miller and Meyers, 1972; Bodsch *et al*., 1986), cats (Hossmann and Kleihues, 1973), and rabbits (Marshall *et al*., 1975).

Neurons that demonstrate particular vulnerability to ischemic challenges have been termed “selectively vulnerable neurons”. Of the entire forebrain, the CA1 neurons of the hippocampus are the most vulnerable (Pulsinelli *et al*., 1982). Apart from the CA1 pyramidal neurons in the forebrain, neocortical layers 3, 5, and 6 are also vulnerable (Sieber *et al*., 1995), as well as the medium spiny neurons of the striatum (as opposed to the large aspiny ones; Chesselet *et al*., 1990; Francis and Pulsinelli 1982). Beside the forebrain neurons, cerebellar Purkinje cells are the most vulnerable neuronal population to ischemic challenges, and in total are second only to CA1 hippocampal neurons (Miller and Meyers, 1972; Pulsinelli, 1985; Cervos-Navarro and Diemer, 1991). This thesis will focus on ischemic responses in both CA1 neurons and Purkinje cells.

Even though transient ischemia affects only selectively vulnerable neurons, selective neuronal loss is sufficient to produce neurological deficits and impairments in learning and memory. An increase in locomotor activity was observed in gerbils after transient forebrain ischemia, and it was correlated with damage in the CA1 region of the hippocampus (Gerdhardt and Boast, 1988; Mileson and Schwartz, 1991). Spatial learning impairments, tested by the water maze, were found in ischemic rats which had incurred significant neuronal loss in the CA1 region (Hartman *et al*., 2005). Rats subjected to 5 and 20 minutes of ischemia showed marked learning impairment in the radial maze task which correlated with neuronal degeneration of CA1 pyramidal cells, suggesting that impairment of memory was a result of hippocampal damage (Kiyota *et al*., 1991). In humans, selective vulnerability was found following cardiorespiratory arrest (Petito *et al*., 1987; Horne and Schlote, 1992). In a detailed case study, a patient with selective bilateral damage in the CA1 region following an ischemic event experienced significant memory impairments (Zola-Morgan *et al*., 1986). Thus, although transient ischemia produces damage in only selective neuronal populations, it is sufficient to cause long-term disabilities and cognitive impairments.

1.1.3 Delayed Cell Death

Because selectively vulnerable neurons take a few days to undergo degeneration, the degenerative process has been termed “delayed neuronal death” (Kirino, 1982). Studies have demonstrated that delayed neuronal death following ischemia is due mainly to apoptosis (MacManus *et al*., 1993; Nitatori *et al*., 1995). Physiological as well as biochemical analyses using animal models have demonstrated that the CA1 pyramidal neurons are normal 24 hours after brief forebrain ischemia (Suzuki *et al*., 1983a,b; Arai *et al*., 1986; Monmaur *et al*., 1986), but then begin to degenerate within 2 to 4 days after transient ischemia, leaving only 5.8% of the neurons to survive after 3 weeks (Kirino, 1982; Bonnekoh *et al*., 1990). According to the morphological criteria of apoptosis, dying cells show chromatin condensation and cell shrinkage, followed by heterophagocytosis (Kerr *et al*., 1972). After 3-4 days of brief forebrain ischemia in the gerbil, the CA1 pyramidal neurons demonstrate increased immunoreactivity for lysosomal cysteine proteinases, cell shrinkage, nick-end labeled nuclei with biotinylated dUTP (TUNEL staining), dense chromatin masses, laddering of DNA, and phagocytosis by microglial cells; all indicative that delayed death of the CA1 pyramidal neurons after brief ischemia is apoptotic (Nitatori *et al*., 1995).

Mitochondria are involved in apoptosis by releasing cytochrome *c* from their intermembrane space to the cytoplasm via the mitochondrial permeability transition pore (Liu *et al*., 1996). Cytochrome *c* initiates the cytochrome *c*-dependent caspase cascade

(Li *et al*., 1997; Kuida *et al*., 1998; Yoshida *et al*., 1998), resulting in release of active caspase-3 (CPP32) which triggers activation of additional caspases and leads to apoptosis (Liu *et al*., 1996; Slee *et al*., 1999). CPP32 is overexpressed in the CA1 region after transient global ischemia, and administration of a CPP32 inhibitor reduces apoptotic cell death (Chen *et al*., 1998). Furthermore, immunohistochemistry revealed cytosolic cytochrome *c*-positive cells exclusively in the CA1 region of the hippocampus as early as 2 hours after 10 minutes of ischemia (Suguwara *et al*., 1999). Cyclosporin A, which blocks the mitochondrial permeability transition pore, effectively protects against 5 minute ischemia-induced CA1 neuronal death, and in the same study, completely blocked cytochrome *c* translocation to the cytosol (Domanska-Janik *et al*., 2004).

1.1.4 Severe or Permanent Ischemia

The molecular and cellular changes that occur following severe or permanent ischemia are strikingly different from those that occur following transient ischemia. Longer and more severe ischemia causes the generalized death of all cell types, including glia and endothelial cells. This type of damage is known as pan necrosis. The most widely used *in vivo* model for severe or permanent ischemia is focal ischemia, as severe or permanent ischemia in a global model would inevitably lead to death. In focal ischemia models, the middle cerebral arteries are occluded by various different methods.

In focal ischemia, two tissue volumes are commonly distinguished: the core of the infarction, and the surrounding zone, known as the penumbra. These are distinguished based on blood flow interruption (Astrup *et al*., 1981). One hour of focal ischemia leads to infarction, or edema, in the core, but if the occlusion is prolonged to 2-3 hours, then infarct develops in the penumbra and is the same size as that seen in permanent ischemia (Garcia *et al*., 1995; Kaplan *et al*., 1991; Memezawa *et al*., 1992). The infarct appears at a much earlier time than delayed cell death, being detected as early as 3 hours after the ischemic insult (Osborne *et al*., 1987). Cerebral infarction is typically characterized by pallor in hematoxylin and eosin staining, in addition to histological features such as vacuolation of the neuropil, alterations in the shape of perikarya and nuclei, and neuronal and astrocytic swelling (Petito *et al*., 1982; Garcia *et al*., 1993). The final stage of the infarct develops into pan necrosis, seen at 72 to 96 hours after the arterial occlusion (Garcia *et al*., 1993; Nagasawa and Kogure, 1989), in which neuronal and glial death is accompanied by endothelial cell death and loss of cellular elements (Plum, 1983; del Zoppo, 1994). Necrotic tissue then triggers an immune response and then is scavenged by macrophages and becomes liquefied (Feuerstein *et al*., 1994; Hallenback, 1996; Rosenblum, 1997). Although distinct mechanisms of cell death can be distinguished between very short ischemic durations and longer ischemic durations, in reality a gradation of both processes is observed (Lipton, 1999).

1.2 An overview of the glutamatergic system

The most well established hypothesis for the mechanism that underlies neuronal damage mediated by ischemia is based on excitotoxicity studies. Glutamate, the main excitatory neurotransmitter in the brain (Mayer and Westbrook, 1987), is neurotoxic when administered at high concentrations (Olney, 1969; Olney and Sharpe, 1969). Thus in order to understand the mechanisms which mediate neuronal damage during ischemia, an overview of the glutamatergic system must be introduced. There are other mechanisms that contribute to neuronal death caused by ischemia; however these are not the subject of this thesis, and will not be discussed here (see Lipton, 1999 for a review).

1.2.1 Vesicular Glutamate Release

Glutamate is present throughout the central nervous system (CNS) in all cell types. It is concentrated into synaptic vesicles in synaptic terminals of glutamatergic neurons (Aas *et al*., 1990; Ottersen *et al*., 1992; Hamori *et al*., 1990; De Biasi and Rustioni, 1990; Storm-Mathisen and Ottersen, 1990) that are released upon the trigger of an action potential (Ikeda *et al*., 1989; Storm-Mathisen and Ottersen, 1990; Aas *et al*., 1990; Laake *et al*., 1993; Nicholls, 1993). Action potentials depolarize presynaptic membranes and activate voltage-gated calcium channels (VGCCs) of the N and P/Q types (Nowycky *et al*., 1985;

Fox *et al*., 1987; Tsien *et al*., 1988; Llinas *et al*., 1989). These allow calcium to enter the cell which triggers the vesicular membrane fusion with the plasma membrane, allowing exocytosis. Glutamate is released in a high concentration into the synaptic cleft and activates glutamate receptors on the postsynaptic membrane, generating an excitatory postsynaptic current (EPSC). The postsynaptic cell consequentially depolarizes, and if the membrane potential reaches threshold, an action potentials is propagated.

The synaptic vesicle population is composed of two distinct pools: the readily releasable pool and the reserve pool (Rosenmund and Stevens, 1996; Murthy and Stevens, 1999), and the two pools do not intermingle (Sara *et al*., 2005). The readily releasable pool is responsible for the action potential-triggered release described above, and undergoes a series of steps that render vesicles to be competent for fusion upon a calcium signal. Vesicles first are docked to the active zones on the membrane, the sites in which vesicle fusion occurs (Akert *et al*., 1971), via rab proteins (Geppert *et al*., 1994a). Vesicles then become primed, or fusion competent, when the SNARE protein complex is assembled (Weber *et al*., 1998; Nickel *et al*., 1999). Assembly of the SNARE complex involves the interaction between the vesicular SNARE (synaptobrevin or VAMP) and target SNAREs (SNAP25 and syntaxin1), which brings the two membranes into close contact (Sollner *et al*., 1993). Fusion is thought to be triggered by calcium binding to the vesicular protein synaptotagmin 1 or 2 (Geppert *et al*., 1991; Geppert *et al*., 1994b). After fusion, vesicles

are retrieved and recycled (Gandhi and Stevens, 2003). Vesicles from the reserve vesicle pool are recruited to the readily releasable pool only during special circumstances that cause the depletion of the readily releasble pool (Rizzoli and Betz, 2005). Reserve vesicles are tethered to a cytoskeletal network of filamentous actin (F-actin) via synapsins (Samigullin *et al*., 2004; Benfenati *et al*., 1992; Bahler and Greengard, 1987; Petrucci and Morrow, 1987; Greengard *et al*., 1994). During repetitive synaptic stimulation, synapsins are phosphorylated either via CaM kinase II or MAP kinase, which releases reserve synaptic vesicles (Chi *et al*., 2003).

Calcium-dependent exocytosis can be regulated at two points: the peak cytosolic calcium concentration ($[Ca^{2+}]_c$) entering the cell, and by variations in the release probability for a given $[Ca^{2+}]_c$ (Sudhof, 2004). The latter component, the release probability for a given $[Ca^{2+}]_c$, depends on the number of release-ready vesicles and their calcium-responsiveness (Sudhof, 2004). The release probability per $[Ca^{2+}]_c$ varies characteristically between different types of synapses (Xu-Friedman *et al*., 2001). The climbing fiber synaptic terminals in the cerebellum have high probabilities of release, and demonstrate very little, if any, augmentation of vesicle release upon consecutive stimulations (Dittman and Regehr, 1998). The parallel fiber synaptic terminals in the cerebellum and the CA3 synaptic terminals in the hippocampus have low probabilities of release, and repetitive stimulation leads to the summation of calcium transients in the

terminals, leading to an augmentation of vesicle release (Dittman and Regehr, 1998; Dittman et al., 2000). Occasionally, vesicles fuse spontaneously with the presynaptic membrane in the absence of action potentials. Because these are small individual quantal events, they have been called miniature excitatory postsynaptic currents (mEPSCs; Katz and Miledi, 1969). The mechanism underlying mEPSC events is not that well understood, but it is regulated by the concentration of calcium in presynaptic terminals (Frerking *et al*., 1997; Doze *et al*., 1995; Sara *et al*., 2005; Li *et al*., 1998; Schoppa and Westbrook, 1997), and by calcium-independent processes as well (Khvotchev *et al*., 2000; Morales *et al*., 2000). It has been demonstrated that defragmenting actin filaments with the actin filament destabilizer latrunculin A causes an increase in the frequency of mEPSCs, presumably by release of vesicles from the reserve pool (Morales *et al*., 2000).

1.2.2 Glutamate Receptors

When glutamate is released in the synaptic cleft, it activates both ionotropic and metabotropic glutamate receptors, which generate responses in the postsynaptic membrane. The ionotropic gluatamate receoptors, or glutamate receptor channels, are subdivided into three main classes named according to their corresponding selective agonists: *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA), and kainate receptors (Davies *et al*., 1982; Monaghan *et al*., 1989;

Watkins *et al*., 1990). Thus, in total, there are 4 types of glutamate receptors, the ionotropic NMDA, AMPA, and kainate receptors, and the metabotropic receptors. This section will further discuss the properties of glutamate receptors and their physiological functions in the CNS.

AMPA and Kainate receptors together are known as the non-NMDA receptors, and are usually nonselectively permeable to monovalent cations while relatively impermeable to divalent cations (Mayer and Westbrook, 1987; Jonas and Sakman, 1992). Thus, in physiological solutions, the direction of current they convey reverses at around 0mV (Mayer and Westbrook, 1987). Their calcium impermeability is determined by their subunit composition. The subunits that comprise AMPA receptors are GluRs 1 through 4 (Keinanen *et al*., 1990; Sommer and Seeburg, 1992; Partin *et al*., 1993; Lomeli *et al*., 1994; Mosbacher *et al*., 1994); whereas those that comprise kainate receptors are GluRs 5 through 7 and KA 1 and 2 (Bettler *et al*., 1992, Egebjerg *et al*., 1991; Werner *et al*., 1991; Herb *et al*., 1992). The GluR 2 subunit in AMPA receptors (Boulter *et al*., 1990; Verdoorn *et al*., 1991; Hollman *et al*., 1991; Mosbacher *et al*., 1994) and the GluR 5 and 6 subunits in kainate receptors are responsible for rendering the receptors impermeable to calcium, although Glu R5 and 6 editing is variable (Herb *et al*., 1992; Egebjerg and Heinemann, 1993; Bernard and Khrestchatisky, 1994; Sommer *et al*., 1991). Under physiological conditions, the AMPA receptors are assembled as heteromers, and usually

contain the GluR 2 subunits (Mayer and Westbrook, 1987; Jonas and Sakman, 1992; Wenthold *et al*., 1992), however in certain synapses GluR 2 subunits are not expressed resulting in calcium-permeable receptors (Gilbertson *et al*., 1991; Burnashev *et al*., 1992; Muller *et al*., 1992; Jonas and Spruston, 1994). The GluR 2 subunit undergoes postranscriptional editing, where a glutamine residue (Q) is switched to a charged arginine (R) at the channel pore region, which impedes the passage of calcium (Hume *et al*., 1991; Burnashev *et al*., 1992; Greger *et al*., 2002). The AMPA receptors in the CA1 neurons of the hippocampus (Keinemen *et al*., 1990) and in Purkinje cells (Linden *et al*., 1993; Tempia *et al*., 1996) contain the edited versions of GluR 2, and are largely impermeable to calcium.

NMDA receptors are highly permeable to calcium in addition to monovalent cations (Mayer and Westbrook, 1987; Ascher and Nowak, 1988; Schneggenburger *et al*., 1993), and their activation requirements are more complex than the non-NMDA receptors. NMDA receptors are relatively non-conductive at potentials negative to -35mV, due to a fast channel block by physiological levels of magnesium (about 1mM; Mayer *et al*., 1984; Ascher and Nowak, 1988). Furthermore, activation of this receptor requires the cofactor glycine in addition to glutamate (Johnson and Ascher, 1987). The NMDA receptor subunits are NR1, NR2 A through D, and NR 3 A and B (Kutsuwada *et al*., 1992; Meguro *et al*., 1992; Monyer *et al*., 1992; Monyer *et al*., 1994; Das *et al*., 1998;

Nishi *et al*., 2001). Native NMDA receptors are formed by 4 subunits (Laube *et al*., 1998), and require the NMDAR1 subunit and an NMDAR2 subunit (Kutsuwada *et al*., 1992; Monyer *et al*., 1992; Monyer *et al*., 1994). The CA1 neurons of the hippocampus express NMDA receptors containing the NR1, NR2 A and NR2 B subunits (Laurie and Seeburg, 1994; Monyer *et al*., 1994). Interestingly, Purkinje cells do not express NMDAR2 subunits beyond postnatal day 8, and thus do not have functional NMDA receptors (Perkel *et al*., 1990; Llano *et al*., 1991; Rosenmund *et al*., 1992).

At most synapses, excitatory postsynaptic currents (EPSCs) are characterized by the rapidly activating non-NMDA receptor-mediated component, and a slower NMDA receptor-mediated component (Lester *et al*., 1993; Jonas and Spruston, 1994). AMPA receptors are rapidly activated and quickly, and almost completely (>90%), desensitize in the continuous presense of glutamate (Trussell and Fischbach, 1989; Colquhoun *et al*., 1992; Raman and Trussell, 1992; Hestrin, 1993; Jonas and Spruston, 1994). Thus they are responsible for the fast excitatory synaptic transmission component in the CNS (Nicoll *et al*., 1990; Wisden and Seeburg, 1993). The NMDA receptors present slower kinetics (Lester and Jahr, 1992, Lester *et al*., 1993) and are responsible for the slower component of glutamatergic synaptic responses. The relative proportions of these components vary considerably across brain regions (Raman *et al*., 1994; Rossi *et al*., 1995).

The fourth glutamate receptor subclass consists of the metabotropic glutamate receptors, and unlike the ionotropic receptors, these receptors are not channels, but are coupled to G-proteins. There are eight metabotropic receptor subtypes, named mGluRs 1 through 8 (Abe *et al*., 1992; Tanabe *et al*., 1992; Nakajima *et al*., 1993; Okamoto *et al*., 1994; Saugstad *et al*., 1994; Duvoisin *et al*., 1995), and these are classified into three groups according to their amino acid sequences, their intracellular coupling mechanisms, and pharmacology (Nakanishi, 1992). mGluRs 1 and 5 comprise group I, and activate G_q, mGluRs 2 and 3 comprise group II and activate G_i, while mGluRs 4, 6, 7, and 8 comprise group III and also activate G_i (Nakanishi, 1992). The activation of these receptors lead to changes in second messenger signals such as inositol-tris-phosphate (IP_3) production, which releases calcium from intracellular stores for group I subtypes (Sugiyama *et al*., 1987), and inhibition of adenylate cyclase which decreases the levels of cAMP for groups II and III (Cartmell and Schoepp, 2000). Group I metabotropic receptors are located in the regions surrounding the synapse and increase cell excitability (Baude *et al*., 1993; Schoepp, 2001; Cartmell and Schoepp, 2000). Group III mGluRs and mGluR 2 from group II are located presynaptically and negatively regulate glutamate release (Schoepp, 2001; Cartmell and Schoepp, 2000). mGluRs 3 and 5 are located postsynaptically in neurons and on glia (Riedel *et al*., 2003; Schoepp, 2001; Schools and Kimelburg, 1999; Cai *et al*., 2000). Purkinje cells predominantly express mGluR 5, and some studies

detected moderate expression of mGluR 7 (Ohishi *et al*., 1995; Kinzie *et al*., 1995; Lein *et al*., 2007). The CA1 neurons of the hippocampus express both group I receptors (Davies *et al*., 1995) and mGluRs 4 and 7 from group III, while mGluR 7 is also expressed presynaptically in Schaffer collaterals (Ohishi *et al*., 1995).

1.2.3 Glutamate Transporters

Once glutamate is released, it must be rapidly removed from the synaptic cleft in order to avoid prolonged excitation. Although non-NMDA receptor responses are rapidly terminated by desensitization (Colquhoun *et al.* 1992; Hestrin, 1992), NMDA receptors may be activated for much more prolonged time in the continued presence of glutamate. NMDA receptors are permeable to calcium and lead to the depolarization of the postsynaptic membrane that can activate VGCCs, permeable to calcium as well. Overactivation of glutamate receptors, leading to an excessive influx of calcium, can have deleterious effects on neurons as will be further discussed under section 3 below. Glutamate transporters play a role in removing excess glutamate from the synaptic cleft to avoid over-activation of glutamate receptors, and to ensure proper CNS function.

There are five glutamate transporters in the central nervous system: Glast or EAAT1, GLT-1 or EAAT2, EAAC1 or EAAT3, EAAT4 and EAAT5 (Stork *et al*., 1992; Kanai and Hediger, 1992; Pines *et al*., 1992; Fairman *et al*., 1995; Arriza, *et al*., 1994).

Transporters are homomers composed of three subunits (Yernool *et al*., 2004; Koch and Larsson, 2005). Glast and GLT-1 are glial transporters, while EAAC1 is most abundantly expressed in glutamatergic neurons (Stork *et al*., 1992; Kanai and Hediger, 1992; Pines *et al*., 1992; Kanai *et al*., 1993), although it has also been detected in cerebellar Purkinje cells, which are GABAergic (Takahashi *et al*., 1996). EAAT 4 is exclusively expressed in Purkinje cells, and is present in higher densities in cerebellar lobule X (Nagao *et al*., 1997), while EAAT5 is expressed in retinal photoreceptors and bipolar cells (Arriza *et al*., 1997)

All glutamate transporters remove glutamate from the extracellular space by coupling the energy to pump glutamate against its concentration gradient to the transport of Na^+ and K^+ down their concentration gradients (Stork *et al*., 1992; Kanai and Hediger, 1992; Pines *et al*., 1992; Kanai *et al*., 1993; Attwell and Mobbs, 1994). The stoichiometry of the uptake cycle is 3 Na^+, 1 H^+ and the counter-transport of 1 K^+ with each glutamate (Zerangue and Kavanaugh, 1996; Levy *et al*., 1998). Thus, glutamate transport is electrogenic, generating the accumulation of 2 positive charges in the cell per cycle (Zerangue and Kavanaugh, 1996). Consequently, the minimum concentration of extracellular glutamate that the glutamate transporter can maintain is dependent on the membrane potential and on the gradients of the transported ions (Attwell *et al*., 1993; Takahashi *et al*., 1996). The concentration of extracellular glutamate in slices has been

estimated to be about 25nM (Herman and Jahr, 2007), about 100-fold lower than the previously reported by microdyalisis studies *in vivo* (Lerma *et al*., 1986; Baker *et al*., 2002; Nyitrai *et al*., 2006).

Uptake by the glutamate transporter also activates a chloride conductance (Wadiche *et al*., 1995a,b). However this is not thermodynamically coupled to the uptake of glutamate as Na^+, K^+ and H^+ are, and the direction of glutamate uptake has no effect on the anion conductance (Billups *et al*., 1996). Since the anion conductance is not necessary for the uptake of glutamate, its role is not well understood. It is speculated that, in the case of the neuronal tranporters EAAT3 and especially EAAT5, it may play a role in hyperpolarizing the presynaptic membrane, terminating the synaptic response (Tzingounis and Wadiche, 2007).

It has been speculated that the function of the glial transporters is to keep the ambient levels of extracellular glutamate low, while neuronal tranporters play a role in regulating synaptic activity (Rothstein *et al*., 1996). The role of glutamate transporters in shaping synaptic responses varies for different synapses. In hippocampal CA3-CA1 synapses, glutamate transport inhibition with the non-selective competitive inhibitor DL-thre-β-benzyloxyaspartate (TBOA) leads to a slight increase in the NMDA receptor meadiated EPSC peak amplitude and a prolongation in its decay kinetics (Diamond, 2001; Arnth

Jensen *et al*., 2002). Glutamate transporters do not play a role in terminating non-NMDA receptor responses in CA3-CA1 synapses (Sarantis *et al.* 1993). In the climbing fiber and parallel fiber synapses on Purkinje cells, glutamate transporters play a role in terminating both NMDA and non-NMDA receptor-mediated responses (Barbour *et al.* 1994; Takahashi *et al.* 1995; Wadiche and Jahr, 2001). The transporter EAAT4 can prevent activation of postsynaptic metabotropic glutamate receptors in Purkinje cells (Brasnjo and Otic, 2001) especially in lobule X, where EAAT4 is present in higher densities (Wadiche and Jahr, 2005). Because the complete transport cycle is relatively slow, it is speculated that the mechanism by which glutamate transporters terminate synaptic responses is by binding, or buffering, of glutamate, rather than uptake (Rusakov and Kullmann, 1998; Tzingounis and Wadiche, 2007).

1.3 Ischemia and Excitotoxicity

Glutamate, the main excitatory neurotransmitter in the CNS, is neurotoxic at high, sustained concentrations, and is the most recognized perpetrator of neuronal damage induced by ischemia. This section will focus on how this came into understanding, the mechanism by which glutamate induces neuronal damage, and how glutamate is released during ischemia. Although much of glutamate-mediated neuronal damage by ischemia has been well documented, there are still many unresolved aspects pertaining to the mechanisms by which glutamate is released. Furthermore, the hippocampus has been the main experimental model for ischemia studies, and thus many aspects pertaining to the ischemic response in other brain regions remain unknown. These unresolved issues are the subject of this thesis.

1.3.1 Cerebral Metabolism

All cerebral activity, including glutamatergic signaling, is critically dependent on the supply of blood borne metabolic substrates, namely oxygen and glucose – which together are the substrates for the production of adenosine triphosphate (ATP). When the supply of oxygen and glucose is compromised, as in an ischemic event, the fine equilibrium between ATP production and consumption is disrupted – the concentrations of ATP and

other substrates for ATP production, such as phosphocreatine and glycogen, rapidly decline as cellular activity requiring this energetic compound continue to consume it (Mc Ilwain, 1955; Lowry *et al*., 1964). Once ATP becomes scarce, all ATP-dependent activity is interrupted. The Na^+/K^+ ATPase has traditionally been viewed as the main energy drain in neurons, and its failure produces a catastrophic cascade of events that lead to neuronal damage as will be further discussed below. However, the changes responsible for neuronal death that lead to the long-term disabilities associated with ischemia are not yet fully understood, and the incomplete understanding of these changes is reflected by the lack of existing treatments for ischemia-induced neurological disorders. Thus, to benefit the thousands of Americans who suffer from long-term disabilities associated with ischemia every year, it is of great interest to further investigate the cellular changes that occur during metabolic inhibition that lead to neuronal damage.

1.3.2 Glutamate is the Trigger for Neuronal Death in Ischemia

The term excitotoxicity was coined to describe the deleterious effects of excess glutamate exposure to neurons (Olney, 1978). Glutamate is a remarkably potent and rapidly acting neurotoxin: exposures to only 100μM for 5 minutes are sufficient to kill large numbers of cultured cortical neurons (Choi *et al*., 1987). Thus, it is possible that the transient release of only a small fraction of glutamate into the extracellular space can damage neurons.

Excitotoxicity studies are far from emulating metabolic inhibition; however there is extensive evidence that suggest that neuronal death caused by ischemia is due to a similar mechanism. The evidence for glutamate being excitotoxic and an important factor in ischemic brain damage comes primarily from 3 consistent findings – in a wide range of experimental models, including cultured cells, brain slices, and animals:

1) Ischemia causes an increase in the ambient levels of glutamate,

2) Eliminating synaptic input, which presumably eliminates the source of glutamate, attenuates the damage caused by ischemia, and

3) Glutamate receptor antagonists significantly attenuate the damage caused by ischemia.

Early anoxia studies provided the first direct indication that excitatory synaptic transmission might influence the sensitivity of neurons to such insults. One of the first *in vitro* anoxia studies demonstrated that the death of two-week cultured hippocampal neurons could be prevented by blockade of synaptic activity, and that newly cultured neurons that had not yet established their synaptic connections were spared from damage (Rothman, 1983). In a following *in vivo* hippocampal study, it was demonstrated that elimination of pre-synaptic input spared neurons from undergoing degeneration. When synaptic input to the CA1 neurons was severed, CA1 neurons were preserved from ischemic damage (Onodera *et al.*, 1986). In a similar model, microdialysis studies

demonstrated that ischemia caused a six-fold increase in exogenous glutamate in the CA1 neurons in the intact hippocampus, and when the CA3 input to the CA1 neurons was severed, the increase in glutamate was only 1.4-fold (Benveniste *et al*., 1989; Butcher *et al*., 1990). The pre-synaptic severing experiments were careful enough to demonstrate that there had not been any changes in the density of glutamate receptors (Onodera *et al*., 1986; Benveniste *et al*., 1989).

A more direct role for glutamate receptor activation in ischemia-induced neuronal death was demonstrated with glutamate receptor antagonists. A variety of NMDA receptor antagonists protect cortical (Goldberg, 1987, Weiss *et al*., 1986) and hippocampal cultures (Rothman *et al*., 1987) from anoxic insults, as well as brain tissue from *in vivo* ischemic insults (Choi, 1988; Kochhar *et al*., 1988; Marcoux *et al*., 1988; Church *et al*, 1988; Boast *et al*., 1988; Steinberg *et al*., 1988; Park *et al*., 1988). Similarly, AMPA receptor antagonists also display neuroprotection against ischemic injury (Buchan *et al*., 1991; Le Peillet *et al*., 1992; Sheardown *et al*., 1990, Gill *et al*., 1992, Smith and Meldrum, 1992). With these and similar experiments, glutamate has been the most widely accepted trigger of ischemic brain injury.

1.3.3 Glutamate as a Neurotoxin

Once it was established that glutamate was excitotoxic, the question then became – by which mechanism? From ion substitution experiments, the mechanism underlying glutamate excitotoxicity was dissected into two components. Glutamate exposure to *in vitro* neuronal preparations produced immediate neuronal swelling that could be prevented by the removal of extracellular sodium ($[Na^+]_e$) or chloride ($[Cl^-]_e$) (Rothman, 1985; Olney *et al*., 1986; Choi, 1985; Rothman and Olney, 1987). In the absence of sodium, neurons still degenerated in a delayed fashion, and this glutamate-induced degeneration was substantially attenuated by the concomitant removal of extracellular calcium ($[Ca^{2+}]_e$) (Choi, 1985; Rothman *et al*., 1987; Choi *et al*., 1987). Glutamate applications also induced the accumulation of $_{45}Ca^{2+}$ by cortical neurons *in vitro* (Berdichevsky *et al*., 1985). These observations suggested that excitotoxicity is made up of two components:

1) An acute, Na^+ and Cl^- dependent component which is marked by immediate cell swelling and

2) A component that is Ca^{2+}-dependent and delayed

The first component is consistent with glutamate activation of AMPA receptors, causing a steady depolarization by means of an influx of Na^+, which increases cell osmolarity and leads to influx of water. Eventually, tension on the cell membrane increases and the neuron lyses (Rothman and Olney, 1987). The second component may be explained by

AMPA receptor activation leading to cell membrane depolarization, which activates NMDA receptors and VGCCs, both which lead to an overload of cytosolic calcium that triggers delayed cell death (Choi, 1988). Calcium is the main signaling molecule that triggers a variety of cell death pathways in cells, including apoptosis in neurons; and thus, it was proposed that an increase in $[Ca^{2+}]_i$ during metabolic deprivation is one of the causes of irreversible neuronal damage (Siesjo, 1981).

Anoxia triggers the rapid translocation of $[Ca^{2+}]_e$ to intracellular spaces of brain tissue (Nicholson *et al*., 1977), and glutamate-induced $_{45}Ca^{2+}$ accumulation by cortical neurons is highly correlated with the resultant neuronal degeneration (Marcoux *et al*., 1988; Deshpande *et al*., 1987). Calcium chelators were demonstrated to be neuroprotective in *in vivo* models of focal ischemia (Tymianski *et al*., 1993; Tymianski *et al*., 1994) as well as over expression of calcium binding proteins (Yenari *et al*., 2001; Fan *et al*., 2007). Consistent with the hypothesis, VGCC blockers are also neuroprotective during ischemia (Leach *et al*., 1993; Zhao *et al*., 1994; Buchan *et al*., 1994; Smith and Meldrum, 1995).

1.3.4 Mechanisms of Glutamate Release During Ischemia

For being the most recongnized perpetrator of ischemic injury, much effort has been geared towards understanding the mechanisms by which glutamate is released during

ischemia; however, many aspects still remain unresolved. Part of the current understanding of the mechanisms of glutamate release during ischemia was founded on observations from *in vivo* changes in extracellular ion concentrations. Ischemic challenges produce a biphasic change in ion gradients; during the first 1-2 min of ischemia onset, there is an increase of $[K^+]_e$ from 3 to approximately 10 mM, while the other ion concentrations remain essentially unchanged (Hansen and Zeuthen, 1981). This initial increase in $[K^+]_e$ (Hansen *et al*., 1982) is due to the activation of K_{ATP} channels (Zhang and Krnjevic, 1993; Jiang *et al*., 1994; Fujimura *et al*., 1997). At this same time, there is a cessation of EEG activity (Suzuki *et al*., 1983b). During the second phase, $[K^+]_e$ exhibits a rapid increase to 55 mM, whereas $[Na^+]_e$ rapidly decreases from about 155 to 60 mM, $[Cl^-]_e$ from 130 to 75 mM, and $[Ca^{2+}]_e$ from 1.3 to 0.08 mM. These changes are accompanied by a rapid negative shift of 20mV in the local extracellular electrical potential (Hansen and Zeuthen, 1981). These findings have been reported to occur in the core in focal ischemia models (Gido *et al*., 1997; Nedergaard and Hansen, 1993; Harris *et al*., 1981; Harris and Symon, 1984). The rapid potential change during the second phase of ischemia is known as the "anoxic depolarization" (Hansen, 1985), and by a real-time microdialysis study, demonstrated to be correlated with the release of glutamate (Satoh *et al*., 1999).

The previously described ion gradient dissipation produced by ATP depletion is caused by the inhibition of the Na^+/K^+ ATPase (Dagani and Erecinska, 1987; Kauppinen *et al*., 1988). Glutamate transporters rely on the co- and counter-transport of Na^+ and K^+ respectively for extracellular glutamate uptake (Bouvier *et al*., 1992). A rise in $[K^+]_e$ and decrease in $[Na^+]_e$, such as that which occurs during ischemia, hinders the transport of glutamate, and the glutamate transporter operates backwards, driven by the large concentration gradient of cytosolic glutamate (Nicholls and Attwell, 1990). Indeed, glutamate is released when the Na^+/K^+ ATPase is blocked with ouabain (Madl and Burgesser, 1993), and the reversal of glutamate transporters was demonstrated to be possible in glial cells under conditions similar to ischemia (Szatkowski *et al*., 1990). Confirmation of the hypothetical mechanism of glutamate release by reversed uptake was achieved with *in vitro* and *in vivo* experiments. Ischemic release of endogenous glutamate from the hippocampal slices was blocked by the competitively transported substrates D,L-threo-β-hydroxyaspartate (THA) and L-*trans*-pyrrolidine-2,4-dicarboxylic acid (PDC) (Roettger and Lipton, 1996). The non-transported blocker of the EAAT 1–3 transporters, DL-*threo*-β-benzyloxyaspartate (DL-TBOA) prevented a substantial portion (42%) of extracellular glutamate increase *in vivo* (Phillis *et al*., 2000).

Voltage-clamp recordings of individual neurons from *in vitro* slice models of ischemia reveal, after a delay of several minutes, a large, sustained glutamate receptor–mediated

current. In current clamp recordings, at the same time frame there is a membrane depolarization to near the reversal potential for nonspecific cation channels (Rossi *et al*., 2000). Because the large glutamate-mediated inward current is analogous to the anoxic depolarization seen in *in vivo* models of ischemia, it is termed the "anoxic depolarization current" (Rossi *et al*., 2000). This glutamatergic response was demonstrated to be caused by the reversal of glutamate transporters by pre-incubating slices with PDC (Rossi *et al*., 2000), and simulations of the following positive feedback loop predicted changes in current very similar to those obtained in the study:

1) Ischemia depletes the ATP supply to the Na^+/K^+ ATPase, and K^+ accumulates in the extracellular space.
2) Higher $[K^+]_e$ causes cells to depolarize.
3) $[Na^+]$ gradient driving glutamate uptake is reduced.
4) Glutamate is released by reversed uptake.
5) When the membrane potential reaches -60mV, NMDA receptors activated by glutamate start to generate current, depolarizing the cells further.
6) More K^+ and and glutamate is relased

Thus, *in vitro* ischemia simulations in slices recapitulate ischemic responses observed in *in vivo* models. Although it is has been well demonstrated that a large portion of glutamate release is caused by the reversal of glutamate transporters, the tranporter

responsible for reversing has still not been resolved. Dihydrokainate (DHK), a non-transported blocker of reversed uptake by GLT-1, the main glial transporter in the hippocampus, had no effect on the anoxic depolarization current (Rossi *et al*., 2000). Consistent with this, the time course and amplitude of the anoxic depolarization current in hippocampal slices from GLT-1 knock-mice was not significantly different from that in wild-type siblings (Hamann *et al*., 2002b). Thus it is thought that in the hippocampus, glutamate is released by reversal of neuronal tranporters on glutamatergic presynaptic terminals, which are expressed in higher densities than glial cell transporters, and which are driven by higher internal glutamate concentrations. While the ischemic response has been well documented in the hippocampus, it is unknown in other brain regions.

Glutamate transporter reversal is not the only mechanism by which glutamate is released during ischemia. Voltage-clamp recordings revealed that, prior to the anoxic depolarizaton current, there is an increase in the frequency of mEPSCs; previously undetectable by other research techniques. The increase in mEPSC frequency is the earliest detectable glutamate-mediated change to occur during ischemia (Hershkowitz *et al*., 1993; Fleidervish *et al*., 2001). This mechanism has been observed to occur in the hippocampus (Hershkowitz *et al*., 1993; Katchman and Hershkowitz, 1993), cortex (Fleidervish *et al*., 2001), spiny neurons of the striatum (Zhang *et al*., 2006). An increase in miniature end-plate potentials were also observed in the neuro-muscular junction

during anoxia (Nishimura, 1986). This increase in frequency is caused by presynaptic mechanims, as the amplitude of mEPSCs and responses to pressure ejected glutamate remain unchanged throughout the duration of the transient ischemic episode (Hershkowitz *et al*., 1993; Katchman and Hershkowitz, 1993). The increase in mEPSCs was determined to be independent of action-potentials and $[Ca^{2+}]_e$ influx (Katchman and Hershkowitz, 1993; Fleidervish *et al*., 2001) but caused by calcium release from dantrolene-sensitive intracellular stores (Katchman and Hershkowitz, 1993). Indeed $[Ca^{2+}]_i$ rises during ischemia, in part by an arrest in calcium extrusion mechanisms (Kass and Lipton, 1986), and part by release in from intracellular stores (Dubinsky and Rothman, 1991; Mitani *et al*., 1990; Mitani *et al*., 1993). However, these calcium imaging studies were not correlated with electrophysiological recordings, and it is not clear if $[Ca^{2+}]_i$ rises at the same time the increase in mEPSC frequency occurs. Furthermore, treating slices with the ryanodine receptor antagonist dantrolene causes an increase in the baseline mEPSC frequency, complicating the interpretation of its effect during subsequent ischemia. Thus, it still remains unclear if the increase in mEPSC frequency is caused by calcium release from intracellular stores. One of the main goals of this thesis is to address the mechanism underlying the increase in mEPSC frequency during ischemia.

1.4 The Hippocampus and Cerebellum Compared

The hippocampus is the most widely used model for studying brain ischemia, and as such has generated general pathophysiological concepts thought to represent the entire CNS. However, some reservations must be applied when results in the hippocampus are generalized to ischemia *per se*, as mechanisms discussed in the hippocampus may not be applicable to other brain areas. The cerebellum is a frequenct site of focal ischemia, accounting for 2-3% of strokes annually (Kelly *et al*., 2001). The Purkinje cells of the cerebellum, have a very unusual configuration of many of the key molecules implicated in ischemia-induced excitotoxicity in the hippocampus, yet, they are also extremely sensitive to ischemia (Brasko *et al*., 1995; Welsh *et al*., 2002), ranking second behind CA1 hypocampal neurons (Pulsinelli, 1985). Despite their extreme sensitivity to ischemia, very little is known about how Purkinje cells responsd to to ischemia. Since this thesis is concerned with differences between ischemic responses in the hippocampus and cerebellum, this section is dedicated to compare these two brain structures anatomically and molecularly.

1.4.1 Microcircuitry of the Hippocampus

The hippocampus plays a role in learning and memory (Squire, 1992; Squire *et al*., 2004; O'Keefe and Dostrovsky, 1971; Burgess *et al*., 2002). The microcircuitry of the hippocampus is relatively simple and very well understood compared to other regions of the CNS, and for such reason it has been the major model system for synaptic physiology studies. The hippocampus is characterized by a unidirectional, excitatory trisynaptic circuit. It has 3 major subdivisions: the dentate gyrus, the CA3 subregion, and the CA1 subregion; and each of the synapses of the circuit are:

1) Entorhinal cortex neuron – Dentate granule cell (synapse 1)
2) Dentate granule cells – CA3 pyramidal cell (synapse 2)
3) CA3 pyramidal cell – CA1 pyramidal cell (synapse 3)

The dentate gyrus receives cortical input from the superficial layers of the entorhinal cortex (Steward and Scoville, 1976), also known as the perforant pathway; and a minor portion from the presubiculum and parasubiculum (Kohler, 1985). The principal cells of the dentate gyrus, the granule cells, give rise to axons called mossy fibers that connect with pyramidal cells of the CA3 subregion (Blackstad *et al*., 1970; Gaarskjaer, 1978; Swanson *et al*., 1978; Claiborne *et al*., 1986) through *en passant* synapses (Amaral and Dent, 1981). The pyramidal cells of the CA3 region, in turn, are the source of the major input to the CA1 hippocampal field, via the Schaffer collateral axons (Ishizuka *et al*., 1990). It is estimated that a CA3 axon contacts a single CA1 neuron in no more than 10

synapses (Sorra and Harris, 1993; Trommald *et al*., 1996), through *en passant* boutons (Ishizuka *et al*., 1990; Sorra and Harris, 1993). And finally the CA1 neurons project to the subiculum (Amaral *et al*., 1991) and also back to the deep layers of the entorhinal cortex (Witter *et al*., 1988; Naber *et al*., 2001; Kloosterman *et al*., 2003).

1.4.2 Microcircuitry of the Cerebellum

The cerebellar cortex plays an important role in the signal processing that underlies motor control and learning (Albus, 1971; Yeo, 1987; Thach *et al*., 1992; Steinmetz *et al*., 1992; Perret *et al*., 1993; Khater *et al*., 1993). The cerebellum has a highly ordered morphology with two afferent systems: the climbing fibers and the mossy fibers; and only a single output neuron, the Purkinje cell. The climbing fibers form a powerful, direct, all-or-none excitatory synapse with Purkinje cells by multiple innervations over the whole extent of the dendritic tree (Eccles *et al*., 1966a). Mossy fiber inputs are recoded by an enormous number of excitatory interneurons, the granule cells, which excite the Purkinje cells via the parallel fibers. Three types of inhibitory interneuron also receive mossy fiber inputs and contribute to the local circuit (Eccles *et al*., 1966b,c). It is estimated that each Purkinje cell receives a single synaptic input from up to 200,000 parallel fibers (Llinas and Sugimori, 1992; Barbour, 1993), and thus stimulation of parallel fibers produce a

graded response. Since Purkinje cells are the sole output neuron of the cerebellar cortex, they are the final integrator of all signal processing done by the cerebellar cortex.

1.4.3 The Molecular Make-up in the Cerebellum

Purkinje cells expresse a relatively high density of glutamate transporters (Lehre and Danbolt, 1998), even though they are GABAergic. Furthermore, cerebellar Purkinje cells express EAAT4, an unusual glutamate transporter subtype in the CNS that activates a high chloride conductance during transport cycle (Fairman *et al*., 1995). For being GABAergic and thus having a lower $[Glu]_i$ (Storm-Mathisen *et al*., 1992), the glutamate transporters in Purkinje cells may play a protective role during ischemia by removing extracellular glutamate. EAAT4 may also be protective as its uptake of glutamate leads to a hyperpolarization of the plasma membrane which could further increase the driving force for glutamate uptake during ischemia. Interestingly, in the cerebellum, ischemia-induced Purkinje cell death occurs in parasagittal bands where Purkinje cell survival coincides with the expressiom of EAAT4 (Welsh *et al*., 2002) suggesting that EAAT4 plays a protective role during ischemia. However, it is possible that survival is attributed to other parasagittal band restricted molecules, such as the glycolytic enzyme Aldolase-C, which could also lead to different susceptibilities to ischemia (Welsh *et al*., 2002; Sarna and Hawkes, 2003).

Other difference in Purkinje cells responses during ischemia could arise from their absence of functional NMDA receptors (Llano *et al*., 1991; Rosenmund *et al*., 1992; Hausser and Roth, 1997). NMDA receptor activation is thought to be the major mediator of ischemia-induced excitotoxic cell death by allowing excess calcium influx into cells (Gill *et al*., 1987; Goldberg and Choi, 1993). Because AMPA receptors are rapidly desensitizing, NMDA receptors mediate most of the glutamate-induced current during simulated ischemia in hippocampal CA1 pyramidal cells (Rossi *et al*., 2000). Thus, it is unclear how ischemia results in Purkinje cell damage. Despite these differences, however, Purkinje cell damage induced by ischemia is also via an excitotoxic mechanism. AMPA receptor blockade during ischemia reduces Purkinje cell damage (Balchen and Diemer, 1992), as well as surgical removal of climbing fiber inputs to Purkinje cells (Welsh *et al*., 2002). Prolonged exposures to AMPA leads to calcium-dependent damage of Purkinje cells (Garthwaite and Garthwaite, 1991; Strahlendorf *et al*., 1998). Finally, $[Ca^{2+}]_c$ increases in Purkinje cells during ischemia, both via calcium influx and release from intracellular stores (Mitani *et al*., 1995). It is possible that AMPA receptor activation and/or metabotropic glutamate receptor activation leads to damage in Purkinje cells during ischemia, as activation of these receptors lead to increases in $[Ca^{2+}]_c$ (Linden *et al*., 1994; Gruol *et al*., 1996; Tempia *et al*., 2001). These differences in the key players of the second phase of the ischemic reponse compared to other ischemia-sensitive

neurons, may signifiy very different ischemic responses in these cells. This thesis will address the electrical response of Purkinje cells to simulated ischemia in the following chapter.

1.5 Objectives

Ultimately, the development of treatments for various forms of brain ischemia will require a detailed understanding of how each particular brain region responds to ischemia, but the lack of such information regarding Purkinje cells seems particularly deleterious given their extreme sensitivity and atypical properties. Furthermore, not much research has been devoted to the first phase of the ischemic response generally, which could determine the detrimental outcome for transient ischemic attacks and the penumbra region in focal ischemia. Thus, the goal of this thesis is to characterize early and late responses to ischemia in the two brain regions discussed above: the hippocampus and cerebellum. Chapter two will discuss the second phase of the ischemia response in Purkinje cells and compare it to what is understood in hippocampal CA1 neurons. Chapter three will discuss the early responses to ischemia in the hippocampus and chapter four will compare the early responses in the hippocampus with the cerebellum.

Chapter 2:

The electrical response of cerebellar Purkinje neurons to simulated ischemia

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2.1 Introduction

The cerebellum and hippocampus are particularly vulnerable to brain anoxia or ischemia, showing loss of Purkinje cells and pyramidal cells, respectively (Pulsinelli, 1985; Cervos-Navarro and Diemer, 1991). There have been numerous studies of the events evoked by hippocampal ischemia, in which the cessation of ATP production inhibits the Na/K pump, generating a rise of extracellular potassium concentration (Hansen, 1985). This leads to a release of glutamate by reversed uptake which activates N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA) receptors (Rossi *et al.*, 2000), triggering an excessive cation influx and $[Ca^{2+}]_i$ rise which lead to pyramidal cells dying (Choi, 1987; Deshpande *et al.*, 1987; Andine *et al.*, 1988; Abdel-Hamid and Tymianski, 1997; Zhang and Lipton, 1999).

By contrast, little is known about the electrical response of cerebellar neurons to ischemia. Purkinje cells die necrotically like hippocampal pyramidal cells, despite lacking (after 8 days post-natally) the NMDA receptors which usually mediate a large glutamate-evoked Ca^{2+} influx into neurons (Yue *et al.*, 1997; Martin *et al.*, 2000). Glutamate, acting at Purkinje cell AMPA receptors, must be involved because AMPA receptor blockers prevent ischemia-evoked Purkinje cell death in vivo (Balchen and Diemer, 1992). Since Purkinje cell AMPA receptors have a low Ca^{2+} permeability (Tempia *et al.*, 1996), the $[Ca^{2+}]_i$ rise occurring in ischemic Purkinje cells (Mitani *et al.*, 1995) may be generated by voltage-gated Ca^{2+} channels in response to the depolarization produced by incompletely desensitizing AMPA receptors (Brorson *et al.*, 1995).

In this study we provide the first detailed description of the electrophysiological response of cerebellar Purkinje neurons to ischemia. We show that glutamate release is a key determinant of the cells' response to ischemia and, by using the cells' glutamate receptors to sense the rise in extracellular glutamate concentration, we assess the source of the glutamate release which triggers Purkinje cell death.

2.2 Methods

2.2.1 Brain slices and extracellular solution

Sprague–Dawley rats 12–19 days old, were killed by cervical dislocation in accordance with UK animal experimentation regulations. Patch-clamp recordings from visually

identified Purkinje cells in thin (160–220 mm) parasagittal cerebellar slices were performed as previously described (Hamann *et al*., 2002a). Slices were from animals of either sex. Recordings were made at 32 ± 2^{o}C. Normal extracellular solution contained (mM): NaCl 126, $NaHCO_3$ 24, NaH_2PO_4 1, KCl 2.5, $CaCl_2$ 2.5, $MgCl_2$ 2, D-glucose 10 (gassed with 95% O_2/5% CO_2), pH 7.4. For experiments with no extracellular Ca^{2+}, $CaCl_2$ was replaced by 2.5 mM $MgCl_2$ and 2 mM EGTA to chelate trace calcium. Kynurenic acid (1 mM) was included in the dissection and incubation solution (to block glutamate receptors, to reduce potential excitotoxic damage) but was omitted from the superfusion solution. To simulate the energy deprivation that occurs in ischemia, glucose was replaced by 7mM sucrose, 95% O_2/5% CO_2 was replaced with 95% N_2/5% CO_2 and 2 mM sodium iodoacetate and 1mM sodium cyanide were added to block glycolysis and oxidative phosphorylation (Reiner *et al*., 1990).

2.2.2 Preloading slices with PDC

To block reversed transport of glutamate, slices were preloaded with the slowly transported glutamate analogue L-*trans*-pyrrolidine-2,4-dicarboxylic acid (PDC) (Longuemare and Swanson, 1995; Roettger and Lipton, 1996; Rossi *et al*., 2000). Slices were soaked in PDC (1 mM) containing solution for 1 h, in the presence of 1 mM kynurenate to block glutamate receptors and prevent neuronal death. Control slices were soaked in 1 mM kynurenate alone. Roettger and Lipton (1996) have shown that this procedure accumulates PDC within the cells of hippocampal slices without leading to a loss of glutamate. Rossi *et al*., (2000) showed that it reduced the activity of glutamate

transporters in hippocampal slices without affecting glutamate receptors (as we also show below for cerebellar slices).

2.2.3 Patch-Clamp Recording From Cerebellar Slices

For recording, slices were placed under flowing solution on the stage of an upright microscope and viewed with a x40 or x60 water immersion objective with differential interference contrast and infrared optics. Whole-cell voltage-clamp recordings were made using an Axopatch 200B amplifier (Axon Instruments, USA) from the somata of visually identified Purkinje neurons. Patch pipettes were constructed from thick-walled borosilicate glass capillaries. For current-clamp recordings of membrane potential and for voltage-clamp experiments with E_{Cl} set to -65 mV, the solution contained (mM) K-gluconate 120, KCl 7.7, NaCl 4, HEPES 10, BAPTA 10, MgATP 4 and Na_2GTP 0.5, pH adjusted to 7.2 with KOH. For voltage-clamp recordings of membrane current with E_{Cl} set to 0 mV they were filled with (mM) CsCl 130, NaCl 4, HEPES 10, BAPTA 10, MgATP 4, Na_2GTP 0.5 and QX-314 10 (to block voltage-gated sodium currents and $GABA_B$ receptors), pH set to 7.2 with CsOH. Corrections for electrode junction potentials were made.

2.2.4 Series Resistance Voltage Errors

In whole-cell mode, after series resistance compensation by ~75%, the residual series resistance was ~1MΩ. The large size of the current changes evoked by ischemia (up to 10

nA) at the peak of the anoxic depolarization (AD) means that, even after compensation, significant series resistance voltage errors (up to 10 mV) will inevitably occur (a detailed analysis of the effect of these errors for hippocampal pyramidal cells is presented in Hamann *et al*., 2002a). Data are presented in this paper without correction for this, because the series resistance was similar in different experimental conditions, and correcting for series resistance voltage errors would not alter the conclusions reached.

2.2.5 Statistics

The effects of drugs on the ischemic responses were assessed by comparing data from interleaved slices studied in the absence or presence of each drug (or transporter). Data are presented as mean ± standard error of the mean and significance of changes was assessed with a two-tailed Student's t-test or X^2-test as appropriate.

2.3 Results

2.3.1 The Voltage Response of Purkinje cells to Ischemia

Purkinje cells were clamped using the whole-cell configuration with electrodes containing a mock-physiological solution (K^+ as the main cation and E_{Cl} set to -65 mV), and had an input resistance at -70 mV of 135 ± 33 MΩ (7 cells). When not voltage-clamped, they tended to fire action potentials spontaneously; the voltage response of cells to solution mimicking ischemia was studied after spontaneous spiking in control solution

had been terminated by the injection of hyperpolarizing current to bring the resting potential to -75 to -80 mV (mean value -78.3 ± 0.7 mV in four cells).

At the onset of simulated ischemia, Purkinje cells initially hyperpolarized to -87 mV (mean hyperpolarization after 1–2.5 min in ischemic solution was 8.5 ± 1.3 mV in four cells), but after ~8 min (504 ± 8 s) this was followed, after a short burst of action potentials, by a rapid prolonged depolarization to -14.3 ± 1.6 mV (Fig. 1A, mean data are shown in Fig. 1C). The initial hyperpolarization and subsequent prolonged depolarization are analogous to the voltage changes seen in ischemic hippocampus, where the hyperpolarization may reflect an activation of Ca^{2+}- and ATP-gated K^{+} channels when [ATP] falls following metabolic inhibition (Yamamoto *et al*., 1997; Nowicky and Duchen, 1998) and the sudden and prolonged depolarization is termed the AD (Hansen, 1985).

To test the involvement of glutamate release in this series of events, we repeated this experiment in the presence throughout of the glutamate receptor blockers NBQX (25 µM) and D-AP5 (50 µM). The NMDA receptor blocker D-AP5 was included to guard against the possibility that the Purkinje cell still expressed some NMDA receptors (although these are normally absent after ~8 days post-natally: Llano *et al*., 1991; Rosenmund *et al*., 1992; Hausser and Roth, 1997). In the presence of these blockers the Purkinje cell showed only the hyperpolarization to ~-87 mV produced by ischemia (12.3 ± 1.5mV, from a resting potential of -74.7 ± 0.9mV in three cells: Fig. 1B and C) with a subsequent very slow depolarization but no AD. The absence of the AD when ionotropic

glutamate receptor blockers are present, in recordings lasting 20 min in ischemia, demonstrates that the AD is produced by glutamate release, and that current generated by metabotropic glutamate receptors (Crepel *et al.*, 1991; Vranesic *et al.*, 1991; Glaum *et al.*, 1992; Linden *et al.*, 1994) is not involved in generating the AD. The possible mechanism of the ischemia-evoked hyperpolarization remaining when glutamate receptors are blocked is considered in the Discussion.

2.3.2 Reversibility of the Anoxic Depolarization

To determine for how long glutamate needs to depolarize Purkinje cells to produce irreversible damage, we carried out experiments in which the superfusion solution was switched back from ischaemia solution to normal solution either ~1 min (67 ± 6 s in 14 cells) or ~2.5 min (147 ± 10 s in 16 cells) after the AD (the timing of which was defined as the time of maximum rate of change of the potential), and observed whether the membrane potential was able to recover from the AD. Some cells recovered towards the normal resting potential, although they tended to show some instability of the resting potential even after this recovery (Fig. 1D), while others failed to show a significant recovery (Fig. 1E).

When cells fail to recover it is sometimes difficult to be certain that the lack of recovery does not result from movement of the cell relative to the electrode when the slice swells after the AD, so we rejected all cells in which the apparent potential fell to 0 mV and the capacity transient produced by a voltage step indicated complete loss of the cell. Having

rejected these lost cells, to quantify the degree of irreversible depolarization we defined 'recovered' cells as those which hyperpolarized to at least -50 mV after ischemia, while 'no-recovery' cells were those that failed to hyperpolarize beyond -30 mV(there were no cells which adopted potentials between -30 and -50 mV). Membrane potential recovery was greater when switching back to normal solution 1 min after the AD (10 out of 14 cells recovered) than when switching 2.5 min after the AD (4 out of 16 cells recovered), and this difference was significant ($P = 0.03$ by a X^2-test with Yates correction; Fig. 1F). These data suggest that when glutamate is released and produces the AD it evokes irreversible damage to the cell in the first few minutes after the AD.

2.3.3 The Current Response of Purkinje Cells to Ischemia

To study the mechanism of glutamate release, we voltage-clamped Purkinje cells, and used their glutamate receptors to sense the rise of glutamate occurring. Figure 1G shows voltage-clamp data obtained with the same K^+-containing internal solution as in the voltage recording experiments of Fig. 1A–F. On applying ischemic solution the current at -40 mV initially became more outward, reflecting the initial hyperpolarization seen in Fig. 1A, but then a large inward current suddenly developed, reflecting the AD in Fig. 1A. Applying NBQX and D-AP5 during the plateau of this current led to an almost complete block of the current (81 ± 3% in five cells, calculated assuming a current baseline at the level of the outward current before the AD), demonstrating that the depolarizing current is generated largely by glutamate release activating ionotropic receptors (Fig. 1H).

Having established that glutamate is the main agent producing the AD, subsequent experiments to define the receptor types generating the AD and to investigate the mechanism of glutamate release were performed in the presence of bicuculline (40 μM) plus picrotoxin (100 μM), to block $GABA_A$ receptor-mediated currents and thus help to isolate glutamate-mediated currents. In addition, Cs^+ was used as the main intracellular cation and QX-314 was included in the patch pipette (see Methods) to improve voltage uniformity in the Purkinje cell dendritic tree and to block $GABA_B$ receptors. Figure 2A shows a specimen current response to ischemia solution recorded at -33 mV. When Cs^+ replaced K^+ as the main internal cation and $GABA_A$ receptors were blocked, the initial ischemia-evoked outward current was absent and was replaced by a slowly developing inward current which may partly reflect K^+ entry through K^+ channels activated in ischemia. After ~8 min, 55 of the 56 cells studied showed an AD current that rapidly reached a peak and then decayed slowly (Fig. 2A), and sometimes increased again after that (e.g. Figs 3C and 4C). The time to the AD did not differ significantly ($P = 0.44$, Fig. 2B) when voltage-clamping with Cs^+ in the pipette as in Fig. 2A, or recording in physiological conditions with K^+ in the pipette and the membrane potential unclamped as in Fig. 1A. When ischemia solution containing NBQX and D-AP5 was applied, no AD current was seen in 5 out of 7 cells (Fig. 2C, cf. Fig. 1B) while the remaining 2 out of 7 cells showed a smaller and more transient AD current than normal: overall the peak inward current generated was only 7.5% of that seen in 4 interleaved cells and 5% of that seen in all 56 cells studied (Fig. 2D).

2.3.4 The AD is Generated Largely by AMPA Receptors

Purkinje cells lack NMDA receptors after ~8 days post-natally (Llano *et al.*, 1991; Rosenmund *et al.*, 1992; Hausser and Roth, 1997), but NMDA receptors on granule cells might contribute to controlling the release of glutamate onto Purkinje cells in ischemia. However, applying D-AP5 to block the effects of glutamate on NMDA receptors after the AD had no effect on the ischemia-evoked AD current (Fig. 3A, B and E). Superfusing the AMPA/kainate receptor blocker NBQX, either on top of D-AP5 (Fig. 3B) or alone (Fig. 3C) resulted in most of the post-AD inward current being blocked.

To distinguish the possible contributions of AMPA- and kainate-receptors to the maintained depolarization produced by ischemia, we studied the effect of the AMPA receptor blocker GYKI 53655 [20 μM, which blocks AMPA receptors by >90% but blocks kainate receptors by <5% (Wilding and Huettner, 1995; Bleakman *et al.*, 1997)]. GYKI 53655 blocked most of the ischemia-evoked inward current after the AD (Fig. 3D and E) and superimposing NBQX produced only a small further block, some of which may be block of the small fraction of AMPA receptors remaining unblocked by the GYKI 53655. On average GYKI 53655 blocked ~80% of the total current blockable by GYKI 53655 and NBQX together (Fig. 3F). Thus, the great majority of the ischemia-evoked, glutamate-mediated current in Purkinje cells is mediated by AMPA receptors which do not completely desensitize in the maintained presence of glutamate.

2.3.5 Glutamate Release is Action Potential Independent

The occurrence of ischemia-evoked action potentials in cells that are not voltage-clamped (Fig. 1A) suggested that the AD and glutamate release may be triggered by action potentials. To test this we applied ischemic solution containing 1 µM TTX to block voltage-gated Na^+ channels (Fig. 3G). TTX had no effect on the time to the AD, the magnitude of the AD current, or the current suppressed by ionotropic glutamate receptor blockers after the AD (I_{glu}) (Fig. 3H).

2.3.6 Glutamate Release is Initially Calcium-Dependent

When ischemia solution lacking calcium (and containing 2 mM EGTA to chelate trace Ca^{2+}: see Methods) was applied (Fig. 4B and D), although an AD current was present at the normal latency (Fig. 4E), it was smaller in amplitude ($P < 0.01$) than in interleaved slices in the control solution (Fig. 4A, C and F). Applying glutamate receptor blockers 10 min after the start of ischemia suppressed a glutamate-mediated current that was only 30% of the amplitude seen in normal calcium containing solution (Fig. 4A, B and G, $P = 0.016$ compared with control solution). However, by 15 min after the start of ischemia, the ischemia-evoked inward current in zero calcium solution had become much larger (Fig. 4D), and the glutamate-mediated current was not significantly different from that seen after 15 min in normal solution (Fig. 4C and H, $P = 0.36$). The response of Purkinje cells to superfused AMPA (1 µM, in 40 µM bicuculline, 50 µM D-AP5 and 1 µM TTX) was not affected by calcium removal (Fig. 4I and J). Consequently, the suppression of ischemia-induced currents produced by removing calcium (Fig. 4A–D) reflects reduced

glutamate release. These results indicate that Ca^{2+}-dependent exocytosis generates glutamate release for at least the first few minutes after the AD, and that 15 min after the start of ischemia the Ca^{2+}-dependent release has stopped.

The use of zero-calcium solution to block exocytotic release of glutamate assumes that exocytosis is indeed Ca^{2+}-dependent. This is a reasonable assumption (which is supported by the reduction of glutamate release seen in Fig. 4); although it has been suggested that early ischemia-evoked spontaneous exocytosis of transmitter is Ca^{2+}-independent (Katchman and Hershkowitz, 1993; Fleidervish *et al.*, 2001), these studies did not use EGTA to chelate trace Ca^{2+}, and subsequent work has suggested that this protocol did not lower $[Ca^{2+}]_o$ sufficiently to block Ca^{2+}-dependent exocytosis (Allen and Attwell, 2004).

2.3.7 Glutamate Release is Partly by Reversed Uptake

The run-down of ion gradients occurring during hippocampal ischemia has been shown to lead to release of glutamate by reversal of glutamate transporters (Madl and Burgesser 1993; Roettger and Lipton, 1996; Phillis *et al.*, 2000; Rossi *et al.*, 2000). A similar run-down of gradients occurs in ischemic cerebellum, with $[K^+]_o$ rising to 40 mM (Kraig *et al.*, 1983). To test whether this $[K^+]_o$ rise (and the associated depolarization and fall of $[Na^+]_o$) leads to significant glutamate release by transporter reversal, we used the approach of Longuemare and Swanson (1995), Roettger and Lipton (1996) and Rossi *et al.*, (2000) to block transporter function. We preloaded slices with the slowly transported glutamate analogue PDC (see Methods), and then washed extracellular PDC out of the

slice. The aim was to accumulate PDC intracellularly, so that if the run-down of ion gradients in ischemia reverses the operation of glutamate transporters, the slowly transported PDC will bind preferentially, occupying the transporter and preventing glutamate release.

To assess whether this procedure succeeded in blocking transporter function, we compared the response of Purkinje cells in non-ischemic slices to superfused glutamate (100 μM), the extracellular concentration of which is normally reduced by uptake, and to the non-transported glutamate analogue AMPA (1 μM). If PDC preloading blocks transporters then superfused glutamate should penetrate further into the slice and generate a larger response, relative to the response produced by AMPA which should be unaffected by block of uptake. Figure 5A and B show responses to glutamate and AMPA in a control slice and a slice preloaded with PDC. While the responses to AMPA were similar in control conditions and after PDC preloading (Fig. 5C), the ratio of (response to glutamate/response to AMPA) was significantly greater ($P = 0.04$) in six slices preloaded with PDC than in five control slices (Fig. 5C), confirming that the activity of transporters was reduced by the preloading procedure.

Figure 5D shows the response to ischemia of two PDC preloaded slices, with NBQX and D-AP5 applied at ~10 and ~15 min after the AD (cf. the control data in Fig. 4A and C). The PDC-preloaded slices show no AD current and a much smaller glutamate-mediated current. Out of 12 PDC preloaded slices studied, 7 showed complete abolition of the AD current (while only 1 out of 35 interleaved control slices failed to show an AD current;

significantly different, $P = 7 \times 10^{-5}$ by X^2-test; Fig. 4E). The remainder did show an AD current (presumably the PDC preloading was less successful in blocking reversed uptake, possibly because some PDC was lost from the cells during the time needed to locate and record from a cell), but the subsequent glutamate mediated current was reduced by 60–80% in magnitude. Averaging over all the slices, PDC-preloading reduced the glutamate-mediated current measured 10 and 15 min after the start of ischemia by 88 and 83%, respectively (Fig. 4E).

These data, together with those recorded in 0 Ca^{2+} solution, suggest that in the first 10 min of ischemia glutamate is released both by exocytosis and by the reversed operation of glutamate transporters, but that by 15 min in ischemia exocytotic glutamate release has stopped and the remaining release is almost entirely by reversed uptake.

2.4 Discussion

We have characterized for the first time the early electrical responses of cerebellar Purkinje cells to ischemia, with the aim of understanding the mechanism of the ischemia-evoked rise of extracellular glutamate concentration which leads to Purkinje cell death and motor dysfunction (Balchen and Diemer, 1992).

2.4.1 Ischemia Initially Hyperpolarizes Purkinje Cells

When the cerebellum is made ischemic, the loss of the ATP supply leads to the Na^+/K^+ pump being inhibited and, as a result of the lack of pumping back into the cell of K^+ which leaks out because the membrane potential is more positive than E_K (Rossi ***et al.***, 2000), there is an initial slow rise of extracellular potassium concentration, $[K^+]_o$, to ~12 mM, followed by a more rapid rise to ~40 mM (Kraig ***et al.***, 1983). We find that Purkinje cells initially hyperpolarize when exposed to ischemic solution. Whole-cell voltage-clamping the cells using a K-gluconate based pipette solution revealed an outward current corresponding to the initial hyperpolarization (Fig. 1G). This was absent when using a CsCl based internal solution and $GABA_A$ receptors were blocked (Fig. 2). The hyperpolarization cannot be produced by ischemia-evoked GABA release activating $GABA_A$ receptors (Allen ***et al.***, 2004) because the initial resting potential (-78 mV) was more negative than E_{Cl} (-65 mV). It may, therefore, reflect the activation of a Ca^{2+}- or ATP-gated K^+ current in Purkinje cells, as seen previously in hippocampal neurons (Nowicky and Duchen, 1998), which could contribute to the initial rise of $[K^+]_o$ to ~12 mM (Kraig et al., 1983).

2.4.2 The AD of Purkinje Cells is Generated by Glutamate and Rapidly Causes Irrevocable Malfunction

After the initial hyperpolarizing phase of the response to ischemia, Purkinje cells exhibit a large and rapid AD which probably correlates with the rise of $[K^+]_o$ to ~40 mM (Kraig ***et al.***, 1983). The Purkinje cell AD was blocked by glutamate receptor blockers, and so reflects ischemia-evoked glutamate release. By using blockers of NMDA, AMPA and

AMPA plus-kainate receptors, we have shown that the great majority of the glutamate-mediated current is generated by AMPA receptors (Fig. 3A–F). The rapid speed of the AD suggests that it is a regenerative event produced by a positive feedback loop like that in the hippocampus, where ionic gradient rundown releases K^+ which depolarizes cells and thus releases glutamate, which in turn depolarizes cells further and releases more K^+. Recovery of the resting membrane potential after termination of simulated ischemia was prevented if the ischemia was maintained for even just a few minutes after the AD occurred (Fig. 1D–F).

The complete abolition of the AD that we see with glutamate receptors blocked is similar to the effect of receptor blockers in abolishing the AD in hippocampal pyramidal cells (Rossi *et al*., 2000). The different types of glutamate receptors expressed in different brain areas will determine which receptor blockers are most effective at preventing the AD. In hippocampal pyramidal cells, where both NMDA and (to a lesser extent) AMPA receptors generate the ischemia-evoked inward current (Rossi *et al*., 2000), it is essential to have both receptor types strongly blocked to prevent the AD; blocking only one type delays, but does not prevent, the AD (Tanaka *et al*., 1997a). Interestingly, some authors report an AD occurring even with blockers of both receptors present (Lauritzen and Hansen, 1992; Muller and Somjen, 2000) but in some cases at least (Jarvis *et al*., 2001) the blocker concentrations used were lower than those Rossi *et al*., (2000) found necessary to block the AD in the hippocampus: the positive feedback mechanisms involved in generating the AD require that a strong block of all glutamate receptors is achieved to prevent the AD. In the neocortex, blocking NMDA receptors alone is

sufficient to block spreading depression (which has some similarities to the AD) and AMPA receptor block is ineffective (Nellgard and Wieloch, 1992), probably because of the fairly complete desensitization of AMPA receptors in the face of a maintained glutamate concentration rise. In contrast, in cerebellar Purkinje cells, which lack NMDA receptors but have incompletely desensitizing AMPA receptors (Brorson *et al*., 1995), it is essential to block AMPA receptors to prevent the AD (Fig. 3).

2.4.3 Incompletely Desensitizing AMPA Receptors Generate the AD

Rossi *et al*., (2000) used a simple model of the ischemic hippocampus to predict the rise of $[K^+]_o$, membrane depolarization and rise of extracellular glutamate concentration produced by ischemia, based on the idea that cutting off the ATP supply to the Na^+/K^+ pump leads to run-down of the transmembrane ionic gradients, reversal of glutamate transporters and activation of glutamate receptors. In that simulation, activation of NMDA receptors by glutamate played a key role in producing a sustained depolarization of cells, because hippocampal AMPA receptors desensitize almost completely (Spruston *et al*., 1995). Cerebellar Purkinje cells, by contrast, lack NMDA receptors after postnatal day 8, so the fact that ischemia-evoked glutamate release generates a sustained depolarizing current (as in Figs 1–3) depends on Purkinje cell AMPA receptors desensitizing incompletely, as suggested by Brorson *et al*., (1995).

2.4.4 Glutamate is Released Both by Exocytosis and by Reversal of Glutamate Transporters

In hippocampal slices, blocking glutamate release by reversed uptake can completely abolish the AD and subsequent glutamate release, whereas removing extracellular Ca^{2+} has little effect (Rossi *et al.*, 2000). However, in cerebellar slices reversed uptake is not the only significant means of glutamate release. Blocking Ca^{2+}-dependent exocytosis by removing extracellular Ca^{2+} reduces glutamate release and thus reduces the glutamate-mediated membrane current by ~70% early after the AD (10 min after the start of ischemia; Fig. 4). Later than 15 min after the start of ischaemia, however, removing extracellular Ca^{2+} has no effect on the ischemia evoked glutamate release. This situation is similar to that in striatum, where microdialysis experiments *in vivo* have shown that in ischemia there is a brief period of Ca^{2+}-dependent glutamate release, after which glutamate release is Ca^{2+}-independent (Wahl *et al.*, 1994).

Even with exocytosis blocked there is still substantial glutamate release in cerebellar ischemia (Fig. 4). Blocking reversed uptake by preloading with PDC produces a larger reduction of early (10 min) ischemic glutamate release than does blocking exocytosis (Fig. 5). Further, blocking reversed uptake produces a similar reduction of late glutamate release (15 min after the start of ischemia; Fig. 4), but late glutamate release is unaffected by removing calcium (Fig. 5). Thus, the major fraction of ischemic glutamate release is by reversed uptake. Interestingly, after 10 min ischemia, removal of calcium blocks ~70% of the glutamate-mediated current, whereas blocking reversed uptake blocks

~88%, implying (since the sum of these blocks is >100%) that these two release mechanisms do not have independent effects on the glutamate-mediated current. This could result simply from non-linearity in the conversion of extracellular glutamate concentration to AMPA receptor-mediated current. Alternatively, it could result from a synergy between the effects of release by Ca^{2+}-dependent exocytosis and reversed uptake, as follows. Glutamate released by exocytosis will activate AMPA receptors and promote uptake reversal by raising $[Na^{+}]_i$ and depolarizing cells, and in addition the resulting ATP consumption on ion pumping (Attwell and Laughlin, 2001) will accelerate the run-down of ion gradients that reverses transporters. Thus, blocking exocytosis will reduce reversed uptake as well. Conversely, glutamate released by reversed uptake will depolarize cells and release more glutamate by exocytosis, so that blocking reversed uptake will reduce exocytotic glutamate release as well.

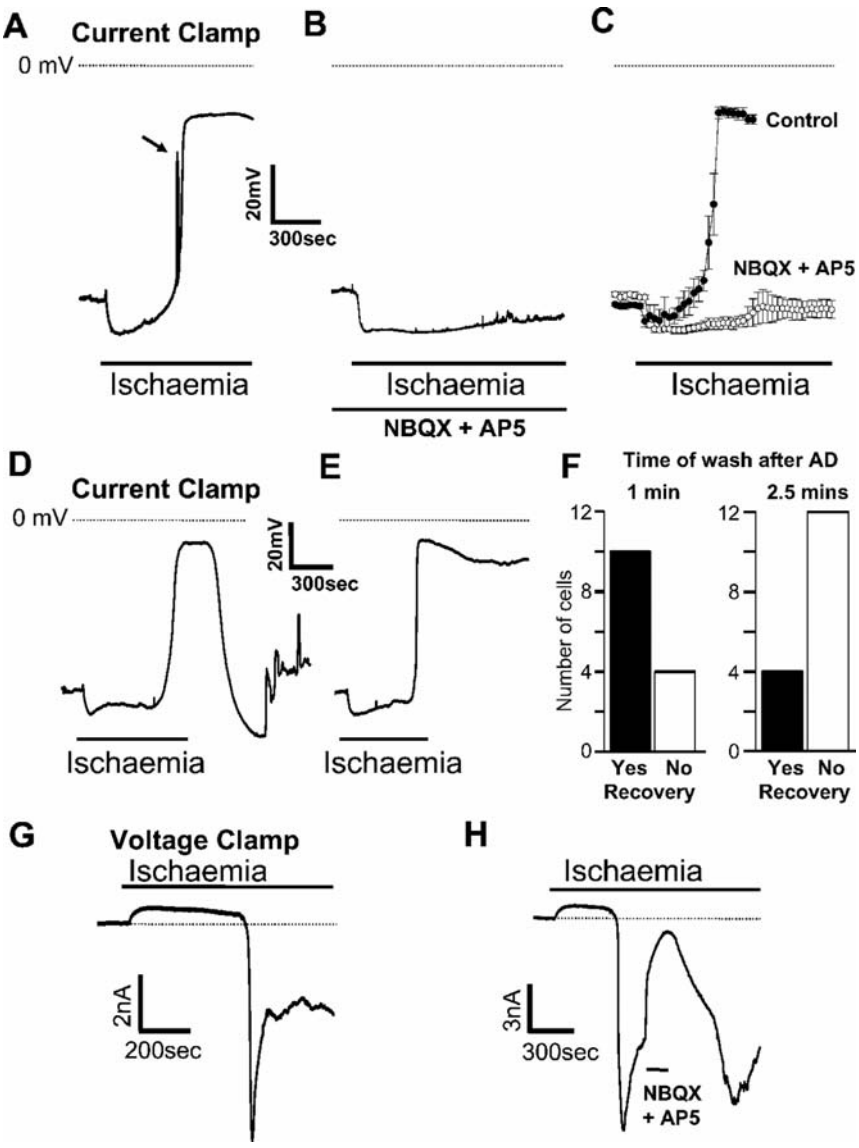

Figure 1. Simulated ischemia evokes a small hyperpolarization followed by a large depolarization in current-clamped cerebellar Purkinje cells. (**A**) Voltage response of a Purkinje cell to ischemia solution. Before the large AD there is a burst of action potentials (arrow). Resting potential before ischemia was -78 mV. (**B**) In ionotropic glutamate receptor blockers (NBQX and D-AP5), the AD is blocked, leaving the ischemic hyperpolarization. Resting potential before ischemia was -75 mV. (**C**) Mean data from experiments like **A** and **B** (four control cells, filled circles; three cells in

blockers, open circles). (**D**) Specimen response to ischemia of limited duration of a Purkinje cell which recovered its resting potential after the ischemia. (**E**) Specimen response to ischemia of limited duration of a Purkinje cell which failed to recover its resting potential after the ischemia. (**F**) Number of cells recovering a resting potential more negative than -50 mV or failing to recover more negative than -30 mV, after termination of ischemia 1 or 2.5 min after the AD. (**G**) Current response to ischemia at -40 mV shows an outward current followed by a large inward current which declines to a smaller plateau. (**H**) Applying ionotropic glutamate receptor blockers greatly reduces the inward current. All records from cells studied with K-gluconate based internal solution.

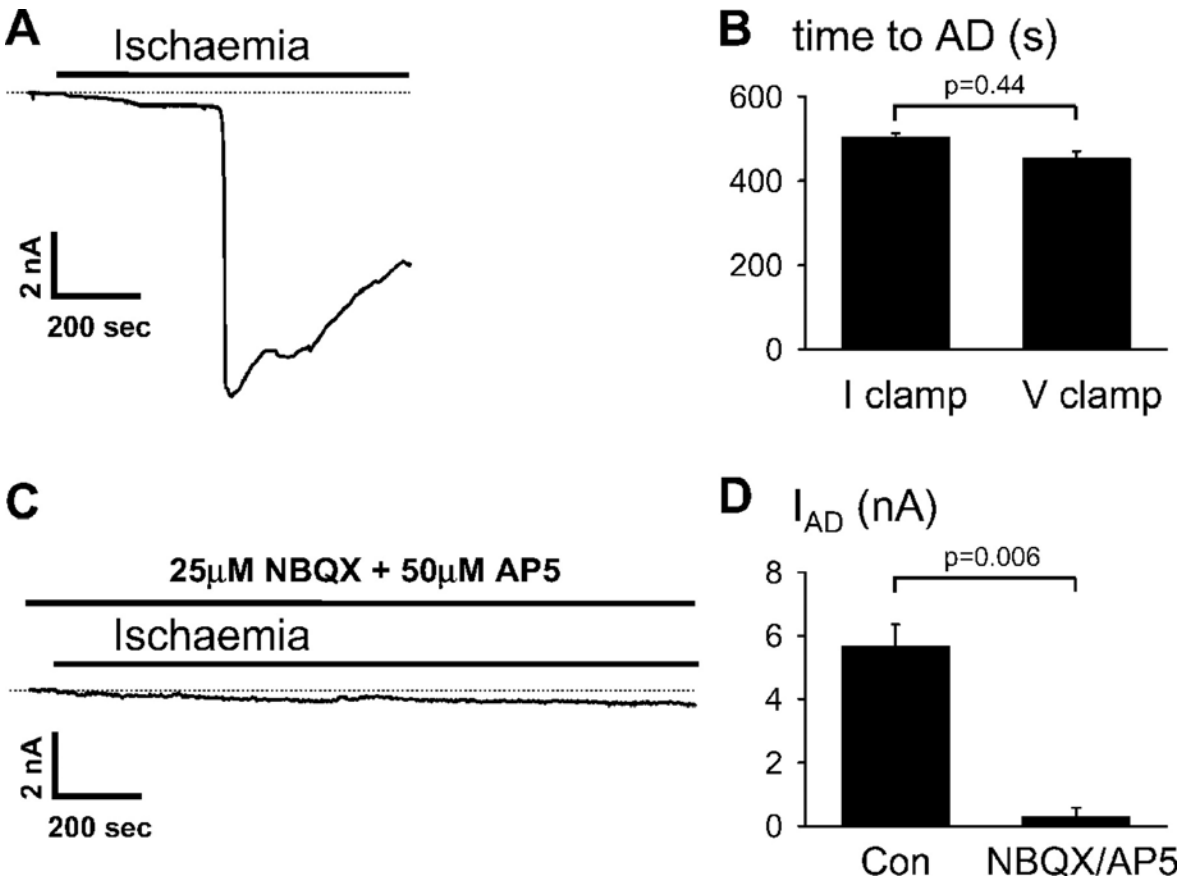

Figure 2. With K+ omitted from the pipette solution and GABAA receptors blocked, the ischemia-evoked current is purely inward. (**A**) Current response of a Purkinje cell at -33 mV to ischemia solution, recorded with a CsCl-based internal solution and bicuculline (40 mM) plus picrotoxin (100 mM) in the external solution. (**B**) Mean time to the AD voltage or current for 4 cells recorded in current clamp (I clamp) with a K-gluconate internal as in Fig. 1A and 55 cells voltage-clamped (V clamp) with a CsCl internal solution and $GABA_A$ receptors blocked as in A. (**C**) Response to ischemia as in A but with ionotropic glutamate receptors blocked with NBQX and D-AP5. (**D**) Comparison of the peak inward current produced by 10 min ischemia solution in the absence (56 cells, con) and the presence (7 cells) of NBQX and D-AP5.

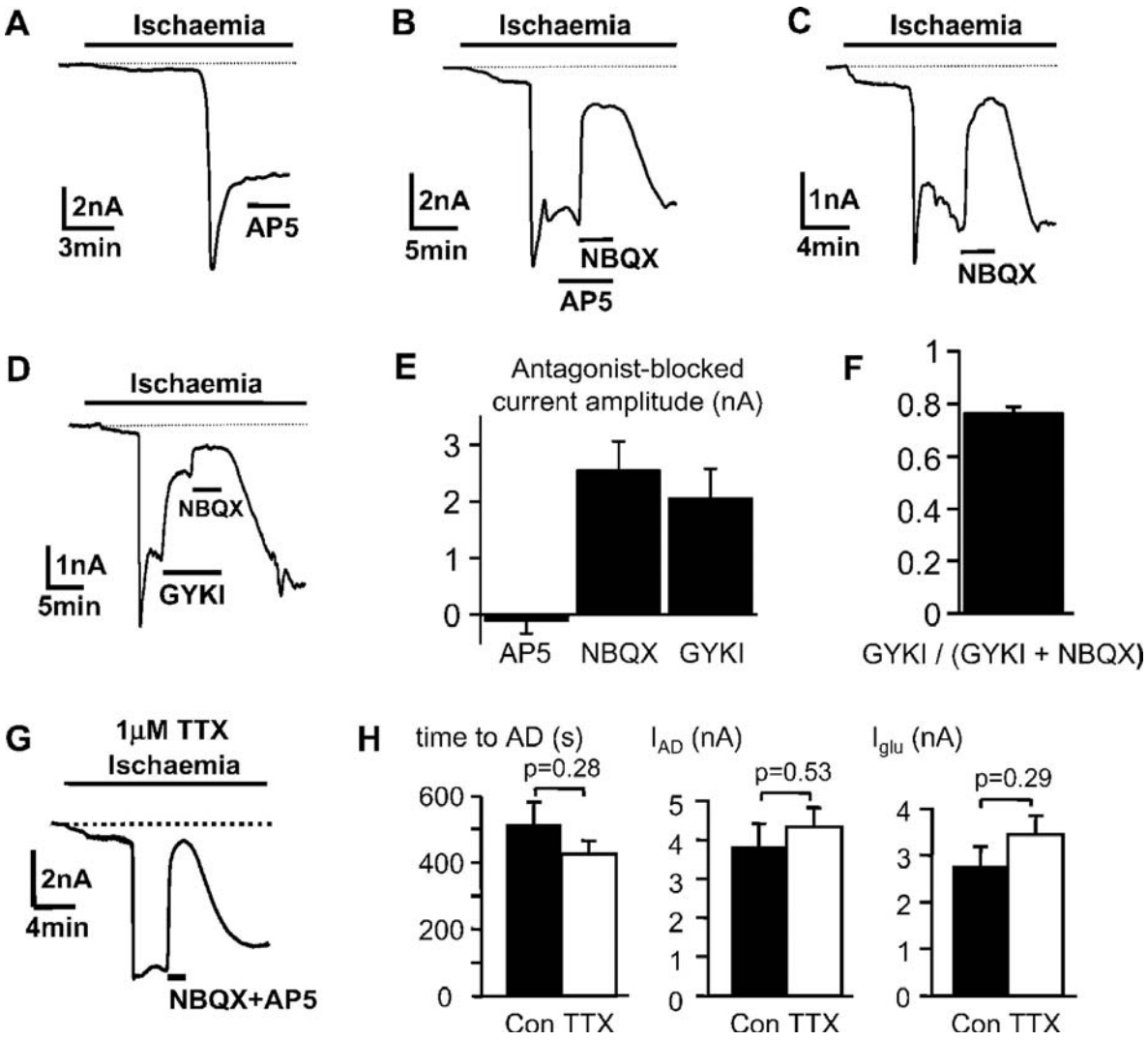

Figure 3. AMPA receptors generate the AD and action potentials do not contribute significantly to glutamate release in cerebellar ischemia. (**A**) Current response to ischemia, and lack of suppression of the post-AD current by D-AP5 (50 μM). (**B**) Superimposing NBQX (25 μM) on AP5 suppresses most of the post-AD current. (**C**) NBQX alone suppresses most of the post-AD current. (**D**) The AMPA receptor blocker GYKI 53655 (20 μM) blocks most of the post-AD current. (**E**) Mean current suppressed after the AD by D-AP5, NBQX and GYKI 53655, in 12, 8 and 7 cells, respectively. (**F**) Fraction of the current blocked by GYKI 53655 plus NBQX that was blocked by GYKI

53655 alone, in six cells. (**G**) Current response to ischemia in the presence of TTX. (**H**) TTX did not significantly affect (in four cells in TTX interleaved with four control cells) the time to the AD, the amplitude of the current change at the time of the AD (I_{AD}), or the glutamate-mediated current after the AD, *Iglu*, defined as the current suppressed by 25 µM NBQX and 50 µM D-AP5. All data recorded at -33 mV with a CsCl-based internal solution with $GABA_A$ receptors blocked.

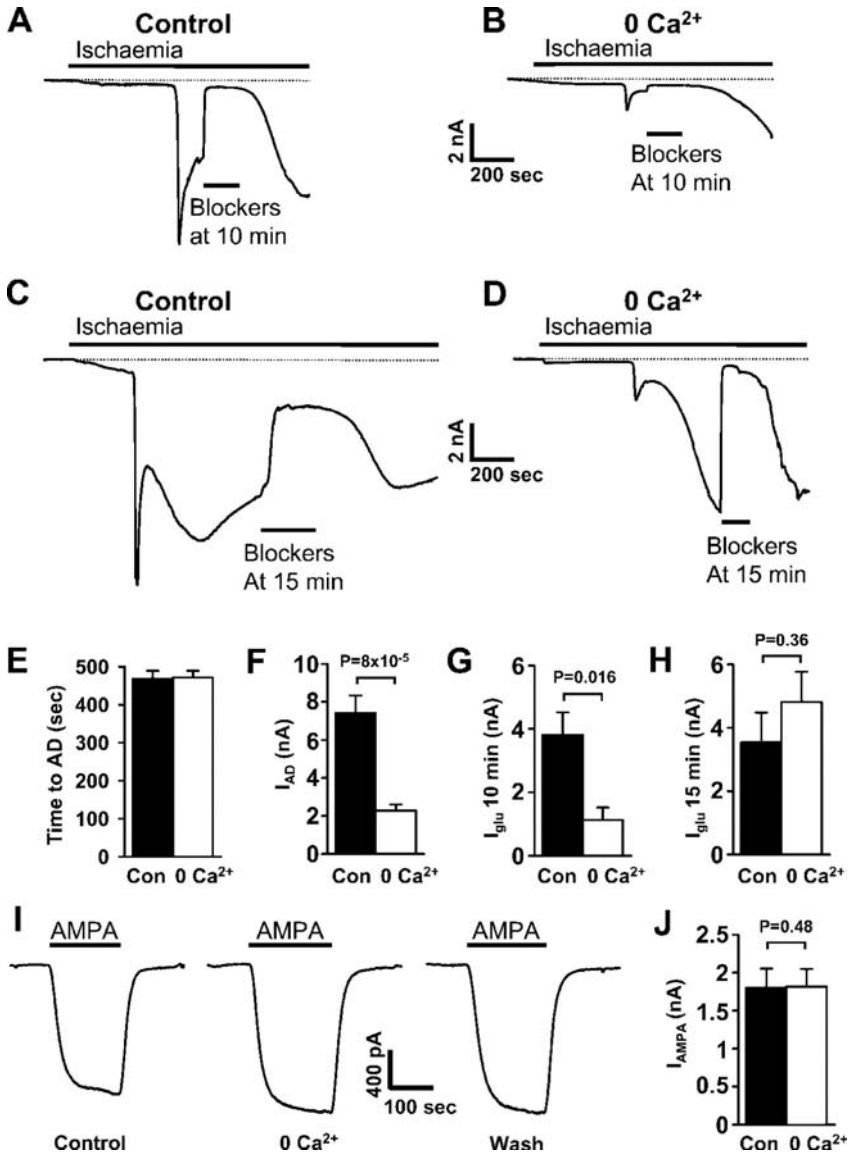

Figure 4. Removal of external calcium reduces early but not late glutamate release evoked by ischemia. (**A**) Specimen response to ischemia in normal solution at -33 mV, with 25 µM NBQX and 50 µM D-AP5 (blockers) applied to measure the glutamate-mediated current 10 min after the start of ischemia. (**B**) Response to ischemia in solution lacking calcium (and containing EGTA). (**C**) As in **A** but with blockers applied to measure I_{glu} after 15 min. (**D**) As in C but in zero-calcium solution. (**E–H**) Comparison of time to the AD (**E**), AD current (**F**), and glutamate-mediated current (I_{glu}) after 10 min

(**G**) or 15 min (**H**) ischemia, in 36 cells in normal solution (of which 18 and 10 cells provided I_{glu} data at 10 and 15 min) and 21 interleaved cells in calcium-free solution (of which 9 and 8 cells provided I_{glu} data at 10 and 15 min). (**I**) Effect of zero-calcium solution on the response to 1 µM AMPA (in 50 µM D-AP5, 40 µM bicuculline and 1 µM TTX) at -33 mV. (**J**) Average data from experiments as in **I** on three cells.

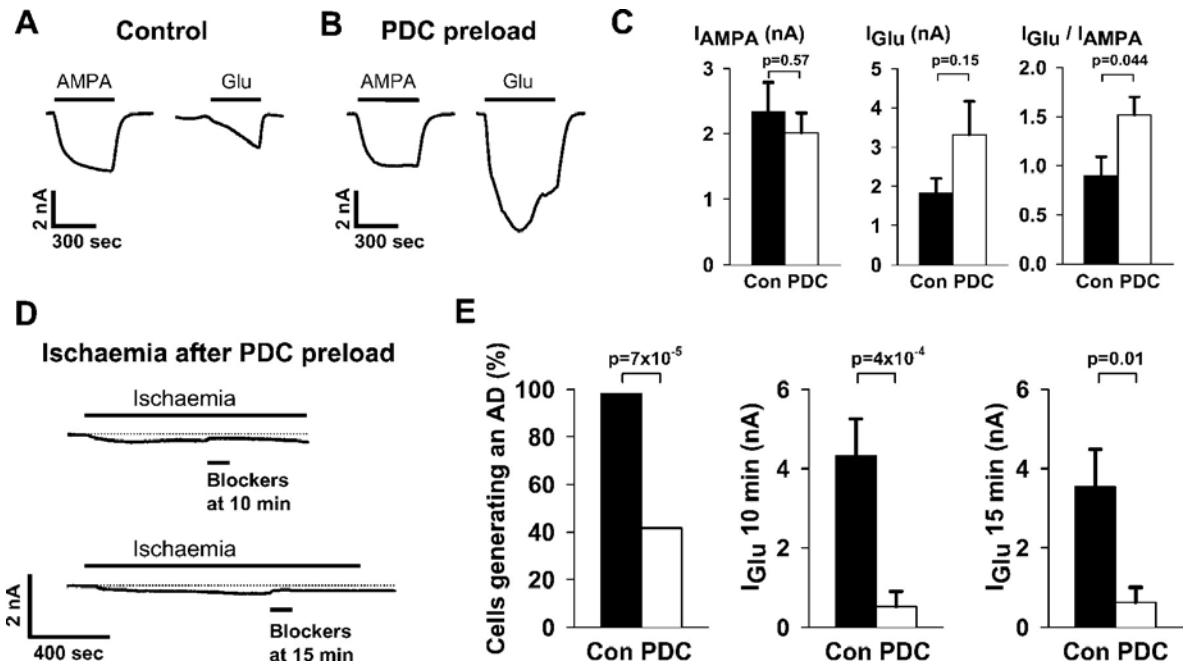

Figure 5. Preloading with the glutamate transport blocker PDC reduces the fraction of cells showing an AD and greatly reduces glutamate release after the AD. (**A–C**) Effects of PDC-preloading on the current response of Purkinje cells (at -33 mV) to glutamate (100 µM) and AMPA (1 µM). (**A**) Specimen response of a cell to AMPA and glutamate in control solution. (**B**) Specimen response to AMPA and glutamate after PDC preloading: the AMPA response is similar to that in control solution, but the glutamate response is increased. (**C**) Mean data from experiments as in **A** and **B**, on five control cells and six cells after PDC preloading. (**D**) Specimen responses of two different Purkinje cells (at -33 mV) to ischemia after PDC preloading, with 25 µM NBQX and 50 µM D-AP5 (blockers) applied after 10 or 15 min. No AD and little glutamate-mediated current are seen in these cells. (**E**) Comparison of the response to ischemia in 36 control cells (of which 18 and 10 cells provided I_{glu} data at 10 and 15 min) and 12 interleaved cells after PDC preloading (of which 5 and 5 cells provided I_{glu} data at 10 and 15 min).

Chapter 3:

Simulated ischemia induces Ca2+-independent glutamatergic vesicle release through actin filament depolymerization in area CA1 of the hippocampus

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3.1 Introduction

Ischemia-induced brain damage is a leading cause of death and disability. *In vivo* studies have identified two main phases of response to severe brain ischemia (Astrup *et al.*, 1977; Hansen and Nedergaard, 1988; Rossi *et al.*, 2007). The first phase, lasting 1-2 minutes, is characterized by an isoelectric EEG and a gradual elevation of extracellular K^+, with little change in the concentration of other principal ions or electrical potential. The second phase is characterized by a rapid and large ischemic depolarization (ID), loss of principal ionic gradients, and build up of glutamate and other neurotransmitters in the extracellular space. If the latter phase persists for more than a couple of minutes, pan necrosis ensues. In contrast, shorter transient episodes of ischemia can either lead to ischemic preconditioning, whereby brain tissue becomes more resistant to subsequent episodes of

ischemia (Obrenovitch, 2008), or to selective cell death in ischemia-sensitive neurons (Pulsinelli, 1985). While much is known about how the ID and release of transmitters damages tissue during the second phase of ischemia (Lipton, 1999; Rossi *et al.*, 2007), less is known about the earlier events of ischemia and their relationship to preconditioning or transition to selective neuronal death.

In vitro brain slice studies have determined that one of the earliest manifestations of brain ischemia/anoxia is an increase in the frequency of miniature excitatory postsynaptic currents (mEPSCs; (Katchman and Hershkowitz, 1993; Fleidervish *et al.*, 2001; Allen *et al.*, 2004). This early increase in glutamatergic excitation could contribute to either ischemic preconditioning (Jiang *et al.*, 2003; Miao *et al.*, 2005; Lin *et al.*, 2008), or to selective neuronal death in ischemia-sensitive neurons (Gill *et al.*, 1987).

An early *in vivo* study using Ca^{2+}-sensitive electrodes detected an early, small rise in $[Ca^{2+}]_c$ in hippocampal CA1 neurons that precedes the ID (Silver and Erecinska, 1990), raising the possibility that increased mEPSC frequency is due to elevated presynaptic $[Ca^{2+}]_c$. However, in a study of hippocampal slices, the anoxia-induced increase in mEPSC frequency was independent of both action potentials and external Ca^{2+} (Katchman and Hershkowitz, 1993). In the same study, dantrolene, a ryanodine receptor antagonist, prevented the increase in mEPSC frequency, leading the authors to suggest that the increased mEPSC frequency is triggered by Ca^{2+} release from intracellular stores in presynaptic terminals. However, this conclusion was complicated somewhat by an

effect of dantrolene on basal mEPSC frequency, and by lack of information on the Ca^{2+} dynamics during anoxia in these experiments. Thus, it remains uncertain whether the ischemia-induced increase in mEPSC frequency is triggered by Ca^{2+}.

We used patch-clamp recording with simultaneous Ca^{2+}-imaging in hippocampal slices during simulated ischemia. Ischemia caused an early increase in $[Ca^{2+}]_c$, mediated by Ca^{2+} influx and release from intracellular stores, that paralleled the increase in mEPSC frequency. Surprisingly, preventing the rise in $[Ca^{2+}]_c$ by removing external Ca^{2+} and soaking slices in BAPTA-AM, did not affect the increase in mEPSC frequency. The increase in mEPSCs was, however, prevented by pre-treating slices with the actin filament stabilizer Jaspamide, suggesting that ischemia-induced vesicle release is mediated by actin filament depolymerization in presynaptic terminals.

3.2 Materials and Methods

3.2.1 Reagents

Fura-2-AM (Invitrogen, Carlsbad, CA), BAPTA-AM (Sigma, St. Louis, MO), AP5, TTX, NBQX, (S)-MCPG (Ascent scientific, Great Britain), Jaspamide (Alexis Biochem, Plymouth Meeting, PA).

3.2.2 Preparation of Brain Slices

The hippocampus was obtained from Sprague-Dawley albino rats (Rossi and Slater, 1993; Rossi and Hamann, 1998; Rossi *et al.,* 2000). Rats (18-21 days old) were anaesthetized with Isoflurane and killed by decapitation. The whole brain was rapidly isolated and immersed in ice cold (0-2ºC) artificial cerebrospinal fluid (ACSF) containing (in mM): 124 NaCl, 26 $NaHCO_3$, 1 NaH_2PO_4, 2.5 KCl, 2.5 $CaCl_2$, 2 $MgCl_2$, 10 D-glucose, and bubbled with 95%O_2/5% CO_2 (pH 7.4). The hippocampus was dissected out of the brain and mounted in a slicing chamber filled with ice cold (0-2ºC) ACSF. Slices (225µm) were made with a vibrating tissue slicer (Vibratome). Slices were incubated in warmed ACSF (33±1ºC) for one hour after dissection and then held at 22-23ºC until used. Kynurenic acid (1 mM) was included in the dissection, incubation and holding solution (to block glutamate receptors to reduce potential excitotoxic damage) but was omitted from the experimental solutions.

3.2.3 Visualized Patch-Clamp Recording from Cells in Brain Slices

Slices were placed in a submersion chamber on an upright microscope, and viewed with an Olympus 60X (0.9 numerical aperture) water immersion objective with differential interference contrast and infrared optics. Slices were perfused with heated (32-34ºC) ACSF at a rate of ~4ml/min. Drugs were dissolved in ACSF and applied by bath perfusion. Whole-cell recordings were made from the somata of visually identified CA1 pyramidal cells. Patch pipettes were constructed from thick-walled borosilicate glass

capillaries and filled with an internal solution containing (mM) Csgluconate 130, NaCl 4, $CaCl_2$ 0.5, HEPES 10, EGTA 5, MgATP 4, Na_2GTP 0.5, QX-314 5 (to suppress voltage-gated sodium currents), pH adjusted to 7.2 with CsOH. Electrode resistance was 1.5 to 2.5 MΩ. Cells were rejected if access resistance was greater than 5 MΩ. Cells were also rejected if the access resistance, monitored with –5mV voltage steps, changed by more than 20% during the course of an experiment.

3.2.4 Simulating Ischemia in Brain Slices

We simulated severe brain ischemia by exposing slices to solution in which glucose and oxygen were replaced with sucrose and nitrogen, and supplemented with iodoacetic acid (2mM) to block glycolysis. Previously we also supplemented our ischemia simulation solution with cyanide (1mM) to block oxidative phosphorylation (Rossi *et al.*, 2000; Hamann *et al.*, 2005). Because cyanide may have chemical interactions with either glutamate receptors or glutamate transporters independent of cellular responses to energy deprivation, in this project we did not include cyanide. The only significant difference that we observed between these two methods of simulating ischemia was a slightly more rapidly developing response when cyanide was included (Data not shown).

3.2.5 Loading Slices with Fura 2-AM and BAPTA-AM

Stock solutions of the acetoxymethyl (AM) ester of Fura 2 (10mM) or BAPTA (20mM) were made in DMSO with 20% pluronic acid, and sonicated for ~1 minute on the day of experiments. Stock solutions were added to our standard incubation solution at final concentrations of 100μM for Fura 2-AM and 200μM BAPTA-AM, followed by brief sonication. Slices were incubated in final solution for 1 hour, and experiments were conducted within 45 minutes of removal from the incubation solution.

3.2.6 Quantifying Changes in Fura-2 Fluorescence Emission

All values are expressed as a percent change in the ratio of Fura-2 fluorescence emission when excited at 340nm and 380nm with the equation $(F_t-F_0)/F_0$, where F_t is the emission ratio at time t and F_0 is the emission ratio at time 0. For all experimental conditions, a separate series of experiments was conducted with slices that were not loaded with Fura 2, and the autofluorescence emission at each excitation wavelength was averaged for at least 5 slices. The resultant mean autofluorescence signal for each excitation wavelength was subtracted from the experimental emission signal for each individual Fura 2 loaded slice. For $0Ca^{2+}$ experiments, because switching to $0Ca^{2+}$ ringer resulted in a gradual but continuous decline in $[0Ca^{2+}]_c$, control experiments were conducted to obtain an average waveform of the Fura 2 emission ratio upon switching to $0Ca^{2+}$ ringer. The resultant mean Fura 2 ratio waveform was subtracted from each slice exposed to ischemia in $0Ca^{2+}$ to isolate the ischemia induced response.

3.2.7 Data Acquisition, Analysis and Statistics

CA1 pyramidal cells were voltage-clamped at -60mV with an Axoclamp 700b patch clamp amplifier (Axon Instruments), and mEPSCs were filtered at 5kHz and acquired at 20kHz with pClamp software (Axon Instruments). Synaptosoft mini analysis software (Synaptosoft Inc.) was used to analyze mEPSC frequency and amplitude. Whole field (1343X780 pixels after 600X magnification) Fura 2 fluorescence was acquired and analyzed using Slidebook software (Olympus microscopes LTD.). All data are presented as means±S.E.M., and statistical significance was defined as $P<0.05$ for paired or unpaired students t-test.

3.3 Results

3.3.1 Ischemia Increases $[Ca^{2+}]_c$ and mEPSC Frequency in Parallel

To determine the influence of ischemia on $[Ca^{2+}]_c$ and mEPSC frequency in the hippocampus, we pre-incubated hippocampal slices with the membrane permeant Ca^{2+} indicator, Fura-2-AM, and voltage-clamped CA1 pyramidal cells in the Fura-2 loaded slices. We used Fura-2-AM because it has a high affinity for Ca^{2+} (Kd=145 nM), enabling us to detect small tonic changes in $[Ca^{2+}]_c$ with better resolution than lower affinity indicators such as rhod-2-AM (Mitani *et al.*, 1993) (Kd=570 nM), and because Fura-2's

affinity for Ca^{2+} and emission characteristics are not affected by pH changes that occur during ischemia (Grynkiewicz *et al.*, 1985). To ensure that any changes in Fura-2 fluorescence that we observe are due to changes in $[Ca^{2+}]_c$, and not changes in NADH autofluorescence (Brooke *et al.*, 1996), for all experimental conditions, we have quantified and subtracted autofluorescence signals mediated by NADH and other endogenous fluorophores from the Fura-2 signal (see methods for details).

Before determining the impact of simulated ischemia, we wanted to ensure that the Fura-2 signal we record included $[Ca^{2+}]_c$ in presynaptic terminals in addition to other subcellular compartments. To do this we electrically stimulated the Schaeffer collateral axon track, recorded the corresponding changes in Fura-2 fluorescence ratio over the entire field of view, and pharmacologically identified the subcellular makeup of the resultant global Ca^{2+} signal (Fig. 1A&C). Tetanic stimulation of the Schaeffer collaterals (400 µA, 50Hz, 4 secs) evoked summating EPSCs (Fig. 1B) and an associated transient increase in $[Ca^{2+}]_c$, as evidenced by an increase in the Fura-2 fluorescence ratio (Fig. 1A). Bath application of ionotropic glutamate receptor antagonists (D-AP5, 50µM; NBQX, 25µM) abolished the summating EPSCs (Fig. 1B) but only partially reduced the amplitude of the Ca^{2+} transient (Fig. 1A&C, Fura-2 emission ratio= 19.8±7.7% of control, n=6); similarly, a residual Ca^{2+} signal persisted in the presence of both these ionotropic glutamate receptor antagonists and the broad spectrum metabotropic glutamate receptor (mGluR) antagonist (S)-MCPG (500µM) (Fig. 1C, Fura-2 emission ratio= 14.1±4.8% of control, n=2). The residual Ca^{2+} signal, when glutamate receptors were blocked, was

abolished by the sodium channel antagonist, TTX (0.5 μM) (Fig. 1A&C, Fura-2 emission ratio= 1.6±0.3% of control, n=6, significantly reduced compared to glutamate antagonists alone, $P<0.05$) suggesting that it reflects action potential-evoked elevations of $[Ca^{2+}]_c$ in presynaptic terminals. Thus, the Fura-2 signal in our experiments reflects the $[Ca^{2+}]_c$ in both pre and post synaptic compartments.

We next examined the effects of solutions designed to mimic severe brain ischemia (*in vitro* ischemia, see methods for details) on $[Ca^{2+}]_c$ in the CA1 region and pyramidal cell mEPSCs (all experiments were done in the presence of TTX and the $GABA_A$ receptor antagonist GABAzine). Ischemia caused an early gradual increase in $[Ca^{2+}]_c$, followed after several minutes by a rapid, large increase in $[Ca^{2+}]_c$, which corresponds to the large inward current recorded simultaneously in a CA1 pyramidal cell, as shown in a representative experiment (Fig. 2A). Our previous work has shown that the large inward current is generated by glutamate receptor-gated channels (Rossi *et al.*, 2000), which *in vivo* studies indicate underlies the large increase in $[Ca^{2+}]_c$ (Silver and Erecinska, 1990). Although there was no major change in pyramidal cell macroscopic membrane current during the early rise in $[Ca^{2+}]_c$ (Fig. 2A), there was a gradual increase in the frequency of mEPSCs (Fig. 2B & 3A, Mean mEPSC frequency in ischemia= 1.2±0.2Hz, n=8, significantly greater, $P<0.05$, than baseline frequency= 0.4±0.08) that paralleled the early gradual increase in $[Ca^{2+}]_c$ (Fig. 3B&C, Fura-2 peak emission ratio in ischemia = 27.9±8.0% greater than baseline ratio, $p<0.05$, n=8). While mEPSC frequency increased

approximately three fold (Fig. 3B&C), the mean amplitude was not affected (Fig. 3D&E).

The increase in mEPSC frequency was not affected by either Fura-2 loading or by imaging with UV light (Fig. 3F), demonstrating that our imaging procedure did not have spurious effects on spontaneous or ischemia-evoked glutamate release. Thus, simulated ischemia causes an early, parallel increase in $[Ca^{2+}]_c$ and mEPSC frequency that precedes by several minutes the ID and massive glutamate release that causes pan necrosis.

3.3.2 Removing Extracellular Ca^{2+} Reduces the Ischemia-Induced Increase in $[Ca^{2+}]_c$ But Not the Increase in mEPSCs

A previous study of hippocampal slices showed that anoxia-induced increases in mEPSC frequency persist in nominally Ca^{2+}-free bathing solution (Katchman and Hershkowitz, 1993). However, even trace amounts of extracellular Ca^{2+} in nominally Ca^{2+}-free solutions might represent a residual source of Ca^{2+} influx; indeed, we have observed that evoked synaptic responses are reduced but not abolished in nominally Ca^{2+} free solutions, unless a chelator is added to remove trace Ca^{2+} (not shown). In extracellular solutions in which we replaced Ca^{2+} with Mg^{2+} in the bath and added EGTA (2mM) to chelate trace residual Ca^{2+}, simulated ischemia still caused an early rise in $[Ca^{2+}]_c$, but with a significantly reduced magnitude when compared to the rise in Ca^{2+} seen in control solutions (Fig. 4A, Peak increase in Fura-2 emission ratio=10.7±2.2%, n=11, $P<0.05$

compared to ischemia in normal Ca^{2+}). Despite the reduced magnitude of the increase in $[Ca^{2+}]_c$, the increase in mEPSC frequency was not affected (Fig. 4B, n=8, $P>0.05$, compared to ischemia in normal Ca^{2+} for all time points). These data indicate that some of the ischemia-induced rise in $[Ca^{2+}]_c$ is via Ca^{2+} influx across the plasma membrane; however, the increase in mEPSC frequency is not dependent on this Ca^{2+} influx.

3.3.3 Preventing the Rise of $[Ca^{2+}]_c$ Does Not Prevent the Ischemia-Induced Increase in mEPSCs

The data in figure 5 indicate that a portion of the rise in $[Ca^{2+}]_c$ is due to release from intracellular stores, and it has been suggested that Ca^{2+} release from intracellular stores in presynaptic terminals is the trigger for vesicle release during anoxia (Katchman and Hershkowitz, 1993). To test this idea, we loaded slices with the membrane permeant Ca^{2+} chelator BAPTA-AM to buffer $[Ca^{2+}]_c$ released from intracellular sources (Abdel-Hamid and Tymianski, 1997). To confirm that this would effectively buffer $[Ca^{2+}]_c$, we examined the effects of BAPTA loading on spontaneous and stimulus-evoked EPSCs. Pre-incubation of slices with BAPTA-AM (see methods for details) abolished stimulus evoked EPSCs at all but the highest stimulus intensities, and even under those conditions the EPSCs were profoundly reduced in amplitude (Figure 5A, Mean eEPSC amplitude at 1mA stimulation was 123.8±71.6pA, n=4 for BAPTA-AM soaked slices, and 494.7±83.8pA, n=12 for control slices, $P<0.05$). Paired pulse facilitation (PPF) of these residual EPSCs was abolished in BAPTA loaded slices (Fig. 5B, Paired pulse ratio was

0.91±0.13, n=4 for BAPTA-AM soaked slices and 1.31±0.04, n=8 for control slices, $P<0.05$), as expected given that PPF is $[Ca^{2+}]_c$-dependent (Regehr et al., 1994; Xu-Friedman and Regehr, 2004). BAPTA loading also significantly reduced the basal mEPSC frequency (Fig. 5C, mEPSC frequency was 0.12±0.02Hz, n=4 for BAPTA-AM soaked slices and 0.37±0.12Hz, n=12 for control slices, $P<0.05$). Taken together, these data indicate that soaking slices in BAPTA-AM effectively loads presynaptic terminals with BAPTA, reducing basal $[Ca^{2+}]_c$, and strongly buffering Ca^{2+} transients.

We next repeated our ischemia experiments in the absence of external Ca^{2+}, in slices that were pre-incubated in BAPTA-AM. Under these conditions, the ischemia induced increase in $[Ca^{2+}]_c$ was abolished (Fig. 6A, Peak increase in Fura-2 emission ratio = 3.3±1.2%, n=16, $P<0.05$ compared to ischemia in control slices and normal Ca^{2+}). However, despite preventing the rise in $[Ca^{2+}]_c$, BAPTA-AM did not prevent the ischemia-induced increase in mEPSC frequency (Fig. 6B&C, ischemia-induced increase in mEPSC frequency was 5.1±1.5 fold, n=8 for BAPTA-AM soaked slices in $0Ca^{2+}$and 2.9±0.4 fold, n=8 for control slices in normal Ca^{2+}, $P>0.05$). Thus, the ischemia-induced increase in mEPSC frequency is not triggered by intracellular Ca^{2+} from either influx or release from intracellular stores.

3.3.4 Ischemia-Induced Vesicle Release is Mediated by Actin Filament Depolymerization

Recent studies indicate that transient episodes of ischemia cause actin filament depolymerization in dendritic spines, resulting in spine retraction that contributes to ischemic preconditioning (Gisselsson *et al.*, 2005; Meller *et al.*, 2008). Since depolymerizing actin filaments in presynaptic terminals with Latrunculin A causes Ca^{2+}-independent vesicle release (Morales *et al.*, 2000), we reasoned that ischemia might increase vesicle release by depolymerizing actin filaments in presynaptic terminals, as it does in dendritic spines (Gisselsson *et al.*, 2005; Meller *et al.*, 2008). To test this hypothesis, we pre-treated slices with the actin filament stabilizer, Jaspamide (20μM), which prevents the vesicle release induced by Latrunculin A (Morales *et al.*, 2000). In agreement with our hypothesis, Jaspamide pretreatment prevented the ischemia-induced increase in mEPSC frequency (Fig. 7, Mean mEPSC frequency in ischemia was 0.13±0.03Hz, n=12 for Jaspamide soaked slices and 1.1±0.32Hz, n=9 for interleaved control slices). The data suggest that the ischemia-induced, increase in mEPSCs is due to actin depolymerization in presynaptic terminals, resulting in increased vesicle fusion.

3.3.5 The Ischemia-Induced Early Rise in $[Ca^{2+}]_c$ is Not Mediated by Glutamate Receptors

Although our data indicate that the increase in mEPSC frequency is not caused by the early rise in $[Ca^{2+}]_c$, given the permeability of glutamate receptors to Ca^{2+} and the parallel temporal relationship between mEPSC frequency and $[Ca^{2+}]_c$, it remains possible that the increase in glutamatergic mEPSCs causes the rise in $[Ca^{2+}]_c$. However, blocking

ionotropic glutamate receptors alone or in conjunction with blocking metabotropic glutamate receptors, using the same pharmacological agents shown in Figure 1, did not prevent the ischemia-induced early rise in $[Ca^{2+}]_c$ (Fig. 8, Mean peak increase of Fura-2 emission ratio was 31.57±0.04%, n=4 and 44.14±8.56%, n=3 for D-AP5+NBQX and DAP5+NBQX+(S)-MCPG, neither different from control ischemia, $P>0.05$). Thus, despite their temporal coincidence, the ischemia-induced increase in mEPSCs and $[Ca^{2+}]_c$ are independent of each other.

3.4 Discussion

The principle findings of this study are that simulated ischemia causes an early rise in $[Ca^{2+}]_c$ that parallels an early increase in mEPSC frequency, both of which precede the ischemic depolarization (ID) by several minutes. The ischemia-induced rise in $[Ca^{2+}]_c$ is mediated in part by Ca^{2+} influx across the plasma membrane and in part from release from intracellular stores. However, the two processes are independent of each other as preventing the increase in $[Ca^{2+}]_c$ does not affect the increase in mEPSC frequency, and blocking the glutamate receptors that underlie the mEPSCs does not affect the rise in $[Ca^{2+}]_c$. In contrast, the ischemia-induced increase in mEPSCs was prevented by blocking actin filament depolymerization with Jaspamide, indicating that the vesicle release is triggered by actin filament depolymerization in presynaptic terminals.

Our observation of an early increase in mEPSC frequency confirms previous reports examining anoxia/ischemia in a variety of brain slice preparations (Katchman and Hershkowitz, 1993; Fleidervish *et al.*, 2001; Allen *et al.*, 2004). Our observation of an early increase in $[Ca^{2+}]_c$ agrees with a previous study of ischemic hippocampus *in vivo* (Silver and Erecinska, 1990), but conflicts with a previous study of simulated ischemia in hippocampal slices (Mitani *et al.*, 1993). The apparent contradiction may reflect the different affinities of the Ca^{2+}-sensitive fluorophores used (rhod-2 in the latter study and Fura-2 in our own), and suggests that the early rise in $[Ca^{2+}]_c$ is relatively modest, requiring a high affinity probe such as Fura-2 to be detected. Alternatively, the difference may be explained by the different methods for simulating ischemia. Mitani *et al.*, (1993) simply removed oxygen and glucose (OGD), whereas we supplemented OGD with iodoacetic acid (IAA) to block glycolysis. If this methodological difference underlies the different Ca^{2+} responses in the respective slice studies, then our study's agreement with the *in vivo* study (Silver and Erecinska, 1990) suggests that for simulating ischemia *in vitro*, supplementing OGD with IAA, which is presumably more severe than OGD alone, more closely replicates the conditions of ischemia *in vivo*.

A previous study with hippocampal slices showed that the ryanodine receptor antagonist, dantrolene, prevented the anoxia-induced increase in mEPSC frequency (Katchman and Hershkowitz, 1993). Based on that finding, the authors suggested that Ca^{2+} release from intracellular stores in presynaptic terminals triggered the early increase in mEPSCs. While we do observe an early release of Ca^{2+} from intracellular stores that parallels the

increase in mEPSC frequency (Fig. 2&3), this calcium is unlikely to be the trigger for the increase in mEPSCs, under our conditions, since it can be blocked without preventing the ischemia-induced increase in mEPSCs (Fig. 6). It is possible that there are simply different triggers during anoxia and ischemia, with ischemia likely leading to a more rapid decline in ATP levels. An alternative explanation is that dantrolene interferes with anoxia-induced vesicle release either indirectly or via mechanisms other than blocking the ryanodine receptor (Salinska *et al.*, 2008; Krnjevic and Xu, 1996).

3.4.1 Source of Early Rise in $[Ca^{2+}]_c$

Our imaging protocol detects $[Ca^{2+}]_c$ signals in multiple subcellular compartments (Fig. 1), making it difficult to clearly define which compartment(s) lead to the schemia-induced increase in $[Ca^{2+}]_c$ we have observed. However, *in vivo* studies of ischemia have shown that hippocampal pyramidal cells exhibit an early rise in $[Ca^{2+}]_c$, similar in magnitude and time course to the rise that we observe in our bulk loaded slices (Silver and Erecinska, 1990). Furthermore, since the relationship between mEPSC frequency and presynaptic $[Ca^{2+}]_c$ is steep near the resting $[Ca^{2+}]_c$ (Frerking *et al.*, 1997), if ischemia caused early changes in presynaptic $[Ca^{2+}]_c$ we would expect it to affect mEPSC frequency. Moreover, preventing those changes by buffering Ca^{2+} should reduce the impact of ischemia on mEPSC frequency, which we did not observe (Fig. 6). Accordingly, while the particular subcellular compartment in which the rise in $[Ca^{2+}]_c$ occurs remains obscure, we think it unlikely that this increase takes place in presynaptic terminals.

Removal of external Ca^{2+} reduced the ischemia-induced early rise in $[Ca^{2+}]_c$ (Fig. 4A), demonstrating that some of the rise is due to Ca^{2+} influx across the plasma membrane. This is in agreement with *in vivo* ischemia studies showing that the early rise in $[Ca^{2+}]_c$ in pyramidal cells is accompanied by a parallel decrease in $[Ca^{2+}]$ in the extracellular space (Silver and Erecinska, 1990). There are many potential mechanisms by which Ca^{2+} might enter pyramidal cells or astrocytes during ischemia (Duffy and MacVicar, 1996; Zhang and Lipton, 1999; Lipton, 1999; Xiong *et al.*, 2004; Bondarenko *et al.*, 2005; Thompson et al., 2006; Rossi et al., 2007), but we can definitively rule out glutamate-gated channels for the early rise in $[Ca^{2+}]_c$ because blocking these channels did not affect the rise in $[Ca^{2+}]_c$ (Fig. 8). The fact that the early rise in $[Ca^{2+}]_c$ was not accompanied by a detectable membrane current in voltage-clamped pyramidal cells also argues against a role for voltage-gated channels, or for channels with a large unitary conductance such as hemi-gap junctions (Thompson *et al.*, 2006). Silver and Erecinska suggested that the early influx of Ca^{2+} was mediated by the Na^+-Ca^{2+}-exchanger pumping out Na^+ ions that entered via the Na^+-H^+-exchanger (Silver and Erecinska, 1990). While the Na^+-Ca^{2+}-exchanger is electrogenic (Hinata and Kimura, 2004), its slow rate of transport compared to channels would generate much smaller currents that would be difficult for us to detect.

While removal of external Ca^{2+} reduced the ischemia-induced early rise in $[Ca^{2+}]_c$, it did not abolish it (Fig. 4A), indicating an early release of Ca^{2+} from intracellular stores as well. To our knowledge, ischemia-induced changes in $[Ca^{2+}]_c$ and mEPSC frequency

have not been directly compared previously, so it is difficult to directly compare results from previous Ca^{2+} imaging studies to the early increase that we report here. Nonetheless, several studies have examined ischemia-induced release of Ca^{2+} from intracellular stores, and the processes discovered could contribute to the Ca^{2+} release observed with our preparation (Mitani *et al.*, 1993; Duffy and MacVicar, 1996; Zhang and Lipton, 1999; Henrich and Buckler, 2008).

3.4.2 Ischemia Induces Ca^{2+}-Independent Vesicle Release Due to Actin Filament Depolymerization in Presynaptic Terminals

Under physiological conditions, vesicle release is triggered by a rise in $[Ca^{2+}]_c$ in presynaptic terminals, and the spontaneous vesicle release rate is tightly coupled to resting $[Ca^{2+}]_c$ (Frerking *et al.*, 1997). Our data demonstrates that the early phase of ischemia induces vesicle release independently of changes in $[Ca^{2+}]_c$ (Fig. 6). Both hypertonic sucrose and α-latrotoxin can also induce vesicle release independent of changes in presynaptic $[Ca^{2+}]_c$ (Khvotchev *et al.*, 2000), but the underlying mechanisms are not well understood. The Ca^{2+}-independent actions of α-latrotoxin probably involves binding to latrophilin (Volynski *et al.*, 2003), but we are not aware of any reports regarding latrophilin and ischemia. In contrast, Latrunculin A has a well-understood effect as an actin depolymerizing agent, and is known to cause a Ca^{2+}-independent release of vesicles, presumably by depolymerizing actin filaments that normally restrain vesicle fusion (Morales *et al.*, 2000). Thus, an ischemia-induced reorganization of the

actin cytoskeleton might underlie the Ca^{2+}-independent increase in mEPSCs observed here.

The previous literature is consistent with this idea, as early events of brain ischemia include both depolymerization of actin filaments (Gisselsson *et al.*, 2005) and a repositioning of glutamatergic vesicles toward the plasma membrane (Williams and Grossman, 1970). Moreover, we have found that preventing actin filament depolymerization by presoaking slices in Jaspamide, an actin filament stabilizing agent which prevents vesicle release triggered by Latrunculin A (Morales *et al.*, 2000), abolished the ischemia-induced increase in mEPSCs (Fig 8). Thus, the early increase in mEPSC frequency during ischemia is due to actin filament depolymerization in presynaptic terminals and a corresponding release of vesicles, similar to that which occurs in response to Latrunculin A.

3.4.3 Ischemia-Induced Increases in mEPSC Frequency May Trigger Ischemic Preconditioning

Either transient episodes of ischemia or exogenously applied glutamate analogues can trigger actin depolymerization-mediated postsynaptic spine retraction (Hasbani *et al.*, 2001), and a resultant protected state (Halpain *et al.*, 1998; Hasbani *et al.*, 2001; Ikegaya *et al.*, 2001; Graber *et al.*, 2004). Furthermore, at least in some preparations, glutamate receptor antagonists can prevent the ischemia-induced spine retraction (Jourdain *et al.*,

2002). These observations suggest that a sub-toxic release of glutamate during the early stages of ischemia can be a trigger for spine retraction and ischemic preconditioning. The increase in glutamatergic vesicle release reported here and elsewhere is an obvious candidate trigger for this process.

Given the role of glutamate receptor activation in delayed cell death (Simon *et al.*, 1984b; Gill *et al.*, 1987; Pivovarova *et al.*, 2004), it is possible that the ischemia-induced increase in mEPSCs could trigger delayed cell death rather than preconditioning. However, for CA1 pyramidal cells, delayed cell death is dependent on Ca^{2+} influx through NMDA receptors (Pivovarova *et al.*, 2004), and in our study blocking glutamate receptors did not affect the ischemia-induced elevation in $[Ca^{2+}]_c$ (Fig. 8). Presumably, the reason the ischemia-induced increase in mEPSCs does not affect $[Ca^{2+}]_c$ is because cells remain hyperpolarized, which prevents Ca^{2+} influx through NMDA receptor channels (Mayer *et al.*, 1984). Furthermore, in hippocampal slices, removing extracellular Ca^{2+} can prevent glutamate receptor triggered cell death without preventing changes in dendritic morphology or preconditioning, the latter effects being triggered by Na^{+} influx (Ikegaya *et al.*, 2001). Accordingly, we favor our preconditioning trigger hypothesis. Thus, actin filament depolymerization may play a dual role in ischemic preconditioning, with presynaptic depolymerization driving vesicular glutamate release, which then triggers glutamate induced-depolymerization postsynaptically to induce spine retraction and subsequent protection.

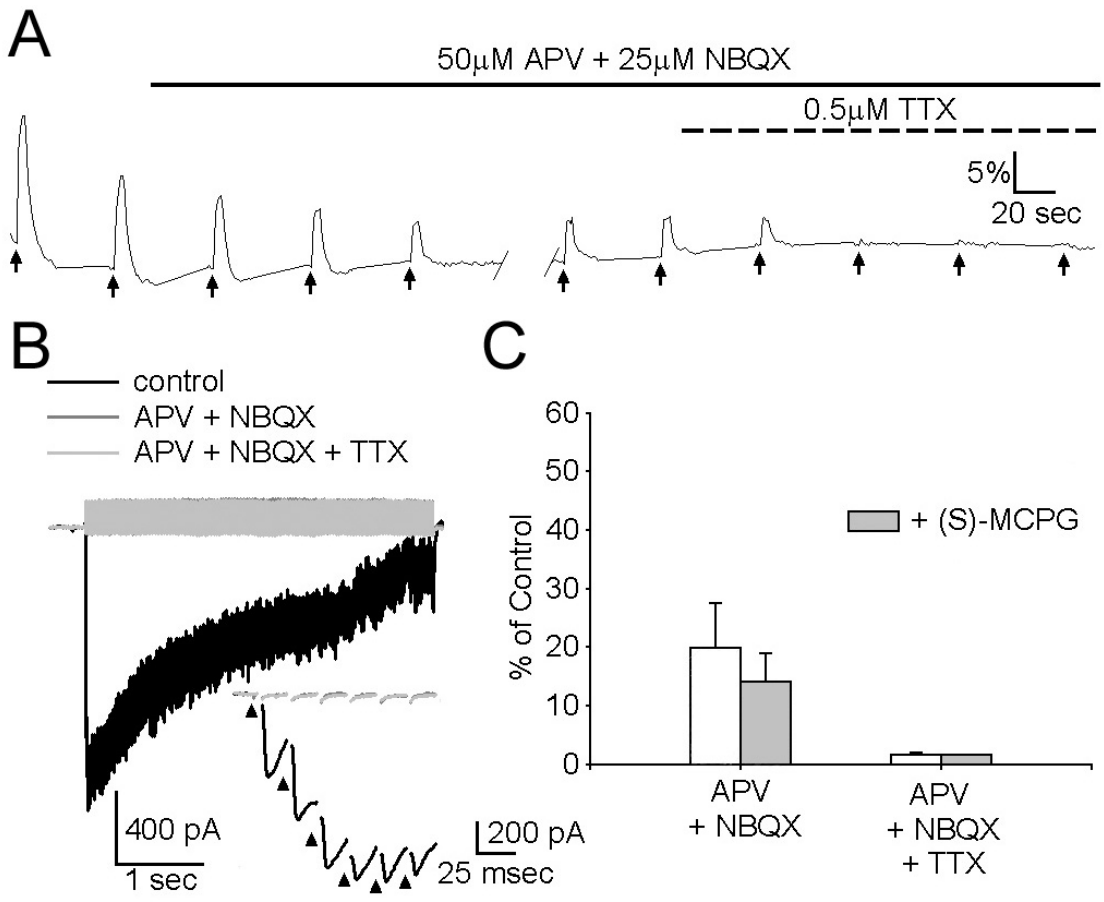

Figure 1. Fura-2 signal in bulk loaded slices reflects $[Ca^{2+}]_c$ in pre- and post synaptic compartments. A. Ratio of the whole field fluorescence emission at 340/380nm excitation during trains of stimulation under various pharmacological conditions. Solid bar indicates application of AP5 (50μM) and NBQX (25μM), and dashed bar indicates application of TTX (0.5μM). Arrows indicate time of tetanic stimulation. **B.** Summating EPSC in a voltage-clamped (Vh=-60mV) CA1 pyramidal cell, evoked by tetanizing the Schaeffer collateral pathway (black), is abolished by AP5 and NBQX (dark grey) or AP5, NBQX and TTX (light grey). Inset shows an expanded time frame of the rising phase of

the response, allowing visualization of individual EPSCs and complete block by AP5 and NBQX. Arrowheads indicate individual stimuli. **C.** Bar chart depicting the mean of the peak percent change of fluorescence emission ratio under various pharmacological conditions (expressed as a percent of the peak response under control conditions). Conditions are D-AP5 (50µM) and NBQX (25µM) either alone (left, white) or combined with (S)-MCPG (500µM, left, grey), or D-AP5, NBQX, and TTX (0.5µM) either alone (right, white), or combined with (S)-MCPG (right, grey).

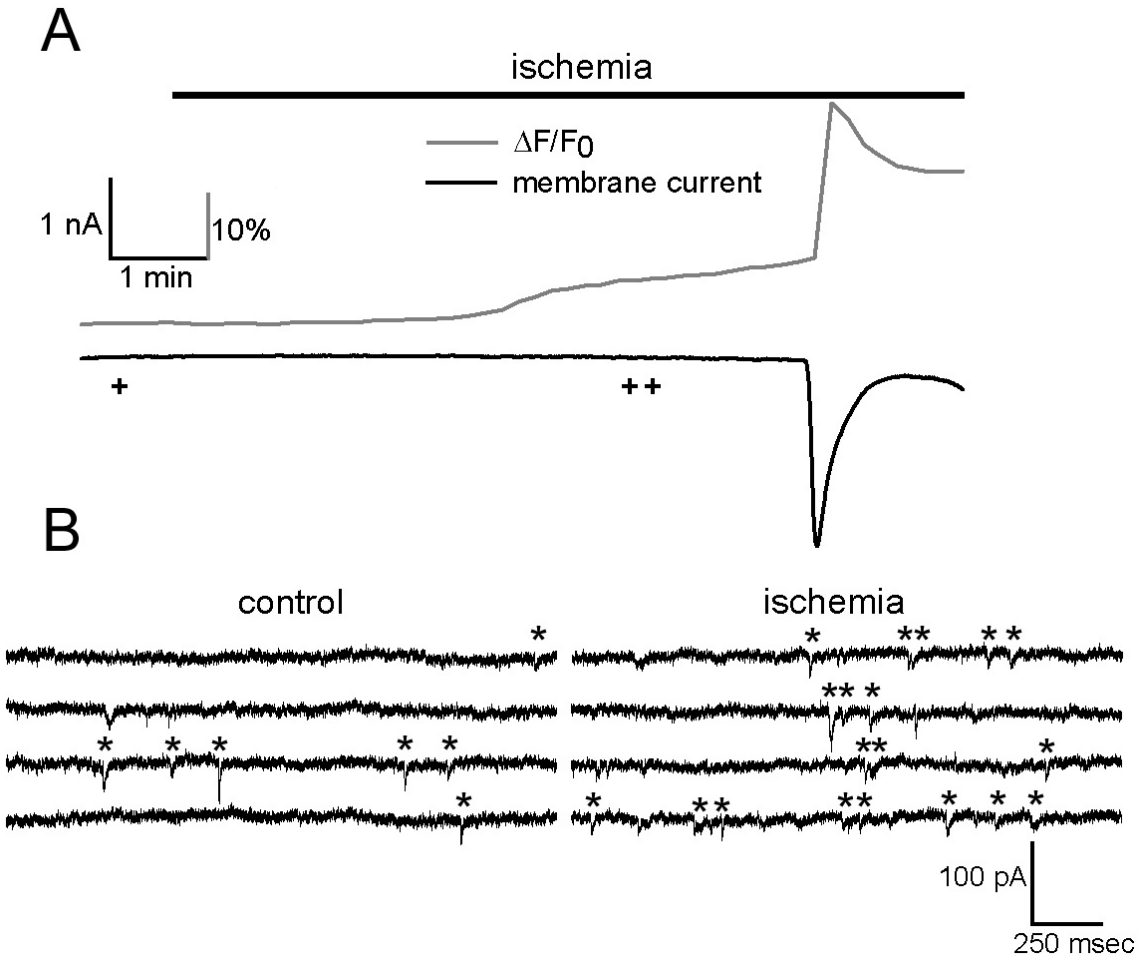

Figure 2. Simulated ischemia causes an early rise in $[Ca^{2+}]_c$ and an increase in mEPSCs prior to the ischemic depolarization. A. Whole field fluorescence emission ratio (grey, top trace, expressed as percent change) and the corresponding membrane current (black, bottom trace) of a voltage-clamped (Vh= -60mV) CA1 pyramidal cell, in the field of fluorescence measurement. Note, + and ++ indicate regions of membrane current used for expanded time and amplitude in B. **B.** mEPSCs in expanded time scale plot of membrane current from the regions of the macroscopic current indicated by the + corresponding to control, and during the early stage of simulated ischemia ++, prior to

the ID current. Individual mEPSCs are indicated by asterisks. Note, the 4 sweeps in each set are temporally contiguous.

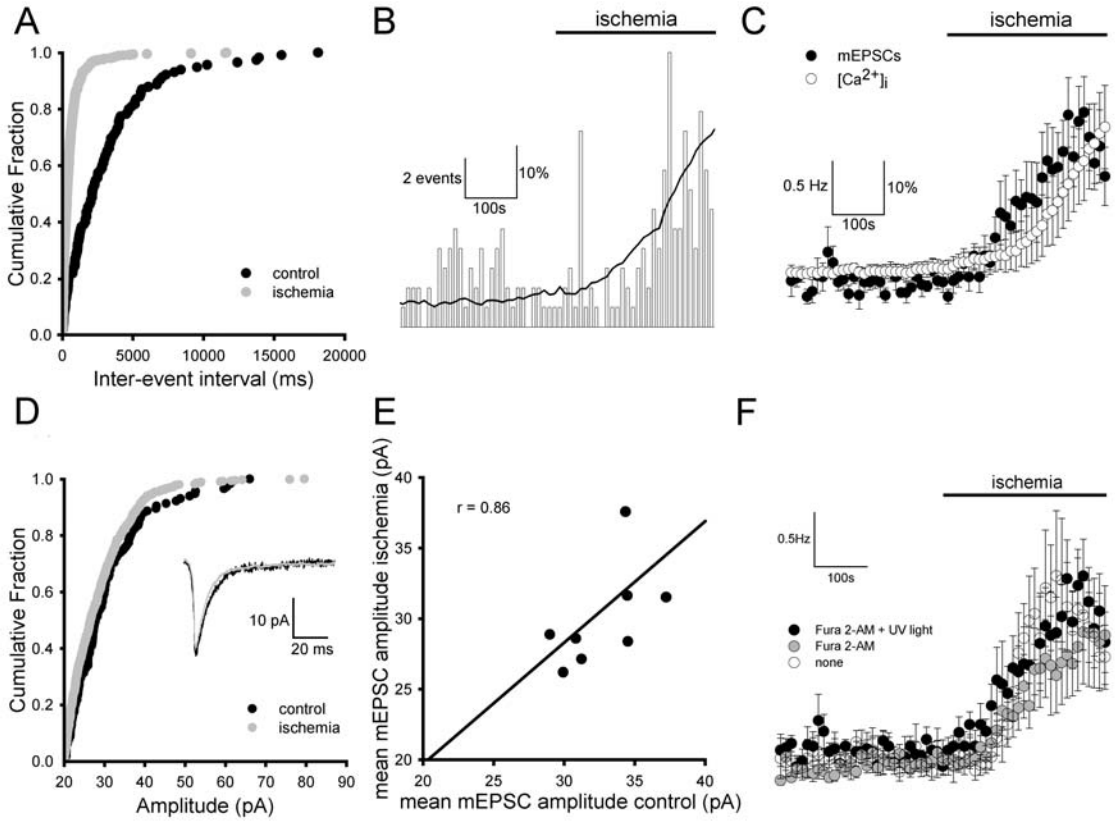

Figure 3. Ischemia-induced increase in mEPSCs parallels ischemia-induced rise in $[Ca^{2+}]_c$. A. Cumulative fraction plot of mEPSC inter-event interval for a single cell, under control conditions (black) and during the early stages of ischemia (as in Fig. 2, grey). **B.** Plot of mEPSC frequency (in 10 second bins) aligned with mean percent change in Fura-2 fluorescence ratio for a single experiment. **C.** Plot of mean mEPSC frequency aligned with mean percent change in Fura-2 fluorescence ratio (n=8). **D.** Cumulative fraction plot of mEPSC amplitude for a single cell, under control conditions (black) and during the early stages of ischemia (as in Fig. 2, grey). Inset shows mean mEPSC waveform aligned by rise time in control (black) and in ischemia (grey). **E.** Plot of mEPSC amplitude under control conditions versus mEPSC amplitude during ischemia. **F.**

Plot of mean ischemia induced increase in mEPSC frequency in cells from slices that were: loaded with Fura-2-AM and exposed to UV light as in B&C (black), loaded with Fura-2-AM without UV exposure (grey), neither loaded with Fura-2-AM nor exposed to UV light (white). The mean mEPSC frequency in Fura-2 loaded slices or Fura-2 loaded and UV exposed slices were not significantly different from the mEPSCs frequency in unloaded, unexposed slices at any time point during control or ischemia.

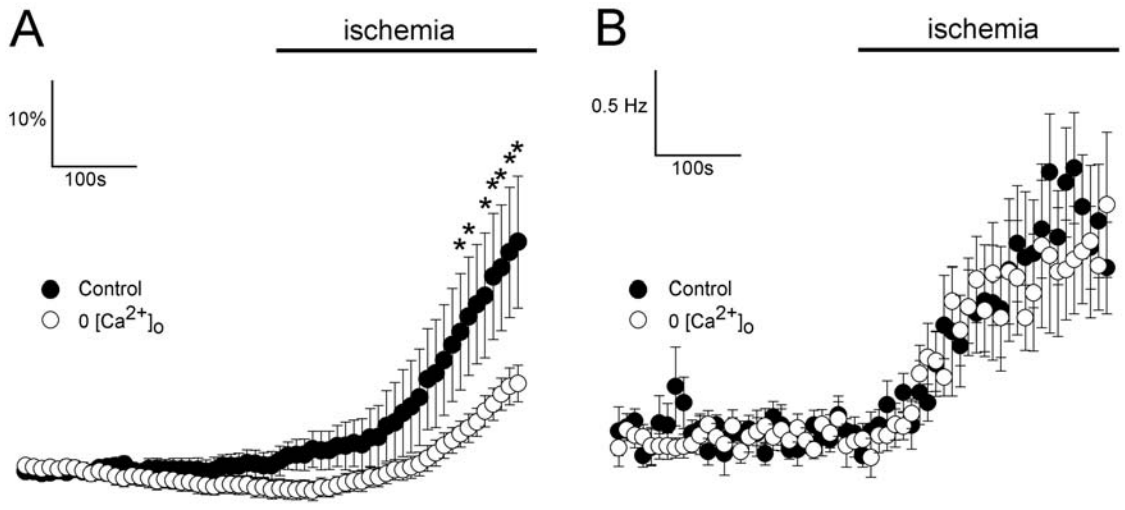

Figure 4. Removing extracellular Ca^{2+} reduces the ischemia-induced rise in $[Ca^{2+}]_c$ without affecting the increase in mEPSCs. A. Plot of mean percent change in Fura-2 emission ratio under normal ischemia conditions (black) and when extracellular Ca^{2+} is replaced with equimolar Mg^{2+} and 2mM EGTA to chelate trace residual Ca^{2+} (white). Asterisks indicate time points where the percent increase is significantly different between conditions (P<0.05, t-test, n=11). **B.** Plot of mean mEPSC frequency under normal ischemia conditions (black) and with extracellular Ca^{2+} removed (white) as in A. The mEPSC frequencies were not significantly different at any time point.

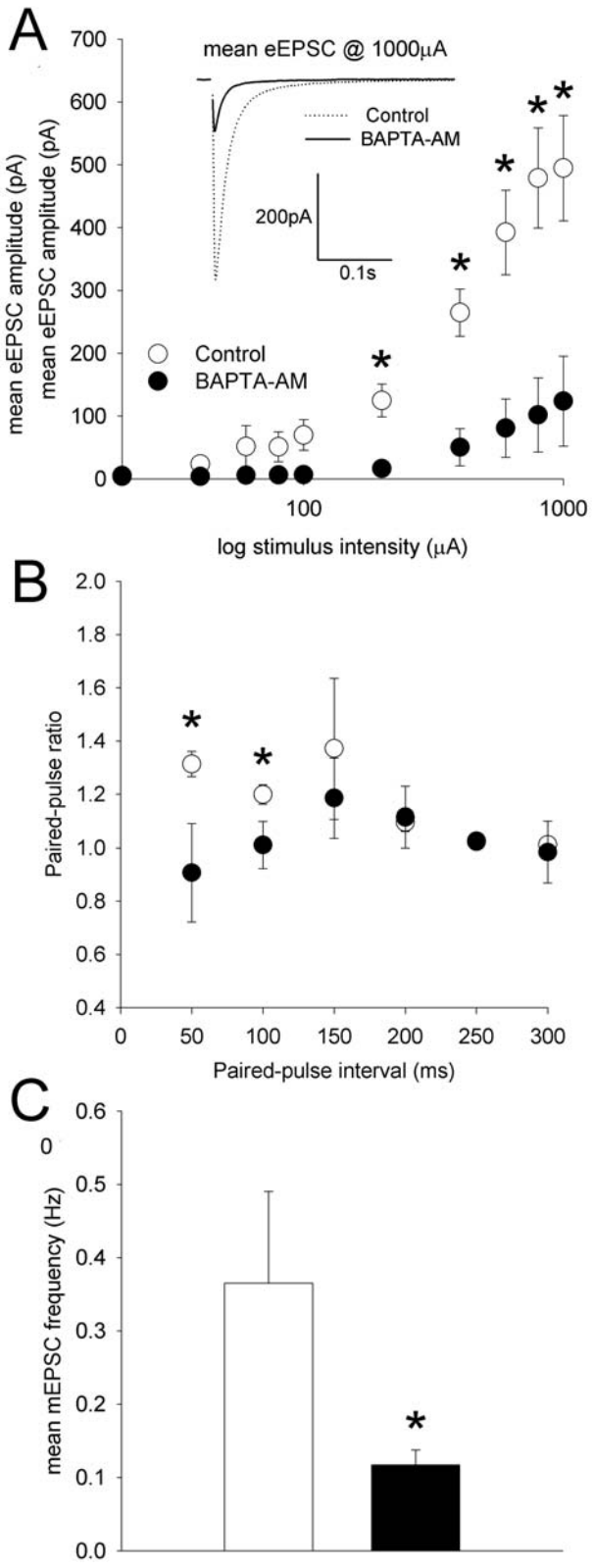
A
mean eEPSC @ 1000μA
Control
BAPTA-AM
200pA
0.1s
mean eEPSC amplitude (pA)
mean eEPSC amplitude (pA)
Control
BAPTA-AM
log stimulus intensity (μA)
B
Paired-pulse ratio
Paired-pulse interval (ms)
C
mean mEPSC frequency (Hz)

Figure 5. BAPTA-AM loading reduces basal $[Ca^{2+}]_c$ and strongly buffers Ca^{2+} transients in presynaptic terminals. A. Plot of eEPSC stimulus intensity response curves for control slices (white) and slices pre-soaked in BAPTA-AM (black). For both conditions, eEPSC amplitudes did not increase for stimulus strengths greater than 1mA (up to 10mA). Asterisks indicate stimulus intensities for which the amplitude of the eEPSCs are significantly different ($P<0.05$, t-test, n=4). Inset shows representative mean EPSC evoked by 1mA stimuli in a control slice (dotted) and a BAPTA loaded slice (black). **B.** Plot of paired pulse ratio evoked with maximal stimulus strength (1mA) for control slices (white) and slices pre-soaked in BAPTA-AM (black). Asterisks indicate significant differences between conditions ($P<0.05$, t-test, n=4). **C.** Plot of mean mEPSC frequency in control slices (white) and slices pre-soaked in BAPTA-AM (black). Asterisk indicates that mEPSC frequency was significantly reduced ($P<0.05$, t-test, n=8) in BAPTA-AM soaked slices.

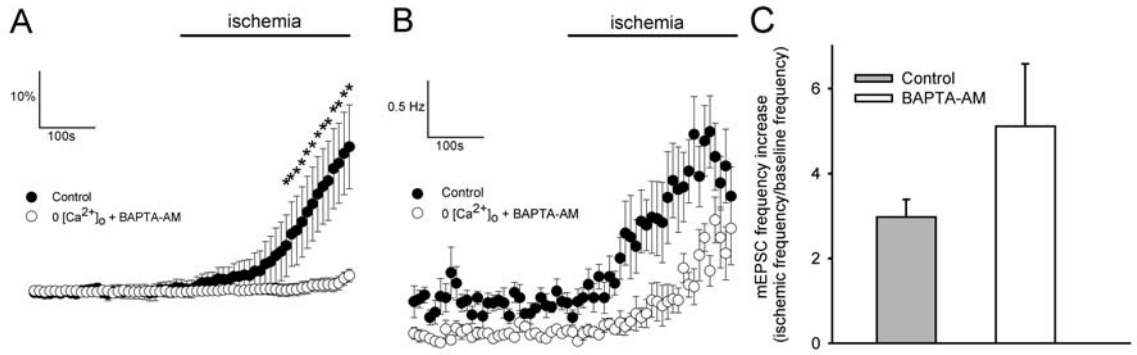

Figure 6. Soaking slices in BAPTA-AM and removing extracellular Ca^{2+} abolishes ischemia-induced increases in $[Ca^{2+}]_c$, but does not prevent ischemia-induced increases in mEPSC frequency. A. Plot of mean percent change in Fura-2 emission ratio under normal ischemia conditions (black) and when BAPTA-AM presoaked slices are exposed to ischemia with extracellular Ca^{2+} removed as in Fig. 4 (white). Asterisks indicate time points where the percent increase is significantly different between conditions (P<0.05, t-test, n=16). **B.** Plot of mean mEPSC frequency under normal ischemia conditions (black) and when BAPTA-AM presoaked slices are exposed to ischemia with extracellular Ca^{2+} removed as in Fig. 4 (white). **C.** Bar chart depicting mean ischemia-induced increase in mEPSC frequency (expressed as a ratio to control mEPSC frequency for each condition) for slices under normal ischemia conditions (grey) and for BAPTA-AM presoaked slices exposed to ischemia with extracellular Ca^{2+} removed as in Fig. 4 (white).

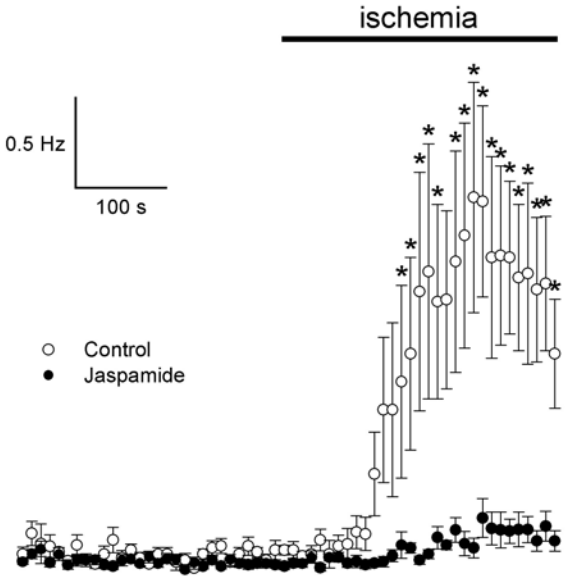

Figure 7. Preventing actin filament depolymerization blocks the ischemia-induced increase in mEPSC frequency. Graph depicting mEPSC frequency change induced by ischemia in control slices (white) and slices pre-soaked in Jaspamide (20µM, black). Asterisks indicate time points where the mEPSC frequency is significantly different between conditions ($P<0.05$, t-test, n=9 and 12 respectively).

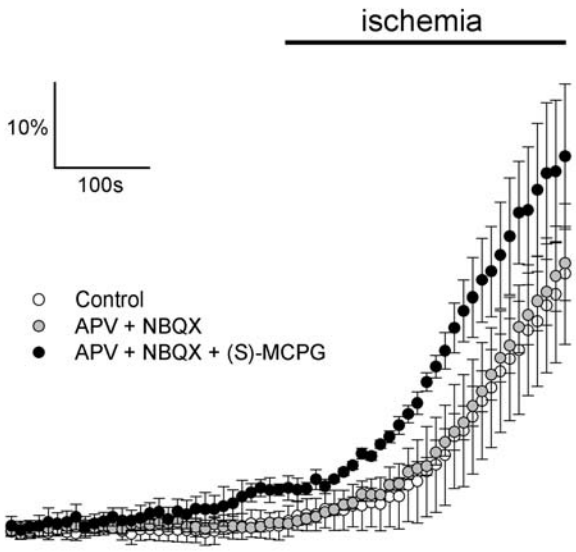

Figure 8. Blocking glutamate receptors does not affect the early ischemia-induced rise in $[Ca^{2+}]_c$. Plot of mean percent change in Fura-2 emission ratio during ischemia alone (white), in the presence of D-AP5 (50µM) and NBQX (25µM, grey), and in the presence of D-AP5 (50µM), NBQX (25µM) and (S)-MCPG (500µM, black). There were no significant differences (n=8, 4, and 3, respectively).

Chapter 4:

Simulated ischemia causes an increase in $[Ca^{2+}]_i$ but not an increase in mEPSC frequency in cerebellar synapses during the first 5 minutes of the ischemia

Adriana L. Andrade and David J. Rossi

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4.1 Introduction

Brain ischemia causes damage to neural tissue by triggering a sequence of events which can be classified in two phases. The first of these is characterized by a small rise in extracellular K^+ with little change in the other principal ion concentrations, the cessation of EEG activity, and an increase in the frequency of miniature excitatory post-synaptic potentials (mEPSCs). The second phase is marked by a severe disregulation of all principal ions, a rapid and large ischemic depolarization, and a massive increase in extracellular glutamate concentration caused by the reversal of glutamate transporters (Astrup et al., 1977; Hansen and Nedergaard, 1988; Rossi et al., 2007). Studies characterizing the two phases of the ischemic response have focused primarily on the hippocampus and other forebrain structures in the brain.

Transient episodes of ischemia can either lead to ischemic preconditioning, whereby brain tissue becomes more resistant to subsequent episodes of ischemia (Obrenovitch, 2008), or to selective cell death in ischemia-sensitive neurons (Pulsinelli, 1985). Presumably, either outcome is triggered primarily by the early events of ischemia that occur during the first phase described above, because the onset of the second phase typically leads to pan necrosis. In CA1 neurons, the earliest response to ischemia is an increase in the frequency of miniature excitatory postsynaptic currents (mEPSCs) paralleled by an increase in the cytosolic calcium concentration ($[Ca^{2+}]_c$) (Hershkovitch *et al.*, 1993; Andrade and Rossi, unpublished results). It is possible that this mechanism of increased glutamatergic excitation could contribute to either ischemic preconditioning (Jiang *et al.*, 2003; Miao *et al.*, 2005; Lin *et al.*, 2008), or to selective neuronal death in ischemia-sensitive neurons (Gill *et al.*, 1987). The cerebellum, like the hippocampus, is particularly vulnerable to brain anoxia or ischemia, showing selective loss of Purkinje cells with a sensitivity second only to the CA1 neurons of the hippocampus (Pulsinelli, 1985; Cervos- Navarro and Diemer, 1991).

While the second phase of the ischemic response has been characterized in Purkinje cells in the cerebellum (Hamann *et al.*, 2005), the earlier phase has not yet been examined in this brain region. It is possible that selective cell death of Purkinje cells is due to glutamatergic ischemic responses that occur prior to the catastrophic ion deregulation associated with the ischemic depolarization (Haman *et al.*, 2005). We recently

demonstrated that the increase in mEPSC frequency in the hippocampus is due to depolymerization of actin filaments in presynaptic terminals (Andrade and Rossi, unpublished results). We therefore sought to determine if the early response to ischemia in the Purkinje cells of the cerebellum was similar to that of the CA1 neurons in the hippocampus. Here we report that, contrary to what occurs in CA1 neurons, there is no increase in mEPSC frequency in Purkinje cells during the first 5 minutes of ischemia, while there is an increase in $[Ca^{2+}]_c$. We also found that while in CA3 synaptic terminals actin filament dynamics influences Ca^{2+}-triggered exocytosis, the same is not true for the parallel-fiber and climbing-fiber synaptic terminals. Thus, the increase in mEPSC frequency is a phenomenon associated with synapses where Ca^{2+}-triggered vesicle release is modulated by actin depolymerization.

4.2 Materials and Methods

4.2.1 Reagents

Fura-2-AM (Invitrogen, Carlsbad, CA), BAPTA-AM (Sigma, St. Louis, MO), AP5, TTX, NBQX, (S)-MCPG (Ascent scientific, Great Britain), Jaspamide (Alexis Biochem, Plymouth Meeting, PA).

4.2.2 Preparation of Brain Slices

The hippocampus and cerebellum were obtained from Sprague-Dawley albino rats (Rossi and Slater, 1993; Rossi and Hamann, 1998; Rossi *et al.*, 2000). Rats (18-21 days old) were anaesthetized with Isoflurane and killed by decapitation. The whole brain was rapidly isolated and immersed in ice cold (0-2ºC) artificial cerebrospinal fluid (ACSF) containing (in mM): 124 NaCl, 26 $NaHCO_3$, 1 NaH_2PO_4, 2.5 KCl, 2.5 $CaCl_2$, 2 $MgCl_2$, 10 D-glucose, and bubbled with 95%O_2/5% CO_2 (pH 7.4). The hippocampus or cerebellum was dissected out of the brain and mounted in a slicing chamber filled with ice cold (0-2ºC) ACSF. Slices (225µm) were made with a vibrating tissue slicer (Vibratome). For cerebellar parallel-fiber stimulation experiments, slices were made on the coronal plane, whereas the sagital plane was used for all other experiments. Slices were incubated in warmed ACSF (33±1ºC) for one hour after dissection and then held at 22-23ºC until used. Kynurenic acid (1 mM) was included in the dissection, incubation and holding solution (to block glutamate receptors to reduce potential excitotoxic damage) but was omitted from the experimental solutions.

4.2.3 Visualized Patch-Clamp Recording from Cells in Brain Slices

Slices were placed in a submersion chamber on an upright microscope, and viewed with an Olympus 60X (0.9 numerical aperture) water immersion objective with differential interference contrast and infrared optics. Slices were perfused with heated (32-34ºC) ACSF at a rate of ~4ml/min. Drugs were dissolved in ACSF and applied by bath

perfusion. Whole-cell recordings were made from the somata of visually identified CA1 pyramidal cells. Patch pipettes were constructed from thick-walled borosilicate glass capillaries and filled with an internal solution containing (mM) Csgluconate 130, NaCl 4, $CaCl_2$ 0.5, HEPES 10, EGTA 5, MgATP 4, Na_2GTP 0.5, QX-314 5 (to suppress voltage-gated sodium currents), pH adjusted to 7.2 with CsOH. For stimulation experiments in the cerebellum, the internal solution contained (in mM): Csgluconate 130, NaCl 4, HEPES 10, BAPTA 5, MgATP 4, Na_2GTP 0.5, QX-314 5, paxilene 0.1, TEA 10. Electrode resistance was 1.5 to 2.5 MΩ. Cells were rejected if access resistance was greater than 5 MΩ. Cells were also rejected if the access resistance, monitored with –5mV voltage steps, changed by more than 20% during the course of an experiment.

4.2.4 Simulating Ischemia in Brain Slices

We simulated severe brain ischemia by exposing slices to solution in which glucose and oxygen were replaced with sucrose and nitrogen, and supplemented with iodoacetic acid (2mM) to block glycolysis. Previously we also supplemented our ischemia simulation solution with cyanide (1mM) to block oxidative phosphorylation (Rossi *et al.*, 2000; Hamann *et al.*, 2005). Because cyanide may have chemical interactions with either glutamate receptors or glutamate transporters independent of cellular responses to energy deprivation, in this project we did not include cyanide. The only significant difference that we observed between these two methods of simulating ischemia was a slightly more rapidly developing response when cyanide was included (Data not shown).

4.2.5 Loading Slices with Fura 2-AM and jaspamide

Stock solutions of the acetoxymethyl (AM) ester of Fura 2 (10mM) or Jaspamide (5mM) were made in DMSO with 20% pluronic acid, and sonicated for ~1 minute on the day of experiments. Stock solutions were added to our standard incubation solution at final concentrations of 100µM for Fura 2-AM and 20µM for jaspamide, followed by brief sonication. Slices were incubated in final solution for 1 hour, and experiments were conducted within 45 minutes of removal from the incubation solution.

4.2.6 Quantifying Changes in Fura-2 Fluorescence Emission

All values are expressed as a percent change in the ratio of Fura-2 fluorescence emission when excited at 340nm and 380nm with the equation $(F_t-F_0)/F_0$, where F_t is the emission ratio at time t and F_0 is the emission ratio at time 0. For all experimental conditions, a separate series of experiments was conducted with slices that were not loaded with Fura 2, and the autofluorescence emission at each excitation wavelength was averaged for at least 5 slices. The resultant mean autofluorescence signal for each excitation wavelength was subtracted from the experimental emission signal for each, individual Fura 2 loaded slice.

4.2.7 Data Acquisition, Analysis and Statistics

CA1 pyramidal cells and Purkinje cells were voltage-clamped at -60mV with an Axoclamp 700b patch clamp amplifier (Axon Instruments), and mEPSCs were filtered at 5kHz and acquired at 20kHz with pClamp software (Axon Instruments). Synaptosoft mini analysis software (Synaptosoft Inc.) was used to analyze mEPSC frequency and amplitude. Whole field (1343X780 pixels after 600X magnification) Fura 2 fluorescence was acquired and analyzed using Slidebook software (Olympus microscopes LTD). For stimulating experiments in the cerebellum, Purkinje cells were voltage-clamped at -20mV to improve voltage control. All data are presented as means±S.E.M., and statistical significance was defined as $P<0.05$ for paired or unpaired students t-test.

4.3 Results

4.3.1 Ischemia in the cerebellum cause an early rise in $[Ca^{2+}]_c$ but no increase in the frequency of mEPSCs

In the hippocampus, the first phase of the ischemic response is characterized by an increase in mEPSC frequency paralleled by a rise in $[Ca^{2+}]_c$, beginning at approximately 2 minutes of ischemia (Chapter 4). To examine the Purkinje cell response during the same phase of ischemia, we whole-cell voltage-clamped Purkinje cells from acute cerebellar slices and simulated ischemia as in our previous study (Chapter 4). Glutamatergic mEPSCs were isolated by inclusion of tetrodotoxin (0.5µM) and gabazine

(10μM) in the extracellular solutions. In stark contrast to the CA1 neurons of the hippocampus, in all cells analyzed, the initial 5 minutes of ischemia had no effect on the frequency of mEPSCs (Fig. 1A). To determine if there are any change in $[Ca^{2+}]_c$ in the cerebellum during the initial response to ischemia, we employed calcium imaging in a broad region of interest in the cerebellum, including the granule cell layer, molecular layer and Purkinje cell layer. Slices were pre-incubated with the acetoxymethyl (AM) ester derivative of the calcium dye, Fura-2 (100uM; Grynkiewicz *et al.*, 1985), for 1 hour, and washed for about 15 minutes with ACSF prior to experimentation. We found that in the cerebellum, although there is no increase in mEPSC frequency in the early response to ischemia, there is a rise in $[Ca^{2+}]_c$ very similar to the CA1 region of the hippocampus (Fig. 1B).

4.3.2 Actin stabilization in the CA3 synaptic terminals of the hippocampus reduces the amplitude of Ca^{2+}-triggered exocytosis in CA1 neurons by decreasing the probability of release

Since the increase in mEPSC frequency in the hippocampus occurs through the destabilization of actin filaments, we wanted to see what role actin plays in the evoked responses in both CA1 neurons and Purkinje cells. To determine the effects of actin filament stabilization in evoked responses, we pre-incubated slices with the actin filament stabilizer Jaspamide (20μM) for 1 hour prior to experimentation. In the hippocampus we stimulated the Schaffer collaterals by placing the stimulating electrode in the CA3

subregion, and recorded the evoked responses in CA1 neurons. We found that jaspamide significantly reduced the size of the evoked responses (Fig. 2A1 and 2), suggesting that actin filament depolymerization promotes vesicle release during Ca^{2+}-triggered exocytosis. To rule out the possibility that jaspamide reduced glutamate receptor conductances, we compared the amplitudes of mEPSCs in both control slices and slices pre-incubated with jaspamide. We found that jaspamide did not have an effect on the amplitude of mEPSCs, suggesting that the reduction in the amplitude of the evoked EPSCs is due to a presynaptic mechanism (Fig. 2C). Thus, to determine if the reduction in evoked EPSC amplitudes by actin filament stabilization was due to a reduction in the probability of release, we examined paired-pulse responses in both control slices and slices pre-incubated with jaspamide. Indeed, jaspamide reduced the probability of release in CA3 synaptic terminals, as paired-pulse ratios were increased at all paired-pulse intervals tested (Fig. 2B).

4.3.3 Actin stabilization in the parallel-fiber and climbing-fiber synaptic terminals of the cerebellum does not affect Ca^{2+}-triggered exocytosis

To examine the role of actin filament stabilization in Ca^{2+}-triggered exocytosis in Purkinje cell synaptic inputs, we examined the effects of jaspamide pre-incubations on Purkinje cell responses to climbing-fiber and parallel-fiber stimulations. To improve voltage control, Purkinje cells were held at -20mV and an intracellular solution

containing paxilline and TEA to block cycling currents from BK and SK channels (Sanchez and McManus, 1996). Climbing-fiber responses were identified by their large size and all-or-nothing response at threshold stimuli. We found that jaspamide pre-incubations did not affect the amplitude of the evoked climbing-fiber response or its paired-pulse ratio (Fig. 3A and B). Parallel-fiber responses were identified by their smaller amplitude and graded responses to increasing stimulus strength. As in the climbing-fiber response, jaspamide pre-incubations did not affect the amplitude of the evoked parallel-fiber response or its paired-pulse ratio (Fig. 4A and B). Together, the data presented here demonstrate fundamental differences in the role of actin filament dynamics at CA1 pyramidal cell and Purkinje cell synapses, with actin filament stabilization reducing vesicle release probability in CA3 synaptic terminals but not in climbing-fiber and parallel-fiber synaptic terminals. Thus, our data suggests that the increase in mEPSC frequency is a phenomenon associated with synapses where Ca^{2+}-triggered vesicle release is modulated by actin filament depolymerization.

4.4 Discussion

We have demonstrated that in contrast to the CA1 neurons of the hippocampus, the Purkinje cells of the cerebellum do not exhibit an increase in mEPSC frequency during the first five minutes of the ischemic response. Even though there is no increase in mEPSC frequency in Purkinje cells during early ischemia, there is a rise in calcium similar to the hippocampus (Fig. 1B). Although the specific mechanisms leading to the

increase in $[Ca^{2+}]_c$ is not known, in the hippocampus it is due to Ca^{2+} influx and release from intracellular stores (Chapter 4). To our knowledge, Purkinje cells are the only cells described so far which do not respond to ischemia with an early increase in mEPSCs (Hershkowitz *et al*., 1993; Fleidervish *et al*., 2001; Zhang *et al*., 2006; Nishimura, 1986). This is not the only difference in ischemic responses in these two brain region. We have previously found that during the second phase of ischemia, the glutamatergic response in Purkinje cells is mediated by non-NMDA receptors, primarily a desensitization-resistant AMPA receptor, while in the CA1 hippocampal neurons it is mediated mainly by NMDA receptors (Hamann *et al.*, 2005). In the same study, we also found that in the cerebellum, glutamate release is drastically reduced by calcium removal during the initial 10 minutes of ischemia in contrast to the hippocampus where glutamate release is unaffected by calcium removal. Thus even though the hippocampus and cerebellum are regions that are very ischemia-sensitive, many of the physiological processes that occur in these regions during metabolic deprivation are very different.

Purkinje cell death occurs via a glutamatergic process, as it can prevented by administration of AMPA receptor antagonists (Balchen and Diemer, 1992). Since the Purkinje cells do not demonstrate an increase in mEPSCs during the first phase of the ischemic response, glutamate release by the reversal of transporters is the most likely source of glutamate that is responsible for triggering cell death in Purkinje cells. As previously discussed, the increase in mEPSC frequency could potentially play a role in ischemic preconditioning, whereby the brain becomes more resistant to subsequent

ischemic episodes. It would be interesting in future studies to see if Purkinje cells precondition, as this is yet unknown.

The ischemia induced increase in mEPSC frequency is not related to the release probability of synapses. Parallel-fiber terminals have a low probability of release and show facilitation upon repeated stimulation, similar to the CA3 terminals (Dittman *et al.*, 2000), however they do not generate an increase in mEPSC frequency during ischemia. It is understandable why probability of release is unrelated to the phenomena of increased vesicle release during early ischemic responses, as probability of release is a trait that is dependent on $[Ca^{2+}]_c$ (Dittman *et al.*, 2000), whereas the ischemia-induced increase in mEPSC frequency is calcium-independent (Chapter 4).

The ischemia-induced increase in mEPSC frequency in CA1 pyramidal cells is caused by the depolymerization of actin filaments in synaptic terminals (Chapter 4). The current understanding of the role of actin in presynaptic terminals is two-fold. Actin acts as a scaffold to which reserve vesicles are tethered (Greengard *et al.*, 1994; Hilficker, 1999; Evergren *et al.*, 2007) and it is also a major constituent of the active zone (Hirokawa *et al.*, 1989; Bloom, 2003; Phillips *et al.*, 2001). Thus actin can play a role in sequestering vesicles (Morales *et al.*, 2000), where actin filament depolymerization promotes vesicle release; and in vesicle recruitment for fusion, were compromising actin will have an inhibitory effect on release (Kuromi and Kidokoro, 1998; Delgado *et al.*, 2000; Watanabe *et al.*, 2005). These effects are synapse-specific and vary with the intrinsic requirements

for vesicular release. We found that actin filament stabilization reduced the amplitude of eEPSCs in the hippocampus (Fig. 2), consistent with previous studies demonstrating that actin plays a role in vesicle sequestering in the CA3-CA1 synapse which is antagonized by actin filament depolymerization with latrunculin A (Morales *et al.*, 2000).

To our knowledge, this is the first time the role of actin filament dynamics has been tested in cerebellar synapses. We found that actin stabilization has no effect on Ca^{2+}-triggered exocytosis in parallel-fiber and climbing-fiber synaptic terminals (Figs. 3 and 4). It is possible that in these terminals, actin plays neither a vesicle-sequestering role nor a vesicle-recruitment role. Alternatively, both processes could have been affected by actin filament stabilization and canceled each other out, as might be expected if actin is important for both processes (Cingolani and Goda, 2008). In a similar manner, it is possible that the lack of an increase in mEPSC frequency during ischemia in Purkinje cells is due to a limited role played by actin in parallel-fiber and climbing-fiber synaptic terminals; or it is possible that ischemia-induced actin filament depolymerization affects both a vesicle-sequestering process and a vesicle-recruitment process that cancels each other out.

Chapter 5:
Concluding Remarks

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5.1 Conclusions

Based on the work presented in this thesis, the following conclusions related to brain ischemia were derived:

1. Similar to CA1 pyramidal cells, ischemia rapidly causes Purkinje cell depolarization, and the ischemic depolarization is generated by glutamate receptors.

2. In contrast to CA1 pyramidal cells, the Purkinje cell ischemic glutamate-mediated current is generated entirely by non-NMDA receptors, primarily of a desensitization-resistant AMPA receptor subtype.

3. In contrast to the hippocampus, during the first 10-15 minutes, ischemic glutamate release in the cerebellum is strongly dependent on $[Ca^{2+}]_e$ influx.

4. Similar to the hippocampus, ischemic glutamate release in the cerebellum is strongly inhibited by preloading slices with the transportable glutamate analogue, PDC.

5. In the CA1 region of the hippocampus, the ischemic early rise in $[Ca^{2+}]_i$ is partly due to $[Ca^{2+}]_e$ influx and partly due to Ca^{2+} release from intracellular stores.

6. In the CA1 region of the hippocampus, the ischemic early rise in $[Ca^{2+}]_i$ is not mediated by glutamate receptor activation.

7. In CA1 pyramidal cells, the ischemia-induced increase in mEPSC frequency is calcium-independent.

8. In CA1 pyramidal cells, the ischemia-induced increase in mEPSC frequency is caused by actin filament depolymerization.

9. In contrast to CA1 pyramidal cells, there is no ischemia-induced increase in mEPSCs in Purkinje cells during the first 5 min of ischemia.

10. Similar to the hippocampus, ischemia triggers an early rise in $[Ca^{2+}]_i$ in the cerebellum, supporting that the rise in calcium is not caused by mEPSCs.

11. The differences in mEPSC responses in CA1 pyramidal cells and Purkinje cells could be due to instrinsic differences in synapses. In the CA3 synaptic terminals, actin filament defragmentation plays a role in evoked vesicle release, while it does not in the parallel-fiber terminals and climbing-fiber terminals in the cerebellum.

5.2 Concluding Remarks

Via a decline in the concentration of intracellular ATP levels, ischemia rapidly triggers a progression of events in brain tissue which eventually lead to neuronal damage. These events have been classified into two phases according to the time of their occurrence: an earlier response, where the frequency of mEPSCs increases in the hippocampus, and no severe ionic disregulation occurs; and a later response, where there is a severe ionic disregulation and a massive glutamate-generated current. While the mechanisms underlying glutamate release in the later phase of the ischemic response are well understood, not much was known about the mechanisms underlying the earlier phase of the response. My work has contributed to the elucidation of the mechanism of glutamate release during the first phase of the ischemic response. While much of ischemia research has focused on the hippocampus, other areas of the brain have received less attention. The cerebellum has a different organization and molecular make-up than the hippocampus, and could potentially exhibit very different responses than the hippocampus. My work has also contributed to the distinction between the early and late

ischemic responses in the hippocampus and cerebellum. Together, these findings may aid in the search for therapies aimed at salvaging ischemic brain tissue.

5.2.1 The Early Glutamatergic Response to Ischemia

It is known that glutamate is the main trigger of cell death in focal ischemia models, which simulate severe or permanent ischemia; and in transient global ischemia models, which simulate brief episodes of ischemia. These two models produce different patterns of cell death in brain tissue, where mild insults (5 minutes) are more likely to induce apoptosis (MacManus *et al.,* 1993; Nitatori *et al.,* 1995); whereas longer durations of ischemia induce necrotic cell death (Garcia *et al.,* 1995; Kaplan *et al.,* 1991; Memezawa *et al.,* 1992). In both cases, the administration of glutamate receptor antagonists significantly reduced cell death (Choi, 1988; Kochhar *et al.,* 1988; Marcoux *et al.,* 1988; Church *et al.,* 1988; Boast *et al.,* 1988; Steinberg *et al.,* 1988; Park *et al.,* 1988; Buchan *et al.,* 1991; Le Peillet *et al.,* 1992, Sheardown *et al.,* 1990, Gill *et al.,* 1992, Smith and Meldrum, 1992), supporting an excitotoxic mechanism of cell death. I found a mechanism of glutamate release in the hippocampus during the initial phase of ischemia that increases the mEPSC frequency but is completely unrelated to changes in $[Ca^{2+}]_i$. The absence of mEPSC involvement in the increase in $[Ca^{2+}]_i$ also raises the question of whether mEPSCs contribute to the apoptotic trigger. Transient episodes of ischemia or exogenously applied glutamate analogues can also trigger actin depolymerization-mediated postsynaptic spine retraction (Hasbani *et al.,* 2001), a mechanism thought to be involved in ischemic preconditioning (Halpain *et al.,* 1998; Hasbani *et al.,* 2001; Ikegaya

et al., 2001; Graber *et al.*, 2004). This mechanism mediated by glutamate receptor activation does not require the influx of calcium (Ikegaya *et al.*, 2001). Thus, it is possible that the ischemia-induced increase in mEPSC frequency is a neuroprotective mechanism, triggering spine retraction and ischemic preconditioning. The source of glutamate that leads to cell death remains unknown, and further experimentation would be necessary to clarify the role of the ischemia-induced increase in mEPSC frequency.

Surprisingly, the Purkinje cells of the cerebellum do not exhibit the early phase of the glutamatergic response to ischemia. This demonstrates that different brain regions respond differently to ischemia. Purkinje cell damage has been demonstrated to occur via activation of AMPA receptors (Balchen and Diemer, 1992), and thus, the glutamatergic system is involved in ischemia-induced Purkinje cell damage. Our finding that Purkinje cells do not exhibit the increase in mEPSC frequency suggests, therefore, that Purkinje cell death is triggered by the second phase of the ischemic response. The reason behind these distinct responses in the hippocampus and cerebellum is unknown; however it could be due to intrinsic differences in synaptic terminal properties. Stabilizing actin filaments with jaspamide decreased the amplitude of evoked responses by decreasing the probability of release in CA3 synaptic terminals, whereas jaspamide had no effect in the parallel-fiber and climbing-fiber terminals. Since the ischemia-induced increase in mEPSC frequency is caused by actin filament depolymerization, it is plausible that it is not observed in the cerebellum due to a reduced role of actin filaments in synaptic activity in the cerebellar synapses.

5.2.2 The Late Glutamatergic Response to Ischemia

My work has also elucidated a number of aspects pertaining to the late glutamatergic response to ischemia in the Purkinje cells of the cerebellum. Some aspects of the ischemic response are very similar to the hippocampus, such as a large glutamate-mediated current caused by the reversal of glutamate transporters. Other aspects, however, are remarkably different. The major difference is that the glutamate-mediated response is generated mainly by non-desensitizing AMPA receptors. This is not surprising, as Purkinje cells do not express functional NMDA receptors after postnatal day 8. The second major difference is that in cerebellar slices, reversed uptake is not the only significant means of glutamate release. There is a calcium-dependent component to glutamate release in the feed-forward steps leading to transporter reversal at around 10 minutes of ischemia, which could be either calcium-dependent exocytosis, or an indirect synergistic effect of calcium-dependent membrane depolarization. At later than 15 minutes of ischemia, however, removing extracellular Ca^{2+} has no effect on glutamate release. Thus, it has been demonstrated here that not all areas of the brain respond equally to ischemia, and it is possible that the design of treatments for brain ischemia may need to be region-specific.

5.2.3 *In vitro* Ischemia Simulations in Acute Brain Slices

Although acute brain slices resemble the intact brain in terms of connectivity and spatial relationships (Glaum *et al.,* 1994; Billups *et al.,* 1998) in many aspects it is different from

working with *in vivo* ischemia models. One of these differences is that in simulated ischemia, tissue is continually exposed to the flow of extracellular solution with constant ionic concentrations and pH. Extracellular ionic concentration changes are somewhat insignificant during the early response to ischemia, but change dramatically during the second phase. These could lead to unexpected differences in our study, as in our preparation the ionic disturbances are less severe; however it should be less of an issue for the strict comparison between hippocampal and cerebellar slices.

Another difference between *in vitro* ischemia simulations and *in vivo* ischemia is that acute slices are in a more compromised metabolic state, with lower ATP values and elevated aerobic glycolysis, and thus could be hypersensitive to ischemic insults (Lipton, 1999). Despite these caveats, the well described late phase of the glutamatergic response in *in vivo* studies has been successfully reproduced in *in vitro* studies (Rossi *et al.*, 2000). The early phase of the glutamatergic response, the increase in mEPSC frequency, however, has never been studied in *in vivo* models, as this phenomena may only be detected through patch-clamping techniques. As *in vivo* patch-clamping techniques become more commonplace, perhaps this issue will soon be evaluated.

There are many methods for simulating ischemia *in vitro*, including simple oxygen and glucose deprivation (OGD); and more severe chemical simulations including the further addition of cyanide, to inhibit complex IV of the electron transport chain or iodoacetic acid, an inhibitor of glycolysis. The response to simulated ischemia is qualitatively similar with all methods, but in the absence of metabolic inhibitors, the various responses

develop more slowly and with a greater temporal variability, making experimentation more difficult (Rossi personal communication, and Brady, Mohr and Rossi in preparation). The inclusion of cyanide only slightly increases the rate at which these events occur compared to iodoacetic acid only. One of the reasons for the discrepancy between the timing of events *in vivo* and with simple OGD *in vitro* could be because slices are in a quiescent state of activity compared to an intact brain in living animals, so ATP consumption is at a reduced rate. Including metabolic inhibitors in the ischemia simulating solution prevents ATP production completely and immediately, thereby causing ATP to be consumed more rapidly, even in a relatively quiescent slice. Another possibility is that our setup allows for oxygen to diffuse through the interface between the air and the bathing solution in the recording chamber; however, this is minimized by a fast flow rate (5ml/min) and second nitrogen bubbling step.

The solutions I used for simulating ischemia included iodoacetic acid (Reiner, *et al.*, 1990; chapters 3 and 4), or iodoacetic acid in conjunction with cyanide (chapter 2) instead of OGD because the response to chemical ischemia more closely resembles the timing of events seen in *in vivo* studies, in terms the timing of changes in cellular [ATP] (Lowry *et al.*, 1964; Madl and Burgesser, 1993), elevation of $[glu]_o$ (Phillis *et al.*, 2000), and consequently, the electrical response and cellular damage (Reiner *et al.*, 1990; Muller and Ballanyi, 2003).

My intracellular recording solution includes ATP and GTP. Thus, when simulating metabolic inhibition the actual concentration of ATP in the patched cell is unknown, and

could be anywhere from the concentration in the pipette to largely depleted, where the probability that it is depleted increases in the direction towards distal dendrites. We interpret the patched cell as having near normal ATP concentrations, as we are able to hold the recordings for very long periods of time in the presence of glutamate receptor antagonists during ischemia. This is a more ideal condition to accurately monitor changes in glutamate, because it circumvents postsynaptic modifications that could potentially occur to receptors, such as desensitization which depends on phosphorylations, and which could artifactually affect our assessment of changes in extracellular glutamate concentration (Banke *et al.,* 2000; Derkach *et al.,* 1999).

Despite these potential caveats associated with the *in vitro* study of simulated ischemia in acute slices, the responses we observe are remarkably similar to those occurring in *in vivo* ischemia. *In vivo* studies of ischemia have shown that hippocampal pyramidal cells exhibit an early rise in $[Ca^{2+}]_c$, similar in magnitude and in time course to the rise that we observe in our bulk loaded slices (Silver and Erecinska, 1990), and the timing at which the anoxic depolarization current occurs is also very similar to that in *in vivo* studies (Rossi *et al.,* 2000). Thus I do not believe that the potential caveats described above are a major confounding influence of our experiments.

5.2.4 The Failure of ATP-Dependent Processes During Ischemia

There are two mechanisms of glutamate release during ischemia. The first is an increase in the frequency of exocytosis of glutamate-containing synaptic vesicles, as a

consequence of actin filament depolymerization. The second is the reversal of glutamate transporter uptake, which occurs when Na^+ and K^+ ion gradients dissipate as a result of the failure of the Na/K ATPase. The Na/K ATPase has traditionally been viewed as the main ATP consumer for neurons, and most sensitive cellular process to be affected by ischemia (Bernstein and Bamburg, 2003). Here, I have discovered a role for actin filaments. Actin turnover in cells is ATP dependent (Atkinson *et al.,* 2004), however its role in ischemic responses has been largely overlooked. Recently, actin filament ATP consumption has been determined to equal to the Na/K ATPase in neurons (Bernstein and Bamburg, 2003). The rise in $[Ca^{2+}]_c$ is due to calcium influx across the plasma membrane and calcium release from intracellular stores. This mechanism is completely independent from the increase in mEPSC frequency observed at the same time, and could be caused by another, yet unknown, ATP-dependent process. Alternatively, it could be indirectly related to the failure of the Na/K ATPase or actin filament depolymerization.

5.2.5 Clinical Implications and Future Directions

The results from my thesis have covered several gaps in the existing knowledge in the ischemia field. Perhaps these results will shed some light in the development of the long-felt need for a treatment to reduce neural tissue damage resulting from ischemia. It is hoped that the findings pertaining to the ischemic response in the cerebellum reported here will lead to the further research for therapeutics targeted to this region, perhaps an antagonist to block the calcium influx that plays a role in glutamate release. Alternatively, perhaps it could lead to more rigorous clinical trials in testing for

treatments for ischemia by distinguishing patients with cerebellar and forebrain ischemia. It is also hoped that the elucidated mechanism for the increase in mEPSC frequency is further investigated in *in vivo* models for its potential role as a cell death mechanism or in ischemic preconditioning. The elucidation of this mechanism may shed some light in the design of therapeutics targeted to treat ischemia-induced delayed cell death in transient ischemic episodes, and in the penumbra of focal ischemia.

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