Cellular Mechanisms of Neurodegeneration and Neuroprotection in the Mammalian Cortex

a dissertation
by
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presented to the Neuroscience Graduate Program and the
Oregon Health & Science University School of Medicine in
partial fulfillment of the requirements for the degree of
Doctor of Philosophy

submitted June of 2011

School of Medicine
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CERTIFICATE OF APPROVAL

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Abbreviations

ANOVA	**|**	analysis of variance
CNS	**|**	central nervous system
CyD	**|**	cyclophilin D
CyD KO	**|**	cyclophilin D knockout
DETA-NO	**|**	2,2'-(Hydroxynitrosohydrazono)bis-ethanimine
DIV	**|**	day *in vitro*
DMSO	**|**	dimethylsulfoxide
DMEM	**|**	dulbecco's modified eagle's medium
DNA	**|**	deoxyribonucleic acid
ER	**|**	estrogen receptor
E2	**|**	17β-estradiol
ICI	**|**	ICI 182,780
IsoPC	**|**	isoflurane preconditioning
LDH	**|**	lactate dehydrogenase
MCAO	**|**	middle cerebral artery occlusion
MK-801	**|**	dizocilpine
NO	**|**	nitric oxide
OGD	**|**	oxygen and glucose deprivation

PCR	**|**	polymerase chain reaction
ShamPC	**|**	sham preconditioning
WT	**|**	wild-type

Acknowledgement

This thesis dissertation would not have been possible – and therefore the prospect of me fulfilling my lifelong ambitions to become a scientist would not have been possible – without the steadfast mentorship of Dr. Stephanie Murphy. The quality of mentorship that Dr. Murphy has bestowed in the mere 29 months that I worked with her is reflected in my ability to secure a funded predoctoral fellowship award, submit 2 first-author manuscripts, present a poster at a Society for Neuroscience meeting, and now defend a Ph.D. thesis dissertation. This accomplishment requires more than simply diligence; it requires absolutely exceptional mentorship. Her gift of mentorship is further realized upon considering that I am her first graduate student. In many regards, this thesis dissertation is as much Dr. Murphy's accomplishment as it is my own. Moreover, catering to my oft quixotic, intractable creativity, Dr. Murphy's pragmatism and fastidious demeanor taught me the value in grounding a curious mind just enough to actualize creativity into resolute scientific approach, and therefore resolute scientific discovery. This is a skill that an independent scientist requires, yet a skill that cannot be taught in lecture. Dr. Stephanie Murphy: your mentorship is inspirational; it is mentorship that I admire and will attempt to mimic. Thank you.

I thank my thesis advisory committee, Dr. Nabil Alkayed, Dr. Mary Stenzel-Poore, Dr. Gary Westbrook, and Dr. Ansgar Brambrink for their scientific prowess and, considering the brevity of my project with Dr. Stephanie Murphy, their acumen in promoting a successful thesis project. I must also thank Dr. Michael Forte. While his mentorship during my initial years as a graduate student investigating cyclophilin D came to a premature end, my investment in this project remains an important

contribution to the data, interpretations, and implications of this thesis dissertation, as well as to the broader neurodegenerative field.

When it comes to additional names to acknowledge, there are too many. I have always respected a philosophy that invests heavily in human relationships, assuming every altruistic being possesses talents and knowledge worth sharing. This perspective is how our species mimicked its way out of the genetic doldrums of Africa and continues to plead this ephemeral human existence to invest in a benevolent legacy, to which I believe science and technology can contribute. Hence, I have sought a network of relationships in Portland that enrich me with experiential texture beyond that of only the scientific discipline. From performance and fine art, to music, to sport, to love (and consequently to loss) – these are the textures by which every relationship I've invested in over recent years has contributed not to the literal substance of this thesis dissertation, which I appreciate as modest, but rather to the value of the skills and knowledge acquired in the *pursuit* of this dissertation so that I may someday have to privilege to reciprocate a small, yet benevolent drop of legacy into the greater ocean of the human experience.

Alas, there are those relationships that must be named. These are the relationships where I have come to define myself not *with* someone else, but *in* someone else. Bridget Robinson, Christina Lorentz, Jamin Aasum, Susie Walsh – you are my Portland family; you are the ground in which these frail roots of mine have taken hold, nourished, and sprouted new growth. Without you I wither. "Thank You" is not enough, if not a disservice. And so I'll leave it to our shared music, photos, and memories – as well as our future – to tell the story of our ever-evolving relationships. The prescribed bounds of the English language (or more likely my fumbling misuse of it) simply cannot synthesize such beauty.

And to my South Dakota family, the words are also difficult to find but for very different reasons. Your influence has certainly imprinted the stamps of moral obligation, diligence, compassion, and, perhaps more recently, forgiveness. These are learned skills that I contribute to any perceived success of mine, both within and outside of the scientific arena. Yet I had the un-mentored role of being the oldest child of 4, as well as the first in our family to pursue a college degree, let alone a doctorate degree. Add to this recipe my voracious appetite for knowledge and experience to a backdrop of familial instability, and the result is an ungrounded pariah, clinging to independent thought and action for stability. Ironically, this is in itself a skill – while painfully learned – that behooves the aspiring scientist seeking independent thoughts through creative ingenuity and independent action through sagacious methodology. Nonetheless, your presence in my life is at a new optimistic horizon – one of support and stability. And for this, I thank – and truly love – all of you.

Abstract

Neurodegeneration can be described as the progressive decline in central nervous system (CNS) function resulting from cell death or cellular dysfunction within nervous tissue, most notably the brain. There is no cure for neurodegenerative disorders, largely due to the post-mitotic nature of neurons and to the difficulty in reestablishing disruptions within the highly-integrated CNS circuitry of mammals. Hence, neurodegeneration is a major contributor to morbidity and mortality world-wide. A better understanding of the cellular mechanisms that lead to cell death in the CNS, as well as a better understanding of processes that prevent cell death, is crucial towards developing more effective neuroprotective strategies.

This thesis takes two different approaches to investigate neurodegenerative and neuroprotective mechanisms at the cellular level. The first approach (Chapters 2 and 3) begins from the observations that sex is a risk factor in ischemic brain injury and that a potential neuroprotective strategy, isoflurane preconditioning, may in itself demonstrate sexually dimorphic efficacy. Investigations in isolated, sex-segregated astrocytes and neurons tested whether innate cell sex dictates differential responses to isoflurane preconditioning and subsequent oxygen and glucose deprivation, an *in vitro* model for ischemia. In Chapter 3, I tested whether the female sex steroid, 17β-estradiol, modulates the neuronal response to isoflurane preconditioning and subsequent oxygen and glucose deprivation via nuclear estrogen receptors. Isoflurane preconditioning increased cell survival in astrocytes and neurons following subsequent oxygen and glucose deprivation regardless of innate cell sex, whereas 17β-estradiol attenuated the protective response to isoflurane preconditioning only in female neurons independent of nuclear estrogen receptors. Additionally, independent of

preconditioning, female neurons had increased cell survival following oxygen and glucose deprivation compared to males, and 17β-estradiol addition increased neuronal survival following oxygen and glucose deprivation regardless of preconditioning or innate cell sex.

The second approach of this dissertation (Chapter 4) begins from the observation that the mitochondrial matrix protein, cyclophilin D, is an important nexus among multiple cell death signaling pathways. I tested whether cyclophilin D also mediates cell death following insults endemic to neurodegeneration in isolated neurons and astrocytes. Neurons and astrocytes genetically lacking cyclophilin D had increased cell survival compared to wild-type cells following oxidant treatment, but not following excitotoxic (glutamate) or energetic challenges (modeled by oxygen and glucose deprivation).

I conclude that the cellular default in astrocytes and neurons is a protective response to isoflurane preconditioning and subsequent oxygen and glucose deprivation independent of innate cell sex. The mechanisms by which female and male neurons respond to isoflurane preconditioning in the presence of 17β-estradiol may be different because 17β-estradiol attenuated IsoPC protection only in female neurons. In addition, cyclophilin D is an important mediator of oxidant insult in neurons and astrocytes, but not excitotoxic or energetic insult in neurons. These findings advance our understanding of how neurodegeneration and potential neuroprotective strategies may differ between female and male brain, and how convergent mediators in cell death, such as cyclophilin D, may facilitate specific cellular insults endemic to neurodegeneration processes.

Chapter 1 | Introduction

I. Overview and Approach

This thesis dissertation takes two different *in vitro* approaches to investigate neurodegenerative mechanisms and identify potential neuroprotective strategies. The first approach considers the rather specific observation that sex is a risk factor in ischemic stroke and that potential neuroprotective strategies for ischemic stroke, such as anesthetic preconditioning, may in themselves have sexually dimorphic efficacies. The second approach considers the observation that the mitochondrial protein, cyclophilin D, mediates brain cell death and that targeting this protein may be an effective neuroprotective strategy for neurodegenerative disorders. Both approaches include an analysis of astrocytes, in addition to neurons, to better appreciate the cellular heterogeneity of the mammalian cortex, and therefore more thoroughly characterize potential neuroprotective strategies.

II. The Problem of Neurodegeneration

Neurodegeneration can be described as the progressive decline in central nervous system (CNS) function resulting from the dysfunction and loss of neurons or physiologically associated cells[1,2]. Prominent examples of neurodegeneration include diseases such as Parkinson's disease, Alzheimer's disease, Huntington's disease, multiple sclerosis, stroke, and traumatic brain injury[1,3,4]. Neurodegeneration can afflict individuals regardless of socio-economic status, race, age, or sex, and is

associated with high morbidity and mortality. There is no cure for neurodegeneration and effective treatment is severely limited, partially due to the post-mitotic nature of neurons and the difficulty in reestablishing damaged connections within the highly complicated neural networks of the mammalian CNS[5]. Hence, neurodegeneration is a significant contributor to diminished quality and duration of human life and represents a major economic burden world-wide.

Developing effective therapeutic strategies to prevent and treat neurodegeneration will require a thorough understanding of the cellular mechanisms in the CNS that eventually lead to neuronal cell death. However, these neurodegenerative cellular mechanisms are multifarious and remain poorly understood. Nearly every cell death process and mediator has been linked to neurodegeneration, including apoptosis and necrosis[6,7], oxidative stress[2,7,8], excitotoxicity[6,8] and energetic dysregulation[2,9]. All of these processes manifest throughout the spectrum of neurodegenerative disorders, yet the distinct roles of each process may vary depending on the specific neurodegenerative disorder as well as genetic and environmental factors. Furthermore, these processes can elicit a cascade of signals that propagate in parallel and/or interact with other processes to contribute to unique neurodegenerative pathology[1,2,6,10-13]. Consequently, a better understanding of the complexity of neurodegeneration – and therefore the ability to develop more effective neuroprotective strategies – will require investigations and analyses from multiple experimental approaches.

One such approach is to begin with a risk factor in neurodegeneration, such as male sex in ischemic stroke[12], and then identify more convergent downstream mechanisms, such as sex steroid signaling. This forward-screen approach can help identify therapeutic targets and neuroprotective strategies that may be more specific

for a particular neurodegenerative condition and therefore more likely to be effective in treating that pathology[12]. In Chapters 2 and 3, I used this approach to explore the cellular basis for the sexually dimorphic response to isoflurane preconditioning (IsoPC), a potential neuroprotective strategy, and to explore downstream mechanisms, such as sex steroids, that may mediate specific responses to IsoPC.

The converse approach is to begin with a convergent mechanism for brain cell death, such as cyclophilin D (CyD) mediation of permeability transition[14], and then subsequently identify upstream neurodegenerative disorders that may share this mechanism, such as multiple sclerosis[15] and ischemic stroke[16]. The approach in Chapter 4 tested if CyD mediates cell death in neurons following oxidative injury, and then tested if CyD mediates more upstream cellular insults endemic to neurodegeneration such as excitotoxicity (glutamate) and energetic failure (oxygen and glucose deprivation [OGD]). The role of CyD following oxidant injury in astrocytes was also explored in Chapter 4.

Employing both experimental approaches to neurodegenerative investigations may illuminate previously obscured relationships between mechanism and disease whereby novel neuroprotective strategies can then be identified.

III. Approach 1: From Disease Towards Mechanism

A. Sex as a Risk Factor in Neurodegeneration

Known neurodegenerative risk factors include genetic polymorphisms, such as those associated with the parkin gene in Parkinson's disease[17]; age, which is considered the highest risk factor for neurodegeneration[2]; and sex, where females and males present unique risks to the neurodegeneration associated with ischemic

stroke[18,19] and multiple sclerosis[10,13,20]. Each risk factor represents a degree of upstream divergence in terms of genetic and/or environmental cause, yet they likely share some convergence because the ultimate result is neuronal dysfunction and cell death[3,6,8].

The role of sex as a risk factor in neurodegeneration is of particular interest because current research employing animal models of neurodegeneration is typically biased towards a single sex[19,21,22]. This is unfortunate because differences between women and men exist in not only the risk to develop certain neurodegenerative conditions, but also in prognosis[13,22]. For example, females typically experience a decreased risk for ischemic stroke and enjoy better outcomes following stroke[18,19]. In multiple sclerosis, females are more susceptible to developing multiple sclerosis, yet females have a better prognosis[10,13]. Animal models of ischemic stroke and multiple sclerosis have been generally consistent with the observed sexual dimorphism in human disease[13,18,19]. Yet the cellular mechanisms underlying sex as a neurodegenerative risk factor require better understanding if better neuroprotective strategies are to be developed[21,23].

Limiting neurodegenerative research to a particular sex, while simplifying experimental variables, limits data interpretations. For example, key mechanisms that have long been linked to neurodegeneration, such as nitric oxide in ischemic stroke[24], have unique, even contrasting roles, between female and male animals[25]. Findings such as these may call for reexamination of reports that were limited to a single or unidentified the sex.

Sexually dimorphic responses in neurodegenerative models also manifest at the cellular level. *In vitro* reports have identified different cell death mechanisms and sensitivities to toxic insults between isolated female and male brain cells[26-28]. The

precise mechanisms and implications of these innate cellular responses in neurodegeneration, however, remain to be better understood. Nonetheless, in chorus with their *in vivo* counterparts, these *in vitro* findings limit the interpretations from culture systems in which cells and tissue are typically pooled from female and male brains.

Sexual dimorphism may influence disease mechanisms and outcomes, and thus alter the efficacy of neuroprotective strategies[21,23]. In chapters 2 and 3 of this dissertation, I consider innate cell sex as a potential factor eliciting specific cellular responses in an in vitro model of neuroprotection and ischemia.

B. General Review of Preconditioning

Preconditioning is an endogenous process in cells, including those within the brain, whereby a noxious stimulus below the threshold that causes damage initiates a cellular program that allows those cells to withstand a subsequent, more noxious stimulus[29-31]. Initial descriptions of preconditioning resulted from brief ischemic episodes to cardiac[32] and brain tissue[33] to protect against subsequent, prolonged ischemic episodes. This represents what is now considered "ischemic preconditioning". Following the early characterizations of ischemic preconditioning, numerous other stimuli have been identified that induce a preconditioning program in brain cells, including pharmacologic agents, bacterial endotoxins, reactive oxygen species, and volatile anesthetics[30,31,34]. These preconditioning stimuli are usually identified by their ability to protect against an array of subsequent insults including ischemia or a more noxious exposure to the preconditioning stimulus itself, such as volatile anesthetic preconditioning protection from a toxic dose of the same volatile anesthetic[35]. The ability of preconditioning stimuli to protect from a variety of subsequent insults

suggests a downstream convergence among the cellular programs of preconditioning, although the identity of such convergent mediators remains to be fully characterized[30,31].

There are two major paradigms of preconditioning that differ in the temporal window between the preconditioning stimulus and its induced response in cells. Rapid, or "classical" preconditioning manifests within minutes of the preconditioning stimulus and lasts for only a few hours. The protection provided by classical preconditioning is often modest[29,31,36]. Delayed preconditioning, on the other hand, requires 1-3 days following the preconditioning stimulus before a response is evident. This form of preconditioning can provide robust protection and can persist for longer periods of time compared to rapid preconditioning[29,31].

Despite the well-characterized protective responses elicited by various preconditioning stimuli and protocols, most investigations have exclusively used male animals or mixed-sex cultured cells. Unlike male responses, female responses to preconditioning in cardiac tissue are highly variable[37], demonstrating a protective response following ischemic preconditioning[38], no response following isoflurane preconditioning[39], or an exacerbated response following chemical preconditioning that was partially mediated by sex steroids[40]. Sexually dimorphic preconditioning responses have also been observed in the brain. The protective efficacy of hypoxic and chemical preconditioning has been shown to vary with the estrous cycle in females[41], which may result from different sex-specific mechanisms via the progesterone receptor[42]. The specific cellular response in the brain to preconditioning, however, remains to be investigated. Chapters 2 and 3 employ an *in vitro* model of delayed preconditioning induced by the volatile anesthetic isoflurane to determine whether the response to

preconditioning and subsequent ischemia is different between male and female astrocytes and neurons.

C. Volatile Anesthetic Preconditioning as a Potential Neuroprotective Strategy for Perioperative Ischemic Stroke

The use of volatile anesthetics to precondition the brain before an ischemic stroke is an example of a potential neuroprotective strategy that can have sexually dimorphic outcomes. Volatile anesthetic preconditioning occurs when brief exposure to a volatile anesthetic, such as isoflurane, alters the response in cells and tissues to a subsequent insult like ischemia. This preconditioning response can be neuroprotective and is therefore of interest in specific clinical applications, namely in perioperative settings associated with a high risk for ischemic stroke[43,44]. Carotid endarterectomy and coronary artery bypass grafting are two examples of surgical procedures that have some of the highest risks for perioperative ischemic stroke[45,46]. These surgical procedures require general anesthesia such as isoflurane, thus the choice and management of an anesthetic could serve a neuroprotective role should a stroke occur during or following surgery[43,47].

Until recently, preconditioning induced from volatile anesthetics such as isoflurane, halothane, sevoflurane, desflurane, and xenon has consistently been shown to be neuroprotective in animal models of ischemic stroke[43,47]. Yet previous studies were limited to assessing the response in male animals. Interestingly, when animal sex was considered, isoflurane preconditioning (IsoPC) induced different responses between female and male mice following subsequent brain ischemia[48]. Compared to the neuroprotective response to IsoPC in male mice following subsequent middle cerebral artery occlusion (MCAO), an *in vivo* model for ischemic stroke, similarly

treated female mice demonstrated a detrimental response[48] (**Figure 1.1**). Hence, sex as a risk factor in ischemic stroke may not only translate to different outcomes between female and male tissue and cells, but also to different neuroprotective interventions between women and men. In Chapters 2 and 3, I used cortical cells from female and males animals to examine how IsoPC may induce sexually dimorphic cell survival outcomes following a subsequent ischemic insult.

D. Innate Cell Sex as a factor in Cell Death

Despite the importance of sex as a factor in neurodegenerative risk and outcome, as well as in the efficacy of potential neuroprotective strategies, female neuroprotection from stoke in humans can persist before puberty and after menopause[18,19]. Likewise, sexually dimorphic response to IsoPC can still persist when circulating sex steroids are removed through castration, where males lose any response to IsoPC[49], or ovariectomy, where females demonstrate a protective response to IsoPC[50]. These observations suggest that the sexual dimorphism of ischemic stroke risk and outcomes, as well as responses to IsoPC, may in part result from innate differences between female and male brain cells independent of sex steroids.

Indeed, innate cell sex is emerging as an important mediator of cell death and neurodegeneration. Du *et al*.[26] chronicled the most extensive examination of innate cell sex responses following excitotoxic, oxidative, and apoptotic challenges. These authors demonstrated that female and male cells (neuronal and non-neuronal) can differ in their sensitivities to specific challenges as a result of sex-specific cell death mechanisms. Specifically in neurons, female hippocampal and cortical cells were generally protected from oxidative damage and excitotoxicity via reduced cytochrome c and apoptosis-inducing factor (AIF) protein mobilization, and an increase in

intracellular antioxidant glutathione compared to male cells. Exceptions were hydrogen peroxide treatment, where there were no differences between female and male cells, and the intrinsic apoptosis-inducing compound, staurosporine, where female neurons were more sensitive compared to male neurons because of cytochrome c mobilization. Interestingly, 17ß-estradiol (E2) had a detrimental response in female and male neurons following excitotoxic challenge, while E2 offered no protection (if not slight detrimental response in female cells) following nitrosative challenge.

The role of innate cell sex in cell survival outcomes has been demonstrated in other neurodegenerative contexts. Female cortical neurons have an increased resistance to cell death via autophagy in energetic crises because of unique lipid profiles and dynamics[27] and have also demonstrated decreased sensitivity to hypoxic and energetic challenges compared to male neurons[28]. Li *et al.*[51] have reported similar findings in sex-segregated hippocampal slice cultures following OGD – perhaps a more complete model of ischemic stroke compared to hypoxia – and excitotoxicity. Female slices were less sensitive to OGD compared to male slices, whereas neuronal nitric oxide synthase mediated the increased OGD sensitivity in male slices. Preincubation with E2 protected both male and female slices from OGD and eliminated the sexually dimorphic outcome. Furthermore, female slices were also less sensitive to *N*-methyl-*D*-aspartate (NMDA)-induced excitotoxicity. It is important to note, however, that these data resulted from hippocampal slice cultures that did not distinguish the response of particular cell types. Regional differences might be additional factors contributing to sex-specific neuronal cell death mechanisms and outcomes. Contrary to what was shown in cortical neurons (**Figure 3.2**), female cerebellar granular neurons have been shown to be more sensitive to OGD compared to male neurons that was the result of differential cell ATP levels and apoptotic mechansims[52].

Innate cell sex has is an important factor in the outcome of cell types in the brain other than neurons. Astrocytes, a crucial cell type in the mammalian brain facilitating proper CNS function, show distinct differences in cell survival between isolated female and male cells following hydrogen peroxide treatment and OGD[53,54]. Similar to sex-segregated isolated neurons in other cell injury paradigms, isolated female astrocytes have a reduced sensitivity to OGD compared to male astrocytes. This finding, together with those from Du *et al*.[26] and Heyer *et al*.[28], further illuminate the complex dialog between innate cell sex and sex steroids that can influence cell survival outcomes following insults associated with neurodegenerative diseases. However, the role of innate cell sex specifically in neurons following OGD has not been assessed.

I examined innate cell sex in astrocytes and in neurons in Chapters 2 and 3 as a factor that could dictate specific cellular responses to IsoPC and subsequent OGD, as well as a potential factor that may dictate specific cell survival outcomes following OGD independent of IsoPC. These are the first investigations to make such considerations in either astrocytes or neurons.

E. Sex Steroids as Factors in Volatile Anesthetic Preconditioning

Sex steroids are obvious candidates mediating the sexually dimorphic response to IsoPC considering that sex steroids have been linked to a panoply of sexually dimorphic responses to brain injury[19], including responses following ischemic stroke[18,55-57]. Indeed, the major female sex steroid, E2, is largely responsible for the detrimental response to IsoPC and subsequent MCAO in females[50]. This report demonstrated that ovariectomized female mice, which have reduced circulating sex steroids, including E2, show a protective response to IsoPC similar to what has been

observed in male mice (**Figure 1.2**). When E2 was reintroduced to ovariectomized females via subcutaneous implants, the deleterious response to IsoPC and subsequent MCAO returned (**Figure 1.2**). Male mice, which normally show a protective response to IsoPC, have low endogenous levels of E2 compared to females[48]. Yet, the neuroprotective response to IsoPC in males requires circulating androgens[49], the major male sex steroid in mice. This latter finding, together with the findings linking E2 to the detrimental IsoPC response in females, suggests that E2 may act differently in female and male brain to elicit sexually dimorphic responses to IsoPC and subsequent MCAO.

The observation that E2 induces a deleterious response to IsoPC in females is paradoxical to the largely protective role of E2 in numerous animal models of ischemic stroke[18,19,58]. For example, *in vivo* preadministration of E2 for 7 days can protect male and female brain from subsequent ischemic injury in a variety of different animal stroke models[55,56]. Similar protection has also been demonstrated in female and male brain following acute E2 administration before subsequent brain ischemia[59].

In vitro experiments largely substantiate the *in vivo* findings by demonstrating E2 protection from a variety of neurodegenerative-associated insults including oxidative stress[60,61], excitotoxicity[62], and energetic failure[62], among others[58]. However, similar to the *in vivo* reports, current *in vitro* reports are largely biased towards tissues and cells from a single sex or from mixed-sex culture systems, thereby overlooking potentially important sex-dependent mechanisms and outcomes.

Despite some sex bias in E2 investigations, mechanisms describing how E2 protects from ischemic injury, as well as E2's exacerbation of IsoPC in females, have been described. In models of ischemic stroke, E2 has both immediate, non-genomic mechanisms and slower, genomic mechanisms that can each induce protective

responses in brain tissue and cells[18,19]. The more immediate responses include moderate (i.e. beneficial) Ca^{2+} spikes that induce downstream pro-survival processes such as phosphorylation of Src/extracellular signal kinase (ERK) and cyclic-AMP response element binding protein (CREB), Bcl-2 upregulation[63], activation of PKC[64] and protein kinase B (Akt)[65], as well as E2's innate antioxidant properties[58,66]. Delayed neuroprotective responses of E2 largely involve transcriptional effects mediated through E2's cognate nuclear estrogen receptors (ERs), ERα and ERβ, which upregulate numerous cell survival genes, including those coding for brain-derived neurotrophic factor (BDNF) and vascular endothelial growth factor (VEGF)[18,19]. Unfortunately many of these non-genomic and genomic mechanisms have been described without strict regard for animal sex. It remains to be more thoroughly tested if E2 operates via different mechanisms between female and male cells.

The protective response of IsoPC shares some of the mechanisms of preconditioning-independent E2 neuroprotection, most notably phosphorylation of Akt[48,65]. In males with normal sex steroid levels, Akt phosphorylation was increased following IsoPC, while genetic ablation of Akt1 eliminated the protective IsoPC response[48]. Females lacking Akt1, on the other hand, show no changes in their response to IsoPC, or any changes in phosphorylated Akt in wild-type females.

Furthermore, E2-mediated neuroprotection[18,19] and E2's effect on the response to IsoPC[50] involve the nuclear ERs, although the precise neuronal mechanisms of nuclear ERs action in these processes remain inconclusive. The role of the nuclear ERs in E2-mediated ischemic neuroprotection appears to depend on the ischemic model, brain region, and animal sex[19], while the role of the nuclear ERs in E2-mediated responses to IsoPC have only been tested in female brain[50]. And unlike the largely

neuroprotective role of the nuclear ERs in ischemic injury, the role of the nuclear ERs in female brain subjected to IsoPC in the presence of E2 can be deleterious.

Chapter 3 of this dissertation addresses the potential role of E2 as a modulator in the neuronal response to IsoPC and subsequent OGD. These investigations also consider the nuclear ERs as potential downstream mediators of E2's action on the IsoPC response in female neurons.

F. Summary

Starting from the perspective of a specific "risk factor" in neurodegeneration, such as male sensitivity to ischemic brain damage, mediators such as innate cell sex and sex steroids can be identified in this process that may also be important to other neurodegenerative disorders. Perhaps more importantly, potential neuroprotective strategies for a specific neurodegenerative disorder may in themselves have specific efficacies, such as a sexually dimorphic response to isoflurane preconditioning subsequent to brain ischemia where female mice show a deleterious response compared to the protective response in males. In the case of anesthetic preconditioning, further investigation of how volatile anesthetics such as isoflurane affect female and male astrocytes and neurons will not only reveal the utility of specific anesthetic protocols as a neuroprotective strategy in the perioperative setting, but may also advance our understanding of the role of innate cell sex and cell type in the pathology of ischemic stroke and neurodegenerative disorders.

IV. Approach 2: From Mechanism Towards Disease

A. Overview

In contrast to starting with rather upstream, specific risk factors such as sex in ischemic stroke, an alternative approach towards better understanding cell death and identifying potential neuroprotective strategies is to begin with more downstream mediators that may be shared by multiple disorders. Such an approach was applied in Chapter 4. This approach has the advantage of being applicable to a broad spectrum of disorders that ultimately converge at neuronal cell death. The weakness of this approach, however, is that it may result in less effective neuroprotective strategies because convergent points may come late in cellular signaling cascades with multiple upstream parallel cell death pathways[2,6,12]. Regardless, convergent loci in brain cell death remain of interest as potential therapeutic targets in neurodegeneration.

B. The Mitochondrion in Neurodegeneration

In terms of cellular organelles, the mitochondrion serves as one of the most promiscuous convergence points for cell death in the brain. This organelle, in addition to its crucial role providing the human brain with ample adenosine triphosphate via oxidative phosphorylation, is a major convergence point for many modes of cell death[2,3,67].

Perhaps the most characterized cell death mode mediated by the mitochondrion is apoptosis where numerous pro-death and pro-survival proteins compete to portend cell fate. These tightly-regulated processes, mediated largely by the Bcl-2 family of proteins, are crucial towards balancing controlled tissue growth and cellular removal without resulting in cancer or degenerating diseases.

Dysregulation of these and other related proteins favors a degenerative outcome in the CNS given the post-mitotic nature of neurons[2,6]. Necrosis, a less-regulated cell death process compared to apoptosis, also involves action at the mitochondrion and is common – if not synchronous with apoptosis – in neurodegeneration[6,68].

Regardless of specific proteins involved or modes of cell death, the mitochondrion is an important nexus for cellular events mediating many different neurodegenerative conditions. Oxidative damage[2,8,69], excitotoxicity[8], and energetic failure[9,12,69] are some of the most common injurious cellular processes that converge or reside at the mitochondrion and can lead to neurodegeneration. Furthermore, molecules that can mediate neurodegeneration or neuroprotection such as E2 have been linked to mitochondrial integrity and ATP production[70], thereby further illustrating the importance of this organelle as a downstream convergence point mediating cell death processes.

C. Permeability Transition and Cyclophilin D in Neurodegeneration

An additional, albeit less-characterized mechanism of mitochondrial cell death manifests through mitochondrial permeability transition. Permeability transition can be described as a sudden, non-selective increase in inner mitochondrial membrane permeability to all solutes less the 1.5 kilodaltons[14] (**Figure 1.3**). Although ephemeral, stochastic permeability transition is thought to mediate homeostatic regulation[71,72], sustained permeability transition leads to cell death[14,73]. Sustained permeability transition induces mitochondrial depolarization and the inhibition of oxidative phosphorylation, increased production of reactive oxygen species, the release of cytochrome c and subsequent initiation of apoptosis, and the release of sequestered mitochondrial matrix Ca^{2+} into the cytosol. All of these phenomena conspire to

uncontrollably and irreversibly dysregulate cellular homeostasis that ultimately kills the cell.

Permeability transition (at least *ex vivo*) is promoted by excessive Ca^{2+} sequestering into the mitochondrial matrix via the Ca^{2+} uniporter, free inorganic phosphate, fatty acids, and reactive oxygen species (which potentially initiates a positive feedback cycle since reactive oxygen species are also products of permeability transition). Despite the known promoters and consequences of permeability transition, the molecular components mediating permeability transition remain unclear. Dogma has traditionally speculated that protein complexes form a "pore" in the inner and possibly outer mitochondrial membranes that results in permeability transition. Although there is not direct evidence for a proteinacious pore, there are positive regulators of permeability transition, which if genetically or pharmacologically inactivated increase the threshold required to induce permeability transition. However, the threshold to induce permeability transition can still be reached independent of these positive regulators[14], leaving the molecular identification of a proteinaceous permeability transition "pore" elusive[74,75].

The outer mitochondrial membrane proteins, voltage-dependent anion channel and the peripheral benzodiazapine receptor, the inner mitochondrial membrane proteins, adenine nucleotide translocator, and the mitochondrial matrix protein, cyclophilin D (CyD), represent the best characterized positive regulators of permeability transition[75]. Of these proteins, CyD shows the most promise as a therapeutic target in that its inhibition does not obstruct any known physiologic functions important for cellular homeostasis, and that there is an expanding spectrum of CyD inhibitors that inhibit permeability transition[74,76], prevent cell death[74,77], and mitigate neurodegeneration[14].

CyD is a peptidyl-prolyl *cis-trans* isomerase protein found exclusively in the mitochondrial matrix. The only known function of CyD is to regulate mitochondrial permeability transition, although other functions are likely given the multiple cyclophilin isoforms in mammals and conserved cyclophilin isomerase activity throughout evolution[78]. Furthermore, mice lacking the CyD gene (*Ppif*) develop behavioral abnormalities and early-onset obesity[79], suggesting that CyD has other direct or indirect roles that may not be related to cell death.

The role of CyD in permeability transition began with the discovery that the fungal-derived immunosuppressant, cyclosporin A, inhibited permeability transition in isolated heart mitochondria[80]. It was later discovered in isolated liver mitochondria that cyclosporin A inhibited permeability transition by binding to CyD[81]. Immediate therapeutic applications for cyclosporine A were tempered, however, because cyclosporine A also binds calcineurin, a functionally unrelated peptidyl-prolyl *cis-trans* isomerase important for activating T cells in immune responses.

Consequently, CyD knockout (KO) mice[16,82-84] have provided a clearer picture of the role of CyD in cell death and disease[14], including neurodegeneration[15,16,44], and have reinforced the perspective that the mitochondrion – and more specifically CyD – represents an important convergence point mediating cell death. CyD KO mice demonstrate robust neuroprotection in models of ischemic stroke[16], multiple sclerosis[15], and more recently Alzheimer's disease[44]. Moreover, CyD has been linked to many cellular processes associated with neurodegeneration such as oxidative damage[15,16,44,82,84], excitotoxicity[85,86], and energetic failure[77].

Despite these observations, the role of CyD in neurons and other brain cells has not been tested independent of non-selective pharmacologic inhibition, which, in addition to CyD, can also inhibit proteins such as calcineurin[14,74]. Using isolated brain

cells from CyD KO animals to determine the response following cellular insults associated with neurodegeneration would advance our understanding of how CyD mediates cell death in the brain, as well as further assess the potential of CyD inhibitors as a neuroprotective strategy. In Chapter 4, I isolated cortical cells from mice genetically lacking CyD to more specifically test how CyD mediates cell survival outcomes in neurons and astrocytes following cellular insults associated with neurodegeneration.

D. Summary

Approaching neurodegeneration from a more downstream, convergent perspective in brain cell death, such as CyD and mitochondrial permeability transition, has the advantage of unveiling mechanisms that may apply to multiple neurodegenerative disorders because cell death processes themselves often converge at specific organelles and proteins regardless of the specific disorder. Such a perspective, however, has the disadvantage of missing potential upstream environmental and/or genetic neurodegenerative initiators, whereby parallel cell death signaling cascades may be activated that render a single downstream therapeutic target – such as CyD – insufficient to prevent cell death.

Nonetheless, the development of CyD-specific pharmacology continues to progress as a potential avenue towards preventing neurodegeneration[76,77]. However, many details regarding the role of CyD in specific brain cell types following specific cellular insults must first be described. Addressing our gap in knowledge about the specific role of CyD in neurons and astrocytes following cellular insults specifically associated with neurodegeneration is essential before targeting CyD can become a promising neuroprotective strategy. Chapter 4 helps overcome this gap in knowledge

by characterizing cell survival outcomes in isolated CyD KO neurons and astrocytes following oxidant, excitotoxic, and energetic challenges.

V. The Importance of Astrocytes in Neurodegeneration

Neurodegeneration has historically been described in the context of neurons. This is expected because neurons provide the computational and functional output of the CNS through synaptic contacts, and because neurons are post-mitotic rendering neuronal degeneration as irreversible. However, it is becoming increasingly appreciated that neuroglia are crucial for proper CNS function[87,88]. These cells include oligodendrocytes which myelinate CNS axons, microglia which facilitate immune and developmental function, and astrocytes which coordinate multiple functions, from neurotransmitter uptake and synthesis to synaptogenesis and blood flow regulation. Indeed, astrocytes comprise the majority of brain cells in rodents and humans and are becoming increasingly appreciated for their role in not only CNS function, but also CNS dysfunction[88]. Astrocytes are indispensable for proper neuronal function and overall CNS physiology and have been linked to nearly every neurodegenerative disorder, from Parkinson's and Alzheimer's disease[88], to multiple sclerosis[88,89] and ischemic stroke[90,91]. The precise roles of astrocytes in mediating neurodegeneration, however, are not well understood.

Similar to neurons, astrocytes have a sexually dimorphic sensitivity in models of ischemic stroke, with male astrocytes being more sensitive than female cells to OGD[53,54]. Although massive, irreversible neuronal death occurs in the severely compromised core of ischemic brain, astrocyte integrity may have an important role in the neuronal outcome in the peri-infarcted brain region or "penumbra" following

ischemic stroke[90]. Therefore, a better understanding of how astrocytes may respond to potential neuroprotective strategies, such as IsoPC, is equally important as understanding how neurons may respond if a therapy is to be effective among the heterogeneous cell population of the mammalian brain[87].

Astrocytes have also been largely overlooked in the context of neurodegenerative convergence points such as the mitochondrion. The role of CyD and permeability transition in neurodegeneration has gained increased attention in recent years[3,15,75], yet most reports using genetic ablation of CyD have focused on whole brain tissue. More recently, genetic ablation of CyD has shown that astrocytic Ca^{2+} dynamics indirectly mediate neuronal excitotoxicity[92]. However, it remains to be determined if CyD can mediate astrocyte survival following an insult associated with neurodegeneration. Assessing the role of CyD in astrocyte survival would advance our understanding of CyD-mediated neurodegeneration in the brain, as well as better characterize the potential of pharmacologically targeting CyD to preserve brain tissue.

Astrocytes are considered in this dissertation as potential contributors to neurodegenerative and neuroprotective processes, both in the context of IsoPC and subsequent OGD (Chapters 2 and 3) and in CyD KO cells following oxidative injury (Chapter 4).

VI. Thesis Objectives and Hypotheses

Taking an approach from both a known specific risk factor in neurodegeneration, such as female sex in ischemic stroke, and from a known general nexus in multiple neurodegenerative disorders such as CyD, the complexities of neurodegenerative processes may become more tractable towards identifying

potential neuroprotective strategies. Moreover, extending each experimental approach to include cells other than neurons, such as astrocytes, provides a more thorough assessment of neurodegenerative and neuroprotective mechanisms among the heterogeneous cell population of the mammalian cortex.

The action of sex steroids in the brain remains incomplete to explain the sexually dimorphic response to IsoPC and subsequent brain ischemia where females experience exacerbated injury compared to the protective response in males[43,47-50]. Hence, this thesis uses isolated cortical brain cells to investigate the overarching hypothesis that the innate cell sex of astrocytes (Chapter 2) and neurons (Chapter 3) contributes to the observed sexually dimorphic response to IsoPC *in vivo*. Chapter 2 focuses on astrocytes to investigate the specific hypothesis that IsoPC protects male astrocytes while having no effect or even a deleterious effect in female astrocytes following subsequent OGD. Chapter 3 investigates a similar hypothesis in neurons, and, based upon the results of the initial investigations in Chapter 3, also investigates the hypothesis that E2 attenuates any protective effect from IsoPC in neurons via nuclear ERs. Ancillary to the primary hypotheses, the experimental design of Chapters 2 and 3 allow additional assessments of how innate cell sex may dictate the survival outcome in astrocytes and neurons following OGD independent of IsoPC.

Chapter 4, on the other hand, expands current evidence that CyD mediates neurodegeneration *in vivo*[74] by investigating the hypothesis that CyD mediates cell death specifically in neurons and astrocytes following insults associated with neurodegeneration, such as oxidative damage, excitotoxicity, and energetic failure.

Figure 1.1 | Isoflurane preconditioning (IsoPC) protects young male (YM) brain from middle cerebral artery occlusion, a mouse model for ischemic stroke, compared to the detrimental response in young female (YF) brain[48]. Sham PC, sham preconditioning. This figure has been modified from Kitano *et al.*, J Cereb Blood Flow Metab **27**(7): 1377-1386 (2007).

Figure 1.2 | Estradiol mediates the detrimental response to isoflurane preconditioning (IsoPC) in female brain following middle cerebral artery occlusion[50]. OVX, ovariectomized; Sham PC, sham preconditioning. This figure has been modified from Wang *et al.*, J Cereb Blood Flow Metab **28**(11): 1824-1834 (2008).

Figure 1.3 | Hypothetical schematic of mitochondrial permeability transition (mPT) in intact neurons. CyD is a positive regulator of mPT in that its absence bestows resistance to mPT in isolated mitochondria. Reactive oxygen species (ROS), excessive glutamate, and oxygen and glucose deprivation (OGD) induce mPT *in vitro*, which likely contributes to neurodegeneration *in vivo*.

Figure 1.1

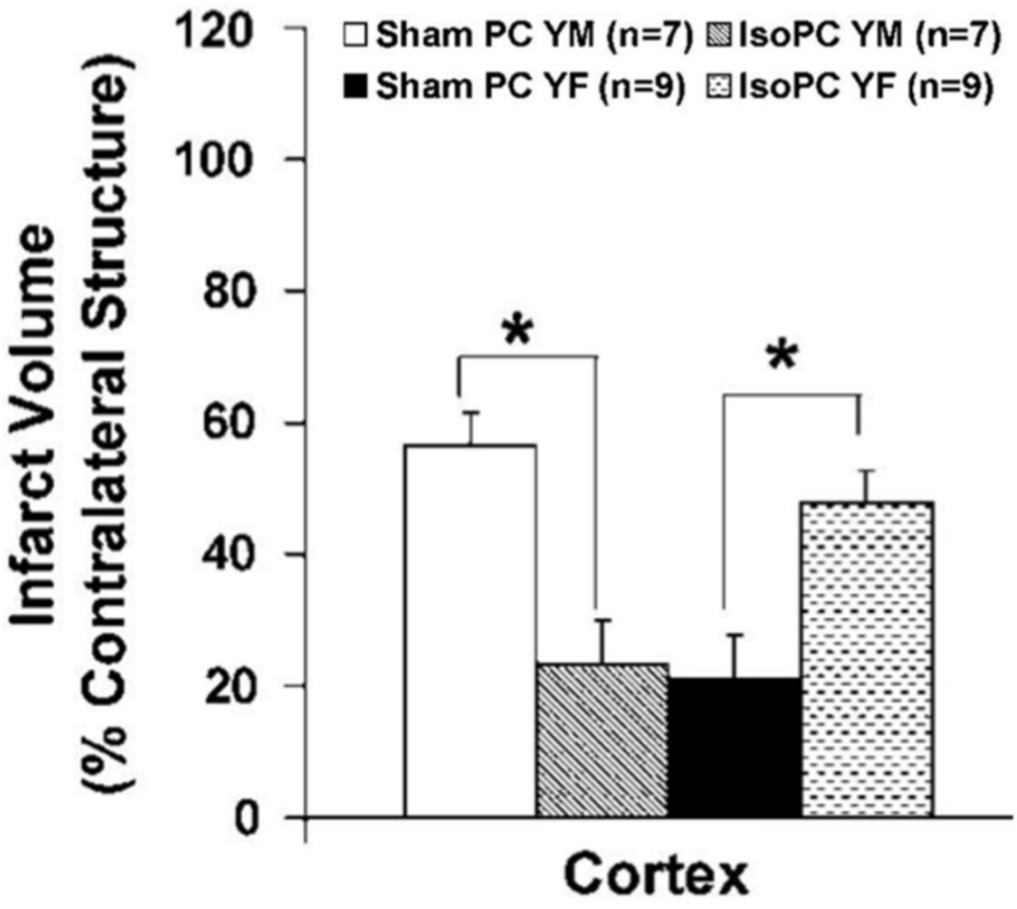

Sham PC YM (n=7)
IsoPC YM (n=7)
Sham PC YF (n=9)
IsoPC YF (n=9)
Infarct Volume
(% Contralateral Structure)
120
100
80
60
40
20
0
*
*
Cortex

Figure 1.2

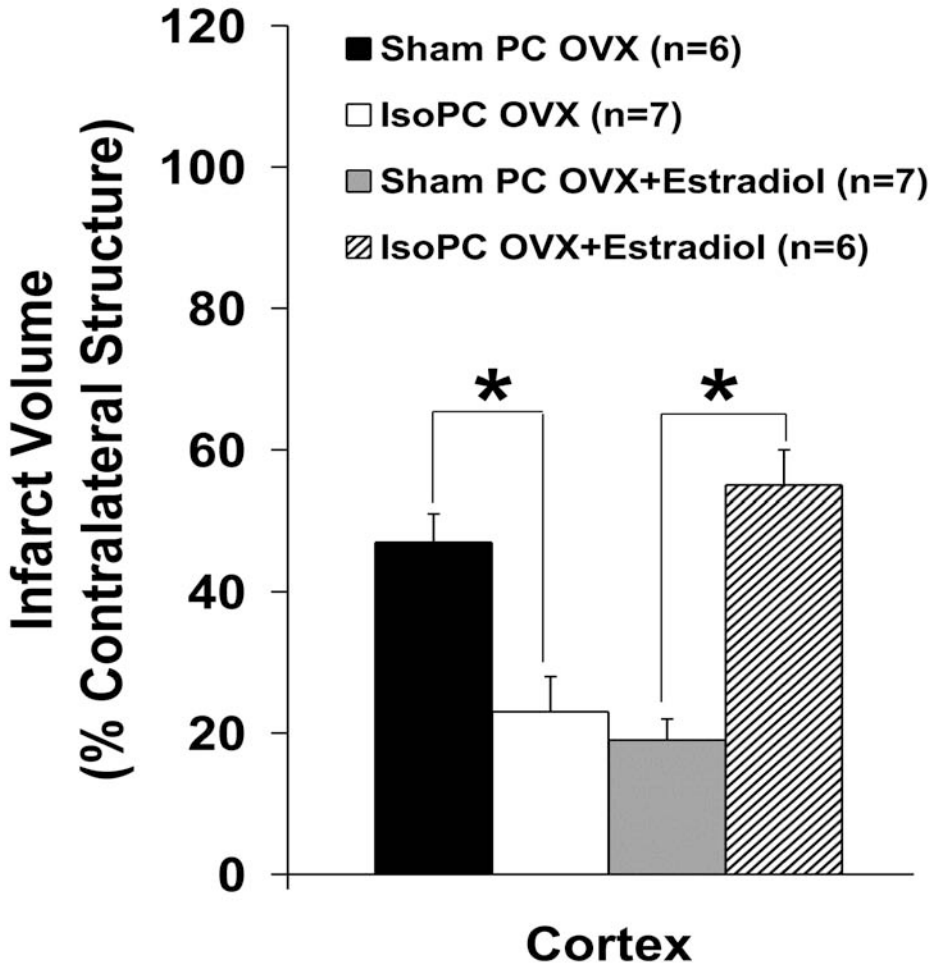
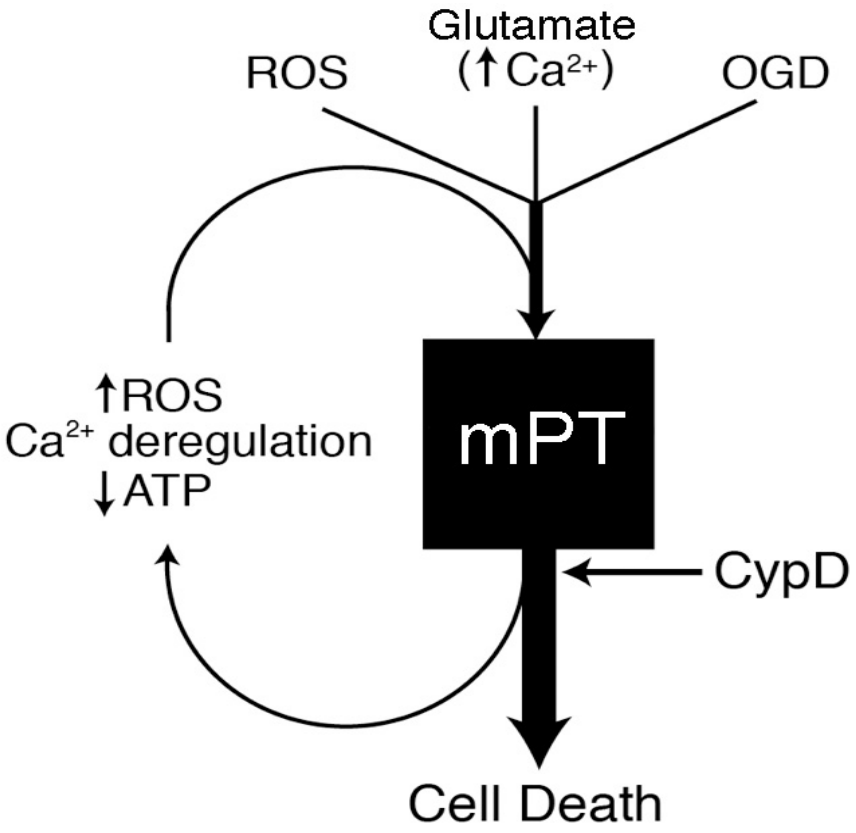

120
100
80
60
40
20
0
Infarct Volume
(% Contralateral Structure)
Sham PC OVX (n=6)
IsoPC OVX (n=7)
Sham PC OVX+Estradiol (n=7)
IsoPC OVX+Estradiol (n=6)
*
*
Cortex

Figure 1.3


Glutamate
(↑Ca2+)
ROS
OGD
↑ROS
Ca2+ deregulation
↓ATP
mPT
CypD
Cell Death

Chapter 2 | Isoflurane Preconditioning Protects Astrocytes from Oxygen and Glucose Deprivation Independent of Innate Cell Sex

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Published: Johnsen D., Murphy SJ. 2011, J Neurosurg Anesthesiol *(in press)*

Abstract

Isoflurane exposure can protect the mammalian brain from subsequent insults like ischemic stroke. However, this protective preconditioning effect is sexually dimorphic, with isoflurane preconditioning decreasing male while exacerbating female brain damage in a mouse model of cerebral ischemia. Emerging evidence suggests that innate cell sex is an important factor in cell death, with brain cells having sex-specific sensitivities to different insults. We used an in vitro model of isoflurane preconditioning and ischemia to test the hypothesis that isoflurane preconditioning protects male astrocytes while having no effect or even a deleterious effect in female astrocytes following subsequent oxygen and glucose deprivation (OGD). Sex-segregated astrocyte cultures derived from postnatal day 0 to 1 mice were allowed to reach confluency before being exposed to either 0% (sham preconditioning) or 3% isoflurane preconditioning for 2 hours. Cultures were then returned to normal growth conditions for 22 hours before undergoing 10 hours of OGD. Twenty-four hours following OGD, cell viability was quantified using a lactate dehydrogenase assay. Isoflurane preconditioning increased cell survival following OGD compared to sham preconditioning independent of innate cell sex. More studies are needed to determine how cell type and cell sex may impact on anesthetic preconditioning and subsequent ischemic outcomes in the brain.

Exposing the brain to volatile anesthetics like isoflurane can induce tolerance, or "precondition" the brain from subsequent injurious insults such as ischemic stroke[43,47]. Protection from isoflurane preconditioning and subsequent ischemia has been shown to be male-specific, with female mice showing exacerbated injury following an identical treatment paradigm[48]. This sexually dimorphic response to isoflurane preconditioning is partially mediated by circulating sex steroids[49,50]; however, it remains possible that innate cell sex in the brain is an additional contributing factor. Indeed, innate cell sex is emerging as an important factor in brain cell death, with neurons and astrocytes exhibiting sex-specific cell death mechanisms and outcomes following exposure to a variety of noxious insults[26-28], including oxygen-glucose deprivation (OGD), an in vitro model of ischemic stroke[53,54].

Moreover, most reports investigating anesthetic preconditioning at the cellular level in the brain have been limited to mixed-sex primary neuronal cultures[93-97], leaving the role of astrocytes unexplored. Astrocytes comprise the majority of brain cells in mammals and are crucial in central nervous system function[87,98]. They also have roles facilitating the neurodegeneration that results from insults like ischemic stroke[87,90,91]. Furthermore, astrocytes have also been shown to respond to anesthetics, including isoflurane, by modifying glutamate uptake[99] and gap junction permeability[100]. However, it remains unknown if preconditioning by a volatile anesthetic can affect astrocyte cell death outcomes following OGD, and if male and female astrocytes respond differently. In the current investigation, we used an effective *in vitro* model of isoflurane preconditioning and ischemia in astrocytes to test the hypothesis that isoflurane preconditioning protects male astrocytes while

having no effect or even a deleterious effect in female astrocytes following subsequent OGD.

Material and Methods

Establishing sex-segregated cortical astrocyte cultures

Animal procedures were conducted in compliance with the National Institutes of Health guidelines for the care and use of animals in research, and experimental protocols were approved by the Institutional Animal Care and Use Committee.

Sex-segregated astrocyte cultures were established and maintained as previously described[54] (**Appendix Figure A2.1**) with the addition of a rinsing step to help remove non-adherent cells (described below). Male and female C57BL/6 (Charles River, Wilmington, MA) mouse pups (postnatal day 0 to 1) were segregated by sex based upon a larger genital papilla and longer ano-genital distance in males versus females. To confirm the sex-segregation technique for the experiments conducted here, tail tissue from randomly-selected male and female postnatal mouse pups was collected for polymerase chain reaction (PCR) analysis. Genomic DNA was extracted and prepared for PCR analysis using the DNeasy kit according to the manufacturer's instructions (Qiagen, Venlo, Netherlands). Two PCR reactions were conducted contemporaneously using the male-specific Y chromosome gene, *Sry*, to identify male animals and the gene for myogenin (*Myog*) as an autosomal control in both males and females[101]. Validation of sex assignment was confirmed using the following primers: *Sry*, 5'–TCATGAGACTGCCAACCACAG–3' and 5'–CATGACCACCACCACCACCAA–3'; *Myog*, 5'–TTACGTCCATCGTGGACAGC–3' and 5'–TGGGCTGGGTGTTAGTCTTA–3'. The expected sizes of the PCR products are 441 base pairs for *Sry* (male) and 245 base pairs for *Myog*

(males and females). The PCR reactions were carried out with one 72°C period for 2 minutes, 30 cycles (94°C for 5 sec, 65°C for 30 sec, 72°C for 30 sec), and one 72°C period for 7 minutes. **Figure 2.1** shows representative PCR products from six randomly-selected male and female pups. Additional PCR rounds from other litters were conducted with 100% accuracy for male and female segregation.

Using the techniques described above, male and female pups from the same litter were segregated by sex and the brains from each sex were pooled to establish male or female cortical astrocyte cultures. These littermate cultures followed an identical, concurrent paradigm throughout the course of subsequent treatments to represent a given n = 1, where the sex-dependent and preconditioning-dependent paired analysis compared means from the same litter (see *Statistics*). Cortices were dissected in an ice-cold Hanks Balanced Salt Solution buffer followed by dissociation in 0.125% trypsin for 10 min at 37°C. The trypsin reaction was stopped with the addition of 0.04 mg/ml of soybean trypsin inhibitor and 0.04 mg/ml of DNase was added to prevent clumping. The cells were washed twice in growth media (Dulbecco's Modified Eagle's Medium without phenol red), supplemented with 10% fetal bovine serum, 2 mM glutamine, and 100 units of penicillin and streptomycin to reduce the probability of bacterial contamination. Cells were then plated at a density of 1.5×10^5 cells/cm^2. The cells were allowed to settle overnight before being gently swirled and rinsed with fresh media to remove non-adherent, spherical, phase bright cells that settled on top of the astrocyte cell layer, thereby contributing to a more morphologically homogenous astrocyte culture. Cultures consisted of approximately 90-98% astrocytes as determined by immunostaining with antibodies specific for the astrocyte marker glial fibrillary acidic protein and with the nuclear stain 4',6-diamidino-2-phenylindole ("DAPI") to label all cells (**Appendix Figure A2.1**).

Male and female cells from the same litter were plated into separate rows across each of 3 different culture plates (24-well plates, 4 to 6 well replicates per row) with one row having male cells and another row having littermate female cells. Each experiment required 3 culture plates subjected to isoflurane preconditioning without OGD, sham preconditioning + OGD, and isoflurane preconditioning + OGD. The astrocytes were maintained in a humidified incubator at 37°C and 5% CO_2 until confluent, usually 10 to 14 days *in vitro*.

Cell culture reagents, including Hank's Balanced Salt Solution, Dulbecco's Modified Eagle's Medium, fetal bovine serum, trypsin, soybean trypsin inhibitor, glutamine, and streptomycin and penicillin are from Life Technologies (Invitrogen), Carlsbad, CA.

In vitro isoflurane preconditioning of astrocytes

Instead of dissolving liquid isoflurane into solution, which can result in highly variable anesthetic concentrations through rapid evaporation[102,103], we modified previously reported designs[95,100,104] for exposing culturing cells to a volatile anesthetic for application to astrocytes. Similar designs have been reported for exposing cultured cells to a volatile anesthetic[97,99,104], although our system and treatment paradigm (described below) is the first to modify such designs to apply to astrocytes. Two air-tight, plastic chambers (Billups-Rothenberg, Del Mar, CA) were filled with medical air and 5% CO_2, with or without 3% isoflurane (**Figure 2.2A**). For isoflurane preconditioning, astrocytes were exposed to 3% isoflurane in one chamber, while sham preconditioned astrocytes were exposed to 0% isoflurane in the other chamber. An anesthetic monitor (POET II, Criticare Systems, Waukesha, WI) was attached to the outflow of the isoflurane preconditioning chamber to determine when the isoflurane

concentration had reached 3% in the chamber. While we did not measure the amount of isoflurane dissolved in the culture media, a 3% gas volume is within the range commonly used for *in vitro* volatile anesthetic studies[96,99,104-107] and has been shown to translate to clinically-relevant concentrations (upper micromolar to lower millimolar)[94,108]. The inflow and outflow ducts were then clamped and each chamber was placed in a standard 37°C incubator during the 2 hour preconditioning period. Following the 2 hour preconditioning period, the culture plates were removed from the chambers and returned to normal growth conditions for 22 hours before OGD. The treatment paradigm is illustrated in **Figure 2.2B**. The 3% isoflurane concentration, 2 hour preconditioning period, and 24 hour post-preconditioning period were chosen based upon similar models demonstrating volatile anesthetic-induced protection in isolated brain cells[97,99,104].

OGD and cell death assessment

OGD and cell death quantification was carried out similarly as described[54]. Astrocytes were rinsed twice in DMEM without glucose and supplements and placed in a chamber (Coy Laboratory Products, Grass Lake, MI) deprived of oxygen (< 0 ppm) at 37 °C for 10 hours. Cells were then returned to normal growth conditions with normal growth media for 24 hours before cell death assessment. Cell death was determined indirectly from dead or damaged astrocytes by quantifying lactate dehydrogenase (LDH) released into the culture media. The LDH assay was conducted according to the manufacture's instructions (Roche, Basel Switzerland). Four to 6 replicate wells from each row, per sex, per preconditioning treatment were averaged to generate the percentage of LDH release that experiment. Two replicate wells in the control group (isoflurane preconditioning without OGD) were treated with 0.01% Triton X-100 to

determine a maximum "100% LDH Release" value. LDH release was quantified using a Victor-3 plate reader (PerkinElmer, Waltham, MA).

Statistics

Data are expressed as mean ± standard error of the mean (SEM). Two-way ANOVA of the difference of paired means (i.e. "repeated measures") was used for paired littermate analysis, comparing the factors of "preconditioning group" and "sex". This analysis controlled for variability in absolute cell death values among cultures. The ANOVA compared innate cell sex-independent outcomes (pooled male and female cell death outcomes) and preconditioning-independent outcomes (pooled sham and isoflurane preconditioned cell death outcomes), followed by the Student Newman-Keuls post-hoc test to analyze differences between and within preconditioning and innate cell sex groups. Each culture from a given litter represented an n = 1; there was a total of 11 paired male and female cultures. *p < 0.05 was considered significant. Statistical analyses were conducted using SigmaStat [version 2.0] software (SPSS Inc., Chicago, IL).

Results

In male astrocytes, isoflurane preconditioning resulted in 32.9 ± 6.2% LDH release vs. 46.0 ± 6.6% LDH release in the sham preconditioned group (**Figure 2.3A**, *p < 0.05). In female astrocytes, isoflurane preconditioning resulted in 29.9 ± 3.6% LDH release vs. 44.2 ± 5.8% in the sham preconditioned group (**Figure 2.3A**, *p < 0.01). However, there was no significant difference between the 32.9% LDH release in the isoflurane preconditioned male astrocytes and the 29.9% LDH release in the isoflurane preconditioned female astrocytes, nor was there a significant difference between the

46.0% LDH release in sham preconditioned male astrocytes and the 44.2% LDH release in sham preconditioned female astrocytes. Within each sex, isoflurane preconditioning reduced OGD-induced cell death as assessed by LDH release from damaged or dead cells similarly in male and female astrocytes (**Figure 2.3A**, male, 13.0 ± 4.6% vs. female, 14.3 ± 5.2%, p = 0.86 for statistical interaction between preconditioning treatment and innate cell sex).

The cell sex-independent analysis (pooled male and female cell death outcomes) showed that isoflurane preconditioning reduced LDH release 13.7 ± 3.4% compared to sham preconditioning regardless of innate cell sex (**Figure 2.3B**, sham preconditioning, 45.1 ± 5.5% vs. isoflurane preconditioning, 31.4 ± 4.5%; *p < 0.01). In contrast to the sex-independent reduction in cell death in isoflurane preconditioned astrocytes, the differences in LDH release as determined by the preconditioning-independent analysis (pooling the outcomes of the sham and isoflurane preconditioning groups) revealed no difference between male and female astrocytes (**Figure 2.3C**, male 39.4 ± 6.6% vs. female 37.0 ± 5.0%; n=11, p = 0.52).

Discussion

Using an *in vitro* model of isoflurane preconditioning and ischemia in isolated, sex-segregated primary astrocytes, we demonstrated two important findings: (1) isoflurane protects isolated astrocytes from OGD-induced cell death independent of innate cell sex, and (2) male and female astrocytes have similar cell death profiles following OGD independent of preconditioning treatment.

Previous reports investigating *in vitro* anesthetic preconditioning in brain cells have been limited to isolated neuron or glia/neuron mixed cultures which were not

stratified by cell sex. In addition to using sex-segregated cultures, this report is the first to design an *in vitro* experimental paradigm to precondition isolated astrocytes with a volatile anesthetic. Since astrocytes are emerging as important contributors to the pathology of brain ischemia and subsequent brain degeneration[87,90,91], it is likely that astrocytes also play a role in processes that protect the brain, including those elicited by anesthetics and other forms of preconditioning[109]. Hence, characterizing isoflurane and other forms of volatile anesthetic preconditioning in isolated astrocytes will provide a more complete assessment of the beneficial potential of anesthetics in the brain.

It is not surprising that astrocytes, like most cell types in the brain and other organs, are protected from an insult such as OGD if the cells are pre-exposed to a volatile anesthetic. For example, the protective preconditioning effect of volatile anesthetics has been demonstrated in neurons[93-97], cardiac cells[110,111], epithelial cells[112], and kidney cells[113]. Until now, however, no reports had considered innate cell sex in the response to anesthetics despite the sex differences reported in isoflurane preconditioning in vivo[48-50]. Although isoflurane exposure itself has been shown to be neurotoxic in some experimental paradigms, the cytoprotective properties of isoflurane preconditioning in vivo and in vitro have typically been associated with lower isoflurane doses and shorter exposure times similar to what was used in the current study[35,43]. For example, in vitro preconditioning with 1.2% or 2.4% isoflurane for 60 minutes was observed to be protective against the toxic consequences of continuous exposure to 2.4% isoflurane for 24 hours in rat primary cortical neuronal cultures[35]. Furthermore, in this study, paired male and female control groups consisting of astrocytes exposed to isoflurane preconditioning alone with no OGD had less than 0.5% LDH release compared to male and female Triton X-100-treated

controls. All of these observations would suggest that any intrinsic toxic effect of isoflurane exposure alone under the conditions utilized in this study did not likely contribute to the lack of innate cell sex differences observed in the response of astrocytes to isoflurane preconditioning and OGD.

Interestingly, the results presented here also show that cell death following OGD is similar between male and female astrocytes regardless of preconditioning treatment. This suggests that the *in vivo* sexual dimorphism resulting from isoflurane preconditioning, where females sustain *exacerbated* brain injury following isoflurane preconditioning and subsequent ischemia compared to the protection seen in males[48], is not likely a direct function of astrocyte cell sex and may instead be the result of neuron cell sex, multiple cell type interactions, and/or other physiologic factors in the brain such as circulating sex steroids. Indeed, differences in cell death mechanisms and outcomes have been observed between isolated male and female neurons[26-28,114], but survival outcomes following an OGD insult were not tested in these studies.

In contrast to our findings, other studies have shown sex differences in cell death outcomes following OGD in isolated male and female astrocytes derived from rats and mouse strains different from the strains used here[53,54]. These studies, however, may not have removed non-adherent, mostly non-astrocytic cells following initial plating as was thoroughly done in this study, which could affect astrocyte purity and alter cell death outcomes. The presence or absence of phenol red, serum and other estrogen-like or estrogen containing media components in astrocyte or other types of cell culture that can act on sex steroid receptors and affect cell survival[115] may also explain differences in cell sex-specific outcomes between our study and others. However, this is unlikely as we used similar media free of serum and phenol red free during OGD as was done in other studies in which sex differences were

observed[53,54]. It is also possible that the lack of sex differences observed in this study may be due to limitations in the measures used to detect cell death. In this study, LDH release was the only method used for evaluation of cell death outcomes. Our findings would be further strengthened had we observed similar outcomes using more than one method for detection of cell outcomes such as propidium iodide for cell death or calcein AM for cell survival. Regardless, the increasing number of reports demonstrating that differences in cell death mechanisms and outcomes can exist between isolated male and female cells is in itself a strong argument to consider innate cell sex when designing and interpreting *in vitro* data from primary cell systems.

Independent of preconditioning, the overall action of volatile anesthetics on astrocytes remains grossly understudied compared to neurons. Astrocytes dramatically influence synaptic transmission by regulating the extracellular milieu within the synaptic cleft[87,98], thereby altering neuronal function. A small body of evidence suggests that volatile anesthetics can alter glutamate uptake and ionic stasis in astrocytes[99,100,116] to an even greater extent than in neurons under some circumstances[99]. Furthermore, we demonstrated in this study that isoflurane can alter the cell death outcome in male and female astrocytes. Hence, further characterization of the impact of volatile anesthetics like isoflurane on astrocytes as well as on neurons is imperative towards understanding the complicated, yet potentially beneficial effects of volatile anesthetics in the brain.

Finally, this report employed a two-way difference of paired means ("repeated measures") ANOVA and post-hoc test as a statistical approach to normalize inherent variability in absolute cell death among cultures (i.e. between different litters). This approach differs from most reports which compare absolute cell death or survival

values. While comparing absolute values is effective in low-variable settings, absolute cell death in primary cell cultures can be highly variable despite rigid procedural uniformity (as evident in **Figures 2.3B** and **C**). Therefore, analyzing the differences in paired cell death means within each culture internally controls for variability in absolute cell death, thus allowing for better resolution of differences when comparing across multiple cultures. Employing this type of analysis may illuminate potentially important, albeit otherwise overlooked data trends in investigations that take an *in vitro* approach to neurodegenerative research.

To our knowledge, we are one of the first laboratories to investigate anesthetic preconditioning in isolated astrocytes and to examine the role of innate cell sex in anesthetic preconditioning. We demonstrated that isoflurane preconditioning protects isolated cortical astrocytes from subsequent OGD. This protection is, however, independent of innate cell sex. These findings together with our previous report provide a more complete understanding of how cell type and cell sex may influence anesthetic preconditioning in the brain, and thereby how anesthetic choice may reduce ischemic brain damage in men and women at risk for perioperative stroke during cardiovascular surgeries.

Acknowledgements

The authors thank Rochelle Fu, Ph.D., Public Health & Preventative Medicine Department, Oregon Health & Science University, Portland, Oregon, for her assistance with statistical analyses. This work was supported by grants from NIH/NINDS (NS054684 - SJM) and the Pacific Mountain Affiliate of the American Heart Association (Predoctoral Fellowship – DJ).

Figure 2.1 | Representative PCR products verifying sex segregation of postnatal day 0 to 1 mouse pups. Tail tissue from 6 randomly-selected female and male pups was digested and assayed for the presence of the male-specific Y chromosome gene, *Sry*, and the autosomal gene, myogenin (*Myog*), as a positive control. Primers without DNA, and DNA without primers, were used as negative control reactions. The asterisks indicate the 500 bp marker. The expected *Sry* and *Myog* products are at 441 and 245 bps, respectively.

Figure 2.2 | Model for *in vitro* volatile anesthetic preconditioning of astrocytes. **(A)** Apparatus for *in vitro* anesthetic preconditioning: (a) anesthetic monitor, (b) medical air input, (c) gas line split with flow meters, (d) isoflurane vaporizer, (e) sham preconditioning (ShamPC) and isoflurane preconditioning (IsoPC) chambers, (f) charcoal filter for IsoPC chamber exhaust, (g) a gas line connection. Once the ShamPC and IsoPC chambers were filled with medical air and 5% CO_2, with or without 3% isoflurane, these chambers were then clamped and disconnected from the air inputs and exhausts and placed into a standard 37°C incubator (not shown) during the 2 hour preconditioning period. (**B**) Schematic of the preconditioning treatment paradigm ("OGD," oxygen and glucose deprivation).

Figure 2.3 | Isoflurane preconditioning (IsoPC) reduced cell death as assessed by lactate dehydrogenase (LDH) release from damaged or dead cells in astrocytes independent of innate cell sex. **(A)** Mean LDH release from sham preconditioning (ShamPC) and IsoPC-treated male and female astrocytes (*$p < 0.05$ ShamPC vs. IsoPC in

male cells, *p < 0.01 ShamPC vs. IsoPC in female cells; p = 0.86 for statistical interaction between preconditioning treatment and innate cell sex, n = 11). **(B)** Sex-independent paired difference analysis of LDH release between ShamPC and IsoPC groups. Each line connects cell death outcomes from the same litter (n = 1); * p < 0.01, n = 11. **(C)** Preconditioning-independent paired difference analysis of LDH release outcomes between male and female astrocytes. Each line connects the LDH release outcome from the same litter (n = 1); p = 0.52, n = 11.

Figure 2.1

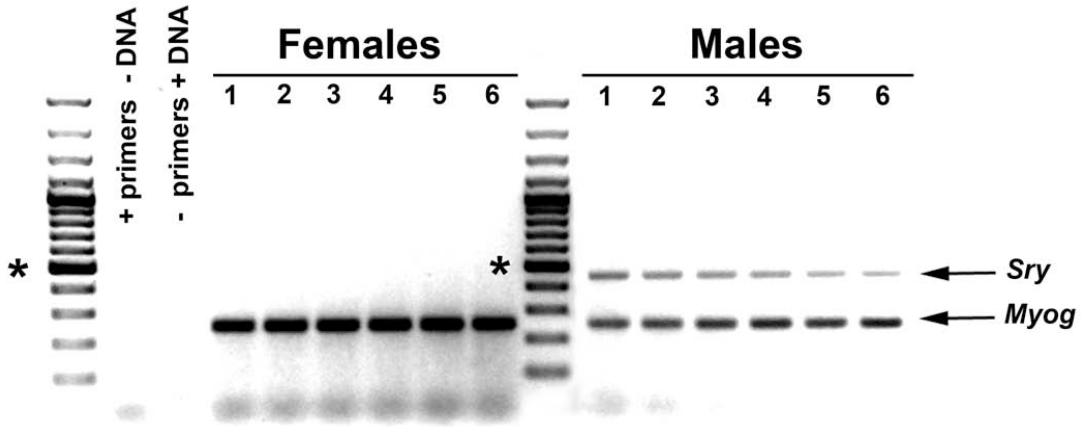
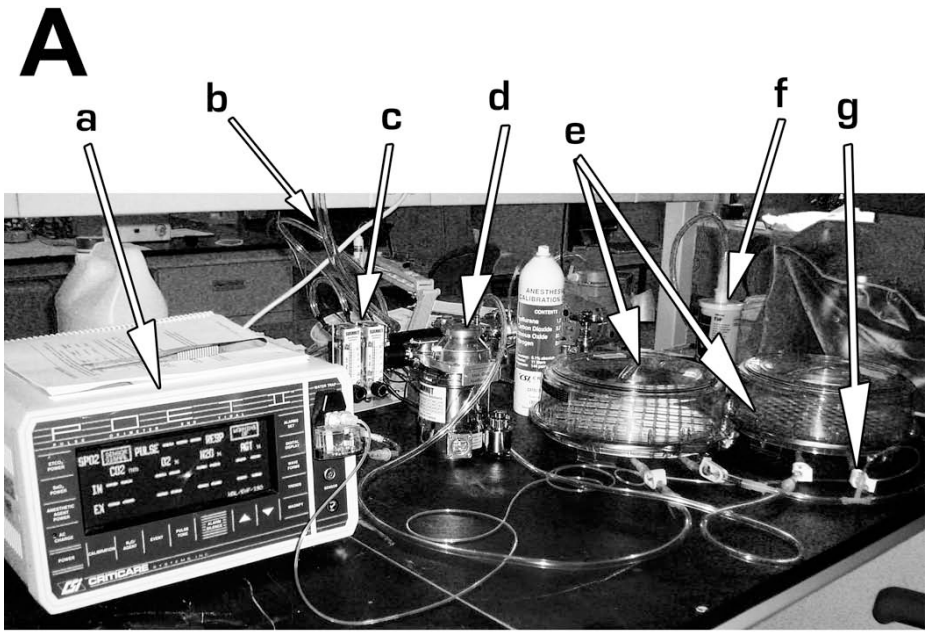

+ primers - DNA
- primers + DNA
Females
Males
1 2 3 4 5 6
1 2 3 4 5 6
*
*
Sry
Myog

Figure 2.2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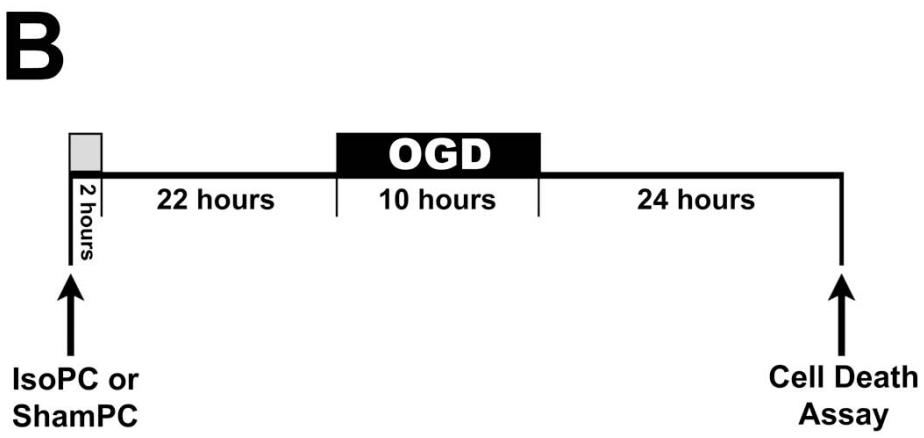
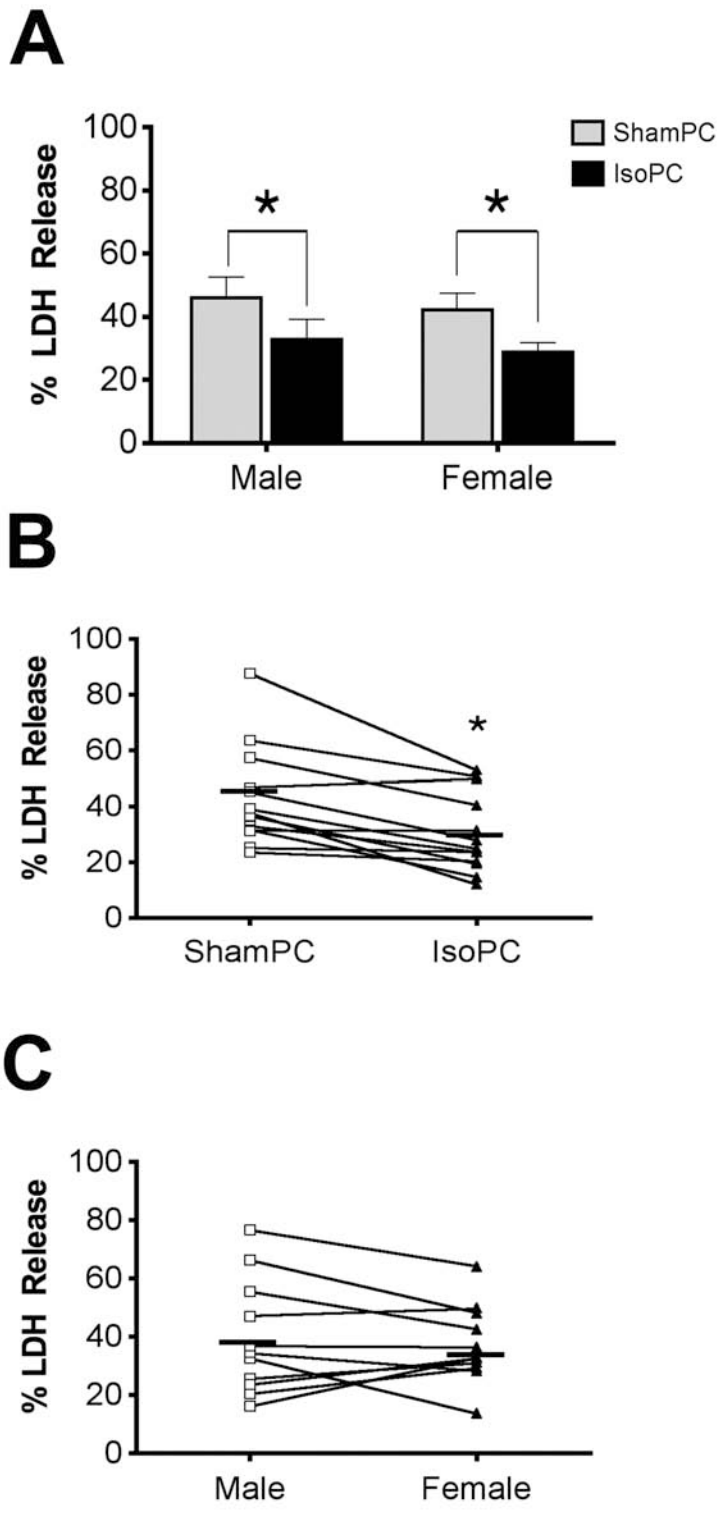

B
2 hours
22 hours
OGD
10 hours
24 hours
IsoPC or
ShamPC
Cell Death
Assay

Figure 2.3


A
% LDH Release
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80
60
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ShamPC
IsoPC
*
*
Male
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B
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80
60
40
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ShamPC
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C
% LDH Release
100
80
60
40
20
0
Male
Female

Chapter 3 | Isoflurane Preconditioning Protects Male and Female Neurons from Oxygen and Glucose Deprivation and is Modulated by Estradiol Only in Female Neurons

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Abstract

The volatile anesthetic, isoflurane, can protect the brain if administered before an insult such as an ischemic stroke. However, this protective "preconditioning" response to isoflurane is specific to males, with females showing an *increase* in brain damage following isoflurane preconditioning (IsoPC) and subsequent ischemia. Innate cell sex is emerging as an important player in neuronal cell death but the role of innate cell sex in the sexually dimorphic response to IsoPC has not been investigated. We used an *in vitro* model of IsoPC and ischemic stroke (oxygen and glucose deprivation, OGD) to test the hypotheses that 1) innate cell sex dictates the response to IsoPC and that 2) 17β-estradiol (E2) attenuates any protective effect from IsoPC in neurons via nuclear estrogen receptors. Using isolated, sex-segregated cultured cortical neurons from postnatal mice, we observed that IsoPC increased cell survival following subsequent OGD regardless of innate cell sex, but that the presence of E2 before and during IsoPC attenuated this protection only in female neurons independent of nuclear estrogen receptors. We also found that female neurons were less sensitive to OGD compared to male neurons, and that transient treatment with E2 protected neurons from subsequent OGD regardless of innate cell sex.

Introduction

Isoflurane has been shown to protect brain tissue[48] and isolated neurons if administered before insults that model ischemic stroke[97,104,106,107]. However, this "preconditioning" protection is specific to male brains, with female brains showing *increased* brain damage following isoflurane preconditioning and subsequent ischemia[48]. The specific neuronal component of this sexually dimorphic response has not been investigated, as current literature assessing neuronal responses to isoflurane preconditioning has relied on mixed-sex cultures. Indeed, innate cell sex is emerging as an important factor in neuronal cell death following a myriad of insults associated with neurodegeneration[26-28,51,117], but the role of innate neuronal cell sex in isoflurane preconditioning has not been investigated.

The major female sex steroid in women and rodents, 17β-estradiol (E2), has primarily been shown to be neuroprotective if administered before or during an ischemic insult[18,117-120]. E2 has also been shown to protect cortical neurons from oxygen and glucose deprivation (OGD), an *in vitro* model of ischemia; however, current *in vitro* reports have been limited to neuronal cultures that were not stratified by animal sex[121] and/or hippocampal slice cultures that could not resolve specific neuronal responses[51,120]. The nuclear estrogen receptors (ERs), subtype α and β, can mediate E2 neuroprotection depending on innate cell sex[122], the specific cellular insult[61,65,122-124], or a combination of these and other factors. We have previously shown that E2 attenuates the brain's protective response to isoflurane preconditioning via the nuclear ERs[50], yet whether E2 exerts this effect on isoflurane preconditioning protection at the specific neuronal level remains unknown.

We used isolated male and female cortical neurons and an *in vitro* model of ischemic stroke, OGD, to determine (1) if innate cell sex dictates the response to isoflurane preconditioning and (2) whether E2 attenuates any protective response to isoflurane preconditioning in neurons via nuclear ERs.

Experimental Procedures

Establishing sex-segregated cortical neuron cultures

Animal procedures were in compliance with the National Institutes of Health guidelines for the care and use of animals in research. All experimental protocols were approved by the Institutional Animal Care and Use Committee.

Male and female C57BL/6 (Charles River, Wilmington, MA) mouse pups (postnatal day 0 to 1) were segregated by sex based upon a larger genital papilla and ano-genital distance in males versus females. Our laboratory has previously validated this sex-segregation technique using PCR analysis for the male-specific gene, *Sry*, and the sex-independent autosomal gene, *Myogenin*[54,125].

Male and female mice within each litter were segregated and the brains from each sex were pooled to establish separate male and female cortical neuron cultures. Cortices were dissected in an ice-cold Hanks Balanced Salt Solution buffer followed by dissociation in papain for 20 min at 37°C. The digestion reaction was stopped with the addition of 0.04 mg/ml of soybean trypsin inhibitor and 0.04 mg/ml of DNase was added to prevent clumping. The cells were washed twice in growth media consisting of Neurobasal A without phenol red to prevent phenol red-induced estrogen signaling[115], 2% B27, 2 mM Glutamax, and 100 units of penicillin and streptomycin, and plated at a density of 1.5 x 10^5 cells/cm^2 onto plastic wells (Costar, Sigma-Aldrich, St. Louis, MO). The wells were pre-treated with 50 μg/ml poly-D-lysine for 3 hours at 37°C and later

rinsed in sterile water and air-dried. The cells were allowed to settle for 2 hours before being gently swirled and rinsed with fresh media to remove non-adherent cells. Cells from each sex of a given litter were plated across 3 different culture plates designated as isoflurane preconditioning without OGD, sham preconditioning + OGD, and isoflurane preconditioning + OGD. Each 24-well plate (6 wells across 4 rows) had 3-4 well replicates per row plated with cells, with each row designated for a different drug treatment depending on the experiment (e.g. untreated; vehicle; E2; the ER antagonist, ICI 182,780; or E2 + ICI 182,780). The neurons were maintained in growth media in a humidified incubator at 37°C and 5% CO_2 for the duration of the experimental period, except during preconditioning and OGD.

Cell culture reagents including Neurobasal A, B27, soybean trypsin inhibitor, Glutamax, streptomycin and penicillin, and poly-D-lysine were from Life Technologies (Invitrogen), Carlsbad, CA. The papain was from Worthington Biochemical Corporation, Lakewood, NJ.

Preconditioning neurons with isoflurane

Neurons were preconditioned with isoflurane using a gas exchange system. A gas exchange system was chosen as opposed to adding liquid isoflurane to the culture media in order to better maintain the gas:liquid anesthetic equilibrium[102,103] and thereby better model *in vivo* anesthetic preconditioning. Similar designs have been reported for exposing neurons and other cell types to volatile anesthetics[97,99,104]. Two air-tight, plastic chambers (Billups-Rothenberg, Del Mar, CA) were filled with medical air and 5% CO_2, with or without 3% isoflurane. For preconditioning, neurons were exposed to either 3% isoflurane in one chamber or sham preconditioned with 0% isoflurane in the other chamber. An anesthetic monitor (POET II, Criticare Systems,

Waukesha, WI) was attached to the outflow of the isoflurane preconditioning chamber to determine when the isoflurane concentration had reached 3% in the chamber. The inflow and outflow tubing was then clamped and each chamber was placed in a 37°C incubator during the 1 hour preconditioning period. Following the preconditioning period, the culture plates were removed from the chambers and returned to normal growth conditions for 23 hours before OGD. The treatment paradigm is illustrated in **Figure 3.1**. The 3% isoflurane concentration, 1 hour preconditioning period, and 23 hour period following preconditioning were chosen based upon reports using similar models of volatile anesthetic preconditioning in isolated brain cells[97,99,104].

17β-Estradiol and ICI 182,780 pretreatments

The effects of E2 (Sigma-Aldrich) and the non-selective nuclear estrogen receptor (ER) α and β antagonist[62], ICI 182,780 (ICI, Tocris Bioscience, Bristol, UK), were examined by dissolving these compounds in either 100% ethanol (for E2) or 100% sterile DMSO (for ICI) to generate 1 mM stock concentrations of each compound. Serial dilutions were then made to achieve the working concentrations. For E2, 1 μl of 1 mM E2 solution was added per 10 ml of growth media (0.01% volume) to achieve a 100 nM E2 solution, which was further diluted separately 1:10 to achieve a 10 nM E2 solution. For ICI, a 1 μM solution was achieved by diluting 10 μl of 1 mM stock solution in DMSO into 10 ml of growth media (0.1% volume). Each "vehicle" control received the same volume dilutions of either ethanol (E2), DMSO (ICI), or both (E2 + ICI). On day *in vitro* 8, E2 and/or ICI were added 24 hours before preconditioning and then removed with two washes in growth media immediately following preconditioning on day *in vitro* 9 (**Figure 3.1**).

OGD and cell survival assessment

OGD was conducted when neurons reached day *in vitro* 10. The cells were gently rinsed twice in Neurobasal A *without* glucose or other nutrients and placed in a chamber deprived of oxygen (< 0 ppm, Coy Laboratory Products, Grass Lake, MI) at 37°C for 2 hours. Control cells (isoflurane preconditioned *without* OGD, "100% Cell Survival") were rinsed alongside the experimental isoflurane preconditioned + OGD and sham preconditioned + OGD groups but then returned to normal growth media and conditions. Following the 2 hour OGD period, the treated cells were returned to normal growth media and conditions.

Cell survival was assessed 24 hours following the end of OGD using calcein-AM (Life Technologies) with an automated plate reading protocol as previously reported[15]. Calcein-AM is a membrane-permeable non-fluorescent molecule that, upon entering an intact living cell, is cleaved by endogenous esterases to produce the highly-fluorescent, membrane impermeable molecule, calcein. This fluorescence can be quantified to represent "cell survival". Calcein was excited by 490 nm light and the emitted fluorescence was filtered at 535 nm for quantification. Each well was scanned at 9 different points to account for intra-well density differences, with 3-4 replicate wells per row. The average fluorescent value from all these points in a row (27 to 36 points total) was used to generate the final fluorescent value for that particular treatment. In each experimental round, the final fluorescent average of the isoflurane preconditioned *without* OGD plate was designated as "100% Cell Survival" by which the other corresponding fluorescent averages from the sham preconditioned + OGD and isoflurane preconditioned + OGD plates were divided to generate "% Cell Survival". This design has the advantage of being an unbiased, high-throughput,

internally-controlled method of quantifying cell survival while compensating for cell density differences within wells, among different wells, and throughout different cultures.

Statistics

Two-way analysis of variance (ANOVA) of the difference of paired means (i.e. "repeated measures") was used to allow for a paired littermate analysis, where compared factors were "cell sex" and "preconditioning" (*Results 3.1*) or "drug treatment" and "preconditioning" (*Results 3.2*). This analysis controlled for inherent variability in absolute cell survival values throughout different cultures. In initial experiments examining innate cell sex and preconditioning, the two-way difference of paired means ANOVA compared innate cell sex-independent, preconditioning-dependent outcomes (pooled male and female cell survival outcomes) and innate cell sex-dependent, preconditioning-independent outcomes (pooled sham and isoflurane preconditioned cell survival outcomes). In experiments examining the effect of E2 on preconditioning, the ANOVA compared E2-independent, preconditioning-dependent outcomes and E2-dependent, preconditioning-independent outcomes separately in male neurons and in female neurons. In experiments examining the effects of nuclear ER receptor blockade by ICI on preconditioning in female neurons, the ANOVA compared drug treatment-independent, preconditioning-dependent outcomes, and drug treatment-dependent, preconditioning-independent outcomes in female neurons. Every ANOVA was followed by the Student Newman-Keuls post-hoc test to analyze differences between and within factors of cell sex, preconditioning, and/or drug treatment (vehicle, E2, ICI, or E2 + ICI).

Each culture from a given litter represented an n = 1. There were 11 paired male and female cultures for the sham preconditioned vs. isoflurane preconditioned experiments (**Figure 3.2**), 9 paired male and female cultures for the E2-treated experiments (**Figure 3.3**), and 5 paired female cultures for the E2 with or without ICI experiments. *p < 0.05 was considered significant; data is given as the mean ± standard error of the mean. Unless otherwise indicated in the text as the ANOVA output, significance in the figures and text represents the post-hoc Student Newman-Keuls output. SigmaStat [v2.0] statistical software (SPSS Inc., Chicago, IL) was used for all statistical analyses.

Results

The effect of innate cell sex following isoflurane preconditioning and OGD

Using our model of *in vitro* isoflurane preconditioning with sex-segregated neuron cultures, we found that isoflurane preconditioning protected both male (**Figure 3.2A**, sham preconditioned, 47.7 ± 4.3% cell survival vs. isoflurane preconditioned, 58.4 ± 5.9% cell survival, *p < 0.05) and female neurons (**Figure 3.1A**, sham preconditioned 61.7 ± 3.0% vs. isoflurane preconditioned 71.6 ± 4.0%, *p < 0.01) from subsequent OGD. However, there was no interaction between cell sex and preconditioning treatment (p = 0.25). The cell sex-independent ANOVA (pooled male and female cell survival outcomes) revealed that isoflurane preconditioning increased cell survival 8.5 ± 3.4% compared to sham preconditioning regardless of innate cell sex (**Figure 3.2B**, sham preconditioned vs. isoflurane preconditioned, 54.7 ± 3.3% vs. 63.2 ± 4.8%, respectively, *p < 0.01). Independent of preconditioning, we found that female neurons showed a 15.4 ± 3.4% increase in cell survival following OGD compared to male

neurons (**Figure 3.2C**, male vs. female, 51.2 ± 5.4% vs. 66.6 ± 3.6%, respectively, *p < 0.001).

The effect of E2 on the response of male and female neurons following isoflurane preconditioning and OGD.

We found that E2 attenuates isoflurane preconditioning-induced protection from OGD in female but not male neurons. In male neurons, the effect of different preconditioning treatments (sham or isoflurane preconditioning) was not altered by the level of E2 present (interaction between preconditioning and E2, $p = 0.74$). The difference in the mean values between the sham and isoflurane preconditioning groups was greater than would be expected by chance after allowing for effects of differences in E2 ($p = 0.03$), indicating that male neurons continued to demonstrate isoflurane preconditioning-induced protection from subsequent OGD regardless of E2 treatment. Conversely, the difference in the mean values among the different levels of E2 was greater than would be expected by chance after allowing for effects of differences in preconditioning treatments ($p = 0.04$). This indicates that independent of preconditioning treatment, E2 protected male neurons from subsequent OGD. Individual preconditioning and E2 means with post-hoc statistical analyses are represented in **Figure 3.3A**.

In contrast to male neurons, the effect of the different preconditioning treatments in female neurons did depend on the level of E2 present (interaction between preconditioning and E2, $p < 0.001$), where E2 attenuated isoflurane preconditioning protection from OGD. This was evident in that the difference in the mean values between sham preconditioning and isoflurane preconditioning was not great enough to exclude the possibility that this difference is simply due to random sampling variability after allowing for the effects of differences in E2 ($p = 0.45$).

Conversely, the difference in the mean values among the different levels of E2 was greater than would be expected by chance after allowing for effects of differences in preconditioning treatments ($p = 0.02$). This indicates that independent of preconditioning treatment, E2 addition protected female neurons from subsequent OGD. Individual preconditioning and E2 means with post-hoc statistical analyses are represented in **Figure 3.3B**.

We used the non-selective nuclear ER antagonist, ICI, to determine if E2's attenuation of the protective response to isoflurane preconditioning in female neurons is mediated through ERα and ERβ. Interestingly, E2 continued to attenuate isoflurane-induced protection in female neurons in the presence of ICI (sham preconditioning + E2 + ICI vs. isoflurane preconditioning + E2 + ICI, 74.0 ± 5.9% vs. 77.4 ± 6.5, respectively; $p = 0.15$; $n = 5$), as did ICI alone (sham preconditioning + ICI vs. isoflurane preconditioning + ICI, 75.3 ± 3.4% vs. 79.8 vs. ± 6.8%, respectively; $p = 0.21$; $n = 5$).

Discussion

Using sex-stratified isolated cortical neurons, we have shown that isoflurane preconditioning protects male and female cortical neurons from subsequent OGD, and that the sex steroid, E2, attenuates the protective isoflurane preconditioning response only in female neurons independent of the nuclear estrogen receptors. Ancillary to our primary hypotheses, we also found that female neurons are less sensitive to OGD compared to male neurons regardless of preconditioning, and that transient E2 exposure protects both male and female neurons from subsequent OGD.

Isoflurane preconditioning protects neurons from subsequent OGD independent of innate cell sex

We have demonstrated that isoflurane preconditioning similarly protected isolated male and female cortical neurons from OGD (**Figure 3.2**). Considering recent findings from our lab demonstrating that isoflurane preconditioning protects both male and female astrocytes from subsequent OGD (Johnsen et al., 2011, *pending review*), our data suggest that the cellular default in the brain in the absence of E2 is a protective response to isoflurane preconditioning regardless of innate cell sex, and that sexually dimorphic responses to isoflurane preconditioning may result from other factors in the intact brain[50]. This notion is supported by the general consensus of current *in vitro* isoflurane preconditioning studies demonstrating a protective response in mixed-sex neuronal cultures that are removed from a sex steroid milieu[97,104,106,107]. Further support that the cellular default in the brain may be a protective response to isoflurane preconditioning is provided by *in vivo* experiments demonstrating a protective response to isoflurane preconditioning following subsequent brain ischemia in ovariectomized females, which have reduced circulating female sex steroids, compared to a detrimental isoflurane preconditioning response in young intact females[50].

E2 attenuates the protective isoflurane preconditioning response only in female neurons independent of nuclear estrogen receptors

Since the presence of E2, the primary estrogen in women and female rodents, has been linked to a deleterious response of female mouse brain to isoflurane preconditioning[50], we hypothesized that E2 would attenuate the apparent default protective response to isoflurane preconditioning in male and female neurons. Interestingly, we found that the presence of E2 before and during isoflurane preconditioning attenuated the protective response only in female neurons, while

male neurons continued to show a protective response to isoflurane preconditioning compared to sham preconditioning. These findings are consistent with previous *in vivo* reports showing that E2 attenuates isoflurane preconditioning protection in ovariectomized female mice[50] and that male mice, which normally have low circulating E2, demonstrate a protective response to isoflurane preconditioning[48].

One interpretation of these findings is that while the neuronal default is a protective response to isoflurane preconditioning regardless of innate cell sex, the mechanisms of this protective response may be different between male and female neurons, of which E2 alters only the mechanism in female neurons. There have been no investigations directly linking sex-specific isoflurane preconditioning mechanisms with E2, although one report has demonstrated differences in Akt phosphorylation between gonadally-intact male and female brain following isoflurane preconditioning[48]. Here it was demonstrated that isoflurane preconditioning increased Akt phosphorylation – a protective response – in male mouse brain compared to female brain, but the role of E2 in this sexually dimorphic Akt phosphorylation was not assessed. Moreover, the volatile anesthetic, xenon, has been to shown to protect rodent brain independent of animal sex that may involve Akt and HIF-1α[126], though many of the mechanisms of xenon preconditioning in the brain appear to be substantially different from those of isoflurane[47,127]. Furthermore, xenon's high cost and limited approval continues to limit its clinical application.

In addition to isoflurane preconditioning inducing different protective mechanisms between male and female neurons, it is equally possible that E2 induces different mechanisms between male and female neurons, of which the mechanism in female neurons alters the default protective isoflurane preconditioning response. Reports investigating differences in E2 signaling between male and female neurons are

scarce, although it has been shown that neuronal cell death-inducible putative kinase, a negative Akt modulator[128] and E2-responsive gene[129], is increased in female cortex compared to males[48]. Expression of this kinase, however, was not modulated by isoflurane preconditioning[48].

The nuclear estrogen receptors (ERs) α and β, on the other hand, have been linked to the E2-mediated attenuation of isoflurane preconditioning in female animals[50] and to differential responses following glutamate exposure to isolated male and female neurons[122], but whether E2 acts through the ERs in female neurons to attenuate isoflurane preconditioning has not been directly tested. By using the ER antagonist, ICI[62], which bind strongly to both nuclear ERs and prevents the dimerization required for most genomic and non-genomic ER action[130], we found that the ERs do not exclusively mediate E2's attenuation of isoflurane preconditioning in female neurons. This is not likely due to differential α and β ER expression as both receptor subtypes are expressed in female neurons[122] and are functionally antagonized by ICI[130]. It may be that E2 attenuates isoflurane preconditioning in female neurons via non-nulcear ERs such as GPR30[131], although this and other non-nuclear ERs have not yet been investigated for a potential role in anesthetic or non-anesthetic preconditioning.

Interestingly, we also found that ICI independently attenuated isoflurane preconditioning similar to that of E2. E2 and ICI share a similar chemical structure that allows both compounds to act as antioxidants[18,60,61]. Considering that antioxidants are known to attenuate many forms of preconditioning[47,93], including anesthetic preconditioning[97,132], it is also possible that E2 may attenuate the default protective response to isoflurane preconditioning in female neurons via antioxidant mechanisms. Further investigations are therefore necessary to address these possibilities.

Female neurons are less sensitive to OGD compared to male neurons independent of preconditioning treatment

The role of innate cell sex in ischemic injury remains under-investigated despite increasing evidence that innate cell sex is important in the execution of various neuronal cell death pathways following various cellular insults[26-28,122]. This report is the first to demonstrate that isolated female cortical neurons are less sensitive to OGD compared to male cells independent of preconditioning (**Figure 3.2C**). Similar sex-specific neuronal sensitivities have been reported following oxidative and excitotoxic damage[26], hypoxia[28], autophagy[27], and OGD in brain slices[51], although until now the role of innate neuronal cell sex in models of ischemic stroke had not been addressed. Furthermore, it has been appreciated for some time that female rodent brain is less sensitive to ischemia compared to males, but most investigations have focused on the role of sex steroids as primary mediators in this sexually dimorphic outcome[18,19,65]. The results presented here suggest that innate neuronal cell sex may have an important role in male versus female ischemic brain injury outcomes.

Transient pretreatment with E2 protects neurons from subsequent OGD independent of preconditioning in male and female neurons

Independent of isoflurane preconditioning, transient pretreatment with E2 protected both male and female neurons (pooled sham preconditioned and isoflurane preconditioned cell survival outcomes, $p < 0.05$). E2 pretreatment is known to protect brain tissue and isolated brain cells from subsequent OGD[62] and other insults[26,28]; however, the results presented here are the first to show that E2-mediated protection from ischemic insult in isolated cortical neurons can persist for up to 24 hours

following E2 removal from the cellular environment in isolated male and female neurons. While these findings were ancillary to our investigation of E2's effect on isoflurane preconditioning, they do have important implications towards expanding our current understanding of cellular responses to E2 in the brain. Similar results have been reported showing that transient E2 exposure protects female hippocampal slices[118] and brain tissue[119] from ischemic insult via intra- and extracellular Ca^{2+}, calcium/calmodulin-dependent kinase II, cAMP response element-binding protein. Our findings extend such E2 protection in models of ischemic injury to isolated male and female cells.

Conclusions

There is considerable clinical interest in applying the neuroprotective potential of isoflurane preconditioning during the perioperative period so as to reduce the risk of brain damage following surgeries at risk for ischemic stroke, such as coronary artery bypass grafting and carotid endarterectomy[43,47]. However, a better understanding of the sexual dimorphic response to isoflurane preconditioning is crucial before such neuroprotective strategies can be implemented in women and men. Our results have important implications towards increasing our understanding of the sex differences in the response to isoflurane preconditioning, as well as potential implications towards ischemic stroke outcomes in women versus men.

Acknowledgements

The authors thank Rochelle Fu, Ph.D., Public Health & Preventative Medicine Department, Oregon Health & Science University, Portland, Oregon, for her assistance with statistical analyses. This work was supported by grants from NIH/NINDS (NS054684 - SJM) and the Pacific Mountain Affiliate of the American Heart Association (Predoctoral Fellowship – DJ).

Figure 3.1 | Schematic of the preconditioning and drug treatment paradigm in sex-segregated cortical neuron cultures from postnatal day 0 - 1 day mice. E2, 17β-estradiol; ICI, ICI 182,780; IsoPC, isoflurane preconditioning; OGD, oxygen and glucose deprivation; ShamPC, sham preconditioning.

Figure 3.2 | Isoflurane preconditioning (IsoPC) increased cell survival in male and female cortical neurons, while female neurons were protected from OGD regardless of preconditioning. **(A)** Mean cell survival of sham preconditioning (ShamPC) and IsoPC-treated male and female cortical neurons following OGD. *$p < 0.05$ ShamPC vs. IsoPC in male cells, *$p < 0.01$ ShamPC vs. IsoPC in female cells; $n = 11$. **(B)** Sex-independent paired difference analysis of variance (ANOVA) of cell survival outcomes between ShamPC and IsoPC groups. Each line connects cell survival outcomes from the same litter ($n = 1$); *$p < 0.05$; $n = 11$. **(C)** Preconditioning-independent paired difference ANOVA of cell survival outcomes between male and female cortical neurons. Each line connects the cell survival outcome from the same litter ($n = 1$); *$p < 0.001$; $n = 11$.

Figure 3.3 | 17β-estradiol (E2) attenuates isoflurane preconditioning (IsoPC)-induced protection from oxygen and glucose deprivation (OGD) in female but not male neurons. **(A)** IsoPC protects male neurons from subsequent OGD independent of E2. *$p < 0.05$, ShamPC vs. IsoPC; $^{++}p < 0.05$, E2 IsoPC vs. vehicle IsoPC; $n = 9$. **(B)** E2 attenuates IsoPC protection following subsequent OGD in female neurons. *$p < 0.01$, ShamPC vs. IsoPC; $^{+}p < 0.05$, E2 ShamPC vs. vehicle ShamPC; $n = 9$.

Figure 3.1

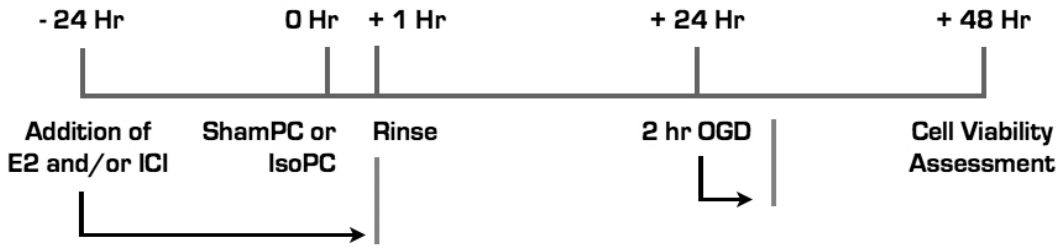
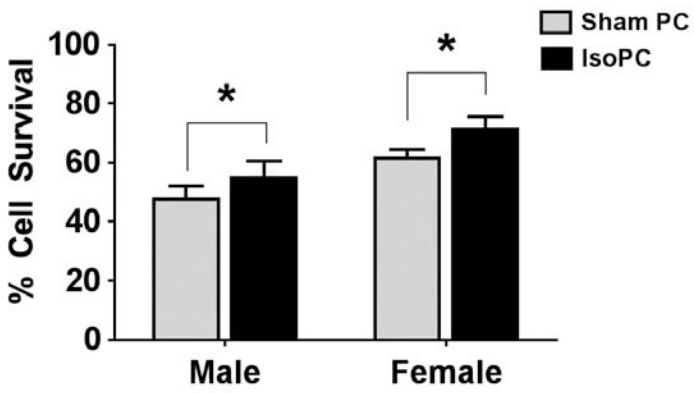

- 24 Hr
0 Hr
+ 1 Hr
+ 24 Hr
+ 48 Hr
Addition of E2 and/or ICI
ShamPC or IsoPC
Rinse
2 hr OGD
Cell Viability Assessment

Figure 3.2

A



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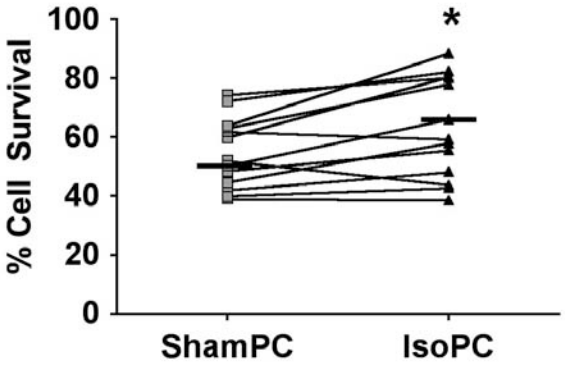

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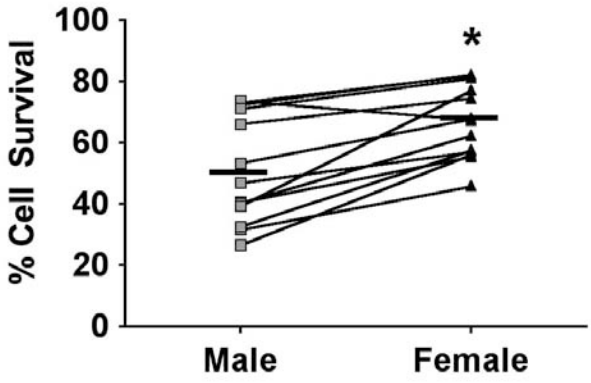
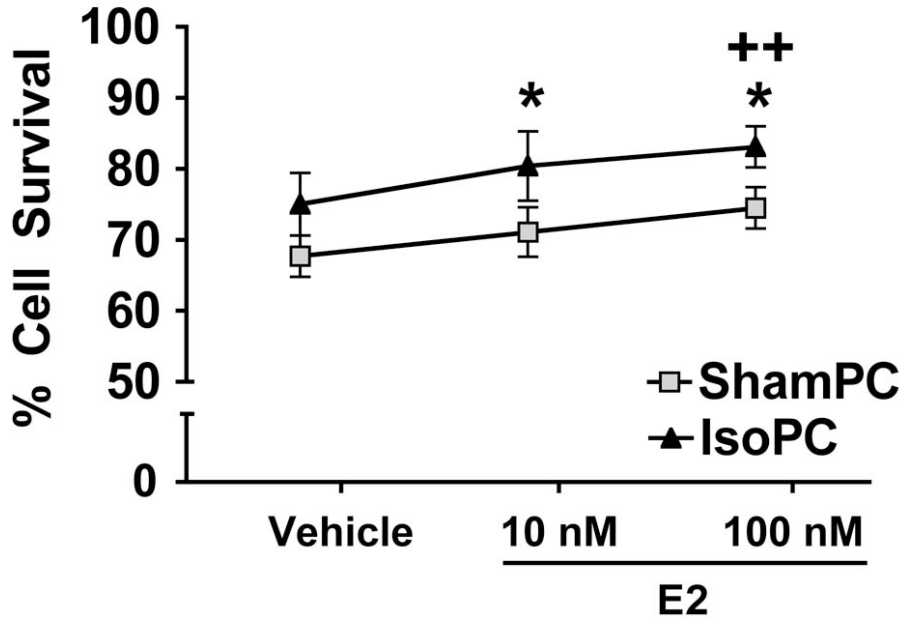


Figure 3.3

A


100
90
80
70
60
50
0
% Cell Survival
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ShamPC
IsoPC
Vehicle
10 nM
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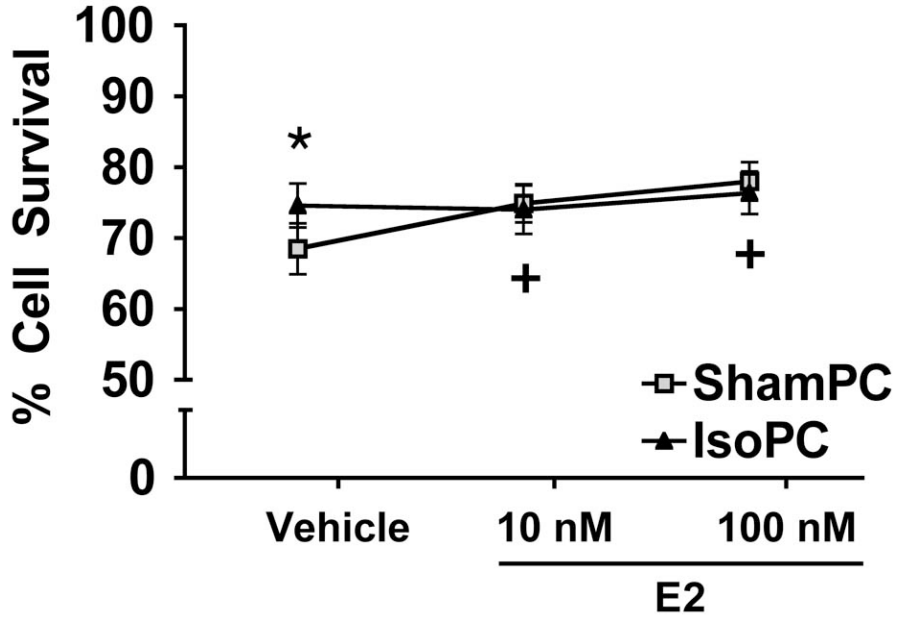
E2

B


100
90
80
70
60
50
0
% Cell Survival
*
+
+
ShamPC
IsoPC
Vehicle
10 nM
100 nM
E2

Chapter 4 | Cyclophilin D Mediates Primarily Oxidant Injury in Isolated Neurons and Astrocytes

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Published in Part: Forte, F., Gold, B., Marracci, G., Chaudhary, P., Basso, E., **Johnsen, D.**, Xiaolin, Y., Fowlkes, J., Rahder, M., Stem, K., Bernardi, P., Bourdette, D. Cyclophilin D inactivation protects axons in experimental autoimmune encephalomyelitis, an animal model of multiple sclerosis. *PNAS*. **104**(18), 7558–7563 (2007)[15]

Abstract

Mitochondrial permeability transition is increasingly being appreciated as a major mediator in neurodegeneration. The mitochondrial matrix protein, cyclophilin D (CyD), has been identified as an important regulator of permeability transition in the brain and in isolated non-neuronal cells, but the specific role of CyD following cellular insults endemic to neurodegeneration in neurons and astrocytes remains uninvestigated. We took a genetic knockout approach to eliminate CyD (CyD KO) in primary cultured brain cells to first test the hypothesis that CyD mediates neuronal survival following oxidative damage, excitotoxicity, and oxygen and glucose deprivation (OGD, an *in vitro* model of ischemic stroke). CyD KO neurons demonstrated increased cell survival following nitric oxide and hydrogen peroxide treatment compared to wild-type (WT) controls, but not following glutamate-induced excitotoxicity or OGD. Since CyD KO neurons were protected from oxidants, we then tested the hypothesis that CyD KO astrocytes would be similarly protected following oxidant treatment. Indeed, CyD KO astrocytes were protected from hydrogen peroxide compared to WT astrocytes. Our results suggest that CyD may be an important mediator of oxidant-induced cell death in the brain, but is likely not exclusively involved following excitotoxic or energetic insults. These findings further characterize the specific roles CyD may play in cell death in the brain and continue to support CyD as an important pharmacologic target in treating neurodegenerative diseases.

The mitochondrion is increasingly being recognized as an important contributor to neurodegeneration[3,69,133,134]. This organelle is essential to adequately fuel the significant energy demand of the brain, as well as buffer intracellular Ca^{2+}, a crucial ion regulating enzyme function, synaptic transmission, and cellular homeostasis. The mitochondrion is also a major coordinator of cell death, namely the intrinsic apoptotic pathway and necrosis. Hence, mitochondrial dysfunction can diminish energy production, dysregulate intracellular Ca^{2+}, disrupt cellular homeostasis, and ultimately lead to cell death. In recent years, mitochondrial dysfunction has been linked to many neurodegenerative diseases including Alzheimer's disease[133,134], Parkinson's disease[133,135], multiple sclerosis[3], and ischemic stroke[9,69,134]. Processes endemic to these diseases at the cellular level, such as oxidative damage, excitotoxicity, and loss in ATP production, are processes known to involve the mitochondrion at the subcellular level. This "mitochondrial view" of neurodegeneration is emerging as an important, albeit under-investigated perspective on the mechanisms of cell death in the brain.

While the mitochondrion can employ an arsenal of mechanisms to elicit cell death, one such mechanism, permeability transition, is of particular interest due to its reliance upon the activity of a single protein with available pharmacologic inhibitors, cyclophilin D (CyD)[14,74]. CyD is a nuclear-encoded peptidy-proly *cis-trans* isomerase that resides exclusively in the mitochondrial matrix and facilitates permeability transition, a process defined by non-selective permeability of the inner mitochondrial membrane to solutes less than 1.5 kDa. Sustained permeability transition reduces the mitochondrial voltage potential, uncouples oxidative phosphorylation, expels

sequestered matrix Ca^{2+}, releases cytochrome c into the cytosol, and leads to cell death. Inactivating CyD through pharmacologic inhibition or genetic deletion prevents permeability transition in isolated mitochondria, possibly by unmasking an inhibitory phosphate binding regulatory site[136], and can protect cells from toxic insults. Indeed, inactivating CyD protects isolated fibroblasts[16,82,84], cardiomyocytes[82,84], and neurons from oxidative damage[15], as well as preserves brain tissue in disease models of multiple sclerosis[15], ischemic stroke[16], and Alzheimer's Disease[44].

Despite the broad protection bestowed by inactivating CyD, the precise role of CyD in mediating toxic insults endemic to neurodegenerative diseases remains uncertain. Thus far, the majority of research investigating how CyD mediates cell death has focused on oxidative damage in non-neuronal cells, leaving cells of the central nervous system, as well as non-oxidative insults such as excitotoxicity and energetic failure largely unexplored. Moreover, the role of CyD in astrocytes has received little experimental consideration compared to other cell types. Astrocytes, long relegated as quiescent "support" cells in the brain, are emerging as a having dynamic roles mediating multifarious processes in the CNS, including metabolic regulation, synaptic plasticity, and immune responses, among others[87,90]. With a better appreciation of astrocytes' role in CNS function, however, comes an increased awareness of astrocytes' role in CNS dysfunction, including neurodegeneration. Hence, determining if CyD differentially regulates neuronal and astrocytic cell death will be an important advance in understanding the complex cellular events that manifest during neurodegeneration.

The experiments described here take a genetic approach to test the hypothesis that **(1)** CyD is an integral mediator of neuronal cell death in the brain following oxidative damage, excitotoxicity, and oxygen and glucose deprivation (OGD), an *in*

vitro model for ischemia. The results of these studies demonstrated that CyD mediates exclusively oxidant-induced cell death, therefore we tested the additional hypothesis that **(2)** CyD also mediates oxidant-induced cell death in astrocytes. Indeed, we found that, as in neurons, CyD mediates oxidant-induced cell death in astrocytes.

Materials and Methods

Animals:

Mice lacking the gene coding for CyD (*Ppif* gene knock-out, CyD KO) were developed by Dr. Mike Forte (Vollum Institute, Portland, OR) and Dr. Paolo Bernardi (University of Padova, Padova, Italy) as previously described[83]. This strain originated on a B6J;129Sv background and has been backcrossed to C57BL/6J for at least 8 generations before being made homozygous. WT C57BL6/J were purchased from Jackson Laboratories (Bar Harbor, Maine). Independent breeding colonies of the WT and CyD KO mice were established and maintained to provide P0 – P2 litters from which the neuron and astrocyte cultures were generated.

Polymerase chain reaction for genotyping:

Every few months, toe and tail tissue samples from 4 randomly-selected euthanized WT and CyD KO adult mice (retired breeders) were used to verify CyD deletion (*Ppif* gene inactivation)[83]. DNA was extracted and purified using the Dneasy kit (Qiagen, Valencia, CA). Initial melting was carried out at 96°C for 5 min., followed by 33 polymerase chain reaction (PCR) cycles of 96°C (1 min.), 56°C (1 min.), and 72°C (3 min.). The WT forward primer used was 5'-CAG CTG ATC AGA ACC ATC ATG–3' and the reverse primer was 5'-GTT GAC CAG AGT GGC GTA GG-3' with a 500 bp product.

The *Ppif* (CyD KO) forward primer recognized the inserted neomycin cassette with a 5'-CCG CTT CCA TTG CTC AGC GG-3' sequence, while the reverse primer for *Ppif* KO insert was the same as the reverse WT primer. The *Ppif* product was a 1.6 kB product. Since the CyD KO primer set shared the WT reverse primer, 2 separate PCR reactions for each animal were necessary to maximize band clarity. **Figure 4.1** represents a typical PCR product. WT and CyD KO genotypes were consistently confirmed with 100% accuracy.

Neuron and astrocyte cortical cultures:

Cortical, mixed-sex neuron and astrocyte cultures were established from postnatal days 0-2 (P0 - P2) pups. Cortical cells were chosen because the mammalian neocortex is among the more vulnerable brain regions in many neurodegenerative diseases, including multiple sclerosis[137] and ischemic stroke[9].

Neuronal cultures were prepared as published[15]. Briefly, neocortical tissue was trimmed from newborn pup brains, dissociated in modified Hank's Balanced Salt Solution with 0.25% trypsin (Gibco, Carlsbad, CA) for 30 minutes, and grown in Neurobasal A (Gibco) with 2% B-27 serum-free supplement (Gibco), 0.5 mM L-glutamine (Sigma-Aldrich, St. Louis, MO) and 100 units of penicillin/streptomycin (Sigma-Aldrich). Neurons were plated onto poly-D-lysine-coated (1 μg/ml, Sigma-Alrich) 24-well culturing plates (Corning, Lowell, MA) at a density of 1.1 X 10^5 cells/cm^2; each culture plate contained neurons from a mixed-sex, pooled litter of a single genotype (n = 1 per litter). Arrangement of cell plating among the 24-well plates depended upon the experimental design for a given set of experiments. For oxidant and glutamate-treated cells, each row consisted of a different treatment, with 3-6 well replicates per row. For oxygen and glucose deprivation (OGD), each

plate was subjected to either control or OGD conditions, with the wells within each plate allowing for different treatments and/or replicates. In all cases, cells from different genotypes were plated separately. Following plating, fresh growth media was added to the cultures after the first 24 hours in culture. Experiments using oxidants were conducted between day-*in vitro* (DIV) 4 and 6; excitotoxicity and OGD experiments were conducted between day-*in-vitro* (DIV) 9-11. A subset of excitotoxicity experiments used DIV 16-18 neurons (see Results and **Appendix Figure A4.2**).

Astrocyte cultures were prepared identically as the neuron cultures except for minor modifications following initial plating onto plastic wells. For astrocytes, the plastic plates were not coated with poly-D-lysine and the growth media used was Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 10% fetal calf serum (HyClone, Logan, UT) and 100 units of penicillin/streptomycin (Sigma-Alrich). Following 24 hours in culture, the plates were gently swirled on a platform rotator at room temperature for 30 minutes to dislodge non-adherent, mostly non-astrocytic cells. Fresh growth media was added and the media was changed every 3-4 days in cultures. Astrocytes were treated with oxidants for experimentation when the culture was confluent, typically DIV 10-14.

All treatments doses, treatment times, and post-treatment recovery times for neurons and astrocytes (described in detail in Results) were based upon preliminary experiments determining paradigms that maximized potential differences in cell survival between WT and CyD KO cells.

Oxidant treatments in neuron and astrocyte cultures:

Oxidative damage was induced in neurons by exposing the cells to 0, 500, or 1000 μM of the nitric oxide (NO) donor, 2,2′-(Hydroxynitrosohydrazono)bis-ethanimine

(DETA-NO, Sigma-Alrich), or hydrogen peroxide (H_2O_2, Sigma-Alrich) dissolved in growth media. Neurons were treated with DETA-NO for 3 hours, washed twice in growth media and then incubated in normal conditions for 16 hours before cell survival assessment; H_2O_2 treatment was continuous for 16 hours before cell survival assessment. Control wells consisted of identical procedures with the addition of "0 μM" oxidants in solution (essentially only rinsing the cells). For astrocytes, 0, 500, or 750 μM H_2O_2 (1000 μM resulted in near 0% cell survival, preliminary data) was dissolved in growth media and incubated with the cells for 3 hours, followed by two rinses in growth media. The cells were then returned to normal growth conditions for 24 hours before cell survival assessment. DETA-NO was not used for astrocyte experiments.

Glutamate treatments in neuron cultures:

Excitotoxicity was induced in neuron cultures (DIV 9-11 to allow for mature synapse formation in culture[138]) with the addition of L-glutamic acid dissolved in growth media at 5, 10, 15 μM ("low dose") or 100 μM ("high dose") for 10 minutes. A low and high dose was investigated here since CyD mediation of neuronal survival has recently been shown to be sensitive to glutamate concentration[85,86]. Five hundred nanomolar tetrodotoxin was present in all treatment solutions to isolate the effects of exogenous glutamate addition from presynaptic release. The neurons were then rinsed twice in warm growth media and returned to normal conditions for 18-24 hours before cell survival assessment. Control wells consisted of identical procedures with the addition of "0 μM" glutamate in solution. Low dose glutamate-induced excitotoxicity was also tested in more mature neuronal cultures (DIV 12-18, see Results and **Appendix Figure A4.2**).

Oxygen and glucose deprivation (OGD) In neuron cultures:

OGD was induced in neurons (DIV 9-11) as similarly described by Liu, M., *et al*.[53] Upon OGD treatment, cells were rinsed twice in warm PBS to remove residual nutrients from the growth media. Glucose and supplement-free media was then added and the cells were placed in an anoxic chamber (Coy Laboratories Products, Grass Lake, MI) for 2 hours. The anoxic chamber is maintained at 37°C with < 1 part per million O_2, and 92% N_2, 3% H_2, and 5% CO_2. Normal growth conditions were restored to the cells following OGD until cell survival assessment 18-24 hours later (pilot experiments determined that maximal cell death occurred by 18 hours following OGD in both genotypes).

To ensure the OGD experimental paradigm was sufficient to detect potential CyD KO protection, positive control experiments were conducted by pretreating neurons with dizocilpine hydrogen maleate (MK-801, Sigma-Aldrich), a non-reversible NMDA receptor blocker known to protect neurons from OGD-induced cell death[139,140]. Neurons were pretreated with MK-801 24 hours prior to a 2 hour OGD treatment, whereby the cells were then returned to normal growth conditions. Cell survival in these experiments was assessed 48 hours following OGD to ensure that any protection from MK-801 was not simply delaying cell death.

The "untreated" plates in all OGD experiments consisted of identical procedures with normal growth media being added to rinsed wells instead of nutrient-depleted media. These control plates were returned to normal growth conditions until cell survival assessment.

Assessment of cell survival:

Cell survival in neurons and astrocytes was determined as previously described[15]. Calcein-AM (Biotium, Inc., Hayward, CA) was used as a quantitative fluorescent indicator of intact, "surviving" cells. Cells were rinsed in warm PBS to remove growth media, followed by the addition of 2 μM calcein-AM in PBS. Cells were incubated for 20 minutes at room temperature. Each culture plate well was scanned at a 485 nm_{ex}/535 nm_{em} spectra and in a 3X3 grid to account for variations in intra-well cell density. Those 9 values were averaged to generate the fluorescent value for that well. Further, there were 3-6 well replicates per treatment (usually across a given row on a culture plate), and the fluorescent values for those well replicates were then averaged to generate the final, non-standardized value representing the cell survival for that particular treatment.

To standardize the fluorescent value for each culture, a designated plate from each culture was subjected to all rinsing and media exchanges but was treated with 0 μM oxidants, 0 μM glutamate, or normal growth conditions (no OGD). The calcein fluorescence of these wells from each experiment was calculated as described above and assigned "100% Cell Survival," by which all treatments in sister culture plates were compared to generate the final, standardized "% Cell Survival" outcome. This method allows comparisons across different cultures since it is internally controlled.

Statistical analysis:

Data are expressed as "% Cell Survival," ± S.D. or S.E.M. A two-way ANOVA was used to determine differences among genotypes and treatments, with Student Newman-Kuels post-hoc determining significance between group pairs. Cells from a

given litter were cultured across multiple sister plates and represented an n = 1; significance was assigned when $p < 0.05$.

Results

Neurons lacking CyD demonstrate increased cell survival following NO and H_2O_2 oxidant treatments compared to WT neurons:

Oxidants such as nitric oxide (NO) and hydrogen peroxide (H_2O_2) are known to contribute to neurodegenerative pathologies[9,141,142], as well as play a pivotal role in CyD-mediated cell death in non-CNS cells[16,82,84,143]. To test if oxidants also play an important role in mediating CyD-dependent cell death in neurons, WT and CyD KO neurons were treated with DETA-NO, an NO donor, or H_2O_2, and cell survival was quantified 16 hours later. CyD KO neurons were robustly protected from DETA-NO and H_2O_2, showing approximately 40% more cell survival compared to WT neurons following 1000 μM DETA-NO or 1000 μM H_2O_2 treatments (**Figure 4.2**)[15].

A 30 minute treatment of the detergent digitonin (0.002%) was used as a positive control for maximal cell death (i.e. minimal calcein fluorescence). As expected, digitonin treatment resulted in cell survivals of 8.6 ± 3.6% and 8.9 ± 0.4% in WT and CyD KO neurons respectively (**Appendix Figure A4.1**). Furthermore, since 1000 μM H_2O_2 did not reduce the average cell survival in CyD KO neurons (**Figure 4.2C**), 1500 μM H_2O_2 was used to demonstrate at least some loss in cell survival in CyD KO neurons and thereby ensure model integrity. A 16 hour treatment with 1500 μM H_2O_2 resulted in 32.2% cell survival in WT neurons and 55.5% cell survival in CyD KO neurons (n = 1).

Neurons lacking CyD are not protected from glutamate-induced excitotoxicity or OGD compared to WT neurons:

Like oxidant-induced cell death, excitotoxicity and ATP loss, or "energetic failure," are common deleterious phenomena linked to neurodegeneration[8,9], but the roles of neuronal CyD in excitotoxicity and energetic failure are largely unexplored. Accordingly, WT and CyD KO neurons (DIV 9-11) were treated for 10 minutes with glutamate and cell survival was assessed 18 hours later. Unlike oxidant treatment, there was no difference in neuronal cell survival following low-dose (5, 10, and 15 μM, **Figure 4.3A**) or high-dose (100 μM, **Figure 4.3B**) glutamate-induced excitotoxicity between WT and CyD KO neurons. Low dose glutamate excitotoxicity was also tested in more mature neuron cultures (DIV 12-18) to determine if CyD-mediated responses could be a function of cell age, which is known to affect neuronal sensitivity to excitotoxicity[138] and some cyclophilin D-mediated processes[144,145]; however, there remained no differences between WT and CyD KO cell survival following glutamate treatment in these older cultures (**Appendix Figure A4.2**).

CyD has also been implicated in animal models of ischemic stroke[16,144], but investigations into the specific response of neuronal CyD in energetic failure is largely unexplored. Hence, WT and CyD KO neurons were subjected to 2 hours of oxygen and glucose deprivation (OGD), an *in vitro* model for ischemic stroke, and cell survival was assessed 18-24 hours later. There was no difference between WT (76.0 ± 3.5%, n = 2) and CyD KO (73.2 ± 3.1%, n = 4) neuronal survival following OGD (**Figure 4.4**). As a positive control for neuronal protection from OGD, WT and CyD KO neurons were pretreated with 1 μM of MK-801 in normal growth media 24 hours prior to OGD. MK-801 elicited strong protection from OGD (CyD KO 111.0 ± 5.5% vs. WT 91.0 ± 8.8%, n = 3)

independent of CyD expression (**Figure 4.4**), thereby verifying model integrity in detecting cellular protection from OGD.

Astrocytes lacking CyD show increased cell survival following H_2O_2 treatment compared to WT astrocytes:

Astrocytes are emerging as an important mediator in neurodegenerative process[87,90], but the role of astrocytic CyD in these processes has not been thoroughly investigated. Since the above experiments using neurons suggest that CyD mediates specifically oxidant-induced cell death, cultured WT and CyD KO astrocytes were treated with 250, 500, and 750 μM H_2O_2 for 3 hours and cell survival was assessed 24 hours later (750 μM H_2O_2 was used because pilot experiments found that a 1000 μM treatment resulted in near 0% cell survival in both genotypes). CyD KO astrocytes demonstrated increased cell survival following 250 μM H_2O_2 treatment compared to WT cells (WT vs. CyD KO, 36.7 ± 11.4 vs. 64.6 ± 5.3% cell survival, respectively, **Figure 4.5**).

Discussion

The results presented here demonstrate that neurons lacking CyD are protected from cell death following NO and H_2O_2 oxidant treatments compared to WT neurons[15]. Interestingly, CyD does not appear to independently regulate neuronal survival following more complex insults such as excitotoxicity and oxygen and glucose deprivation (OGD), where WT and CyD KO neurons exhibited similar reduced cell survival following either treatment. Similar to neurons, astrocytes lacking CyD were also protected from oxidant-induced cell death. The cell survival data from neurons

and astrocytes suggests that CyD may be more important in mediating oxidant-induced cell death in the mammalian brain, but is either not involved or is masked by parallel, non-CyD-mediated cell death mechanisms following neuronal insults such as excitotoxicity and energetic failure.

Our results showing CyD KO protection from oxidant-induced cell death in isolated neurons (**Figure 4.2**) and astrocytes (**Figure 4.5**) are in agreement with other reports showing similar protection in non-CNS cells and brain tissue lacking CyD[16,82,84]. Oxidants such as NO and H_2O_2 are known to both induce CyD-dependent permeability transition, as well as be produced by CyD-dependent permeability transition[143]; hence, the oxidative damage common in neurodegenerative pathology likely involves CyD, thereby further increasing the relevance of this protein as a novel target to prevent and treat neuronal loss in disease. Furthermore, astrocytes are a crucial cell type facilitating proper CNS function, including antioxidant defense in the brain[87,90]. Taken together, these results suggest that CyD may be a nexus of oxidant-induced cell death in the brain whereby pharmaceutical inhibitors of CyD may provide some protection from neurodegenerative processes resulting from oxidative damage.

Interestingly, the data in this study does not support CyD as an independent regulator of neuronal cell death induced by excitotoxicity or energetic failure, which is in agreement with other recent reports using CyD inactivation to investigate related hypotheses *in vitro*[77,85,86]. Compared to WT, CyD KO neurons demonstrated a similar reduction in cell survival following treatment with glutamate or OGD. This is somewhat surprising considering that, at the *in vivo* level, animals lacking CyD show smaller tissue lesions in a variety of neurodegenerative disease models, including middle cerebral artery occlusion (ischemic stroke)[16,144] and experimental autoimmune encephalomylitis (multiple sclerosis)[15] – of which both disease models also show

evidence of neuronal loss via excitotoxicity[3,8]. Yet, a recent report has linked CyD inactivation to protection from energetic failure[77] (OGD) in hippocampal neurons, although this study relied on pharmacological CyD inhibition where extramitochondrial cyclophilins and/or calcineurin inactivation cannot be ruled out as an experimental artifact.

One possible explanation for the inconsistencies among current descriptions of CyD-mediated cell death is that CyD may facilitate unique functions among different cell types in the brain that coordinate specific responses depending on the disease model. Without such multi-cellular coordination in the *in vitro* systems, it is plausible that the role of CyD may change in isolated brain cells. For example, CyD has been shown to elicit both protective or deleterious processes depending on the animal age[144]. This is an important consideration since most animal models of neurodegeneration use adult mice, where genetic ablation of CyD is protective, while cell culture systems using brain cells rely almost exclusively on embryonic or neonatal tissue, where genetic ablation of CyD is deleterious. Therefore, it is possible that CyD deletion protects neurons from excitotoxicity and energetic failure only in neurons from animals beyond the postnatal day 2 developmental stage.

It is also possible that sex may influence CyD's role in cell death. While the influence of sex has not been reported in CyD literature, animal sex and innate cell sex (*in vitro* preparations free from exogenous sex steroids) are known to dramatically alter the disease profile in animals and cell death paradigms respectively. This includes animal models of ischemic stroke[18], as well as *in vitro* studies reporting innate sex differences in neuronal survival following treatments with glutamate, apoptosis-inducing molecules, and oxidants in neurons[26], and in astrocyte survival following OGD[53,54]. The current investigations did not segregate male and female pups and

therefore may have overlooked potential sex differences in CyD-mediated cell survival outcomes.

Finally, compared to oxidant-induced cell death, excitotoxicity and energetic failure may elicit multiple cell death programs that parallel concomitant CyD-mediated process. Recent evidence supports such a hypothesis as genetic and pharmacological ablation of CyD function did not protect hippocampal neurons from glutamate-induced excitotoxicity[77,85,86], while OGD protection in hippocampal neurons was seen only in the context of other, non-mitochondrial cyclophilins (such as cyclophilin A and B) and calcineurin[77].

Comparing the results presented here among the extant body of CyD-associated literature, CyD emerges most consistently as a facilitator of oxidant-induced cell death, including neuronal and astrocytic cell death. Despite some potential age-dependent roles of CyD[145], nearly every experimental test investigating CyD's role in oxidant-induced cell death have demonstrated protection in CyD KO tissues and cells regardless of preparation, cell type, or disease model. And since nearly every neurodegenerative disease has been linked to oxidative damage, CyD remains as a potential drug target, especially when considering the accumulating evidence that CyD inactivation is protective in an array of neurodegenerative disease models[3,14,74] and that the availability of CyD-specific inhibitors is expanding[14,76,146]. Yet, CyD in the brain seems selective in its protection (at least *in vitro*) since this report and others have found a more limited role for CyD mediating cell death following excitotoxic[77,85,86] and metabolic[77] challenges. Further research into CyD's role beyond oxidant damage in the brain is essential since insults such as excitotoxicity and energetic failure can be equally common and equally deleterious in disease pathology.

Figure 4.1 | Representative PCR product confirming WT and CyD KO breeding lines. Two separate PCR reactions (WT and CyD KO primer set) for each randomly selected animal were conducted and resolved on a single gel. Bands near the 500 bp mark indicate WT genotype, while bands near the 1.6 kb mark indicate CyD KO genotype. Faint 500 bp bands using the CyD KO primer set in WT animals represent low-level WT gene amplification since the CyD KO primer set includes the WT forward primer.

Figure 4.2 | CyD KO neurons show increased cell survival following oxidant treatment compared to WT. **(A)** Representative qualitative images of neuronal calcein fluorescence in culture 16 hours after a 3 hour DETA-NO treatment. Magnification is 20X**. (B)** Neuronal survival 16 hours following a 3 hour DETA-NO treatment. Survival is expressed as a ratio to the control "0 μM" group = 100%. **(C)** Neuronal viability following continuous 16 hour H_2O_2 treatment. For B and C, n = 3-6; + S.D., *p < 0.05 CyD KO vs. WT. This data has been published: Forte, M., *et al*. PNAS (104):7558-63, 2007[15].

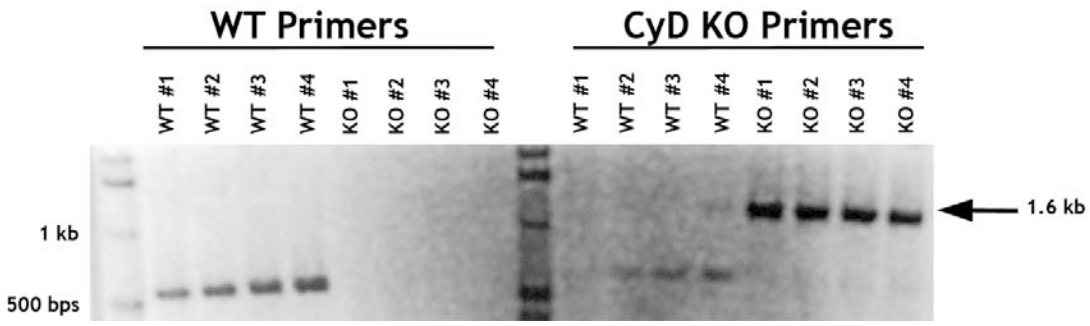
Figure 4.3 | CyD KO neurons are not protected from low or high dose glutamate treatment compared to WT. **(A)** Neuronal survival 18-24 hours following a 10 minute low dose (5, 10 and 15 μM) exposure to glutamate. **(B)** Neuronal survival 18-24 hours following a 10 minute high dose (100 μM) exposure to glutamate. There were no

differences in cell survival between WT and CyD KO neurons in any treatment groups. n = 2-4; + S.E.M.

Figure 4.4 | CyD KO neurons are not protected from oxygen and glucose deprivation (OGD) compared to WT. Neuronal survival 18-24 hours following 2 hour OGD treatment. As an experimental model control, MK-801 addition successfully preserved cell survival in both genotypes up to 48 hours following OGD. n = 2-4; + S.E.M., *p < 0.05 OGD + MK-801 vs. OGD; +p < 0.05 CyD KO vs. WT.

Figure 4.5 | CyD KO astrocytes show increased cell survival following oxidant treatment compared to WT. Astrocyte survival 24 hours following a 3 hour H_2O_2 treatment. n = 4-5; + S.E.M., *p < 0.05 CyD KO vs. WT.

Figure 4.1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CyD KO Primers
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Figure 4.2

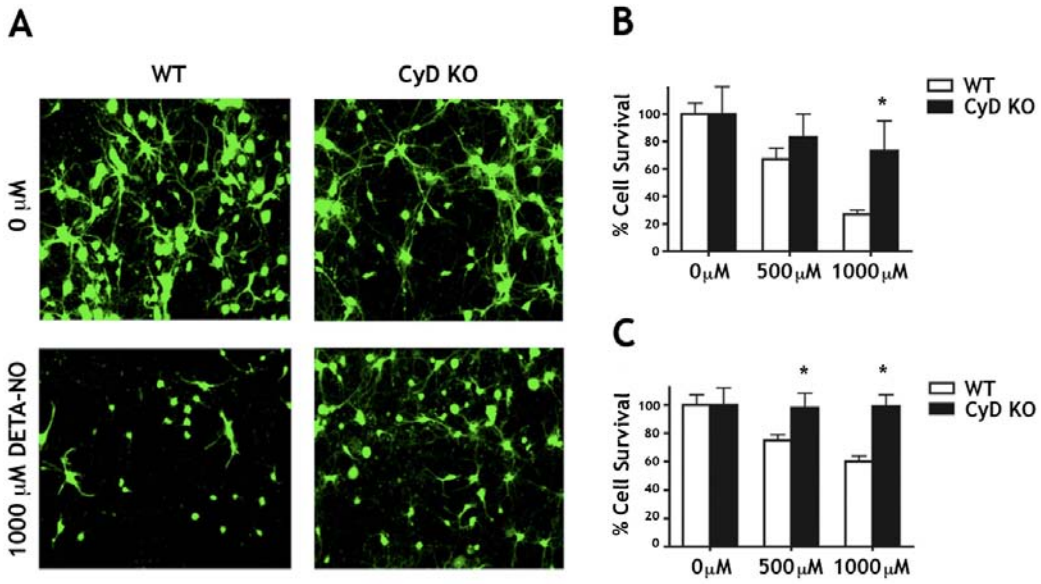
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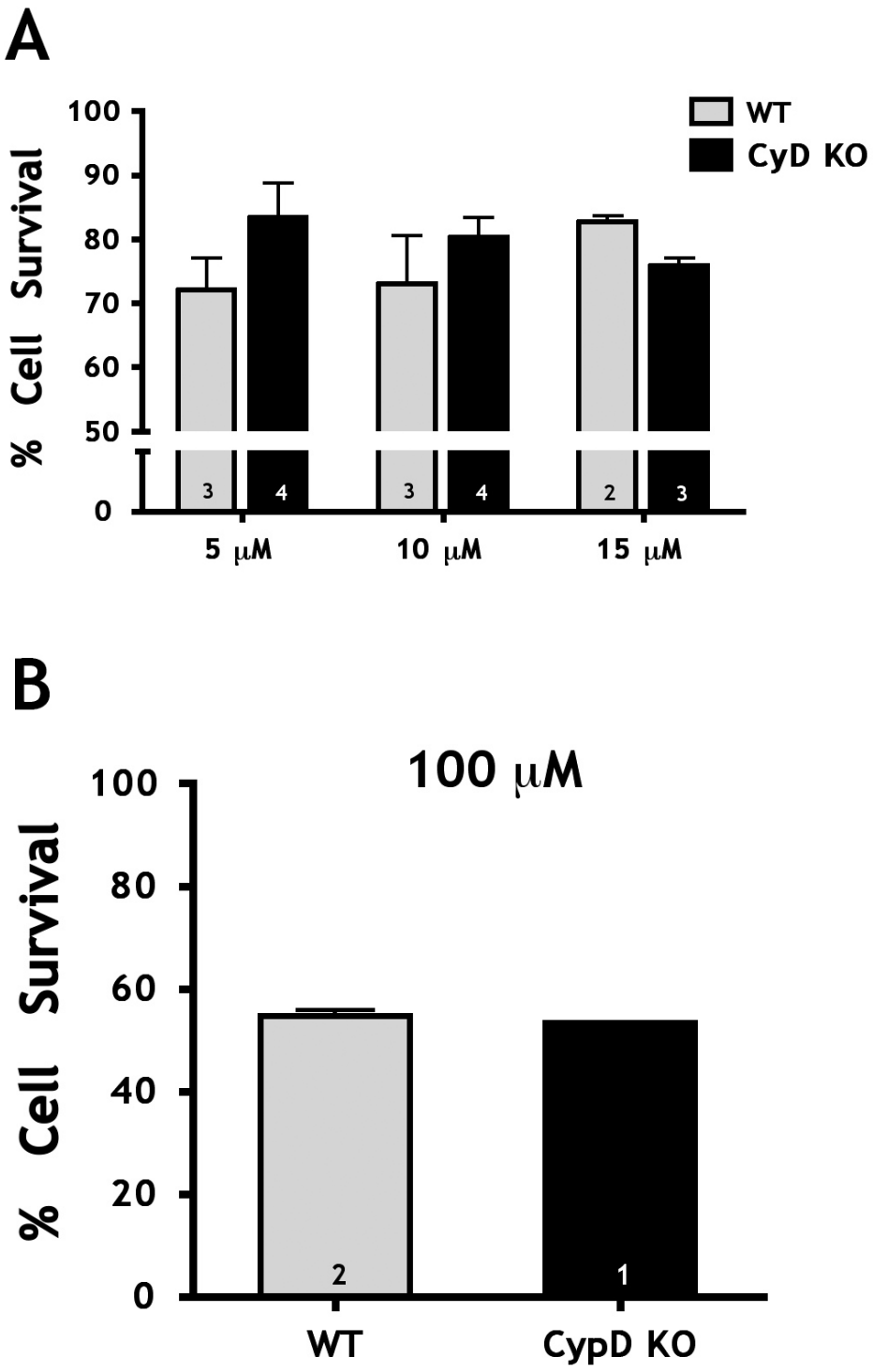
Figure 4.3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3
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80
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40
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1
WT
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Figure 4.4

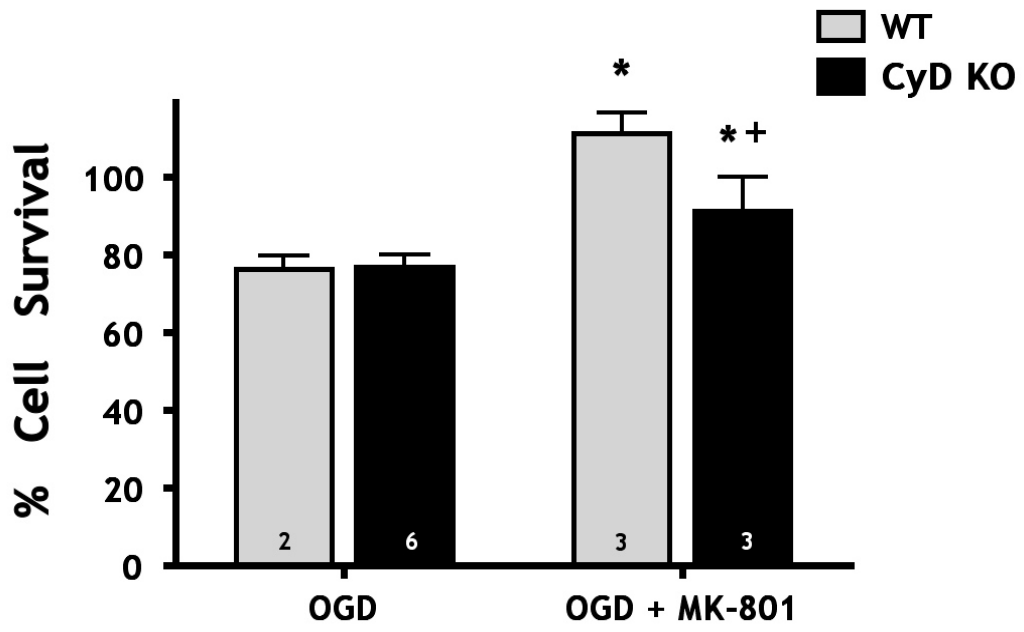
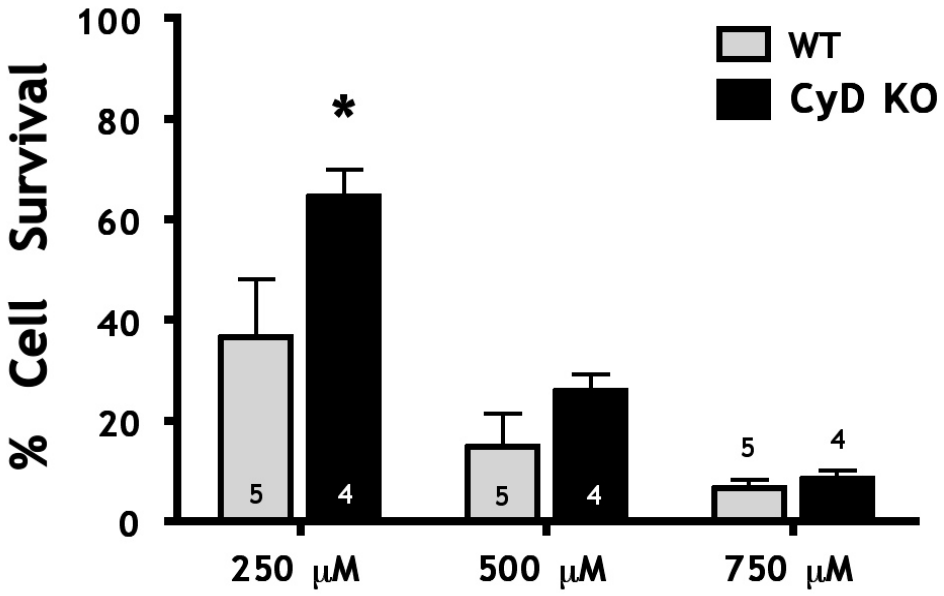


Figure 4.5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100
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4
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4
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4
250 μM
500 μM
750 μM

Chapter 5 | Concluding Remarks

I. Summary of Results

The results presented in this dissertation indicate that: **1**) isoflurane preconditioning (IsoPC) protects isolated astrocytes and neurons regardless of innate cell sex following oxygen and glucose deprivation (OGD); **2**) the sex steroid, 17β-estradiol (E2), attenuates the protective IsoPC response in female but not male neurons independently of the nuclear estrogen receptors (ERs); **3**) innate cell sex is a factor contributing to cell survival outcomes following OGD in neurons but not astrocytes; and **4**) cyclophilin D (CyD) mediates cell survival outcome following oxidant exposure in astrocytes and neurons, but not following glutamate or OGD in neurons.

II. The Role of Innate Cell Sex in the Response of Brain Cells to IsoPC and Subsequent OGD

Exposure to volatile anesthetics such as isoflurane can protect the mammalian brain from subsequent ischemia[43,47]. However, the protective response to IsoPC is sexually dimorphic, with the female brain having a detrimental outcome compared to similarly-treated male animals[48,50]. The investigations described in Chapters 2 and 3 provided the first description of the role of innate cell sex and cell type (astrocytes and neurons) in the response to IsoPC. Regardless of cell type and innate cell sex, IsoPC protected cells from subsequent OGD (**Figures 2.3** and **3.2**). This suggests that (at least in the treatment paradigm used for these studies) female and male cells in the brain have an innate default favoring a protective response to IsoPC, and that

attenuated or detrimental responses to IsoPC may instead result from circulating sex steroids, interactions among multiple cell types, or a combination of these and other unidentified mechanisms. This more complex picture of IsoPC in the mammalian brain is supported by reports demonstrating only partial sex steroid mediation of IsoPC responses in females and males[48-50], and by evidence demonstrating synergistic interactions between astrocytes and neurons that may have implications in anesthetic and non-anesthetic preconditioning [87,88,90], as well as the actions of sex steroids[147].

E2 modulated the IsoPC response only in female neurons, although the effect of IsoPC was eliminated altogether (**Figure 3.3**) rather than having a deleterious response as reported *in vivo*[50]. It is unlikely that this sexually dimorphic response was due to the known increased expression of nuclear ER subtype α in female versus male neurons[122] because the nuclear ER antagonist, ICI, which inhibits both subtypes α and β, did not restore a protective IsoPC response in female neurons (Chapter 3 Results). Additionally, transient pretreatment of female neurons with ICI independently attenuated the protective IsoPC response in female neurons similar to that of E2. This estrogen-like effect of ICI has been reported in the brain[61,130,148] and does not require ER-mediated genomic or non-genomic actions[130], thereby further implicating non-nuclear ER or ER-independent mechanisms in E2's attenuation of the protective IsoPC response.

The suggestion of non-nuclear ER mechanisms mediating the response to IsoPC in female neurons is incongruent with *in vivo* evidence. Both nuclear ER subtypes α and β have been linked to the IsoPC responses in female mice[50]; however, these findings could not resolve the specific neuronal component of the IsoPC response as described here. It remains possible that E2 attenuates IsoPC via other non-nuclear ERs such as GPR30[131], although the role of non-nuclear ERs in IsoPC has not been tested.

ICI did not restore a protective response to IsoPC in female neurons in the presence of E2. Given that ICI and E2 share similar chemical structures to act as antioxidants[18,60,61], it is possible that E2 may attenuate IsoPC in female neurons through antioxidant action since antioxidants can attenuate protective responses to volatile anesthetic preconditioning[47,93]. This scenario, however, would require E2 and/or IsoPC to have different mechanisms between male and female neurons since E2 did not interfere with the protective response to IsoPC in male neurons as it did in female neurons. Such conclusions remain to be directly tested.

III. The Role of Innate Cell Sex in the Response of Brain Cells to OGD Independent from IsoPC

Ancillary to our primary investigations concerning the role of innate cell sex in the response to IsoPC and subsequent OGD, the experimental design of the investigations in Chapters 2 and 3 allowed additional preconditioning-independent analyses of sex differences in astrocytes and neurons following OGD. These analyses are valuable because sexually dimorphic responses are known to exist in models of ischemic stroke, both *in vivo*[18,19] and *in vitro*[28,52], and because women and men have different risk factors for ischemic stroke and stroke outcome independent of circulating sex steroids[18,19].

As reported in Chapter 2, female and male astrocytes had similar sensitivities to OGD independent of preconditioning. This finding contrasts with other reports demonstrating reduced sensitivity of female astrocytes to OGD that is dependent upon P450 aromatase activity[53,54]. While the reasons for this disparity are unclear, a likely explanation resides within different astrocyte culturing procedures. The investigations

in Chapter 2 used an additional culture preparation step (plate swirling) to more thoroughly remove non-adherent, non-astrocytic cells such as microglia. Although not directly tested here, microglia secrete oxidants as a part of their normal function[142] which may alter the cell survival outcome in neighboring astrocytes following OGD as oxidants are known to mediate sex-specific responses to ischemia in the brain[25,149].

The data in Chapter 3 demonstrate that isolated female cortical neurons are less sensitive to OGD compared to male neurons independent of preconditioning (**Figure 3.2**), which is consistent with other reports in the literature. Female rodents have long been appreciated to be less sensitive to models of ischemic stroke compared to age-matched males[18,19], brain slices from female mice are less sensitive to hypoxia compared to males[28], and isolated female and male neurons have sexually dimorphic sensitivities depending upon the specific cellular insults[26,27,122] and brain region[52]. The investigations in Chapter 3 were the first to evaluate the role of innate neuronal sex in the cortex following OGD.

The finding that female neurons are less sensitive to OGD compared to male neurons is an important contribution towards advancing our understanding of neurodegeneration. Innate cell sex is increasingly being identified as an important mechanism in itself that mediates sex-dependent outcomes in models of neurodegeneration[26-28,52]. Innate cell sex in astrocytes, however, may or may not contribute to survival outcome following energetic failure (OGD) depending upon as-yet unidentified additional mediators. Further investigations are required to better resolve the role of innate cell sex in astrocyte survival following OGD.

IV. The Role of Cyclophilin D in Isolated Brain Cells Following Specific Cellular Insults

The investigations described in Chapter 4 further clarify how CyD may mediate neurodegeneration by showing oxidant-specific protection in isolated neurons and astrocytes lacking CyD protein. Previous studies of CyD have largely used isolated mitochondrial preparations or whole tissue[14,74]. Thus, the results in Chapter 4 expand our knowledge of CyD by demonstrating how CyD may be important on a cellular level in the brain.

Neurons from CyD KO mice resisted oxidative insults (H_2O_2 and NO) but not excitotoxic insults (glutamate) or energetic failure (OGD) (**Figures 4.2, 4.3,** and **4.4**), consistent with reports using non-neuronal cells from CyD KO animals[16,44,82,84]. Moreover, the results in Chapter 4 further implicate CyD as an important mediator of oxidant-induced cell death by showing that isolated cortical astrocytes resist H_2O_2-induced cell death (**Figure 4.5**) similar to CyD KO neurons (**Figure 4.2**). This is an important result considering that hippocampal astrocytes have been shown to differ from neurons in their sensitivity to H_2O_2 depending in part on pharmacologic CyD inhibition[150].

Yet it is interesting that CyD KO neurons are equally vulnerable to excitotoxic and energetic challenges compared to WT (**Figure 4.3** and **4.4**) given that CyD KO animals resist multiple models of neurodegeneration *in vivo* that are known to involve excitotoxic and energetic challenges in the brain[2,15,16,44]. One explanation for this discrepancy may lie in the age of the animals and derived cells used for experimentation since CyD activity has been shown to have either pro-survival or pro-death effects depending upon the developmental stage of the animal[144]. However, this

particular report did not consider oxidant injury. Innate cell sex could be another factor mediating differential responses to cellular insults in CyD KO cells since the cultures used in Chapter 4 were not sex-segregated. Sex-specific roles for CyD following oxidant, excitotoxic, or energetic challenges have not been investigated despite the known sexually dimorphic responses to these insults outside the context of CyD[26,28,51,52,122] (and **Figure 3.2**). Nonetheless, the data in Chapter 4 further support existing data[15,16,44] that suggests CyD, presumably via mitochondrial permeability transition, mediates specifically oxidant signaling in cell death processes.

V. Experimental Novelty and Limitations

The experimental designs described in this dissertation uniquely contribute to our understanding of the complex neurodegenerative processes in the brain. The use of isolated female and male cells in Chapters 2 and 3 resulted in the first descriptions of not only sex-specific responses to IsoPC and subsequent OGD, but also sex-specific responses to OGD independent of IsoPC. Furthermore, in contrast to previous reports comparing absolute cell survival data, the novel use of the difference of paired means statistical analysis ("2-way repeated measures ANOVA") in Chapters 2 and 3 increased the resolution of differences between ShamPC, IsoPC, female, and male data sets, as well as potential interactions therein between preconditioning and innate cell sex. In settings that allow for a paired littermate analysis *in vitro*, this statistical approach may help identify important data trends that would otherwise be overlooked in more traditional statistical approaches. Chapter 4 represents the first use of isolated brain cells with genetic ablation of CyD to more specifically assess the role of CyD in cell survival outcomes following specific insults associated with neurodegeneration. Both

of the major themes developed and investigated in this dissertation – that of the cellular response to IsoPC and subsequent OGD and that of CyD following specific cellular insults – consider the role of astrocytes, in addition to neurons, as potentially important contributors among the cellular mechanisms of neurodegeneration and neuroprotection.

As with all *in vitro* investigations, however, an inherent limitation of the investigations described here was reliance upon a simplified cellular environment removed from the intact brain. Yet, this limitation is also a strength in that the *in vitro* experimental designs used here provided important information about how specific brain cells, whether neurons or astrocytes or whether innately female or male, may respond differently to specific insults. This level of resolution is not easily accomplished in the intact brain and provides important information that can advance our understanding of neurodegeneration as well as to advance the development of potential neuroprotective strategies.

An additional limitation of the current investigations was the reliance upon terminal cell death or cell survival outcomes – that of lactate dehydrogenase release into the media (Chapter 2) or calcein-AM fluorescence within intact cells (Chapters 3 and 4). These two outputs represent endpoints in neurodegenerative processes and overlook potentially important intermediate processes, such as specific protein phosphorylation and translocation events, that may distinguish different neurodegenerative disorders and potential neuroprotective targets. However, a terminal output of cell viability is valuable because ultimately all neurodegenerative processes converge at a loss of neurons and/or astrocytes. Therefore any potential neuroprotective strategies that can ultimately extend survival of brain cells, such as anesthetic preconditioning or ablation of CyD function, deserve attention.

VI. Implications for Future Directions

There remains much to be understood about the mechanisms of IsoPC-mediated neuroprotection and about the physiologic function of CyD as CyD KO mice display only mild phenotypes in the absence of major insults[74]. One potential link between these seemingly disparate biological processes is oxidant signaling. Indeed, evidence supports reactive oxidative species as signaling molecules for a variety of protective preconditioning stimuli in that sub-lethal levels of oxidants are generally induced by preconditioning[151] while antioxidants typically block protective preconditioning responses[47,93,97,151-156]. Furthermore, sub-lethal oxidant exposure in the absence of preconditioning stimuli elicits a protective response comparable to that of preconditioning[34,154,156-161].

The mitochondrion, which is the largest intracellular producer of oxidant species, has emerged as a potential convergence point mediating preconditioning stimuli[30,162]. Mitochondria produce oxidants through multiple mechanisms, including uncoupling of the electron transport chain, opening of the mitochondrial ATP-sensitive potassium channel[30,133], and CyD-mediated permeability transition[3,14], all of which could facilitate downstream preconditioning adaptations[30]. Most reports investigating CyD function have focused on the lethal effects of massive oxidant production following sustained permeability transition[3,14]. However, recent evidence indicates that stochastic, sub-lethal superoxide bursts or "flashes" are produced in quiescent myocytes and neurons resulting from coupling between CyD-sensitive permeability transition and the electron transport chain[72]. These superoxide flashes can lead to the production of low levels of other oxidant species which collectively may serve important physiological functions[34,163,164].

Hence, based on the existing literature, a potential model emerges whereby various preconditioning stimuli may converge at the mitochondrion to elicit oxidant bursts through CyD-mediated permeability transition as shown in **Figure 5.1**. In further support of the model proposed in **Figure 5.1**, Chapter 4 of this dissertation demonstrated that CyD KO neurons and astrocytes were selectively protected from oxidant injury compared to other cellular insults. Similar oxidant-specific protection has been reported in CNS and non-CNS CyD KO cells[16,44,82,84]. Considering that sub-lethal pharmacologic and hypoxic stress elicits increased CyD-mediated superoxide bursts[72], and that protective preconditioning stimuli can in themselves be considered a "sub-lethal stress"[71,152,162,165,166], a possible function for CyD in quiescent cells is to communicate potentially noxious environmental changes to the cell via oxidant signaling to promote adaptive responses and encourage survival.

Protection of the brain via CyD-mediated oxidant signaling induced by preconditioning could be tested in CyD KO mice using a protocol that normally induces a protective preconditioning response, such as IsoPC, in a male mouse[48], with the expectation that the protective response would be abolished in the CyD KO animal versus a matched WT control. Indeed, male rat hearts lacking CyD or treated with cyclosporine A to inhibit CyD-dependent permeability transition show no benefit from ischemic preconditioning compared to WT or untreated control hearts[152,166]. Moreover, CyD-dependent oxidant production was required to phosphorylate Akt and ERK1/2[166], proteins that mediate protective preconditioning in a number of different models[30,34], including anesthetic preconditioning in the brain[48,126]. The reports by Hausenloy *et al.*[152,166], however, represent the only investigations identifying CyD-mediated permeability transition as a convergent signal/sensor mechanism (or "module" as

defined by Dirnagl and Meisel[30]) in cardiac preconditioning. There have been no similar investigations in brain preconditioning models.

This proposed model requires CyD-dependent oxidant signaling to differ between female and male cells in order to be consistent with the observed sexually dimorphic responses to IsoPC[39,48-50] (and **Figure 3.3**) and other forms of preconditioning *in vivo*[37,38,40-42]. It may be that these oxidant bursts promote sex-specific adaptive downstream cellular responses via direct regulation of transcription factors like nuclear factor κB[167], which can be neuroprotective[168] and has been linked to preconditioning, E2 signaling, and sex-specific actions[130,168,169]. Another potential sex-specific adaptive downstream mechanism might be Akt phosphorylation. Unfortunately, the only investigation of CyD-mediated preconditioning mechanisms[166] has been limited to hearts from male animals. However, this study did demonstrate that CyD-mediated oxidant signaling is required for Akt phosphorylation. Akt phosphorylation in turn has been induced by IsoPC in male but not female brain and implicated in the protective preconditioning response in male brain following IsoPC[48].

It is also possible that the oxidant profile induced by CyD-mediated preconditioning mechanisms may differ between the sexes. Superoxide is a likely candidate oxidant species for mediating sex differences in protective preconditioning responses in that has been identified as a signal promoting a protective response to IsoPC in male rabbit myocardium[151]. Furthermore, preliminary data generated as a part of this dissertation suggests that IsoPC induces greater superoxide production in male brain compared to female brain (**Figure A5.1**). However, superoxide levels induced by IsoPC appear to be much lower than the damaging levels induced by transient focal cerebral ischemia (**Figure A5.1**). Most importantly and of relevance to

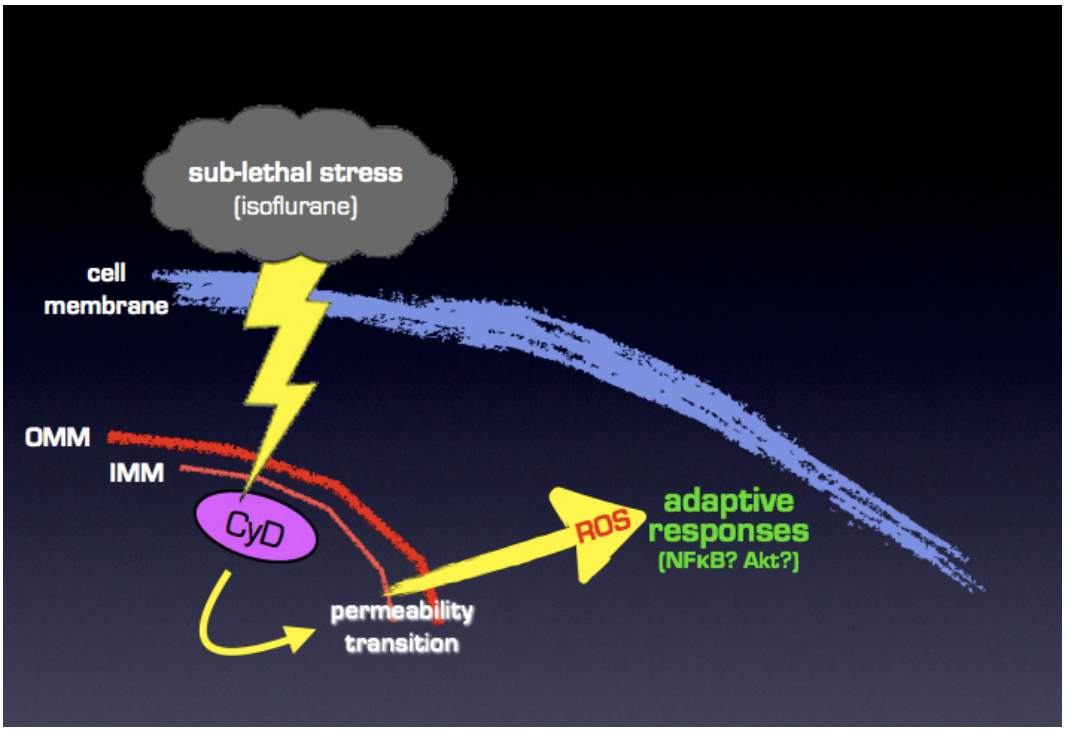
the proposed model in **Figure 5.1**, superoxide production is facilitated by CyD-mediated stochastic permeability transition[72].

Future experiments could take an *in vivo* and *in vitro* approach to confirm sexually dimorphic superoxide production following IsoPC in female and male brains, and in isolated cells, as well as use CyD KO animals to test whether CyD is required to generate the superoxide signal that may be responsible for the male-specific protective response to IsoPC. Similar experiments *in vitro* could test the superoxide profile in isolated female and male astrocytes since these cell types also demonstrated a protective response to IsoPC (**Figure 2.3**), although the effect of E2 on the astrocytic response to IsoPC has not yet been tested.

Investigating the potential links among IsoPC, CyD, oxidant signaling, and sexually dimorphic responses to ischemia would not only advance our understanding of the mechanisms of neurodegeneration and potential neuroprotective strategies, but also advance our understanding of rudimentary cellular physiology as the teleological function of CyD remains a mystery[74].

Figure 5.1 | Model of how cyclophilin D (CyD) may facilitate adaptive responses following sub-lethal environmental insults, including modest isoflurane exposure, by promoting mitochondrial permeability transition and associated reactive oxygen species (ROS). OMM, outer mitochondrial membrane; IMM, inner mitochondrial membrane.

Figure 5.1


sub-lethal stress
(isoflurane)
cell
membrane
OMM
IMM
CyD
ROS
adaptive
responses
(NFκB? Akt?)
permeability
transition

Appendix

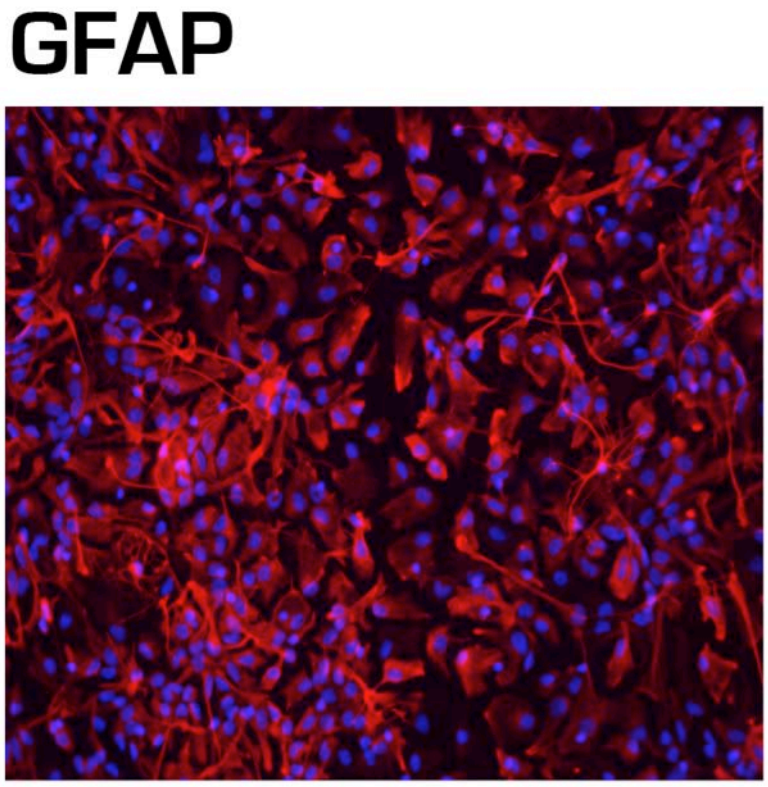


Figure A2.1 | Representative confluent postnatal astrocyte culture using glial fibrillary acid protein (GFAP) as an astrocyte-specific marker and 4',6-diamidino-2-phenylindole ("DAPI") as a nuclear stain.

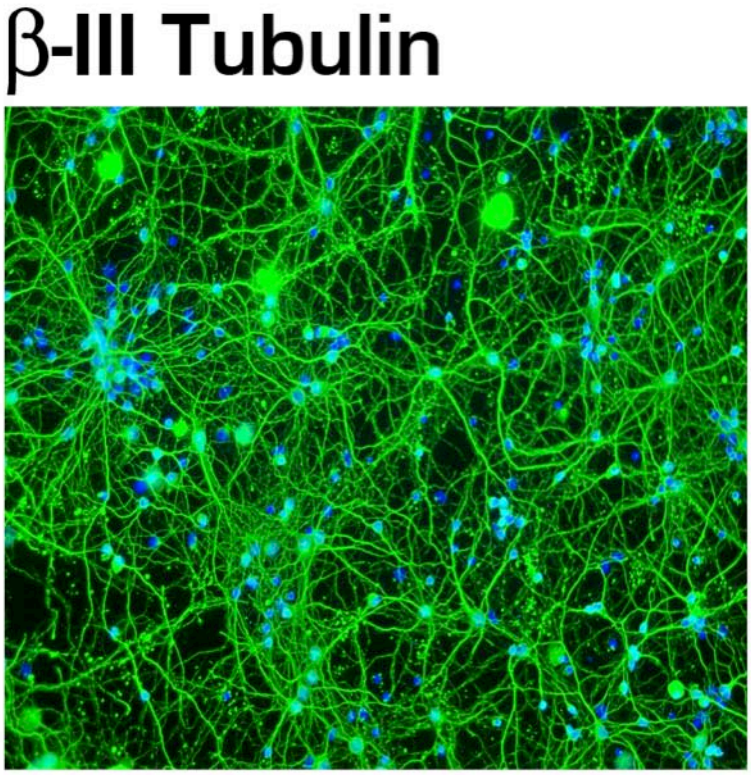


Figure A3.1 | Representative postnatal neuron culture (10 days *in vitro*) using β-III tubulin as a neuron-specific marker and 4',6-diamidino-2-phenylindole ("DAPI") as a nuclear stain.

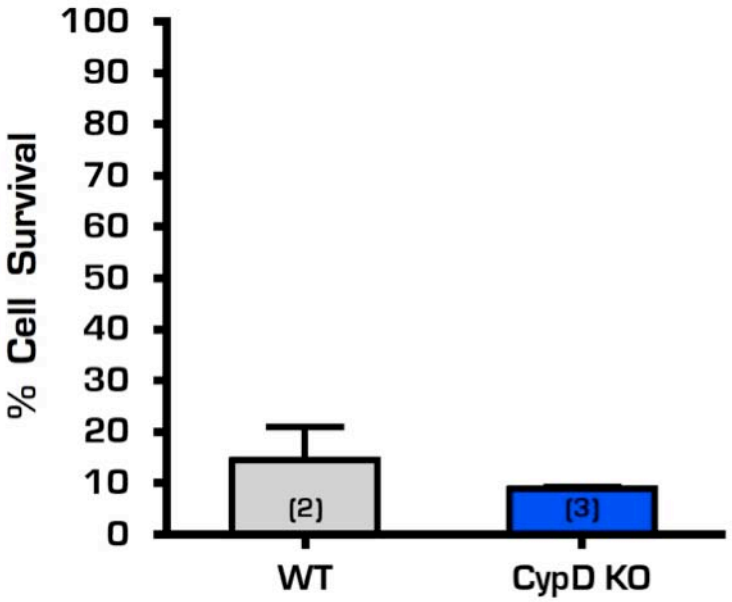


Figure A4.1 | Calcein-AM fluorescence is significantly reduced by addition of digitonin as a positive control for maximal cell death (minimum cell survival). 0.002% digitonin was added to neuronal cultures for 10 minutes, followed by a 24 hour recovery period before cell survival assessment.

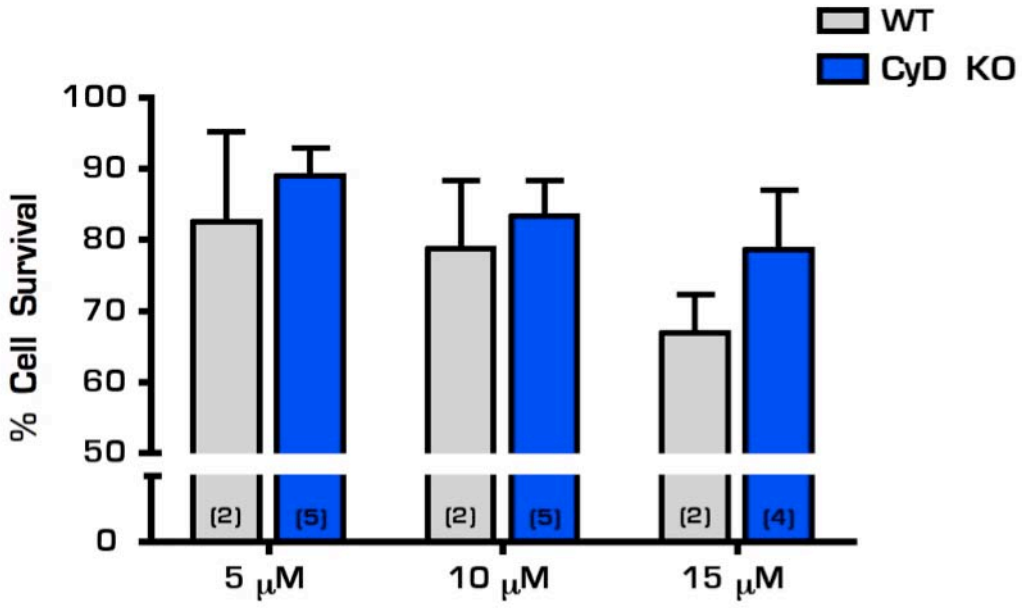


Figure A4.2 | Day-*in-vitro* (DIV) 12-18 cyclophilin D knockout (CyD KO) neurons are not protected from low-dose glutamate-induced excitotoxicity compared to wild-type (WT). DIV 12-18 CyD and WT neurons were exposed to 5, 10, or 15 μM glutamate for 10 minutes followed by a 24 hour recovery period before cell survival assessment.

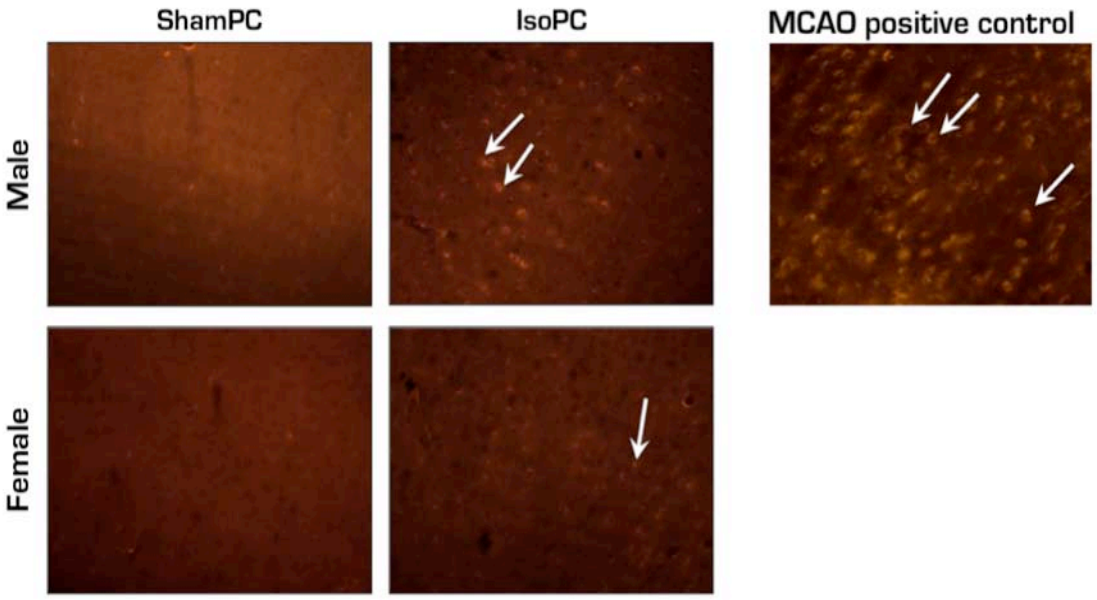


Figure A5.1 | Demonstration of increased superoxide production in male versus female cerebral cortex following isoflurane preconditioning (IsoPC). Adult male and female C57BL/6 mice were injected via the jugular vein with hydroethidine, which is selectively oxidized by superoxide to the fluorescent molecule, ethidium, 30 min before a 4 hour sham preconditioning (ShamPC) or 1% IsoPC period. Mice were then anesthetized with isoflurane and euthanized by transcardial perfusion with saline and 3.7% paraformaldehyde 1 hour following preconditioning. Fluorescence was assessed in 30 μm sections at λ_{ex} = 510-550 nm and λ_{em} > 580 nm. One hour of middle cerebral artery occlusion (MCAO) in unconditioned male cortex, which is known to induce superoxide production, was used as a positive control. Arrows indicate cells positive for accumulated superoxide.

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