Are There Sex-Linked Differences Across the Longitudinal Axis of the Rat Hippocampus Following Ischemic Injury?

by

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Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

Ischemic strokes occur when an occluded blood vessel limits the delivery of oxygen and nutrients to neurons, and can result in widespread cell death. Although stroke is the third leading cause of death and a major cause of disability among Canadians, the ability to treat this condition is limited, in part, due to an incomplete understanding of the underlying factors that influence cell death. The hippocampus, a structure intimately involved with learning and memory, is highly vulnerable to ischemic brain injury; however, evidence suggests that the various regions of the hippocampus do not respond homogenously to insult. In addition, previous work has shown that differences in response to injury also exist between male and female brain tissue. As a result, the present study aimed to investigate how various factors, including hippocampal region and sex, may influence variability in response ischemic insult. Acute hippocampal slices prepared from the septal and temporal poles of male and female Sprague-Dawley rats were subjected to an *ex vivo* model of stroke, oxygen-glucose deprivation (OGD). Cell viability assays, which measured mitochondrial function (*2,3,5*-triphenlytetrazolium chloride metabolism) and cell membrane integrity (lactate dehydrogenase release), were used to examine post-OGD slice viability. To better understand the role that the primary sex hormones, estrogen and testosterone, play in response to neuronal damage, tissue from rats at post-natal day 26-29 (pre-adolescence) was compared to tissue from rats at post-natal day 61-64 (post-adolescence). The findings from this study suggest that hippocampal tissue harvested from the septal pole of male, pre-adolescent, rats displayed the greatest sensitivity to ischemic insult.

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4VO	4 Vessel Occlusion
ACSF	Artificial Cerebral Spinal Fluid
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor
ATP	Adenosine Triphosphate
BBB	Blood Brain Barrier
CA1	cornu Ammonis 1
CA3	cornu Ammonis 3
CAMKII	Calcium-Calmodulin-Dependent Protein Kinase
CNS	Central Nervous System
CREB	Cyclic-AMP Response Element Binding Protein
DG	Dentate Gyrus
ERα	Estrogen Receptor Alpha
fEPSP	Field Excitatory Post Synaptic Potentials
GluA1-4	Glutamate AMPA receptor 1-4
GluN1-4	Glutamate NMDA receptor 1-4
GPx	Glutathione Peroxidase
iLTP	Ischemic Long-Term Potentiation
LDH	Lactate Dehydrogenase
LTED	Long-Term Estrogen Deprivation
LTP	Long-Term Potentiation
MAPK	Mitogen Activated Protein Kinase
MCAO	Middle Cerebral Artery Occlusion
MWM	Morris Water Maze
NAD	β-nicotinamide Adenine Dinucleotide
NMDA	N-methyl D-aspartate
NMDAR	N-methyl D-aspartate receptor
OGD	Oxygen-Glucose Deprivation
OVX	Ovariectomized
PFC	Prefrontal Cortex
PND	Postnatal Day
PTM	Post-translational Modifications
RNS	Reactive Nitrosylative Species
ROS	Reactive Oxygen Species
rtPa	Recombinant Tissue Type Plasminogen Activator
SBDPs	Spectrin Breakdown Products
SOD	Superoxide Dismutase
TBI	Traumatic Brain Injury
tLTP	Tetanic Long-Term Potentiation
TTC	*2,3,5*-Triphenlytetrazolium chloride
VS	Ventral Striatum
VF	Ventricular Fibrillation

1.0 Literature Review

1.1 Introduction to Ischemic Stroke

Ischemic stroke is characterized by the reduction of cerebral blood flow, leading to an insufficient delivery of oxygen and nutrients to neurons (Heart and Stroke Foundation, 2015). Approximately 80% of all strokes are ischemic in nature, and have two main forms: global and focal (Columbia Neurosurgery, 2015; Heart and Stroke Foundation, 2015). Global ischemia is regarded as the reduction of blood flow to a wide range of neuronal tissue, whereas focal ischemia is restricted to a specific area of brain (Columbia Neurosurgery, 2015). With focal ischemia, the majority of damage is located within the ischemic core, a region of permanently infarcted tissue where blood flow is 10-25% of its normal perfusion rate (Internet Stroke Center, 2015). Surrounding this area is a region of salvageable tissue, known as the ischemic penumbra. The loss of cerebral blood flow in the penumbra is less dramatic (25-50% of normal perfusion), thereby providing a greater chance of recovery from injury (Internet Stroke Center, 2015). Due to the delicate nature of brain tissue, the severity of ischemic injury is largely influenced by the duration of insult. Thus, if the ischemic insult persists over a sustained period of time, the ischemic core will expand into the penumbra and increase the extent of irreversible damage (Internet Stroke Center, 2015).

In Canada, stroke is the third leading cause of death, and a major cause of disability. While more than 14 000 Canadians will die from a stroke each year, another 315 000 will suffer from its debilitating side effects (Heart and Stroke Foundation, 2015). Impairments in cognition, memory and motor coordination are common after-effects of stroke, and the Canadian economy is estimated to spend approximately 3.6 billion dollars annually in stroke related costs, including hospital and physician care (Heart and Stroke Foundation, 2015). Despite the severe socio-

economic burden that stroke imposes, however, the ability to treat this condition remains limited, primarily due to an incomplete understanding of the underlying mechanisms driving stroke-related cell death (Kostandy, 2012).

1.2 Pathobiology of Ischemic Stroke

Following ischemic stroke, a number of cellular pathways are activated, and contribute to neuronal toxicity (*See Figure 1 for a schematic summarizing the pathophysiology of stroke*). Initially, levels of oxygen and glucose within the brain are unable to meet the metabolic demands of brain tissue, which results in a depletion of ATP (Szydlowska and Tymianski, 2010; Kostandy, 2012). Energy dependent processes, including function of the Na^+/K^+ ion pump, begin to fail, causing membrane depolarization. With depolarization, there is a Ca^{2+}-mediated release of glutamate, the primary CNS excitatory neurotransmitter, from pre-synaptic vesicles (Szydlowska and Tymianski, 2010; Kostandy, 2012). Released glutamate acts upon three known ionotropic receptors; however, the two most related to cellular damage during ischemia are the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor (AMPAR) and the N-methyl D-aspartate receptor (NMDAR) (Szydlowska and Tymianski, 2010; Chang et al, 2012; Parsons and Raymond, 2014).

Both AMPARs and NMDARs are tetrameric heteromers that play a role in mediating fast excitatory synaptic transmission (Michaelis, 1998; Traynelis, 2010). While AMPARs are comprised of subunits GluA1-4 (Michaelis, 1998), NMDARs are composed of two obligatory GluN1 subunits, as well as two of six additional subunits, GluN2 (A-D) and GluN3 (A and B) (Michaelis, 1998; Traynelis, 2010). Under basal conditions, the majority of AMPARs contain the Ca^{2+}-impermeable GluA2 subunit; a subset of receptors are, however, GluA2 lacking (Ca^{2+} permeable), and promote Ca^{2+}-mediated signalling events (Traynelis, 2010; Blanco-Suarez and

Hanley, 2013). Also, at a resting membrane potential, the NMDAR channel is blocked by Mg^{2+} ions, which reduces Ca^{2+} permeability; however, following high frequency stimulation, the Mg^{2+} ion is expelled from the pore and Ca^{2+} influx into the post-synaptic cell is permitted (Traynelis, 2010; Parsons and Raymond, 2014).

During normal physiological conditions AMPARs and NMDARs mediate cognitive functions including learning and memory; however, following ischemia these receptors become overstimulated and initiate a number of negative cellular mechanisms that eventually trigger a phenomenon known as excitotoxicity (Nikonenko et al., 2009; Szydlowska and Tymianski., 2010; Kostandy, 2012). Initially, glutamate induced excitotoxicity involves an influx of Ca^{2+} and Na^{+}, which can indirectly damage neurons through the activation of certain cellular cascades. For example, excess Ca^{2+} will stimulate the production of reactive oxygen species (ROS) and nitrosylative species (RNS), which leads to free radical damage and mitochondrial dysfunction. Proteases, lipases and endonucleases are also activated, which can trigger the breakdown of cytoskeletal components and reduce cell membrane integrity. In addition, the activation of microglia can also cause damage through the release of cytotoxic molecules, including proinflammatory cytokines, which trigger an inflammatory response and eventually results in cell death (Kostandy, 2012). The form of cell death undergone depends on the level of glutamate exposure and degree of neuronal insult experienced (Lipton and Nicotera, 2000; Szydlowska and Tymianski, 2010). Injured neurons located within the penumbra will often go through apoptotic cell death, in order to remove damaged tissue in a regulated manner (Lipton and Nicotera, 2000). In contrast, neurons located within the ischemic core may undergo a more immediate form of cell death, known as necrosis (Lipton and Nicotera, 2000). Although the direct cellular mechanisms of Ca^{2+} -mediated excitotoxicity are incompletely understood, two theories are often

considered. The first is the "calcium-overload hypothesis," which suggests that when concentrations of intracellular Ca^{2+} surpass a particular threshold, cytotoxicity is triggered (Szydlowska and Tymianski, 2010). A second theory, the "source specificity hypothesis," argues that it is not the quantity of Ca^{2+}, but the specific path of entry and second messenger pathways activated, which lead to neurodegeneration (Szydlowska and Tymianski, 2010).

Due to the detrimental outcomes associated with excitotoxicity, pharmacological therapies have targeted the glutamate system, specifically through the use of glutamate receptor antagonists, in order to evoke neuroprotection following ischemic stroke (Santangelo et al., 2012). Unfortunately, nearly all of these treatments have failed during clinical trials due to a limited window of opportunity and suppression of the normal physiological functions of the glutamate receptors (Kalia et al., 2008). Despite attempts at designing glutamate-based neuroprotective therapies, the only current FDA approved treatment for stroke is a thrombolytic agent known as recombinant tissue type plasminogen activator (rtPa), which catalyzes the transformation of plasminogen into plasmin to promote fibrinolysis and restore blood flow (Montagne et al., 2012; Ali et al., 2014; Suzuki et al., 2016). However, the thrombolytic properties of this drug can also enhance the risk of intracranial bleeding through increasing permeability of the blood-brain barrier (BBB) (Suzuki et al., 2016). In addition, rtPa has been shown to modify NMDARs such that they display greater sensitivity towards excitotoxicity, potentially exacerbating ischemic damage in patients receiving treatment (Montagne et al., 2012; Suzuki et al., 2016). While most research in the field has focussed on the pharmacological treatment of stroke, more work surrounding its basic pathogenesis needs to be done in order to improve our understanding of this condition.

1.3 Ischemic Stroke and Regional Vulnerability

1.3.1 Regional Vulnerability between Hippocampal Subfields

Global ischemia disrupts blood flow to a broad range of neuronal tissue; however, it is well recognized that certain areas of brain are more susceptible to ischemic injury than others. In 1922, the term "pathoclisis" was coined to describe the selective vulnerability to pathological insult observed across anatomical regions (Vogt and Vogt, 1922). Based on the Vogt's notion of pathoclisis, the hippocampus, a structure involved in the formation and consolidation of long-term and spatial memory, is often given considerable attention (Schmidt-Kastner and Freund, 1991; Grivas et al., 2003; Nikonenko et al., 2009; Nunez, 2012; Lalonde and Mielke, 2014). Located in the medial temporal lobe, the hippocampus is composed of varying subfields, including the *cornu Ammonis 1* (CA1), *cornu Ammonis 3* (CA3) and dentate gyrus (DG); however, even among these neuronal subpopulations there appears to be heterogeneity in susceptibility to cellular damage (Schmidt-Kaster and Hossman, 1988; Gee et al., 2006; Lalonde and Mielke, 2014). Previous animal studies have shown that, while the CA region, particularly CA1 pyramidal cells, are the most sensitive to brain injury, cells in the DG appear to be more robust (Akai and Yanagihara, 1993; Gee et al., 2006; Lalonde and Mielke, 2014).

While the fundamental mechanisms for these differences remain unclear, one explanation may be differences in the function and distribution of glutamate receptors among hippocampal sub-fields. Gee et al. (2006) observed that, after global ischemia, CA1 pyramidal neurons underwent delayed cell death, coincident with tyrosine kinase activity that caused an upregulation in NMDAR responses. On the other hand, the CA3 region displayed a decrease in NMDAR function after cerebral ischemia, driven by protein phosphatase signalling. These findings suggest that neurons in possession of characteristics leading to a decrease in NMDAR

activity contribute to a more robust phenotype (Gee et al., 2006). Literature surrounding this topic remains controversial, however, as studies have also found there to be a decrease in the expression and functionality of the NMDARs in the CA1 region, but an increase in expression in the DG region (Zhang et al., 1997; Hsu et al., 1998). Similarly, research has also suggested that ischemia promotes selective AMPAR trafficking within the CA1 region; internalization of GluA2 subunits is followed by the insertion of Ca^{2+}-permeable GluA2-lacking subunits onto the plasma membrane (Soundarapandian et al., 2005; Liu et al., 2006; Blanco-Suarez and Hanley, 2013). Ultimately, increased Ca^{2+} permeability within CA1 pyramidal cells may account for their enhanced vulnerability to ischemic insult (Soundarapandian et al., 2005; Blanco-Suarez and Hanley, 2013).

1.3.2 The Longitudinal Axis of the Hippocampus: Anatomical Differences

Apart from the functional differences that exist between hippocampal subfields, there is a growing body of literature to support the notion that notable differences are also present across the longitudinal axis of the hippocampus (Nadel et al., 1968; O'Keefe and Nadel, 1978; Ashton et al., 1989; Czerniawski et al., 2009; Fanselow and Dong, 2010; Isaeva et al., 2015; Strange et al., 2014; Bekiari et al., 2015). Although a distinct anatomical definition is not agreed upon, in general, the hippocampal long-axis is segmented into two poles, the septal pole (dorsal/posterior) and the temporal pole (ventral/anterior). The two poles are arbitrarily separated by an intermediate portion of tissue; however, intermediate hippocampal slices are thought to behave more like those obtained from the septal region (Strange et al., 2014; Maggio et al., 2015). *See Figure 2 for a diagram of the longitudinal axis of the hippocampus.*

The idea that neuronal connectivity along the septo-temporal axis of the hippocampus is heterogeneous dates back as early as 1934 (Lorente de No). A recent review by Strange et al.

illustrates that projections from the cingulate cortex, a primary component of the limbic system, to the hippocampus, are distinct between the septal and temporal pole. For instance, the infralimbic and prelimbic cortices, involved in emotional processing, send input through the entorhinal cortex, the point of intersection between the neocortex and the hippocampus, to the temporal CA1 region. In contrast, the retrosplenial and anterior cingulate cortex, implicated in the regulation of visuospatial information and cognitive reasoning, project more septally within the CA1 (Fanselow & Dong, 2010; Strange et al., 2014). The lateral septum, a subcortical structure associated with reward and reinforcement, has also been shown to receive projections from the hippocampus in a graded fashion. Specifically, while the temporal CA1 and CA3 subfields appear to project to more ventral regions of the lateral septum, the septal CA1 and CA3 subfields send projections to more dorsal areas (Fanselow & Dong, 2010). Notably, the graded projections are maintained when travelling from the lateral septum to the hypothalamus; thus, output from the septo-temporal axis indirectly influences different areas of the hypothalamus responsible for autonomic and neuroendocrine related functions (Fanselow & Dong, 2010). Similarly, the nucleus accumbens and the amygdala, both structures involved in reward, decision making and emotional processing, also receive innervations from the hippocampus in a graded manner. In this case, output to the medial portion of both structures is received from the temporal pole of the hippocampus (Fanselow and Dong, 2010; Strange et al., 2014). Likewise, the ventral striatum (VS), implicated in the reward pathway, and the prefrontal cortex (PFC), which plays a role in decision making and executive function, receive projections solely from the temporal hippocampus (Bannerman et al. 1999; de Hoz et al., 2003). Furthermore, the temporal CA1 has also been found to directly innervate the olfactory bulb and related primary olfactory cortical structures (Fanselow and Dong, 2010).

1.3.3 The Longitudinal Axis of the Hippocampus: Functional Differences

While the distinct function of the hippocampus remains a topic of debate, differences in neural connections along the longitudinal axis are thought to be reflective of the functional differences observed between the septal and temporal poles. A study by Moser et al. (1995) examined the effects of hippocampal lesions, spanning across the longitudinal axis, on spatial navigation. In order to assess hippocampal-dependent spatial learning and memory, the Morris Water Maze (MWM), a widely accepted behavioural task performed by rodents, is often utilized (Morris, 1981). In this task, animals are initially placed into a large circular pool and must find their way to a hidden platform using visuospatial cues; the idea being that the lower the latency to escape the maze, the better their spatial memory. When lesions to the septal pole were greater than 40% of the entire hippocampus (60% of the hippocampi intact) impairments in task performance were observed; however lesions isolated to the temporal region did not impact spatial learning (Moser et al., 1995; Fanselow and Dong, 2010). These results suggest that the septal hippocampus is necessary for the induction of spatial navigation and memory. Moreover, performance does not appear to rely on a specific sub-section of the septal pole, all connections within this region are able to permit spatial learning (Moser et al., 1995). The role of the septal hippocampus in spatial cognition is thought to be due to a greater density of small, fine-tuned place cells, which are necessary for environmental navigation and episodic memory. In support of this theory, Jung et al. (1994) found that place cells located in the temporal hippocampus are larger and less fine-tuned than those located septally.

While the septal hippocampus appears to be important for cognitive behavioural tasks, evidence suggests that the temporal hippocampus is required for more emotion-based responses, including anxiety and unconditioned fear behaviour (Kjelstrup et al., 2002). In order to measure

levels of anxiety in animal models, a behavioural task known as the elevated plus maze is often used (Walf and Frye, 2007). The maze consists of two open and two enclosed arms, elevated 40-70 cm off the floor, with rats originally placed in the center of the apparatus (Kjelstrup et al., 2002; Walf and Frye, 2007). Under normal circumstances, rats are initially reluctant to enter the open arms of the maze, due to their preference for dark enclosed spaces; however, Kjelstrup et al. (2002) found that lesions isolated to the temporal hippocampus reduced this anxiogenic behaviour, as rats entered the open arms more frequently. While the expression of unconditioned-fear responses was not influenced by lesions to the septal hippocampus, it appeared that contextual fear conditioning was associated with the septal pole (Kjelstrup et al., 2002; Fanselow & Dong, 2010). Along with these findings, data from numerous studies support the argument that septally located hippocampal tissue, primarily in the CA1, is responsible for spatial/contextual memory, whereas temporal hippocampal tissue is involved in non-spatial/innate emotional responses and affect (Nadel et al., 1968; Moser et al., 1995; Bannerman et al., 1999; Kjelstrup et al., 2002; Maren & Holt, 2004; Pothuizen et al., 2004; Rogers et al., 2006; McEowen and Treit, 2009, 2010).

The notion that the septal and temporal poles of the hippocampus are completely discrete in function is not universally accepted. A collection of data would argue that, although lesions to the hippocampus leaving the septal region intact permit spatial learning, the retrieval and retention of spatial memory involves networks within the temporal pole (de Hoz et al., 2003, 2014; Ferbinteanu et al., 2003; Louriero et al., 2011). When lesions to the hippocampus are given prior to any MWM training, only septally intact rats are able to learn the position of the platform; however, when lesions are given 1-2 days after the initial task training, spatial learning is only displayed in temporally intact rats (de Hoz et al., 2014). The role of the temporal hippocampus in

memory retrieval may be due to its distinct anatomical connections to the PFC and VS. When areas of the septal pole are damaged, associations between the PFC and VS may compensate by correlating visuospatial cues with reward and goal-oriented behaviour, thus leading to successful retention of memory (Louriero et al., 2011; de Hoz et al., 2014). Therefore, while the septal pole appears to play a greater role in spatial learning and memory, the evidence suggests that the temporal pole may also be important under certain circumstances.

1.3.4 Response to Brain Injury across the Hippocampal Longitudinal Axis

Despite extensive work concerning the structural and functional properties of the hippocampus, there is still an incomplete understanding regarding the variation that exists along the longitudinal axis. More specifically, the disparity between septal and temporal poles is often a concern in the context of brain injury, where vulnerability to cell death displays anatomical variation. A selection of *in vitro* and *in vivo* studies have found that the temporal hippocampus shows greater susceptibility to epilepsy compared to the septal hippocampus, which may be attributable to a larger release of glutamate that promotes excitatory synaptic transmission (Racine et al., 1977; Lothman and Collins, 1981; Gilbert et al., 1985; Bragdon et al., 1986; Borck and Jefferys, 1999; Lee et al., 1990; Becker et al., 1997; Derchansky et al., 2004; Papatheodoropoulus et al., 2005). The release of glutamate from presynaptic terminals can be regulated by a number of different factors; one, in particular, being adenosine, via A1 receptors. The temporal CA1 region is known to contain significantly lower levels of A1 receptors; thus, the increase in epileptic activity is presumed to be caused by a higher glutamate-induced NMDAR response (Papatheodoropoulos et al., 2005).

Furthermore, when assessing animal models of depression, the temporal pole displays greater neuronal impairments compared to the septal pole. Within the temporal region of the DG,

there is a profound loss of neuronal proliferation, presumably due to an unequal distribution of radial glial-like progenitor cells along the septo-temporal axis, which makes the septal pole more resilient to affective disorders (Tanti and Belzung, 2013; Bekiari et al., 2015). In contrast, diseases involving neuro-inflammatory processes and de-myelination of axons, including multiple sclerosis, appear to negatively impact the septal hippocampus more readily than the temporal hippocampus, indicated by deficits in spatial and working memory (Bekiari et al., 2015).

Neuronal response to ischemic injury in the CA1 subfield also appears to follow a septo-temporal gradient, with higher susceptibility to insult located septally (Smith et al., 1984; Ashton et al., 1989; Akai and Yanagihara, 1993; Rami et al., 1997; Maggio et al., 2015). Using histological methods to assess cell survival, Ashton et al. (1989) found that changes in coagulative cell morphology after ischemia increased from 10% to 90% from the temporal to septal pole, respectively. Electrophysiological data is also in agreement with this finding, whereby loss in synaptic transmission followed a similar gradient; slices from the temporal region displayed a 10% loss in field excitatory post synaptic potentials (fEPSP), while slices from the septal region showed a 70% loss (Ashton et al., 1989).

A previous study by Maggio and Segal (2007) indicated that the temporal hippocampus is less capable of producing tetanic LTP (tLTP), a marker of synaptic plasticity, when compared to the septal hippocampus. Similarly, recent evidence suggests that ischemic LTP (iLTP), a pathological phenomenon that occurs following ischemic insult, also varies across the longitudinal axis (Maggio et al., 2015). The expression of iLTP is greater within the septal pole compared to the temporal pole. The enhanced expression of iLTP was associated with a higher density of post-synaptic NMDARs, specifically containing GluN2A/B subunits, linked to

excitotoxicity, within the septal hippocampus (Maggio et al., 2015). Similarly, Pandis et al. (2006) also observed differences in the expression of glutamate receptor subunits between the septal and temporal poles. The mRNA levels of GluN2A and GluN2B subunits were greater within the CA1 and CA3, which corresponded to higher levels of subunit protein expression, within the septal pole compared to the temporal pole (Pandis et al., 2006). In addition, the overall level of AMPARs was lower in the temporal hippocampi compared to the septal hippocampi; thereby suggesting differences in functionality and excitatory transmission, which may explain the differences in response to brain injury observed across the septo-temporal axis (Pandis et al., 2006).

1.4 Sexual Dimorphism in Ischemic Stroke

1.4.1 Epidemiology

Apart from the anatomical differences that exist in response to neuronal damage, emerging evidence now supports the claim that the occurrence of, and the susceptibility to, brain injury is also sexually dimorphic (Alkayed et al., 1998; Du et al., 2004; Li et al., 2005; Liu et al., 2009; Cheng and Hurn, 2010; Manwani and McCullough, 2011). From an epidemiological perspective, the incidence of ischemic stroke is approximately 33% higher in men than in women (Scott et al., 2012). However, while men have a higher frequency of ischemic episodes across their lifespan, stroke-induced mortality is 45% greater in women (Manwani and McCullough, 2011; Heart and Stroke Foundation, 2015).

There are particular stages throughout a woman's life when she becomes more susceptible to stroke related injury, including pregnancy, childbirth, perimenopause (~45-55 years old) and advanced age (>85 years old) (Scott et al., 2012). In addition, after menopause, the risk of stroke is higher in women than in men (Scott et al., 2012). Since the age at which the

risk of stroke increases is higher in women (74.5) than in men (69.2), women often experience a greater number of co-morbidities and a lower functional outcome from stroke (Manwani and McCullough, 2011; Scott et al., 2012). The percentage of recurrent strokes is also greater in women; while 10% of men aged 45-64 experience a second stroke 5 years after their first ischemic episode, this value is doubled in age-matched women (Manwani and McCullough, 2011). Furthermore, in men and women aged 65 years and older, the risk of a recurring stroke is 20 and 25%, respectively, indicating that susceptibility to recurrent ischemic episodes is higher in women across various age sectors (Manwani and McCullough, 2011).

1.4.2 Sexual Dimorphism in Experimental Models of Ischemia

Sex differences in both the incidence and recovery from ischemic brain injury have also been reproduced in animal studies (Carswell et al., 1999; Alkayed et al., 1998; Vagnerova et al., 2008; Cheng and Hurn, 2010; Manwani and McCullough, 2011). *In vitro* and *in vivo* models of ischemic insult propose that male brain tissue takes on an "ischemia sensitive" phenotype, whereas female brain tissue displays an "ischemia protective" phenotype (Cheng and Hurn, 2010; Manwani and McCullough, 2011). Young (3-4 months) and middle aged (12-14 months) female rodents were less likely to develop spontaneous stroke lesions and displayed reduced vulnerability to neuronal damage following global and focal ischemia (Alkayed et al., 1998; Chung and Hurn, 2010; Manwani and McCullough, 2011). The trend was also displayed when rodents expressed co-morbidities including type 2 diabetes and hypertension (Carswell et al., 1999; Cheng and Hurn, 2010). Differences in susceptibility to ischemic insult between male and female animals also persist with age. Liu et al. (2009) uncovered that aged female mice (16 months) had increased tissue infarction compared to young female mice (9-12 weeks) following middle cerebral artery occlusion (MCAO; model of transient cerebral ischemia). However, aged

male mice displayed reduced levels of infarction relative to young male mice, despite having lower functional outcome and survival rates. These studies are in-line with what is observed clinically, whereby the risk of stroke is greater in women than in men, with age (Shapira et al., 2002; Liu et al., 2009). While the biological mechanisms influencing these sex-based differences in response to neuronal damage are not well understood, differences in the primary sex hormones, estrogen and testosterone, are thought to play an important role.

1.4.3 Estrogen as a Neuroprotectant

17β-Estradiol (estrogen) is a steroid hormone synthesized most readily in the female ovaries via the enzyme aromatase. Estrogen is able to travel through the bloodstream to act on effector tissues, including the brain, to initiate downstream cellular processes (Brann et al., 2012). During the reproductive stages of life, serum levels of estrogen are high, particularly during the follicular phase of the menstrual cycle (analogous to the pro-estrous stage in rodents) (Nussy and Whitehead, 2001; Raval et al., 2009). However, following menopause, a decline in follicular tissue causes estrogen levels to decline considerably (Nussey and Whitehead, 2001). Changes in endogenous estrogen levels throughout the lifespan are suggested to contribute to the age related differences observed in vulnerability to ischemic brain injury (Etgen et al., 2011; Brann et al., 2012).

Numerous *in vitro* and *in vivo* studies have provided evidence to support the role of estrogen as a neuroprotectant (Alkayed et al., 1998; Toung et al., 1998; Wang et al., 1999; Miller et al., 2005; Li et al., 2005; Jover et al., 2002, 2007; De Butte-Smith et al., 2009; Gerstner et al., 2009; Novotny et al., 2009; Etgen et al., 2011; Cai et al., 2014). Surgical removal of the ovaries in young, sexually mature, female rodents is a common procedure used to eradicate circulating levels of estrogen within the body. Ovariectomized (OVX) female rodents have been found to

suffer from greater cerebral infarction when compared to age matched gonadally-intact females (Alkayed et al., 1998). Removal of the ovaries also eliminated the sex-linked differences between male and female adults observed after ischemic insult (Alkayed et al., 1998). Consistent with these findings, Cai et al. (2014) reported that, following MCAO, female OVX adult mice displayed a 27.2% increase in infarct tissue and experienced higher cognitive impairments when compared to sham-control mice. Several laboratories have also revealed that both pre and post treatment with exogenous estrogen (25-300 μg) is capable of alleviating the neurodegenerative effects associated with brain ischemia, including CA1 pyramidal cell loss, infarct volume and functional outcome, in male and female adult rodents (Alkayed et al., 1998, 2000; Li et al., 2004; Miller et al., 2005; Plamondon et al., 2006; Gulinello et al., 2006 Cai et al., 2014; Li et al., 2011).

While the natural depletion of circulating estrogen levels associated with menopause is known to increase susceptibility to ischemic brain injury, work by Alkayed et al. (2000) has suggested that treatment with exogenous estrogen is able to reverse these effects in middle aged, reproductively inactive, female rats exposed to MCAO. Pre-treatment with estrogen for seven days decreased cerebral infarction by 16% in the cortex and 44% in the striatum (Alkayed et al., 2000). Similarly, De Butte-Smith et al. (2009) demonstrated that, in middle aged female rats pretreated with estrogen, 50% of CA1 pyramidal cells survived global ischemia, compared to CA1 neurons from untreated animals. Data pertaining to both the removal and supplementation of estrogen in animal models at various stages of the lifespan support the notion that circulating levels of this primary sex hormone are important in protecting cells against cerebral damage and contribute to the "ischemia protective" phenotype displayed in women.

1.4.4 Estrogen and Neurodegeneration

In contrast to work characterizing estrogen as a neuroprotectant, several studies have found estrogen to exacerbate neuronal damage following brain injury (Harukuni et al., 2001; Bingham et al., 2005; Theodorsson and Theodorsson, 2005; Yong et al., 2005; Macrae and Carswell, 2006; Brann et al., 2012). Harukuni et al. (2001) observed that gonadally-intact adult female rats subjected to a 4 vessel-occlusion (4VO) model of ischemia displayed greater CA1 pyramidal cell death in comparison to their OVX counterparts. However, the seemingly protective effects of reduced circulating levels of estrogen, seen in OVX animals, were diminished upon treatment with exogenous hormone, whereby the loss in CA1 cell survival was correlated with the dose of estrogen administered (Harukuni et al., 2001). Coinciding with these data, work by Theodorsson and Theodorsson (2005) showed that treatment with estrogen after MCAO increased cerebral infarction within the cortex and lateral striatum of adult female rats by approximately 82%. While these findings contradict studies showing a beneficial role of estrogen against neuronal damage, one explanation for the observed differences may be related to variability in the severity of ischemic injury endured. Presumably, with a greater duration of insult, there will be an expansion of the ischemic core; thus neuronal tissue will be more resistant to the neuroprotective effects of estrogen (Harukuni et al., 2001). In addition, it is possible that estrogen influences response to ischemia to a certain extent; however, once the degree of insult exceeds a particular threshold, the protective/detrimental effects of this hormone are no longer observed. This notion was demonstrated by Harukuni et al. (2001), wherein OVX and intact female rats that underwent 5 minutes of 4VO displayed differences in the percent of CA1 cell loss, but these differences were no longer present between groups following exposure to 10 minutes of 4VO.

Studies which test the dose-dependent effects of estrogen during brain injury are often conducted in adult OVX female animals. However, due to the aforementioned epidemiology of stroke, aged female animals may provide a more clinically relevant model to study the influence of reproductive senescence on susceptibility to ischemic damage in women after menopause (De Butte-Smith et al., 2009). While the loss of endogenous estrogen after menopause has been linked to an increased risk of cerebral ischemia, several studies have reported worse functional outcome and larger volumes of infarction in response to estrogen replacement therapy in aged animals (Wappler et al 2010; Strom et al., 2011; Leon et al., 2012).

The disparities in estrogen efficacy observed between young and aged animal models following neuronal damage may be due to variabilities in the dose of estrogen administered between studies. Such methodological differences bring into play the notion of hormesis, whereby sex hormones may exert a diverse range of functions at different concentrations (Strom et al., 2011). While physiological doses of estrogen (5-65 pg/mL) are thought to be associated with neuroprotection, supra-physiological doses (300-600 pg/mL) are affiliated with increased pro-inflammatory pathways and subsequent neurodegeneration (Strom et al., 2011). Despite this concept of hormesis, Cai et al. (2014) witnessed that in 22 month old female rats, both low and high doses of estrogen, 100 μg/mL and 300 μg/mL respectively, failed to improve neurological deficit scores following ischemia.

Numerous researchers have now suggested that there may be a "critical period" during which the effects of exogenous estrogen are protective against ischemic injury, and administration after this point may exacerbate cerebral damage (Brann et al., 2012; Scott et al., 2012). In support of this theory, treatment with estrogen immediately following OVX was able to minimize neural impairments in the CA1 after global ischemic stroke. However if estrogen was

replaced 10 weeks after OVX, representing a period of long-term estrogen deprivation (LTED), typically associated with postmenopausal women, neuroprotection within the hippocampus was not observed (Suzuki et al., 2007; Zhang et al., 2009). While the mechanism remains unclear, it is known that LTED causes a decline in the estrogen receptor, ERα, which is associated with the hormone's neuroprotective effects. Therefore, the lengthy period of estrogen deprivation may have prevented the receptor-mediated activation of beneficial cellular mechanisms, including programmed oxidative death pathways, such as ROS and NADPH oxidase (Zhang et al., 2009). Interestingly, despite the "critical period" hypothesis, a study by De Butte-Smith et al. (2009) found that the time at which the ovaries were removed, relative to the time that estrogen was administered, did not have a significant effect on the neuroprotective actions of this hormone. When OVX occurred immediately, 1 week, or 8 weeks before chronic estrogen treatment (beginning 14 days before ischemic insult), neuronal survival rates were the same, indicating no negative effect of LTED on estrogen efficacy (De Butte-Smith et al., 2009).

Although the underlying cellular mechanisms responsible for the age-related discrepancies in vulnerability to ischemic insult are unknown, evidence suggests that glutamate-induced NMDAR activation may play a distinct role (McEwen, 1996; Gazzaniga et al., 2004). Previous evidence suggests that estrogen plays a role in the regulation of dendritic spine density, NMDAR binding, LTP and LTD within the hippocampal CA1 (Hart et al., 2001). In addition, treatment with estrogen is thought to cause GluN1 subunit upregulation on dendrites and cell bodies within the CA1 via post-translational modifications (PTMs), specifically through muscarinic-cholinergic transmission (Gazzaniga et al., 2004). Within young female rats, the increase in GluN1 subunit expression is accompanied by an increase in the production of dendritic spines, so that the GluN1-dendritic spine ratio is equal. However in aged female rats,

the increase in GluN1 subunit density is not accompanied by an upregulation of dendritic spines; thus potentially explaining the greater susceptibility to excitotoxic related ischemic injury (Gazzaniga et al., 2004). Interestingly, work by Hart et al. (2001), using double-label immunohistochemistry, revealed that the expression of estrogen receptors associated with neuroprotection are differentially distributed across the longitudinal axis of the hippocampus. While the septal hippocampus displayed a higher expression of ERα-positive cells within GABAergic neurons (primary inhibitory neurotransmitter), the temporal hippocampus displayed greater expression of ERα within pyramidal cells, thereby theoretically leading to differences in response to estrogen across the hippocampal long axis (Hart et al., 2001).

1.4.5 Testosterone and Ischemic Injury

When examining the sex-linked disparities associated with the onset and recovery from ischemic insult, the potential role that male androgens play is often less considered. However, given that, for the majority of the lifespan, the incidence of stroke is higher in men compared to women, it seems necessary to explore the influence of testosterone on brain injury.

Testosterone is a steroid hormone produced in the testicles of male animals and, to a lesser degree, the ovaries of female animals (Mooradian et al., 1987). Circulating levels of testosterone are approximately 8 times higher in male animals, however, making it their primary sex hormone (Mooradian et al., 1987; Torjesen et al., 2004). Although the majority of research focusses on the *neuroprotective* effects of estrogen following brain ischemia, a branch of the literature suggests that sexual disparities observed during ischemic injury are, instead, associated with the *lethal* effects of testosterone (Cheng and Hurn, 2010). There is a natural decline in the plasma concentration of testosterone attributable to ageing; over time reductions in the level of this hormone may be responsible for the lower risk of stroke found in ageing men, as compared

to women (Cheng and Hurn, 2010). Both clinical and experimental models have displayed a drastic reduction in serum levels of testosterone following stroke, and this stress-induced decline in testosterone could serve as an initial mechanism to protect against ischemic injury (Yang et al., 2002; Cheng and Hurn, 2010).

High levels of testosterone have been shown to significantly increase glutamate-induced neurotoxicity *in vitro*. Administration of 10 μM testosterone to HT-22 cells was shown to escalate cell death from 71.9% to 87.5% (Yang et al., 2002). In addition, primary rat oligodendrocytes treated with 100 nM testosterone displayed augmented cellular damage caused by the over-activation of AMPA and kainate receptors (Caruso et al., 2004). In agreement with these data, *in vivo* studies have revealed that castrated male rodents given testosterone display greater ischemic lesion sizes than their un-treated counterparts (Hawk et al., 1998). At the same time, however, low levels of testosterone have also been affiliated with greater susceptibility to cerebrovascular accidents and worsened functional outcome (Jeppesen et al., 1996; Yeap et al., 2009; Cheng and Hurn, 2010; Fanaei et al., 2014). Similarly, treatment with concentrations of testosterone ranging from 1 nm to 1 μM also seems to protect neurons against toxicity *in vitro* (Ahlborn et al., 2001; Nguyen et al., 2005, 2007).

While the shift from the beneficial to detrimental effects of testosterone following brain injury are not completely understood, the role of this androgen appears to follow a dose-dependent gradient (Cheng and Hurn, 2010). Uchida et al. (2009) observed that while low physiological levels of testosterone (1.5 mg) prior to MCAO reduced tissue infarction from 37% to 26% in castrated male mice, treatment with higher doses (5 mg) amplified infarct volume to 53%. Physiologically relevant doses of testosterone (5-25 mg) may upregulate pro-inflammatory gene expression following MCAO, and account for the neuro-damaging effects of this androgen;

however, more research is required to unveil the underlying biological mechanisms driving testosterone-related neuroprotection and neurodegeneration after ischemic insult (Bhasin et al., 2001; Uchida et al., 2009).

2.0 Study Rationale

Extensive work concerning how to treat ischemic stroke has been completed, with few successful developments (Montagne et al., 2012; Lopez-Valdes et al., 2014; Kafi et al., 2014). Previous literature has often focussed on methods of pharmacotherapy without full consideration of the distinct anatomical and physiological differences that exist among stroke patients. As a result, more research needs to be completed to better understand the underlying biological characteristics that contribute to variation in the brain's response to injury.

Recent work surrounding anatomical differences in response to brain injury have shown variation across the longitudinal axis of the hippocampus. Both histological and electrophysiological data suggest that tissue harvested from the septal pole is more susceptible to ischemic insult when compared to tissue located temporally (Ashton et al. 1989; Maggio et al., 2015) Considering these findings, this research aims to contribute to the literature concerning septo-temporal disparities, and hopes to confirm previous findings regarding selective vulnerability along the hippocampal long axis by using distinct measures of cell viability, including mitochondrial metabolism and cell membrane permeability.

In addition, although the influence which biological sex has on vulnerability to cerebral ischemia remains unclear, sex hormones clearly play a marked role (Jeppesen et al., 1996; Alkayed et al., 1998; Toung et al., 1998; Wang et al., 1999; Harukuni et al., 2001; Jover et al., 2002; Bingham et al., 2005; Jover et al., 2005; Li et al., 2005; Miller et al., 2005; Theodorsson and Theodorsson, 2005; Yong et al., 2005; Macrae and Carswell, 2006; De Butte-Smith et al., 2009; Gerstner et al., 2009; Novotny et al., 2009; Yeap et al., 2009; Etgen et al., 2011; Brann et al., 2012; Cai et al., 2014; Fanaei et al., 2014). Better understanding the functional role played by estrogen and testosterone in brain injury, as well as the basic molecular mechanisms driving

these processes, is crucial to the development of clinical treatments for stroke-related brain damage. Therefore, the proposed research also hopes to contribute to our current knowledge by exploring the potential sexual dimorphism that exists along the septo-temporal axis of the hippocampus following ischemic stroke.

I expected to meet the following objectives:

1. Determine how oxygen-glucose deprivation (OGD; an *ex vivo* model of ischemia) differentially influences cellular viability in slices acutely prepared from the septal and temporal poles by measuring mitochondrial metabolism, cell membrane integrity and proteolytic calpain activity. *2,3,5*-triphenyltetrazolium chloride (TTC) metabolism was used to evaluate mitochondrial function, whereas a lactate dehydrogenase (LDH) release assay was employed to assess membrane permeability. In addition, the assessment of spectrin breakdown products (SBDPs), via Western blot analysis, was used to examine calpain activity and necrotic cell death.
2. Determine potential sex-linked differences in susceptibility to ischemic injury along the septo-temporal axis of the hippocampus, as well as the possible contributing role of sex-hormones. Age was used as a means to naturally study the concentration of circulating hormone levels; while pre-adolescent male and female rats were expected to share equal concentrations of both hormones, following adolescence male and female rats contained higher levels of testosterone and estrogen, respectively.
3. Determine potential differences in response to ischemic injury between pre-adolescent and post-adolescent stages of life, in male and female rats.

Based on the current literature, I hypothesized that acute hippocampal slices taken from the septal pole would exhibit greater levels of tissue damage compared to slices obtained from

the temporal pole, as indicated by impaired mitochondrial function and compromised membrane integrity. Due to the apparent sexual dimorphism that exists within the brain following injury, I also proposed that hippocampal slices extracted from pre-adolescent male and female animals would show no sex-based differences in response to OGD. However, tissue harvested from sexually mature female rats was expected to display greater resilience to OGD compared to tissue from male animals. Lastly, I proposed that the expression of SBDPs would be greater within septal hippocampal slices relative to temporal hippocampal slices, indicative of their respective cell death profiles.

3.0 Materials and Methods

3.1 Animals

All experiments were conducted on male and female Sprague-Dawley rats, following procedures approved by the animal care committee at the University of Waterloo. All animals were group housed and maintained under a 12 h reverse-light cycle (8:00 a.m. to 8:00 p.m.) with access to food and water *ad libitum*.

3.2 Acute Hippocampal Slice Preparation

Experiments were conducted using acute hippocampal slices prepared from male and female rats at either postnatal day (PND) 26-29 (representing pre-adolescence), or PND 61-64 (representing young adulthood) (*See Figure 3 for a summary of experimental protocol.*)

Rats were anesthetized with CO_2 and sacrificed via decapitation. Brains were removed (~60 s) and placed in chilled (<4°C) artificial cerebral spinal fluid (ACSF) composed of (in mM): 124.0 NaCl, 3.0 KCl, 1.2 NaH_2PO_4/H_2O, 1.0 $MgSO_4/7H_2O$, 2.0 $CaCl_2/2H_2O$, 26.0 $NaHCO_3$, 10.0 Glucose and 10.0 HEPES buffer, equilibrated with carbogen (95% O_2/5% $CO_{2)}$, at pH 7.37-7.43, 310-320 mOsm (these and all remaining reagents were purchased from Sigma Aldrich Canada Co. unless stated otherwise). Both right and left hippocampi were removed from their respective hemispheres and positioned onto the platform of a McIlwain tissue chopper (Mickle Laboratory Engineering Co., Surrey, UK) with the septal pole facing the blade. Slices (350 μm) were prepared in the following manner: the first 4 slices were considered part of the septal region, while the last four slices were considered part of the temporal region. Tissue between these two poles (generally 4-8 slices) were considered part of the intermediate region and discarded. Septal and temporal slices were then positioned on cell-culture inserts (2 slices per insert) placed on either a mesh platform for the TTC assay, or in Petri dishes filled with 1 mL

of ACSF for the LDH assay. All slices were held at interface in an incubation chamber, continuously perfused with carbogenated ACSF at 35°C ± 0.5°C for a recovery period of approximately 60 minutes.

3.3 Oxygen-Glucose Deprivation

A subset of septal and temporal slices were subject to ten minutes of OGD. Inserts were transferred to a mesh platform in a second incubation chamber containing modified ACSF, wherein glucose was substituted for sucrose, and that was continuously perfused with N_2 gas at 35°C ± 0.5°C. Control slices underwent a SHAM procedure to account for any stress experienced during movement. Following OGD, ACSF in all Petri dishes was replaced with 1 mL of fresh carbogenated ACSF, and inserts were returned to their original incubation chamber to initiate a 3 hour recovery period.

3.4 2,3,5-Triphenyltetrazolium Chloride (TTC) Assay

Septal and temporal slices from SHAM and OGD treatment groups were transferred to glass scintillation vials (2 slices per vial) containing 2% (w/v) TTC solution; *2,3,5*- TTC powder was prepared with ACSF and saturated with N_2 for 5 minutes. All vials were covered in foil, to prevent light reaction, and were incubated for 60 minutes at 35°C ± 0.5°C. Following incubation, slices were rinsed twice with aerated ACSF and submerged in 1.5 mL extraction buffer (DMSO and 95% EtOH prepared 1:1) overnight (~18-24 h) in the dark at room temperature. Average formazan absorbance was measured at 485 nm using a spectrophotometer.

3.5 Lactate Dehydrogenase (LDH) Release Assay

LDH release from septal and temporal slices was measured using an LDH Cytotoxicity Assay Kit (Cayman Chemical; Michigan, USA). Following a 3 hour recovery period, 100 µL aliquots of ACSF in Petri dishes from each treatment condition were loaded, in triplicate, onto a

96-well plate; 100 µL of LDH reaction solution was then added to each well. Plates were incubated in an orbital shaker, with gentle shaking, at 37°C for 30 minutes. Average LDH absorbance was measured at 490 nm via spectrophotometry.

3.6 Vaginal Cytology

Estrus cycle staging in sexually mature female rats was done through the assessment of wet vaginal smears using a protocol adopted by Hubscher et al. (2005). Following decapitation, 200 µL of 1X PBS was inserted into the rat's vagina, using a 200 µL pipette tip, and immediately drawn back up. The sample was released onto a clean glass microscope slide and a coverslip was placed on top. Cells in the vaginal epithelium were then examined under a light microscope at 40x magnification to qualitatively determine the stage of the estrus cycle. Proestrus, the stage in which estrogen levels are greatest, was characterized by the presence of densely packed nucleated epithelial cells. In contrast, estrus (ovulation) was distinguished by the presence of cornified epithelial cells, which predominated among other cell types. During the final two stages of the cycle, where estrogen levels are lowest, diestrus was discerned by a large number of leukocytes, which were often surrounded by nucleated epithelial cells, while metestrus contained dispersed populations of leukocytes, in addition to both nucleated and cornified epithelial cells (Long and Evans, 1922; Mandl, 1951; Marcondes et al., 2002) (*See Figure 4 for a representative image of each stage of the estrus cycle.)*

3.7 Blood Serum Collection

Immediately following the decapitation of post-adolescent female rats, trunk blood was collected, using a 5 mL syringe, and subsequently dispensed into two 1.5 mL microcentrifuge tubes. Blood was allowed a 30 minute period of coagulation, followed by 15 minutes of

centrifugation at 2000 x g and 4°C. The resultant supernatants (blood serum) were then stored at -80°C for potential future analysis of serum estrogen levels.

3.8 Tissue Homogenization

In some experiments, following a 10 minute SHAM/OGD challenge, septal and temporal slices prepared from the left hippocampus were mechanically homogenized in 400-500 µL of non-ionizing lysis buffer (100 mM NaCl, 25 mM EDTA, 10 mM Tris, 1% (v/v) Triton X-100, 1% (v/v) NP-40) supplemented with a protease inhibitor cocktail (AEBSF, Aprotinin, Bestatin hydrochloride, E-64, Leupeptin hemisulfate salt, Pepstatin A) and sodium orthovanadate. Homogenates were centrifuged for 10 minutes at 1000 x g and the supernatants were stored at -80°C for later analysis using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Prior to storage, the protein concentration of each hippocampal slice homogenate was determined using a BioRad DC protein assay kit; the optical density of each unknown sample was compared to bovine serum albumin (BSA), which was used as a protein standard.

3.9 SDS-Page and Immunoblotting

Frozen hippocampal homogenates were thawed and loaded in duplicate (5 µg protein/lane) for SDS-PAGE. Proteins were then blotted onto polyvinylidene fluoride (PVDF) membranes using a wet transfer technique (35 V at 4°C overnight). Ponceau S solution was used to confirm a successful protein transfer; membranes were then blocked with 5% skim milk powder (w/v) made in TBS-T [20 mM Tris 140 mM NaCl, 01% Tween-20 (v/v), pH 7.6] for 1 hour at room temperature.

Immunoblotting was performed to probe for biomarkers of necrotic cell death, SBDPs, using the primary antibody, spectrin α II (C-3) (1:1000, mouse monoclonal IgG_1, #sc-48382; Santa Cruz Biotechnology, USA). All blots were incubated with the primary antibody overnight

at 4°C, and then incubated with an HRP-linked anti-mouse secondary antibody (1:5000) for 1 hour. All solutions were prepared in a 5% (w/v) skim milk/TBS-T blocking buffer and secondary antibodies were activated via incubation with enhanced chemiluminescence (ECL) substrate. In order to detect the density of each band, SYNGENE was used with exposure intervals ranging from 30 seconds to 3 minutes (all analyzed points were selected from within the linear range of detection).

3.10 Exclusion Criteria

Based on an *a priori* criterion that slices exposed to OGD would endure greater cellular damage than SHAM treated slices, data points were removed if TTC metabolism in OGD slices was equal to, or higher than, SHAM treated slices; out of a total of 80 animals, nine animals were removed from the final data analysis for violation of the *a priori* criterion (*Table 1*). Likewise data points were removed if LDH efflux in OGD slices were equal to, or lower than, SHAM treated slices. Notably, in those cases where a data point was excluded, data points from the opposite pole were also removed.

3.11 Statistical Analysis

All data were stored in Microsoft Excel worksheets. To examine the influence of the primary sex hormones on response to ischemic injury, SHAM and OGD hippocampal slices obtained from the septal and temporal regions were compared between and within sexes in both pre-adolescent and post-adolescent groups. Differences between groups were assessed through either the calculation of difference scores (mean of group 1 – mean of group 2; $\bar{\mu}_1$ - $\bar{\mu}_2$) followed by the Wilcoxon signed-ranked test (one-sample test), or through the Mann-Whitney U test (two-sample test), both of which are non-parametric statistical tests that do not assume the data are

normally distributed. All calculations were done using GraphPad Prism 7 and statistical significance was set at $p \leq 0.05$.

The effect size index, using Cohen's *d* (standardised mean difference), as well as 95% confidence intervals (CIs) were calculated using the Exploratory Software for Confidence Intervals (ESCI) program. Interpretation of biological relevance was guided by Cohen's conventions: Cohen's *d = 0.2* (small magnitude of effect), *d = 0.5* (moderate magnitude of effect) and *d = 0.8* (large magnitude of effect) (*see Nakagawa and Cuthill, 2007 for more information on effect size)*. The statistical significance (*p* value) and the magnitude of effect *(d* value), along with its 95% CI, were considered together in order to make inferences about the data collected.

4.0 Results

4.1 Lactate Dehydrogenase (LDH) Release Assay Data Analysis

4.1.1 Changes in LDH Release could not be Detected Following OGD

The amount of LDH released from hippocampal slices was intended to be used as an indication of damage to the cell membrane (*see materials and methods section 3.5)*. Although basal levels of LDH release were detected from slices across the longitudinal axis, no statistically significant difference in LDH efflux was observed between SHAM treated slices and slices subjected to 10 minutes of OGD within male septal ($p = 0.22$), male temporal ($p > 0.99$), female septal ($p = 0.15$), or female temporal ($p = 0.22$) pre-adolescent rats (all LDH data collected from cohort 1) (*Figure 5*). Calculation of Cohen's *d* also revealed no effect of a 10 minute OGD challenge on changes in LDH release within pre-adolescent male septal ($d = 0.49$) or male temporal ($d = 0.08$) slices; despite a large effect size calculated in the female septal ($d = 0.84$) and female temporal ($d = 1.1$) group, the direction of effect was opposite to that which would have been expected, as SHAM slices displayed greater LDH efflux than OGD slices.

Within post-adolescent rats, the concentration of LDH released into the surrounding ACSF was not statistically significantly different between SHAM and OGD slices from the male septal ($p = 0.10$), male temporal ($p = 0.22$), female septal ($p = 0.15$), or female temporal ($p = 0.84$) groups. Calculation of Cohen's *d* also confirmed no effect of OGD within the female temporal group ($d = 0.18$), but suggested an effect within post-adolescent male septal ($d = 1.44$), male temporal ($d = 0.71$), and female septal ($d = 1.17$) slices. Despite these values, however, the direction of effect was opposite to that expected, as SHAM slices displayed greater LDH efflux than OGD slices *(Figure 6)*.

In order to determine whether the failure to detect an increase in LDH concentration was due to an insufficient degree of injury, a subset of slices taken from pre-adolescent male rats was subjected to 30 minutes of OGD and a 3 hour recovery period. Two slices were also submerged in a vial containing 1 mL of ACSF (previously saturated with carbogen) and were kept at 35°C ± 0.5°C for 4 hours, to serve as a positive control of LDH efflux. Similar to the results obtained following 10 minutes of insult, 30 minutes of OGD did not cause appreciable changes in LDH release compared to SHAM slices. In contrast, a considerable increase in LDH release was detected in the positive control group (*Figure 7)*. Since the values observed following both 10 and 30 minutes of ischemia violated an *a priori* assumption, which states that slices treated with OGD should have higher LDH efflux than SHAM slices, a decision was made to eliminate the LDH assay as a measure of cellular viability during the second cohort.

4.2 *2,3,5*-Triphenyltetrazolium Chloride (TTC) Assay Data Analysis

4.2.1 Ten Minutes of OGD Reduces TTC Metabolism in Hippocampal Slices

To confirm that 10 minutes of OGD *(see materials and methods section 3.3)* was sufficient as a model of injury in acute hippocampal slices, in preliminary work, the average formazan absorbance was quantified in a subset of male (~PND 60) SHAM and OGD slices taken from across the longitudinal axis *(outlined in materials and methods section 3.4).* Ten minutes of OGD successfully reduced TTC metabolism relative to SHAM treatment in septal *($p = 0.03$)* and temporal *($p = 0.0079$)* slices. Calculation of Cohen's *d* also confirmed that OGD had a large effect on mean formazan absorbance in septal (*d = 2.01*; 95% CI = 0.40, 3.54) and temporal (*d* = 1.86; 95% CI = 0.29, 3.35) slices *(Figure 8).*

4.2.2 Response to OGD across the Hippocampal Longitudinal Axis

In order to assess whether 10 minutes of OGD differentially influenced mitochondrial metabolism across the longitudinal axis, average formazan absorbance in septal and temporal slices was quantified. Injured slices from both poles were taken as a % of the respective SHAM slices in pre and post-adolescent animals and difference scores were constructed for each experimental group (*Table 2*). No significant differences in TTC metabolism were observed between septal and temporal slices taken from male pre-adolescent (*p = 0.20, d = 0.55*), female pre-adolescent *(p = 0.43, d = 0.39),* male post-adolescent *(p = 0.19, d = 0.47)*, or female post-adolescent *(p = 0.49, d = 0.23)* rats *(Figure 9).*

4.2.3 Sex-Linked Differences in Response to OGD

To examine the potential influence of exposure to varying levels of the primary sex hormones, estrogen and testosterone, following 10 minutes of OGD, % of SHAM difference scores were constructed between pre-adolescent male and female (N = 9) and post-adolescent male and female (N = 10) rats, in slices from across the longitudinal axis *(Table 3)*. Within the septal pole of the hippocampus, sex had a large effect *(d = 0.90)* on response to insult between pre-adolescent animals, whereby TTC metabolism in male septal slices was lower than TTC metabolism in female septal slices. Sex also appeared to have a large effect *(d = 0.89)* on TTC metabolism within the temporal pole, with pre-adolescent female rats displaying greater sensitivity to injury than pre-adolescent male rats; however, this difference did not reach statistical significance *(p = 0.07)*. No significant differences in response to OGD were observed between male and female animals during post-adolescence in both septal *(p = 0.63, d = 0.02)* and temporal slices *(p = 0.63, d = 0.05) (Figure 10).*

4.2.4 Age Related Differences in Response to OGD

To determine whether mitochondrial metabolism following 10 minutes of OGD differed between animals at various stages of brain development, % of SHAM difference scores were constructed between pre and post-adolescent male (N = 9) and female (N = 10) animals (*Table 4)*. Within the septal pole of the hippocampus, TTC metabolism was lower in pre-adolescent male slices to a statistically significant degree *(p = 0.05)* relative to post-adolescent male slices. Further confirmation with Cohen's *d* effect size revealed a moderate to large effect of age on response to injury *(d = 0.68)*. However, no significant differences in TTC metabolism were observed between pre and post adolescent male temporal slices *(p = 0.57, d = 0.26)*. Likewise, there were no significant differences in TTC metabolism present between pre and post-adolescent female septal *(p = 0.77, d = 0.17)* or temporal *(p = 0.85, d = 0.03)* slices *(Figure 11)*.

4.3 Estrus Cycle Staging and Circulating Levels of Estrogen

4.3.1 Estrogen Levels and Response to OGD across the Longitudinal Axis

In order to account for potential differences in response to OGD caused by varying levels of circulating estrogen, estrus cycle staging was performed on sexually mature female rats (N = 20) (*materials and methods section 3.6)*. For a number of reasons (violation of an *a priori* assumption, experimental error, estrus cycle could not be discerned) N = 7 rats were excluded from the analysis, and the remaining N = 13 rats were stratified based on their respective stage of the estrus cycle.

To assess how levels of estrogen may influence response to injury across the longitudinal axis, rats were further categorized into high estrogen (proestrus; N = 5) and low estrogen (estrus, metestrus and diestrus; N = 8) groups; % of SHAM difference scores were then calculated between septal and temporal slices for each group *(Table 5)*. High levels of circulating estrogen

did not have an apparent effect *(d = 0.12)* on TTC metabolism across the longitudinal axis, as septal and temporal slices were not different to a statistically significant degree *(p = >0.99)*. Similarly, in animals with low levels of circulating estrogen, no statistically significant differences *(p = 0.31)* in TTC metabolism were present between septal and temporal slices, which was further confirmed by a small Cohen's *d* effect size *(d = 0.35) (Figure 12)*.

4.3.2 Estrogen-Concentration Dependent Differences in Response to OGD

To better understand how fluctuating levels of estrogen may differentially influence response to OGD in post-adolescent female animals, mean % SHAM TTC values were compared between high (N = 5) and low (N = 8) estrogen level groups *(Table 6)*. Although calculation of Cohen's *d* suggested a moderate to large effect of estrogen level on response to injury in both the septal (*d = 0.78)* and temporal (*0.14)* poles, these values did not reach statistical significance (septal: *p = 0.35;* temporal: *p = 0.72)*. Further assessment of the 95% CIs revealed values containing 0; thereby further implying no statistically significant differences between the groups *(Figure 13)*.

4.4 SDS Page and Immunoblotting

4.4.1 Optimal Protein Concentration for Immunoblotting

In order to determine the optimal protein concentration that would generate a clear signal without saturation, the αII-spectrin antibody was probed against hippocampal tissue over a series of various protein concentrations ranging from 2.5 µg – 15 µg. *(See Figure 14 for a standard curve optimization blot conducted with post-adolescent male septal SHAM tissue*.) Based on the results, 5 µg was determined to be the optimal sample loading concentration.

4.4.2 Preliminary Assessment of SBDP Expression across the Longitudinal Axis

To confirm whether 10 minutes of OGD would trigger calpain mediated cleavage of the 280 kDa αII-spectrin protein, indicative of necrotic cell death (Knox et al., 2013), protein expression of 150 kDa SBDPs were measured in homogenates prepared from SHAM and OGD slices across the hippocampal longitudinal axis using Western blot analysis. The assessment of homogenates is ongoing. (*See Figure 15 for a representative blot of SBDP expression within pre-adolescent male tissue.)*

5.0 Discussion

While the majority of research often considers the hippocampus a homogeneous structure, a growing body of literature has proposed that differences in response to brain injury are present across the hippocampal longitudinal axis (Lorente de No., 1934; Smith et al., 1984; Ashton et al., 1989; Akai and Yanagihara, 1993; Rami et al., 1997; Maggio et al., 2015). In an effort to address this possibility, I examined how basic factors, including sex and age, contributed to differences in response to ischemic insult across the longitudinal axis. Cell viability following 10 minutes of OGD was analyzed in septal and temporal hippocampal slices isolated from male and female rats during either the pre-adolescent or post-adolescent stages of life.

5.1 Lactate Dehydrogenase Release Assay

5.1.1 Increased LDH Release could not be detected following OGD

Lactate dehydrogenase (LDH) is a soluble enzyme present in the cytoplasm of almost all cells (Izumi et al., 2001). Following ischemic stroke, the plasma membrane integrity of neurons is compromised, which results in higher amounts of LDH release into the surrounding cerebrospinal fluid (CSF) (Lampl et al., 1990). Based on this phenomenon, LDH release has become a useful measure of cytotoxicity (Koh and Choi, 1987; Chan et al., 2013). Despite efforts to develop a protocol to measure the amount of LDH release from acute hippocampal slices, my results indicate that following 10 minutes of OGD and a 3 hour recovery period, an increase in LDH release could not be detected when compared to SHAM slices. More specifically, the results violated an *a priori* condition, whereby no appreciable rise in LDH levels was shown in slices following an OGD challenge relative to SHAM slices. In an attempt to interpret these

unexpected results, I examined the experimental methods employed in this study, and was left with three possible explanations.

Time of Assessment

Failure to detect significant changes in LDH release may have been caused by the time at which slices were assessed after injury. My study used an acute *ex vivo* preparation of hippocampal slices in order to identify changes in cell viability across the longitudinal axis following OGD. In the context of neuronal insult, the brain slice model is advantageous as it maintains tissue architecture and synaptic circuitry; however, these studies are often restricted in their assessment of response to injury because slices can only remain functional for several hours before tissue degeneration (Cho et al., 2007; Mielke et al., 2007; Lien et al., 2011). As a result, outcome from excitotoxic damage is representative of the sort of acute cell death that would take place in the ischemic core (Taubenfeld et al., 2002; Cho et al., 2007; Lien et al., 2011) In contrast, *in vitro* cell culture models can be maintained for weeks to months, and allow for the detection of delayed cellular events that take place after brain injury (Cho et al., 2007). Although permeabilization of the cell membrane occurs during necrotic cell death, LDH-release from the cytoplasm appears to occur several hours to days after injury, which makes the assay more suitable for cell culture work (Vornov et al., 1991; Romano et al., 1995; Izumi et al., 2001). Clinical studies reported by Lampl et al. (1990) are also in agreement with these observations, whereby LDH activity detected in the CSF of men and women is greatest 48-120 hours following a cerebrovascular accident. The use of acute hippocampal slices in this study limited us to assaying LDH release just 3 hours following a 10 minute OGD challenge. Based on the delayed release of LDH following insult, my results would suggest that analyzing cell damage at this time point is too early to detect any significant effects of OGD on LDH release. Moreover,

the variations in LDH efflux observed between SHAM and OGD slices may be characteristic of normal fluctuations in basal levels of LDH released from cells at any given time.

Model of Ischemic Injury

To induce brain injury, this study employed an *ex vivo* model of ischemia, OGD; for over a decade OGD has been used in numerous experiments involving neuronal insult and neuroprotection, and has been deemed a reliable stroke model (Tasca et al., 2015). While the general protocol for OGD (outlined in the *materials and methods section 3.3)* is usually similar, the duration of OGD may vary between studies, thereby leading to differences in the degree of damage sustained by slices (Tasca et al., 2015). Our study challenged septal and temporal hippocampal slices to 10 minutes of OGD followed by a 3 hour period of recovery; however, based on the results, this level of insult may not have been sufficient to cause changes in LDH release past basal levels. In support of this notion, work by Fernández-López et al. (2005) revealed that the amount of LDH release in forebrain slices was correlated with the length of the ischemic challenge. While 10 minutes of OGD caused an indistinguishable change in LDH release compared to control slices, LDH efflux was significantly increased following 30 minutes of OGD and a 3 hour recovery period (Fernández-López et al., 2005). Similarly, Pérez-Rodríguez et al. (2015) uncovered enhanced LDH activity after 30 minutes of OGD and 3 hours of reperfusion, when compared to slices held at normoxic conditions. In an attempt to replicate these findings, a set of male hippocampal slices was exposed to 30 minutes of OGD; however, following a 3 hour period of recovery, a rise in LDH efflux was not detected. These results suggest that the length of injury may not be the only important factor to consider when modelling ischemic injury.

Several studies, in which cell viability was quantified through LDH activity, have also displayed modifications to the traditional OGD protocol (Izumi et al., 2001; Siqueira et al., 2004; Zhou and Baudry, 2006; Mozes et al., 2012). For instance, Zhou and Baudry (2006) performed an ischemic challenge by placing hippocampal slices in vials containing 2 mL of aglycaemic ACSF that were previously gassed with N_2 for 20 minutes. Following a 1 hour incubation length, LDH efflux was measured in the OGD medium and slices were transferred to vials containing 2 mL of ACSF (saturated with carbogen) for a 2 hour reperfusion period. Following recovery a second measure of LDH was taken, and total LDH release was calculated as the sum of LDH efflux from the 1 and 2 hour incubation lengths (Zhou and Baudry, 2006). This experiment differed from the design of my study in two major ways: first, while acute slices were exposed to 10 minutes of OGD in my study, the authors used a 1 hour length of OGD. Second, while my experiment maintained slices at interface, their study subjected hippocampal tissue to submerged conditions, which has been shown to cause a decrease in metabolic activity and synaptic function (Aitken et al., 1995; Ivanov and Zilberter, 2011). Thus, in addition to the insult endured during OGD, LDH release may have been influenced by cellular damage experienced during the recovery period, wherein, levels of oxygen may not have been sufficient to sustain the metabolic needs of slices (Ivanov and Zilberter, 2011).

The experimental design implemented by Zhou and Baudry (2006) did, however, raise the question of whether slices subjected to OGD under submerged conditions would release a greater amount of LDH compared to those held at interface. To answer this question, a set of hippocampal slices were placed in a vial containing 1 mL of aglycaemic ACSF, previously saturated with N_2, for a 10 minute OGD challenge. A second set of slices were challenged with 10 minutes of OGD using the standard interface approach (*materials and methods section 3.3)*,

and, following OGD, LDH release was quantified in both submerged and interface groups. Slices from each condition were then maintained at interface for a 3 hour recovery period, and a second measure of LDH was taken thereafter; total LDH release was calculated as a sum of the two measures. Despite this modification, no appreciable rise in LDH efflux was observed in slices submerged during OGD, compared to SHAM slices, or slices held at interface during the OGD challenge (data not presented). Although these experiments were conducted on slices from 8-9 month old female rats, as opposed to those from younger male rats, these findings indicate that with the method of brain slice preparation and maintenance used in my study, a significant increase in the level of LDH release cannot be observed following 10 minutes of OGD and a 3 hour period of recovery.

Additional Required Methods

In addition to the induction of OGD, it is possible that the concurrent application of toxic agents may be required in order to promote the release of LDH from acute slices. This idea was demonstrated in a study by Mozes et al. (2012) wherein hippocampal slices subject to 1 hour of OGD did not release significantly higher amounts of LDH compared to control slices. In contrast, when slices were treated with beta-amyloid (Aβ 1-42) peptide, a toxin involved in Alzheimer's disease, for 4 hours following OGD, a significant increase in LDH efflux was observed (Mozes et al., 2012). Treatment with other toxic reagents, including H_2O_2 and KCN following a 1 hour period of OGD also lead to enhanced LDH release (Mozes et al., 2012).

LDH is an intracellular enzyme commonly known to reside within the cell's cytoplasm; however, a large proportion of LDH molecules are also attached directly to the cell membrane (Muronetz et al., 1996; Izumi et al., 2001). The presence of membrane-bound LDH may prevent its release into the extracellular environment, despite a neuronal insult. As such, some

researchers have accompanied their model of injury with additional methods that target degradation of the cell membrane, in order to encourage the release of membrane-bound LDH (Izumi et al., 2001). In attempts to promote structural deterioration and LDH discharge, Izumi et al. (2001) employed a method of brief mild sonication with septal slices of the rat hippocampus. Tissue slices submerged in ACSF were subject to mild sonication pulses at two-second intervals following the administration of a toxin known as iodoacetate (IA). While exposure to the neurotoxin, or sonication alone did not cause significant changes in LDH activity, 20 pulses of mild sonication and 200 μM of IA lead to a considerable release of cytosolic LDH (Izumi et al., 2001).

While the aforementioned study did not examine neuronal damage caused by *ischemic* insult, if their hypothesis holds true, my failure to detect a significant increase in LDH levels following OGD may be explained by its association with the cell membrane. While OGD indirectly influences cell membrane permeability through initiation of the ischemic cascade (Tasca et al., 2015), this method of insult may not be robust enough to disrupt the connection between LDH and the cell membrane. Although liberation of membrane-bound LDH is achieved through sonication, this method is more invasive and caution must be taken with its use, as excessive application can completely lyse the cell membrane and damage slices beyond the extent intended (Ashush et al., 2000). In addition, while exposure to neurotoxic agents may assist in membrane deterioration and LDH release, their effects may not directly reflect the pathophysiology of ischemia. Therefore, in attempts to preserve a clinically relevant model of ischemic injury, employing sonication, or additional toxic agents to hippocampal slices exposed to 10 minutes of OGD would not seem to be suitable methods.

5.2 *2,3,5*-Triphenyltetrazolium Chloride (TTC) Assay and Response to OGD

5.2.1 Response to 10 Minutes of OGD did not differ across the Longitudinal Axis

Ten minutes of OGD successfully caused ischemic injury in slices from across the longitudinal axis, indicated by significant reductions in TTC metabolism relative to SHAM slices. When comparing changes in TTC metabolism between the septal and temporal poles, however, no differences were observed. My results conflict with previous evidence, which suggests that differences in response to injury follow a septo-temporal gradient (Smith et al., 1984; Ashton et al., 1989; Akai and Yanagihara, 1993; Rami et al., 1997; Papatheodoropoulos et al., 2005; Maggio et al., 2015). When male rats were subjected to transient forebrain ischemia, Rami et al. (1997) found that 82% of CA1 hippocampal neurons within the septal region displayed necrotic damage, but necrosis was only present in 16% of neurons from the temporal region; this is in contrast to the present study, where male and female pre-adolescent and post-adolescent rats only showed a 5-10% difference in mitochondrial function between their septal and temporal poles. Although 10 minutes of OGD did not provoke changes in viability across the longitudinal axis as expected, these unanticipated findings may be explained by two possibilities.

First, it is important to consider how cellular response to excitotoxic insult was measured in the various studies reported. Much of the work that has identified septo-temporal differences across the hippocampus used electrophysiological recordings to examine synaptic transmission (Ashton et al., 1989; Papatheodoropoulos et al., 2005; Maggio et al., 2015). Conversely, the present study utilized the TTC assay, which offers high-throughput screening of slice viability through quantifying mitochondrial activity (Watson et al., 1994). Given the differences in methods of quantification, failure to observe changes in response to ischemic damage between septal and temporal slices may be a reflection of the cellular change analyzed.

Evoked field potentials and mitochondrial function were previously shown to be correlated measures of cell viability following OGD in acute hippocampal slices (Mielke et al., 2007); however, this previous report did not account for potential differences between the septal and temporal poles. It is possible that this correlation may not exist across the longitudinal axis, and, while the modulation of synaptic transmission in response to brain injury appears to follow a septo-temporal gradient, changes in mitochondrial metabolism may not. To address this notion, it is worth considering the time at which cell viability was assessed. Viability measures were conducted 3 hours after the induction of OGD; therefore, changes in TTC metabolism would likely indicate an immediate *reduction* in mitochondrial function, rather than complete mitochondrial *dysfunction*, which is characteristic of delayed neuronal cell death (Elmore et al., 2007). As such, it is possible that while both poles initially react equally to this assessment of damage, over time as downstream biological mechanisms take place, these discrepancies may become more pronounced.

As previously discussed *(literature review section 1.2)*, glutamate-mediated excitoxic damage can be explained through the "calcium-overload" hypothesis, where an initial rise in intracellular Ca^{2+}-ions following cellular stress will trigger the mitochondria to store calcium within its matrices; a process known as calcium sequestration (Hossmann et al., 1985; Szydlowska and Tymianski., 2010). During ischemic insult, excessive accumulation of Ca^{2+}-ions within mitochondria has been shown to cause damage to CA1 neurons, and this damage is further amplified by a second increase in calcium conductance following reperfusion, consequently leading to calcium over-load (Hossmann et al., 1985). Previous work has shown that calcium sequestration is capable of causing changes to mitochondrial metabolism within neurons, in areas of irreparable damage (Borgers et al., 1981; Simon et al., 1984), most likely

representative of the ischemic core. However, differences in response to mitochondrial injury may not be observed between areas of varying levels of sensitivity because it is thought that early signalling cascades following ischemia are similar between brain regions (Hossmann et al., 1985). If this hypothesis holds true, then the failure to observe septo-temporal differences in mitochondrial function following 10 minutes of OGD and 3 hours of recovery may be explained by the fact that viability was assessed during the acute phase of injury, when cell death pathways are thought to be analogous throughout the hippocampus. However, if a second measure of outcome was assessed that was not directly reflective of mitochondrial damage, such as electrophysiological or biochemical analyses, differences may become apparent across the longitudinal axis.

A second reason that may explain these findings is that, while differences in response to neuronal damage exist along the hippocampal axis, they were not observed because of a high signal to noise ratio. The majority of studies that examined anatomical disparities across the hippocampus focused on susceptibility to injury within particular subfields of the septal and temporal poles (Ashton et al., 1988; Akai and Yanagihara, 1993; Rami et al., 1997; Papatheodoropoulos et al., 2005; Maggio et al., 2015). Previous evidence suggests that while the CA1 hippocampus displays the greatest sensitivity toward cellular stress, the DG displays the most tolerance (Schmidt-Kaster and Hossman, 1988). Consistent with this notion, it was reported that following experimental stroke, approximately 50-60% of slices failed to undergo synaptic transmission within the septal CA1; however only 2 of 120 slices failed to do so within the septal DG (Ashton et al., 1989). Histopathological changes also indicated that the septal CA1 subfield displayed greater vulnerability to ischemic insult than both the septal DG and the temporal CA1 (Ashton et al., 1989). Similarly, Akai and Yanagihara (1993) reported that susceptibility to brain

ischemia was larger in the "subiculum CA1" region of the septal hippocampus, as compared to the CA3 region.

In this study, TTC metabolism following 10 minutes of OGD was measured in whole hippocampal slices, rather than within the individual subfields, across the longitudinal axis. However, as noted, given the selective vulnerability that exists between hippocampal subfields, potential septo-temporal differences may not have been detected because of varying degrees of ischemic tolerance *within* slices. While the septal CA1 appears to possess greater susceptibility to injury than the temporal CA1, the DG does not share this same pattern of vulnerability across the axis (Ashton et al., 1989). Therefore, the increased sensitivity of particular subfields to neurotoxicity, such as the CA1, may go unnoticed because of signals from regions more resilient to damage, such as the DG. As a result, potential differences in response to OGD would be masked by an overall equal level of TTC metabolism between septal and temporal poles. To address this discrepancy, in the future, the various subfields (CA1, CA3, and DG) should be micro-dissected and analyzed separately, in order to probe for differences in TTC metabolism across the longitudinal axis.

5.2.2 Sex-Linked Differences Exist within the Septal Hippocampus of Pre-adolescent Rats in Response to 10 minutes of OGD

Based on a broad range of literature, it has become widely accepted that response to ischemic injury exhibits sexual dimorphism (Alkayed et al., 1998; Du et al., 2004; Li et al., 2005; Liu et al., 2009; Cheng and Hurn, 2010; Manwani and McCullough, 2011). However, many of these differences are often explained by the presence of the primary sex hormones, estrogen and testosterone (Alkayed et al., 1998, 2001; Uchida et al., 2009; Brann et al., 2012; Fairbanks et al., 2012), with less consideration given to the innate differences that may exist between male and female brain tissue (Arnold et al., 2003; Du et al., 2004; Cheng and Hurn, 2010). To establish

the presence, and underlying cause, of sex-linked differences in response to OGD across the longitudinal axis, this research examined male and female rats at two distinct points of life. First, to assess the possible role of intrinsic sex differences, male and female litter-mates were taken at PND 26-29, which represented a period of pre-adolescence. Animals at this age are presumed to contain low, and equal, levels of sex hormones; therefore any differences in response to brain injury would be thought to reflect chromosomal, or molecular–based differences between sexes (Turtzo and McCullough, 2010).

The results from this study indicated that hippocampal slices harvested from the septal pole of pre-adolescent male rats displayed greater sensitivity to 10 minutes of OGD when compared to their pre-adolescent female counterparts. These findings are in agreement with previous *in vitro* studies which examined differences in response to neuronal injury in sex-specific cell culture models (Du et al. 2004; Li et al., 2005; Hurn, 2014). Li et al. (2005) found that organotypic hippocampal slice cultures derived from male pups at PND 7 sustained greater damage from exposure to OGD than female cultures. Similarly, in primary cultured hippocampal neurons, as well as in astrocytes and microvascular endothelial cell culture models, XY cells displayed increased susceptibility to OGD when compared to XX cells (Du et al., 2004; Heyer et al., 2005; Hurn, 2014). Results from my study are also representative of the disparities regarding brain injury that are observed clinically between male and female patients. While pre-adolescent boys are at a greater risk of developing neural disorders, such as cerebral palsy, pre-adolescent girls experience better functional outcome following traumatic brain injury (Donders and Hoffman, 2002; Vannucci and Hurn, 2009; Cikla et al., 2015). Although the reason for this sexual dimorphism is incompletely understood, the fact that these sex differences are observed

before the onset of puberty, in both laboratory and clinical settings, suggests that hormone-independent mechanisms may influence response to ischemic injury.

The notion of sex-specific molecular pathways following cellular damage was previously described by Du et al. (2004) whereby XX and XY neurons showed varying degrees of susceptibility and underwent distinct cell death pathways upon exposure to cytotoxic chemicals. To support the concept of sexually dimorphic cell death mechanisms, emerging evidence now suggests that while female brain tissue undergoes a caspase-dependent pathway, male brain tissue undergoes a caspase-independent pathway following ischemic insult (Lang and McCullough, 2008; Yuan et al., 2009; Turtzo and McCullough, 2010).

Caspases are a family of enzymes that perform cysteine protease activity and regulate cell death through the initiation of apoptosis (McIlwain et al., 2013). In general, apoptosis is regarded as a form of programmed cell death, whereby intracellular components are disassembled and condensed before being phagocytosed (Elmore, 2007). Within female neurons, the ischemic-induced shift in energy metabolism is thought to trigger a release of cytochrome c from the intramembrane space of the mitochondria (Du et al., 2004; Turtzo and McCullough, 2010; McIlwain et al., 2013). Cytochrome c then binds to an adaptor protein known as apoptotic protease-activating factor-1 (APAF1), which leads to a number of conformational changes in its domain. Following these structural modifications, caspase-9 is bound and activated; resulting in the formation of a complex known as the apoptosome (Cain et al., 2002; Ferraro et al., 2009; McIlwain et al., 2013). With assembly of the apoptosome, downstream effector caspases are eventually recruited and apoptotic cell death is stimulated (Shiozaki et al., 2002; McIlwain et al., 2013).

In contrast to the caspase-dependent pathway, the male-associated caspase-independent pathway appears to be triggered when activation of neuronal nitric oxide synthase (nNOS) leads to the over production of nitric oxide (NO) (Du et al., 2004; Li et al., 2005; Yuan et al., 2009). Excess intracellular levels of NO results in the generation of peroxynitrite ($ONOO^-$), which subsequently leads to breaks in the strands of DNA (Hurn et al., 2005; Li et al., 2005; Yuan et al., 2009). In attempts to restore chromatin integrity, the DNA repair enzyme poly adenosine diphosphate ribose polymerase-1 (PARP-1) is activated (Li et al., 2005). Under normal physiological conditions, PARP-1 utilizes the cell's energy stores to repair single stranded DNA breaks; however during ischemia, PARP-1 becomes over stimulated and diminishes NAD^+ and ATP levels, which leads to a microglia-induced inflammatory response that ultimately results in necrotic cell death (Hagberg et al., 2004; Sairanen et al., 2009).

Based on these distinct molecular pathways, the results from this study may be explained by differences in the form of cell death experienced by male and female brain tissue. While female neurons are shown to display an apoptotic method of cell death; neurons derived from male tissue undergo a more necrotic pathway (Lang and McCullough, 2008; Yuan et al., 2009; Turtzo and McCullough, 2010). In general, necrosis is an uncontrolled process, resulting from a loss of energy, and is characterized by cellular swelling and rupture of the cell membrane. Following membrane deterioration, the release of cytoplasmic components signals an inflammatory response, which often damages a large portion of adjacent cells (Trump et al., 1997). In contrast, as previously mentioned, apoptosis is regarded as a more controlled mechanism that allows damaged cells to be removed without harming neighbouring cells, or causing inflammation (Elmore, 2007). As a result, the progression of apoptotic cell death is much slower than its counterpart, necrosis (Elmore, 2007).

Much of the literature reporting biological sex differences following neuronal injury utilized cell culture systems (Du et al., 2004, Li et al., 2005; Yuan et al., 2009). While culture models are able to maintain viability over an extended length of time, which aids in the assessment of long term cellular damage, they generally use cells from late stage embryos, or neonates (Cho et al., 2007). In contrast, the acute hippocampal slice model is beneficial in examining animals at different stages of life. However, due to the limited lifespan of slices, only short term/immediate cellular processes are able to be analyzed (Cho et al., 2007). In this study, acute hippocampal slices were subject to 10 minutes of OGD, followed by a 3 hour period of recovery. Since cellular viability was assessed a short time after injury, changes in mitochondrial function would be more likely to reflect necrotic cell death, rather than apoptotic cell death. In considering this notion, it is possible that the observed increased sensitivity of pre-adolescent male septal slices to the effects of OGD was caused by increased vulnerability to necrosis. If the slice model allowed one to examine the effects of delayed cell death pathways involving apoptosis, it is possible that TTC metabolism in female slices would have been altered. Nonetheless, my findings suggest that the male septal hippocampus from pre-adolescent rats contains heightened sensitivity to OGD-mediated necrotic cell death pathways, when compared to similar tissue from female animals.

Data from this study also displayed an apparent effect of sex within the temporal pole of pre-adolescent rodents; specifically, female temporal slices responded less well to 10 minutes of OGD than male temporal slices. This finding is opposite to the above mentioned results, in which male pre-adolescent tissue showed higher vulnerability to ischemic injury than female tissue within the septal pole. In addition, these results contradict evidence that reports greater ischemic tolerance within female neurons as compared to male neurons (Du et al., 2004).

Further analysis of this data revealed that the *p* value was 0.07, indicating no statistical significance. *P* values are important in testing the probability that the observed difference between two experimental groups occurred by chance; values of 0.05 or less are interpreted as reflecting true differences, and are regarded as *statistically* significant (McGough and Faraone, 2009). However, these calculations do not provide information on the *clinical* relevance, or the *magnitude of effect* that the differences between the groups display; therefore, in order to better understand the biological significance of the findings, Cohen's *d* effect sizes were also calculated (McGough and Faraone, 2009). Although the calculated *p* value suggested no statistical significance, given that $p = 0.07$ is quite close to the conventional threshold of $p = 0.05$, and, that a large effect size was obtained, it is worth considering the idea that pre-adolescent female temporal slices respond worse to ischemic insult than pre-adolescent male temporal slices. While the exact mechanism behind this phenomenon is currently unknown, it is possible that biochemical differences may exist between the temporal tissue of pre-adolescent male and female animals, which cause female slices to experience greater sensitivity toward 10 minutes of OGD. Further work examining differences in the expression of acute cell death markers, including SBDPs, should be completed in order to address this issue.

5.2.3 Hormone-Dependent Sex-Linked Differences were not present between Post-adolescent Male and Female Rats across the Longitudinal Axis

Apart from innate differences that exist between male and female brain tissue, a large body of literature also supports the claim that the primary sex hormones, estrogen and testosterone, play an intrinsic role in outcome from brain injury (Brann et al., 2007; Liu et al., 2009). More specifically, the influence of estrogen on response to cellular stress has been heavily studied, although findings are often contradictory (*see literature review section 1.4.3 and 1.4.4).* In an attempt to better understanding the effects of sex hormones on response to ischemic insult,

sexually mature male and female rats at PND 60-64 (post-adolescence) were assessed. Results from this study indicated that TTC metabolism following 10 minutes of OGD and a 3 hour period of recovery did not differ between post-adolescent male and female animals across the longitudinal axis of the hippocampus. Given the number of studies reporting an "ischemia sensitive" phenotype in male brain and an "ischemia protective" phenotype within female brain during young adulthood, these results were fairly unexpected (Liu et al., 2009; Cheng and Hurn, 2010; Manwani and McCullough, 2011).

In an effort to explain these findings, we considered potential discrepancies that may have been present within post-adolescent female rodents, specifically with regards to fluctuating levels of estrogen. Throughout the 4 day rodent estrus cycle, concentrations of endogenous estrogen can range between 30 pg/mL (metestrus) to 140 pg/mL (proestrus) (Carswell et al., 2000); therefore, a failure to observe hormone-dependent sex differences between male and female rats may have been caused by a high amount of noise from combining data from animals at different stages of the estrus cycle. Work by Carswell et al. (1999, 2000) demonstrated that, in sexually mature stroke prone spontaneously hypertensive rats, stratifying females based on their estrus cycle stage resulted in different outcomes from ischemic injury when compared to female rats that were not staged. When the estrus cycle was not controlled for, female rats displayed an infarct volume of 13%, 24 hours after MCAO; however, female rats determined to be in metestrus (low estrogen) showed an infarct volume of 30% in the contralateral hemisphere (Carswell et al., 1999). In addition, while the uncontrolled female group appeared to recover better from stroke when compared to male rodents, females in metestrus displayed a worse outcome than their male counterparts (Carswell et al., 1999).

In my study, stratifying post-adolescent female rats into high estrogen (proestrus) and low estrogen (estrus, metestrus and diestrus) groups did not yield appreciable differences in TTC metabolism following OGD within tissue from either the septal, or the temporal poles. This result is in contrast to evidence which found that rats with higher circulating levels of estrogen, in proestrus, displayed improved outcome from ischemia *in vivo* when compared to rats in low estrogen stages, such as metestrus and diestrus (Carswell et al., 2000; Raval et al., 2009). My findings are more in line, however, with research studying cardiac ischemia in isolated rodent hearts (Frasier et al., 2013; Hatcher et al. 2013). Hatcher et al. (2013) found that susceptibility to ischemic ventricular fibrillation (VF) did not differ between hearts isolated from female rodents at various stages of the estrus cycle. Similarly, differences in infarct volume following cardiac ischemic insult were not apparent between the four stages of the estrus cycle, in female rat hearts prepared *ex vivo* (Frasier et al., 2013).

When analyzing studies where fluctuating levels of estrogen were shown to influence response to injury, and, comparing them to studies that did not observe the effects of sex hormones, differences in the experimental design must be taken into consideration. While the present study, as well as others that reported no influence of the estrus cycle, used an *ex vivo* model to induce ischemic injury (Frasier et al., 2013; Hatcher et al., 2013), studies where estrogen levels were shown to have an effect on outcome employed *in vivo* models of ischemia (Carswell et al., 1999, 2000; Raval et al., 2009). Therefore, our failure to observe a hormone-dependent effect on response to OGD may have been caused by a lack of circulating endogenous estrogen at distant sites of injury within the brain. In support of this claim, a review by Brann et al. (2007) highlighted that gonadal-derived estrogen played a significant role in protecting neurons against ischemic injury.

While many cellular pathways are thought to be involved in estrogen-mediated neuroprotection, in general it is thought that estrogen released from the ovaries travels through the circulatory system and activates mitogen activated protein kinase (MAPK) as well as calcium-calmodulin-dependent protein kinase (CAMKII) (Raval et al., 2009). MAPK and CAMKII go on to activate cyclic-AMP response element binding protein (CREB) through phosphorylation, which then initiates a downstream signalling cascade (Raval et al., 2009). Within the hippocampal CA1 subfield, following *in vivo* stroke, the phosphorylation of MAPK increased by 56% and phosphorylation of CAMKII increased by 71% during the shift from diestrus (low estrogen) to proestrus (high estrogen) (Raval et al., 2009). These findings corresponded with a reduction in the number of healthy neurons by 82% within diestrus animals, but a 17% increase in the number of healthy neurons within proestrus animals, 7 days post-injury (Raval et al., 2009). Taken together, these data support the notion that higher circulating levels of endogenous estrogen serve to protect against ischemic damage via phosphorylation of CREB within the *intact* animal (Raval et al., 2009). In light of this concept, the use of an acute hippocampal slice model would have prevented direct exposure of slices to the varying levels of estrogen in the bloodstream; therefore eliminating the activation of certain neuroprotective pathways.

Apart from the effects of gonadal derived estrogen, a growing body of literature also suggests that estrogen can be produced in neurons within the hippocampal formation, following NMDA-mediated excitotoxicity, in order to exert neuroprotection (Hojo et al., 2004). Following MCAO, Carswell et al. (2005) observed an increase in the expression of aromatase, the enzyme critical for the production of estrogen, within the brain of OVX female rodents. In addition, McCullough et al. (2003) found that neuronal impairments after ischemic stroke were greater in

aromatase knock-out mice compared to OVX wild-type mice. Given that these studies indicated both the existence and the importance of locally synthesized estrogen on neuroprotection following brain injury, it was thought that extra-gonadal, brain-derived estrogen may have played a role in response to ischemic insult within acute hippocampal slices. Nonetheless, the failure to observe the effects of estrus cycle stage on 10 minutes of OGD in post-adolescent female brain slices may have been due to the time in which hippocampal tissue was assessed. As mentioned previously, response to OGD measured after a 3 hour period of reperfusion would be reflective of immediate damage within the ischemic core. However, it was reported that the upregulation of aromatase was only observed 8 hours and 24 hours after insult, and was not present 2 hours post-MCAO (Carswell et al., 2005). In addition, aromatase was predominantly expressed within the penumbra; therefore estrogen –facilitated neuronal protection would most likely be seen in areas of reversible damage rather than the ischemic core (Carswell et al., 2005). Together, these data suggest that the experimental design of this study would not allow for the observation of estrogen's protective effects following OGD. If experimental stroke was induced *in vivo*, a longer period of reperfusion would have been permitted, which would allow for the induction of hormone-dependent genomic and non-genomic signalling pathways that are thought to be initiated following brain injury (Brann et al., 2007). Following a more delayed assessment, differences in cell viability from 10 minutes of OGD may have been observed between post-adolescent male and female rats.

5.2.4 Age-related Differences in TTC metabolism are present in the Male Septal Hippocampus Following 10 Minutes of OGD

As previously noted, age was used in this study as a means to determine how naturally circulating levels of the primary sex hormones, estrogen and testosterone, might influence response to brain injury. In order to explore differences in mitochondrial function following 10

minutes of OGD, male and female rodents at ~PND 26, representing pre-adolescence, were compared to rodents at ~PND 60, representing young adulthood. Results from my study revealed no differences in TTC metabolism between pre-adolescent and post-adolescent female rats within the septal, or temporal poles of the hippocampus. Within male animals however, my data indicated that the septal hippocampus in pre-adolescent rats showed increased vulnerability to the effects of excitotoxicity when compared to their post-adolescent counterparts. Although rats at post-adolescence were considered adults, these animals do not represent an *aged* population and therefore would not have experienced the physiological changes that occur with advanced ageing, including reproductive senescence and the development of co-morbidities that often lead to poorer functional outcome after stroke (Manwani and McCullough, 2011). As such, the age-related differences observed in response to neuronal injury may be more appropriately explained by the biological differences that exist between the immature and mature brain.

The idea that the brain displays different levels of susceptibility to injury at various stages of development has been well established (Blomgren et al., 2003; Zhu et al., 2005; Giza et al., 2009; Babikian et al., 2010; Semple et al., 2013). In this study, the use of rats at PND 26 corresponded to the juvenile stage of brain maturation, spanning from the point of weaning (PND 21) to ~PND 45, whereas rats at PND 60 reflected the mature, adult brain (Semple et al., 2013). Between these periods of life, several anatomical and molecular distinctions are thought to exist, which render the immature brain more susceptible to the effects of neuronal insult (Giza et al., 2009; Babikian et al 2010). One mechanism in particular pertains to the upregulation of mitochondrial defense systems following brain injury (Giza et al., 2009; Babikian et al., 2010; Semple et al., 2013). Cytotoxicity and oxidative stress result in excessive ROS generation within the cell. To control for this overproduction of ROS, mitochondrial antioxidant enzymes,

including superoxide dismutase (SOD) and glutathione peroxidase (GPx), are activated, and work to scavenge excess free radicals (Aspberg et al., 1992; Mavelli et al., 1982). Interestingly, work by Fan et al. (2003) found that this increase in antioxidant activity appeared to be an age-related process. While the adult mouse brain expressed a 28% increase in GPx activity 3 hours after traumatic brain injury (TBI), there were no detectable changes in GPx activity present in mice at PND 21 (Fan et al., 2003). Furthermore, this failure to stimulate mitochondrial antioxidants corresponded to an increased susceptibility of the underdeveloped brain to neuronal damage (Fan et al., 2003). Taken together, it is possible that the worsened mitochondrial function observed in pre-adolescent male septal tissue, compared to post-adolescent male tissue, may have been related to an insufficient degree of antioxidant activity.

A second potential factor that may have contributed to the age-related differences observed in response to ischemic challenge are changes in the NMDAR subunit composition, which occur during development (Monyer et al., 1994; Cull-Candy., 2001; Law et al., 2003; Giza et al., 2009; Semple et al., 2013). At birth, there is a dramatic rise in the expression of the GluN1 receptor subunit, which peaks during the third week of life (Law et al., 2003). Conversely, while GluN2A subunit expression is low at birth, GluN2B expression is high, reaching maximal levels around PND 20 in the rat hippocampus and cortex (Giza et al., 2009; Semple et al., 2013). However, as the brain matures, the GluN2A:GluN2B ratio switches; while GluN2B subunits decline following the third/fourth week of life, they appear to be replaced with GluN2A subunits, and, less commonly, GluN2C subunits (Cull-Candy, 2001; Giza et al., 2009).

The GluN2B subunit plays an essential part in excitatory neurotransmission following calcium influx (Lujan and Wan, 2012). During early stages of neuronal development, the increased expression of the GluN2B subunit is necessary for the induction of LTP, associated

with learning and memory; however, evidence also suggests that the GluN2B subunit is heavily involved in mediating excitotoxic cell death pathways leading to neurodegeneration (Giza et al., 2009; Lujan and Wan, 2012). Based on the conflicting roles of the GluN2B subunit, it would appear that while its heightened expression allows for increased synaptic plasticity in the immature brain, it also renders young animals more susceptible to the effects of ischemic insult (Giza et al., 2009).

As previously described, changes in cellular viability observed 3 hours post-OGD would be associated with cell death pathways resulting in necrosis rather than delayed pathways leading to apoptosis (Czogalla and Sikorski, 2005). One of the earliest hallmarks of necrotic cell death is the excessive influx of Ca^{2+}-ions, which trigger the activation of calcium-sensitive proteases known as calpains (Czogalla and Sikorski, 2005). In response to cellular stress calpains will undergo proteolytic activity in order to break down specific substrates associated with cellular maintenance (Czogalla and Sikorski, 2005; Yan and Jeromin, 2012). Spectrin (αII-spectrin), in particular, is a key component of the cytoskeleton that plays a role in the structural integrity and modulation of synaptic components (Yan and Jeromin, 2012). Following neuronal injury, calpain will perform enzymatic cleavage of the 280 kDa αII-spectrin protein, creating 145 kDa and 150 kDa fragments, referred to as spectrin breakdown products (SBDPs) (Yan and Jeromin, 2012). Both *in vitro* and *in vivo* models have provided evidence for the relation between excitotoxicity and calpain activation resulting in the appearance of SBDPs within the hippocampus (Yan and Jeromin, 2012). As such, SBDPs have now become a widely accepted biomarker of early necrotic cell death (Czogalla and Sikorski, 2005; Zhu et al., 2005; Yan and Jeromin, 2012).

Differences in calpain-mediated necrotic cell death have been observed between the mature and immature brain following hypoxic-ischemic (HI) injury (Zhu et al., 2005). A study by Zhu et al. (2005) reported that while protein concentration of calpain remained consistent during the various stages of development, after HI there was an age-dependent decrease in calpain levels, indicative of increased activity and subsequent degeneration. Within the immature brain, male mice at PND 21 displayed a >20% loss in calpain levels; however, mice at PND 60 revealed only a 5% reduction in calpain (Zhu et al., 2005). These results suggest that immediate pathways of necrotic cell death may be more readily activated within the developing brain as compared to the adult brain.

Under pathophysiological conditions, there also appears to be a strong link between the over-activation of the NMDARs and the immediate presence of 145 kDa and 150 kDa SBDPs (Seubert et al., 1988; Siman et al., 1989; Delcerro et al., 1994; Bahr et al., 1995; Vanderklish et al., 1995; Wechsler and Teichberg, 1998; and Czogalla and Sikorski, 2005). Within cultured hippocampal neurons, SBDPs were apparent just 2 minutes after treatment with NMDA (Bahr et al., 1995); however, alternative measures of viability, including propidium iodide staining, could not be detected until 30 minutes later (Czogalla and Sikorski, 2005). Moreover, when exposed to NMDAR-antagonists, calpain-mediated SBDPs were not observed, thereby further supporting the relationship between NMDAR stimulation and calpain activity (DelCerro et al., 1994).

As mentioned earlier in this discussion, response to neuronal insult is thought to be, in part, regulated by the subunit composition of the NMDARs (Giza et al., 2009). In light of this notion, evidence suggests that following exposure to NMDA, calpain will selectively cleave the GluN2B subunit, creating 115 kDa fragments that remain active on the cell surface, and are assumed to be involved in post-ischemic excitotoxicity; a process that is not observed with the

GluN2A subunit (Simpkins et al., 2003). This calpain-mediated GluN2B cleavage was also associated with a greater number of SBDPs within the hippocampus of epileptic rats, as reported by Araujo et al. (2005). When taken together, these findings may help to explain the heightened sensitivity of pre-adolescent male tissue compared to the post-adolescent tissue that was observed in this study. If the immature brain contains a greater concentration of GluN2B NMDAR subunits, when compared to the mature brain, then tissue extracted from young animals would be more prone to calpain-mediated subunit cleavage than tissue extracted from adult animals. Consequently, this would leave pre-adolescent male septal slices more susceptible to mitochondrial impairments and exacerbated damage following 10 minutes of OGD, as compared to post-adolescent septal slices.

6.0 Conclusions and Future Directions

In the present study, both the age and sex of an animal appeared to have an influence on response to OGD, in a region of the brain previously reported to be selectively vulnerable to ischemic insult. When taken together, these results suggest that the septal pole of pre-adolescent male rodents displays greater sensitivity to neuronal injury than both pre-adolescent female rodents, and post-adolescent male rodents. To explore potential differences in necrotic cell death pathways that may contribute to this increased vulnerability, calpain activity and SBDPs will be probed in each experimental group using Western blot analysis. In addition, the results from this study were not in agreement with previous literature, which found differences in response to injury across the longitudinal axis of the hippocampus. To further study these differences, acute hippocampal slices will be sub-dissected into their three main subfields (CA1, CA3 and DG) and response to OGD across the axis will be assessed accordingly. Finally, the use of an *ex vivo* system posed certain limitations with respect to the time of assessment, as well as not allowing for the examination of an intact animal model. In the future, the use of an *in vivo* system will be used in order to examine the effects of delayed cell death, as well as to further understand the role that endogenous hormones, such as estrogen, play on response to OGD across the hippocampal axis.

Region	Age	Sex	N
Temporal	Pre-Adolescent6	Male	1
Temporal	Pre-Adolescent	Female	2
Temporal	Post-Adolescent	Male	3
Temporal	Post-Adolescent	Female	3

Table 1: Summary of data excluded from analysis. Male and female pre and post-adolescent animals excluded from the final data analysis based on TTC metabolism values that violated an *a* priori assumption.

	Mean % SHAM Septal	Mean % SHAM Temporal	D.S.	SEM	N	*p* Value	Effect Size (*d*)	± 95% CI
Male Pre-adolescent	59.99	69.92	-10.00	6.0	9	0.20	0.55	-1.24, 0.17
Female Pre-adolescent	73.88	62.92	10.96	8.8	10	0.43	0.39	-0.26, 1.03
Male Post-Adolescent	71.54	62.49	9.05	6.1	10	0.19	0.47	-0.2, 1.1
Female Post-adolescent	71.69	63.80	5.09	7.0	10	0.49	0.23	-0.41, 0.85

Table 2: Statistical table for regional differences. Data presented as % of SHAM TTC metabolism. Relative mean difference scores (D.S.) ± standard error of the mean (SEM) calculated between septal and temporal slices in male and female pre and post-adolescent animals. Statistical significance represented by *p* value and Cohen's *d* value ± 95% confidence interval (CI).

	Mean % SHAM Male	Mean % SHAM Female	D.S.	SEM	N	*p* Value	Effect Size *(d)*	± 95% CI
Septal Pre-adolescent	59.99	73.88	-13.16	4.89	9	0.04	0.90	-1.66, -0.10
Temporal Pre-adolescent	69.92	62.92	10.86	4.06	9	0.07	0.89	0.09, 1.7
Septal Post-Adolescent	71.54	71.69	-0.15	2.82	10	0.63	0.017	-0.64, 0.60
Temporal Post-adolescent	62.49	63.80	-1.31	8.47	10	0.63	0.049	-0.67, 0.57

Table 3: Statistical table for sex-linked differences. Data presented as % of SHAM TTC metabolism. Difference scores (D.S.) ± standard error of the mean (SEM) calculated between pre-adolescent, as well as post-adolescent, male and female animals in both the septal and temporal poles. Statistical significance represented by *p* value and Cohen's *d* value ± 95% confidence interval (CI).

	Mean % SHAM Pre-Adolescent	Mean % SHAM Post-Adolescent	D.S.	SEM	N	*p* Value	Effect Size *(d)*	± 95% CI
Male Septal	59.99	71.54	-12.03	5.86	9	0.05	0.68	-1.40, 0.06
Female Septal	73.88	71.69	2.19	4.15	10	0.77	0.17	-0.46, 0.79
Male Temporal	69.92	62.49	5.80	7.4	9	0.57	0.26	-0.41, 0.92
Female Temporal	62.92	63.80	-0.88	9.14	10	0.85	0.03	-0.65, 0.59

Table 4: Statistical table for age-related differences. Data presented as % of SHAM TTC metabolism. Difference scores (D.S.) ± standard error of the mean (SEM) calculated between pre-adolescent and post-adolescent male and female animals in both the septal and temporal poles. Statistical significance represented by *p* value and Cohen's *d* value ± 95% confidence interval (CI).

	Mean % SHAM Septal	Mean % SHAM Temporal	N	D.S.	SEM	*p* Value	Effect Size (*d*)	± 95% CI
High Estrogen	63.93	67.89	5	-3.96	14.73	> 0.99	0.12	-0.99, 0.77
Low Estrogen	72.12	65.07	8	7.06	7.07	0.313	0.35	-0.79, 0.61

Table 5: Statistical table for estrogen levels and regional differences. Data presented as % of SHAM TTC metabolism. Difference scores (D.S.) ± standard error of the mean (SEM) calculated between septal and temporal slices from female animals in high estrogen, and low estrogen, groups. Statistical significance represented by *p* value and Cohen's *d* value ± 95% confidence interval (CI).

	Mean % SHAM High E2	**SEM**	**Mean % SHAM Low E2**	**SEM**	***p* Value**	**Effect Size *(d)***	**± 95% CI**
Septal	63.93	5.01	72.12	3.52	0.35	0.78	-0.36, 1.93
Temporal	67.89	10.47	65.52	6.07	0.72	0.14	-0.98, 1.26

Table 6: Statistical table for estrogen-concentration dependent differences. % of SHAM TTC metabolism ± standard error of the mean (SEM) compared between female rats from high and low estrogen groups in both the septal, and temporal, poles. Statistical significance represented by *p* value and Cohen's *d* value ± 95% confidence interval (CI).

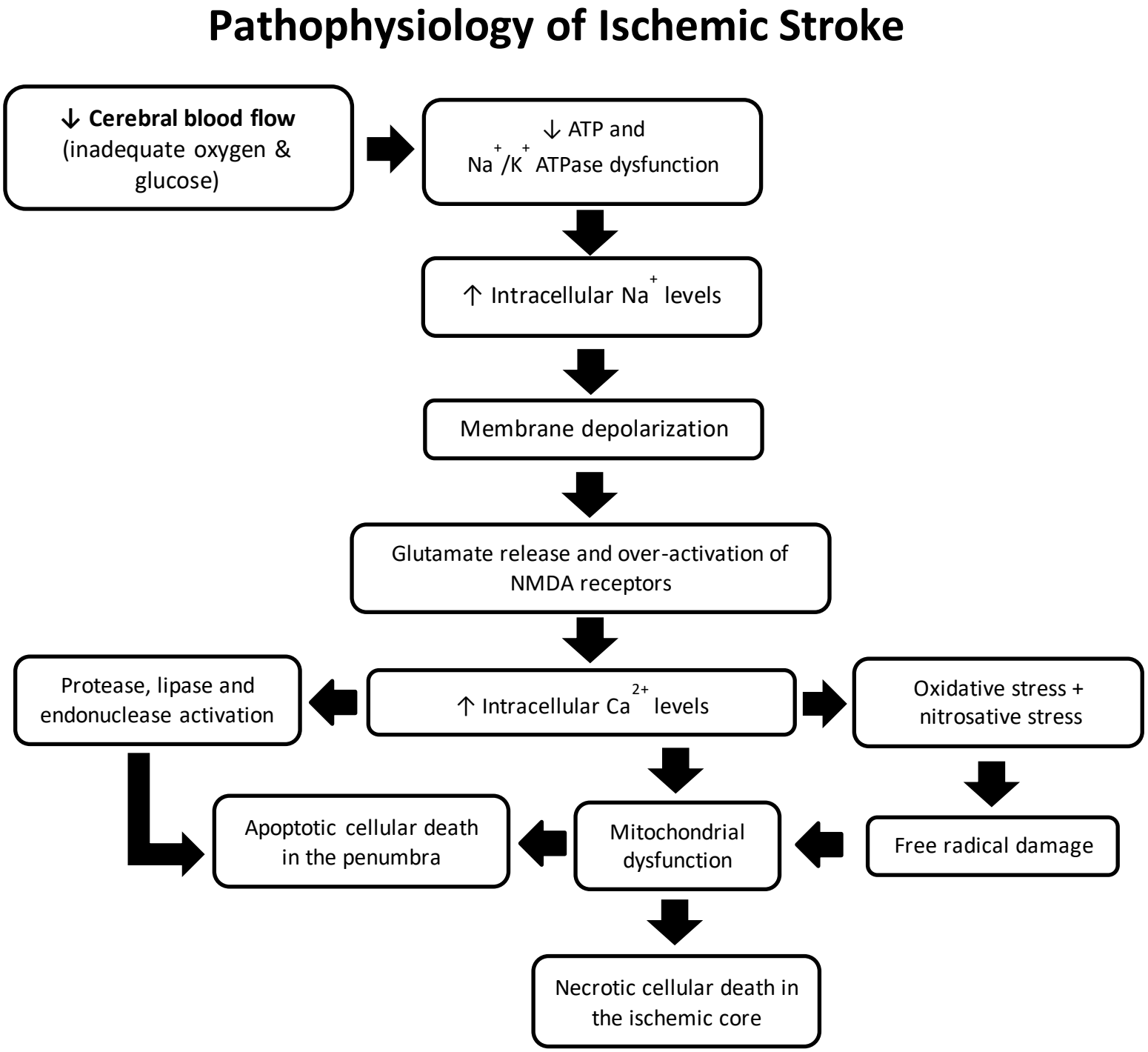


Figure 1: Key cellular elements involved in the ischemic cascade.

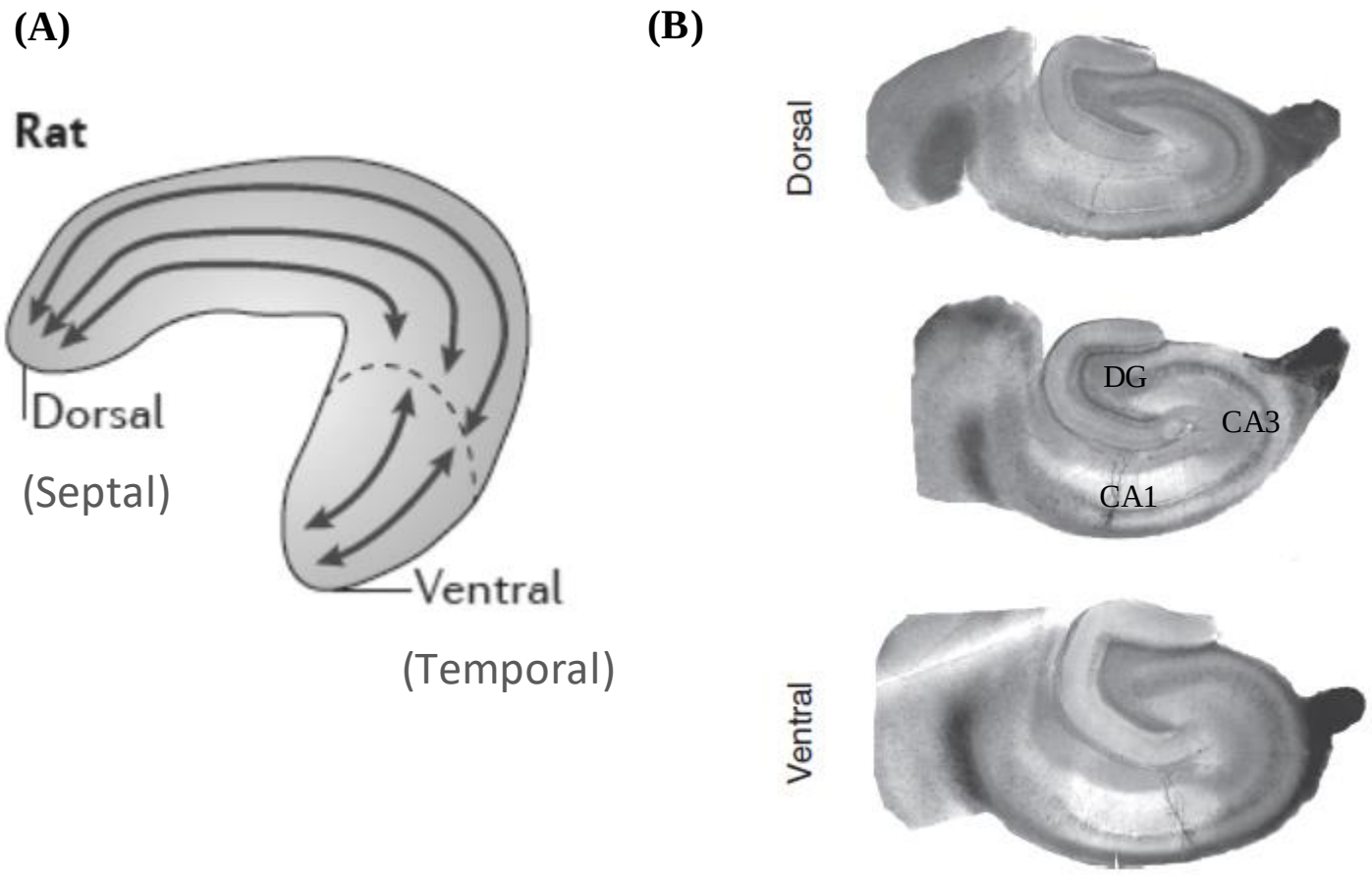


Figure 2: The longitudinal axis of the rat hippocampus. (A) The dorsoseptal and ventrotemporal poles of the rat longitudinal axis are outlined. The lines are indicative of the portion of tissue each region occupies; the septal pole extends across the first two-thirds of the hippocampal long axis, while the temporal pole occupies the remaining one-third of the hippocampus. Figure adapted from "Functional organization of the hippocampal longitudinal axis," by B. A. Strange, M. P. Witter, E.S. Lein and E. I. Moser, 2014, *Nature Reviews, vol 15,* pp. 658. Copyright 2014 Macmillan Publishers. **(B)** Hippocampal slices prepared across the longitudinal axis. Figure adapted from "Anatomical and Electrophysiological Comparison of CA1 Pyramidal Neurons of the Rat and Mouse," by B. N. Routh, D. Johnston, K. Harris and R. A. Chitwood, 2009, *J Neurophysiol, vol 102,* pp. 2291. Copyright 2009 The American Physiological Society.

Experimental Protocol

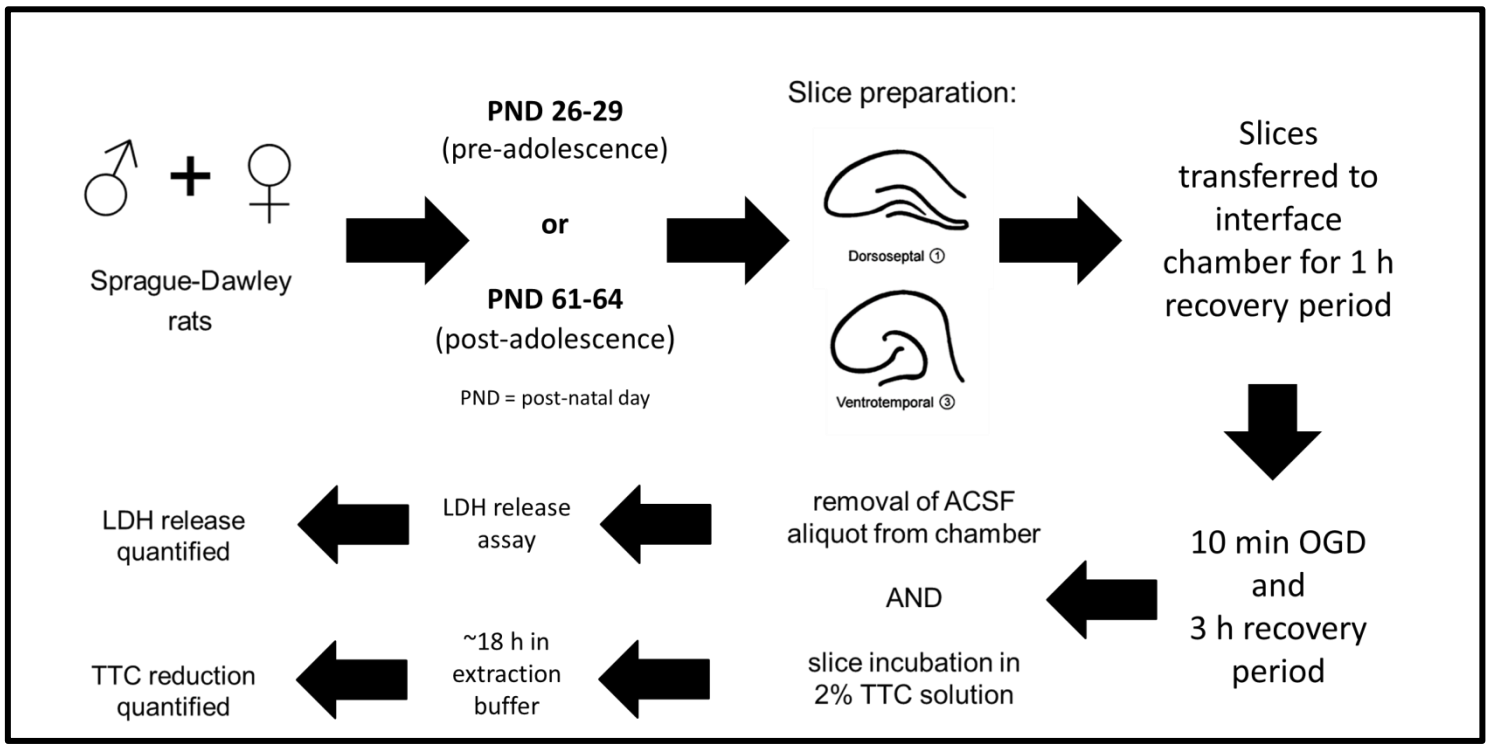


Figure 3: Summary of experimental protocol.

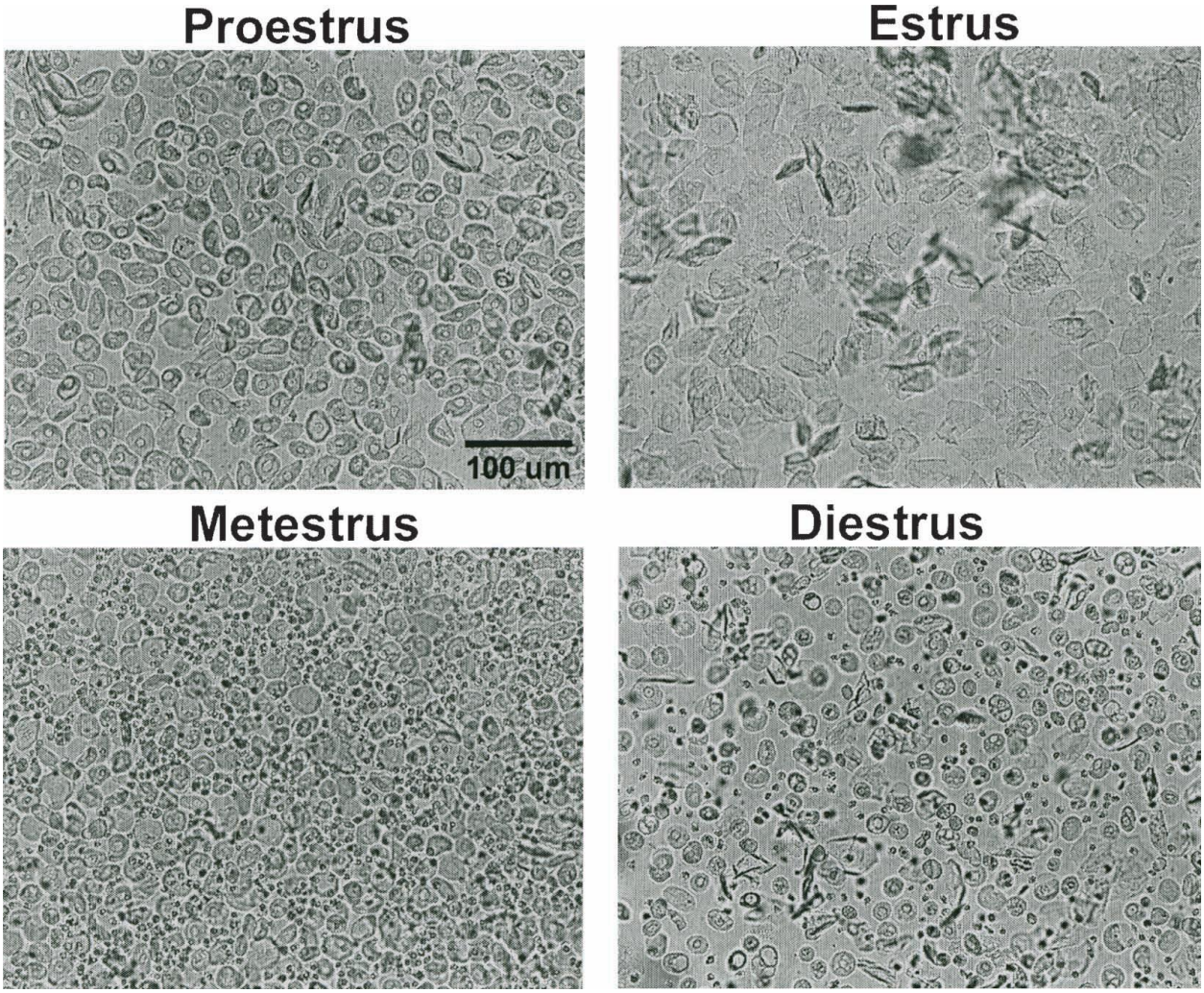


Figure 4: Representative image of wet vaginal smear from each stage of the rat estrus cycle. 200 x. Figure taken, with permission, from "A quantitative method for assessing stages of the rat estrous cycle" by CH Hubscher et al.., 2005, *Biotechnic and Histochemistry ,vol.80:2*, pp.79-87, www.tandfonline.com http://dx.doi.org/10.1080/10520290500138422.

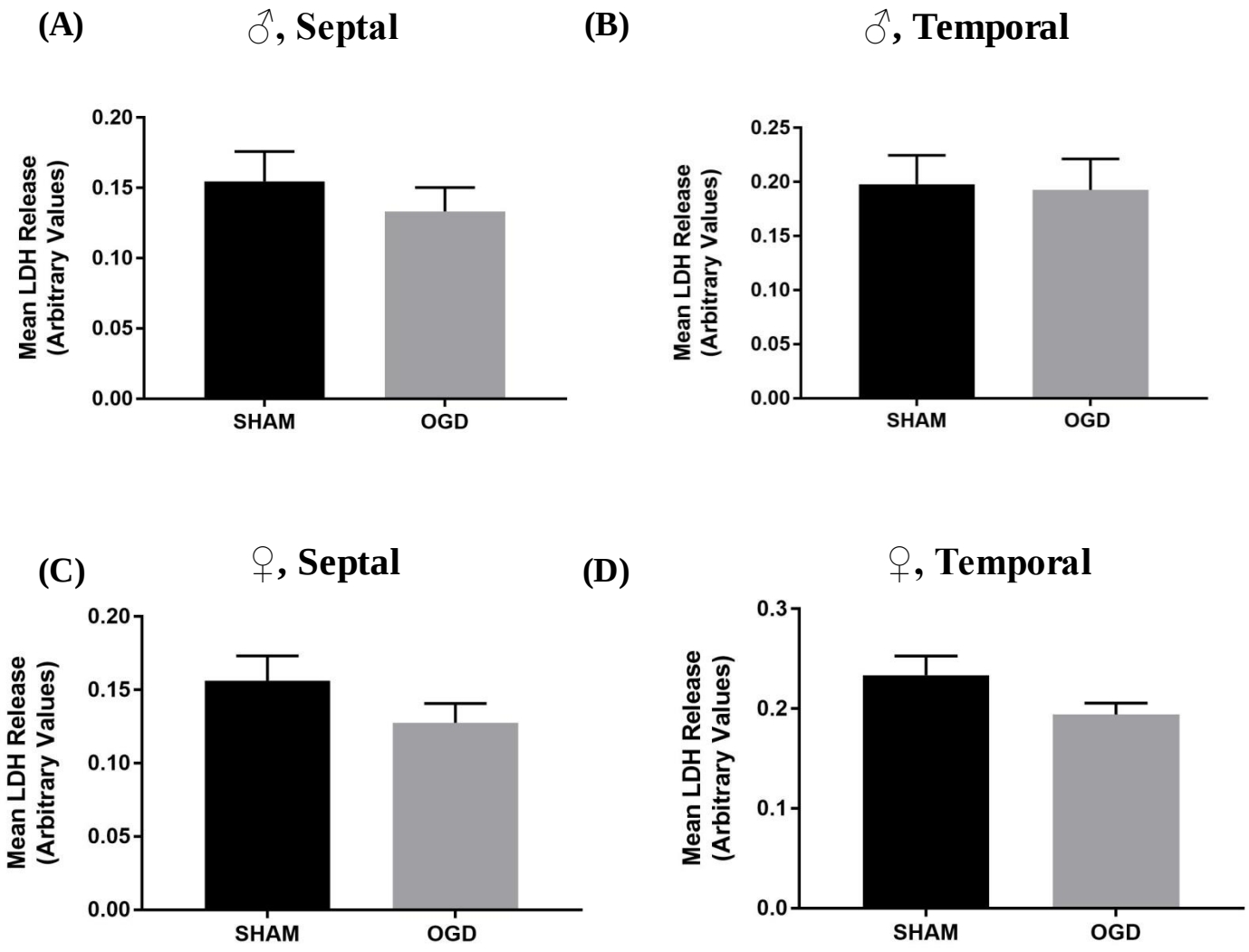


Figure 5: **10 minutes of OGD did not cause significant changes in LDH release in pre-adolescent slices across the longitudinal axis. (A)** Male septal slices (arbitrary units: SHAM, 0.154 ± 0.02 vs. OGD, 0.133 ± 0.02) **(B)** Male temporal slices (arbitrary units: SHAM, 0.198 ± 0.03 vs. OGD, 0.193 ± 0.03) **(C)** Female septal slices (arbitrary units: SHAM, 0.156 ± 0.02 vs. OGD, 0.128 ± 0.01) or **(D)** Female temporal slices (arbitrary units: SHAM, 0.23 ± 0.02 vs. OGD, 0.19 ± 0.01); N = 5 litters with the average of 2 animals from each litter being used. Data are presented as mean ± SEM.

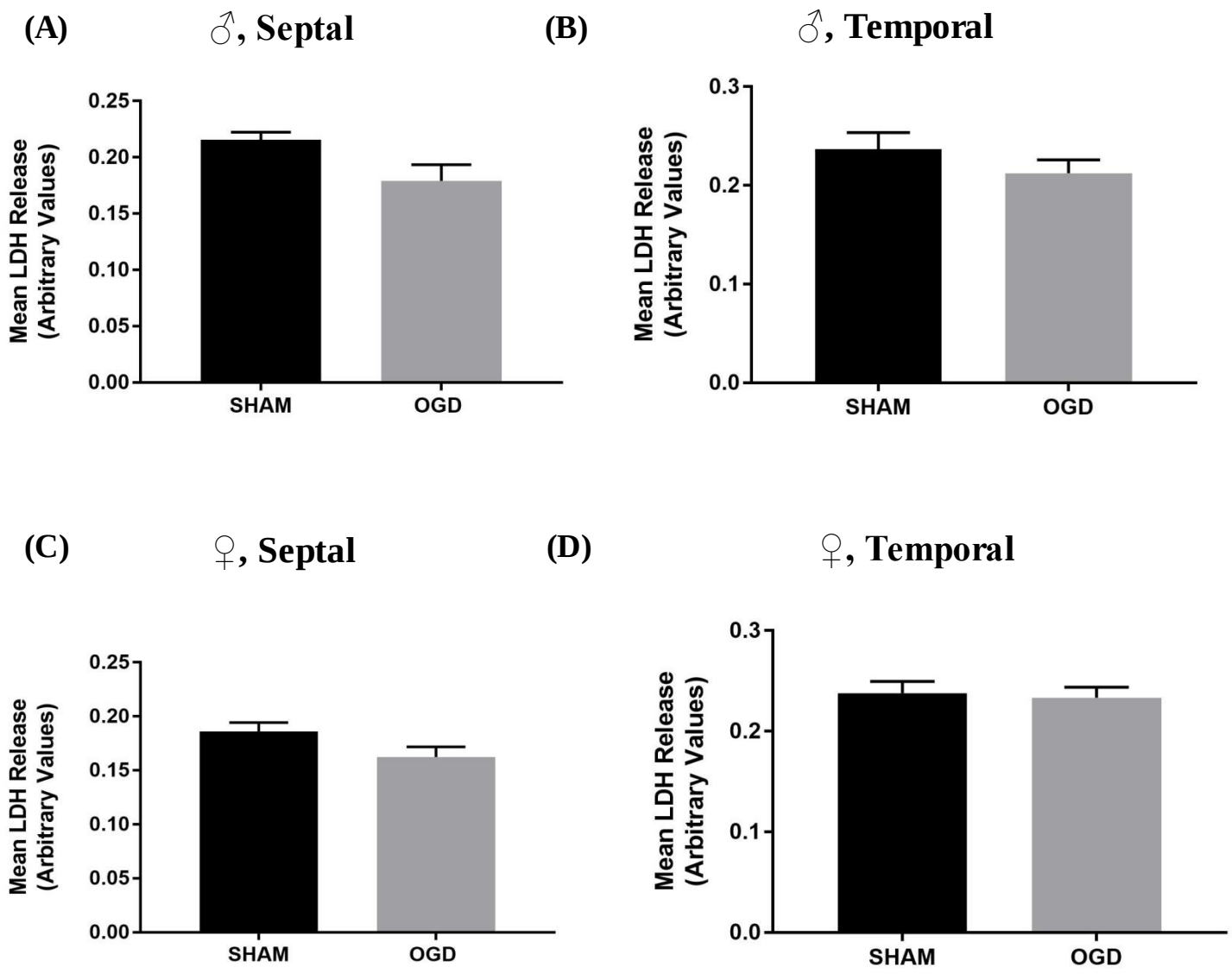


Figure 6: **10 minutes of OGD did not cause significant changes in LDH release in post-adolescent slices. (A)** Male septal slices (arbitrary units: SHAM, 0.216 ± 0.007 vs. OGD, 0.179 ± 0.01) **(B)** Male temporal slices (arbitrary units: SHAM, 0.237 ± 0.02 vs. OGD, 0.212 ± 0.01) **(C)** Female septal slices (arbitrary units: SHAM, 0.186 ± 0.01 vs. OGD, 0.162 ± 0.01) or **(D)** Female temporal slices (arbitrary units: SHAM, 0.238 ± 0.01 vs. OGD, 0.233 ± 0.01); N = 5 litters with the average of 2 animals from each litter being used. Data are presented as mean ± SEM**.**

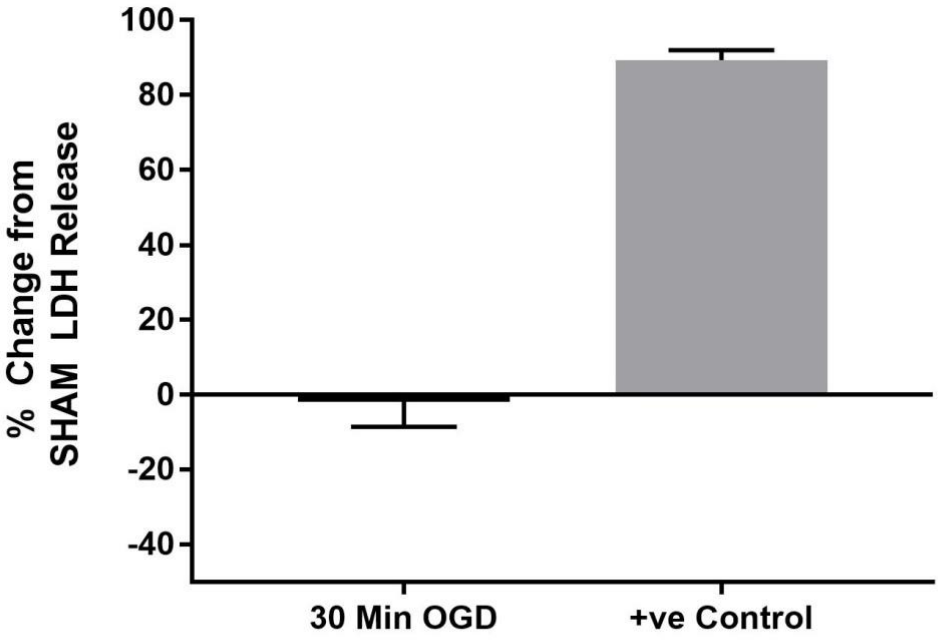
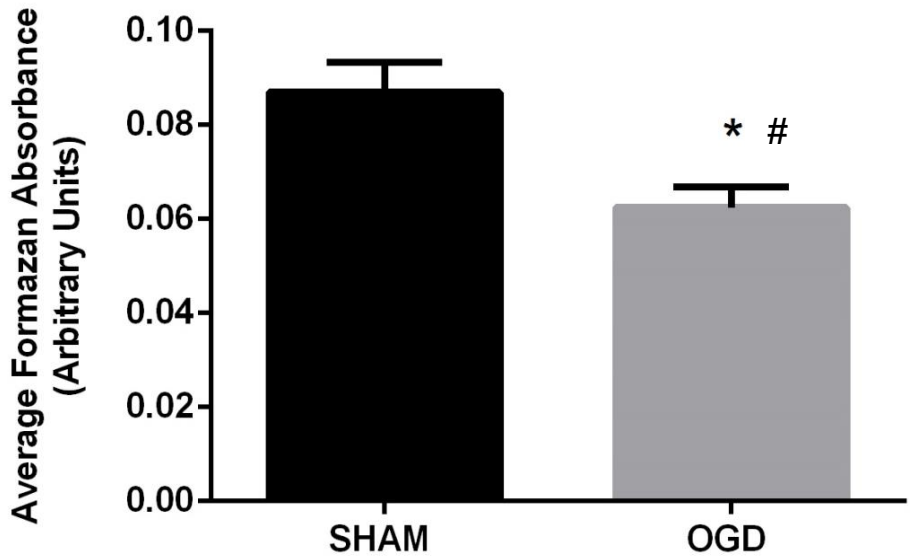


Figure 7: 30 Minutes of OGD did not cause significant changes in LDH release. Data presented as mean ± SEM. Percent change in LDH released from slices subjected to 30 min of OGD and a 3 h recovery (slices held at interface throughout) vs. slices kept in a glass vial with 1 mL ACSF for 4 h at 35.0 ± 0.5^{o}C (slices submerged; positive control). Slices harvested from pre-adolescent male rats.

(A)



(B)

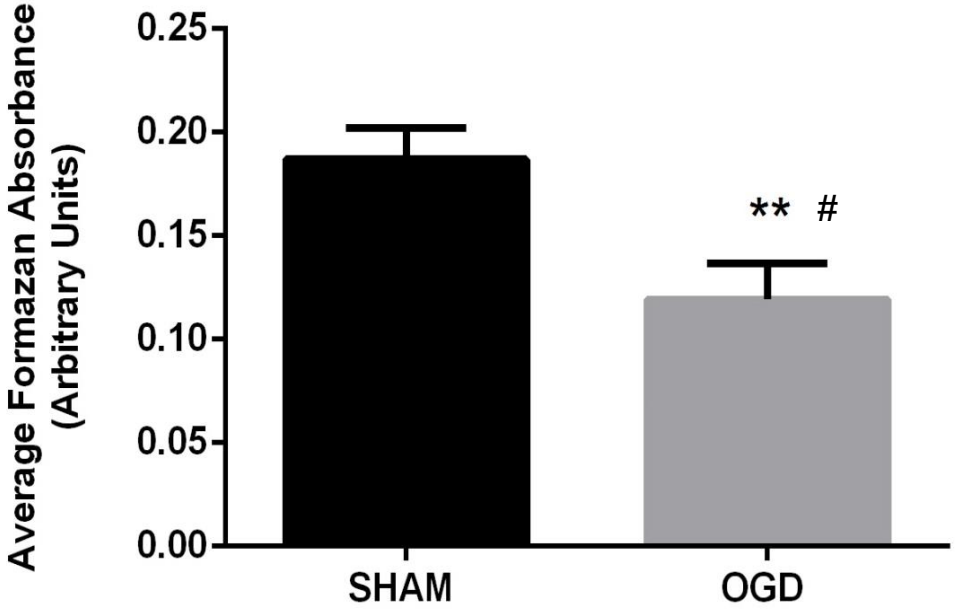


Figure 8: **Ten minutes of OGD has a large effect on TTC metabolism**. Ten minutes of OGD has a large effect on TTC metabolism relative to SHAM treatment in both **(A)** septal (arbitrary units: SHAM, 0.087 ± 0.01 vs. OGD, 0.063 ± 0.01; Cohen's *d* = 2.01 (95% CI = 0.40, 3.54) and **(B)** temporal slices (arbitrary units: SHAM, 0.19 ± 0.02 vs. OGD, 0.11 ± 0.02; Cohen's *d* = 1.86 (95% CI = 0.29, 3.35); n = 2 animals from each of N = 5 litters. All slices were harvested from male rats at ~PND 60. Data presented as mean ± SEM; *p = 0.03, **p = 0.0079 following the Mann-Whitney U test. Effect size calculated using Cohen's *d;* # = large effect size.

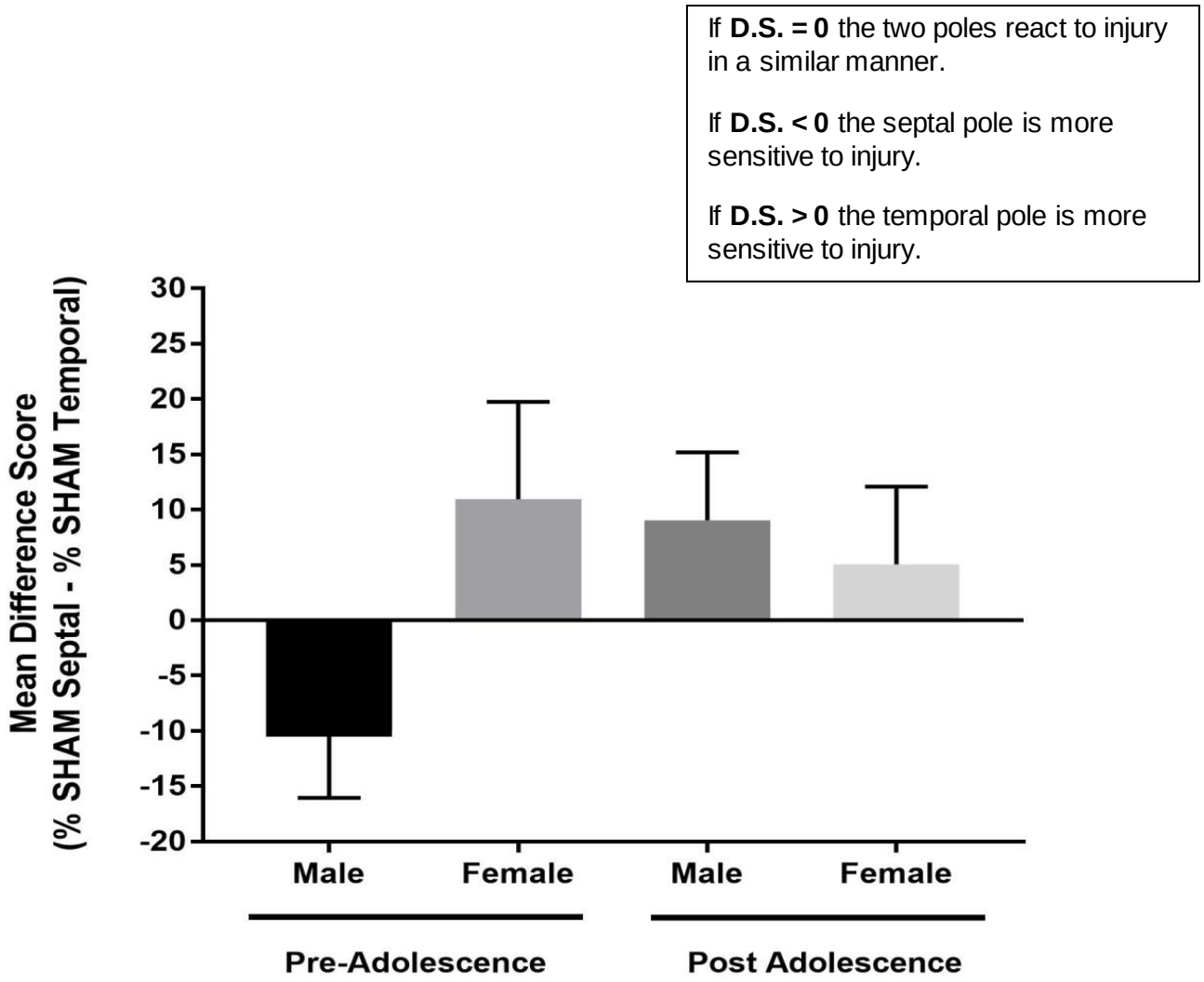


Figure 9: TTC metabolism following 10 minutes of OGD does not differ across the longitudinal axis in male and female rats. Post-OGD TTC metabolism was not appreciably different between septal and temporal slices in pre-adolescent male [p = 0.2; Cohen's *d* = 0.55 (95% CI = -1.24, 0.17)]; n = 2 animals from each of N = 9 litters, pre-adolescent female [p = 0.43; Cohen's *d* = 0.39 (95% CI = -0.26, 1.03)], post-adolescent male [p = 0.19; Cohen's *d* = 0.47 (95% CI = -0.2, 1.11)], or post-adolescent female [p = 0.49; Cohen's *d* = 0.23 (95% CI = -0.41, 0.85)] rats ; n = 2, N = 10. Data presented as mean difference score (D.S.) ± SEM. Statistical significance measured using the Wilcoxon signed-ranked test; $p < 0.05$. Effect size calculated using Cohen's *d*.

If **D.S. = 0** both sexes react to injury in a similar manner.

If **D.S. < 0** the male group is more sensitive to injury.

If **D.S. > 0** the female group is more sensitive to injury.

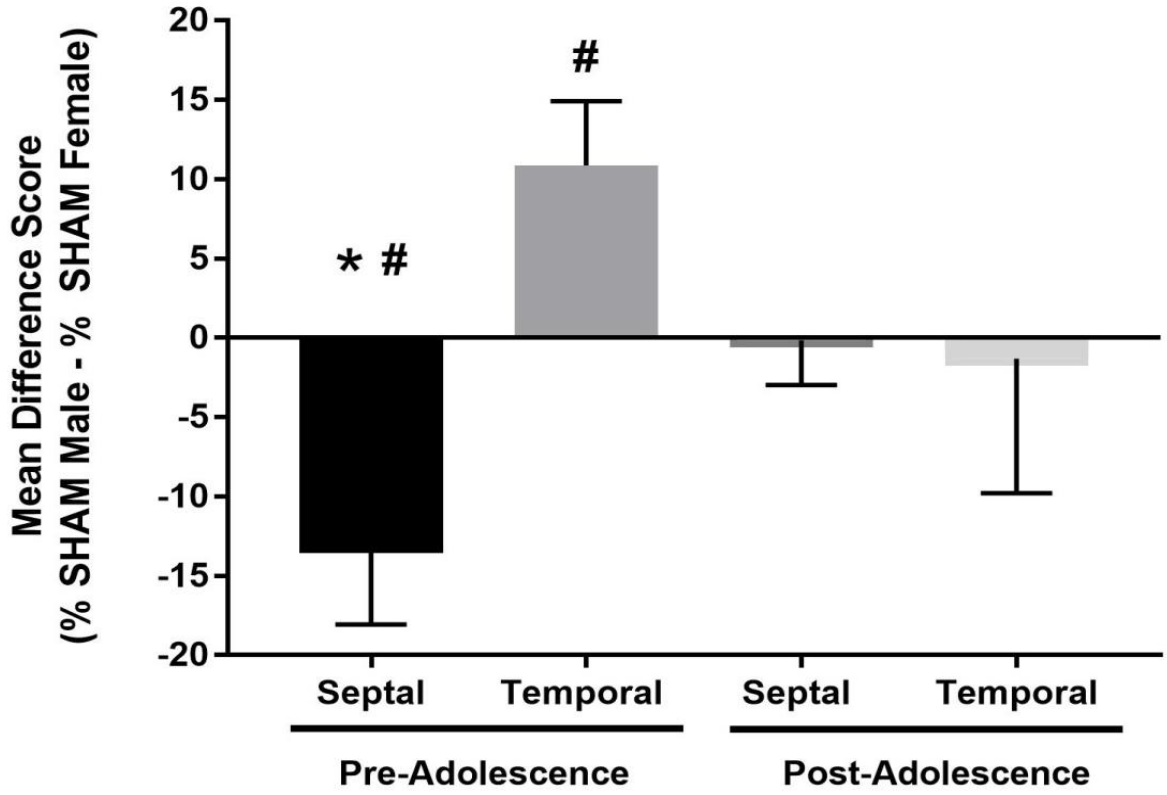


Figure 10: Sex has a large effect on differences in TTC metabolism between pre-adolescent male and female rats. Ten minutes of OGD had a large effect on changes in TTC metabolism between pre-adolescent male and female septal [p = 0.039; Cohen's *d* = 0.90 (95% CI= -1.66, -0.10)] and temporal [p = 0.07; Cohen's *d* = 0.89 (95% CI = 0.09, 1.66)] slices; n = 2 animals from each of N = 9 litters. 10 minutes of OGD did not have a large effect on changes in TTC metabolism between post-adolescent male and female septal [p = 0.63; Cohen's *d* = 0.02 (95% CI = -0.64, 0.60)] or temporal [p = 0.63; Cohen's *d* = 0.05 (95% CI = -0.67, 0.57)] slices; n = 2, N = 10. Data are presented as mean difference score (D.S.) ± SEM. Statistical significance was measured using the Wilcoxon signed-ranked test; *p < 0.05. Effect size calculated using Cohen's *d;* # = large effect size.

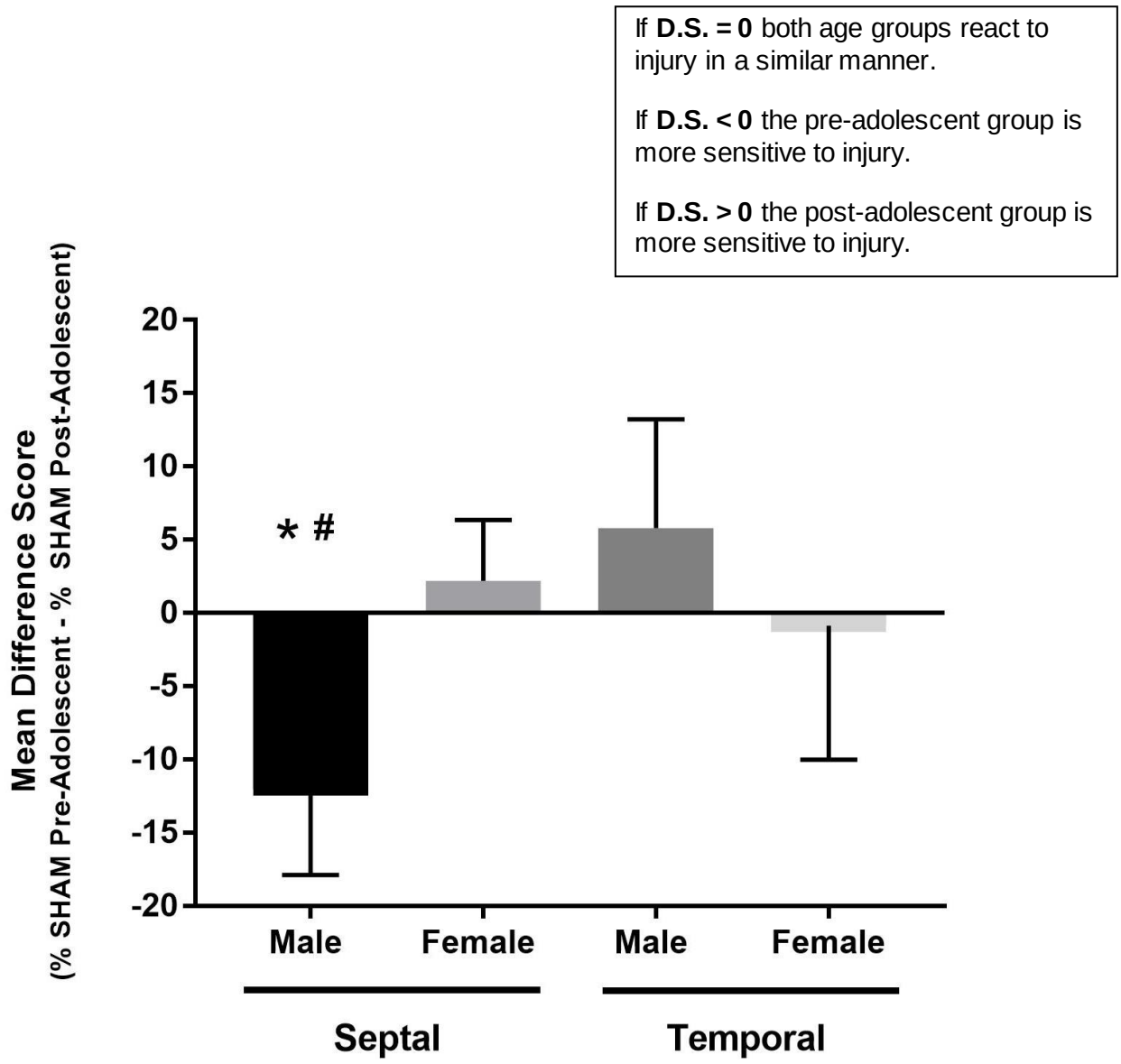


Figure 11: TTC metabolism following 10 minutes of OGD significantly differs between pre and post-adolescent male rats. TTC metabolism following 10 minutes of OGD was significantly different between pre-adolescent and post-adolescent male septal slices [p = 0.05; Cohen's *d* = 0.68 (95% CI= -1.4, 0.06)]; n = 2 animals from each of N = 9 litters. TTC metabolism following 10 minutes of OGD did not appreciably differ between pre-adolescent and post-adolescent female septal slices [p = 0.77; Cohen's *d* = 0.17 (95% CI= -0.46, 0.79)]; n = 2, N = 10, or between male temporal [p = 0.57; Cohen's *d* = 0.26 (95% CI = -0.41, 0.92)]; n = 2, N = 9, or female temporal slices [p = 0.85; Cohen's *d* = -0.03 (95% CI = -0.65, 0.59)]; n =2, N = 10. Data are presented as mean difference score (D.S.) ± SEM. Statistical significance was measured using the Wilcoxon signed-ranked test *p = 0.05. Effect size calculated using Cohen's *d*. # = moderate effect size.

If **D.S. = 0** the two poles react to injury in a similar manner.

If **D.S. < 0** the septal pole is more sensitive to injury.

If **D.S. > 0** the temporal pole is more sensitive to injury.

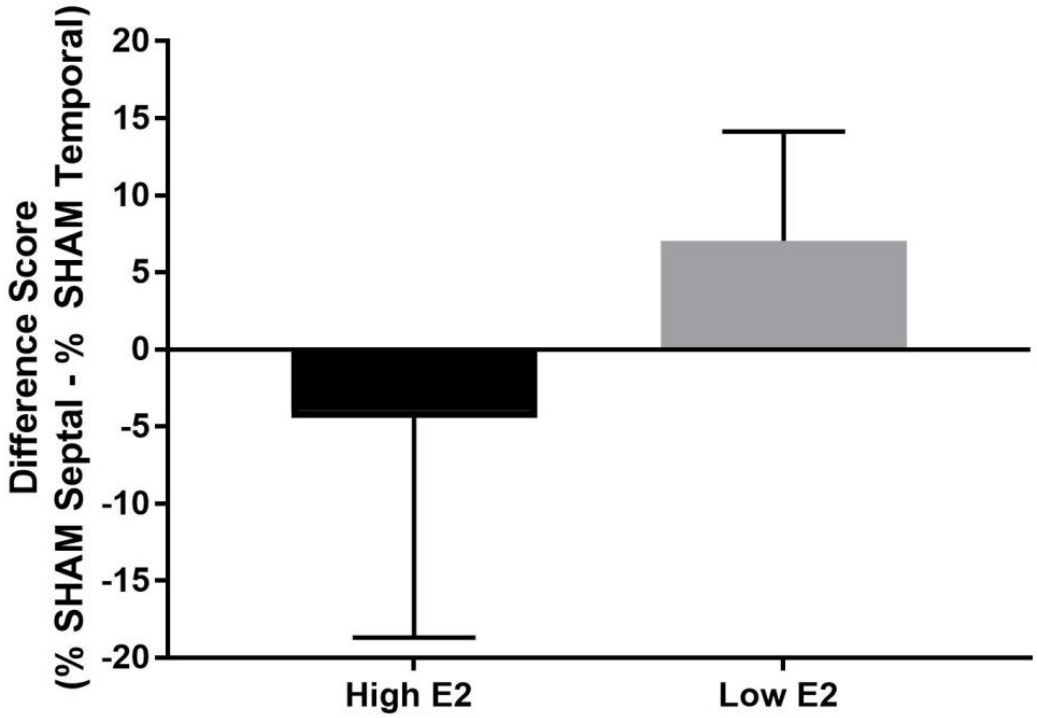


Figure 12: Estrogen levels do not influence TTC metabolism following OGD across the longitudinal axis in sexually mature female rats. TTC metabolism following 10 minutes of OGD was not appreciably different between septal and temporal slices in high estrogen (E2) [$p > 0.99$; Cohen's $d = 0.12$ (95% CI = -0.99, 0.77]; n = 2 slices from each of N = 5 animals, or low E2 [$p = 0.31$; Cohen's $d = 0.35$ (95% CI= -0.79, 0.61)]; n = 2, N = 8, stages of the estrus cycle. Data are presented as mean difference score (D.S.) ± SEM. Statistical significance was measured using the Wilcoxon signed-ranked test; *$p < 0.05$. Effect size calculated using Cohen's *d*.

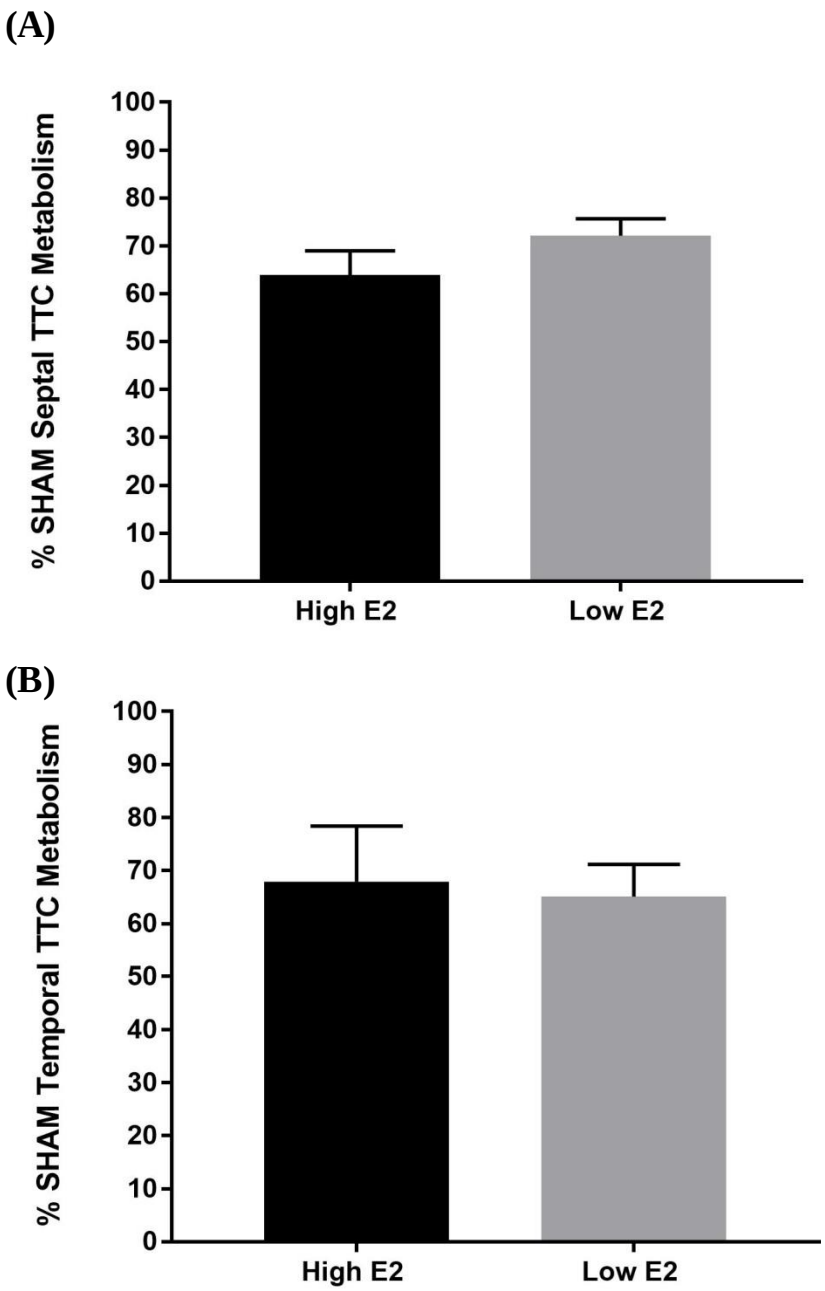


Figure 13: Differences in estrogen levels do not influence TTC metabolism following OGD in sexually mature female rats. **(A)** TTC metabolism following 10 minutes of OGD was not appreciably different between septal slices from post-adolescent female rats with high (63.93 ± 5.01; n = 2 slices from each of N = 5 animals), or low (72.12 ± 3.52; n = 2, N= 8) levels of circulating E2 [*p = 0.35;* Cohen's *d* = 0.78 (95% CI = -0.36, 1.93)]. **(B)** TTC metabolism following 10 minutes of OGD was not appreciably different between temporal slices from post-adolescent female rats with high (67.89 ± 10.47; n = 2 slices from each of N = 5 animals), or low (65.07 ± 6.07; n = 2, N= 8) levels of circulating E2 [*p = 0.72;* Cohen's *d* = 0.14 (95% CI = -0.98, 1.3)]. Data are presented as mean ± SEM. Statistical significance was measured by Mann-Whitney U test.

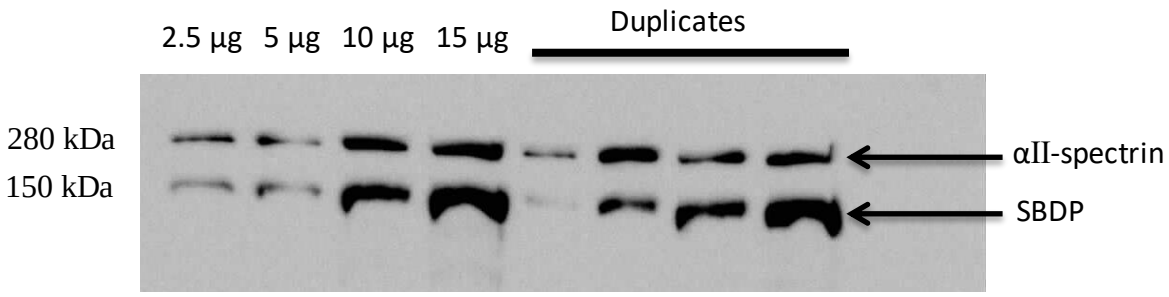


Figure 14: Standard curve protein loading characterization blot. Hippocampal homogenate ranging from 2.5 – 15 µg was loaded for αII-spectrin immunoblotting to determine the optimal protein loading concentration. The 5 µg of protein was determined to yield the best signal. Samples were taken from post-adolescent male septal SHAM tissue. SBDP, spectrin breakdown products.

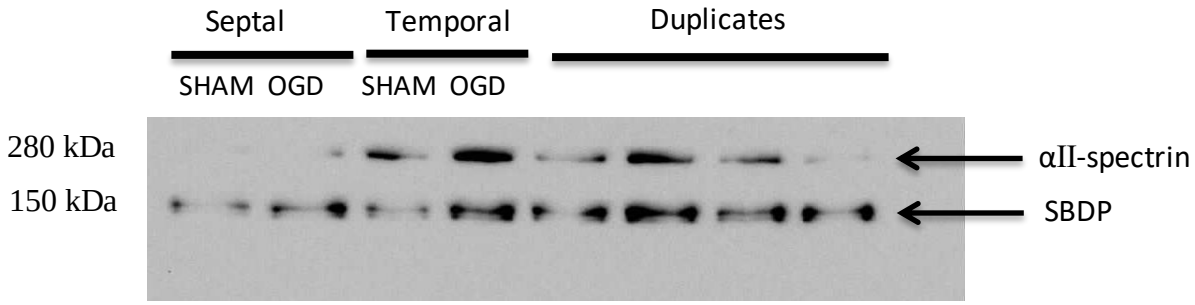


Figure 15: Expression of SBDPs across the longitudinal axis following OGD. Representative blot of 150 kDa SBDPs in septal and temporal slices treated under 10 minutes of SHAM, or OGD conditions. Samples taken from pre-adolescent male tissue.

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