THE EFFECT OF ISCHEMIA ON CEREBELLAR PURKINJE CELL $GABA_A$ RECEPTORS

By

Melissa H. Kelley

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CERTIFICATE OF APPROVAL

This is to certify that the Ph.D. dissertation of

MELISSA HERNANDEZ KELLEY

has been approved on April 1, 2011

Advisor, Paco Herson, PhD

Member and Chair, Gary Westbrook, PhD

Member, James Maylie, PhD

Member, Stephanie Murphy, VMD, PhD

Member, Stephen Smith, MB, BS, PhD

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ABSTRACT

Cerebellar Purkinje cells (PCs) are vulnerable to ischemic injury and excitotoxicity. The large excitatory input from climbing fibers and parallel fibers are thought to underlie the sensitivity of PCs to ischemia. PCs also have uniquely large numbers of GABAergic contacts with inhibitory interneurons, providing an endogenous method of controlling excitation. However, increasing GABAergic neurotransmission as a neuroprotective strategy has not been thoroughly tested in the cerebellum. The goal of this work was to investigate the neuroprotective mechanisms of the GABA-active neurosteroid allopregnanolone and reveal the effects of ischemia on PC $GABA_A$ receptor function. Experiments were performed on primary cerebellar neuronal cultures subjected to *in vitro* ischemia (oxygen-glucose deprivation; OGD), and on an *in vivo* mouse model of cardiac arrest and cardiopulmonary resuscitation to induce global cerebral ischemia.

I initially tested the hypothesis that ischemia causes a rapid down-regulation of $GABA_A$ receptors in PCs, thereby increasing susceptibility to excitotoxicity. Whole cell voltage clamp experiments demonstrated that OGD caused a rapid decline in functional $GABA_A$ receptors. Recordings performed within the first hour of re-oxygenation revealed decreased GABA mediated current density and amplitudes of miniature inhibitory post-synaptic currents were reduced. $GABA_A$ receptor protein levels were also significantly reduced by OGD.

Functional properties of the remaining $GABA_A$ receptors following ischemia were examined using subunit specific modulators of $GABA_A$ receptors. OGD caused decreased sensitivity to the $\beta_{2/3}$ subunit preferring compound, etomidate, but had no effect on the sensitivity to propofol, a compound that modulates β_{1-3} subunits. The responses to the α/γ subunit acting compounds, diazepam and zolpidem were also unaffected by OGD. This pharmacological profile led to the hypothesis that ischemia increases the relative contribution of β_1 containing receptors, however β_1 subunit expression had not been detected in PCs. Contrary to previous reports, single cell RT-PCR revealed PCs contain β_1 subunit mRNA and levels of β_1 subunit protein in cultured PCs were unaffected by OGD. Loreclezole, a compound that can inhibit β_1 containing $GABA_A$ receptors when applied at high concentrations, inhibited PC GABA mediated currents, also indicating that PC $GABA_A$ receptors contain the β_1 subunit. These data suggest that PCs express the β_1 subunit and there is a greater contribution of β_1 containing $GABA_A$ receptors following OGD.

Allopregnanolone (ALLO) protected cerebellar PCs from ischemic damage at least in part by preventing reduced $GABA_A$ receptor current and protein *in vitro*. Additionally, ALLO protected PCs from global ischemia as assessed by FluroJade B staining and prevented the decline in $GABA_A$ receptor protein *in vivo*. The effect of sex on ALLO neuroprotection from global ischemia was also examined. At low doses, ALLO provided greater neuroprotection in females compared to males, whereas high doses protected both sexes. ALLO slowed the decay of PC spontaneous inhibitory postsynaptic currents (IPSCs) to a greater

degree in female compared to males, yet a sex difference that was not observed in miniature IPSCs. Therefore ALLO affects males and females differentially through a mechanism other than binding postsynaptic $GABA_A$ receptors.

In summary, the work presented in this thesis demonstrates that ischemia causes rapid and sustained loss of $GABA_A$ receptors while sparing β_1 containing receptors, ALLO protects PCs from ischemia by preventing the decline in $GABA_A$ receptors, and females are more sensitive to ALLO than males. These findings may aid in the development of therapies for stroke and cardiac arrest.

ABBREVIATIONS USED IN TEXT

ALLO, allopregnanolone

CA/CPR, cardiac arrest/cardiopulmonary resuscitation

DIV, days in vitro

DZP, diazepam

E, embryonic day

GABA, gamma-amino butyric acid

ip, intraperitoneal

IPSC, inhibitory postsynaptic current

MCAO, middle cerebral artery occlusion

mIPSC, miniature inhibitory postsynaptic current

OGD, oxygen-glucose deprivation

PC, Purkinje cell

RT-PCR, reverse transcriptase polymerase chain reaction

TBI, traumatic brain injury

CHAPTER 1:

INTRODUCTION

I. Cardiac Arrest and Cerebral Ischemia

Cardiac arrest (CA) requiring cardiopulmonary resuscitation is experienced by over 300,000 Americans every year and is the major cause of global cerebral ischemia (Roger et al., 2010). Due to limited treatment for cerebral ischemia, clinical outcome for survivors of CA is poor with only 5% of the afflicted able to return to healthy and productive lives (Becker et al., 1991; Manning and Katz, 2000). The neurological and neuropsychological impairments commonly seen in CA survivors include memory loss, executive function deficits, and motor function deficits (Lim et al., 2004).

Cerebral ischemia causes neuronal damage and neuronal death through a cascade of events primarily involving excitotoxicity, oxidative stress, apoptosis, and inflammation (Doyle et al., 2008). This thesis is primarily focused on neuroprotective strategies, and ischemia induced modifications in neuronal physiology, during the early stages of ischemic injury, most attributed to excitotoxicity. Excitotoxicity, excessive and uncontrolled excitation that causes neuronal damage, is an early event in the ischemia reperfusion injury process that begins at the onset of ischemia. Initially, lack of oxygen ceases

mitochondrial production of ATP thereby causing the Na^+/K^+ ATPase and Ca^{2+} ATPase ionic pumps to fail (Balestrino, 1995). The increased intracellular Na^+ and Ca^{2+} depolarize neurons and cause excessive glutamate release, activating a positive feedback loop of depolarization, calcium influx, and subsequent transmitter release (Paschen, 1996). Glutamate transporter reversal from increased intracellular Na^+ also contributes to the uncontrolled glutamate release causing excitotoxicity (Rossi et al., 2000). Following reoxygenation, excitotoxic damage persists and manifests as other forms of injury. For example, glutamate mediated Na^+ influx depolarizes local membranes and activates voltage dependent Ca^{2+} channels. Glutamate can also promote Ca^{2+} influx by activating NMDA receptors or Ca^{2+} permeable glutamate receptor type 2 lacking AMPA receptors. Elevated levels of intracellular calcium can then activate apoptotic signaling pathways leading to cell death (Arundine and Tymianski, 2004). Therefore, excitotoxicity alone causes neuronal damage and it can also lead to other forms of injury through increased intracellular calcium. Not surprisingly, inhibition of glutamate receptors as a neuroprotective strategy for ischemia has been the focus of many studies. However, glutamate receptor antagonists have failed in clinical trials largely due to severe side effects, despite success in experimental models of ischemia (Ginsberg, 2008). Therefore, we chose to focus on augmenting GABAergic neurotransmission as strategy to minimize excitotoxicity.

II. The Cerebellum and Purkinje Cell Vulnerability to Ischemic Damage

Although there are several studies describing memory and executive function deficits following cerebral ischemia, less attention has been paid to motor coordination deficits. The cerebellum processes input from various brain regions, and the spinal cord, in order to produce coordinated and precise movements (Kandel et al., 2000; Apps and Garwicz, 2005). The organized and well characterized cerebellar cortex facilitates the study of individual neuronal populations. It consists of a granule cell layer, primarily containing excitatory granule cells, Purkinje cell (PC) layer, and molecular layer where inhibitory basket and stellate cells reside. Cerebellar PCs receive excitatory input from granule cells and climbing fiber projections from the inferior olivary nucleus of the brain stem. To control levels of excitation, basket and stellate cells form numerous inhibitory synapses onto PCs. As the sole output of the cerebellum, PCs are critical to cerebellar function. Therefore, PC dysfunction or cell death results in ataxia and other motor deficits (Mullen et al., 1976; Sausbier et al., 2004).

Cerebellar ischemia is experienced when blood flow to the posterior inferior cerebellar, anterior inferior cerebellar, or superior cerebellar arteries is disrupted (Barth et al., 1993). Focal cerebellar strokes occur but they are documented with less frequency than strokes in other brain regions (Yamaura et al., 1991). A more common cause of cerebellar ischemia is CA, which results in global cerebral ischemia. Global ischemia differs from focal ischemia (stroke) in that global ischemic insult does not result in a damaged core or penumbra

region. Rather, global ischemia causes select damage to vulnerable populations of neurons including hippocampal CA1 neurons, striatal neurons, and cerebellar PCs. To date there is limited clinical data closely examining cerebellar damage following CA and few animal studies investigating the role of the cerebellum in the pathology and outcome of CA/CPR. Nonetheless, PCs have long been recognized as a neuronal population that is highly sensitive to ischemic damage (Pulsinelli et al., 1982; Sato et al., 1990; Horn and Schlote, 1992; Brasko et al., 1995; Fonnum and Lock, 2000). The high sensitivity of PCs to ischemic damage has largely been attributed to excitotoxicity from glutamatergic synapses onto PCs from climbing fibers and parallel fibers (Welsh et al., 2002). Indeed, inhibition of glutamate receptors can provide neuroprotection of PCs following global ischemia (Brasko et al., 1995; Welsh et al., 2002). However, glutamate receptor antagonists have failed in clinical trials because of severe side effects and the inability of patients to tolerate the doses used in animal studies to achieve neuroprotection (Muir et al., 1994; Albers et al., 1995; Muir, 2006).

III. Functional Role of $GABA_A$ Receptors During Ischemia

Stimulation of $GABA_A$ receptors could provide an alternative approach to blocking excitotoxic injury of PCs by directly hyperpolarizing the neurons to attenuate excitation. Enhanced GABAergic function can reduce ischemic injury in animal models (Schwartz-Bloom and Sah, 2001). For example, muscimol, a $GABA_A$ receptor agonist, reduces damage in rodent models of global cerebral

ischemia (Sternau et al., 1989; Zhang et al., 2007) and focal cerebral ischemia (Lyden and Hedges, 1992; Shuaib et al., 1993). Positive allosteric modulators of $GABA_A$ receptors also reduce damage from focal ischemia (Schwartz et al., 1994; Schwartz-Bloom et al., 2000; Sayeed et al., 2006) and simulated ischemia *in vitro* (Ardeshiri et al., 2006; Ricci et al., 2007). However, some studies have failed to observe beneficial effects of increasing inhibition (Kuhmonen et al., 2002; Davies et al., 2004). Additionally, two positive modulators of $GABA_A$ receptors, clomethiazole and diazepam failed to show an effect of treatment in clinical trials for neuroprotection from stroke (Wahlgren et al., 1999; Lyden et al., 2002; Lodder et al., 2006). Chapter 2 addresses these inconsistencies and describes a mechanism behind the reduced efficacy of $GABA_A$ receptor agonism as a treatment for cerebral ischemia.

$GABA_A$ receptors are ligand gated channels with a pentameric subunit composition, similar to other members of the cys loop family of receptors including the nicotinic acetylcholine receptors (Cromer et al., 2002; Ernst et al., 2003; Unwin, 2003). There are sixteen known subunits including α_{1-6}, β_{1-3}, γ_{1-3}, δ, θ, ε (Olsen and Sieghart, 2009). The most common native $GABA_A$ receptors are composed of 2 α subunits, 2 β subunits, and one γ or δ subunit. Through diversity in subunit composition, $GABA_A$ receptor properties differ in various brain regions and subcellular compartments. These properties allow specialized physiological roles and unique pharmacological sensitivities (Fritschy and Mohler, 1995; Luddens et al., 1995; Olsen and Sieghart, 2009).

IV. PC $GABA_A$ Receptors, Pharmacology and Plasticity

In situ hybridization and immunolabeling have demonstrated that cerebellar PCs predominantly express $GABA_A$ receptors containing the $\alpha_{1,}$ $\beta_{2\text{-}3,}$ and γ_2 subunits (Wisden et al., 1996), the dominant receptor composition in the brain (Wisden et al., 1992). Structure and function analysis of $GABA_A$ receptors expressed in heterologous systems, have revealed the subunit specific site of action for several agonists and modulators. A brief description and site of action for the compounds utilized in these studies is included below for GABA, diazepam, zolpidem, etomidate, propofol, and loreclezole (for schematic representation, please refer to Fig.1).

GABA, the endogenous agonist of $GABA_A$ receptors, binds the interface of the α and β subunits resulting in two GABA binding pockets per receptor (Ernst et al., 2003). Therefore, PC $GABA_A$ receptors presumably bind GABA at $\alpha_1\beta_2$ or $\alpha_1\beta_3$ interfaces.

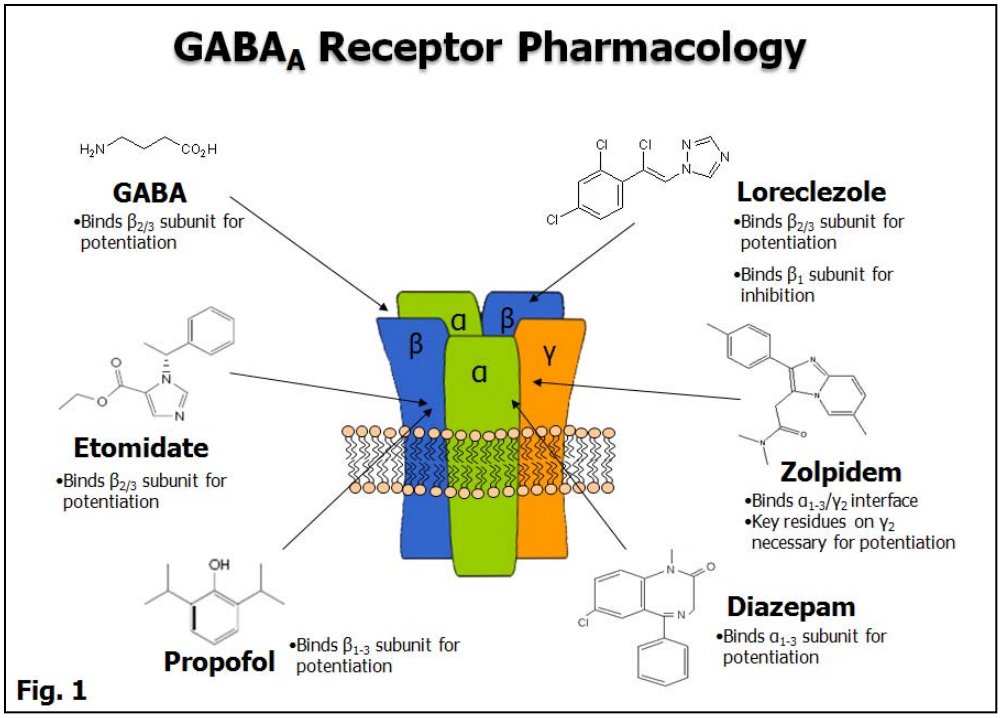

Fig. 1

The α/γ subunit interface is the classical benzodiazepine binding site for drugs such as diazepam (Sigel and Buhr, 1997). However, the α4 and α6 subunits are insensitive to classic benzodiazepines and the γ1 and γ3 subunits are less sensitive than γ2 (Whiting et al., 1995). Zolpidem is a non-benzodiazepine that binds the α/γ subunit interface; however, specific amino acids on the γ_2 subunit are necessary for potentiation, rendering it a γ_2 subunit dependent positive modulator (Buhr and Sigel, 1997). Accordingly, diazepam and zolpidem are predicted to bind PC GABA$_A$ receptors at the α1γ2 interface.

The intravenous anesthetics etomidate and propofol have unique mechanisms of action, binding to the β subunit of GABA$_A$ receptors and potentiating GABA responses. Etomidate has a higher affinity for the β_2 and β_3 subunits than β_1, requiring up to 3 times greater concentration to activate β_1

containing receptors over β_2 and β_3 (Belelli et al., 1997). Alternatively, propofol can strongly modulate all β subunits equally (Sanna et al., 1995).

Loreclezole is a unique drug because it can potentiate or inhibit $GABA_A$ receptors depending on the subunit composition. Specifically, loreclezole acts as a positive modulator of $GABA_A$ receptors containing the β_{2-3} subunits (Wingrove et al., 1994;Wafford et al., 1994) and at concentrations >6 μM, loreclezole inhibits $GABA_A$ receptors containing the β_1 subunit (Fisher et al., 2000).

Changes in $GABA_A$ receptor subunit expression have been observed following exposure to neurosteroids and ethanol (Mody, 2005). In addition, receptor subunit changes have been reported in pathological states, such as epilepsy (Peng et al., 2004). However, $GABA_A$ receptor plasticity following an ischemic insult is not well characterized. Understanding $GABA_A$ receptor subunit composition and any changes caused by ischemia is important for designing protective therapies and may underlie inconsistencies with neuroprotection by GABA mimetic compounds. Therefore, chapter 4 examines ischemia induced differences in PC $GABA_A$ receptor sensitivity to various compounds.

V. Progesterone, Allopregnanolone, and Neuroprotection

The ovarian hormone progesterone can also be synthesized *de novo* in male and female brain. Female sex is protective in cerebral ischemia in large part because of estrogen (Alkayed et al., 1998; Hurn and Macrae, 2000; Liu et al., 2009), and partially by progesterone (Herson et al., 2009). Progesterone is

neuroprotective in experimental animal models of focal and global ischemia (Liu et al., 2010), including middle cerebral artery occlusion (Roof and Hall, 2000; Murphy et al., 2002; Gibson and Murphy, 2004) and cardiac arrest (Gonzalez-Vidal et al., 1998; Cervantes et al., 2002). Following an ischemic insult, animals treated with progesterone have reduced levels of oxidative stress (Ozacmak and Sayan, 2009), decreased edema (Betz and Coester, 1990), and suppression of inflammatory cytokines (Gibson et al., 2005; Jiang et al., 2009). However, the exact mechanism of progesterone neuroprotection remains unclear.

Progesterone is readily metabolized in the brain by the 5 alpha-reductase and 3 alpha-hydroxysteroid dehydrogenase (Mellon and Vaudry, 2001). Neurosteroids, the neuroactive metabolites of progesterone, are potent modulators of $GABA_A$ receptor activity (Lambert et al., 2003). Neurosteroids mediate anesthesia and anxiolysis through interaction with the $GABA_A$ receptor (Gee, 1988). One such compound is ALLO (Belelli et al., 2006). ALLO can be synthesized *de novo* in neural tissues therefore aderenalectomized and ovariectomized animals have detectable levels of ALLO (Purdy et al., 1992).

ALLO enhances the response to GABA by increasing channel open probability (Callachan et al., 1987). At high concentrations (μM range) ALLO binds the α/β interface of PCs and directly activates $GABA_A$ receptors, whereas at lower concentrations (nM range) ALLO binds at a separate site on the α subunit (Hosie et al., 2006). The subunit composition can influence the potency of ALLO. The α_1 and α_3 containing receptors are sensitive to concentrations starting at 3 nM whereas α_2, $\alpha_{4\text{-}6}$ containing receptors require 3-10 fold higher

concentrations (Belelli et al., 2002). Plasma levels of ALLO in male rats were below the detection threshold of 25 pg in a radioimmunoassay (Purdy et al., 1990; Purdy et al., 1991). However, concentrations of ALLO in cortical tissue homogenate have been reported to be 2.7 ng/ml (estimated to be about 10nM) (Purdy et al., 1991). Female rats have higher plasma and brain tissue levels of ALLO that fluctuate according to the estrous cycle (Purdy et al., 1990; Paul and Purdy, 1992).

In animal models, ALLO prevents neuronal damage from seizures (Lonsdale and Burnham, 2007), Alzheimer's disease (Brinton and Wang, 2006), Niemann-Pick C disease (Mellon et al., 2008), kainate excitotoxicity (Ciriza et al., 2004) and traumatic brain injury (TBI) (Djebaili et al., 2004). At comparable doses, ALLO provides greater neuroprotection than progesterone from middle cerebral artery occlusion (MCAO) in rats (Sayeed et al., 2006). In cultured cerebellar neurons subjected to oxygen and glucose deprivation (OGD) to simulate ischemia, progesterone neuroprotection is mediated almost solely by its metabolism to ALLO and requires activation of $GABA_A$ receptors (Ardeshiri et al., 2006). Therefore, ALLO appears to be a more potent neuroprotectant than its parent compound progesterone and may be responsible for the neuroprotective properties attributed to progesterone. Chapters 2 and 3 of this thesis further characterize ALLO neuroprotection though its interaction with $GABA_A$ receptors and sex differences in ALLO neuroprotection.

VI. Summary

Purkinje neurons are sensitive to ischemic injury due to an elevated risk of excitotoxicity from parallel and climbing fiber innervation. Therapies aimed at reducing excitotoxicity by glutamate receptor antagonism have not been successful, and stimulating inhibitory $GABA_A$ receptors to attenuate excessive excitation has yielded inconsistent results. The work presented in this thesis describes changes in $GABA_A$ receptor structure and function that occur in response to an ischemic event. These modifications may explain inconsistencies in neuroprotection studies.

The neurosteroid and metabolite of progesterone, ALLO, is neuroprotective against ischemia and other models of neurodegeration, yet the mechanism of neuroprotection is unknown. ALLO is a well characterized positive modulator of $GABA_A$ receptors and neuroprotection by ALLO has been shown to require $GABA_A$ receptor activation. This thesis explores the mechanism of ALLO neuroprotection by interacting with the $GABA_A$ receptor.

VII. Goals

The goal of this thesis is to expand the understanding of $GABA_A$ receptor mediated inhibition following ischemia and further dissect the mechanism of ALLO neuroprotection. In chapter 2, I examine the net inhibitory potential of PC $GABA_A$ receptors and characterize ALLO neuroprotection through $GABA_A$

receptor interactions. As a metabolite of the ovarian hormone progesterone, the effects of ALLO may be different in males and females. Therefore in chapter 3, I investigate sex differences in ALLO neuroprotection. Lastly in chapter 4, I explore ischemia induced alterations in $GABA_A$ receptors in greater detail, with an emphasis on changes in subunit composition.

CHAPTER 2:

Ischemic Insult to Cerebellar Purkinje Cells Causes Diminished $GABA_A$ Receptor Function and Allopregnanolone Neuroprotection is Associated with $GABA_A$ Receptor Stabilization

Melissa H. Kelley, Norico Taguchi [1], Ardalan Ardeshiri, Masayuki Kuroiwa [2], Patricia D. Hurn[4], Richard J. Traystman[3], and Paco S Herson

Department of Anesthesiology and Perioperative Medicine, Oregon Health & Science University, 3181 SW Sam Jackson Park Road, Portland, OR 97201

[1] Present address: Department of Anesthesia, University of Tsukuba, Ibaraki, Japan

[2] Present address: Department of Anesthesiology, National Hospital Organization, Sagamihara National Hospital, Kanagawa, Japan

[3] Present address: University of Colorado, Denver, CO

[4] Present address: University of Texas, Austin, TX

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Abstract:

Cerebellar Purkinje cells are particularly vulnerable to ischemic injury and excitotoxicity, although the molecular basis of this sensitivity remains unclear. We tested the hypothesis that ischemia causes rapid down-regulation of $GABA_A$ receptors in cerebellar Purkinje cells, thereby increasing susceptibility to excitotoxicity. Oxygen-glucose deprivation caused a decline in functional $GABA_A$ receptors, within the first hour of re-oxygenation. Decreased amplitude of miniature inhibitory post-synaptic potentials confirmed that oxygen-glucose deprivation caused a significant decrease in functional synaptic $GABA_A$ receptors and quantitative Western blot analysis demonstrated the loss of $GABA_A$ receptor current was associated with a decline in total receptor protein. Interestingly, the potent neuroprotectant allopregnanolone prevented the decline in $GABA_A$ receptor current and protein. Consistent with our in vitro data, global ischemia in mice caused a significant decline in total cerebellar $GABA_A$ receptor protein and Purkinje cell specific immunoreactivity. Moreover, allopregnanolone provided strong protection of Purkinje cells and prevented ischemia-induced decline in $GABA_A$ receptor protein. Our findings indicate that ischemia causes a rapid and sustained loss of $GABA_A$ receptors in Purkinje cells, whereas allopregnanolone prevents the decline in $GABA_A$ receptors and protects against ischemia-induced damage. Thus, interventions which prevent ischemia-induced decline in $GABA_A$ receptors may represent a novel neuroprotective strategy.

I. Introduction:

Global cerebral ischemia, as experienced following cardiac arrest, results in cell death in susceptible neuronal populations such as the striatum, CA1 hippocampal neurons and cerebellar Purkinje Cells (PC). Many studies have investigated the mechanisms of ischemic cell death and neuroprotection in the hippocampus, compared to the relatively few studies on PC. Nevertheless PC have long been recognized as neurons that are susceptible to ischemic damage *in vivo* (Horn and Schlote, 1992;Brasko et al., 1995;Fonnum and Lock, 2000) and more recently *in vitro* (Ardeshiri et al., 2006). The robust excitatory input that PC receive likely make them vulnerable to excitotoxicity, a major event that occurs early in ischemia induced neuronal cell death. Consistent with this hypothesis, inhibiting AMPA receptors with the antagonist NBQX or surgically eliminating excitatory climbing fiber input, protects PC from damage following global cerebral ischemia (Brasko et al., 1995;Welsh et al., 2002). Similarly, we have observed that the AMPA receptor antagonist CNQX protects cultured cerebellar PC against *in vitro* ischemia (Ardeshiri et al., 2006). In addition to excitatory input, PC receive strong GABAergic inhibitory input from a variety of interneurons, particularly basket and stellate cells. The inhibitory input is large enough that application of the $GABA_A$ receptor agonist muscimol completely abolishes spontaneous PC firing *in vivo* (Caesar et al., 2003). These synaptic connections make PC an ideal cell type to assess the effects of ischemia-induced

excitotoxicity and the neuroprotective potential of therapies that preserve the balance between inhibition and excitation.

Allopregnanolone (ALLO) is an active metabolite of progesterone that can be generated in the brain via *de novo* synthesis from cholesterol or metabolism of peripheral sources of progesterone. The anesthetic and anxiolytic effects of ALLO, by directly interacting with $GABA_A$ receptors to potentiate currents, are well documented (Gee, 1988;Gasior et al., 1999);Gee, 1988; Lambert et al., 2003;(Mellon and Vaudry, 2001;Stoffel-Wagner, 2001;Lambert et al., 2003;Belelli et al., 2006). In addition, recent studies have shown that ALLO is neuroprotective in several experimental animal models of neurodegeneration, including seizures (Lonsdale and Burnham, 2007), Alzheimers (Brinton and Wang, 2006), Niemann-Pick C disease (Mellon et al., 2008), kainate excitotoxicity (Ciriza et al., 2004), middle cerebral artery occlusion (MCAO) (Sayeed et al., 2006), and traumatic brain injury (TBI) (Djebaili et al., 2004). Although the exact mechanism of ALLO neuroprotection was not determined in these studies, we recently demonstrated that the neuroprotection of cerebellar PC provided by ALLO is dependent on activity of $GABA_A$ receptors (Ardeshiri et al., 2006).

$GABA_A$ receptors are perfectly positioned to reduce excitability and inhibit the positive-feedback loop that constitutes excitotoxicity. Augmenting GABAergic function can reduce ischemic injury in animal models (Schwartz-Bloom and Sah, 2001;Zhang et al., 2007). For example, the $GABA_A$ receptor agonist muscimol reduces damage in rodent models of global cerebral ischemia, (Sternau et al.,

1989;Zhang et al., 2007) and focal cerebral ischemia (Lyden and Hedges, 1992;Shuaib et al., 1993). Similarly, $GABA_A$ receptor allosteric potentiators ALLO and diazepam (DZP) are neuroprotective in focal ischemia (Schwartz et al., 1994;Schwartz-Bloom et al., 2000;Sayeed et al., 2006) and *in vitro* ischemia (Ardeshiri et al., 2006;Ricci et al., 2007). Despite the beneficial effects of increasing inhibition, some studies have failed to observe neuroprotective benefit of GABAergic compounds (Kuhmonen et al., 2002;Davies et al., 2004). A simple explanation for this inconsistency is that ischemia may cause a decline in $GABA_A$ receptor expression, rendering GABA-potentiating compounds ineffective. In fact, there are reports of both increases and decreases in $GABA_A$ receptor expression following ischemia in rodent cortex and hippocampus (Li et al., 1993;Schiene et al., 1996;Inglefield et al., 1997;Qu et al., 1998).

We used an *in vitro* ischemia model, oxygen-glucose deprivation and an *in vivo* global ischemia model, cardiac arrest/cardiopulmonary resuscitation, to test the hypothesis that ischemia induced cerebellar PC damage is preceded by a down regulation of $GABA_A$ receptors that can be prevented by ALLO.

II. Materials and Methods

Primary Cell Culture. Neurons were cultured from the cerebellum of embryonic day (E)18 Sprague–Dawley rats in accordance with National Institute of Health guidelines and experimental protocols approved by the institutional IACUC committee. Cultures were performed as previously described (Ardeshiri et al.,

2006), with the minor modification of the use of DMEM/F-12 1:1 mix ,supplemented with L-glutamine (1.4 mM), penicillin-streptomycin (50 units*µg/ml), and b-27 supplement (1X)(Gibco/Invitrogen), to best support PC growth *in vitro*. Briefly, time-pregnant Sprague–Dawley rats (Charles River, Wilmington, MA) were killed with CO_2 and E18 fetuses removed by caesarean section. Brains were isolated and placed in ice-cold dissection solution composed of Hank's Balanced Salt Solution (Gibco/Invitrogen, Carlsbad, CA, USA) supplemented with 0.03% bovine serum albumin and 10 mM $MgSO_4$ (Sigma-Aldrich, St Louis, MO). Cerebella were dissected, meninges removed with forceps, and tissue digested with 0.2% trypsin at 37˚C in dissection solution. Digestion was halted by washing the tissue with supplemented DMEM/F-12 medium (described above) containing 10% fetal calf serum. Tissue was then triturated, filtered through a 70 µm cell sorting nylon mesh and isolated cells were pelleted by centrifugation at 1000xg and resuspended in supplemented DMEM/F-12 1:1 media without FBS. Cells were plated at a density of $2.8x10^5$ cells per 12 mm round cover slip coated with poly-D lysine and grown in a humidified atmosphere at 37˚C. All neurons used in experiments were grown for 10-14 days *in vitro* (DIV) to allow maturation and formation of synaptic connections prior to use. Cultures consisted of approximately 30-40% PC, determined by immunostaining with PC specific antibody, anti-C28K (data not shown).

Oxygen-Glucose Deprivation. In vitro ischemia was induced by transferring cells to a glucose-free saline (in mM: 140 NaCl, 5 KCl, 0.8 $MgCl_2$, 1 $CaCl_2$, 10 HEPES,

pH 7.35 with NaOH) and placed in an anaerobic incubator for 2 hours. The anaerobic incubator contained an oxygen reacting catalyst to ensure the presence of less than 5 ppm O_2, kept at 37˚ with 5% CO_2 and 95% N_2 (Coy Laboratory Products, Grass Lake, MI). Cells treated with drug (ALLO or DZP) received a 15 min pre-treatment with drug in culture media and drug remained present throughout oxygen-glucose deprivation. Re-oxygenation was initiated by transferring cells to culture media in aerobic incubator or glucose containing saline for electrophysiological recordings (see below).

Electrophysiology. Cells were transferred to a recording chamber mounted on an inverted Leica DM IRB microscope (Leica, Houston, TX). Whole cell voltage clamp experiments were made from the soma of PCs using an Axopatch 200B (Axon Instruments, Union City, CA) amplifier interfaced to a Dell computer (Dell, Round Rock, TX). PC were selected by their large soma, low input resistance, and extensive dendritic arbor. Data was collected at a sample frequency of 20 kHz and analyzed using pCLAMP9 (Molecular Devices, Sunnyvale, CA), Clampfit (Axon Instruments), and Igor Pro software (WaveMetrics, Lake Oswego, OR). Electrodes pulled from borosilicate glass capillaries with inner filaments using a Flaming Brown electrode puller (Sutter Instrument Co, Novato, CA) had resistances of 2-3 MΩ when filled with internal pipette solution (see below). Whole cell capacitance and series resistance were electronically compensated to 60-80%. Adequate whole cell access (R_a<20MΩ) was achieved prior to measuring responses to GABA and verified at end of recording. A wide barrel

manifold (250 µm) pressurized microperfusion system, for solution exchange around the entire cell body and dendritic processes (ALA Scientific Instruments, Westbury, NY), and a gravity fed bath flow rate of 2-5ml saline/min was used to measure PC response to GABA. The amplitude of GABA activated currents was measured using Clampfit analysis software (Axon Instruments, Union City, CA) and normalized to each cell's capacitance to calculate current density (pA/pF) and allow comparison across cells varying in size. For concentration response analysis, individual cell's peak response to each concentration of test compound was measured, plotted, and fit with the Hill equation to determine EC_{50} values. The EC_{50} is presented as the mean±SEM of individual cell's calculated EC_{50}. Global activation of PC $GABA_A$ receptors was achieved by briefly applying a saturating concentration of GABA (1 mM, 1 s). Miniature inhibitory post synaptic potentials (mIPSCs) were measured in the presence of bath applied CNQX (25 µM) and tetrodotoxin (TTX, 1µM) to block AMPA receptors and action potentials respectively. Individual events were detected by a sliding variable amplitude template (Clampfit), using a sum of exponentials function with rise time and decay constants calculated by fitting the average of 10 hand selected events (template). Event detection and fitting were confirmed by eye. The total number of events observed in 5 minute recordings from each cell was used to calculate frequency. The amplitude of all events detected in each cell's recording was used to calculate mIPSC amplitude for that cell and mIPSC amplitudes were reported as mean±SEM of 5-8 separate cells. Synaptic events were fitted with a sum of 2 exponentials to determine the average rise and decay time constants.

Solutions and Drugs. The composition of the bathing Saline solution was (in mM): 140 NaCl, 5 KCl, 0.8 $MgCl_2$, 1 $CaCl_2$, 10 HEPES and 10 Glucose, pH 7.35 with NaOH. Internal pipette solution was (in mM): 140 CsCl, 1 EGTA, 10 HEPES, 1 $MgCl_2$, 5 MgATP, pH of 7.3 with CsOH. CNQX was dissolved in DMSO at a stock concentration of 25 mM. TTX was dissolved in DMSO at a stock concentration of 1 mM. ALLO was dissolved in DMSO at a stock concentration of 10 mM. DZP was dissolved in EtOH at a stock concentration of 10 mM. Components of internal and external solutions, ALLO, DZP and GABA were obtained from Sigma-Aldrich (St Louis, MO). CNQX and TTX were obtained from Tocris (Ellisville, MO).

Western Blot. Total protein was collected from cultures by lysing cells with CelLytic-M lysis buffer (Sigma-Aldrich, St Louis, MO), centrifuged at 10,000 g for 5 min at 4ºC and pellet was discarded to remove nuclei and debris. For *in vivo* experiments, cerebellar tissue was harvested following CA/CPR and 3 or 48 hours recovery (see below). Mice were euthanized with an intaperitoneal injection (ip) of 120 mg/kg pentobarbital, decapitated and brain removed. Cerebella were dissected, cut in half, and tissue frozen in liquid nitrogen. Half cerebellum from each mouse was used for Western blot experiments and the other half for quantitative RT-PCR (see below). Tissue was homogenized in a sucrose based lysis buffer using a dounce homogenizer and centrifuged at 2,000

g for 10min at 4°C to discard nuclei and debris. Total protein content of the supernatant was measured using BCA protein assay (Pierce/Thermo Scientific, Rockford, IL) and 30 µg of total protein from each sample run on a precast 4-12% stacking polyacrylamide gel (Invitrogen, Carlsbad, CA) for 1hr at 200V. Protein was transferred to PVDF membrane (Invitrogen) for 1hr at 30V. Blots were incubated in primary antibody, 1:1000 dilution of rabbit anti-$GABA_A$ receptor α_1 subunit (Millipore, Billerica, MA) overnight at 4°C, followed by secondary antibody, 1:1000 donkey anti-rabbit IgG ECL-HRP (GE Healthcare, Piscataway, NJ) for 1hr at RT. Blots were then stripped and re-probed with primary antibody, 1:7000 mouse anti-β-actin (Sigma-Aldrich) 1hr at RT, followed by secondary antibody, 1:1000 goat anti-mouse IgG ECL HRP linked (GE Healthcare) 1hr at RT. ECL-Plus chemi-luminescent detection system (GE Healthcare) was used to visualize protein levels by exposing auto-radiographic film (PerkinElmer, Waltham, MA) with blots or using a Kodak Image Station 4000R (Carestream Health, Rochester, NY). Bio-Rad Quantity One analysis software (Hercules, CA) was used to quantify protein from film and Kodak 1D image analysis software (Carestream Health) was used to quantify protein from imaged blots. $GABA_A$ receptor protein levels, normalized to β-actin, were expressed as percent of control levels.

Quantitative Reverse Transcriptase-PCR. For qPCR measurement of $GABA_A$ receptor α_1 subunit transcripts, cerebella were harvested at 3hr or 48hr recovery and frozen in liquid nitrogen. Total RNA was isolated using the RNAqueous-4

PCR kit (Ambion, Austin, TX) per the manufacturer's instructions. Briefly, approximately 30 µg tissue (half a mouse cerebellum) was homogenized in 400 µL lysis buffer. Total RNA was isolated and eluted from a column with 50 µL RNase-free elution buffer, and further treated with Turbo DNase (Ambion, Austin, TX, USA,). First strand cDNA was reverse transcribed from 500 ng total RNA with High Capacity cDNA archive Kit (Applied Biosystems, Foster City, CA). qPCR reactions were performed on ABI Prism 7000 sequence detection system in triplicate using 50 ng cDNA. qPCR cycle parameters: first cycle was 50ºC for 2 min, 95ºC for 10 min followed by 40 cycles of 95ºC for 15 s and 60ºC for 1 min. qPCR primers and probe were purchased from Applied Biosystems, (exact primer sequence unavailable, refer to Cat # Mm00439040_m1). 18S RNA was also assayed for each sample using 5 ng total cDNA (Eurogentec North America, Inc., San Diego, CA). Final results were normalized to 18S RNA and expressed as the ratio of target gene to 18S.

Cardiac Arrest & Cardiopulmonary Resuscitation (CA/CPR). CA/CPR was performed on 20-25 g, adult male C57BL/6 mice (Charles River) to simulate global cerebral ischemia as described by our group previously (Kofler et al., 2004). Anesthesia was induced with 3% Isoflurane and maintained with 1.5-2% Isoflurane in O_2 enriched air via face mask. Temperature probes were inserted into left temporalis muscle and rectum to monitor head and body temperature simultaneously. For drug administration, a PE-10 catheter was inserted into the right internal jugular vein and flushed with heparinized 0.9% saline. A second PE-

10 catheter was inserted into the right fermoral artery and connected to a pressure transducer to continuously monitor MAP (mean arterial blood pressure) (Gould Instruments, Valley View, OH). Animals were then endotracheally intubated, connected to a mouse ventilator (Minivent, Hugo Sachs Elektronik, March-Hugstetten, Germany) and set to a respiratory rate of 160 min-1. Tidal volume was adjusted according to body weight to maintain arterial CO_2 tension within physiological range (35-45 mm Hg). CA was induced by injection of 50 µl 0.5 M KCl (4°C) via the jugular catheter, and confirmed by immediate drop in MAP. The endotracheal tube was disconnected from the ventilator and anesthesia stopped. During CA, body temperature was cooled to assure ease of resuscitation by placing the mouse on an ice water filled pad. However, mouse head temperature was maintained at 38.8°C, to assure reproducible injury, by a heated water-filled coil. CPR began 10 min after induction of CA by injecting 0.5 ml warm epinephrine solution (16 µg/ml), chest compressions at a rate of 300 min-1, ventilation with 100% O_2 at a rate of 190min-1, and 25% increased tidal volume. At initiation of CPR, head temperature was cooled to 37°C and the body re-warmed using a heat lamp and pad. Cardiac compressions were stopped when spontaneous circulation is restored to sustained systolic blood pressure of 60 mm Hg. Catheters and temperature probes were removed and skin wounds closed. CPR was stopped and animal excluded from experiment if circulation was not restored within 2 min. CA survivors received post-operative care and survived 3 hr or 48 hr post CA/CPR. ALLO administration protocol was modified from Sayeed et al., 2006. ALLO was administered by ip injection (8 mg/kg) 30

min. prior to CA/CPR, with two subsequent boosting injections (at 6 hr & 24 hr) to ensure ALLO presence throughout the 48 hr of recovery. Vehicle consisted of 20% β-cyclodextran in 0.9% saline and was administered at the same time points used for ALLO.

Cerebellar slice preparation, Histology and Immunohistochemistry. Following CA/CPR and 48 hr recovery, mice were euthanized with an ip injection of 120 mg/kg pentobarbital, perfused through the left ventricle with saline, followed by cold 4% formaldehyde, then removed and post-fixed in formaldehyde for an additional 12 hr. After removal, brains were coded with randomly assigned numbers. Brains were dehydrated, cleared, and embedded in paraffin. Coronal sections were made through the cerebellum at approximately –5.8 mm from Bregma at a thickness of 6 μm. Deparaffinized slices were heated in 0.5 mM sodium citrate buffer (pH 6) to expose antigen. All rinses were made with Phosphate buffered saline (PBS). Sections were incubated in blocking solution (5% normal goat serum in PBS) for 60 min at RT, followed by incubation with rabbit anti-$GABA_A$ receptor α_1 antibody (Millpore, Billerica, MA) diluted 1:1000 in blocking solution, 72 hr at $4^{\circ}C$. Secondary antibody goat anti-rabbit alexa-fluor 546 conjugated secondary antibody (Invitrogen, Carlsbad, CA) was diluted 1:1000 in blocking solution to visualize $GABA_A$ receptor protein. FluoroJade B staining was performed to assess neuronal degeneration (Schmued and Hopkins, 2000) by incubating sections in 0.001% FluoroJade B at 4^0C for 1hr. Fluorescent images were captured on an upright Leica DMIRE2 microscope and

the percentage of FluoroJade positive PC was determined by counting the number of fluorescent and non-fluorescent PC in the vermis of each cerebella. Experimenter performing analysis was blinded to treatment.

Statistical Analysis. All data is presented as mean ± SEM.

Each n represents an individual cell for electrophysiology experiments, individual culture for *in vitro* Western blot experiments, and an individual animal for *in vivo* experiments. Statistical significance was determined using students t-test (unpaired, 2-tailed, if $p<0.05$) or one way analysis of variance (ANOVA) with Neuman-Keuls post hoc analysis, $p<0.05$.

III. Results:

Oxygen-glucose deprivation caused a significant reduction in PC $GABA_A$ receptor current and α_1 subunit protein *in vitro.*

Whole-cell voltage-clamp recordings of cerebellar PC (10-14 DIV*)* contained spontaneous inhibitory post-synaptic currents (IPSCs) and robust GABA activated currents. Initial experiments were performed to determine the dose-dependence of GABA currents. Brief pulses (1 s) of increasing concentrations of GABA (1-3000 µM) evoked currents that reached maximal amplitude at 100-300 µM (Fig.1A). The EC_{50} was 8.8 ± 1.3 µM (n=5; Fig.1B), consistent with previous reports using cultured neurons (Rho et al., 1996;Yang

and Uchida, 1996). In order to examine the effect of ischemia on $GABA_A$ receptor function, PC were exposed to 2hr oxygen-glucose deprivation (OGD), a duration that causes significant PC death (Ardeshiri et al., 2006). OGD did not significantly affect $GABA_A$ receptor affinity for GABA (EC_{50} 7.8 ± 2.6 μM, n=6, Fig. 1B). However, OGD had a significant effect on PC GABA current density, declining from 701 ± 87 pA/pF under control conditions (n=25) to 431 ± 65 pA/pF within 60 minutes of re-oxygenation (n=26; Students t-test, $p<0.05$, Fig.1C, D). These findings suggest that the loss of total $GABA_A$ receptor activity is due to a decrease in the number of $GABA_A$ receptors, not reduced sensitivity of the receptors to GABA. Additionally, amplitude of mIPSCs were significantly smaller following OGD (22.6 ± 2.4 pA; n=8) than under control conditions (34.6 ± 5.2 pA; n=5, $p<0.05$, Student's t-test, Fig.1E, F). Importantly, OGD had no effect on mIPSC kinetics or frequency (data not shown), indicating loss of synaptic $GABA_A$ receptors in cerebellar PC. To determine if the decline in functional $GABA_A$ receptors is associated with loss of protein, western blot analysis of total cellular protein against the $GABA_A$ receptor α_1 subunit was used. The level of $GABA_A$ receptor protein remained stable during OGD and rapidly declined within the 1st hr of re-oxygenation (Fig.2A). Immunocytochemistry confirmed that $GABA_A$ α_1 is highly expressed on PC in culture, with a small amount of expression on non-PC, likely granule cells (Fig.S1).

Ischemia induced loss of $GABA_A$ receptor protein and current is prevented by ALLO*.*

ALLO protects cultured PC against oxygen-glucose deprivation (OGD) in a $GABA_A$ receptor activity dependent manner (Ardeshiri et al., 2006). Therefore, we tested the ability of ALLO to prevent the OGD-induced decline in $GABA_A$ receptor protein and current. OGD caused a significant decline in total $GABA_A$ receptor α_1 subunit protein, reducing to 64±11% of control levels, that was completely prevented by both physiological (10 nM) and pharmacological (1 µM) doses of ALLO, returning to 96±19% and 96±13% of control levels, respectively (n=6 for each group; Fig.2B,C). Exposure to ALLO (10nM or 1µM) for 3hr without OGD did not alter $GABA_A$ α1 protein levels (data not shown). To determine whether ALLO protects against the OGD-induced decline in $GABA_A$ receptor current, a physiological and neuroprotective dose of ALLO (10nM; (Ardeshiri et al., 2006) was tested. ALLO was removed during recordings to avoid the confounding effect of direct potentiation of $GABA_A$ receptors. The GABA current density recorded from cells exposed to OGD in the presence of ALLO was not significantly different than control cells (650 ± 228 pA/pF, n=8 and 701 ± 87 pA/pF, n=25; $p>0.1$ ANOVA; Fig.3A, B). These findings suggest that ALLO neuroprotection is at least in part mediated by its ability to prevent OGD-induced loss of $GABA_A$ receptor current and protein.

ALLO protection of oxygen-glucose deprivation induced decline in $GABA_A$ Receptor current is not activity dependent.

ALLO is a well characterized positive allosteric modulator of $GABA_A$ receptors. To determine whether ALLO preserves $GABA_A$ receptor response by potentiating activity, we tested a different allosteric modulator of $GABA_A$ receptors, diazepam (DZP). Oxygen-glucose deprivation (OGD) did not alter PC $GABA_A$ receptor sensitivity to DZP. The EC_{50} was 2.3 ± 0.4 µM (n=7) under control conditions and 2.8 ± 0.4 µM (n=6) following OGD. We used the same experimental protocol for ALLO with a dose of 2 µM DZP, the approximate EC_{50} value for PC. This concentration falls within the efficacious range (1-5 µM) used to protect rat cortical brain slices from OGD (Ricci et al., 2007). DZP treated cells had an average current density of 321 ± 69 pA/pF (n=11) 1hr after re-oxygenation, which did not significantly differ from OGD cells (355 ± 79 pA/pF; n=14). However, both groups had significantly reduced GABA current density compared to control cells (592 ± 94 pA/pF n=12; $p<0.05$ ANOVA; Fig.3C, D). The inability of DZP to prevent the OGD-induced decline in $GABA_A$ receptor current suggests that ALLO preserves $GABA_A$ receptor function following OGD through a mechanism that is unique to ALLO, and likely independent of its ability to potentiate $GABA_A$ receptor activity.

ALLO prevents decline in $GABA_A$ receptor protein *in vivo* and protects against global cerebral ischemia.

We used a mouse model of global cerebral ischemia, cardiac arrest/cardiopulmonary resuscitation (CA/CPR), to test the effect of ischemia on

PC survival and $GABA_A$ receptor expression in the intact animal. Injection of KCl resulted in immediate systolic cardiac arrest in all mice. Body weight, mean ischemia time, epinephrine dose and survival rate did not differ between groups (Vehicle: 20% β-cyclodextran or 8 mg/kg ALLO). The effect of ALLO treatment on physiological parameters was tested (n=5/group), and no differences in blood gases, pH, blood glucose, lactate, sodium or potassium were observed (data not shown). CA/CPR caused significant damage to cerebellar PC, indicated by bright green labeling with the neuronal damage marker FluoroJade B (Fig.4A), and ALLO provided significant protection to PC, decreasing damage from 22.3 ± 6.9% (n=9) in vehicle treated mice to 9.9 ± 3.3% (n=11; $p<0.05$ Student's t-test; Fig.4B,C). These data present a novel finding that ALLO is a strong neuroprotectant in the cerebellum *in vivo*.

Similar to our findings *in* vitro, cerebral ischemia caused a significant reduction in total cerebellar $GABA_A$ receptor protein. Western blot analysis of cerebellar $GABA_A$ α_1 subunit protein revealed that vehicle treated mice had significantly less protein 48hr after recovery from CA/CPR (55.1 ± 5.5% of control, n=4; $p<0.05$ ANOVA; Fig.5). In addition, ALLO treated mice had significantly more $GABA_A$ α_1 subunit protein than vehicle treated mice (75.4 ± 15% of control, n=4; $p<0.05$, ANOVA; Fig.5). Analysis of $GABA_A$ α_1 subunit protein at an early time point of recovery (3 hr) revealed a similar trend. CA/CPR (vehicle) caused a rapid decline in $GABA_A$ receptor protein (73.6 ± 3.6%; n=4) that was partially prevented by the presence of ALLO (83.1 ± 7.6%; n=4; data not shown). Quantitative real-time RT-PCR analysis of cerebellar mRNA

demonstrated ischemia did not alter transcription of the $GABA_A$ α_1 subunit gene at 3 hr or 48 hr recovery from CA/CPR (data not shown).

Cerebellar granule cells and inhibitory interneurons (basket and stellate cells) also express $GABA_A$ receptor α_1 subunits, although in comparison to PC, the expression level is low. To observe PC specific changes in $GABA_A$ receptors we stained cerebellar sections for total α_1 subunit expression. We observed a qualitative reduction in staining intensity at the soma of PC in vehicle treated animals (Fig.6B) compared to sham (Fig.6A). In addition, the staining intensity was markedly greater in ALLO treated mice (Fig.6C) compared to vehicle. These data confirm global ischemia causes loss of $GABA_A$ receptor α_1 subunits in cerebellar PC that is prevented by ALLO.

IV. Discussion:

Cerebellar PC respond to ischemia by rapidly removing $GABA_A$ receptors from synapses, resulting in significantly less inhibitory potential. Our data show that GABA current density and amplitude of mIPSC are reduced within the first hour of reperfusion following *in vitro* ischemia. Consistent with our electrophysiology data, we show that ischemia causes a concurrent loss of $GABA_A$ receptor protein and the progesterone metabolite ALLO protects PC against ischemia, both *in vitro* and *in vivo,* at least in part by preventing ischemia-induced decline in $GABA_A$ receptor activity. These findings help define the

molecular basis of cerebellar PC susceptibility to ischemia-induced damage and excitotoxicity.

Effects of Ischemia on functionally available $GABA_A$ receptors; comparison to other studies

To our knowledge we are the first to assess the function of $GABA_A$ receptors in PC during the early reperfusion stage following a lethal dose of ischemia. Our findings using cerebellar PC are consistent with studies which report depressed IPSCs following ischemia in other neuronal populations (Khazipov et al., 1995; Luhmann et al., 1995; Zhan et al., 2006). Previous studies differed in their experimental design and did not rule out pre-synaptic effects on neurotransmitter release. However, all observed a sustained decline in GABAergic neurotransmission. Another cause of reduced inhibitory transmission could be from loss of GABAergic synapses, as reported in hippocampal CA3 neurons after global ischemia (Epsztein et al., 2006). Our data show mIPSC frequency in cerebellar PC is not altered by ischemia, indicating that the number of synapses remains unchanged during the early phase of reperfusion. The decline in amplitude of mIPSCs observed in our study indicates that ischemia causes loss of synaptic $GABA_A$ receptors. In addition, it is possible that ischemia causes a reduction in the amount of GABA released at each synapse (decreased quantal size). However, we did not observe ischemia-induced changes in mIPSC

kinetics, indicating synaptic $GABA_A$ receptors are exposed to saturating GABA before and after ischemia.

$GABA_A$ receptors expressed on cerebellar PC are almost exclusively comprised of $\alpha_1\beta_{2/3}$ and γ_2 subunits (Fritschy et al., 1992). In fact, genetic deletion of $GABA_A$ α_1 subunits completely abolishes synaptic GABAergic transmission in cerebellar PC (Fritschy et al., 2006). Therefore, we used levels of $GABA_A$ α_1 subunits as a measure of total $GABA_A$ receptor levels. We believe the ischemia-induced reduction of $GABA_A$ receptor current and α_1 subunit protein in PC is due in large part to protein degradation. Three separate results lead us to conclude that ischemia causes $GABA_A$ receptor degradation. First, western blots performed on total cellular protein, including membrane and cytoplasmic fractions, indicate $GABA_A$ receptor protein is reduced after ischemia. Second, the decline in protein occurs rapidly, during the first hour of re-oxygenation, but not during ischemia, excluding the possibility of reduced protein from ATP depletion and subsequent reduction in translation of new protein. Finally, quantitative real-time PCR indicates that ischemia does not alter transcription of $GABA_A$ receptors. Taken together, we conclude ischemia causes decreased $GABA_A$ receptor protein without altering transcription or translation, implicating protein degradation.

Our results contrast somewhat with studies in cortical and hippocampal neurons which report that ischemia causes $GABA_A$ receptor internalization, but not degradation (Alicke and Schwartz-Bloom, 1995). An explanation for this discrepancy may be that different neuronal populations have distinct

mechanisms of $GABA_A$ receptor trafficking in response to ischemia. Alternatively, additional mechanisms may contribute to the loss of functional $GABA_A$ receptors observed in our study. For example, loss of neuronal chloride gradient after ischemia has been proposed as a mechanism of ischemia-induced decline in GABAergic inhibition (Sah and Schwartz-Bloom, 1999) and measurements of chloride ion levels indicate that CA1 hippocampal neurons have increased intracellular chloride after one hour of reperfusion (Galeffi et al., 2004). However, the current study was designed to avoid this confounding effect by using conventional whole-cell patch clamp configuration with symmetrical chloride concentrations in the recording pipette and saline bath. Therefore, the decline in $GABA_A$ receptor activity observed following ischemia was due to decreased receptor availability and not alterations in the chloride gradient. Phosphorylation of $GABA_A$ receptors can also alter GABAergic neurotransmission, both increasing and decreasing activity (Sigel et al., 1991; Kano and Konnerth, 1992; Leidenheimer et al., 1992; Moss et al., 1995;Wan et al., 1997) and ischemia can affect the activity of various protein kinases (Bright and Mochly-Rosen, 2005). It is possible then that a portion of the ischemia-induced reduction in $GABA_A$ receptor activity observed in the current study is a result of altered $GABA_A$ receptor phosphorylation.

Implications of ALLO neuroprotection of PC

Consistent with recent reports demonstrating ALLO neuroprotection against damage induced by MCAO or TBI (Sayeed et al., 2006;Djebaili et al., 2004), we found ALLO significantly decreased cerebellar PC damage following global ischemia. The mechanism of neuroprotection afforded by ALLO is not completely clear. Multiple mechanisms have been postulated to underlie the beneficial effects of ALLO, including neurogenesis (Wang et al., 2005), inhibition of apoptosis by activation of PKB/Akt kinase (Xilouri et al., 2007), and potentiation of $GABA_A$ receptor activity. In fact, ALLO is among the strongest potentiators of $GABA_A$ receptor activity (Belelli and Lambert, 2005) and ALLO neuroprotection from ischemic cell death is dependent on potentiation of $GABA_A$ receptor activity (Ardeshiri et al., 2006). Our results indicate that ALLO prevents ischemia-induced loss of $GABA_A$ receptor current in cultured PC and loss of $GABA_A$ α_1 protein *in vitro* and *in vivo*, suggesting that ALLO augments inhibitory neurotransmission following ischemia via multiple mechanisms. One mechanism involves the well-characterized direct interaction of ALLO with $GABA_A$ receptors resulting in potentiation of receptor activity. A second involves a previously unknown mechanism of stabilizing synaptic $GABA_A$ receptors, maintaining GABAergic activity following ischemia. In addition, application of ALLO concurrent with re-oxygenation provides robust protection against OGD (Ardeshiri et al., 2006), suggesting that ALLO may be effective as a treatment following cerebral ischemia.

Although the exact mechanism by which ALLO stabilizes $GABA_A$ receptors following ischemia remains unknown, a simple hypothesis is that ALLO

potentiation of $GABA_A$ receptor activity during ischemia/reperfusion prevents ischemia-induced loss of $GABA_A$ receptors. We tested this hypothesis directly by treating PC with diazepam, another well-characterized $GABA_A$ receptor potentiator. Diazepam failed to prevent the OGD-induced decline in $GABA_A$ receptors, indicating that ALLO has a unique effect on $GABA_A$ receptor homeostasis. ALLO has a lipophilic structure that may allow it to incorporate into cell membranes where it can affect membrane-associated endocytotic and trafficking machinery. Alternatively, binding of ALLO to $GABA_A$ receptors may cause a conformational change in the receptor exposing a phosphorylation site or other regulatory site altering $GABA_A$ receptor stability (Fancsik et al., 2000).

Cerebellar PC have long been understood to be particularly sensitive to ischemia and excitotoxicity by an elusive cellular mechanism. This study implicates the rapid loss of inhibitory GABAergic signaling as a contributor to PC sensitivity to ischemia, a finding which has ramifications for the use of GABAergic compounds as neuroprotectants. The efficacy of $GABA_A$ receptor potentiators would decrease with ischemia-induced loss of $GABA_A$ receptors, thereby diminishing their effectiveness as neuroprotectants. Therefore, treatments aimed at stabilizing $GABA_A$ receptor protein at the membrane, preserving receptor function, represent an exciting new therapeutic approach to protect neurons from ischemia/reperfusion injury.

Figures:

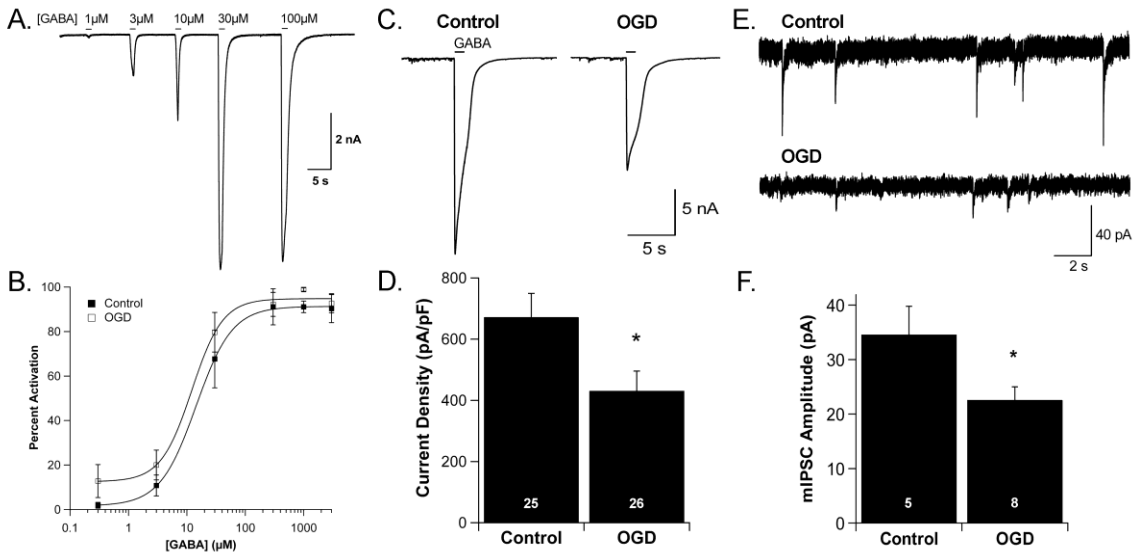


Fig.1 Oxygen-glucose deprivation causes a significant reduction in synaptic and total $GABA_A$ receptor current.

A.) Representative trace depicting response of a single cell to 1s applications of increasing concentrations of GABA, indicated by solid bar. B.) Concentration-response relationships for control and OGD cells. Each concentration-response relation was normalized to individual cell's maximal current response, data presented are the average of 5-6 cells. C.) Representative responses to 1s application of 1mM GABA from control (left) and OGD (right). D.) Quantification of $GABA_A$ receptor current density. E.) Representative traces of mIPSCs, recorded in the presence of CNQX and TTX under control (top) and OGD (bottom) conditions. F.) Quantification of mIPSC amplitude. OGD treated cells

exposed to 2hr OGD and data collected within initial 1hr of re-oxygenation. The number of experiments is indicated in each bar. Data are mean ± SEM. * $P < 0.05$ vs. control cells.

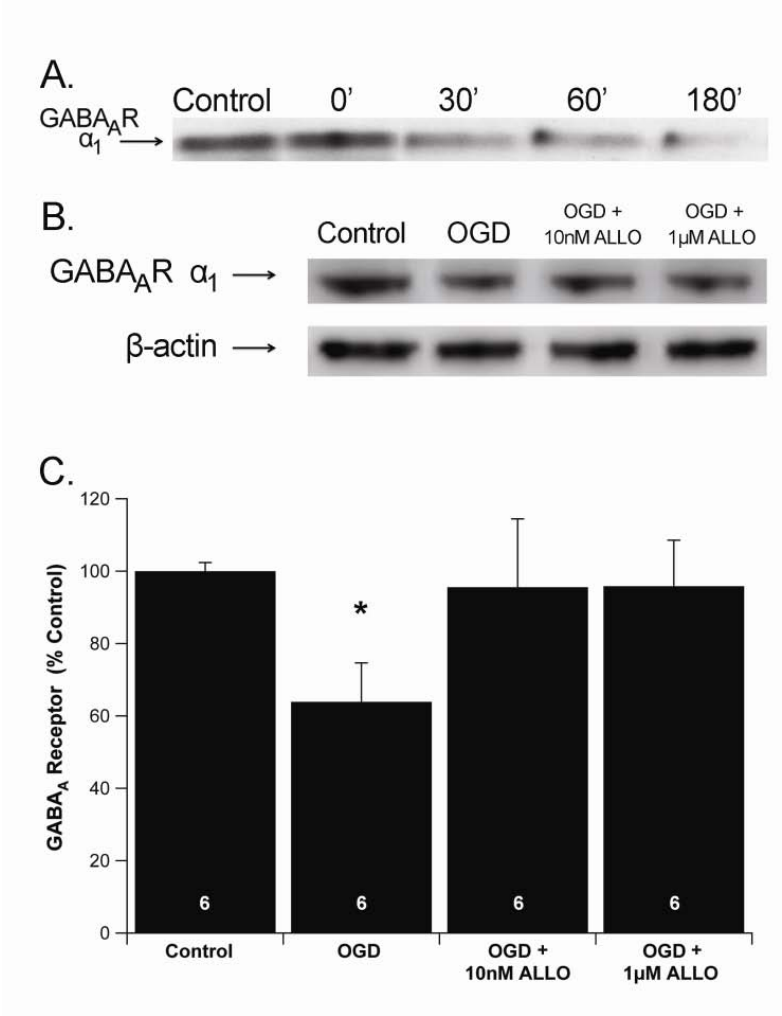


Fig. 2 Oxygen-glucose deprivation causes a rapid and sustained loss of GABA$_A$ receptor protein that can be prevented by ALLO. A.) Representative Western blot for GABA$_A$ receptor α$_1$ subunit protein from a control cell and OGD cells after 0, 30, 60, or 180 minutes re-oxygenation, indicated above each lane. B.) Representative Western blot for GABA$_A$ receptor α$_1$ subunit protein from control, OGD and OGD+ALLO treated samples following 1hr re-oxygenation. ALLO cells received 15min pre-treatment with 10nM or 1μM ALLO, and ALLO

remained present during OGD and re-oxygenation. β-actin loading control shown in lower panel C.) Quantification of normalized $GABA_A$ receptor α_1 subunit protein, normalized to β-actin loading control and expressed as percent of average control level. The number of experiments is indicated in each bar. Data are mean ± SEM. * $P < 0.05$ vs. control cells.

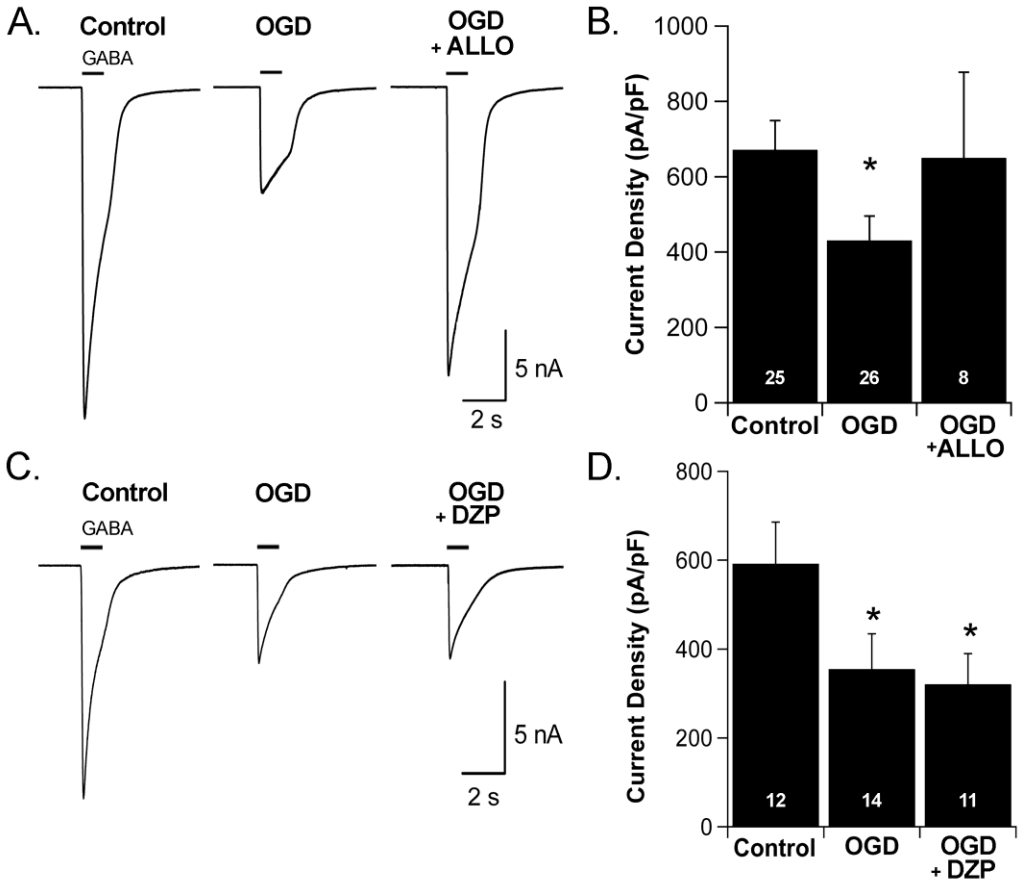


Fig.3 ALLO prevents the oxygen-glucose deprivation induced loss of $GABA_A$ receptor current but diazepam does not. OGD treated cells exposed to 2 hr OGD and data collected within initial 1 hr of re-oxygenation. A.) Representative responses to 1s application of 1mM GABA from a control, OGD and OGD+ALLO cell. ALLO cells received 15 min pre-treatment with 10 nM ALLO, and ALLO remained present during OGD and removed prior to recording. B.) Quantification of measured current density for conditions in A. C.) Representative current responses to 1s application of 1mM GABA from a control, OGD, and OGD+DZP cell. DZP cells received 15min pre-treatment with 2μM

DZP, and DZP remained present during OGD and removed prior to recording. B.) Quantification of measured current density for conditions in C. The number of experiments is indicated in each bar. Data are mean ± SEM. * $P < 0.05$ vs. control cells.

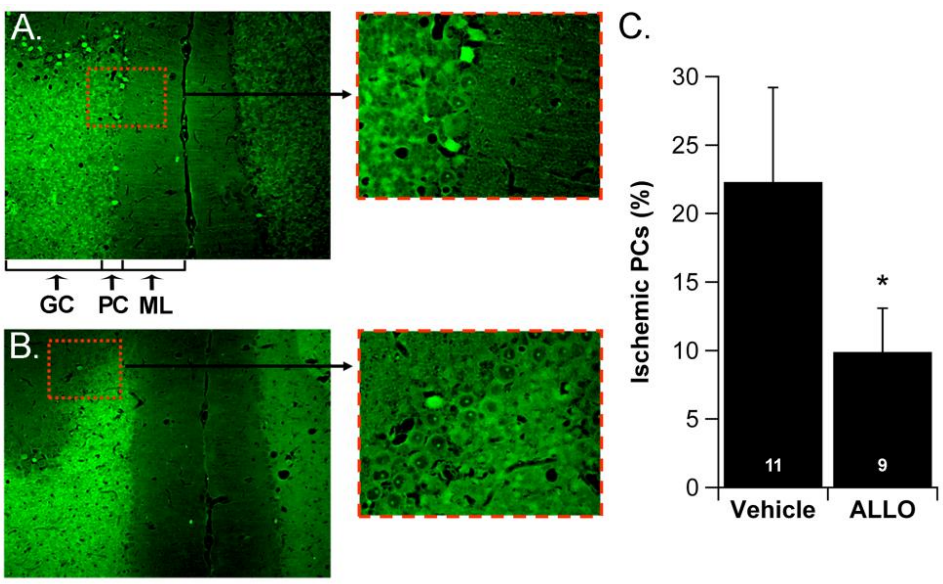


Fig. 4 ALLO protects cerebellar PC against global cerebral ischemia *in vivo*.

A.) Representative FluoroJade B staining of cerebellar section from a vehicle treated mouse 48hr after CA/CPR. Injured PC stain bright green and red box indicates region expanded to right. GC= granule cell layer, PC= Purkinje cell layer, ML= molecular layer. B.) Representative FluoroJade B staining of cerebellar section from an ALLO treated mouse 48hr after CA/CPR. Injured PC stain bright green and red box indicates region expanded to right. C.) Quantification of ALLO protection of cerebellar PC. Percentage of FluoroJade positive PC determined by dividing the number of bright green PC by total number of PC in the section. The number of experiments is indicated in each bar. Data are mean ± SEM. * $P < 0.05$ vs. control cells.

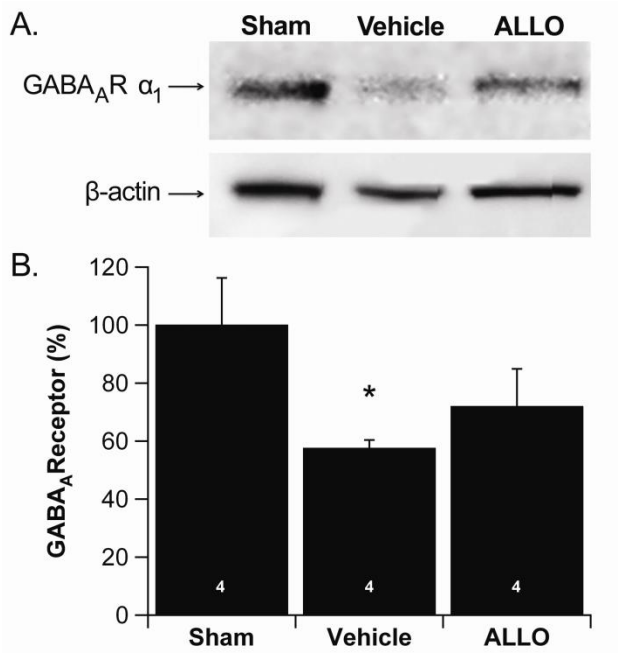


Fig. 5 ALLO prevents ischemia-induced decline in cerebellar GABA$_A$ receptor α$_1$ protein A.) Representative Western blot of cerebellar GABA$_A$ receptor α$_1$ subunit protein from control (sham), vehicle and ALLO treated mice 48hr after CA/CPR. β-actin loading control shown in lower panel B.) Quantification of effect of ALLO on ischemia-induced decline in GABA$_A$ receptor α$_1$ subunit protein, normalized to β-actin loading control and expressed as percent of control level. The number of experiments is indicated in each bar. Data are mean ± SEM. * $P < 0.05$ vs. control cells.

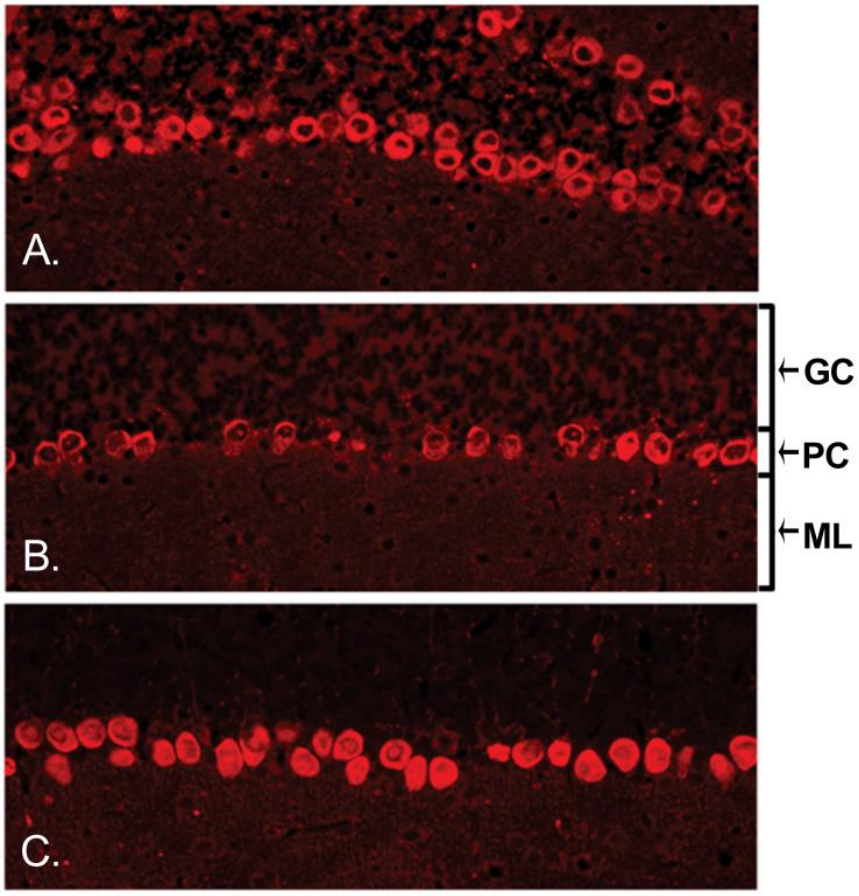
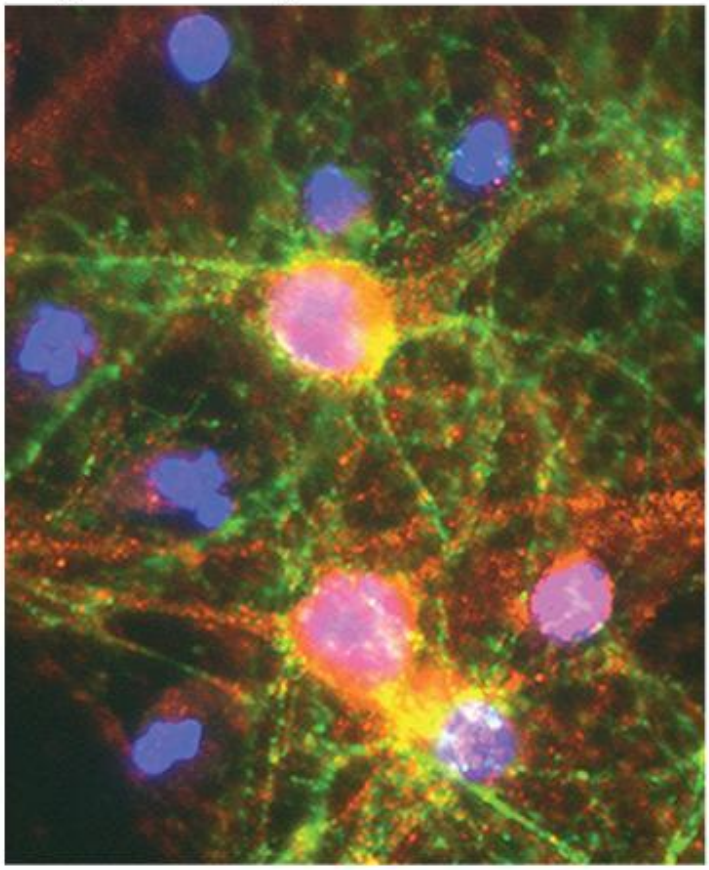


Fig.6 ALLO prevents ischemia-induced decline in PC $GABA_A$ receptor α_1 protein

A.) Representative staining with $GABA_A$ receptor α_1 subunit antibody of cerebellar section from sham operated mouse. B,C.) Representative staining with $GABA_A$ receptor α_1 subunit antibody of cerebellar sections from vehicle (B) or ALLO (C) treated mice 48hr after CA/CPR. GC= granule cell layer, PC= Purkinje cell layer, ML= molecular layer.

Supplemental Fig. 1



Cultured cerebellar PC's predominantly express $GABA_AR$ α_1 subunit

Red= C28K (PC marker)
Green= $GABA_AR$ α_1 subunit
Blue= DAPI

CHAPTER 3:

Sex Difference in Sensitivity to Allopregnanolone Neuroprotection in Mice Correlates with Effect on Spontaneous Inhibitory Post Synaptic Currents

Melissa H. Kelley, Masayuki Kuroiwa[1], Noriko Taguchi[2], Paco S. Herson[*]

Department of Anesthesiology and Perioperative Medicine

Oregon Health and Science University,

3181 SW Sam Jackson Park Rd.

Portland, OR USA

[1] Present address: Department of Anesthesiology, National Hospital Organization, Sagamihara National Hospital, Kanagawa, Japan

[2] Present address: Department of Anesthesia, Univeristy of Tsukuba, Ibaraki, Japan

Keywords: allopregnanolone; $GABA_A$ receptor; Purkinje cell; sex difference; ischemia; neuroprotection

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Abstract

Allopregnanolone (ALLO) is a neurosteroid that has many functions in the brain, most notably neuroprotection and modulation of gamma-amino butyric acid (GABA) neurotransmission. Using a mouse model of cardiac arrest and cardiopulmonary resuscitation, we have previously demonstrated that ALLO protects cerebellar Purkinje cells (PCs) from ischemia in a $GABA_A$ receptor-dependent manner. In this study we examined the effect of sex on ALLO neuroprotection, observing that low dose ALLO (2 mg/kg) provided greater neuroprotection in females compared to males. At a higher dose of ALLO (8 mg/kg), both sexes were significantly protected from ischemic damage. Using an acute cerebellar slice preparation, whole cell voltage clamp recordings were made from PCs. Spontaneous inhibitory postsynaptic currents (IPSCs) were analyzed and the response to physiological ALLO (10 nM) was significantly greater in female PCs compared to male. In contrast, recordings of miniature IPSCs, did not exhibit a sex difference in response to ALLO, suggesting that ALLO affects males and females differentially through a mechanism other than binding postsynaptic $GABA_A$ receptors. We conclude that the female brain has greater sensitivity to ALLO mediated potentiation of GABAergic neurotransmission, contributing to increased neuroprotection.

1. Introduction

Cardiac arrest requiring cardiopulmonary resuscitation (CA/CPR) is a leading cause of death and disability in the United States, affecting over 300,000 adults and children in the United States each year (Roger et al., 2010). Gender is a significant factor influencing incidence and possibly outcome, with women being relatively protected compared to men (Kim et al., 2001; Wigginton et al., 2002; Vukmir, 2003; Rosamond et al., 2008). Animal models of cerebral ischemia, including CA/CPR, mimic the human epidemiology, exhibiting sex-specific differences in tissue damage and long-term behavioral recovery (Herson and Hurn, 2010). Innate protection from ischemic damage in the female is due in large part to endogenous levels of sex steroids, the estrogens and progesterone. In addition to the estrogens and progesterone, there is emerging evidence that metabolites of these hormones have important physiological and pathophysiological roles in the brain (Liu et al., 2010).

The neurosteroid allopregnanolone (ALLO) is a metabolite of progesterone that is neuroprotective in several animal models of neurodegenerative diseases including Alzheimer's (Brinton and Wang, 2006), traumatic brain injury (TBI) (Djebaili et al., 2004), stroke (Sayeed et al., 2006), and recently cardiac arrest (Kelley et al., 2008). Cerebral ischemia, as experienced following CA/CPR results in selective damage to vulnerable neuronal populations, including hippocampal CA1 neurons (Pulsinelli et al., 1982) and cerebellar Purkinje cells (Horn and Schlote, 1992; Brasko et al., 1995; Fonnum and Lock, 2000; Ardeshiri et al., 2006). We have previously demonstrated that ALLO can protect cultured

PCs from *in vitro* ischemia, oxygen-glucose deprivation, and global ischemia *in vivo*, in part by potentiating $GABA_A$ receptor activity (Ardeshiri et al., 2006) and also by preserving $GABA_A$ receptor protein and function (Kelley et al., 2008). To date, the interaction between animal sex and ALLO has not been examined in the context of neuroprotection. In the current study, we tested whether there is a sex difference in ALLO neuroprotection of PCs and we examined the ability of ALLO to enhance GABAergic neurotransmission in the cerebellum.

2. Methods

2.1. Cerebellar Slice Preparation

All animal experiments were performed in accordance with the National Institutes of Health guidelines for care and use of Laboratory animals and approved by the Oregon Health and Science University Animal Care and Use Committee. Adult (8-16 wk old) C57/BL6 male and female mice (Charles River) were anesthetized by i.p. injection of a ketmine/xylazine (80/12 mg/ml, Sigma Aldrich) cocktail and transcardially perfused with ice cold oxygenated cutting solution (see 2.3). Mice were decapitated, cerebellum removed, and sagittal slices (400 μm thick) were cut from the vermis of the cerebellum with a vibroslicer (Vibratome or Leica). Slices were incubated at 37ºC in warm oxygenated artificial cerebrospinal fluid (ACSF, see 2.3) for 30 minutes then stored at room temperature. All experiments were performed at room temperature and completed within 4-5 hours of slicing to ensure cell viability.

2.2. Electrophysiology

Whole cell voltage clamp recordings were made from the soma of PCs using an Axopatch 200B (Axon Instruments, Union City, CA) amplifier interfaced to a Dell computer (Dell, Round Rock, TX). Data was collected using pCLAMP9 (Molecular Devices, Sunnyvale, CA) at a sample frequency of 20 kHz, with lowpass filtering at 2 kHz. Electrodes pulled from borosilicate glass capillaries with inner filaments using a Flaming Brown electrode puller (Sutter Instrument Co, Novato, CA) had resistances of 2-4 MΩ when filled with a CsCl internal pipette solution (see 2.3). Whole cell capacitance and resistance were electronically compensated. Adequate whole cell access (R_a < 30 MΩ) was verified at the beginning of the recording, before recording in the presence of ALLO, and at the end of recording. Slices were continuously perfused with aerated ACSF using a gravity fed perfusion system with a flow rate of 1-2 ml/minute.

Purkinje cells were voltage-clamped at a membrane potential of -60 mV and GABA-mediated spontaneous inhibitory post synaptic currents (sIPSCs) were recorded as inward currents (Konnerth et al., 1990; Herson et al., 2003). IPSCs were recorded in 3 minute sweeps. TTX (250 nM) was bath applied for 6 minutes prior to recording baseline miniature IPSCs (mIPSCs) and TTX remained present during ALLO application. ALLO (10 nM) was bath applied for 20 minutes prior to recording its effect on IPSCs. Individual cells were used separately to test the effect of ALLO on sIPSCs or mIPSCs. Recordings were analyzed using Clampfit (Axon Instruments), and Igor Pro software (WaveMetrics, Lake Oswego, OR). Analysis of IPSC amplitude, frequency and

kinetics were analyzed as described previously (Herson et al., 2003). Briefly, kinetic analysis was performed by detecting individual IPSCs using a sliding variable amplitude template. 20-30 events from each cell per condition were chosen at random, event baseline was adjusted by subtracting the mean baseline of the events, events were aligned by rise time, and event traces were averaged (Clampfit). Using a sum of exponentials function, decay constants were calculated (Igor). Event detection and fitting were confirmed by eye. The total number of events observed in 3 minute recordings from each cell was used to calculate frequency (Igor). The amplitude of all events detected for each cell during a 3 minute recording was used to calculate average IPSC amplitude and cumulative probability of amplitude for that cell, and data are reported as mean ± SEM. Unless otherwise noted, n represents the number of recordings of individual cells from separate animals. Additionally, recordings were only made from slices that were not previously exposed to exogenous ALLO.

2.3. Solutions and Drugs.

Cutting solution was composed of (in mM) 110 choline chloride, 2.5 KCl, 7 $MgSO_4$, 0.5 $CaCl_2$, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 25 Dextrose, 11.6 Na-ascorbate, 3.1 Na-pyruvate. The composition of the ACSF solution was (in mM): 119 NaCl, 2.5 KCl, 1 NaH_2PO_4, 26.2 $NaHCO_3$, 1.3 $MgCl_2$, 2.5 $CaCl_2$, and 10 Dextrose, aerated with 95% O_2/ 5% CO_2. Internal pipette solution was (in mM): 140 CsCl, 1 EGTA, 10 HEPES, 1 $MgCl_2$, 5 MgATP, pH of 7.3 with CsOH. TTX was dissolved in DMSO to make a stock concentration of 1 mM, working concentration was 250 nM. For electrophysiology recordings, ALLO was dissolved in DMSO at a stock

concentration of 10 mM for storage. Fresh working solution of ALLO (10 nM) was made by serial dilution in ACSF daily. ALLO for in vivo CA/CPR experiments was dissolved in 20% β-cyclodextran in 0.9% saline. ALLO was obtained from Calbiochem/EMD Chemicals (Gibbstown, NJ). TTX was obtained from Tocris (Ellisville, MO).

2.4. Cardiac Arrest & Cardiopulmonary Resuscitation (CA/CPR).

CA/CPR was performed on adult (20-25g) male and female C57BL/6 mice to simulate global cerebral ischemia as described by our group previously (Kofler et al., 2004; Kelley et al., 2008). Anesthesia was induced with 3% isoflurane and maintained with 1.5-2% isoflurane in O_2 enriched air via face mask. Temperature probes were inserted into left temporalis muscle and rectum to monitor head and body temperature simultaneously. For drug administration, a PE-10 catheter was inserted into the right internal jugular vein and flushed with heparinized 0.9% saline. Animals were then endotracheally intubated, connected to a mouse ventilator (Minivent, Hugo Sachs Elektronik, March-Hugstetten, Germany) and set to a respiratory rate of 160 min-1. Tidal volume was adjusted according to body weight to maintain arterial CO_2 tension within physiological range (35-45 mm Hg). CA was induced by injection of 50 µl KCl (0.5 M, 4°C) via the jugular catheter, and confirmed by EKG. The endotracheal tube was disconnected from the ventilator and anesthesia stopped. During CA, body temperature was cooled to assure ease of resuscitation by placing the mouse on an ice water filled pad. However, mouse head temperature was maintained at 38.5°C, to assure reproducible injury, by a heated water-filled coil. CPR began 10 min. after

induction of CA by injecting 0.5 ml warm epinephrine solution (16 µg/ml), chest compressions at a rate of 300/min, ventilation with 100% O_2 at a rate of 190/min, and 25% increased tidal volume. At initiation of CPR, head temperature was cooled to 37^{o}C and the body re-warmed using a heat lamp and pad. Cardiac compressions were stopped when spontaneous circulation was restored. Catheters and temperature probes were removed and skin wounds closed. CPR was stopped and animal excluded from experiment if circulation was not restored within 2 minutes. CA survivors received post-operative care and survived 48 hr post CA/CPR. ALLO was administered by ip injection (either 2 mg/kg or 8 mg/kg) 30 minutes prior to CA/CPR, with two subsequent boosting injections (at 6 hr & 24 hr), as described previously (Kelley et al., 2008). Vehicle (see 2.3) was administered at the same time points used for ALLO.

2.5. Histology.

Following CA/CPR and 48 hr recovery, mice were euthanized with isoflurane, perfused through the left ventricle with saline, followed by cold 4% formaldehyde, then brains were removed and post-fixed in formaldehyde for an additional 12 hr. After removal, brains were coded with randomly assigned numbers. Brains were dehydrated, cleared, and embedded in paraffin. Coronal sections were made through the cerebellum at approximately –5.8 mm from Bregma at a thickness of 6 µm. FluoroJade B staining was used to label injured neurons (Schmued and Hopkins, 2000) as previously described by Kelley et al., 2008. Experimenter performing analysis was blinded to treatment. PC damage was expressed as percentage of FluoroJade positive PCs in the vermis of each cerebellum. N

represents the number of mice examined under each condition. For each n, all PCs in a single 6 μM section (approximately 300 cells) were analyzed to determine the percentage of FluoroJade positive PCs.

2.6. Statistical Analysis.

All data is presented as mean ± SEM. Each n represents an individual cell for electrophysiology experiments and an individual animal for *in vivo* experiments. Statistical significance was determined using students t-test (unpaired, 2-tailed, if $P<0.05$) or two way analysis of variance (ANOVA) with Neuman-Keuls post hoc analysis, $P<0.05$ to assess sex versus drug interaction.

3. Results

3.1. ALLO neuroprotection following CA/CPR

We have previously demonstrated that ALLO can protect PCs from global ischemia in male mice at a dose of 8 mg/kg (Kelley et al., 2008). However, ALLO's neuroprotective effects have not been examined in female mice. Using our mouse CA/CPR model we tested whether ALLO is neuroprotective in females and if the dose dependence is similar to males. Two doses of ALLO were tested, either 2 mg/kg or 8 mg/kg, administered by ip injection 30 minutes prior to CA/CPR followed by two boosting injections of the same dose at 6 hr & 24 hr recovery. We found that both males and females were significantly protected with the 8 mg/kg dose of ALLO. The percentage of FluoroJade positive PCs for males decreased from 20.09 ± 3.94% with vehicle treatment (n=11) to 9.87 ± 2.06% with 8 mg/kg ALLO (n=13, Fig. 1). The percentage of

FluoroJade positive PCs for females decreased from 13.25 ± 2.39% with vehicle (n=6) to 5.64 ± 2.18% with 8mg/kg ALLO (n=6, Fig. 1). With a 2mg/kg dose of ALLO males showed almost no protection (19.00 ± 5.12%, n=8), while females were strongly protected (5.43 ± 2.84% n=7; P= 0.06 when compared to female vehicle; Fig. 1). Post-hoc analysis revealed that response to 2 mg/kg was significantly affected by sex (P> 0.05, 2-way ANOVA). While not significant, our data indicates that vehicle treated females suffered less PC damage compared to males, consistent with female neuroprotection attributed to estrogen and progesterone (Herson et al., 2009; Liu et al., 2010; Herson and Hurn, 2010).

3.2. Effects of ALLO on sIPSCs

ALLO is a well-characterized $GABA_A$ receptor potentiator (Belelli and Lambert, 2005). In acute brain slice recordings, ALLO prolongs IPSC decay kinetics (Cooper et al., 1999; Vicini et al., 2002; Harney et al., 2003; Koksma et al., 2003), resulting in increased overall inhibitory neurotransmission. To test sex-specific sensitivity of cerebellar PCs, a physiological concentration of ALLO (10 nM) (Purdy et al., 1991) was applied to PCs recorded from adult male and female mice. Spontaneous IPSCs were recorded from PCs in acute brain slices and individual synaptic events were analyzed to determine decay kinetics. In all recordings, bath application of ALLO slowed the decay kinetics of IPSCs (Fig. 2a), consistent with previous studies. In male PCs, sIPSC decay kinetics were best fit by a single exponential (τ: 9.85 ± 1.08 ms, n=5 cells from 3 mice) and ALLO significantly altered the kinetics of sIPSC decay, resulting in sIPSCs best fit with a double exponential (τ1 = 7.54 ± 0.60 ms and τ2 = 25.42 ± 3.25 ms, %τ2

= 18± 0.02, n=5 cells from 3 mice). Similarly, under control conditions IPSCs recorded from female PCs had decay kinetics best fit by a single exponential (10.15 ± 0.51 ms, n=5). ALLO significantly slowed the kinetics of sIPSC decay in female PCs, resulting in IPSCs fit by a double exponential (τ1 = 7.76 ± 0.79 ms and τ2 = 42.26 ± 5.75 ms, %τ2 = 19 ± 0.04, n=5). While no difference in IPSC kinetics was observed between male and female PCs under control conditions, IPSCs recorded from female PCs had a significantly greater response to ALLO; the slow component of the IPSC (τ2) was significantly different between sexes in the presence of ALLO, where males were 25.42 ± 3.25 ms and females were 42.26 ± 5.75 ms (Fig. 2c). The relative contribution of each tau was not different between male and female cells, and after ALLO. Frequency and amplitude of sIPSCs were not significantly different between males and female PCs under control condition and interestingly, ALLO had no effect on frequency or amplitude of IPSCs (Table 1; Fig 2 e, f).

3.3. Effects of ALLO on mIPSCs

Miniature IPSCs were recorded (250 nM TTX) to assess the role of post-synaptic $GABA_A$ receptors in response to ALLO observed in sIPSCs. Under control conditions, decay kinetics of male and female mIPSCs were best fit with a single exponential and were not significantly different at baseline (τ1 was 9.59 ± 0.62 ms, n=8; 11.33 ± 0.58 ms, n=7 for males and females respectively; Fig. 3c). Similar to ALLO's effect on sIPSCs, ALLO significantly altered the kinetics of mIPSCs in both sexes, resulting in mIPSCs best fit with a double exponential. Interestingly, the effect of ALLO on mIPSC kinetics was not significantly different

between sexes (τ1 was 6.31 ± 0.47 ms, 7.09 ± 0.27 ms for male and females respectively; τ2 was 34.21 ± 4.29 ms, 39.91 ± 2.78 for males and females respectively; n=8 for males, n=7 for females; Fig. 3a-c). The relative contribution of each tau was not dramatically different (<5%) in male and female cells after ALLO. Frequency and amplitude of mIPSCs were not significantly different between males and females, and as described for sIPSCs, ALLO had no effect on frequency or amplitude of mIPSCs in either sex (Table 1; Fig.3 e.f).

4. Discussion

Our experiments demonstrate that sex modifies the response of GABAergic neurotransmission to physiological levels of ALLO in cerebellar Purkinje cells. This finding correlates with our in vivo data demonstrating that females are protected from global ischemia at a dose of ALLO four times lower than that required to protect males. Our mIPSC data were surprising and suggest that the differences we observed, comparing male and female sIPSCs, are likely not at the level of the postsynaptic receptors.

ALLO has been proven to be a strong neuroprotectant against focal ischemia in male rats at a dose of 8 mg/kg (Sayeed et al., 2006). Using the same experimental paradigm, we previously demonstrated that 8 mg/kg ALLO reduces cerebellar Purkinje cell damage following global cerebral ischemia induced by CA/CPR in male mice (Kelley et al., 2008). Data in the current study are consistent with previous reports, where ALLO significantly reduced PC damage in both male and female mice following CA/CPR. Few studies utilize both sexes

in the same set of experiments, yet it is of great clinical relevance to do so. Comparison of male and female response to CA/CPR revealed that females exhibit less damage compared to males, consistent with the large body of literature demonstrating that females are relatively protected compared to males due to the well characterized neuroprotection by ovarian hormones, estrogen and progesterone (Herson et al., 2009; Liu et al., 2010; Herson and Hurn, 2010). Despite the less severe damage observed in females, ALLO provided further protection, decreasing damage by over 50%. Our most striking finding was that male and female mice responded differently to a lower dose (2 mg/kg) of ALLO, having no effect in males following CA/CPR, but providing significant protection in females, reducing damage by 60%. This surprising observation suggests that male and female cerebellum respond differently to ALLO, with female being more sensitive.

ALLO can be derived by metabolizing progesterone or may be synthesized *de novo* in the brain from cholesterol (Mellon and Vaudry, 2001). Therefore, the concentration of ALLO in neural tissue may vary greatly, but plasma levels have been estimated to range between 3-10 nM under normal physiological conditions (Purdy et al., 1991). The current study utilized 10 nM ALLO in physiological recordings to directly assess the relative sensitivity of male and female PCs to ALLO. We tested the effect of a physiological concentration of ALLO on its well documented ability to potentiate GABAergic transmission using acute brain slice recordings from male and female mice. Consistent with many studies in other brain regions, we observed that ALLO potentiated GABAergic neurotransmission,

observed as a slowing of the decay kinetics of sIPSCs in cerebellar PCs. Previous studies have used relatively high concentrations of ALLO (100 nM -1 μM), in order to observe large effects on IPSC kinetics (Cooper et al., 1999; Vicini et al., 2002; Harney et al., 2003). The current study demonstrated that low, physiological levels of ALLO potentiate GABAergic neurotransmission, resulting in significant changes in the decay kinetics of sIPSCs. Our data indicates that sIPSCs recorded from male and female PCs respond differently to low concentrations of ALLO, with females having a significantly greater response compared to males.

$GABA_A$ receptor sensitivity to neurosteroids, such as ALLO, has been shown to be modulated by several factors, including subunit composition (Lambert et al., 2003) and protein kinases PKC and PKA (Jones and Westbrook, 1997; Poisbeau et al., 1999). Therefore, it is possible that male and female PCs are different either in subunit composition or post-translational modification. To begin to address this issue we recorded GABAergic neurotransmission in the presence of TTX (mIPSCs), to isolate action potential independent neurotransmitter release and observe synaptic activity from release of single vesicles (Collingridge et al., 1984). Therefore, data from mIPSCs supplied information regarding postsynaptic GABA receptors while data from sIPSCs revealed changes in presynaptic release as well as postsynaptic activation of receptors. We expected to observe a sex difference in mIPSC kinetics, suggesting distinct expression of $GABA_A$ receptor subunits in male and female PCs. ALLO significantly potentiated mIPSCs, slowing the decay kinetics in PCs from both male and

female mice. However, mIPSC kinetics were not significantly different between sexes and the degree of slowing was similar to that observed in male sIPSCs. Therefore, our data suggest that ALLO has an additional effect on female sIPSCs on a site other than the post-synaptic receptor.

One explanation for the extra prolongation of decay kinetics observed in female sIPSCs that is absent in mIPSCs could be that in addition to directly interacting with post-synaptic $GABA_A$ receptors, ALLO can alter pre-synaptic release in female PCs. However, ALLO did not affect PC sIPSC amplitude or frequency in either sex, making it unlikely that ALLO alters release probability. Interestingly, Puia et al. observed that reducing endogenous ALLO by inhibiting the 5α-reductase enzyme decreased the sensitivity of sIPSCs to ALLO (Puia et al., 2003). This observation led to the hypothesis of the existence of a "receptor on the receptor," which allows endogenous levels of ALLO to calibrate the brain's sensitivity to ALLO. In the context of our current study, we would hypothesize that female cerebellum is exposed to higher levels of ALLO than male. This would sensitize females to consequent exposure to ALLO, as we observed in both our neuroprotection studies as well as sIPSC recordings. Further work is warranted to elucidate the mechanism underlying enhanced sensitivity of female PCs to ALLO potentiation of GABAergic neurotransmission. Nonetheless, our electrophysiological data is consistent with our histological data and indicates that the enhanced sensitivity of GABAergic neurotransmission in female PCs likely contributes to increased neuroprotection observed at low doses of ALLO.

5. Conclusions

The current study provides the first evidence that ALLO protection against global cerebral ischemia is significantly different in male and female mice. Consistent with the neuroprotection results, ALLO potentiates GABAergic neurotransmission in PCs in a sex specific manner. In summary, our data suggests that ALLO is strongly neuroprotective and that sex influences sensitivity to ALLO.

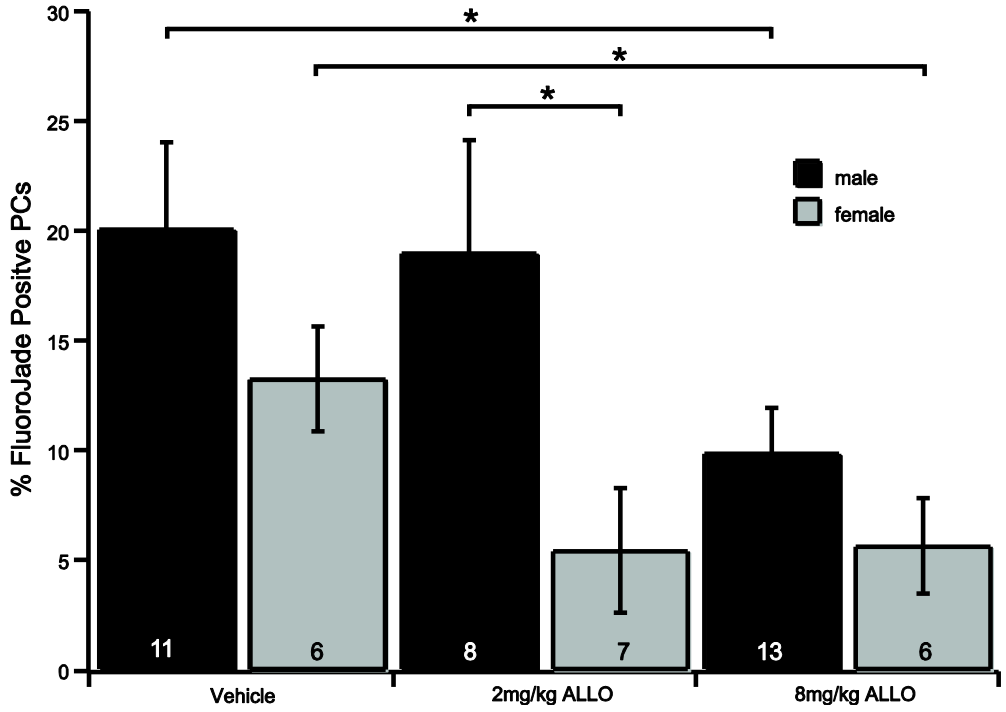


Fig. 1 ALLO reduces Purkinje cell damage following CA/CPR in male and female mice.

Quantification of Purkinje cell damage in mice 48 hr after 10 min CA/CPR. Male and female mice were treated with either vehicle (20% β-cyclodextran in 0.9% saline), 2 mg/kg ALLO, or 8 mg/kg ALLO via i.p. injection given 30 min prior to CA and following 6 hr and 24 hr recovery. FluoroJade positive PCs were counted and expressed as a percentage of total PCs counted in sagittal sections of cerebellum at -5.8 bregma. The data demonstrates that male mice (black bars) were significantly protected compared to vehicle with 8 mg/kg ALLO. Female mice (gray bars) were significantly protected compared to males with 2 mg/kg ALLO, and compared to vehicle with 8 mg/kg ALLO. The number of mice analyzed in each condition is indicated in each bar. Statistical significance was determined by 2-way ANOVA with Neuman-Keuls post hoc analysis *= P≤ 0.05.

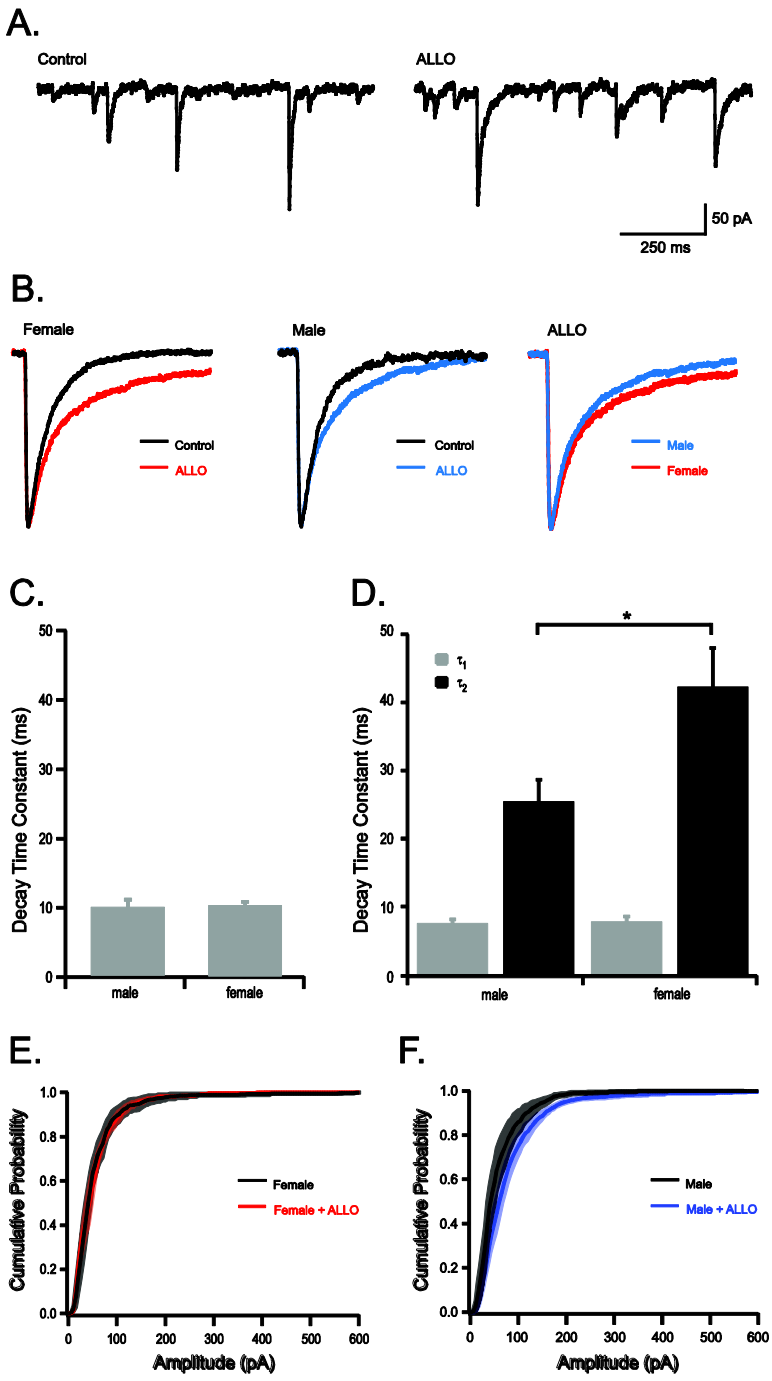


Fig. 2 ALLO prolongs sIPSC decay kinetics in PCs and has a greater effect in females.

(a) Representative recordings illustrating sIPSCs recorded from PCs in acute brain slices from a male mouse under control conditions and 20 min after

exposure to 10nM ALLO. (b) Representative ipsc kinetics acquired by averaging 20-30 events recorded from one female cell (left), and one male cell (middle) under control conditions (black) and in the presence of 10 nM ALLO (colored). Far right traces illustrate difference in male and female response to ALLO. (c, d) Quantification of decay kinetics in PCs recorded from male (n=5) and female (n=5) mice. Under control conditions sIPSCs were best fit by a single exponential (c). In contrast, following exposure to ALLO, sIPSCs were best fit by a double exponential ($\tau 1$ and $\tau 2$; d). (d, e) Cummulative probability of sIPSC amplitudes for females (e), males (f), and in the presence of ALLO (red/blue). (The symbol * indicates statistical significance, determined by students t-test, $p \leq 0.05$.

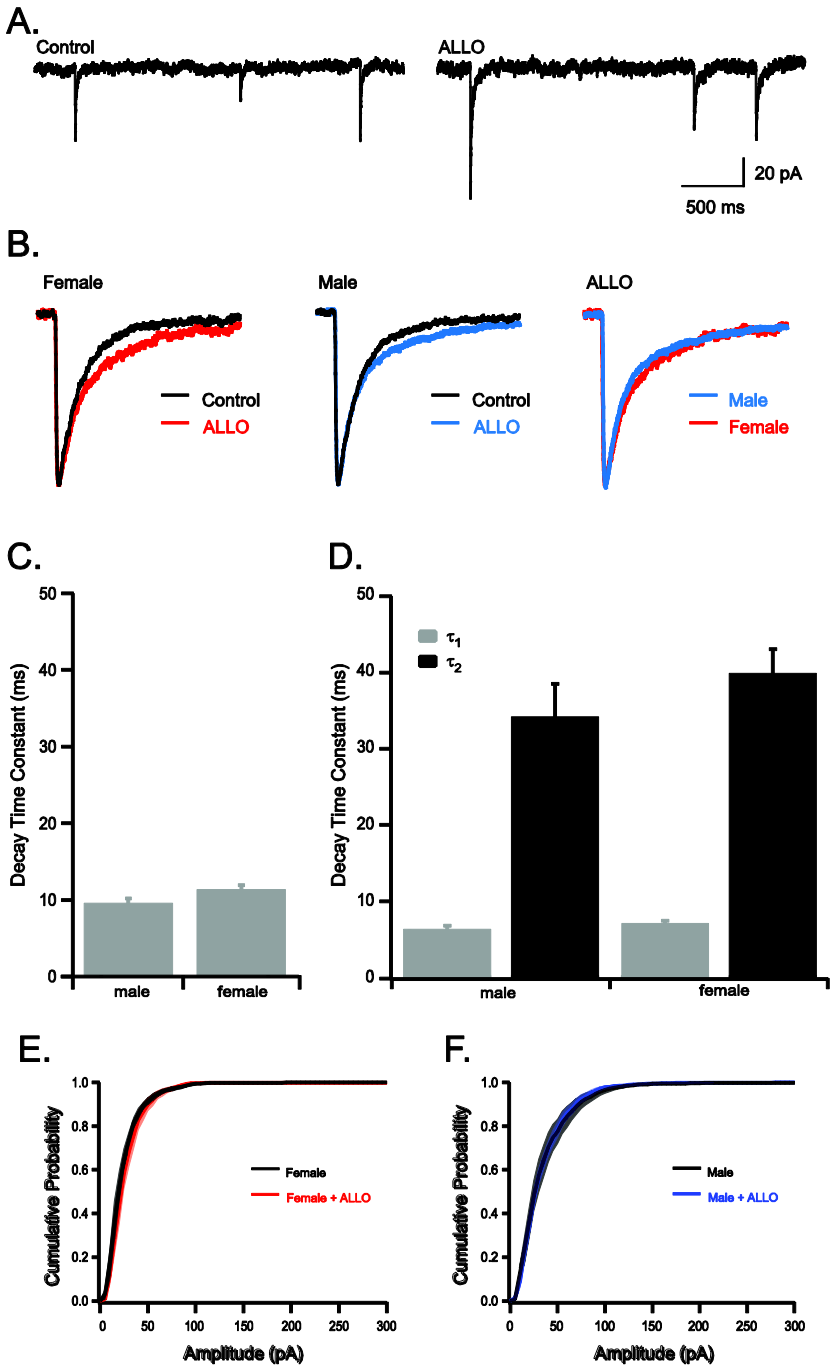


Fig. 3 ALLO prolongs mIPSC decay kinetics but isolating single synaptic events abolishes the sex difference observed in spontaneous activity.

(a) Representative recordings illustrating mIPSCs recorded in the presence of 250 nM TTX from PCs in acute brain slices from a male mouse under control

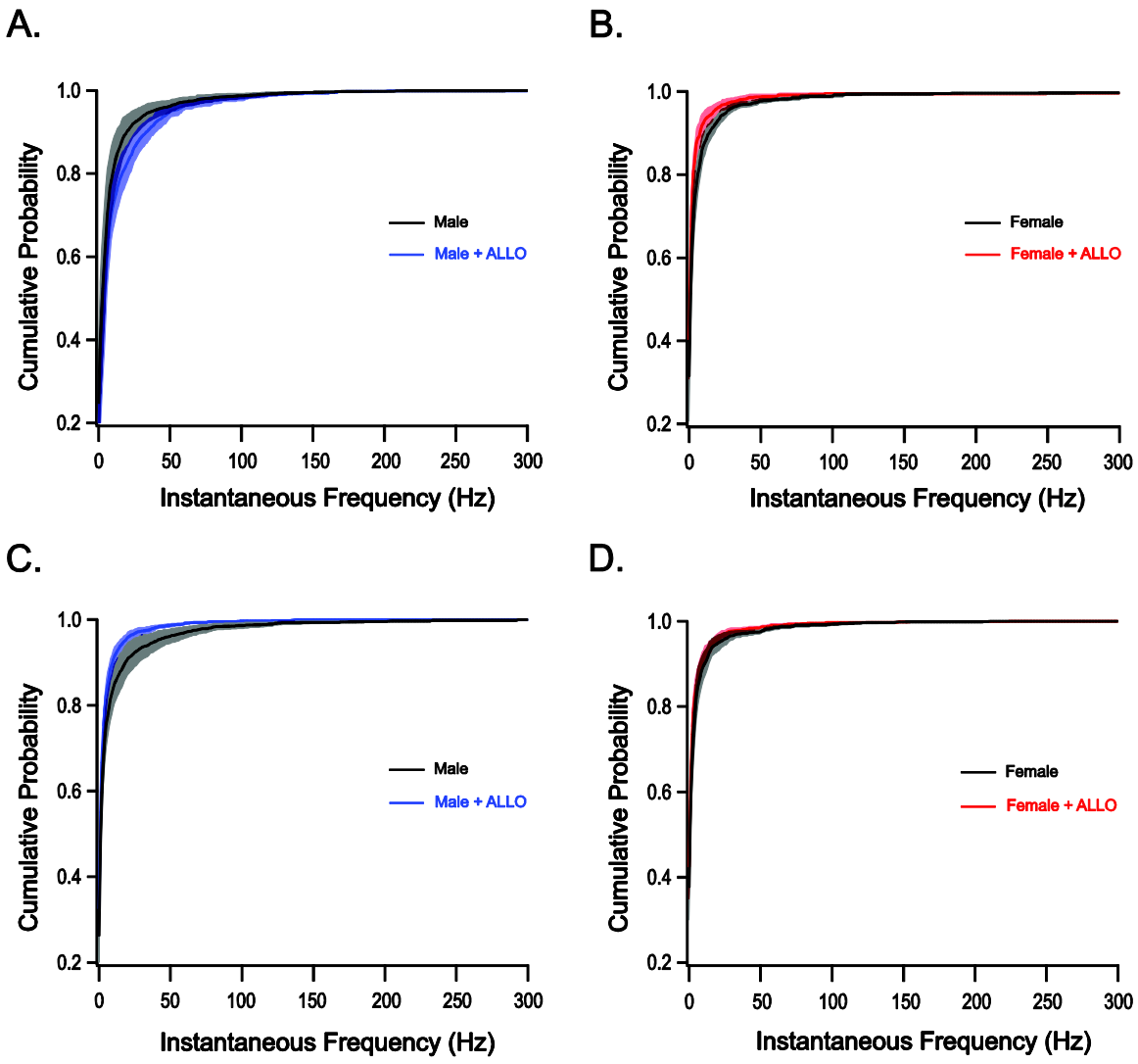
conditions and 20 min after exposure to 10 nM ALLO. (b) Representative averaged events from one female cell (left), and one male cell (middle) under control conditions (black) and in the presence of 10 nM ALLO (colored). Far right traces illustrate difference in male and female response to ALLO. (c, d) Quantification of decay kinetics in PCs recorded from male (n=8) and female (n=7) mice. Under control conditions mIPSCs are best fit by a single exponential (c). In contrast, following exposure to ALLO, mIPSCs were best fit by a double exponential (d). Cumulative probability of mIPSC amplitudes for females (e), males (f), and in the presence of ALLO (red/blue). The symbol * indicates statistical significance, determined by students t-test, $p \leq 0.05$.

	Male		Female	
condition	amplitude (pA)	frequency (Hz)	amplitude (pA)	frequency (Hz)
sIPSC	62.00±9.69 (5)	3.13±1.38 (5)	59.80±9.53 (5)	1.48±0.46 (5)
sIPSC+ALLO	63.88±10.34 (5)	2.19±1.22 (5)	61.12±6.64 (5)	1.14±0.37 (5)
mIPSC	39.99±4.59 (8)	1.68±0.32 (8)	29.31±2.10 (7)	1.07±0.25 (7)
mIPSC+ALLO	39.34±2.56 (8)	1.23±0.20 (8)	31.89±3.02 (7)	0.97±0.21 (7)

Table 1. Amplitude and Frequency of spontaneous and miniature IPSCs

[a] Each value is the mean±SEM

[b] The number of neurons studied is in parentheses



Supplemental Fig. 1 ALLO does not alter miniature or spontaneous IPSC frequency. Cumulative probability of sIPSC instantaneous frequency (a, b) and mIPSC instantaneous frequency (c, d). Control males are depicted in black, ALLO males in blue. Control females are depicted in black, ALLO females in red.

CHAPTER 4:

Purkinje Cell $GABA_A$ Receptor Pharmacology is Altered by Ischemia Due to Greater Contribution of β_1 Subunit Containing Receptors

Melissa H. Kelley, Justin Ortiz, Paco S. Herson

Department of Anesthesiology and Perioperative Medicine

Oregon Health and Science University, 3181 SW Sam Jackson Park Rd.

Portland, OR USA

Six keywords: $GABA_A$ receptor, Purkinje cells, oxygen-glucose deprivation, β_1 subunit, electrophysiology, single cell PCR

Abstract:

Cultured Purkinje cells (PCs) are compromised by oxygen-glucose deprivation (OGD), mimicking PC death from ischemia reperfusion injury in animal models of cardiac arrest. Downregulation of $GABA_A$ receptors by ischemia is thought to contribute to PC sensitivity to ischemia induced excitotoxicity. Here we examined the functional properties of the $GABA_A$ receptors that are spared following ischemia. Using subunit specific positive modulators of $GABA_A$ receptors we observed that OGD causes a decrease in sensitivity to the $\beta_{2/3}$ subunit preferring compound, etomidate. Sensitivity to propofol, another β subunit acting compound that modulates β_{1-3} subunits, was not affected by OGD. The α/γ subunit acting compounds, diazepam and zolpidem were also unaffected by OGD. Using single cell PCR with isolated PCs from acutely dissociated cerebellar tissue, we performed RT-PCR for the β subunits. PCs expressed the β_1 subunit, contrary to several previous reports examining $GABA_A$ receptor subunit expression in PCs. $GABA_A$ receptor β_1 subunit protein was also detected in cultured PCs by Western blot and levels remained unaffected by OGD. As previously demonstrated with β_1 subunit containing $GABA_A$ receptors expressed in heterologous systems, high concentrations of loreclezole (30 μM), inhibited PC GABA mediated currents. From our data we conclude that PCs express the β_1 subunit and there is a greater contribution of β_1 containing $GABA_A$ receptors following OGD.

I. Introduction

Cerebellar Purkinje cells (PCs) are the output neurons of the cerebellar cortex and they are strongly innervated by GABAergic and glutamatergic synapses. The powerful excitatory glutamatergic input that PCs receive contributes to their vulnerability to excitotoxic damage after ischemia (Horn and Schlote, 1992;Brasko et al., 1995;Fonnum and Lock, 2000;Ardeshiri et al., 2006;Kelley et al., 2008). Many neuroprotective strategies have been explored using PCs as a model system with variable success. One approach is to increase inhibitory tone by enhancing $GABA_A$ receptor activity thus hyperpolarizing cells and leaving them less vulnerable to excitotoxic damage (Schwartz-Bloom and Sah, 2001). We have recently demonstrated that the neurosteroid allopregnanolone (ALLO) protects PCs from ischemic damage both *in vitro* & *in vivo* via a $GABA_A$ receptor dependent mechanism (Ardeshiri et al., 2006; Kelley et al., 2008). However, it was also observed that the quantity of $GABA_A$ receptor α1 subunit protein is decreased in PCs after ischemic insult (Kelley et al., 2008). Indeed, several studies have demonstrated that following cerebral ischemia, as experienced during a stroke or cardiac arrest, $GABA_A$ receptors are downregulated in various brain regions (Li et al., 1993; Schiene et al., 1996; Qu et al., 1998; Kelley et al., 2008; Arancibia-Carcamo et al., 2009), resulting in increased excitotoxic damage. Therefore, preserving $GABA_A$ receptor function plays an important role in neuroprotection from ischemia (Ardeshiri et al., 2006; Kelley et al., 2008; Arancibia-Carcamo et al., 2009). Yet,

there is currently limited information regarding receptor structural changes and trafficking in pathophysiological states, making the full nature and consequences of these changes in $GABA_A$ receptors unknown. This apparent disease-induced plasticity could contribute to the difficulty in finding $GABA_A$ receptor modulating agents that are effective neuroprotectants.

$GABA_A$ receptors are pentameric proteins thought to be composed of two α subunits, two β subunits, and one γ or δ subunit (or less commonly an ε, π, or θ subunit). Sixteen subtypes of $GABA_A$ receptor subunits have been cloned (Olsen and Sieghart, 2009), allowing for heterogeneous expression profiles across brain regions and distinct channel properties. Subunit composition dictates the specific properties of the receptor, including ligand binding, channel kinetics, and response to allosteric modulators. Cerebellar PCs are thought to express a homogeneous population of $GABA_A$ receptors, consisting of $\alpha_1\beta_{2\text{ or }3}\gamma_2$, that are downregulated by ischemia (Kelley et al., 2008). We have previously demonstrated that *in vitro* ischemia or OGD can cause a rapid and sustained downregulation of $GABA_A$ receptors, thereby reducing overall inhibitory potential and increasing excitotoxic damage (Kelley et al., 2008). In the current set of experiments, we tested whether ischemia alters the remaining functional PC $GABA_A$ receptors by examining their pharmacological profiles following OGD.

We used cultured PCs and a dose of oxygen and glucose deprivation (OGD) known to cause significant damage and loss of $GABA_A$ receptor α_1 subunit protein. With this model, we examined the function of the remaining $GABA_A$ receptors in greater detail by measuring responses to well characterized

subunit specific modulators, etomidate, propofol, diazepam, and zolpidem. Our findings were surprising and led us to hypothesize that PCs express the β_1 subunit as well as β_2 and β_3.

II. Methods

Primary Cerebellar Culture. All experiments were performed in accordance with National Institute of Health guidelines and experimental protocols approved by the institutional animal care and use committee. Cerebellar neurons were cultured from embryonic day (E)18 Sprague-Dawley rats as previously described (Kelley et al., 2008). In brief, time-pregnant Sprague–Dawley rats (Charles River, Wilmington, MA) were euthanized with CO_2 and embryos removed by caesarean section. Embryonic brains were isolated and placed in ice-cold dissection solution composed of Hank's Balanced Salt Solution (Gibco/Invitrogen, Carlsbad, CA) supplemented with 0.03% bovine serum albumin and 10 mM $MgSO_4$ (Sigma-Aldrich, St Louis, MO). Cerebella were dissected, meninges removed, and tissue digested with 0.2% trypsin in dissection solution at 37˚C. Digestion was halted by washing with cerebellar culture medium composed of DMEM/F-12 1:1 mix supplemented with 1.4 mM L-glutamine, 50 units*µg/ml penicillin-streptomycin, b-27 supplement (1X), and 10% fetal bovine serum (Hyclone, Logan, UT). Tissue was triturated, filtered through a 70 µm cell sorting nylon mesh, and suspension centrifuged at 1000xg for 10 min to isolate cells. Cell pellet was resuspended in cerebellar culture

medium without FBS. Cells were plated at a density of $2.8x10^5$ cells per round 12 mm glass coverslip and grown at 37˚ in a humidified incubator containing 5% CO_2. Cultured neurons were grown for 10-14 days *in vitro* (DIV) to allow maturation and formation of synapses.

Oxygen and Glucose Deprivation. Cells were transferred to a glucose-free saline solution composed of (in mM): 140 NaCl, 5 KCl, 0.8 MgCl2, 1 CaCl2, 10 HEPES, pH 7.35 with NaOH, and placed in an anaerobic incubator at 37˚ containing an oxygen reacting catalyst and 5% CO2, 95% N2 (Coy Laboratory Products, Grass Lake, MI) for 2 hours. This duration of OGD was previously determined to cause significant levels of cell death in our cultures following 24 hours of reperfusion (Ardeshiri et al., 2006). Re-oxygenation was initiated by transferring cells to glucose containing saline for electrophysiological recordings (see below).

Electrophysiology. Cells were transferred to a recording chamber mounted on an inverted Leica DM IRB microscope (Leica, Houston, TX) containing normal saline composed of (in mM): 140 NaCl, 5 KCl, 0.8 MgCl2, 1 CaCl2, 10 HEPES, 10 Glucose, pH 7.35 with NaOH, and a gravity fed bath flow rate of 2-5 ml/min. Whole cell voltage clamp recordings were made from the somas of PCs using an Axopatch 200B (Axon Instruments, Union City, CA) amplifier interfaced to a Dell computer (Dell, Round Rock, TX). PCs were selected by their large soma, low input resistance, and extensive dendritic arbor. Electrodes were pulled from borosilicate glass capillaries with inner filaments using a Flaming Brown electrode puller (Sutter Instrument Co, Novato, CA) and had resistances of 2-3

MΩ when filled with internal pipette solution composed of (in mM): 140 CsCl, 1 EGTA, 10 HEPES, 1 $MgCl_2$, 5 MgATP, pH of 7.3 with CsOH. Data were collected at a sample frequency of 20 kHz and using pCLAMP9 (Molecular Devices, Sunnyvale, CA). Whole cell capacitance and series resistance were electronically compensated to 60-70%. Adequate whole cell access (R_a<20MΩ) was achieved prior to measuring responses to compounds and verified at end of recording. A pressurized microperfusion system with a wide barrel manifold (250 µm) was used for solution exchange around the entire cell body and dendritic processes (ALA Scientific Instruments, Westbury, NY) to measure PC response to GABA and subunit specific modulators. The amplitude of currents, in response to GABA and test compounds, were measured using Clampfit analysis software (Axon Instruments, Union City, CA). For concentration response analysis, data from each cell were plotted and fit with the following equation to determine EC_{50} using Igor Pro software (WaveMetrics, Lake Oswego, OR): $f(x) = (I_{max}-I_0)/(1+(EC_{50}/x)^n)+I_0$, where x equals the concentration of drug and n equals the Hill coefficient. The EC_{50} is presented as the mean±SEM for individual cells. Data were expressed as the percent potentiation of 2 or 5 µM GABA (approximate I_{10}) or as the percent maximum GABA mediated current, achieved by applying a saturating concentration of GABA (1mM, 1s). For loreclezole inhibition experiments, 1s pulses of 20 µM GABA were applied every 30 s to establish a stable baseline response, 30 µM loreclezole was then pre-applied by local perfusion for 30s and remained present through three subsequent pulses of

20 µM GABA every 30s. Washout of drug was achieved by local perfusion of saline in addition to bath perfusion.

Solutions and Drugs. Etomidate and zolpidem were dissolved in EtOH at a stock concentration of 10 mM. Loreclezole was dissolved in DMSO at a stock concentration of 5 mM. Diazepam was dissolved in DMSO at a stock concentration of 10mM. Propofol was dissolved in EtOH at a stock concentration of 20 mM. GABA was dissolved in water at a stock concentration of 500mM. Working solutions were prepared by careful serial dilution of stock compounds in Saline. Final concentrations of DMSO and EtOH were < 0.01%. Components of internal and external solutions, propofol,and diazepam were obtained from Sigma-Aldrich (St Louis, MO). Etomidate, loreclezole, GABA and zolpidem were obtained from Tocris (Ellisville, MO).

Acute cerebellar dissociation and Purkinje cell collection.

Cerebellar dissociation for isolation of PCs was performed according to Raman and Bean (Raman and Bean, 1997;Benton and Raman, 2009) with the modification of use of RNAse free H_2O for all solutions. Postnatal day 14-20 C57BL/6 mice (Charles River) were anesthetized with isoflurane, decapitated, and superficial layers of the vermis of the cerebellum were dissected for dissociation. Tissue was minced in ice-cold, oxygenated dissociation solution composed of (in mM): 82 Na_2SO_4, 30 K_2SO_4, 5 $MgCl_2$, 10 HEPES, 10 glucose, and 0.001% phenol red (buffered to pH 7.4 with NaOH). Tissue was then

incubated in 10 ml oxygenated dissociation solution containing 3 mg/ml protease XXIII (Sigma-Aldrich, St. Louis, MO), pH 7.4 with NaOH, at 37°C for 7 min. Enzymatic digestion was halted by washing tissue in warmed, oxygenated dissociation solution containing 1 mg/ml bovine serum albumin and 1 mg/ml trypsin inhibitor, tissue was microdissected to roughly 1 mM pieces, and transferred to oxygenated Tyrode's solution composed of (in mM): 150 NaCl, 4 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, 10 glucose, pH 7.4 with NaOH, at room temperature. To disperse cells, tissue was triturated with fire-polished RNAse free Pasteur pipettes. Dissociated cells were plated onto 35 mm Petri dishes and allowed to settle for 20-30 min. PCs were visualized using an inverted Leica DM IRB microscope (Leica, Houston, TX) and identified by their large size, teardrop shape, and proximal dendritic stump. 2-3 MΩ pipettes were pulled from RNAse free borosilicate glass, filled with RNAse free Tyrodes solution and manipulated with an oocyte injector. Pipettes were positioned near cell and tip broken, by lowering pipette into Petri dish, to create a large enough opening to capture PC with applied negative pressure. Immediately following isolation, 4-6 cells were expelled into each siliconized microcentrifuge tube containing 1.6 µl of 5x Superscript buffer (Invitrogen, Carlsbad, CA), 18 U of RNAsin, 0.8 µl of 100 mM DTT, and DEPC-treated water to 8 µl. Negative control consisted of Tyrodes solution collected from the vicinity of PCs. Samples were frozen at -70ºC until used for RT-PCR.

Single Cell PCR

Each sample, containing 4-6 cells, was thawed on ice and 0.2 µM dNTPs, 100 ng of random hexamers, and 400 ng OligodT, were added. Samples were heat denatured 5 min at 65°C then cooled on ice for 5 min. Cellular RNA was reverse transcribed by adding 100 U of Superscript III reverse transcriptase, 3.4 µl of 5x Superscript buffer, 15.2 U of RNAsin, 1.2 µl of 100 mM DTT, and DEPC-treated water to 14 µl, together with cells for a final volume of 25 µl. RT-reactions were incubated at 25°C for 5 min, transcribed at 50°C for 60 min, and denatured at 70°C for 15 min, and cooled on ice.

Nested PCR was used for all samples, using degenerate outer and inner primers for α and β subunits, with the exception of the reaction for β_1b primer set. PCR Primers were designed using DNA Star software (Madison, WI). For α degenerate primers, sequences corresponded to the α1 subunit (accession number NM_010250, forward primer 347-365 nt, reverse primer 1000-1020 nt) and the α6 subunit (accession number NM_008068, forward primer 290-308 nt, reverse primer 853-872 nt). For β degenerate primers, sequences corresponded to the β_1 subunit (accession number NM_008069, forward primer 201-223 nt, reverse primer 907-929 nt), the β_2 subunit (accession number NM_008070, forward primer 206-227 nt, reverse primer 911-933 nt), and the β_3 subunit (accession number NM_008071, forward primer 305-326 nt, reverse primer 1010-1032 nt). All other primers were as follows: α_1 (273 nt product, accession number NM_010250, forward primer 413-434 nt, reverse primer 674-686 nt); α_6 (287 nt product, accession number NM_008068, forward primer 395-417 nt, reverse primer 661-682 nt); β_1a (206 nt product, accession number NM_008069,

forward primer 464-483 nt, reverse primer 651-670 nt); β_1b (669 nt product, accession number NM_008069, forward primer 1145-1166 nt, reverse primer 1792-1814 nt); β_2 (271 nt product, accession number NM_008070, forward primer 428-445 nt, reverse primer 679-699 nt); β_3 (260 nt product, accession number NM_008071, forward primer 519-535 nt, reverse primer 760-779 nt). PCR was performed using 2 µl of cDNA template from each RT reaction in a 25 µl PCR volume containing 5 µl 5X Mg free buffer, 2µl 25mM MgCl2, 0.5µl 10mM dNTPs, 0.4µl GoTaq Flexi DNA polymerase (Promega), and 0.2 µM each of forward and reverse primers. Thirty-five cycles of amplification were performed using a Gene Amp PCR system 9700 thermal cycler (Applied Biosystems) in 0.2 ml thin-walled PCR tubes with the following protocol: 94°C for 2 min; 35 cycles of 96°C for 30 s, 54–57°C for 0.5-1 min, 72°C for 1 min; with a final 72°C extension for 5 min. PCR products were visualized with ethidium bromide on a 2% agarose gel.

Western Blot. Total protein was collected from cultures by lysing cells with CelLytic-M lysis buffer (Sigma-Aldrich, St Louis, MO), centrifuged at 10,000 g for 5 min at 4ºC and pellet was discarded to remove nuclei and debris Total protein content of the supernatant was measured using a BCA protein assay (Pierce/Thermo Scientific, Rockford, IL) and 30 µg of total protein from each sample run on a precast 4-12% stacking polyacrylamide gel (Invitrogen, Carlsbad, CA) for 1 hr at 200 V. Protein was transferred to a PVDF membrane (Invitrogen) for 1hr at 30 V. Blots were incubated in primary antibody, 1:1000

dilution of rabbit anti-$GABA_A$ receptor β_1 subunit (Novus Biologicals, Littleton, CO) overnight at 4°C, followed by secondary antibody, 1:1000 donkey anti-rabbit IgG ECL-HRP (GE Healthcare, Piscataway, NJ) for 1hr at RT. Blots were then stripped and re-probed with primary antibody, 1:7000 mouse anti-β-actin (Sigma-Aldrich) 1 hr at RT, followed by secondary antibody, 1:1000 goat anti-mouse IgG ECL HRP linked (GE Healthcare) 1 hr at RT. SuperSignal West Dura chemi-luminescent detection system (Thermo Scientific) was used to visualize protein and blots were imaged and analyzed using a FluorChem FC2 gel imager (Alpha Innotech, San Leandro, CA). $GABA_A$ receptor β_1 subunit protein levels were normalized to β-actin and expressed as arbitrary units.

III. Results

3.1 Purkinje cells have decreased sensitivity to etomidate following oxygen and glucose deprivation.

Whole-cell voltage clamp recordings were made from cultured cerebellar PCs under control conditions or within 1 hr of re-oxygenation following 2 hr oxygen glucose deprivation (OGD). To test the effect of ischemia on $GABA_A$ receptor sensitivity to the positive modulator etomidate, peak current amplitudes in response to 1s pulses of 5μM GABA plus increasing concentrations of etomidate (0.3-10 μM) were measured before and after OGD (Fig.1A). Etomidate is a positive modulator of $GABA_A$ receptor activity that preferentially binds to $\beta_{2/3}$-containing receptors. Concentration response relationships were plotted and the

EC_{50} for each individual cell was determined. For control cells the average EC_{50} was 2.9 ± 0.3 µM (n=8). Two hours of OGD caused a significant increase in the average EC_{50}, to 3.8 ± 0.2 µM ($P< 0.05$; n=6; Fig.1B). Additionally, the maximum ability of etomidate to potentiate $GABA_A$ receptors was reduced following OGD (Fig.1B). Therefore, ischemia reduced the sensitivity and efficacy of etomidate on PC $GABA_A$ receptors.

3.2 Oxygen and glucose deprivation does not alter PC $GABA_A$ receptor sensitivity to propofol.

The shift in etomidate sensitivity following ischemia led us to hypothesize that the β subunit of $GABA_A$ receptors is altered following ischemia. We tested propofol, another $GABA_A$ receptor modulator that binds to the β subunit, enhancing the activity of all β-subunit containing receptors (β_{1-3}) equally. Peak current amplitudes in response to 1 s pulses of 2 µM GABA plus increasing concentrations of propofol (1-15 µM) was measured before and after OGD (Fig.2A). Sensitivity and efficacy of propofol potentiation of $GABA_A$ receptors remained unchanged by ischemia (Control EC_{50}=5.6 ± 0.5 µM, n=9; OGD EC_{50}= 5.8 ± 0.5 µM, n=12; Fig.2B).

3.3 Oxygen and glucose deprivation does not alter $GABA_A$ Receptor sensitivity to zolpidem and diazepam.

We tested the effect of ischemia on the α and γ subunits by performing concentration response analysis to diazepam and zolpidem. These compounds bind the α_1 subunit and potentiate $GABA_A$ receptor response to GABA by

increasing current amplitude (Sigel and Buhr, 1997). However, the γ_2 subunit is also necessary for zolpidem potentiation (Buhr and Sigel, 1997), therefore, we utilized diazepam as an indicator of α_1 function and zolpidem for γ_2 function. Responses to 1 s pulses of 5 µM GABA plus increasing concentrations of diazepam (0.1-10 µM) or zolpidem (50-3000 nM) were measured before and after OGD. Simulated ischemia did not significantly alter efficacy or affinity for diazepam (Control EC_{50}= 2.27 ± 0.4 µM, n=7; OGD EC_{50}= 2.81 ± 0.4 µM, n=6; Fig.3A) or zolpidem (Control EC_{50}= 465.7 ± 75.9 nM, n=9; OGD EC_{50}= 482.6 ± 62.4 nM, n=8; Fig.3B).

$GABA_A$ receptor β_1 subunit is expressed in Purkinje cells.

Purkinje cells are thought to express a homogeneous population of $GABA_A$ receptors composed of α_1, $\beta_{2/3}$, and γ_2 subunits. However, the pharmacological shift in sensitivity that we observed with etomidate, but not propofol, is consistent with the expression of the β_1 subunit. We used single cell PCR to test for the presence β_1 mRNA in PCs isolated from acutely dissociated mouse cerebellum (Mintz et al., 1992;Raman and Bean, 1997). The presence of PCs and purity of samples was confirmed by examining α subunit mRNA expression, the presence of α_1 is indicative of PC and α_6 indicates granule cells (Fig.4A). PC pure samples were then tested for β subunit expression. Out of 6 samples, 2 had detectable levels of β_1 mRNA (Fig.4B). β_2 and β3 mRNA was also present in 4 out of 6 samples (Fig. 4B). One PC pure sample did not have detectable levels of any of the β subunits (data not shown). Western blot analysis of protein collected from cultured PCs confirmed the expression of β_1 subunit protein (Fig.4C). Unlike the

α_1 subunit, which is degraded following simulated ischemia (Kelley et al., 2008), β_1 protein remained unaffected by OGD (Fig.4D).

To confirm functional expression of the β_1 subunit, we used Loreclezole, a positive modulator of $GABA_A$ receptors that binds the β_{2-3} subunits (Wingrove et al., 1994; Wafford et al., 1994). At high concentrations (> 6 μM), loreclezole inhibits $GABA_A$ receptors containing the β_1 subunit by decreasing channel open time and increasing the occurrence of a closed channel state (Fisher et al., 2000). Consistent with PC expression of β_1 subunits, we observed that pre-application of loreclezole (30 μM, 30 s) caused an initial 30% reduction in GABA mediated current that maximally decreased with subsequent pulses of GABA by 65.5 ± 6.8% (n=4), and almost completely reversed following 60-120 s washout (92 ± 5.2% recovery) (Fig.4E).

IV. Discussion

Summary of findings

Our experiments demonstrate that ischemia modifies $GABA_A$ receptor response to etomidate in PCs, significantly decreasing sensitivity and efficacy of the drug. Responses to other $GABA_A$ receptor modulators, propofol, diazepam, and zolpidem, remain unaffected by ischemia. We examined β subunit expression in PCs by single cell PCR analysis and detected β_1 subunit transcripts, a subunit previously not known to be present in PCs. Using western blot analysis, β_1 subunit protein was also present in our cerebellar cultures and functional recordings indicate the presence of β_1. Our data lead us to propose that the shift

in etomidate sensitivity observed following OGD in PCs is due to a greater contribution of β_1 subunit containing $GABA_A$ receptors.

Etomidate sensitivity and efficacy are altered by ischemia but propofol is not.

Etomidate acts on the beta subunit of $GABA_A$ receptors with a higher affinity for the β_2 and β_3 subunits compared to β_1 (Belelli et al., 1997). Using heterologous expression systems the EC_{50} for etomidate has been reported to range between 0.6-1.2 µM for $GABA_A$ receptors composed of $\alpha_1\beta_2\gamma_2$ (Hill-Venning et al., 1997), and 4µM for receptors composed of $\alpha_1\beta_3\gamma_2$ (Belelli et al., 2003). The presence of β_1 increases the EC_{50} to range between 6-11 µM (Hill-Venning et al., 1997). Our data is consistent with several previous reports indicating that PC express predominantly $\beta_{2/3}$ containing receptors, exhibiting an EC50 for etomidate of 2.9 µM under control conditions. Following OGD, the EC50 significantly increased to 3.8 µM. One explanation for decreased sensitivity of $GABA_A$ receptors to etomidate is an increased relative expression of β_1-containing receptors. However, the literature strongly suggests that PC $GABA_A$ receptors are composed of $\beta_{2/3}$-containing receptors at the exclusion of β_1. To directly test the hypothesis that OGD increases the relative proportion of β_1-containing receptors, we tested propofol, a $GABA_A$ receptor modulator that potentiates $GABA_A$ receptors expressing β_1 subunits equally as well as those expressing $\beta_{2/3}$-subunits (Sanna et al., 1995). Indeed, we observed an EC_{50} of PC $GABA_A$ receptors within the published range for propofol and most interestingly, OGD had no effect on PC response to propofol. We recently demonstrated that OGD

causes a rapid decline in $GABA_A$ receptor current density and loss of $_1$-subunit protein (Kelley et al., 2008). This observation coupled with the observation that OGD alters etomidate sensitivity, and not propofol, implies that OGD-induced $GABA_A$ receptor decline/degradation is specific to $\beta_{2/3}$-containing receptors, sparing β_1-containing subunits. Indeed, our Western blot data demonstrates that OGD has no effect on β_1 protein. Therefore, we hypothesized that PCs express the β_1 subunit and following OGD, the relative contribution of β_1 containing receptors is increased compared to $\beta_{2/3}$. In order to test the specificity of the OGD-induced change in $GABA_A$ receptor pharmacology, we tested additional compounds known to interact with subunits other than β_2. The lack of effect of OGD on $GABA_A$ receptor sensitivity to diazepam and zolpidem strongly implies that OGD alters the relative expression of β-subunits, while having no effect on relative expression of the other subunits, α and γ.

Loreclezole inhibition of PC $GABA_A$ receptor current

To confirm the data suggesting cerebellar PCs express functional β_1 subunits we performed additional experiments with loreclezole. Loreclezole is a strong potentiator of GABAA receptors containing the β_2 and β_3 subunits, but has substantially diminished ability to positively modulate β_1 containing $GABA_A$ receptors (Wingrove et al., 1994; Wafford et al., 1994). In fact, it is estimated that loreclezole has 300x greater affinity for $\beta_{2/3}$ containing receptors compared to β_1-containing receptors (Wafford et al., 1994). Despite its clear specificity for $GABA_A$ receptor $\beta_{2/3}$ subunit, loreclezole can have multiple effects depending on the concentration and duration of application. Most importantly for this study,

high concentrations of loreclezole (30 μM) strongly inhibit β_1 containing receptors, resulting in reduced peak amplitude in response to GABA (Fisher et al., 2000). We observed that low concentrations of loreclezole potentiate $GABA_A$ receptors in PCs, consistent with expression of $\beta_{2/3}$-subunits (data not shown). However, a high concentration of loreclezole (30 μM) significantly reduced $GABA_A$ receptor currents in PCs, likely by loreclezole inhibition of β_1 containing receptors. Therefore, consistent with our single-cell PCR data, it is clear that cerebellar PCs express $GABA_A$ receptors containing functional β_1-subunits.

Single Cell PCR demonstrates β_1 expression

How is it possible that the literature so strongly indicates that cerebellar PCs do not express β_1-subunits while our functional data suggests otherwise? One explanation is that PCs express the β_1-subunit transcript at very low levels. Single cell PCR is powerful technique allowing detection of gene expression in specific neuronal populations. Unfortunately, one of the biggest challenges to amplifying genes from single cells is the extremely low RNA yield. Abundance of the transcript is another factor that can affect results from single cell PCR, with more abundant transcripts being easier to detect. To avoid false negatives from dilute cDNA, we used the nested PCR method, with degenerate primers for homologous regions on the 3 β (1-3) subtypes. Additionally, we tested a primer set used by the Allen Mouse Brain Atlas that positively labeled PCs by in situ hybridization, though with decreased intensity compared to β_2 and β_3. Indeed, β subunit transcripts were harder to detect compared to α_1, and the level of β_1 transcript in PCs was lower than that of β_2 and β_3, thereby requiring optimized

primers for detection by PCR. This may explain the discrepancy between our findings and other studies which did not detect β_1 in PCs by single cell PCR (Ruano et al., 1997; Sim et al., 2000) or in situ hybridization (Laurie et al., 1992; Wisden et al., 1992). Yet, there are reports describing labeling of β1 mRNA in a distinct band at the interface of the granule cell layer and molecular layer, where PCs reside (Zhang et al., 1991; Zdilar et al., 1992; Lein et al., 2007). We provide molecular and functional data indicating that PC express β_1 subunits and re-evaluation of previous findings may be warranted. The physiological and pathophysiological significance of this observation remains to be determined.

Conclusion

From our data we conclude that PCs express the β1 subunit, contrary to previous reports. The relative contribution of $\beta_1$containing $GABA_A$ receptors appears to increase following ischemia, thereby decreasing sensitivity to etomidate. While β_1 transcript levels may be lower than $\beta_{2/3}$ levels, β_1 protein is easily detectable by Western blot and protein levels remain unaffected by ischemia. The finding of β_1 subunit expression in PCs is novel and controversial, yet important for understanding inhibition in the cerebellum and $GABA_A$ receptor expression and trafficking in ischemia.

Drug	Subunit	Control EC_{50}	OGD EC_{50}	Control v. OGD $P < 0.05$
Etomidate	β_{2-3}	2.9 ± 0.3 μM (8)	3.8 ± 0.2 μM (6)	*
Propofol	β_{1-3}	5.6 ± 0.5 μM (9)	5.8 ± 0.5 μM (12)	-
Zolpidem	α_x, γ_2	466.1 ± 75.8 nM (9)	491.5 ± 55.6 nM (8)	-
Diazepam	α_1, γ_x	2.3 ± 0.4 μM (7)	2.8 ± 0.3 μM (7)	-

Table 1. PC $GABA_A$ receptor sensitivity to subunit specific positive modulators.

[a] Each value is the mean±SEM

[b] The number of neurons studied is in parentheses

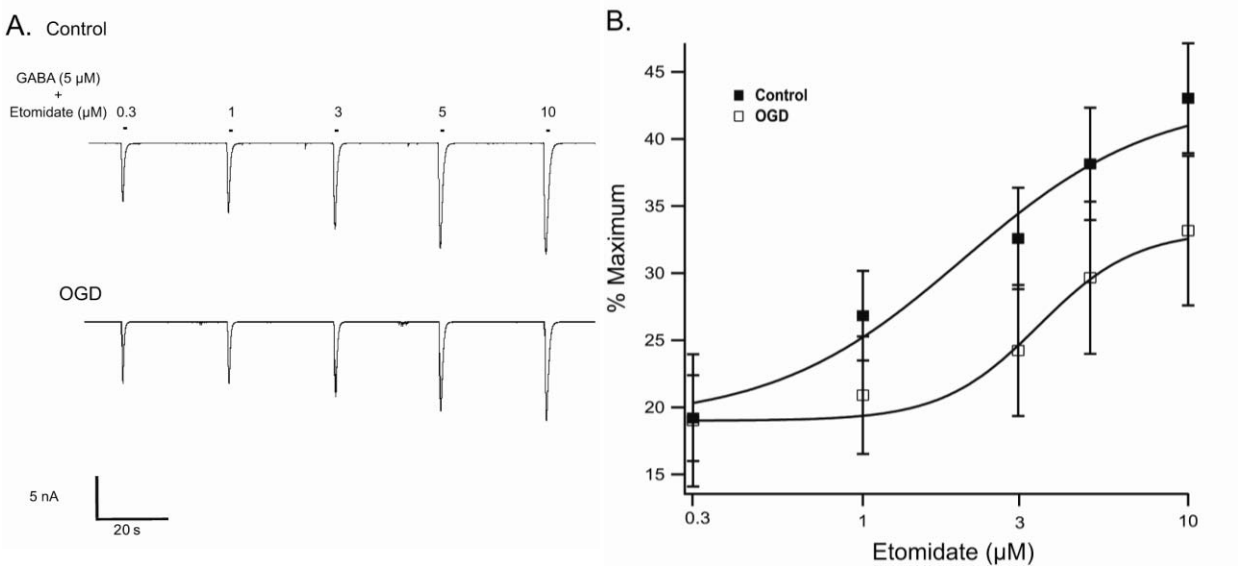


Fig. 1 OGD decreases PC $GABA_A$ receptor sensitivity to etomidate

A) Representative recordings from cultured PCs illustrating responses to 1s applications of 5 μM GABA plus 0.3, 1, 3, 5, or 10 μM etomidate (bars above trace indicate application). B) Average concentration-response relationships for etomidate from control and OGD PCs. The average EC_{50} for control cells was 2.9 ± 0.3 μM. OGD cell average EC_{50}, was 3.8 ± 0.2 μM ($P < 0.05$). Data presented are the average of 8 control cells and 6 OGD cells.

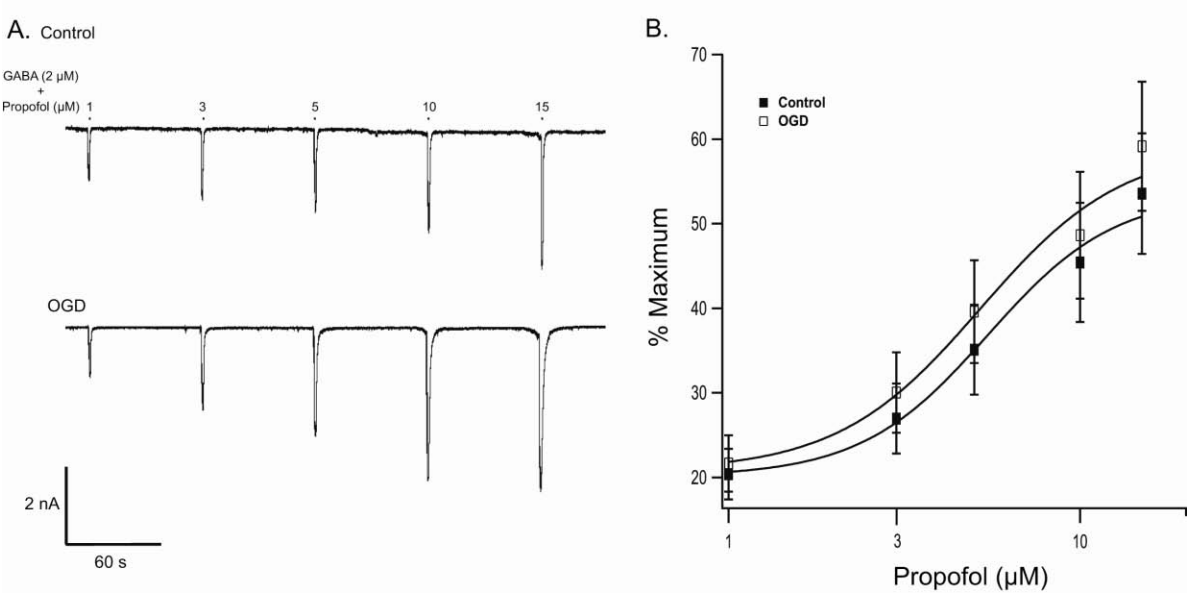


Fig. 2 OGD does not affect PC $GABA_A$ receptor sensitivity to propofol

A) Representative recordings from cultured PCs illustrating responses to 1 s applications of 2 μM GABA plus 1, 3, 5, 10, or 15 μM propfol (bars above trace indicate application). B) Average concentration-response relationships for propofol from control and OGD PCs. The average EC_{50} for control cells was 5.6 ± 0.5 μM, n=9. OGD cell EC_{50} was 5.8 ± 0.5 μM, n=12.

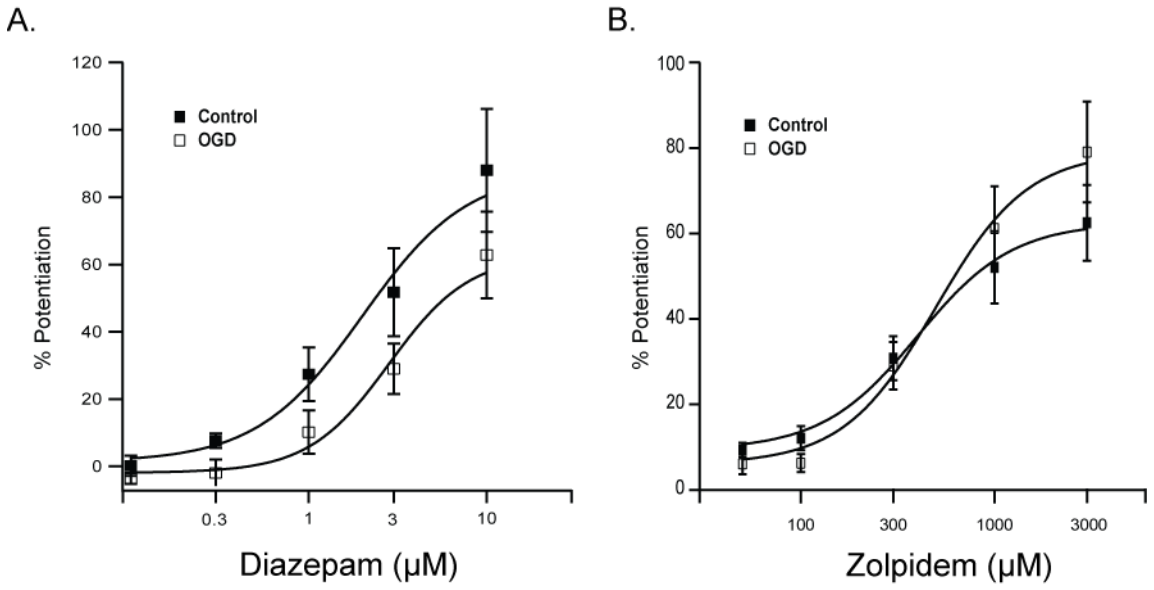


Fig. 3 OGD does not affect PC $GABA_A$ receptor sensitivity to diazepam or zolpidem

A) Average concentration-response relationships for diazepam from control and OGD PCs. The average EC_{50} for control PCs was 2.27 ± 0.4 μM, n=7. For OGD cells the average EC_{50} was 2.81 ± 0.4 μM, n=6. B) Average concentration-response relationships for zolpidem from control and OGD PCs. The average EC_{50} for control cells was 465.7 ± 75.9 nM, n=9; average OGD cell EC_{50} was 482.6 ± 62.4 nM, n=8.

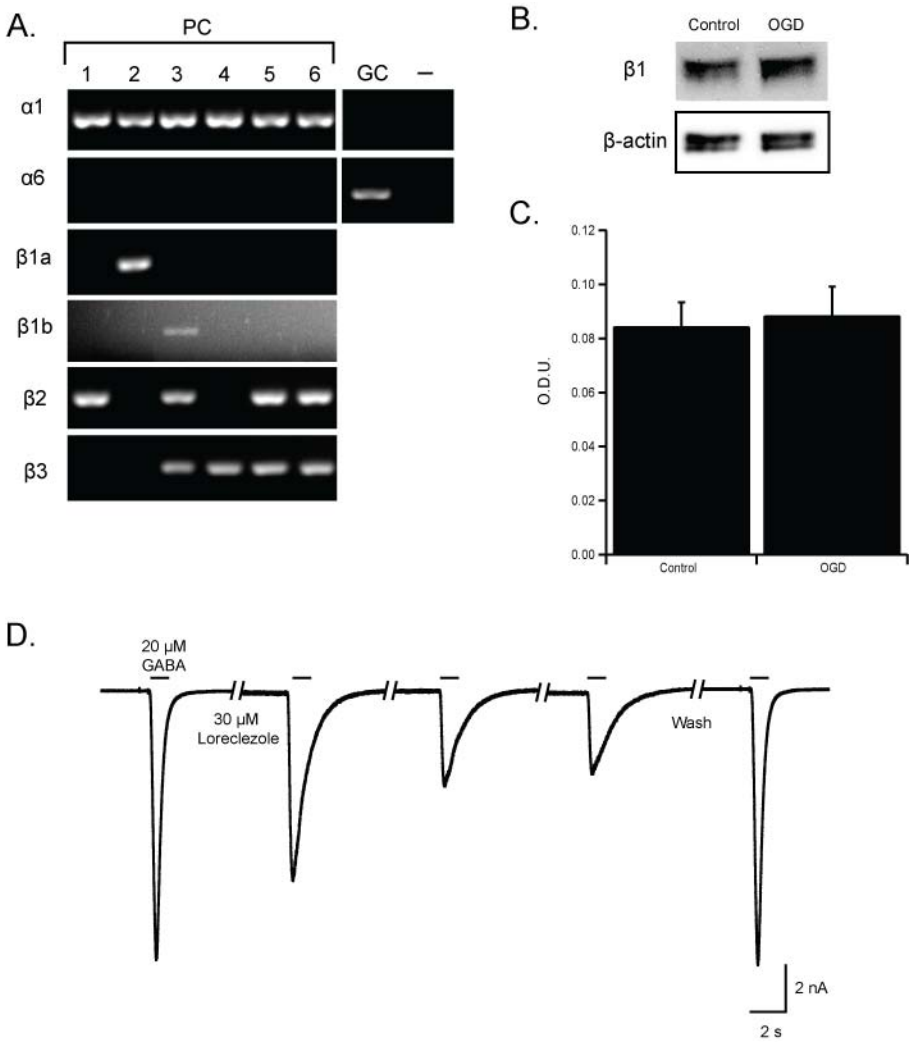


Fig. 4 PCs express the $GABA_A$ receptor β_1 subunit and protein expression of β_1 is not affected by OGD.

A.) Representative gel illustrating the mRNA expression of α_1, α_6, β_1, β_2, and β_3 subunits in Purkinje cells (PC). Each lane (1-6) represents one sample containing 4-6 PCs. Purity of PC samples was confirmed by the absence of α_6. Granule cells (GC) were the positive control for α_6. Negative control (-) was Tyrodes solution collected from vicinity of PCs. B.) Representative Western blot of cerebellar $GABA_A$ receptor β_1 subunit protein from control cultured PCs and

cells that received 2 hr OGD and 1 hr re-oxygenation. β-actin loading control shown in lower panel. C.) Quantification of $GABA_A$ receptor β_1 subunit protein, normalized to β-actin loading control and expressed as optical density units, n=5 for each group. Data are mean ± SEM. D.) Representative recording depicting loreclezole inhibition of $GABA_A$ receptor mediated currents. Each bar represents application of 20 μM GABA. After stable peak amplitude response to GABA was achieved, 30 μM loreclezole pre-application was perfused onto the cell for 30 s and loreclezole remained present through 3 GABA pulses applied in 30 s intervals. Following 60 s wash out of loreclezole, peak amplitude of GABA response was restored.

CHAPTER 5:

CONCLUDING REMARKS

I. Summary of Results

The main finding of this thesis is that OGD caused rapid removal of $GABA_A$ receptors from synapses in Cerebellar PC, leaving them with significantly less inhibitory potential and an increased propensity for excitotoxic damage. Currents evoked by exogenously applied GABA, as well as amplitudes of mIPSCs, were diminished following *in vitro* ischemia within the first hour of reoxygenation. The loss of current is likely due to $GABA_A$ receptor degradation, as indicated by reduced levels of $GABA_A$ receptor α1 subunit protein. While there was a significant loss of $GABA_A$ receptor protein, a subset of $GABA_A$ receptors were not internalized and degraded in response to ischemia. The $GABA_A$ receptors that were conserved had different pharmacological profiles compared to non-ischemic control cells. Specifically, OGD decreased PC $GABA_A$ receptor sensitivity to etomidate, but not propofol, diazepam, or zolpidem. $GABA_A$ receptor β_1 subunit mRNA was detected in PCs, and characteristic inhibition of GABA mediated currents were observed with loreclezole. Additionally, β_1 subunit protein was detected in cerebellar cultures and unlike the α1 subunit, β_1 protein levels were not reduced by ischemia. Therefore the decreased sensitivity to etomidate is likely due an increased contribution of $GABA_A$ receptors containing the β_1 subunit.

Another major finding in this thesis was that ALLO protected PCs from ischemia by preventing the decline in $GABA_A$ receptor function and protein. Sensitivity to ALLO was greater in females compared to males where males required a dose of ALLO four times higher than females to achieve significant neuroprotection from global ischemia. Accordingly, ALLO prolonged sIPSC kinetics to a greater degree in females compared to males. However, there was no sex difference in ALLO's ability to slow mIPSC kinetics. Therefore, the sex difference in ALLO sensitivity is likely not at the level of the postsynaptic receptors.

II. Models of Cerebellar Ischemia

The experiments in this thesis utilized both *in vitro* and *in vivo* models of ischemia. The *in vitro* model consisted of cultured cerebellar neurons exposed to OGD. Prior to experimentation, cells were grown in culture for a minimum of 10 days to allow the neurons to form synapses similar to those formed by PCs *in vivo* (Gruol, 1983; Hirano et al., 1986; Hirano and Kasono, 1993) Additionally, cultured PCs mature in culture and they display simple and complex firing patterns similar to adult PC (Gruol and Franklin, 1987). Primary cultures of cerebellar neurons contained a greater ratio of PCs to other cerebellar neurons than would be observed in adult cerebellum. This was due in part to the stage in development of the cerebellar tissue used in culture and also to the culture

conditions which favor PC growth over granule cells. Despite culture conditions optimized for PCs, our cerebellar cultures contained granule cells and interneurons. In fact, PCs used in this work were previously characterized to receive excitatory input that is blocked by CNQX as well as picrotoxin sensitive inhibitory GABAergic input (Ardeshiri et al., 2006). The use of a mixed cerebellar neuronal culture is vital to studying how ischemia may disrupt the balance between excitation and inhibition as well as how the enhancement of endogenous inhibition may be neuroprotective. Cultured PCs remain vulnerable to ischemia and significant levels of PC death are observed with 2 hours of OGD and 24 hours of re-oxygenation (Ardeshiri et al., 2006). Recordings in thesis were made from cultured PCs within one hour of reperfusion, giving insight to early changes that are occurring in neurons that are compromised by ischemia. Whole cell voltage clamp recordings of cultured PCs allowed rapid solution exchange which facilitated the measurement of responses to various test compounds. These qualities made the primary cerebellar culture system ideal for the study of OGD dependent regulation of PC $GABA_A$ receptors.

Fundamental findings using the *in vitro* model of ischemia were confirmed *in vivo* with mouse CA/CPR. The mouse CA/CPR model is a well established model of global cerebral ischemia (Kofler et al., 2004). Mouse CA/CPR has been used to reveal the effect of global cerebral ischemia in genetically modified mice (Kofler et al., 2005;Kofler et al., 2006), as well as the neuroprotective properties of pharmacological agents (Noppens et al., 2005). Significant damage to PCs is observed with 10 min CA/CPR, making it a useful model for studying ischemic

injury of PCs. It would be of great interest to record from PCs in acute slices of cerebellum, from mice that have survived CA/CPR, to examine functional changes to GABAergic signaling. Future studies may include analyzing functional properties of sIPSCs and evoked IPSCs recorded from CA/CPR mice.

III. $GABA_A$ Receptor Reduction in Response to Ischemia

A major finding of this work was that ischemia significantly reduced the number of functional $GABA_A$ receptors in PCs, thereby decreasing PC inhibition. This conclusion was based on decreased peak amplitudes in response to saturating concentrations of GABA following 2 hrs of OGD. Diminished responses to GABA could also result from decreased channel conductance, receptor desensitization, or internalization. However, we determined that the reduced number of functional $GABA_A$ receptors following OGD was due to rapid $GABA_A$ receptor degradation by measuring $GABA_A$ α_1 subunit protein, an effect that occurred within the first hour of reoxygenation and persisted for several hours to days post ischemia. These experiments were performed under the assumption that PC $GABA_A$ receptors were solely composed of $\alpha_1/\beta_{2\text{-}3}/\gamma_2$ subunits. Therefore, the α1 subunit levels were presumed to indicate total $GABA_A$ receptor protein. However, subsequent experiments revealed novel expression of the β_1 subunit and a shift in the relative contribution of β_1 containing receptors. Thus, PCs express a more heterogeneous population of

$GABA_A$ receptors than previously reported, and each receptor subtype may have unique regulation and a distinct functional role in healthy and diseased states.

$GABA_A$ receptor β and γ subunits contain AP2 recognition sites for clathrin mediated endocytosis (Kittler et al., 2000). The β subunits contain atypical AP2 binding motifs which include serines that are targets for serine/threonine kinases such as PKA or PKC (Kittler et al., 2005). The γ2 subunit contains a more classical tyrosine based AP2 binding motif (Kittler et al., 2008). Phosphorylation of GABA receptor AP2 binding motifs can prevent association of AP2, thereby blocking clathrin mediated internalization. Additionally, the endosomal Huntington associated protein (HAP1) can interact with β subunits to facilitate receptor recycling and inhibit degradation (Kittler et al., 2004). These findings indicate the β and γ subunits are actively engaged in $GABA_A$ receptor trafficking and may play a role in the ischemia induced endocytosis and degradation of $GABA_A$ receptors. It would be of great interest for future studies to investigate the role of these regulatory sites in ischemia-induced $GABA_A$ receptor trafficking. It is also likely that AP2 binding motifs or HAP-1 interactions differ between β_2 and β_1 subunits, providing a possible explanation for unique trafficking and regulation following ischemia. A more detailed examination of β and γ subunit protein following ischemia is warranted for development of therapies aimed at preventing $GABA_A$ receptor internalization and degradation.

IV. Purkinje Cell Pharmacology and β_1 Subunit Expression

Many $GABA_A$ receptor modulating compounds have been extensively characterized in heterologous expression systems. However, by studying $GABA_A$ receptor modulators in neurons, responses are more physiological and they incorporate effects of downstream $GABA_A$ receptor interacting proteins. PCs express a relatively homogenous population of $GABA_A$ receptors assisting in predicting sensitivity to various modulators. In fact, EC_{50} measurements from cultured PCs were similar to values reported in the literature and the increased EC_{50} value for etomidate but not propofol led to the hypothesis that PC $GABA_A$ receptors express the β_1 subunit in addition to β_2 and β_3.

Early studies mapping the distribution of $GABA_A$ receptor subunits have utilized *in situ* hybridization and immunolabeling, techniques which have limitations with detection threshold and specificity. *In situ* hybridization studies have detected a band of β_1 mRNA at the interface of the granule cell layer and molecular layer, where PCs reside (Zhang et al., 1991; Zdilar et al., 1992; Lein et al., 2007). However, mutant mice lacking PCs display similar, yet significantly reduced β1 mRNA labeling where PCs normally reside, leading to the conclusion that PCs do not express β_1 (Zdilar et al., 1992).

The data in this work clearly show β_1 subunit expression in cultured PCs and acutely dissociated PCs. However, based on single cell PCR experiments, β_1 transcript level are likely lower than β_2 and β_3. Despite this, β_1 protein was easily detected in cultured PCs and loreclezole inhibited a comparable

percentage of GABA mediated current as the heterologously expressed $\alpha_1\beta_1\gamma_2$ receptor.

V. ALLO Neuroprotection and Sex difference

This work demonstrated that ALLO provides significant protection from ischemic damage of PCs by preventing the reduction in $GABA_A$ receptors. ALLO is known to increase $GABA_A$ receptor channel open probability thereby potentiating responses to GABA (Callachan et al., 1987). However, diazepam, does not prevent the ischemia induced decline in $GABA_A$ receptors. Therefore, ALLO blocks $GABA_A$ receptor reduction by a mechanism other than receptor activation. One unique effect of ALLO that differs from diazepam is that ALLO can promote phosphorylation of $GABA_A$ receptors (Fancsik et al., 2000). Phosphorylation of the receptors may block AP2 binding to prevent internalization and degradation, thereby preserving $GABA_A$ receptors and better equipping neurons to combat excitotoxicity caused by ischemia.

Another interesting finding of this work is the sex difference observed with ALLO neuroprotection of PCs from global ischemia. Although not significantly different than male mice, control females sustained less damage to PCs than males despite receiving equivalent durations of global ischemia. This finding may be explained by sex differences in endogenous levels of ALLO, because females have higher levels of circulating progesterone. In addition, the cerebellum expresses enzymes required to metabolize progesterone to ALLO and synthesize ALLO *de novo* (Celotti et al., 1997), yet sex differences in

abundance and function of these enzymes are unknown. The closest estimates of ALLO concentrations in male and female brain come from radioimmunoassay for ALLO in brain lysates. The concentration of ALLO in male rat brain is reported to be near detection threshold (25pg) under normal conditions (Purdy et al., 1991), whereas females have higher levels of ALLO that fluctuate according to estrous cycle (Purdy et al., 1992).

In addition to increased endogenous levels of ALLO in females compared to males, $GABA_A$ receptors display variability in the sensitivity to ALLO based on subunit composition. For example, receptors containing the β_1 subunit are more sensitive to ALLO than those containing $\beta_{2/3}$ (Lambert et al., 2001). Based on this data, it is possible that female $GABA_A$ receptors have more β_1 subunit expression compared to males, resulting in ALLO to having a greater effect in females. However, our data is not consistent with this hypothesis because we did not observe differences in mIPSC kinetics recorded in male and female PCs. Alternatively, ALLO may be acting presynaptically, affecting GABA release in females.

Nevertheless, ALLO provided significant neuroprotection in both sexes at a higher dose and has great therapeutic potential for treatment of stroke and CA. In fact, ALLO is neuroprotective in other neurological diseases and forms of brain injury. For example, ALLO is protective in animal models of TBI (Djebaili et al., 2004), Alzheimers disease (Brinton and Wang, 2006), Niemann-Pick C disease (Mellon et al., 2008), kainate excitotoxicity (Ciriza et al., 2004), and focal cerebral ischemia (Sayeed et al., 2006). In addition to neuroprotection by stimulating and

preserving $GABA_A$ receptor response, ALLO can promote neurogenesis (Wang et al., 2005; Brinton and Wang, 2006), decrease inflammation (He et al., 2004), increase Bcl2, anti-apoptotic, gene transcription (Charalampopoulos et al., 2004; Charalampopoulos et al., 2006), and activate the PKB/Akt kinase to decrease apoptosis (Xilouri and Papazafiri, 2006; Xilouri et al., 2007). Therefore, ALLO is a dynamic compound that has great capacity for protecting against neurodegeneration.

Conclusions

The goal of this work was to examine the effect of ischemia on $GABA_A$ receptors in order to design better therapies aimed at attenuating excitotoxicity. Our data demonstrate that $GABA_A$ receptor function is significantly reduced by ischemia due to receptor degradation. Subunit composition of $GABA_A$ receptors may influence their stability in response to ischemia since β_1 containing receptors are not down regulated by ischemia. Finally, significant protection from ischemia was achieved by preserving $GABA_A$ receptors response with ALLO.

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