

The endocannabinoid antagonist AM251 as a method of protection prior to global cerebral ischemia: implications for dopamine function, neuronal survival and behaviour

Masters Dissertation in Psychology with Specialization Neuroscience

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Abstract

Implications for the endocannabinoid system in global cerebral ischemia has not been clearly defined. Ischemia produces an excitotoxic environment that is severely damaging to neurons, causing degradation of cell membrane and ultimately cell death. Contradicting research suggests both the benefits and adverse effects of endocannabinoids on neurological injury. Due to the excitotoxic nature of ischemic injury, and the mechanisms at play with endocannabinoid agonists, such as increased transmission of dopamine and glutamate, it is suspected that endocannabinoid antagonists, such as AM251, may provide cell protection. 40 male Wistar rats were separated into 4 groups (n=10/group). The first group of rats were administered AM251 (2 mg/kg, i.p) 30 minutes prior to global cerebral ischemia (four vessel occlusion), while the second group were given AM251, 30 minutes prior to sham surgery. Finally the last two groups were given a vehicle control instead of AM251 and given either ischemia or the sham surgery. Behavioural testing, open field test and elevated plus maze, took place after a five day recovery period following ischemia. Immunohistochemical analyses were performed using to mark tyrosine hydroxylase (TH) and dopamine receptor 1 (DRD1) to compare dopamine function amongst groups. Cell survival was also evaluated using thionin staining. Ischemia induced significant reduction in dopamine within the mesolimbic circuit, including: ventral tegmental area, nucleus accumbens, CA3 & CA1 of the hippocampus, and basolateral amygdala. These reductions in dopamine transmission by global ischemia were partially or fully reversed when AM251 was given beforehand. Furthermore, cell survival was increased in the CA1 from treatment of AM251. Behavioural results show similar results that AM251 reversed emotional irregularities associated with ischemia insult. The endocannabinoid antagonist AM251 improves

deficits in dopamine function, prevents cell death and regulates emotionality when given prior global cerebral ischemia.

Abbreviations

2AG: 2-arachidonoyl-glycerol
4-VO: Four Vessel Occlusion
ACEA: Arachidonyl-2'-chloroethylamide
AEA: anandamide
AM251: *N*-(Piperidin-1-yl)5(4-iodophenyl)1(2,4-dichlorophenyl)4methyl1*H*-pyrazole-3-carboxamide
BLA: basolateral amygdala
cAMP: cyclic adenosine monophosphate
CB₁: cannabinoid type 1 receptor
CB₂: cannabinoid type 2 receptor
CRF: corticotropin releasing factor
DSE: depolarization-induced suppression of excitation
DSI: depolarization-induced suppression of inhibition
DRD1: Dopamine receptor type 1
eCB: endocannabinoids
EPM: Elevated Plus Maze
FAAH: fatty acid amide hydrolase
IA: ischemia rats treated with AM251
IC: ischemic controls
ip: intraperitoneally
ir: immunoreactivity
LTD: Long term depression
MAGL: monoacylglycerol lipase
NA: nucleus accumbens
NAPE-PLD: *N*-acylphosphatidylethanolamine-hydrolyzing phospholipase D
OFT: Open Field Test
SA: sham rats treated with AM251
SC: sham controls
SN: substantia nigra
SR141716: Rimonabant
STD: short term depression
TH: tyrosine hydroxylase
TRPV1: transient receptor potential cation channel subfamily V member
VTA: ventral tegmental area

TABLE OF CONTENTS

CHAPTER	PAGE
Acknowledgements	ii
Abstract	iii
Abbreviations.....	v
I. INTRODUCTION	
A. Global Cerebral Ischemia.....	1
B. Cerebral Ischemia & Dopamine Function	3
C. Ischemia & Behavior	5
D. Endocannabinoid as Neuromodulators	6
E. Endocannabinoid Antagonists	12
F. Endocannabinoid & Pathology	13
G. Protection by eCB Antagonists	16
H. Endocannabinoids & Behavior	18
I. The Dopaminergic System	20
J. eCBs & Dopamine: Interaction of GABA & Glutamate	26
H. Objective & Hypotheses	30
II. METHODS	
A. Subjects	33
B. Drug Preparation	33

C. Four Vessel Occlusion Model	34
D. Behavioral Testing.....	36
E. Immunohistochemistry	37
F. Analysis of Immunohistochemical Labeling.....	39
G. Analysis of Neuronal Survival.....	39
H. Statistical Analyses	40

III. RESULTS

A. Behavioral Testing.....	41
i. Elevated Plus Maze.....	41
ii. Open Field Test	44
B. Immunohistochemistry	48
i. Ventral Tegmental Area.....	48
ii. Nucleus Accumbens	50
iii. CA3 of the Hippocampus.....	52
iv. CA1 of the Hippocampus	54
v. A11 Dopaminergic Neurons.....	56
vi. Basolateral Nucleus of the Amygdala.....	58
vii. Medial Forebrain Bundle.....	60
C. Neuronal Survival.....	62
i. CA1 of the Hippocampus	62
ii. CA3 of the Hippocampus	64

IV. DISCUSSION	
A. AM251 regulation of anxiety-like behavioral responses.....	65
B. AM251 regularized TH and DRD1 expression post ischemia.....	68
REFERENCES	80
APPENDICES (Optional)	
Appendix A – Extra Figures	99

I. INTRODUCTION

A. Global Cerebral Ischemia

Ischemic injury is the restriction of blood to any part of the body including the heart and brain, causing a depletion of oxygen and nutrients, and is the primary cause of cell death in humans (Hossmann, 2008; Wappler et al, 2009). Ischemic injury within the brain and heart, like: stroke, cardiac arrest and cerebrovascular disease, are the most prevalent causes of neurological impairment and disability within clinical practice, not to mention being the two leading causes of death worldwide (Hossmann, 2008; Krafft et al, 2012; Lopez et al, 2006; Wappler et al., 2009). The need to understand the mechanisms of ischemic injury is crucial in order to provide future prevention and treatment to patients (Hossmann, 2008). Animal models, like the four vessel occlusion model in rats (4-VO), are often used to study the effects of ischemic injury in the brain (Pulsinelli & Brierley, 1979). 4-VO is a form of transient global ischemia where blood flow to the brain is restricted by occlusion of four major arteries, in order to induce ischemic injury, allowing reperfusion following occlusion (Pulsinelli & Brierley, 1979). Acute global ischemia results in widespread bilateral damage in the brain resulting in both cognitive and motor impairment (Krafft et al, 2012; Lim et al, 2004; Traystman, 2003). This model is most often used to mimic the effects of cardiac arrest on the human brain, but is indicative of the damage done by various types of stroke, including: hemorrhage, atherothrombotic disease, cerebral small vessel disease and cardiac emboli (Krafft et al, 2012; Wang-Fischer & Koetzner, 2008).

During development and periodically throughout adulthood programmed cell death, also

called apoptosis, is considered normal and healthy; however, excessive cell death, including necrosis, can lead to neuronal degeneration with pathological consequences (Martin et al, 1998). Global cerebral ischemia causes this excessive cell death, said to be a result of excitotoxicity, target deprivation, and problems in the pathways that regulate programmed cell death and survival (Girbovan, Morin & Plamondon, 2011; Kim, Gonzalez, & Chan, 2005; Martin et al, 1998; Tauofik & Probert, 2008). More generally, necrosis occurs when the integrity of the cell membrane is compromised, from lack of oxygen delivery or traumatic injury, causing an influx of calcium and changes in phospholipid metabolism, which in turns results in a change in membrane permeability, activation of phospholipases and free radicals, pyknosis (uniformly compacted chromatin) and causing changes in mitochondrial function (Farber, Chien & Mitnacht, 1981; Martin et al, 1998). In addition, ischemia involves the activation of caspases, which are enzymes that aid in the breakdown of cell material (Martin et al 2003). Overactivity of caspases leads to nucleus severance, DNA fragmentation and development of cell surface protuberances (apoptotic bodies) eventually leading to cell death by apoptosis (Martin et al 2003; Niquet et al 2003). This rapid departure from a healthy physiological state results in depletion in energy stores, irregular phosphorylation, swelling, complete dissolution of the cell membrane or nucleus and ultimately leading to cell death, which in many cases can be irreversible (Farber, Chien, Mitnacht, 1981; Martin et al, 1998).

Excitotoxicity, initiated by the massive surge in glutamate release, prompts a biochemical cascade of physiological events leading to cell death in different pathological states, including cerebral ischemia (Martin et al, 1998; Paschen, 1996). During ischemia,

glutamate is released in excess, unable to keep up with this influx and the uptake systems breakdown, leading to the accumulation of glutamate in the synaptic cleft (Paschen, 1996). The over supply of glutamate activates the glutamate receptors, in particular the ionotropic subtype *N*-methyl-D-aspartate (NMDA), causing a surge of calcium, at the basis of pathologically induced necrosis and apoptosis, as mentioned above. Under normal physiological conditions, glutamate is involved in panoply of physiological processes including long-term potentiation and brain plasticity essential for learning and memory. However, in the process of a cerebral ischemic event, excessive exposure of neuronal cells to glutamate becomes toxic (Budd & Nicholls, 2002; Paschen 1996; Pellegrini-Giampietro, Mannaioni & Bagetta. 2009). Glutamate's toxicity at high concentrations was verified when rats administered a glutamate antagonist prior to insult showed reduced ischemic damage (Meldrum, 1985; Park et al, 2004). It should be noted that the therapeutic actions of glutamate antagonists are often dose dependent and since these drugs can lower body temperature and hypothermic conditions confer protection this should also be considered (Bust et al, 1987; Paschen, 1996). Apart from glutamate, the release of various other neurotransmitters is increased during and following an ischemic event (Paschen, 1996). Among these, a five-fold increase in dopamine release has been observed following forebrain ischemia (Globus et al, 1987; Globus et al, 1988; Zhang et al, 2008).

B. Cerebral Ischemia & Dopamine Function

Similar to glutamate, dopamine has been shown to have neurotoxic effects when too much is released into the synaptic cleft as in the case with global cerebral ischemia (Globus et al, 1987). In excess, like during ischemia, dopamine is broken down into hydrogen peroxide and

dihydroxyphenylacetate that increases oxidative stress exponentially (Berman & Hastings, 1997; Zhang et al, 1998). Indeed, recent studies have suggested that dopamine is more implicated in ischemic injury than originally proposed. Although the exact effect of cerebral ischemia on the dopaminergic system remains largely unknown, there are indications that dopamine function is dramatically affected (Martin et al, 2012; Zhang et al, 2008). Initial studies identified altered D₁ receptor transmission following global cerebral ischemia, and further investigation has confirmed these results (Benfenati et al, 1989). Recently, a positron emission tomography study done by Martin and colleagues (2012) found significantly reduced dopaminergic D₂ receptor transmission up to 28 days following focal ischemia in rats. Another study found similar results, and following the sudden increase in dopamine release associated with the ischemic event, tyrosine hydroxylation, a necessary step in the synthesis of dopamine, declined on the third day after ischemia and dopamine dysfunction maintained for an additional 28 days (Takagi et al, 1995). There is further evidence suggesting that dopamine neurons, in particular, are more vulnerable to ischemic and hypoxic events (Li et al, 2009; Zhang et al, 1998). In this context, dopamine agonists such as levodopa, which is normally used to treat Parkinson's disease, and amphetamine a psychostimulant drug, have been shown to improve motor and cognitive function when given after an ischemic stroke (Ruscher, Kuric & Wielock, 2012; Sonde et al 2001). Even though during and immediately after ischemia dopamine levels are significantly high, post ischemic long term dopamine transmission is significantly reduced, implying that dopamine function is considerably altered from ischemic insult (Globus et al, 1987; Martin et al, 2012; Ruscher, Kuric & Wielock, 2012).

According to Zhang and colleagues (2008) excess release in dopamine, on top of the excess glutamate, during and immediately following ischemia may contribute to neuronal death of dopamine cells in particular. This has been previously shown in a comprehensive study done by Zhang and colleagues (1998); where dopamine neurons were more vulnerable and prone to degeneration from excess dopamine and related excitatory amino acids. This connection is reinforced by studies showing that cell survival is significantly improved when dopamine transmission is blunted prior to ischemic insult (Globus et al, 1987; Yamamoto et al 1994; Zhang et al 2008). Additionally, dopamine depletion prior to transient ischemia has been shown to confer neuronal protection (Ren, Li & Xu, 1997). Together, this data suggests that similar to glutamate-induced excitotoxicity, excessive dopamine release during and following an ischemic event can be detrimental to the neuronal recovery, and that prevention of dopamine release prior to ischemia may prove beneficial. Being a main focus of this study, the role of dopamine will be further discussed below.

C. Ischemia & Behaviour

Impairment following stroke is not only physiological, but can also have a profound effect on behaviour, cognition and related emotional function (Bueters et al, 2008; Milot & Plamondon, 2009; Nunn & Hodges, 1994; von Euler et al, 2006). For instance, spatial working memory impairment has consistently been observed following global cerebral ischemia, a phenomenon that has been related to neuronal loss within the hippocampus, in particular the more vulnerable neurons of the pyramidal CA1 cell layer of the hippocampus (Bueters et al, 2008; Milot & Plamondon, 2011; Nunn & Hodges, 1994). These deficits have been evaluated

using a variety of land based and water maze tasks, such as the radial arm maze, Barnes maze and Morris water maze. In addition to memory impairments, time-dependent changes in anxiety levels have been noted post ischemia, as measured by the open field test (OFT) and elevated plus maze (EPM), notably increased anxiety in the first few days following ischemia while decreased anxiety compared to sham-operated rats have been observed between 4-7 days (Milot & Plamondon, 2009; Plamondon and Khan, 2005). Locomotor activity is also effected, ischemic animals showing increased locomotor activity compared to sham-operated controls (Kronenberg et al, 2012; Milot & Plamondon, 2008). Finally, differences in grooming behaviour have been noted following four vessel occlusion, such that following ischemia the time spent grooming is markedly increased compared to sham controls (Yan et al, 2007). Depressive-like behaviour has been characterized following global ischemia, and sometimes proposed to be linked to dysfunction in the mesolimbic circuit, including reduced concentrations of dopamine resulting from ischemia-induced neural degeneration in dopaminergic circuits (Kronenberg et al, 2012). Others have suggested that emotional changes be associated to changes in emotional reactivity or neuronal damage in the limbic circuitry (Joseph, 1999; Sapolsky, 2003; Stone et al, 2002).

D. Endocannabinoids as Neuromodulators

The existence of an endogenous cannabinoid system in mammals has only recently been acknowledged (Castillo et al, 2012; Pertwee, 2010; Howlett et al, 2002). Exploration and research of Δ^9 -tetrahydrocannabinol (THC), the psychoactive component found in the marijuana, has led to the discovery of endogenous receptors binding to both THC and endogenous ligands, endocannabinoids (eCBs) (Castillo et al, 2012). Endogenously produced cannabinoids, eCBs,

have since been discovered to act similarly as THC. Of current interest are two endogenous lipophilic ligands anandamide (AEA) (Figure 1A) and 2-arachidonoyl-glycerol (2-AG) (Figure 1B) produced and excreted on demand into the extracellular membrane which act upon cannabinoid receptors (Devane et al, 1992; Melis & Pistis, 2007; Sugiura et al 1995). Currently two main cannabinoid receptors have been identified: cannabinoid type 1 receptors and cannabinoid type 2 receptors, respectively CB₁ and CB₂, both being G protein-coupled seven-transmembrane domain receptors known to regulate synaptic function (Kano et al, 2009; Matsuda et al, 1990; Munro et al, 1993; Pertwee, 2010).

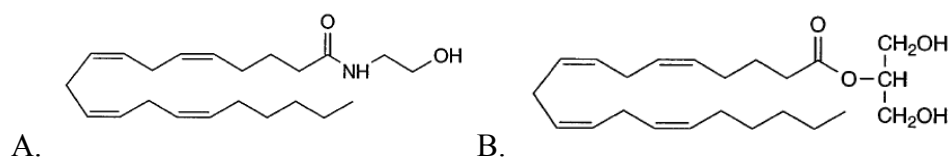


Figure 1. Structural chemical formula of two endogenous cannabinoids, called endocannabinoids (A) anandamide (AEA) and (B) 2-arachidonoyl-glycerol (2-AG) found throughout both the central nervous system and peripheral nervous system, images adapted from Di Marzo & Deutsch (1998).

CB₁ receptors are found extensively throughout the central nervous system (CNS), whereas CB₂ receptors have been identified mostly in the peripheral nervous system (PNS), having a primary role on immune regulation and with new data showing it may also be present in neural cells involved in nociception, however these findings remain preliminary (Beltramo et al, 2006; Kano et al, 2009; Wotherspoon et al, 2005). For the purpose of the current study, focus will remain mostly on the CB₁ receptor as its presence in the brain has been well defined, the CB₁ receptor is the primary target of THC, eCBs and related chemical compounds (Kano et al, 2009). CB₁ receptor distribution throughout the brain was determined using injection of radio-labeled

cannabinoid (Herkenham et al, 1990; Mailleux & Vanderhaeghen, 1992). Areas of high affinity ligand binding include: the hippocampus, lateral striatum, olfactory bulb, substantia nigra pars reticulata, and globus pallidus. Sites of medium affinity ligand binding include: the cerebral cortex, septum, amygdala, hypothalamus, brain stem and spinal cord. CB₁ receptors are differentially distributed amongst both inhibitory and excitatory synapses in various regions of the brain (Kano et al, 2009). As result of the diverse distribution of CB₁ receptors throughout the brain and also the ability for these receptors to induce both excitatory and inhibitory messaging the role of CB₁ receptors is complex and sometimes even conflicting.

As previously discussed, knowledge of the existence and role of CB₂ receptors in the brain is limited. Low levels of CB₂ receptors have been found in various brain regions, but these receptors seem to be play a role in immune function and pain management as they have been identified in microglial cells and leukocytes and increased density observed in rats subjected to chronic pain (Aston et al, 2006; Beltramo et al, 2006). More recently, some studies have proposed a third cannabinoid receptor, denoted CB₃ (Hajos, Ledent & Freund, 2001; Kano et al, 2009; Katona et al, 1999). The evidence for this novel receptor was first noted by Hajos, Ledent & Freund (2001). In this experiment mice lacking CB₁ receptors still showed effect when cannabinoid agonist were given, suggesting such agonist were acting upon receptor other than the CB₁ receptors (Hajos, Ledent & Freund, 2001). The existence of the CB₃ receptor is contentious among researchers, as the more recent research has found no effects of cannabinoid agonists under the same circumstances and using CB₁ knock-out mice (Kawamura et al, 2006). For the purpose of this study our focus will thus remain on CB₁ receptors.

The mechanism of action of cannabinoids and endocannabinoids became clearer when in 2001 their ability to interpose retrograde signaling was discovered (Kano et al, 2009; Kreitzer & Regehr, 2001; Maejima et al, 2001; Ohno-Shosaku, 2001; Wilson & Nicoll, 2001). This discovery came from realizing that eCBs are a major component involved in depolarization-induced suppression of inhibition (DSI) and depolarization-induced suppression of excitation (DSE) both of which are forms of short term synaptic plasticity (Castillo et al, 2012; Diana & Marty, 2004; Kano et al, 2009; Kreitzer & Regehr, 2001; Ohno-Shusoaku, et al 2001; Wilson & Nicoll, 2001). DSI was first seen on GABAergic synapses where an increase in post synaptic calcium levels would result in a decrease in neurotransmitter release from presynaptic terminals (Diana & Marty, 2004; Kano et al, 2009). DSE was identified much later than DSI and involves similar mechanism as DSI often targeting glutamatergic synapses instead of GABAergic ones. Detailed examination of discrete effects on various physiological processes is beyond the scope of the study at hand, and the impact of endocannabinoid on DSE and DSI will be discussed in terms of their function to regulate short term synaptic plasticity, keeping in mind that both DSE and DSI are said to result from postsynaptic depolarization (Diana & Marty, 2004).

Other forms of suppression of synaptic transmission linked to endocannabinoid have subsequently been found in numerous areas of the brain including the hippocampus and cerebellum (Kano et al, 2009; Maejima et al, 2001). Endocannabinoids are retrograde messengers that have been found to act on both glutamatergic and GABAergic presynaptic terminals causing a short lived suppression of excitatory and inhibitory synapses, depending on the specific neurons being targeted (Kano et al, 2009; Varma et al, 2001). Endocannabinoids are

not only involved in short-term depression, but also long-term depression (LTD). Various studies have looked at endocannabinoids as retrograde messenger in LTD affecting both GABAergic and glutamatergic neurons in various regions of the brain including but not limited to: CA1 and CA3 of the hippocampus, nucleus accumbens, and striatum (Zano et al, 2009; Peterfi et al, 2012). For instance, CB₁ receptor agonists lead to reduced GABA release in both the hippocampus and nucleus accumbens (Schlicker & Kathmann, 2001).

Endocannabinoid receptors are widely distributed throughout the brain and are key mediators of various forms of plasticity, suggesting their importance in regulating general synaptic function within the brain (Castillo et al, 2012). Working as retrograde messengers, endocannabinoids are released from the postsynaptic neuron and act on the presynaptic neuron (Castillo et al, 2012; Kano et al, 2009). During LTD, upon binding of the eCB to the G-protein coupled CB₁ receptor the production of adenylyl cyclase ceases, blocking the signaling of cyclic adenosine monophosphate (cAMP) ultimately causing a reduction in neurotransmitter release (Castillo et al, 2012; Pertwee, 2010). The action mechanism in STD is similar with the difference that CB₁ receptors are activated only momentarily to induce a reduction of calcium influx on the presynaptic neuron, inhibiting neurotransmitter release. It should be noted that endocannabinoid are said to be more efficient at reducing GABA release than reducing glutamate release. Once neurotransmitters release is inhibited new eCBs production is stopped by the enzymes monoacylglycerol lipase (MAGL) and existing eCBs are broken down by fatty acid amide hydrolase (FAAH) and *N*-acylphosphatidylethanolamine-hydrolyzing phospholipase D (NAPE-

PLD) (Amantea et al, 2007; Blankman, Simon & Cravatt, 2007; Castillo et al, 2012; Gulyas et al, 2004; Kano et al, 2009; Okamoto et al, 2004).

There is some evidence that endocannabinoids also act in a non-retrograde manner. This is possible with the involvement of transient receptor potential cation channel subfamily V member 1 (TRPV1) channel, part of the vanilloid receptor type 1 (Castillo et al, 2012; De Petrocellis & Di Marzo, 2010; Pertwee, et al 2010). TRPV1 activation is involved in regulation of synaptic transmission associated with pain and sensation in the peripheral nervous system, but TRPV1 channels have also been located in the central nervous system (Caterina & Julius, 2001; Cristino et al, 2006). Of interest, AEA appears to have binding affinity for TRPV1 channels, which when activated mediate a form of postsynaptic LTD (Gibson et al, 2008). Even more interesting concerning the work of the current study is that TRPV1-LTD has been detected on dopamine receptors in the nucleus accumbens (Grueter et al, 2010). In this cascade, it is presumed that AEA is created from activation of mGluR5, as a result of glutamate being released, the AEA then acts on the postsynaptic vesicles in an autocrine manner by activating TRPV1 channels (Castillo et al, 2012). In this case eCBs act as non-retrograde messenger, they are released and act on the same cell, activating TRPV1 channels which results in changes in function like initiating postsynaptic depression (Castillo et al, 2012). Lastly, there is also some evidence that endocannabinoid receptors exist on astrocytes, when eCBs are released and act on astrocytes which then, through gliotransmission, release glutamate in the synaptic cleft (Castillo et al, 2012).

E. Endocannabinoid Antagonists

Several synthetic endocannabinoid antagonists have been developed to aid in further understanding the endocannabinoids, their receptors and functions in the brain. These antagonists block eCB CB₁ receptor activation, their chemical structure is similar to eCBs making them bind to these receptors and ultimately preventing further endocannabinoid action (Pertwee, 2010). Of interest is the antagonist AM251 (Figure 2), *N*-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide, which is a potent CB₁ receptor antagonist, with higher binding affinity to CB₁ receptors than CB₂ receptor by approximately 305 fold ratio based on displacement (K_d) values using radioactive markers (Pertwee, 2010). AM251 is structurally very similar to Rimonabant (SR141716) which has been used in a wide variety of studies. Both AM251 and Rimonabant are classified as diarylpyrazoles (see fig. 2). AM251 itself is not able to activate cannabinoid receptors, but merely block the receptor sites and stops normal eCB function. It should be noted that AM251, along with some other cannabinoid antagonists, has been noted to act as G protein-coupled receptor 55 (GPR55) agonist (Pertwee, 2010). The physiological function of GPR55 is not fully understood at this point but this receptor has been found in both the central and peripheral nervous systems, some terming it the third endocannabinoid receptor (Petitet, Donlan & Michel, 2006; Schich & Storr, 2012). The evaluation of endocannabinoid antagonist on GPR55 and possible effects on physiological function should be clearly defined helping to draw further conclusions on the exact effects of AM251.

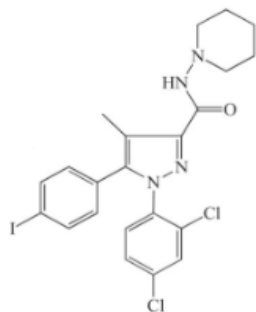


Figure 2. Structural chemical formula of the eCB antagonist AM251, *N*-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide, which binds with high affinity to CB₁ receptors in the central nervous system, images adapted from Pertwee (2010).

F. Endocannabinoids & Pathology

Endocannabinoids have been studied in numerous conditions involving pathological environments and models (Miller & Devi, 2011). They are particularly interesting to study in models of disease because of their ‘on-demand’ synthesis, suggesting their function is important when the physiological environment changes (Kano et al, 2009; Miller & Devi, 2011).

Endocannabinoids have been implicated in a number of pathological states, including: cancer, liver dysfunction, metabolic disease, neuropathic pain, cardiovascular disease, gastrointestinal disease, neuro-inflammation and brain injury (Batkai et al, 2004; Hansen et al, 2001; Miller & Devi, 2011; Teixeira-Clerc et al, 2006; Wang et al, 2008). It appears that cannabinoid receptor expression and activation effects regulatory function, that is; when the physiological system is challenged these receptors act to bring the system back to normal (Miller & Devi, 2011).

Depending on the disease or stressor affecting the system, endocannabinoid receptor expression and activation can either be adaptive or maladaptive (Miller & Devi, 2011). The underlying mechanisms involved in the changes in both eCBs and CB₁ receptor expression during pathological states is still not fully understood. Discrepancies in the literature and scientific

evidence have caused a divide in endocannabinoid research, although such differences should generally be viewed to represent the extensive interaction of endocannabinoids in the CNS, and considerations must be made to site and dose specificity in assessing the role of endocannabinoids.

Endocannabinoids and their receptors, in particular the CB₁ receptor, have been implicated in possible therapies for ischemia (Pellegrini-Giampietro, Mannaioni & Bagetta, 2009). There is much debate as to the role of endocannabinoids in ischemia, both agonists and antagonists have differing effects on outcome (Pellegrini-Giampietro, Mannaioni & Bagetta, 2009). For instance, THC has been shown to have both toxic and neuroprotective effects when chronically administered prior to ischemia insult (Hampson et al, 1998; Scallet, 1991). More recent data continues to be inconsistent, some studies suggesting activation of the CB receptors to be protective, increasing cell survival and cell firing, using various agonists like WIN 55212-2 given following focal or global ischemia (Louw et al, 2000; Melis et al, 2006; Nagayama et al, 1999), whereas other experiments show marked increased in cell survival and protection by eCB antagonists, such as SR141716 and AM251 (Berger et al, 2004; Hansen et al, 2002; Muthian et al, 2004). Additionally, eCB agonists have also shown to have no beneficial effect on outcome, as well as being detrimental to cell survival following ischemia. These differences have sparked a divide in the research leading some to believe agonists offer protective effects for ischemia, and the other side believing that antagonists offer protection (Down et al, 2001; Hansen et al, 2002).

The current bifurcated view of the role of eCBs is limited and often overreaching in terms of the conclusions that can be drawn. A number of factors may be at play when cannabinoid drugs act on the CNS, which need to be considered. Some of these factors include: the dose, as most eCB agonists and antagonists act differently depending on the dose, administration before or after the time of injury, site of injection, and number of doses (Fernandez-Ruiz, Hernandez, & Ramos, 2010; Muthian et al, 2004; Pellegrini-Giampietro, Mannaioni & Bagetta, 2009). Additionally, the role of the agonist versus an antagonist is not necessarily mutually exclusive, any effect of one does not necessarily mean the opposite effect of the other (Muthian, et al 2004). For instance, in some cases CB₁ receptor agonists prove to be beneficial, and such effects are lost when the antagonist is paired with the agonist, but the use of the antagonist alone does not prove to be detrimental, and as previously discussed in some cases improves outcome (Muthian et al, 2004; Panikashvili et al, 2001). Furthermore, some studies have shown that eCB agonists inhibit ATP-induced long-term potentiation and associated strengthening of synapses in hippocampal brain slices, a phenomenon reversed by prior AM251 administration, suggesting the involvement of CB₁ receptor in such modulation (Ievglevskyi et al, 2012). Finally, it has been proposed that the benefits of eCBs are inherent in the structure and property of cannabinoids alone, not necessarily similarly formed synthetic agonists, nor the activation of the receptors by such cannabinoid-like chemicals (Marsicano et al, 2002). Thus eCB agonists and antagonist do not necessarily have opposing roles, as such their mechanisms and functions need to be more fully understood. For the purpose of the current paper the role of the CB₁ antagonists AM251 will be further explored.

G. Protection by eCB Antagonists

There is a substantial research suggesting that endocannabinoid antagonists offer protection during ischemic injury. One theory as to why this may occur is because there is a markedly increase in anandamide, similar to the rapid increase in both glutamate and dopamine, during ischemia and other forms of brain injury (Amantea et al, 2007; Berger et al, 2004; Muthian et al, 2004). In fact during focal ischemia, anandamide was increased about three fold more than controls, this is most likely due to the very nature of eCB release, rapid calcium influx, occurs during ischemic insult (Amantea et al, 2007; Degn et al 2007; Farber, Chien & Mitnacht, 1981; Martin et al, 1998). This sudden increase in AEA resulting in its increased action on its receptors triggers the rapid release of catalyzing enzymes, like FAAH and NAPE-PLD, a process similar to excitotoxicity and may in fact result in acute neurodegeneration (Amantea et al, 2007). This theory stands when rats were given a single dose of endocannabinoid receptor antagonists either SR141716 or LY32135 prior to occlusion, which significantly reduced infarct volume by 40-50% and led to significant improvement in functional recovery using various neurological assessment tools including postural reflex and forelimb placing (Muthian et al, 2004). The hypothesis that AEA can be toxic in ischemic conditions was further supported as the control rats, that were not given the eCB antagonist, showed significantly larger infarct volume and increased motor impairment (Muthian et al, 2004). Furthermore, contrary to reports that cannabinoid agonists serve as neuroprotective during ischemia, the same study found that rats given the CB1 receptor agonist WIN 55212-2 prior to ischemia showed no significant improvements (Muthian et al, 2004). This data suggests that the excess release of AEA

exacerbates the damage done during ischemia and the therapeutic administration of eCB antagonists may be beneficial (Amantea et al, 2007; Degn et al, 2007; Muthian et al, 2004).

Other examples of eCB antagonists improving ischemic outcome have been noted. In particular, AM251 has been effective in preventing excessive cell death during in vivo oxygen-glucose deprivation (Landucci et al, 2011). Additionally the CB₁ agonist Arachidonyl-2'-chloroethylamide (ACEA) worsened outcome in the same study, resulting in Landucci and colleagues (2011) to conclude that not only is eCB antagonist administration beneficial during ischemia, but the role of endocannabinoids released on demand and exogenously administered agonists may be detrimental. This study, as well as others, pose that CB receptor blockade may play a more crucial role in alleviating damage during pathological states, than originally thought and should not be overlooked (Ashton et al, 2007; Landucci et al, 2011; Zhang & Martin et al, 2008).

The endocannabinoid antagonist AM251 is of particular interest for a number of reasons. Not only has it been implicated in protection when administered before ischemia, it has also been shown to regulate cognition function and behaviour in other situations when normal physiological conditions are compromised. For instance, mice pretreated with AM251 had reduced amphetamine-induced behavioural sensitization, this is said to be a result of the interplay of endocannabinoid and dopamine functions, which appear to be extrinsically linked (Thiemann et al, 2008). The link between eCBs and dopamine will be discussed further in upcoming passages. For now, it is efficient to say that AM251 plays a regulatory function for a number of

conditions including ischemia. In some instances, AM251 has also shown effects when administered under normal physiological functions, and low doses around 1 mg/kg has been shown to improve recognition memory in an object recognition test compared to control animals and those treated with higher doses (Bialuk & Winnicka, 2011).

H. Endocannabinoids & Behaviour

The importance of endocannabinoids (eCBs) role in regulating behaviour, and more specifically anxiety-related behaviour, has become more evident with current understanding of its action on CB₁ receptors (Arvelo, de Miguel & Hernandez-Tristan, 2001; Bialuk & Winnicka, 2011; Chaperon & Thiebot, 1999; Roohbakhsh et al, 2009). Preliminary studies have revealed that pharmaceutical agents that enhance CB₁ receptor transmission alleviate anxiety and related stress responses whereas agents that block CB₁ transmission increase anxiety and overall emotional reactivity (Griebel et al, 2005; Rubino et al, 2007). However more recently, this dichotic division of the effects of endocannabinoids and their antagonists on behavioural reactivity has been blurred by conflicting data which revealed that such effects are dependent on dosage, brain region analyzed, and basal stress activity (Kupferschmidt et al, 2012; Hill et al, 2009; Rubino et al, 2008).

The cannabinoid system has also been implicated in learning and memory function (Lichtman, 2000; Pertwee, 2005). Substantial evidence has shown that cannabinoids and their structurally similar agonists impair learning and memory (Chaperon & Thiebot, 1999; Kosiorek et al, 2003, Lichtman, Dimen & Martin, 1995). For instance, in one study, rats who were

administered either delta 9-THC or WIN 55212-2 via cannula injection directly into the hippocampus or systemic intraperitoneal injection, made significantly more errors in a radial maze task, compared to vehicle-injected controls (Lichtman, Dimen & Martin, 1995).

Rimonabant, a potent antagonist, has been shown to reverse deleterious effects of eCBs and their agonists and even improve learning and memory when given prior to various behavioural tests including the match-to position task and social recognition test in rodents (Mallet & Beninger, 1998; Terranova et al, 1996). Similarly, AM251 has been shown to improve recognition memory at a dose of 1 mg/kg using the object recognition test, while having no effect on anxiety (Bialuk & Winnicka, 2011).

The endocannabinoid system is also known to highly interact with the hypothalamic-pituitary-adrenal axis, such that eCB transmission alters Corticotropin Releasing Factor (CRF) levels (Evanson et al, 2010). For instance, using behavioural measures of anxiety, such as the Elevated Plus Maze (EPM), AM251 has been shown to reverse behavioural anxiety induced by CRF injection despite elevating plasma corticosterone levels (Kupferschmidt et al, 2012). Conflicting results have also been presented some studies indicating AM251 to have anxiogenic effects while others showed anxiolytic effects (Arevalo et al, 2001; Haller et al 2002; Kupferschmidt et al, 2012). Furthermore, another study reported no change in EPM performance between saline controls and AM251-injected rats (Roohbakhsh et al, 2009). Importantly, rats that showed increased anxiety from AM251 were usually treated with high dosages, 5 mg/kg or more, whereas lower doses, below 3 mg/kg tended to produce non significant results or decrease in anxiety behaviour (Bialuk & Winnicka, 2011; Thiemann et al 2008). Similarly, anxiety-like and

depression-like behaviour were assessed in rodents given 3 mg/kg ip injection prior to the tail-suspension test and the forced-swim test, AM251 was just as effective as antidepressant medication as reducing immobility (Shearman et al, 2003). More evidence on AM251's anxiolytic ability was shown in a study where AM251-treated rats showed reduced anxiety from exogenous administration of CRF and withdrawal from chronic cocaine exposure (Kupferschmidt et al, 2012). Thus, it appears that the effects of AM251 follow a dose-related curve, with lower doses generally producing anxiolytic effects and higher doses being anxiogenic (Bialuk & Winnicka, 2011; Shearman et al, 2003). It is pertinent to look at the effects of AM251 on other processes that may affect emotional tone or reactivity, other than the HPA axis, such as the dopaminergic reward system.

1. The Dopaminergic System

Catecholamine containing neurons are found throughout the brain, including but not limited to the extensive dopamine (DA) networks discovered by Falck and Hillarp, using a novel monamine identification method (Flack, et al, 1962). This visualization method enabled researchers to map out the various parts of the brain, both human and rodent, which contained dopamine. With the technological progression of immunohistochemistry, as a means of identifying monoamines, came the ability to properly network dopamine mapping within the brain (Bjorklund & Dunnett, 2007). In particular, Tyrosine Hydroxylase (TH) markers in immunohistochemistry are often used to distinguish catecholamine synthesizing neurons. Tyrosine Hydroxylase is the essential rate-limiting enzyme in the reactive creation of catecholamines, where L-dopa is transformed from tyrosine (Marin et al, 2005). TH

immunoreactivity (TH-ir) is used in part largely to distinguish dopamine containing cells. However, these TH-ir positively marked cells can sometimes be an indicator for both dopamine and noradrenaline together, thus making TH-ir a less selective but still effective dopamine marker, as all known catecholaminergic regions in both the mid and forebrain are dopaminergic (Bjorklund & Dunnett, 2007; Marin et al, 2005). Similar to numerous studies in the field and for the purpose of this paper, TH-ir will be synonymous with dopamine immunoreactivity (Agrawal et al, 2012; Choi et al, 2012; Ikemoto, 2002; Li et al, 2012). With the use of TH-ir reactivity both human and rodent dopamine- containing neurons have been networked, of particular interest are the dopaminergic networks within the rodent brain (Figure 3).

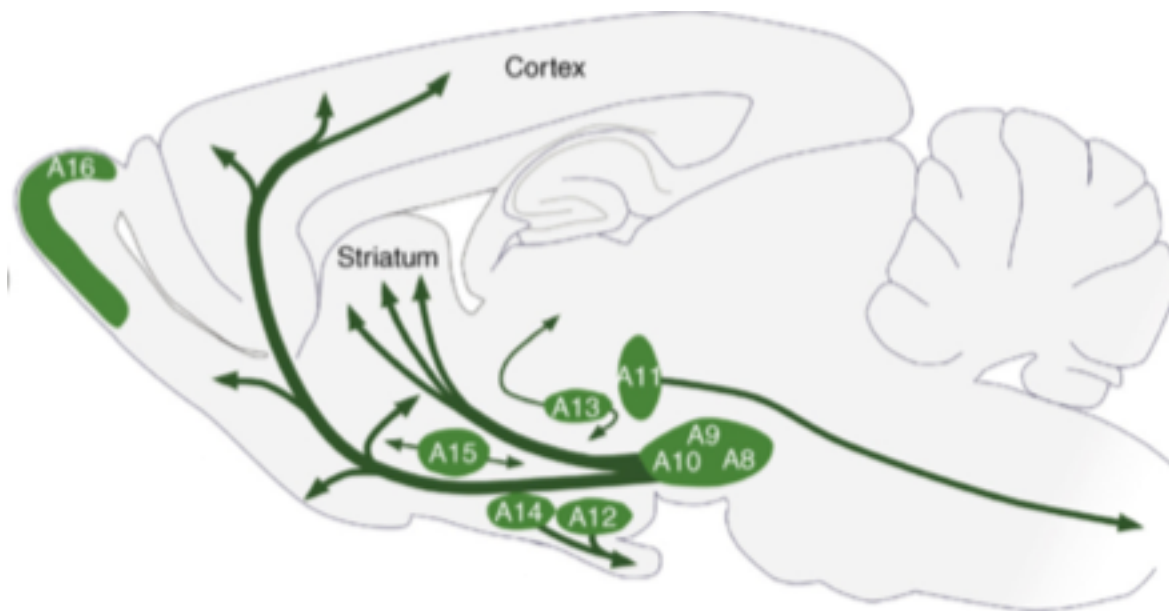


Figure 3. Distribution of dopamine neurons within the adult rodent brain, as shown in green dopamine neurons are subset in nine areas throughout the brain. Arrow indicating sites of transmission from the posterior region including the mesencephalon to the frontal cortical areas and the olfactory bulb (from Bjorklund & Dunnett, 2007).

Some of the numerous structures in the brain associated with dopamine transmission include groups of cells that were identified by Dahlstrom and Fuxe (1964). Such dopamine containing areas include cell clusters denoted A8 to A16 (Figure 1). These dopamine neurons are associated with various functions and their location within the brain is crucial to their role in variety of tasks. For instance the midbrain dopaminergic neurons A8, A9, and A10 are part of the nigrostriatal pathway, which is essential in motor function and has been found determinant in Parkinson's disease (Grealish et al, 2010). With the progression of Parkinson's disease the viability of these dopamine neurons is compromised, neuroblast grafts of these neuron populations is able to reverse motor function. In essence, these midbrain neurons are not homogeneous and their shape, function and transmission vary greatly. For instance, the A9 dopamine neurons are large and more jagged in shape and project to the dorsolateral striatum whereas the A10 neurons are smaller and oval in shape and project to the frontal regions of the brain including the limbic system (Dahlstrom & Fuxe, 1964; Grealish et al, 2010; Kawano, 2006). Functionally, the A10 neurons of the ventral tegmental area (VTA) act on these mesolimbic and cortical areas, including the nucleus accumbens, hippocampus and amygdala, and serve as important regulators in both cognitive and psychological function (Kawano, 2006; Pralong et al, 2002; Steketee 2003). Additionally, the diencephalic A11 dopamine neurons of the hypothalamus serve an important but less characterized role in brain function. The A11 dopaminergic neurons were originally thought to solely act on the spinal cord through inhibitory signaling, thus playing an important role in nociception and movement (Charbit et al, 2009; Holstege et al, 2006; Takada et al, 1988). However, recent research has shown that these neurons project to various regions of the brain including the neocortex and the trigeminocervical

complex, suggesting that these neurons may play a more important role in brain function and behaviour than originally thought (Charbit et al, 2009; Takada et al, 1988). The role of the A9, A10 and A11 also remain elusive and not fully acknowledged. Although some areas are synonymous, such as the A10 and Ventral Tegmental Area, in the current thesis we will refer to these areas by the name of the brain structures themselves, and not their A(X) designation, with exception of the A11 dopaminergic neurons.

More clearly understood and thoroughly examined, is the dopamine reward pathway (Ikemoto, 2007; Pralong et al, 2002). As briefly discussed above, midbrain and subcortical dopamine neurons connect to other areas in the brain including the mesolimbic, medial prefrontal cortex and the frontal cortex (Ikemoto, 2007; Steketee, 2003). To further clarify the exact connections and projections one must understand how the more posterior, and midbrain structures connect to the more anterior and frontal regions. The ventral tegmental area neurons project to the nucleus accumbens (NA) and olfactory tubercles, which project directly to the ventral striatum, amygdala and hippocampus (Oades & Halliday, 1987; Pralong et al, 2002). This connection has been primarily implicated in motivation and reward, and distinctly involved the processes of drug abuse (Fibiger & Phillips, 1986; McBride et al, 1999; Ikemoto, 2007). This was supported by the observation that blockade of dopamine transmission within the nucleus accumbens significantly attenuated the rewarding effects of self-administered cocaine, and other reinforcing drugs (Gerrits & Van Ree, 1996). Moreover, repeated consumption of cocaine and related drugs has been directly linked to originating in the dopamine containing neurons of the VTA (Robinson & Beck, 1985; White et al 1995). Evidence has shown that dopamine

transmission from the VTA triggers activation in the shell of the NA, more so than the core (Rodd-Hendricks, 2002; Ito et al, 2004). As discussed thus far, the dopaminergic neurons within the VTA project to the NA, and this transmission is associated with reward and referred to as the ventral striatum stream (Ikemoto, 2007).

The dopaminergic neurons of the VTA project to areas other than the NA, such as the hippocampus, amygdala, and prefrontal cortex, and this pathway is known as the mesocorticolimbic system (Fallon & Moore, 1978; Lindvall et al, 1974; Pralong et al, 2002; Swanson, 1982). These structures are grouped together; hippocampus, amygdala, thalamus, and also the cingulate gyrus, in what is termed the limbic system, all structures situated within close proximity, and are linked together in regulating emotion, memory and cognition (Heimer & Alheid, 1991; Papez, 1937; Pralong et al, 2002). Within the amygdala, the basolateral amygdala (BLA) in particular, dopamine release increases as a direct influence of increased release from the nucleus accumbens (Howland, Taepavarapruk & Phillips, 2002). However, these areas receive less dopaminergic input directly from the VTA, compared to the innervation from the VTA to the nucleus accumbens (Ikemoto, 2007). These frontal and limbic areas also receive dopaminergic inputs from the SN, and together with DA influx from the VTA and the SN, they are significantly influenced by dopaminergic innervation within the A8, A9 and A10 dopaminergic neurons.

Changes in dopamine levels can stand as an important indicator of the physiological state, and health of the individual at hand. Dopamine irregularities have been linked to numerous mood

disorders, low tonic levels of dopamine function have been linked to depression and bipolar disorder (Maji, 2001, Pralong et al, 2002; Willner, 1995). Similarly, the dopamine imbalance has been implicated in the neuropathological progression of schizophrenia, data suggesting an over active dopamine transmission may be a primary cause (Abi-Dargham et al, 2000; Patel et al, 2010; Shen, Liao & Tseng, 2012). Beneficial effects of dopamine antagonists as effective treatment and the detrimental effects of dopamine enhancing drugs, like amphetamine, reassert this hypothesis (Pralong et al, 2002; Shen, Liao & Tseng, 2012). Dopamine transmission has also been largely implicated in Parkinson's Disease, as the dopamine agonist levodopa has been found to be an effective treatment for various symptoms associated with the disease, including motor fluctuations and dyskinesias (Fritsch et al, 2012). Activation of the dopamine reward system, as a whole, has also been implicated in broad spectrum of various forms addiction and reward seeking behaviour (Koob et al, 1994; Koob, 1996; Pralong et al, 2002). Finally, as previously discussed, dopamine plays an important role during stroke and related ischemic events. Excitotoxic events associated with ischemia create an immediate surge of dopamine release throughout the brain, resulting in surrounding necrosis and ultimately in blunted dopamine function (Benfenati et al, 1989; Globus et al, 1987; Kronenberg et al 2012; Li et al, 2009; Martin et al, 2012; Takagi et al, 1995; Ruscher, Kuric & Wielock, 2012).

Dopamine stands as an extremely important neurotransmitter, effecting numerous brain areas. Nonetheless, it is important to consider that dopamine's function is interwoven with other small molecule function, including serotonin, noradrenaline, GABA and glutamate, whose importance must not be overlooked (Pralong et al, 2002). For instance, in the hippocampus

glutamate neurons act on both excitatory and inhibitory dopamine neurons in the dentate gyrus thus may have profound influence on dopamine function (Smialowski & Bijak, 1987). Similarly, both GABA and glutamatergic inputs from the limbic system act directly on the reward pathway, including the VTA and NA (Pralong et al, 2002).

J. Endocannabinoids & Dopamine: Interaction of GABA and Glutamate

Of particular interest is the interaction between the endocannabinoid system and dopamine system. As briefly discussed, modulation of dopamine in various pathological states can have beneficial effects. The effect of endocannabinoid agonists and antagonists on influencing dopamine transmission is apparent. As stated however, numerous factors influence the way dopamine is modulated by these chemicals, including dosage, physiological state, site of injection, and so forth (Khoury et al, 2012). Numerous studies have found that cannabinoid receptor activation increases extracellular dopamine and dopamine neuron activity, particularly in the mesolimbic circuit including the nucleus accumbens, prefrontal cortex and ventral tegmental area (French, Dillon & Wu, 1997; Fernandez-Ruiz, Hernandez & Ramos, 2010; Khoury et al, 2012; Marinelli et al, 2007; Melis & Pistis, 2012; Seif et al, 2011; Tanda et al, 1997). This effect of eCBs to increase dopamine in the mesolimbic system is intuitive with the drug addiction hypotheses associated with cannabis (Melis & Pistis, 2012). This stimulation of dopamine release by cannabinoid agonists is not direct, but involves CB₁ receptors located on both GABAergic and glutamatergic neurons, which then interact with dopamine neurons (Fernandez-Ruiz, Hernandez & Ramos, 2010; Gerdeman & Fernandez-Ruiz, 2008; Khoury et al, 2012; Melis & Pistis, 2007; Pralong, Magistretti & Stoop, 2002). The existence of the CB₁

receptors on GABAergic and glutamatergic neurons render them able to regulate both inhibitory and excitatory messages. However, it has been discovered that the majority of the time activation eCB acts on inhibitory CB₁ receptors located on GABAergic neurons which causes a disinhibition of dopamine activation, thus increasing dopamine transmission (Gerdeman & Fernandez-Ruiz, 2008; Katona et al, 1999; Khoury et al, 2012). Numerous studies suggest that eCB transmission favors diminished GABA inhibition, thus CB₁ receptor activation not only initiate release of dopamine but also alters the balance between glutamatergic and GABAergic activity: as GABAergic neurons become inhibited, glutamate action increases (Khoury et al, 2012; Patel, Rademacher, & Hillard, 2003).

It is also proposed that cannabinoid agonists act on excitatory TRPV1 located on glutamatergic terminals, thus the resulting imbalance and increase in glutamate may be a contributing factor to the increase in dopamine transmission as, increases in glutamate activity also tends to trigger dopamine release (Khoury et al, 2012; Marinelli et al, 2007; Segovia & Mora, 2001). Supporting this mechanism of action it was found that THC also decreases extracellular GABA, while increasing both extracellular glutamate and dopamine in rats (Pistis et al, 2002). These results are inclusive to the striatum and cortex, including the mesolimbic circuit (Khoury et al, 2012). Again, it should be noted that these results tend to be dose dependent, and most consistent using low to medium doses depending on the drug. For instance, THC average dose is 1 mg/kg ip, and any extremely high doses can have opposite effects (Khoury et al, 2012).

In general, endocannabinoid antagonists result in opposing effects as those obtained with endocannabinoid agonists (Khoury et al, 2012). Numerous studies have shown that endocannabinoid antagonists have been effective in reducing the addictive quality and associated behavioural deficits, like compulsive drug seeking and the effects of withdrawal of various drugs, including alcohol, THC, and cocaine (Colombo et al, 2005; Corbille et al, 2007 De Vries et al 2001; Khoury et al, 2012). These addictive properties are said to be mainly a result of the dramatic increase dopamine release in the mesolimbic pathway, thus antagonism of the endocannabinoid system seems to work by attenuating this dopamine surge (Khoury et al, 2012; Sidhpura & Parsons, 2011). In one study the effect of AM251 on dopamine was evaluated based on a lever-pressing simulation, where more lever presses were indicative of normal or high dopamine transmission in the nucleus accumbens and low lever pressing was indicative of altered or reduced dopamine (Randall et al, 2012). Rats treated with AM251 had significantly reduced lever pressing, similar results were seen with rats given a direct dopamine antagonist (Randall et al, 2012). Similarly, hyper-dopaminergic rodents that normally have poor performance on cognitive tasks, performed significantly better when given AM251 compared to saline-treated counterparts, suggesting that AM251 has effect to regulate excess dopamine (Khoury et al, 2012). Of interest, the interaction between dopamine and glutamate has been implicated in excitotoxicity. For instance, blocking dopamine lead to reduced glutamate-induced lesions during excitotoxicity, reasserting that dopamine release intensifies the excitotoxic effects (Filloux & Wamsley, 1991). Of interest, excess dopamine was found to inhibit glutamate uptake, thus perpetuating the amount of glutamate in the synapse and increasing cell damage and death (Berman & Hastings, 1997; Zhang et al, 2008).

Finally it was recently discovered, through immunohistochemical analyses, that CB₁ receptors are co-localized with tyrosine hydroxylase in the nucleus accumbens, ventral tegmental area, and striatum, suggesting that endocannabinoids may indeed act directly on dopamine neurons (Wenger, Moldrich & Furst, 2003). Indeed, these new findings would suggest a more direct relationship between eCBs and dopamine function although further investigation is required to confirm these results.

One needs to emphasize the existence of numerous discrepancies in current literature on the effects of eCBs and their antagonists on glutamate. For instance, Hill and colleagues (2010) proposed that under steady state conditions tonic levels of AEA and 2-AG act to regulate the stress response and glutamate within the basolateral amygdala, and that under such conditions eCB antagonists create increase in HPA reactivity and glutamate. However, the same authors also reveal that activation of the HPA axis, under physiological stress, causes an increase in both AEA and 2-AG, which in turn results in a rapid increase in FAAH which catalyzes AEA ultimately leading to a reduction in tonic endocannabinoid release and further increases in HPA activity (Hill et al, 2010). Thus although eCBs play a regulatory function under steady state conditions, when the system is challenged it appears that eCB release may indeed exacerbate stress and that CB₁ antagonism may reverse this cycle (Hill et al, 2010). The role of antagonists under stressful conditions may indeed prove to prevent this damaging cycle from causing HPA over-reactivity. From these findings, it can be suggested that discrepancies in the effect eCB antagonists, including AM251, in various studies is because their action is state-dependent, and

benefits are often only seen when physiological conditions are challenged, or when AM251 is given to non-healthy subjects (Hill et al, 2010; Khoury et al, 2012).

There is some remaining contention as to how endocannabinoid antagonists alter glutamate activity and ultimately dopamine transmission (Tzarvara et al, 2009; Xi et al, 2006). For instance, there remains discrepancy among the effect of eCBs in altering glutamatergic activity. Endocannabinoids have been shown to decrease glutamatergic activity, thus contraindicating the previously discussed findings showing that endocannabinoid antagonists decrease glutamatergic activity (Hill et al 2010). This is why the proposition of state-dependent effects need to be considered (Hill et al, 2010; Khoury et al, 2012). Another explanation may be that those studies which found that eCB antagonists increase glutamatergic activity, may be limited as they only looked at the very brief effect of the antagonist (Hill et al, 2010). Some studies proposed that, alternatively, CB₁ receptor antagonists alter glutamate receptor activation in a different manner (Tzarvara et al, 2009; Xi et al, 2006). One such study found that AM251 increases momentarily glutamate release, however, this is quickly reversed as AM251 increases binding on mGluR2/3 autoreceptors which then prevents further glutamate release, ultimately resulting in reduced glutamate release (Xi et al, 2006).

H. Objective & Hypotheses

The current study intends to further explore the role of endocannabinoids, using the CB₁ receptor antagonist AM251, in protecting the brain against ischemia and stroke. It is also the intention to further clarify the effects of stroke on dopamine transmission and the effect AM251

has on restoring ischemia-induced deficits in dopamine. Since it is acknowledged that endocannabinoids and their antagonists target areas within the dopamine reward system, it is suspected that AM251 alter the hypo-dopaminergic state following ischemia, by a mechanism thought to prevent excitotoxic events.

More specifically, the current study aimed to analyze the effect of administering the endocannabinoid antagonist AM251 (2mg/kg, i.p.) prior to global cerebral ischemia to examine its effects on dopamine transmission, cell survival, and behavioural outcome. It is also intended to more carefully characterize the effect of global cerebral ischemia on the dopamine transmission through the mesocorticolimbic dopamine pathways.

Based on previous studies suggesting that up-regulation of dopaminergic release at short interval is replaced by reduced dopaminergic function at longer term interval following brain ischemia, it is expected that the current study will observe a blunted TH expression in reward pathways seven days following global cerebral ischemia. It is hypothesized that treatment with AM251 before ischemia will improve such outcome as suggested by previous research supporting benefits of eCB antagonists on ischemic insult.

Moreover, from studies suggesting that excessive glutamate, dopamine and AEA release during ischemia contribute to damaging neurons, we predict that AM251 pretreatment by acting on these processes will attenuate ischemia-induced neuronal degeneration.

The current study is taking a novel approach by evaluating AM251's ability to protect against dopaminergic dysfunction caused by ischemia. This will be done by assessing dopamine function, using TH and DRD1 immunoreactivity (ir) within the mesocorticolimbic pathways. It is anticipated that AM251 will improve dopamine-ir. Additionally, cell survival in the hippocampus will be assessed in order to confirm AM251's ability to protect against neuronal death when administered prior to cerebral ischemia. Cell survival will be analyzed by counting and comparing CA1 neuronal density using thionin staining. Considering the demonstration of endocannabinoids effect in regulation of emotional responses, including anxiety, behavioural tests have also been used to validate a possible impact of eCBs on mediation of anxiety and hyperactivity post ischemia. It is expected that vehicle-treated ischemic rats will display reduced anxiety and locomotor hyperactivity when tested 5 days post ischemia (Kronenberg et al, 2012; Milot & Plamondon, 2008; Milot & Plamondon, 2009). This will be assessed using the elevated plus maze and open field test. We predict that considering effects of the endocannabinoids on anxiety and stress response, treatment with AM251 will alter these behavioural outcomes. In sum, the current literature suggest that acute administration of AM251 prior to global cerebral ischemia will have effects to alter post ischemic dopaminergic expression, CA1 neuronal survival and behaviour.

II. METHODS

A. Subjects

Forty male Wistar rats weighing between 300-350g were obtained from Charles River Laboratory (Rochefort, Quebec, Canada). They were individually housed and maintained on a 12 hour light/dark cycle, lights on at 7:00 am, room temperature (21-23°C) with 60% relative humidity, and free access to water and standard Purina rat chow. Once they arrived at the animal facility at the University of Ottawa (Vanier Building), rats were habituated to the new environment 2 weeks prior to surgery. All procedures were carried out in accordance with the Canadian Council of Animal care and approved by the University of Ottawa Animal Care Committee.

Rats were randomly assigned to one of four experimental groups (n=10/group). The first group designed as **IA** was composed of ischemic rats pretreated with a 2 mg/kg dose of the CB1 antagonist AM251 administered intraperitoneally (ip) 30 minutes prior to ischemic surgery. The second group designed as **SA** was composed of AM-251 pretreated sham-operated rat. The third group **IC** (Ischemic Control) was composed of ischemic rats ip injected with the vehicle solution 30 minutes prior to ischemic surgery. Lastly, the fourth group **SC** (Sham Control) consisted of rats ip injected with the vehicle solution 30 minutes prior to sham surgery.

B. Drug Preparation

The CB1 receptor antagonist AM251, IUPAC name: *N*-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide (Tocris Biosciences,

Bristol, UK) was stored at room temperature until the date of injections when it was solubilized using DMSO, Tween 80 and 0.9% saline (1:1:8) as previously done in other studies (Newsom et al, 2012). Rats were weighed throughout the study and before drug administration to ensure proper administration volume by weight (1ml/kg). The 2mg/kg dose was selected based on different studies showing an effective range between 1mg/kg to 3 mg/kg (Bialuk & Winnicka, 2011; Tallett Blundell & Rogers, 2007; Thiemann et al, 2008). The injection was performed 30 minutes prior to occlusion to ensure that the drug had reached physiological targets when ischemia was induced. Control rats were administered the vehicle solution (1ml/kg) following the same administration regimen.

C. Four Vessel Occlusion Model

The Four Vessel Occlusion model (4VO) developed by Pulsinelli & Brierley (1979) was used to induce global cerebral ischemia in rats. The 4VO method mimics cardiac arrest and leads to restriction of blood flow to the forebrain. Briefly, under isoflurane anesthesia (dissolved in 2% oxygen) an incision was made about 1 cm in length behind the occipital bone, and the paraspinal muscles were separated in order to expose the left and right alar foramina. Using a 0.5 mm electrocauterizing needle, the two vertebral arteries were then permanently occluded. Immediately after, the common carotid arteries were isolated, without restricting blood flow, and a silk thread was loosely looped around each of the arteries until subsequent occlusion on the following day. Both sites of incision were then stitched up using surgical staples. The rats were allowed to recover for twenty-four hours in their individual cages. After the twenty-four hour recoveryThe following day, both carotids were tightened using microvascular clamps in freely

ventilating animals to induce global cerebral ischemia. The carotid arteries were occluded for a total of 10 minutes. Clips were removed after ten minutes to allow restoration of blood flow. Sham-operated animals underwent anesthesia, and received the same dorsal and ventral surgical incisions as the ischemic group with the exception of electrocoagulation of the vertebral arteries. Twenty-four hours later, carotid arteries were exposed but not clamped. The core temperature was regulated throughout the surgery by means of a feedback regulated heating blanket connected to a rectal thermometer (Homeothermic Blanket Control Unit, Harvard Instruments, Natick, MA), and supported with a heating pad during vessel occlusion and in the hours following surgery and reperfusion.

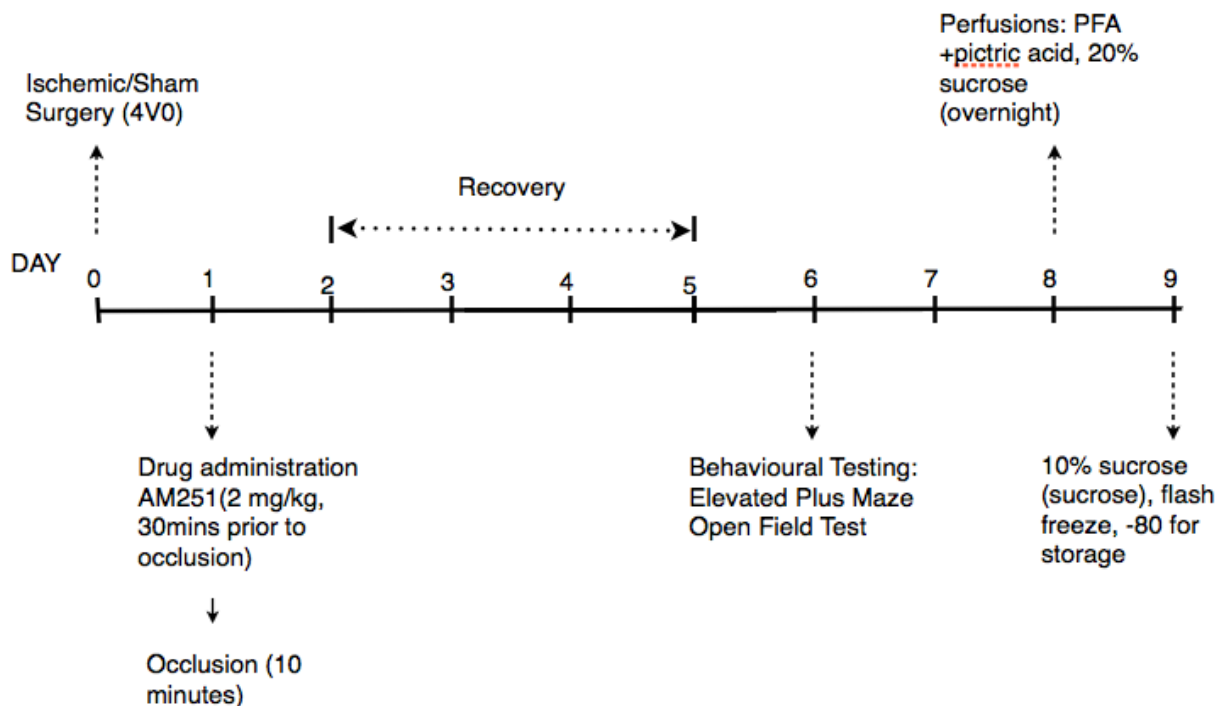


Figure 4. Timeline of study: time of surgery, occlusion, recovery period, behavioural testing, and termination of the study are outlined.

D. Behavioural Testing

Behavioural testing took place at day 6 following occlusion during the light period of the light/dark cycle (see figure 4). Both the Elevated Plus Maze (EPM) and open field test (OFT) were administered. To avoid possible testing sequence effects, half of the rats were tested on the elevated plus maze first while the other half were tested in the open field first. Rats were habituated to the testing room for at least 30 minutes before being tested.

Elevated Plus Maze (EPM): The EPM was used to assess anxiety-like behaviour. The test consisted of an X shaped plexiglass structure, all four arms measured 50cm x 10cm, the open arm had a 5mm plexiglass lip and the closed arm had 40 cm walls. The entire maze was raised above the floor by 60cm, and surrounded by plain white walls. The observer was separated from the test by a white curtain and viewed the EPM activity using a video camera. Rats were placed in the closed arms facing outwards, and were allowed to roam freely for a five minute period while the number of open arm entries, number of closed arm entries, number of non-walking movement (counted as dipping head in open arms as well as rearing), time spent in the open arms, time spent in the closed arms and number of times crossing the center into an opposing arm were calculated using ODlog. The apparatus was cleaned with 70% ethanol and dried in between testing for each animal.

Open Field Test (OFT): The OFT was used to assess anxiety-like behaviour and locomotion. The test consisted of a square observation arena made of plexiglas with dimensions of 75 cm x 75 cm (with 15 cm walls). On the bottom of the arena was a painted grid of 36 equally sized squares (20 making the peripheral zone and 16 the center zone of the arena). The test was surrounded by

plain white walls and the observer was separated from the test by plain white curtains and viewed OFT activity using a video camera. Rats were placed in the arena facing a corner of the test and then allowed to roam freely for a 10 minute period while the number of entries in the peripheral squares (as measured per line crossing into a new square), number of entries in the center squares (as measured per line crossing into a new square), rearing frequency, and time spent grooming were all assessed using ODlog. The test was cleaned with 70% ethanol and dried in between testing for each animal.

Nine days following reperfusion, the rats were deeply anesthetized with pentobarbital and intracardiacally perfused using 0.9% saline followed by 4% paraformaldehyde with picric acid solution. The brains were extracted from the skull and immersed in a 4% paraformaldehyde with picric acid solution for 1 hour, and then placed in a 20% sucrose solution for 24 hours, and finally transferred in 10% sucrose solution for a 4 hour period before being frozen using carbon dioxide. The brains were subsequently stored at -80°C until sectioned. Cryostat cut brain sections (14µm thickness) were collected on polylysine slides and kept at -80 °C until processed.

E. Immunohistochemistry

Immunoreactivity for selected endogenous markers was assessed using fluorescent immunohistochemical detection. Brain slices were initially bathed in 10 mM Phosphate buffered solution (PBS) at room temperature for a minimum five minutes to allow for defrosting and rehydration of brain tissue.

The brain slices were then treated with one of the following primary antibodies:

Tyrosine Hydroxylase (TH): The monoclonal mouse Anti-Tyrosine Hydroxylase (Millipore, Massachusetts, USA) was diluted to 1/1000 with PBS-triton (0.02%) and incubated at 4°C for 24 hours. Slides were rinsed three times for five minutes using PBS, then treated with the secondary antibody Alexa Fluor 488 donkey anti-mouse IgG from Invitrogen (Canada) diluted to 1/500, with 0.02% PBS-triton and incubated for 30 minutes at 37°C. Slides were rinsed three times for five minutes and then treated with the DNA binding marker Hoeschst, diluted to 1/20,000 with PBS, for 30 minutes at room temperature covered to avoid light contamination. Slides were washed three times for five minutes using PBS. Slides were then dried, an antifade solution was applied to preserve immunofluorescent signal, and coverslips were placed and sealed with nail polish.

DRD1 Dopamine Receptor: The polyclonal goat antibody D1DR [C-20] was purchased from Santa Cruz Biotechnology (Dallas, Texas, USA). It was diluted to 1/400 with PBS-triton (0.02%) and incubated at 4°C for 60 hours. Slides were rinsed three times for five minutes using PBS, then treated with the secondary antibody Alexa Fluor 488 donkey anti-goat IgG from Invitrogen (Canada) diluted to 1/500, with 0.02% PBS-triton and incubated for 30 minutes at 37°C. Slides were rinsed three times for five minutes and then treated with the DNA binding marker Hoeschst following an identical procedure as previously described. The subsequent steps were also the same as the ones performed for TH immuno labeling.

note: Upon completion of the procedure, the slides were either directly visualized or stored back at -80 °C until analysis using a fluorescence equipped microscope.

F. Analysis of Immunohistochemical Labeling

Assessment of changes in dopamine and dopamine receptor 1 expression was evaluated using both TH and DRD1, respectively. The immunofluorescent signal detection was accomplished using an Olympus DX51 microscope (Center Valley, PA, USA). Digital images of immunofluorescence were obtained using the Progress Pro 2.7.6 software under 20× magnification. For all regions of interest, immunoreactive cell bodies or processes were quantified using Image J software (Image J, National Institutes of Health) and the method described by Hayes and colleagues (Hayes et al., 2005). Percentages of optical densities (Mean Grey Values: estimates of the staining intensity) from a selected brain region relative to a subthreshold background were obtained. This technique required to initially subtract the background and then measure intensely labeled area. Four anatomically matched pictures of the left and right hemispheres of the brain were used to produce an average immunoreactivity score for each brain region in each animal. Data are presented as background corrected standardized image densities for each brain (Schneider, Rasband & Eliceiri, 2012).

G. Analysis of Neuronal Survival

Neuronal density was assessed in the Ammon's horn CA1 and CA3 pyramidal cell layers of the hippocampus using thionin stain to reveal Nissl bodies, as per described by Kirino and colleagues (1991). Coronal hippocampal slices were collected between 3.14 and 4.16 mm posterior to bregma (Paxinos & Watson, 1986). The number of intact neurons were counted manually using a LEICA DAS microscope attached to a SONY camera and the image analysis software Norton Eclipse (v 6.0; Empix Imaging, Mississauga, Ontario). Only neurons that had

normal morphology were counted, cells that were small, uneven or far away from the CA1 or CA3 band were not counted. Both sides of the hippocampus were assessed for both the CA1 and CA3, and a mean from six counts per structure obtained for each rat. Neuronal density is reported per mm linear tissue length. The person counting the cells was blind to the treatment conditions.

H. Statistical Analyses

Statistical analyses were performed using IBM SPSS Statistics 20 software. Significance was considered when P value was less or equal to 0.05. On the rare occasion, rats were excluded if they were showing two standard deviations from the group average. Homogeneity of variance and sphericity were always verified prior to analyses. Two-way ANOVAs were performed for all analyses comparing all four experimental groups, the two factors being surgery (either ischemic or sham) and drug (vehicle control or AM251). Simple effects tests were used when significant interactions were found in the initial two-factor ANOVA. Confidence intervals of 95% were used for all analyses. Bonferroni correction was used to adjust alpha per comparison (in all cases 4 comparisons were made so an alpha of 0.0125 was used).

III. RESULTS

A. Behavioural Testing

i. Elevated Plus Maze

Open Arm Time: Time spent in the open arms was compared between all four experimental groups. Significant main effects of the surgery [$F(1,36)=84.77, p<0.000$] and the drug [$F(1,36)=33.30, p<0.000$] was found as well as a significant surgery X drug interaction [$F(1,36)=28.02, p<0.000$]. Simple effect tests indicated that vehicle-treated ischemic rats spent significantly more time in the open arm than the sham control rats ($p<0.000$) and AM251-pretreated ischemic rats spent significantly more time in the open arm than AM251 sham rats ($p=0.007$). Notably, vehicle- and AM251-treated sham rats did not significantly differ on that measure ($p=0.740$). See figure 5.

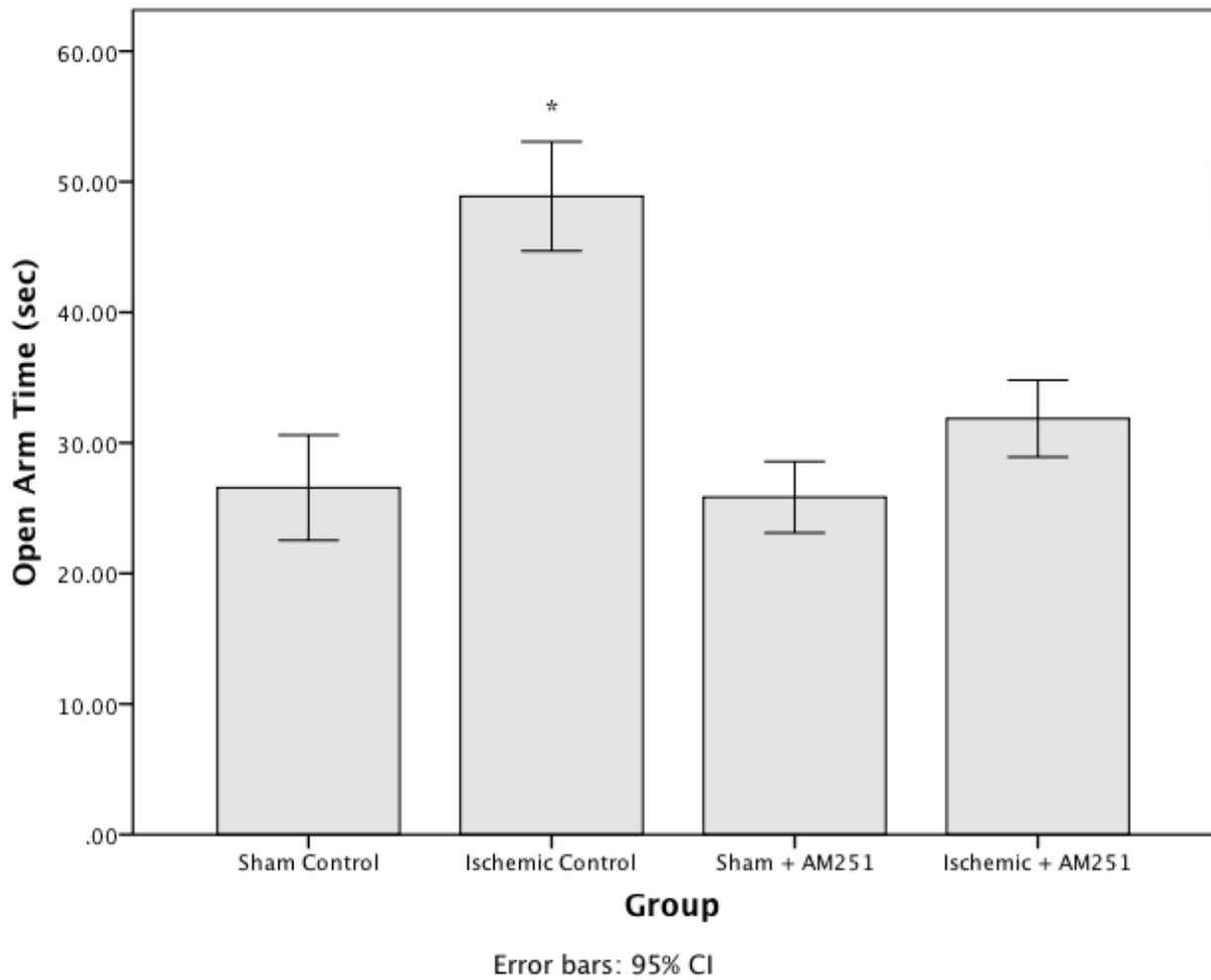


Figure 5. Mean time spent in the open arm of the EPM for each of the experimental groups. Vehicle-treated ischemic rats spent significantly more time in the open arm than all other groups, indicating an anxiolytic behaviour ($P<0.007$).

Open Arm Entry: Frequency of open arm entry showed a similar profile as that observed for time in the open arm. A significant main effect of the drug [$F(1,36)=7.87, p=0.008$] and a surgery X drug interaction [$F(1,36)=6.86, p=0.013$] were observed but no main effect of the surgery [$F(1,36)=1.03, p=0.317$]. Simple effect tests indicated that vehicle-treated ischemic rats made more entries in the open arm than the sham control rats ($p<0.017$). There was no difference

between vehicle and AM251-treated shams ($p=0.896$) nor between AM251-treated ischemic and sham animals ($p=0.741$). However, vehicle-treated ischemic rats made significantly more open arm entries than the AM251-treated ischemic rats ($p<0.000$). See figure 6.

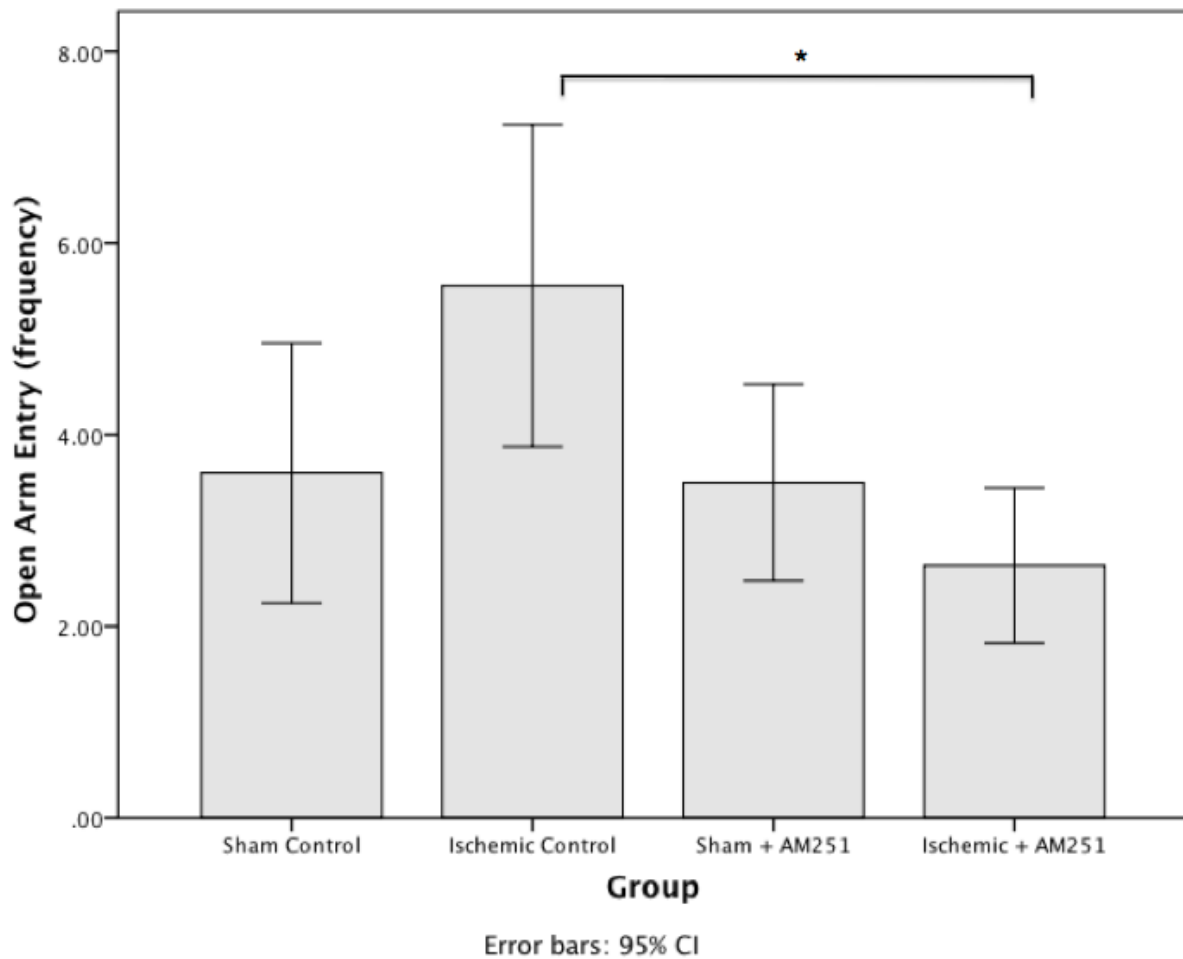


Figure 6. This graph illustrates the mean frequency of open arm entries for all four experimental groups. The ischemic controls had significantly more open arm entries than the Ischemic + AM251 ($p<0.000$).

Closed Arm Time: Analysis of the time spent in the closed arm revealed a significant main effect of the surgery [$F(1,36)=5.5, p=0.024$] and a surgery X drug interaction [$F(1,36)=11.72, p=0.002$] but no significant effect of the drug [$F(1,36)=2.92, p=0.096$]. Simple effect tests indicated that vehicle-treated sham rats spent significantly more time in the closed

arm than vehicle-treated ischemic rats ($p<0.000$) and no differences was found between AM251 ischemic rats and sham rats ($p=0.444$). Both groups of sham rats spent comparable time in the closed arm ($p=0.233$) while AM251-treated ischemic rats spent more time in the closed arm than the vehicle-treated ischemic rats ($p=0.001$). (See Appendix Figure i)

Risk assessment: Risk assessment in the EPM was measured as the amount of head dips, specifically that when their two front forepaws were out into the open arm as well as rearing. No main effect of the surgery [$F(1,36)=2.38$, $p=0.131$], drug [$F(1,36)=0.21$, $p=0.643$], or surgery X drug interaction [$F(1,36)=2.62$, $p=0.114$] were observed. (See Appendix Figure ii).

Crossing: Frequency of crossing the center line from one arm to the opposing arm was compared between all four experimental groups. No significant main effect of the surgery [$F(1,36)=104$, $p=0.749$], drug [$F(1,36)=0.19$, $p=0.658$] or surgery X drug interaction [$F(1,36)=2.15$, $p=0.151$] were observed. (See Appendix Figure iii).

ii. Open Field Test

Locomotion in the Center Zone: Frequency of walking in the center zone of the open field test, measured as each time a rat entered one of the 16 center squares of the arena (measured as line crossing frequency), was compared between the rat groups. A main effect of surgery [$F(1,36)=48.05$, $p=0.000$] and a surgery X drug interaction [$F(1,36)=12.11$, $p=0.001$] were found. The analysis revealed no effect of the drug treatment [$F(1,36)=1.29$, $p=0.262$]. Simple effect tests indicated that vehicle-treated ischemic rats spent more time walking in the center zone compared to vehicle-treated sham rats ($p<0.000$) and AM251-treated ischemic

animals ($p=0.002$). Both sham groups showed comparable walking frequency in the center zone ($p=0.106$). Analysis also revealed a no significant difference between drug-treated sham and ischemic rats ($p=0.17$). See Figure 7.

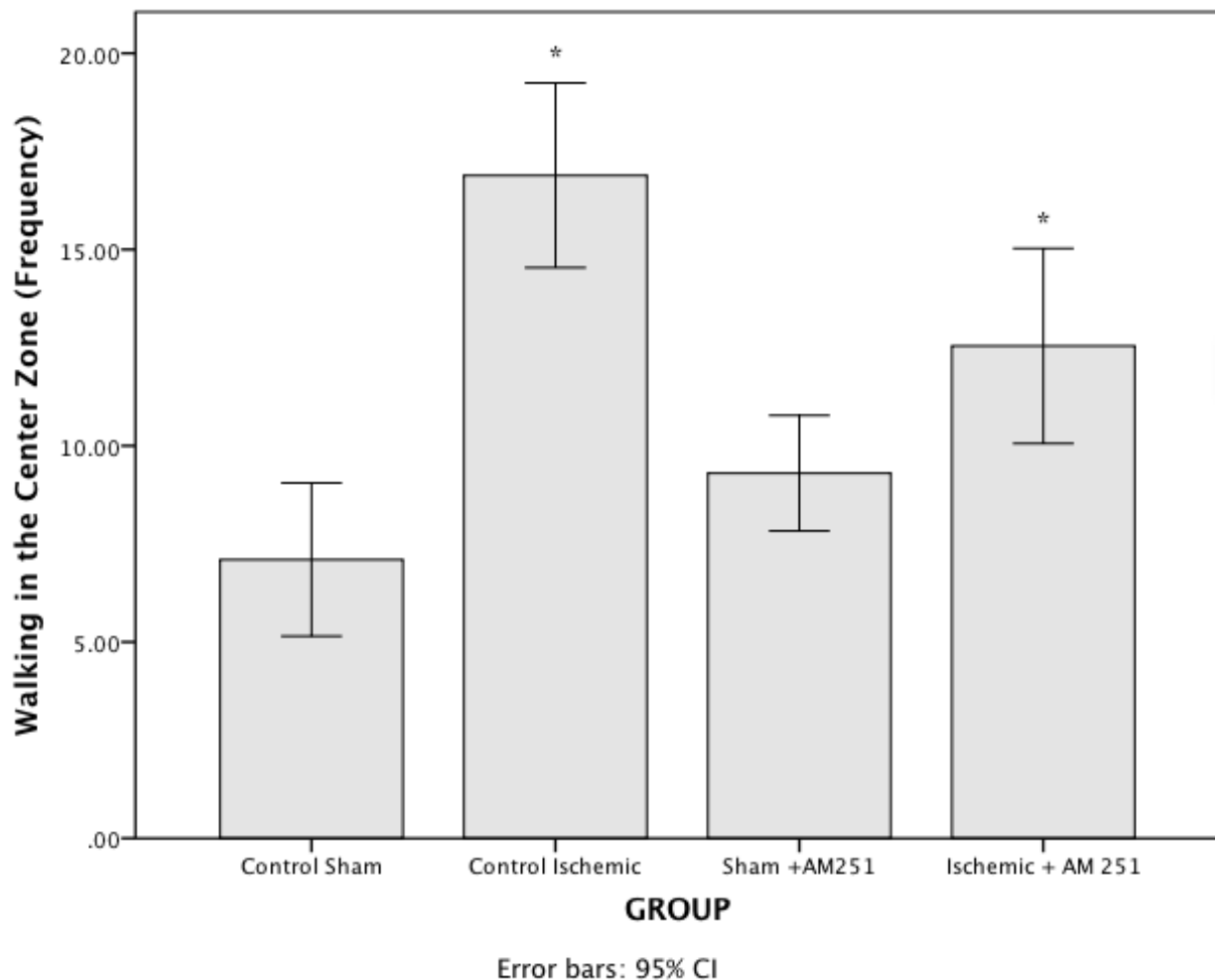


Figure 7. This graph illustrates the mean frequency of walking in the center zone in all four experimental groups. Ischemics were showed increased locomotion in the center zone compared to sham groups ($p<0.000$). This was attenuated in AM251-treated ischemic animals that showed reduced center zone walking compared to vehicle-treated ischemic rats ($p=0.002$).

Grooming in the OFT: Time spent grooming over the duration of the open field test was compared between the groups. Analysis revealed a main effect of surgery [$F(1,36)=4.87$,

$p=0.034$], drug [$F(1,36)=12.98$, $p=0.001$) and a surgery X drug interaction [$F(1,36)=12.59$, $p=0.001$]. Simple effect tests revealed that this was partly attributable to increased grooming frequency in the vehicle-treated ischemic compared to vehicle-treated sham ($p<0.000$) and AM-251 ischemic ($p=0.000$) rats. AM251-treated sham and ischemic rats also did not differ ($p=0.337$) nor the two sham groups ($p=0.969$).

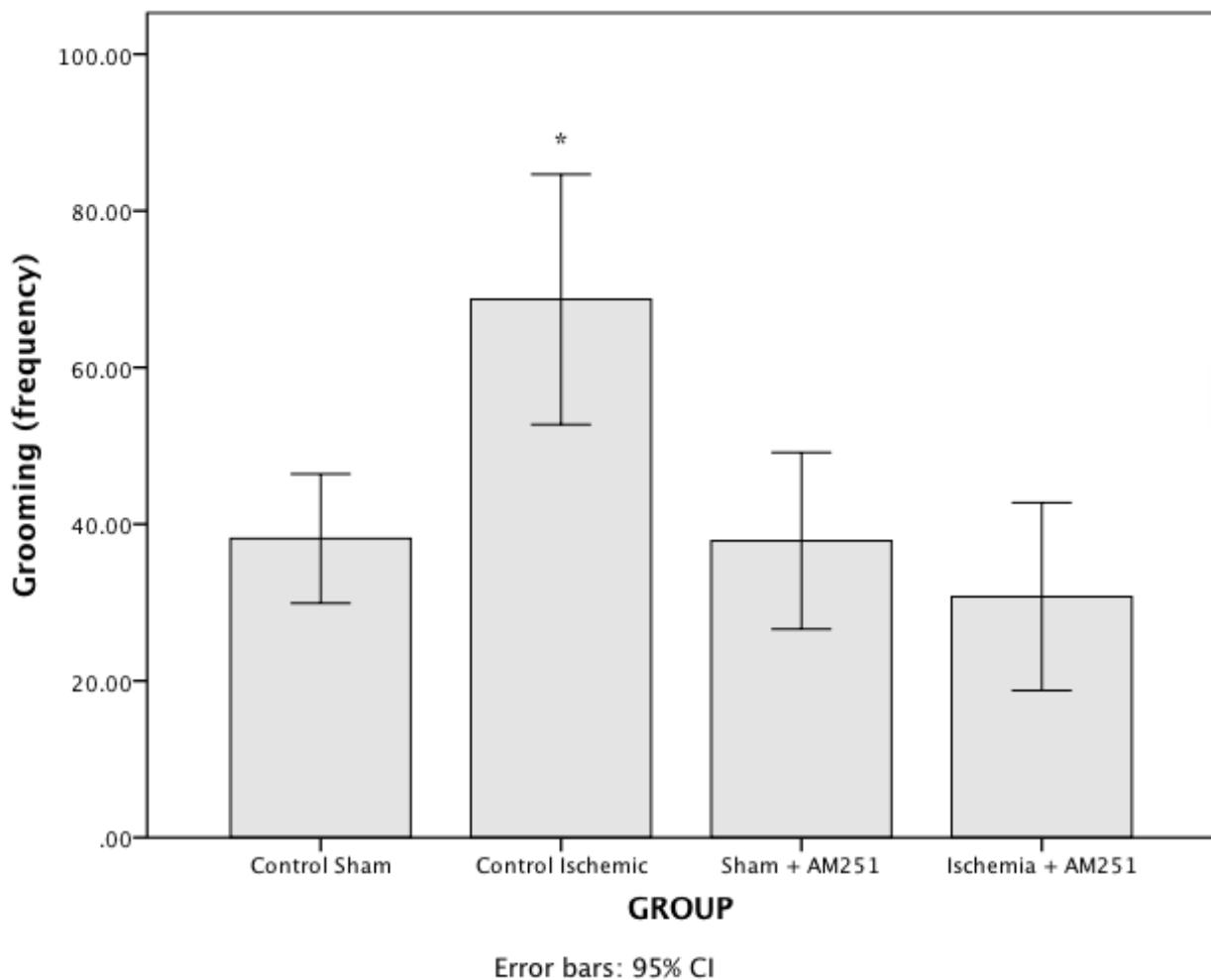


Figure 8. This graph illustrates the mean frequency of walking in the center zone in all four experimental groups. The control ischemic group spent significantly more time grooming, measured in seconds, than all other experimental groups ($p<0.000$).

Rearing in the OFT: Frequency of rearing in the open field was compared between all experimental groups. No significant effects were found. (See Appendix Figure iv).

Walking in the Periphery: Frequency of walking in the periphery of the OFT measured each time a rat entered one of the 20 squares directly adjacent to the four arena walls was compared between all experimental groups. No significant main effects of surgery [F(1,36)=0.12,p=0.725], and drug [F(1,36)=2.57, p=0.118] were found nor a surgery X drug interaction [F(1,36)=0.42, p=0.518].

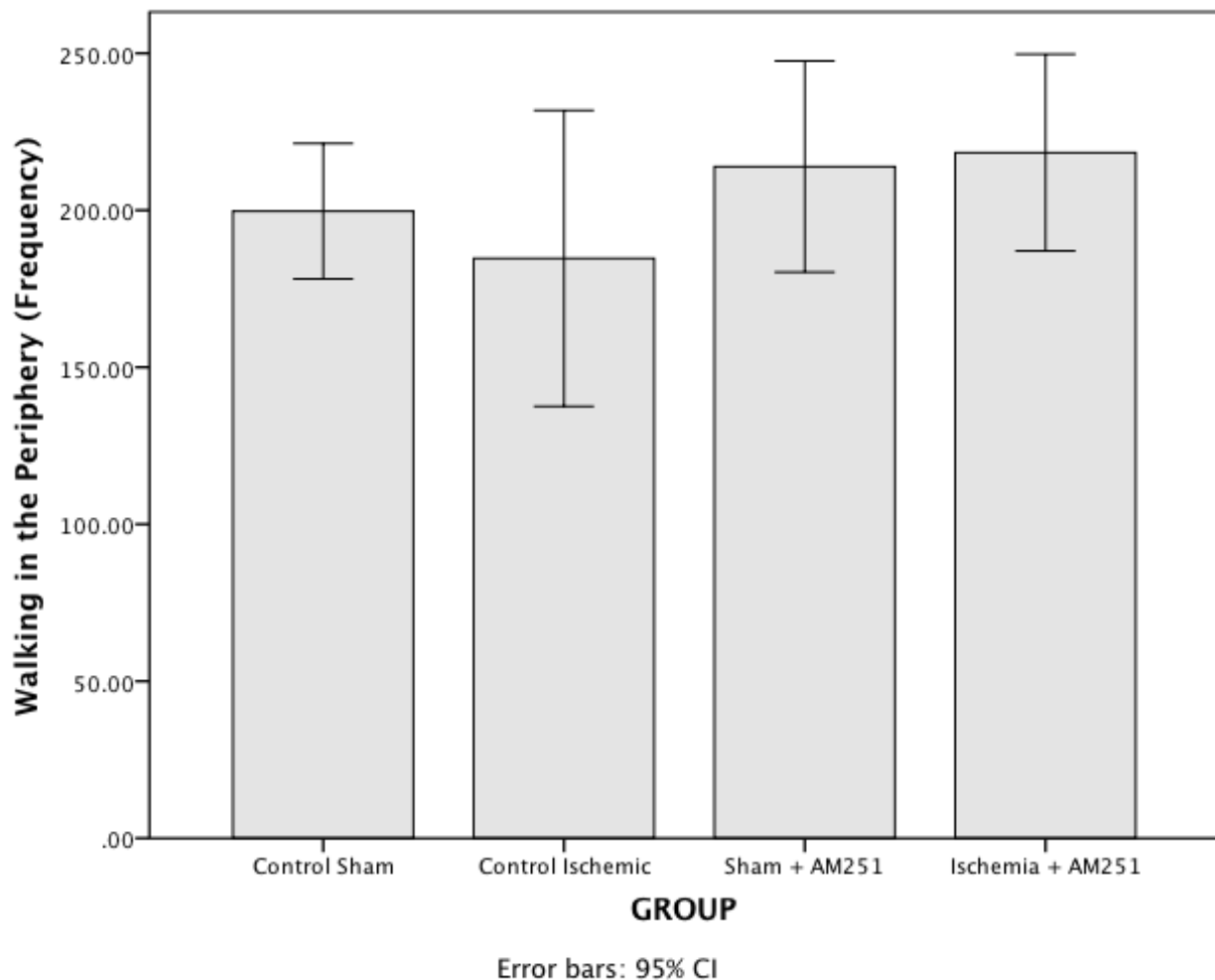


Figure 9. This graph illustrates the mean frequency of walking in the peripheral zone of the open field test in all four experimental groups. No significant differences were found ($p > 0.05$).

B. Immunohistochemistry

i. Ventral Tegmental Area

The amount of TH-IR was compared between all four experimental groups. Two-way ANOVA analyses revealed main effects of surgery [$F(1,36)=82.49$, $p=0.000$], drug [$F(1,36)=13.71$, $p<0.001$] and a surgery X drug interaction ($F(1,36)=14.15$, $p<0.001$). Simple effect tests indicated that this was due to increased TH-IR in vehicle- and drug-treated sham animals compared to the both ischemic rat groups ($p<0.000$). However, AM251 pretreated ischemic rats showed enhanced TH expression compared to vehicle-treated ischemic rats, supporting partial reversal of ischemia-induced TH-IR attenuation by the CB1 antagonist ($p<0.000$). Vehicle- and AM251-treated sham groups did not differ in VTA TH-IR ($p=0.967$). See figure figures 10 and 11.

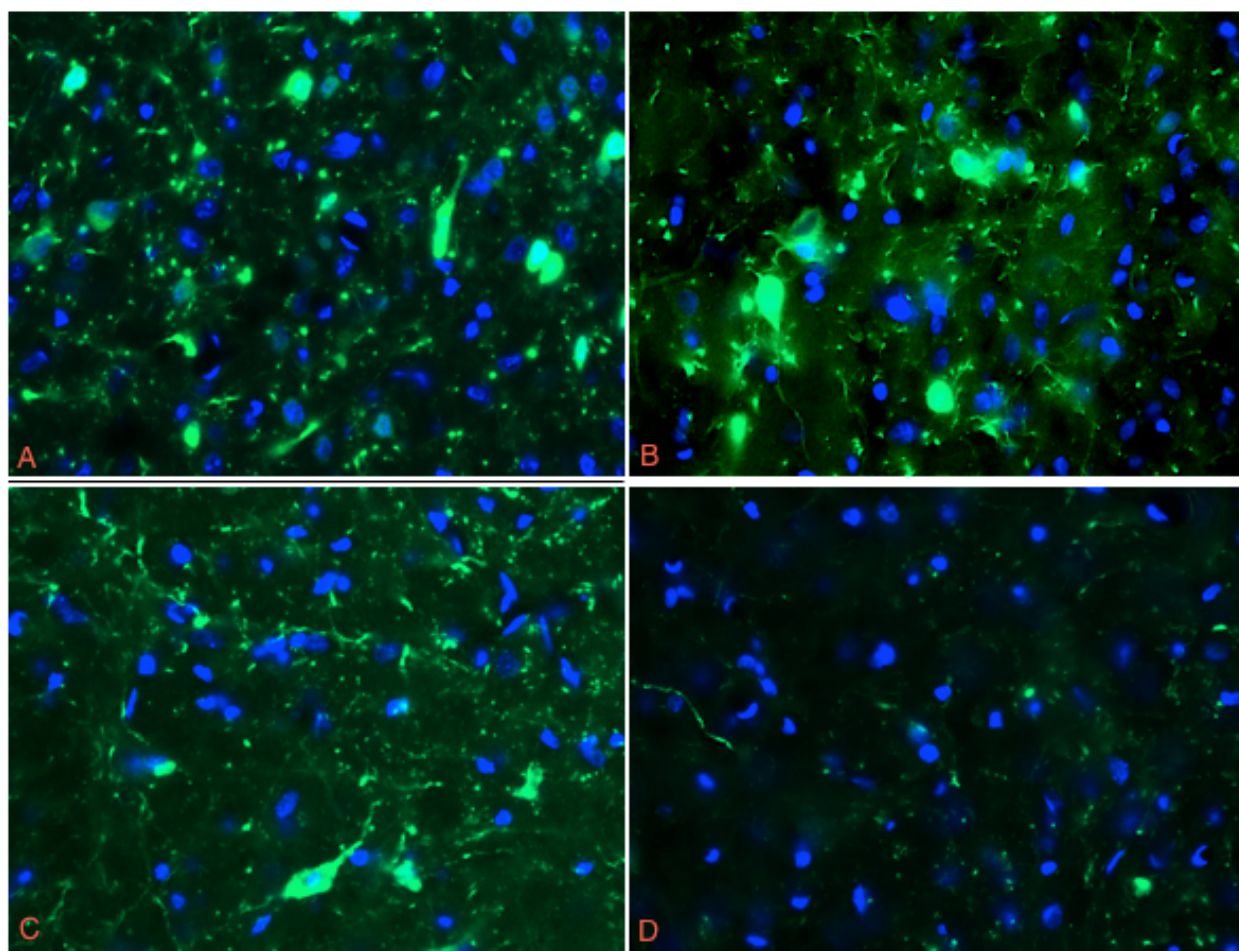


Figure 10. Representative photomicrographs of immunohistochemical staining of TH-IR in the Ventral Tegmental Area of (A) SC group (B) SA group (C) IA group and (D) IC group at 40x. AM251 administration led to improved TH-IR compared to vehicle-treated ischemic rats.

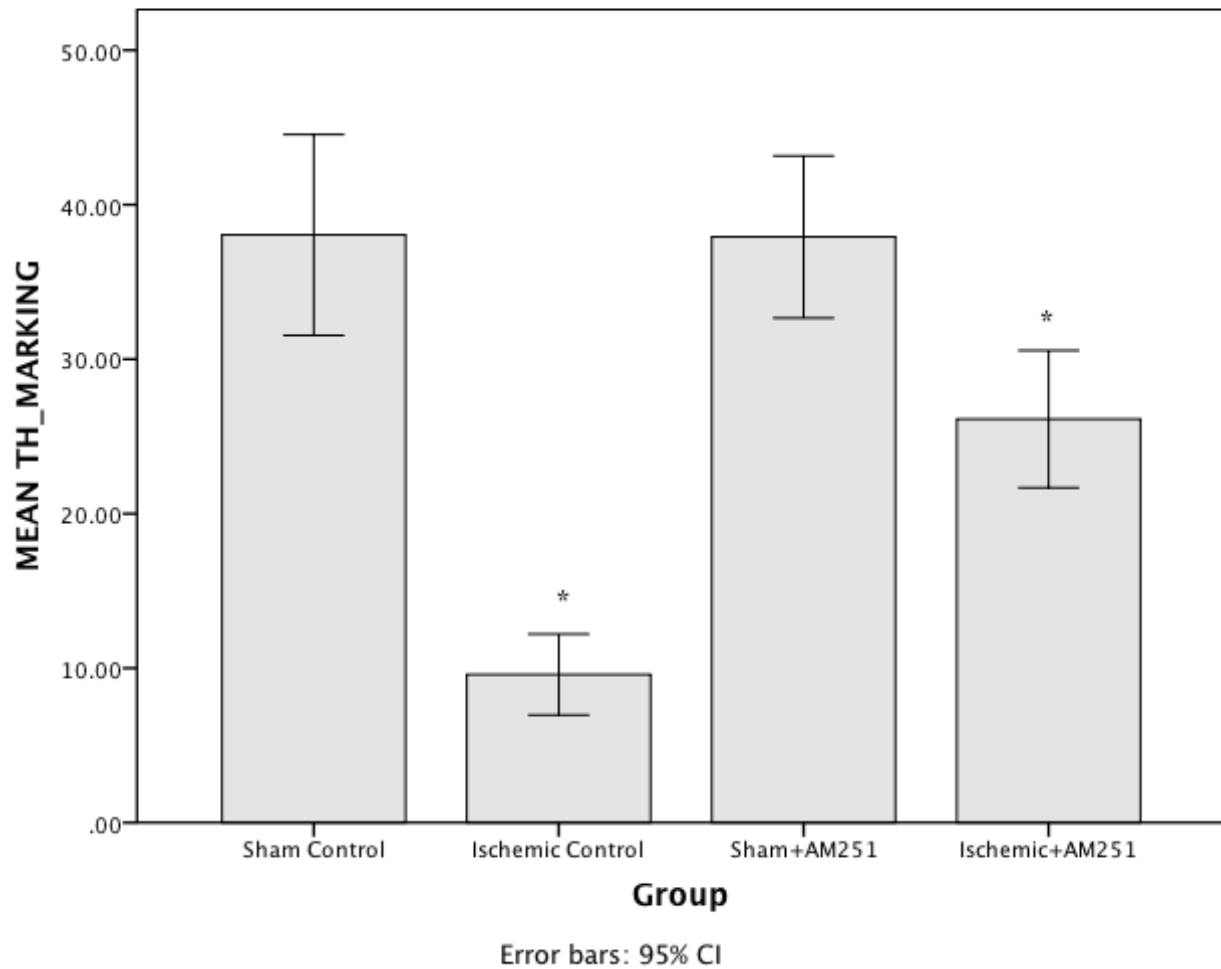


Figure 11. Histogram representation of the TH-ir optical density values obtained at the VTA for the different groups. * Indicates a significant reduction in TH-IR compared to the sham groups. The IC group had significantly less TH-IR than the SC, SA and IA rat groups ($p<0.00$). The IA had significantly less staining than the SC and SA but significantly more than the IC ($p<0.00$).

ii. *Nucleus Accumbens*

The amount of TH-IR was compared between the experimental groups in the shell portion of the Nucleus Accumbens. Two-way ANOVA analyses revealed a main effect of surgery [$F(1,35)= 104.74$ $p=0.000$], drug [$F(1,35)=42.65$, $p<0.000$] and a surgery X drug interaction ($F(1,35)= 51.58$, $p<0.000$). Simple effect tests indicated that this was attributable to vehicle-treated sham rats having increased TH-IR than the vehicle-treated ischemic rats ($p<0.000$).

AM251-treated ischemic rats also showed increased TH-IR compared to vehicle-treated ischemics ($p<0.000$). No significant difference was found between the AM251-treated sham and ischemic groups, using an adjusted alpha value of 0.0125 considering the amount of comparisons ($p=0.036$). Both sham groups showed comparable TH-IR ($p=0.652$). See figure figures 12 and 13.

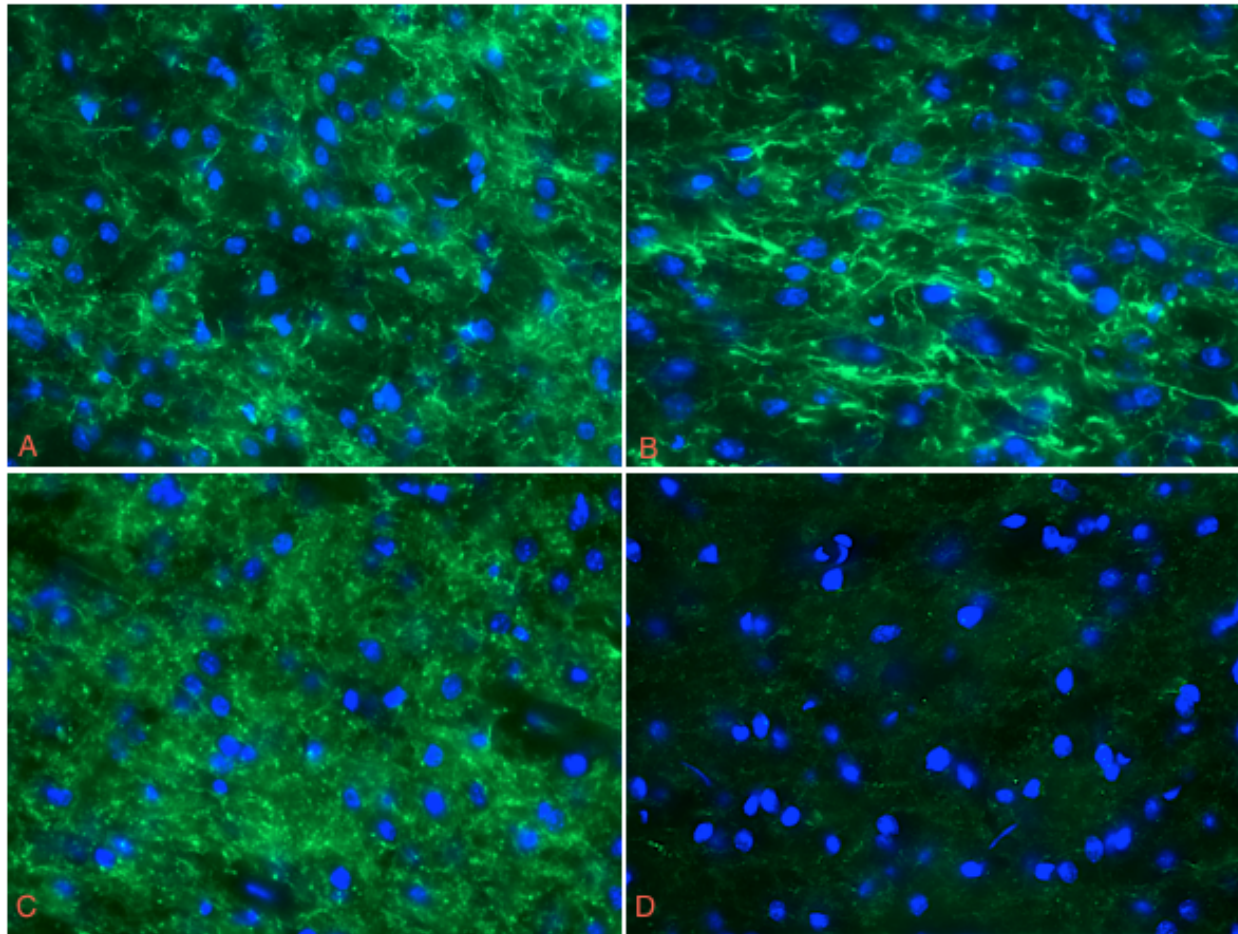


Figure 12. Representative photomicrographs of immunohistochemical staining of TH-IR in the Nucleus Accumbens shell for the (A) SC group (B) SA group (C) IA group and (D) IC group at 40x. AM251 treated ischemic rats showed increased TH-IR compared to vehicle-treated ischemic rats ($p<0.000$).

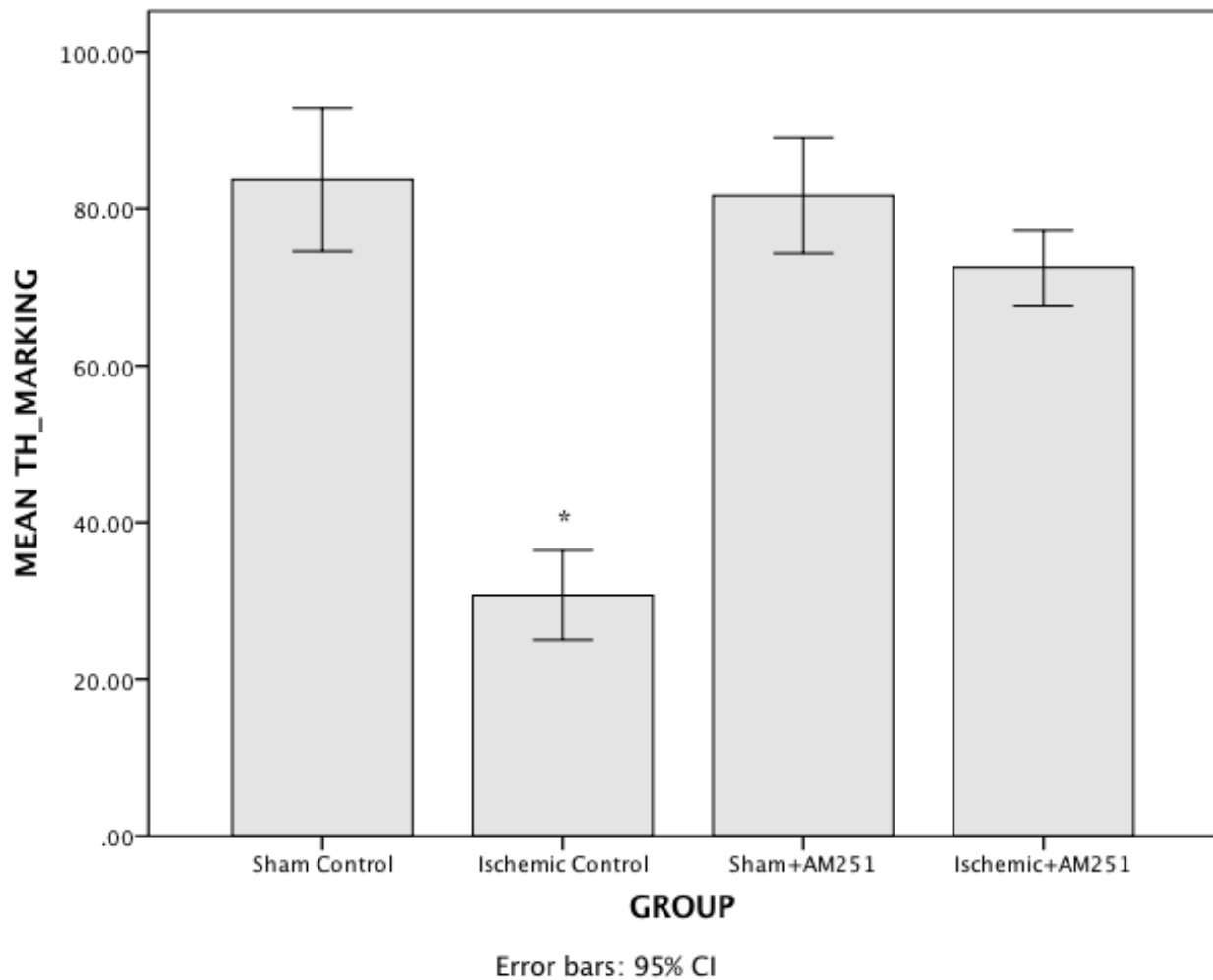


Figure 13. Histogram representation of the TH-ir optical density values measured at the Nucleus Accumbens shell for the different groups. The IC group had reduced TH-IR staining than all other groups, SC, SA and IA ($p < 0.00$). AM251 pretreatment reversed the ischemia induced reduction of TH expression ($p < 0.00$).

iii. CA3 of the Hippocampus

Two-way ANOVA analyses of TH-IR expression at the hippocampal CA3 revealed main effects of the surgery [$F(1,36) = 7.37$, $p = 0.01$], drug treatment [$F(1,36) = 77.56$, $p < 0.000$] as well as a surgery X drug interaction ($F(1,36) = 59.36$, $p < 0.000$). Simple effect tests indicated that sham controls had significantly more TH-IR than the ischemic controls ($p < 0.000$). Ischemia-induced TH reduction was prevented by AM251 treatment ($p < 0.000$), this animal group even showing

increased TH-IR compared to sham rats ($p=0.001$). Both sham groups showed comparable TH-immunostaining ($p=.440$). See figures 14 and 15.

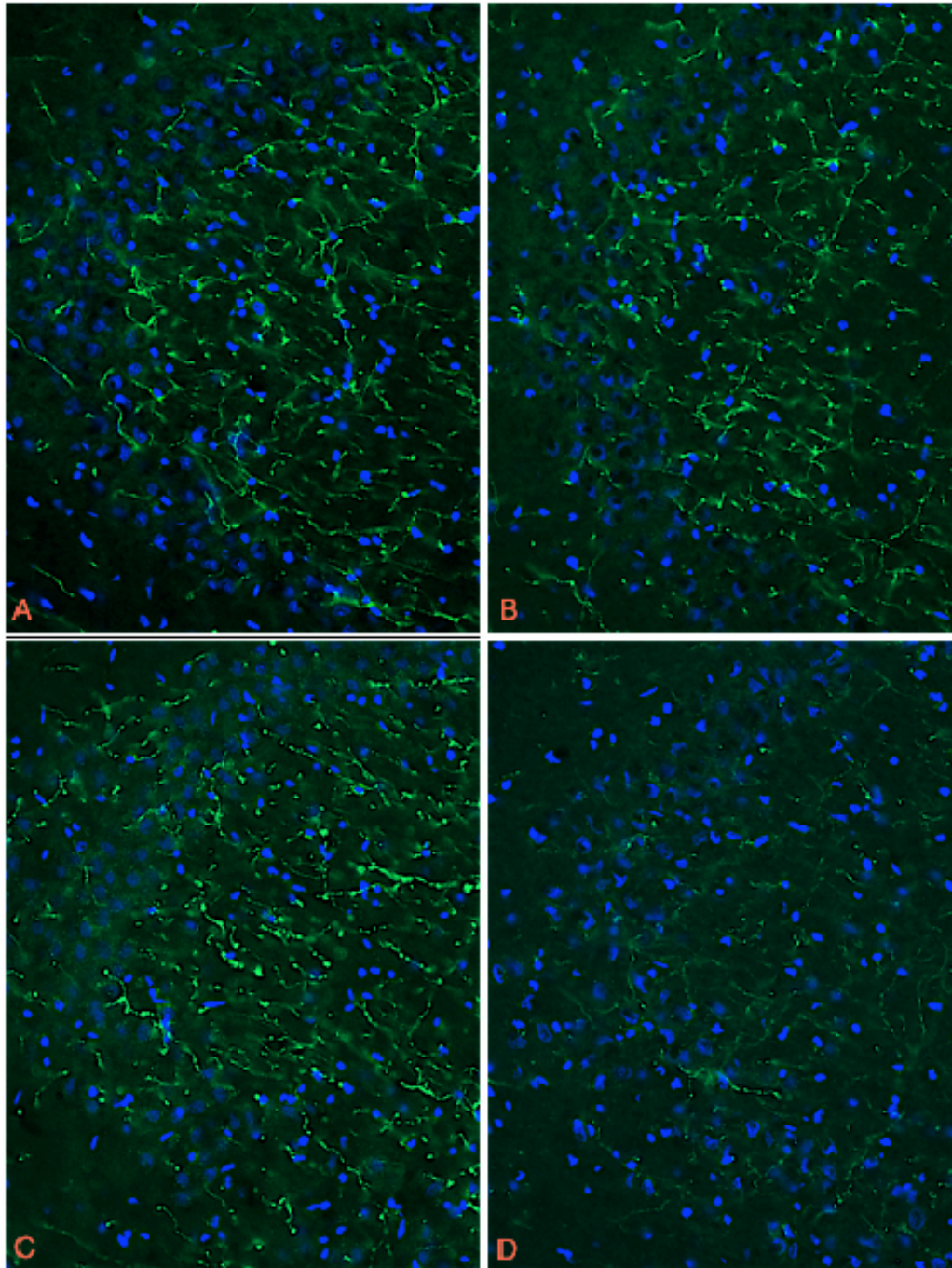


Figure 14. Representative photomicrographs of TH-IR in the CA3 pyramidal layer of the hippocampus for the different groups (A) SC (B) SA (C) IA and (D) IC at 20x magnification. AM251 treated ischemic rats showed increased TH-IR compared to ischemic animals treated with the vehicle ($p<0.000$).

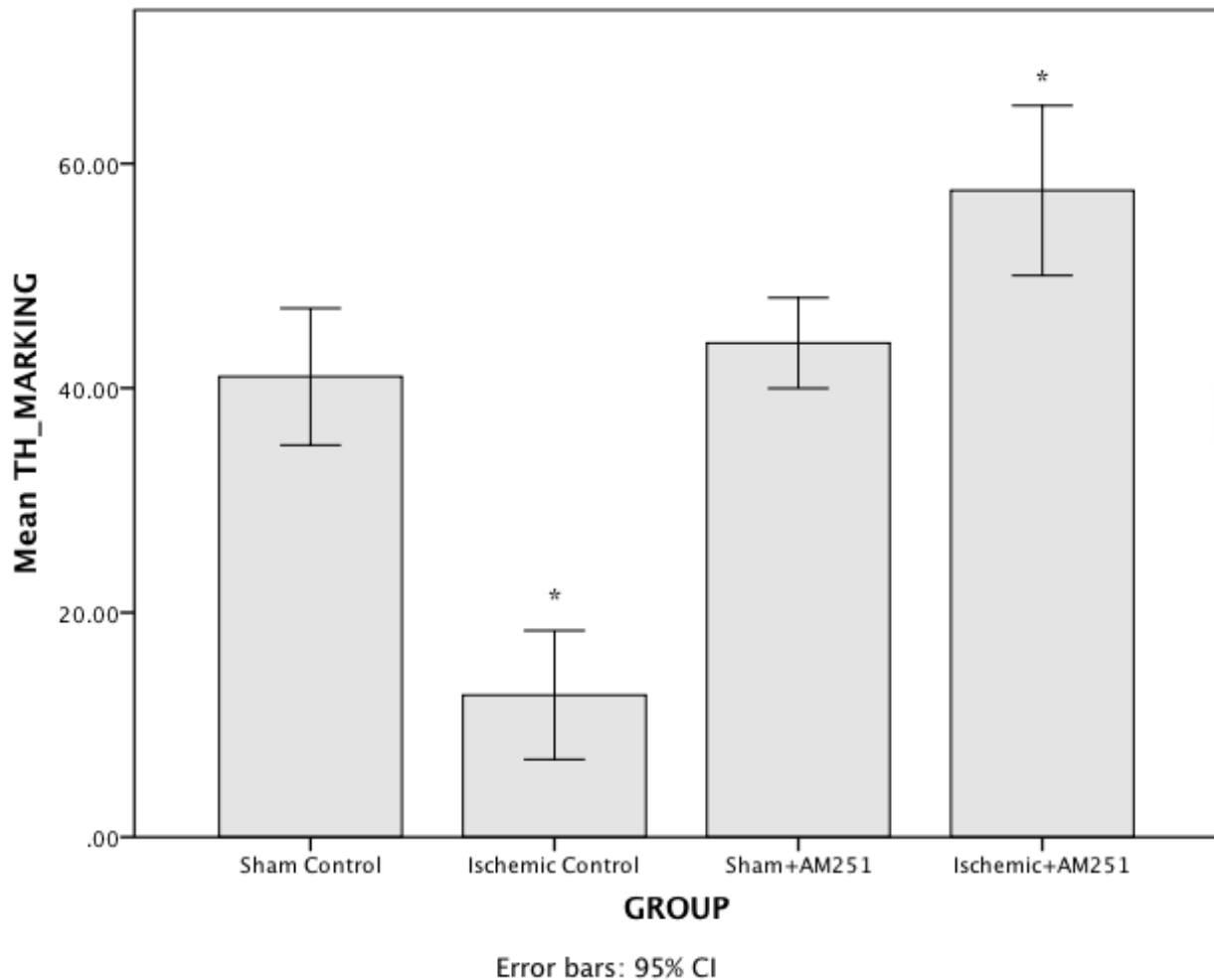


Figure 15. Histogram representation of the TH-ir optical density values measured at the CA3 of the Hippocampus. TH-IR was significantly reduced in vehicle-treated ischemic rats compared to all other groups ($p<0.000$). In contrast, AM251 treatment prior to ischemia prevented this reduction and was associated with a slight but significant increased in TH expression compared to all other groups ($p<0.001$).

iv. CA1 of the Hippocampus

The amount of TH-IR was compared between all four experimental groups in the CA1 of the hippocampus. Two-way ANOVA analyses revealed a main effect of the surgery [$F(1,36)=20.63$, $p<0.000$], drug treatment [$F(1,36)=33.22$, $p<0.000$] and a surgery X drug interaction ($F(1,36)=28.92$, $p<0.000$). Simple effect tests indicated that sham rats and AM251-treated sham

and ischemic rats were not different ($p=0.574$) and had significantly more TH-IR than the ischemic controls ($p<0.000$). Sham-operated groups showed comparable TH-IR ($p=.743$) (See figure 16 and 17).

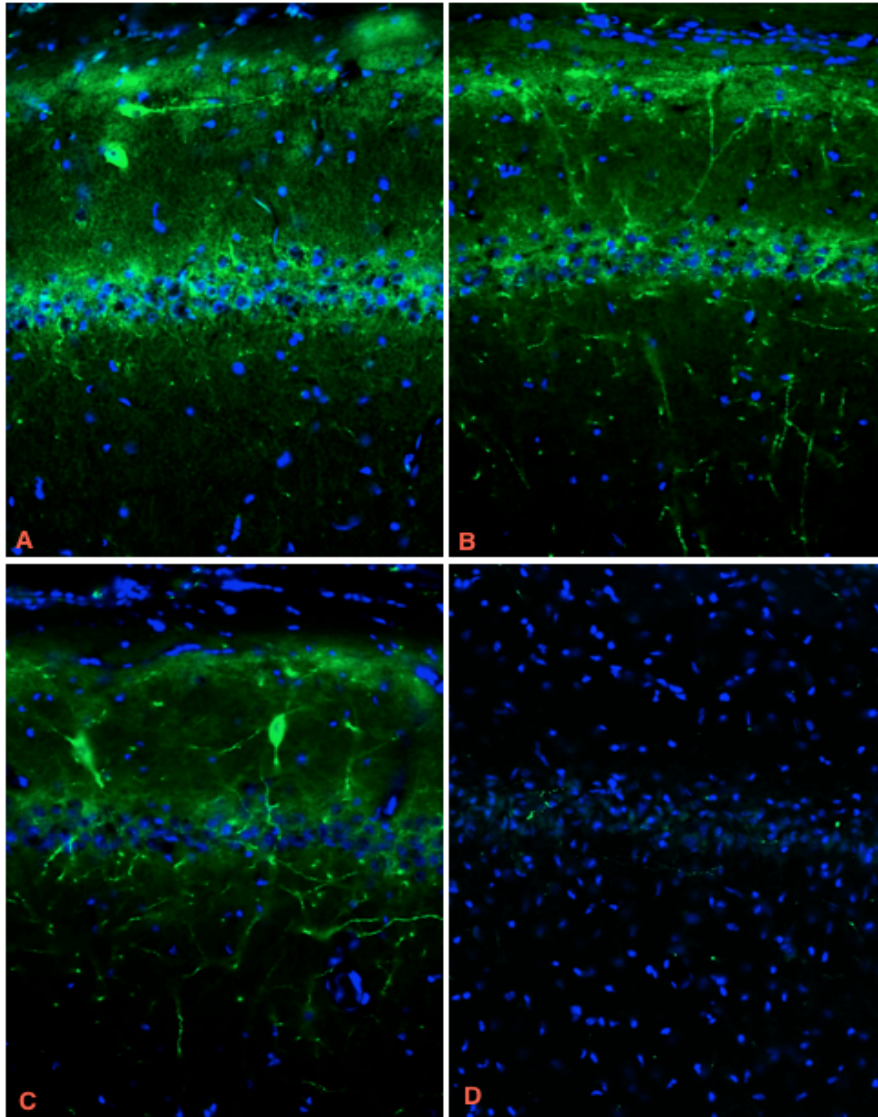


Figure 16. Representative photomicrographs of immunohistochemical staining of TH-IR in the CA1 of the Hippocampus for the (A) SC group (B) SA group (C) IA group and (D) IC group at 20x magnification. AM251-treated ischemics showed increased TH-IR compared to vehicle-treated ischemic rats ($p<0.000$).

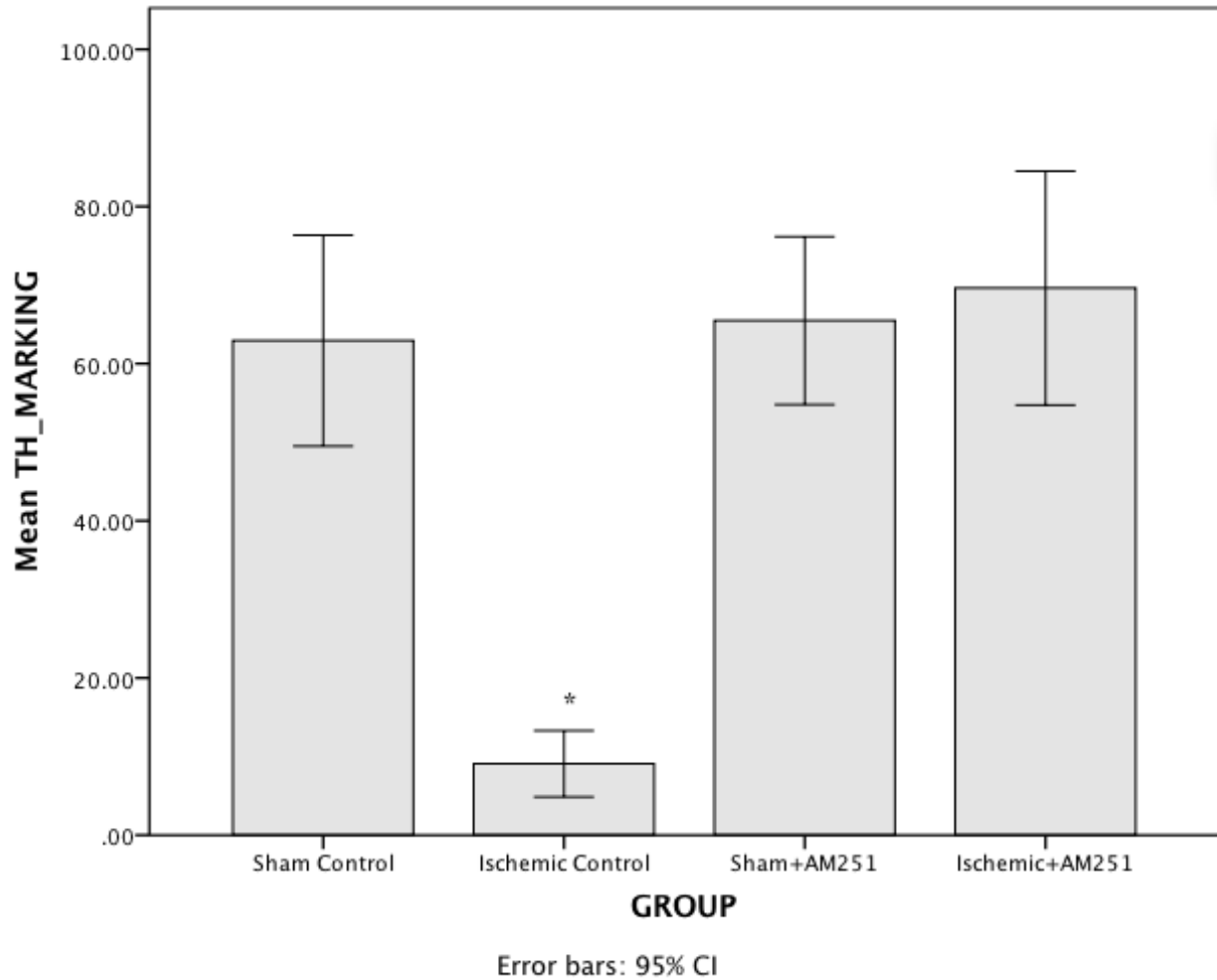


Figure 17. Histogram representation of TH-IR optical density values measured at in the CA1 of the Hippocampus for each experimental group. The IC group had reduced TH-IR than all other groups ($p < 0.000$).

v. A11 Dopaminergic Neurons

TH-IR was assessed in the A11 dopaminergic neurons of the hypothalamus. Two-way ANOVAs revealed a significant main effect of the surgery [$F(1,36) = 55.08$, $p = 0.000$] but no drug effect [$F(1,36) = .74$, $p = 0.396$] or surgery X drug interaction ($F(1,36) = 1.29$, $p = 0.245$). See figure 18 and 19.

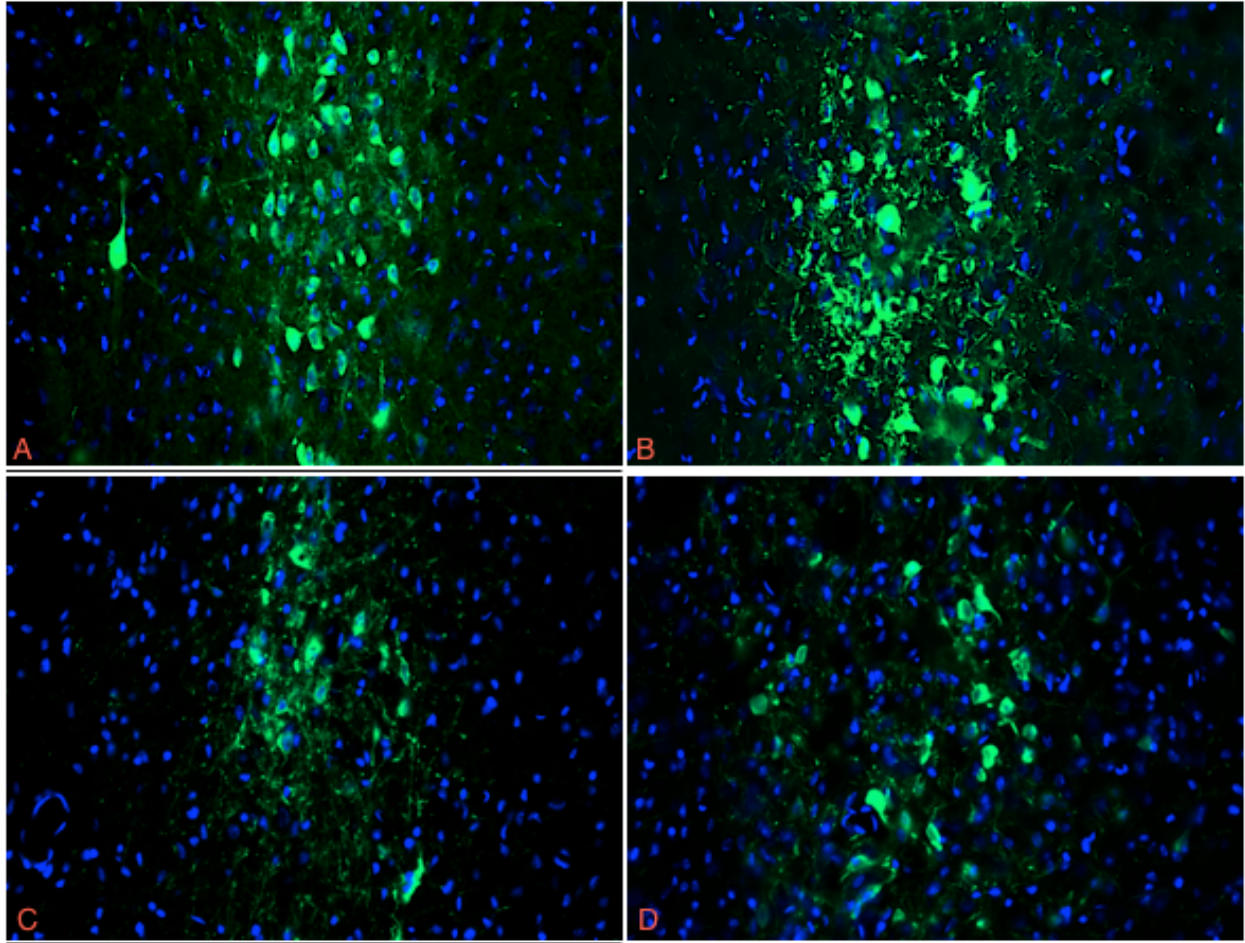


Figure 18. Representative photomicrographs of TH-IR staining in the A11 dopamine neurons of the hypothalamus (A) SC group (B) SA group (C) IA group and (D) IC group at 20x magnification. Both ischemic groups (IA & IC) had significantly less TH-IR than the sham operated animals (SC & SA) ($p < 0.000$). There was no effect of the drug ($p = 0.396$) at altering TH-IR.

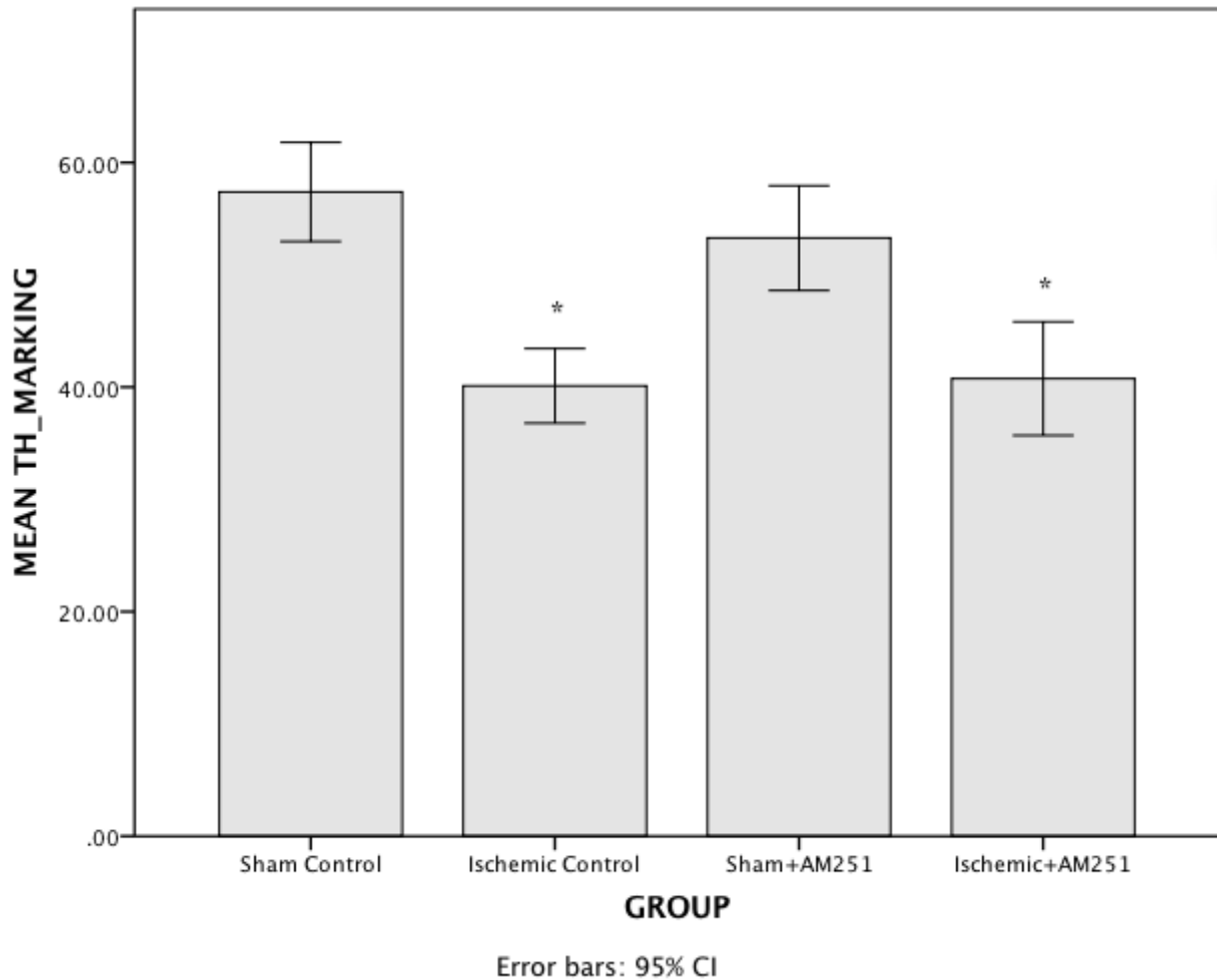


Figure 19. Histogram representation of the TH-IR optical density values measured in the A11 dopamine neurons of the hypothalamus for each experimental group. Both ischemic groups had significantly less TH-IR than the sham-operated animals ($p < 0.000$).

vi. Basolateral Nucleus of the Amygdala (BLA)

Two-way ANOVA analyses of the BLA revealed significant a main effect of the surgery [$F(1,34) = 64.49, p = 0.000$], drug treatment [$F(1,34) = 9.94, p = 0.004$] and a surgery X drug interaction between the [$F(1,34) = 15.17, p < 0.000$]. Simple effect tests indicated increased TH-IR in both sham control rats compared to ischemic groups ($p \leq 0.005$). Additionally, TH-IR was increased in the AM251-treated ischemic rats compared to vehicle-treated ischemics ($p < 0.000$).

Both sham groups showed comparable TH-IR ($p=577$). See figure 20 and 21.

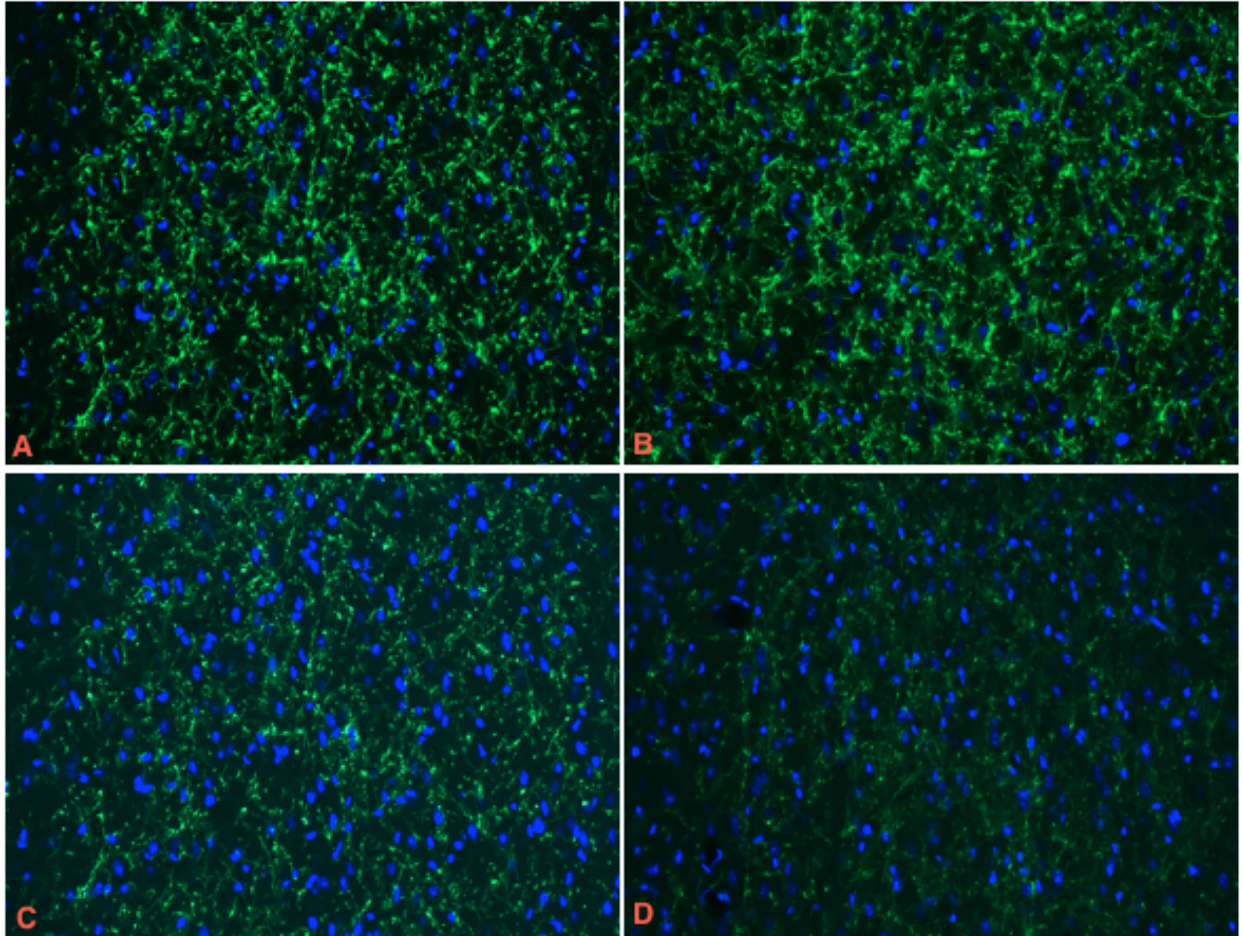


Figure 20. Representative photomicrographs of TH-IR in the basolateral amygdala. (A) SC group (B) SA group (C) IA group and (D) IC group at 20x magnification. Increased TH-IR was observed in sham-operated groups compared to ischemic counterparts ($p<0.000$). Additionally, AM251 pretreatment partially blocked ischemia-induced TH-IR depletion.

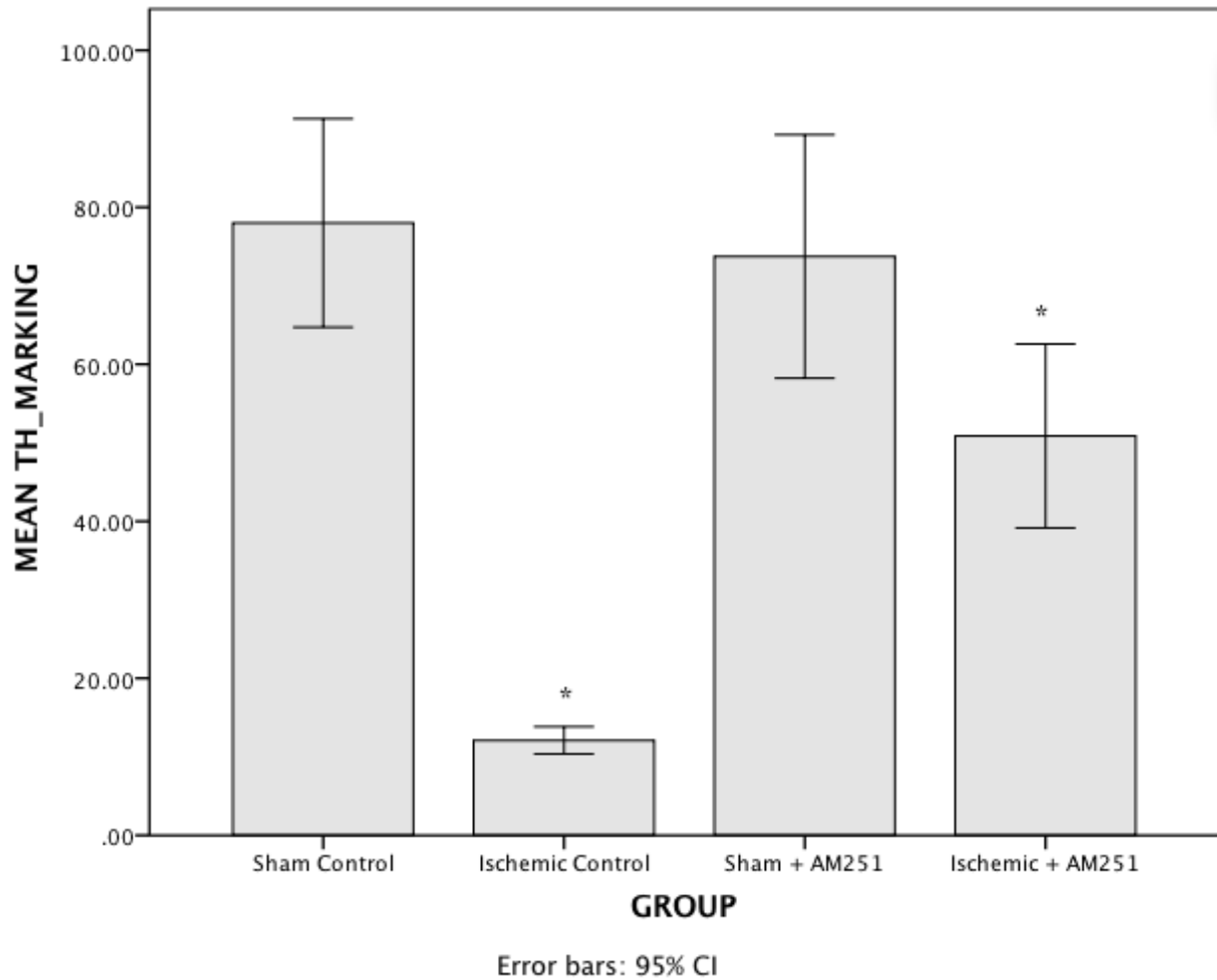


Figure 21. Histogram representation of TH-IR at the basolateral amygdala. Control ischemic rats showed reduced TH-IR compared to all experimental groups ($p < 0.000$), a reduction that was partially prevented by AM251 treatment prior to ischemia.

vii. Medial Forebrain Bundle

Two-way ANOVA analyses assessing DRD1-IR in the medial forebrain bundle revealed main effects of the surgery [$F(1,35) = 44.44$, $p = 0.000$], drug treatment [$F(1,35) = 75.42$, $p = 0.000$] and a surgery X drug interaction [$F(1,34) = 62.62$, $p < 0.000$]. Simple effect tests indicated that sham control rats had significantly more DRD1-IR than the ischemic controls ($p < 0.000$). However, there was no difference in DRD1-IR between AM251-treated sham and ischemic rats

($p=0.585$). AM251 treatment prevented ischemia-induced decrease in DRD1-IR expression ($p<0.000$). Sham-operated groups did not differ ($p=0.620$). See figure 22 and 23.

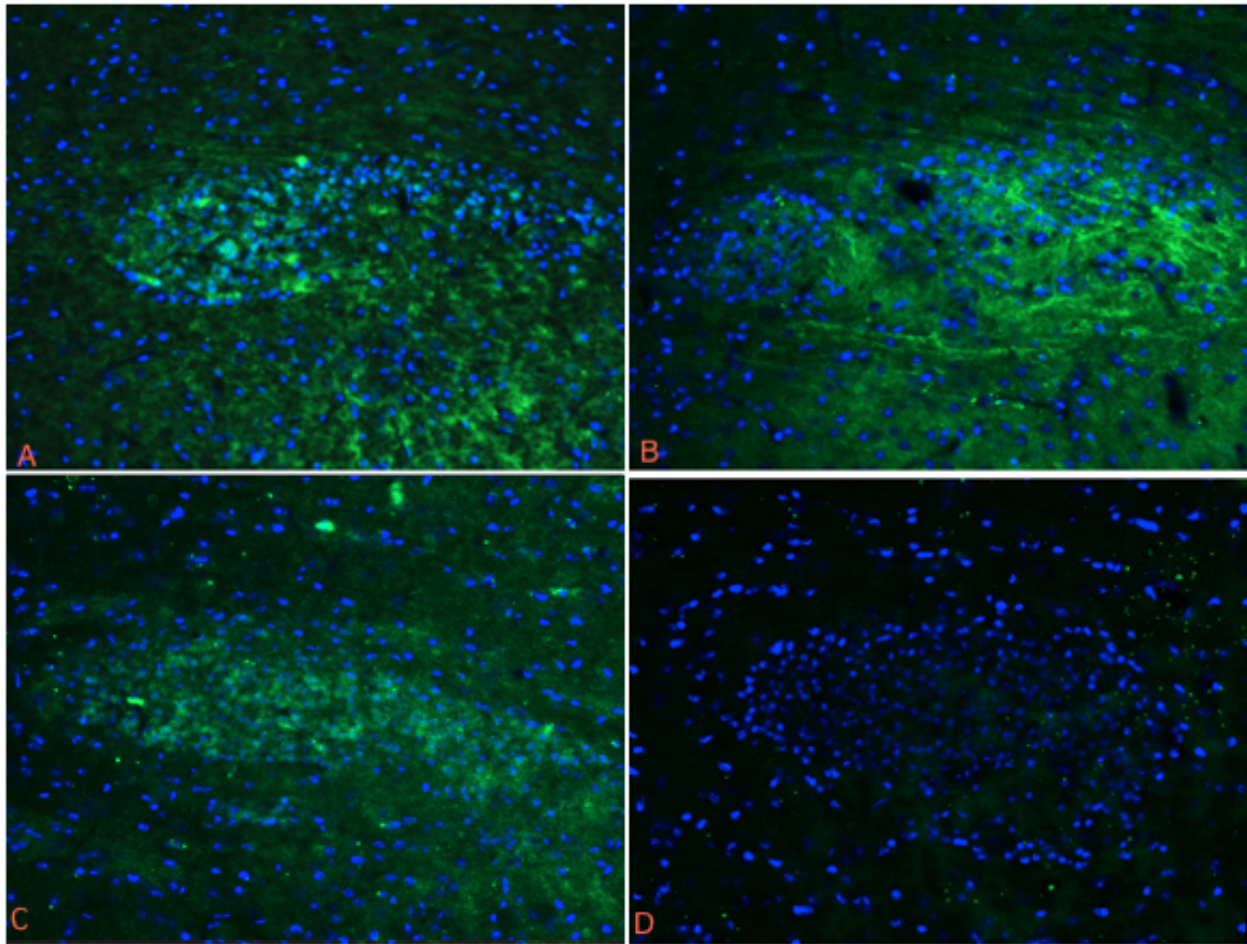


Figure 22. Representative photomicrographs of DRD1-IR staining in the medial forebrain bundle (A) SC group (B) SA group (C) IA group and (D) IC group at 20x magnification. The sham controls and shams treated with AM251 had significantly more DRD1 expression than the ischemic counterparts ($p<0.000$). Additionally the AM251-treated sham and ischemic rats showed comparable DRD1-IR ($p=0.585$), which was increased compared to that of vehicle treated ischemic rats ($p<0.000$).

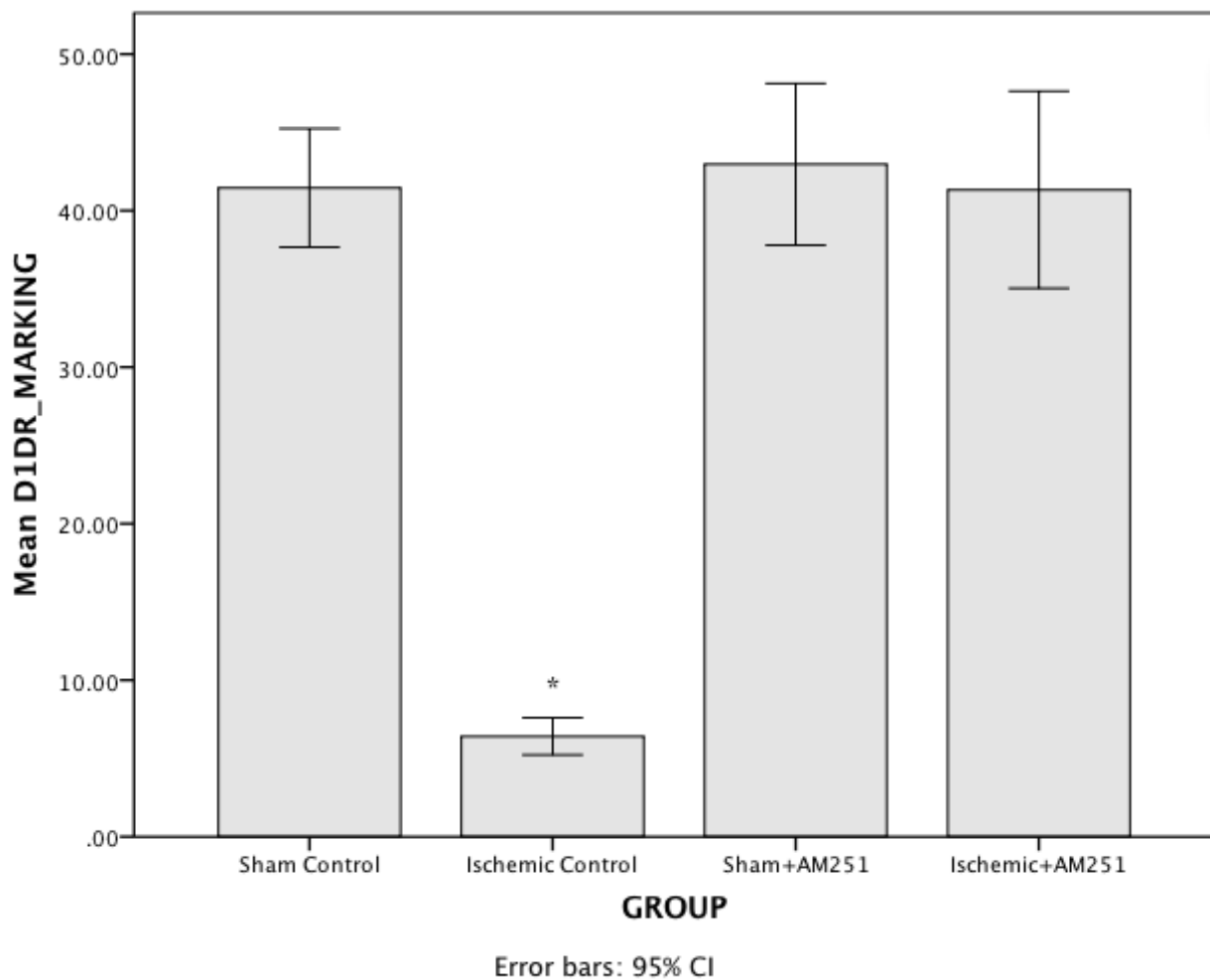


Figure 23. Histogram representation of DRD1-IR expression in the medial forebrain bundle. Reduced D1DR-IR post ischemia was prevented by AM251 treatment. Vehicle-treated ischemic rats had significantly less DRD1-IR than all other groups ($p<0.000$).

C. Neuronal Survival

i. CA1 of the Hippocampus

Neuronal damage in the CA1 pyramidal layer of the hippocampus was compared between the groups using a two-way ANOVA analysis. Main effects of surgery [$F(1,35)=36.35$, $p<0.000$], drug [$F(1,35)=10.24$, $p=0.003$] and a surgery X drug interaction [$F(1,35)=27.44$, $p<0.000$] were revealed. Simple effect tests indicated significantly increased CA1 neuronal injury in ischemic

compared to sham controls ($p<0.000$). Additionally, the AM251-treated ischemics showed reduced CA1 neuronal damage compared to vehicle-treated ischemic rats ($p<0.000$) and did not differ from AM251-treated sham rats ($p=0.574$). Both sham groups had comparable CA1 neuronal density ($p=0.153$). See figure 24 and 25.

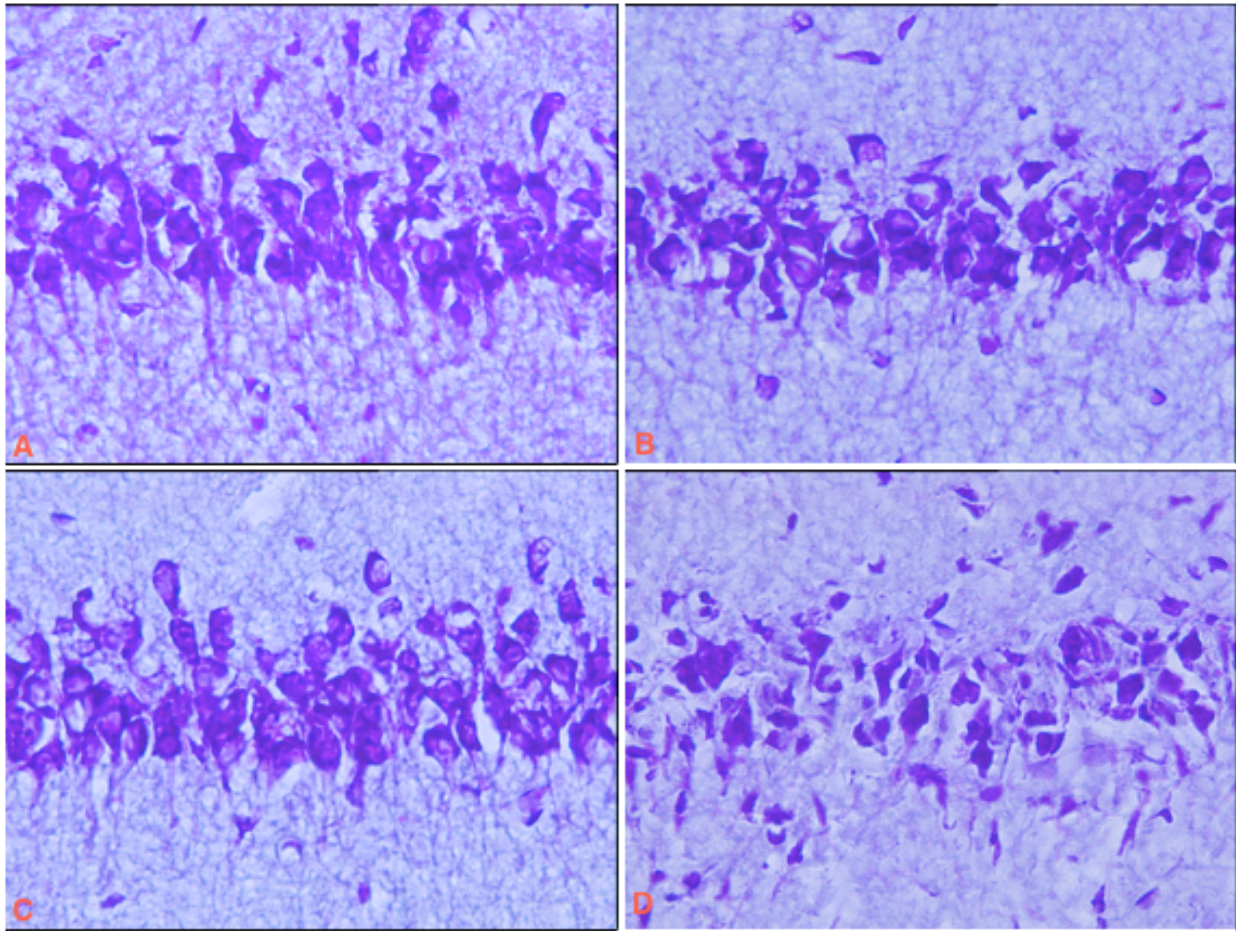


Figure 24. Representational photomicrographs of neuronal density in the CA1 of the Hippocampus. (A) SC group (B) SA group (C) IA group and (D) IC group at 20x magnification. The IC group had significantly reduced viable neurons when compared to all other groups ($p<0.000$).

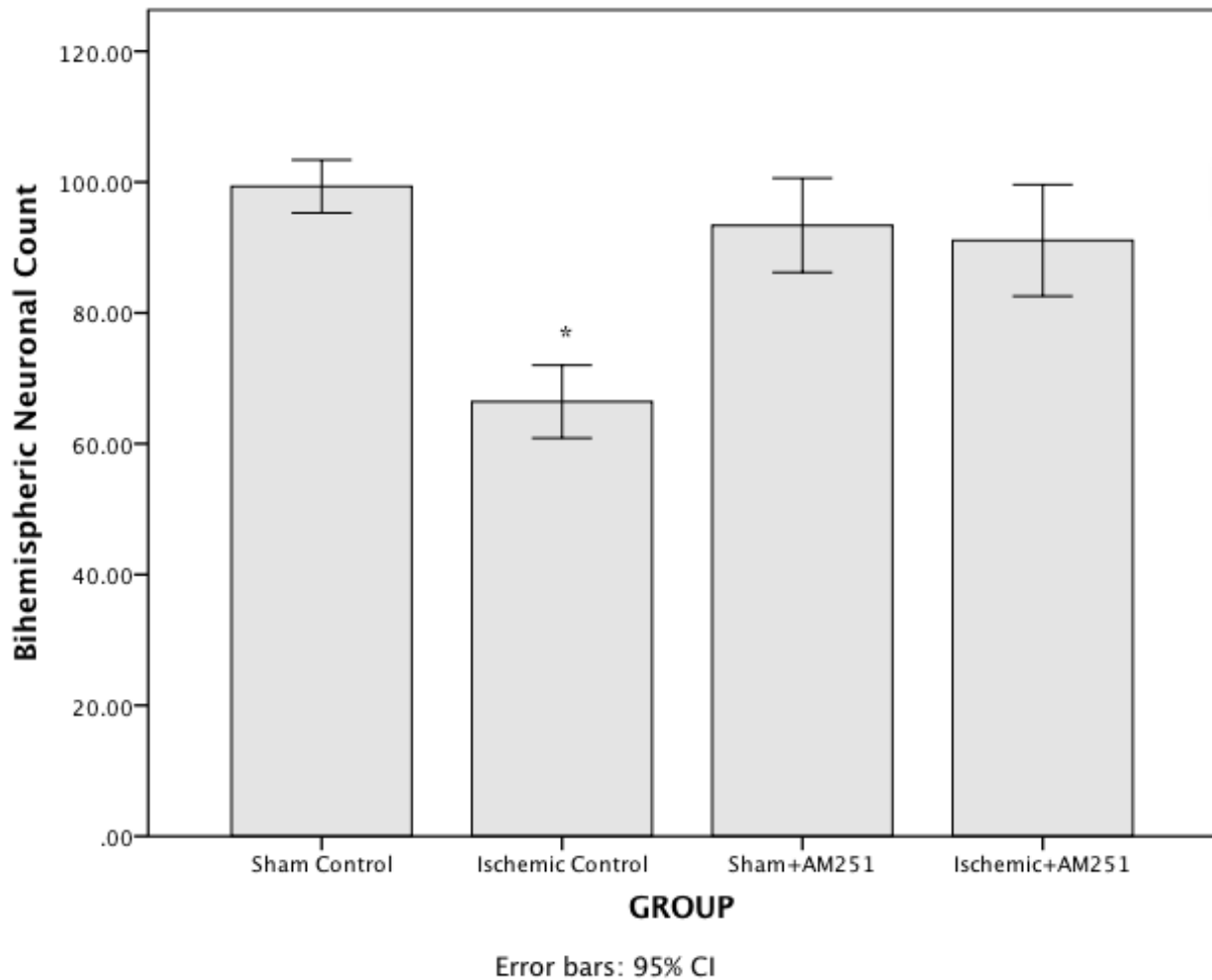


Figure 25. Histogram representation of CA1 neuronal density. The IC group showed increased neuronal death compared to all other groups, SC, SA and IA ($p < 0.000$).

ii. CA3 of the Hippocampus

Two way ANOVA analyses of CA3 neuronal injury revealed no main effects of the surgery [$F(1,36)=0.005$, $p=0.944$), drug treatment [$F(1,36)=1.75$, $p=0.194$] and no surgery X drug interaction [$F(1,36)=0.510$, $p=0.48$]. (See Appendix figure v)

IV. Discussion

The current study has evaluated the action of AM251 administration on ensuing physiological responses following an ischemic insult, as well as delayed impact on behaviour. To our knowledge, this is the first study to test the effects of acute administration of AM251 prior to global cerebral ischemia on neurochemical alterations in various brain regions including motivational pathways, as well as regulating effects anxiety-like behaviour in the elevated plus maze and open field test.

A. AM251 regulation of anxiety-like behavioural responses following ischemia.

Overall our findings support reduced anxiety-like behaviour in ischemic controls which spent more time in the center zone of the open field and open arm of the elevated plus maze. The observations are consistent with different studies showing that ischemia induces behavioural changes in emotional reactivity (Bueters et al, 2008; Milot & Plamondon 2011; Milot & Plamondon, 2009; Nunn & Hodges, 1994; Kronenberg et al, 2012; de la Tremblaye and Plamondon, 2011, Plamondon and Khan, 2005). It was consistent with temporal patterns showing augmented anxiety-behaviour following ischemia, starting with increased anxiety up to 48 hours following ischemia and then in a compensatory fashion decreased HPA and anxiety up to day 7 (Bueters et al 2008; Milot & Plamondon, 2011). Considering the effects of endocannabinoids to regulate glutamate release and modulate physiological and behavioural responses following different stressors, the effect of the CB1 endocannabinoid antagonist AM251 on these behavioural changes was evaluated (Hill et al, 2010).

Of most interest, the treatment with AM251 prior to global ischemia prevented ischemia-induced effects on anxiety-like behaviour in the elevated plus maze, as measured by time spent in the open arm and number of entries into the open arm. AM251 pretreatment led to decreased time spent in the open arm and entries bringing them back to comparable levels as those observed in vehicle- and AM251-treated sham rats. It appears that in the current study, AM251 acted to regulate altered anxiety level following ischemia bringing this response closer to that observed in sham animals. Such action would be consistent with data showing that treatment with AM251 increased plasma corticosterone levels (Kupferschmidt et al, 2012). AM251, as well as other endocannabinoid antagonists, has also been shown effective in reducing cognitive impairments as well as improving learning and memory (Mallet & Beninger, 1998; Terranova et al, 1998; Thiemamm et al, 2008; Bialuk and Winnicka, 2011). It is speculated that the action of AM251 is regulatory in function and brings physiological function to basal levels. In the present study, it would appear that when AM251 is given prior to ischemic insult it protects against induced behavioural deficits. In the upcoming paragraphs, we will address possible endogenous mechanisms involved in such effects. It also should be noted that the time of administration of AM251 and behavioural testing is five days apart in our study, making direct comparisons with other studies testing AM251 effects on behavioural responses minutes following its administration more difficult but allowing to study lasting impact of a single drug treatment.

Consistent with observation in the EPM, AM251-treated ischemic rats showed reduced time spent in the center zone of the open field compared to vehicle-treated ischemic animals. However, this effect appeared partial as level of center field exploration of AM251-treated

ischemic rats remained elevated compared to that of vehicle- and AM-251-treated sham rats. Interestingly, ischemic control rats spent more time grooming in the OFT than all other groups. Grooming is often interpreted as an indirect effect of sympathetic nervous system activation (Moody and Merali, 2004). Concomitant decreased anxiety and increased grooming might appear difficult to reconcile. However, different endogenous changes might mediate these behavioural responses post ischemia and help reconcile these observations. For example we and others have demonstrated reduced CRH expression in the central nucleus of the amygdala as well as reduced neuronal survival in the basolateral nucleus of the amygdala following global ischemia (McCarthy et al., 2009; de la Tremblaye et al., 2011), which could lead to reduced anxiety in these animals. In contrast, ischemia is known to lead to heightened HPA reactivity and increased CRH and noradrenergic expression or release lasting for weeks post ischemia, which could induce increased grooming post ischemia (Khan et al, 2004; Stevens et al, 2003; Weidenfeld et al, 2011). Either of these processes might have been prevented by AM251 treatment prior to ischemia although this study cannot confirm this. Nonetheless, considering improved TH-ir and DRD1-ir in AM251 treated rats compared to vehicle-treated ischemic rats, neurochemical alterations combined with cell death could mediate such alterations. Our data provides some insight into the effect of ischemia on the brain and the possible preventive therapeutic action of AM251. Stroke and cardiac arrest are associated with a number of behavioural changes, and the use of AM251 provide of anxiety-like behaviour through dopamine regulation, as will be discussed below, and may provide new insight on post-stroke depression (Kronenberg et al, 2012).

2. AM251 regularized TH and DRD1 expression post ischemia.

Tyrosine hydroxylase expression was evaluated as a means of determining changes in dopamine transmission within various regions of the brain. Although TH is the rate-limiting enzyme of all catecholamines and in this respect can be considered a less selective marker, it is widely used to measure dopamine function in brain pathways involved in reward where it is the main expressed catecholamine (Agrawal et al, 2012; Choi et al, 2012; Ikemoto et al, 2002; Li et al, 2012; Martine et al, 2005). Our findings indicate a significantly blunted TH-ir expression within the VTA, seven days following ischemia. This is consistent with declined tyrosine hydroxylation observed following ischemia (Takagi et al, 1995).

The Ventral Tegmental Area is believed to be the point of entry and gatekeeper of dopaminergic activity within the mesolimbic circuit (Melis, Muntoni & Pistis; 2012). This area is particularly vulnerable during ischemia because the large amount of glutamate to dopamine synapses found within the VTA. Stimulation of excitatory glutamate afferents that project directly to the VTA during excitotoxicity makes this area particularly susceptible (Lui et al, 2010). Interestingly, cannabinoids and endocannabinoids have shown direct action on these synapses, creating potent glutamate signaling originating from the cerebral cortex to the VTA (Liu et al, 2010; Nugent et al, 2008). These connections are mainly implicated in synaptic augmentation, like LTP and LTD, however during ischemia can result in an excitotoxic cascade particularly targeting this area (Liu et al, 2010). This cortical connection to the VTA in particular may help explain why we observed a pronounced reduction in dopamine function in this area following ischemia.

The VTA appears to be an area that is susceptible to ischemia which is most likely a result of the afferent glutamate neurons which synapse with dopamine neurons directly in this area (Liu et al, 2010; Nugent et al, 2008). As the start of the mesolimbic circuit, the VTA dopaminergic neurons project directly to the nucleus accumbens shell (Everitt & Robbins, 2005; Pralong et al, 2002). The nucleus accumbens shell dopamine projections in particular are responsible for stimulant reinforcement in the reward pathway (Everitt & Robbins, 2005). The VTA also sends projections to the hippocampus, important for context memories, and which then progress to the basolateral amygdala, which is important for conditioned emotional responses (Everitt & Robbins, 2005). We found significant reduction in TH expression throughout the reward system, namely the nucleus accumbens, dorsal hippocampus, and basolateral amygdala. Blunted TH-IR in these areas fits the idea that cerebral ischemia causes excitotoxicity from excess glutamate afferents which project directly from the VTA all the way to the basolateral amygdala.

In a similar fashion, DRD1 receptor immunoreactivity was significantly reduced within the medial forebrain bundle one week following 10 min global ischemia. Notwithstanding widespread TH-ir reduction including in the A11 dopamine neurons of the hypothalamus post ischemia, AM251 failed to prevent such reduction in this particular region, suggesting that changes in this brain locus are not mediated by eCBs. From these observations, it appears that dopamine function is altered in various loci of the brain at a delayed interval post ischemia. However, transmission appeared more substantially diminished in structures associated to the reward/motivational pathway in our study. Together these observations nicely corroborate studies showing that dopamine function is dramatically reduced following ischemia (Benfenati et al,

1989; Li et al, 2009; Martin et al, 2012; Takagi et al, 1995; Zhang et al, 2008). One of the proposed mechanisms behind this phenomenon relates to excitotoxic events that occur during ischemia, to which excess dopamine release is proposed to contribute, and which result in cell death in surrounding cells, in particular dopaminergic cells (Globus et al, 1987; Martin et al, 2012; Ruscher, Kuric & Wielock, 2012; Zhang et al, 2008). This is supported by studies showing that substantia nigra lesion protect against ischemic damage in the striatum (Globus et al., 1987) and levodopa treatment improves functional recovery following stroke (Rusher et al, 2012).

The current study is the first to evaluate the effect of the CB1 endocannabinoid antagonist AM251 on dopamine function when administered immediately prior global cerebral ischemia. Of most interest, we were able to detect persisting changes in the brain resulting from blockade of CB1 endocannabinoid receptors before induction of a potent physiological stressor. Within the ventral tegmental area, ischemic rats treated with AM251 showed partial recovery of TH-IR expression compared to ischemic controls, levels remaining significantly different in this group compared to that observed in either of the sham-operated groups. The effect appeared greater in the shell portion of the nucleus accumbens, where AM251 prevented ischemia-induced reduced dopamine expression. Interestingly however, we observed that in this region, TH-ir labeling appeared slightly different in AM251-treated ischemic rats compared to that observed in sham animals. For example, immunostaining observed in sham rats appears to not only label cells but also axonal connections giving a meshed like appearance to the staining, a phenomenon observed with TH immunostaining. However, the IA group's labeling presented as slightly more diffuse staining most centered on cell bodies (Figure 11). Additional techniques including in vivo

voltammetry could be used to assess the functional relevance of these differences on actual recovery of dopamine transmission in this brain region.

Within the A11 dopaminergic cells of the hypothalamus global ischemia reduced TH-IR although in this region, AM251 failed to alter this effect. The diencephalic A11 dopamine neurons is an area implicated in sensorimotor processing and pain control within the spinal cord, associated with restless leg syndrome and migraine headaches (Barraud et al, 2010). Moreover, the A11 area has been shown to largely express dopaminergic D2 receptors, with minimal D1 receptors, and are not part of the dopaminergic reward pathway (Barraud et al, 2010). Differential effect of the AM251 treatment in this brain region support specific effect of AM251 to prevent dopaminergic dysfunction following global ischemia, possibly mainly involving brain regions associated with the mesolimbic pathway. This can be supported by the fact that there is a large amount of glutamatergic-dopamine synapses found in this area, which are in particular sensitive to eCBs and eCB antagonists (Everitt & Robbins, 2005) These subtleties in the action of AM251 in specific areas remain to be further assessed. It should also be noted that structures of the mesolimbic pathway were not equally affected. For instance, within the basolateral amygdala a partial effect was seen, as AM251 treated ischemic rats displayed increased TH-ir although not reaching comparable levels as the shams treated with AM251 or the sham controls.

Two-way ANOVA analyses of the immunohistochemical data demonstrate significant interactions between the drug treatment and surgical conditions in most analyses. This is attributable to the impact of AM251 treatment prior to global cerebral ischemia in preventing or

reducing ischemia-induced alterations in TH and/or DRD1 expression. A proposed mechanism for this physiological change when CB1 receptors are blocked prior to ischemia involves ensuing attenuation of the cascade of excitotoxic events associated with the ischemic injury (Globus et al, 1987; Martin et al, 2012; Ruscher, Kuric & Wielock, 2012; Zhang et al, 2008). It is proposed that AM251 has opposing effects of endocannabinoid agonists, by altering dopamine levels by blocking CB₁ receptor activation of GABAergic and glutamatergic neurons (Fernandez-Ruiz, Hernandez & Ramos, 2010; Gerdeman & Fernandez-Ruiz, 2008; Khoury et al, 2012; Melis & Pistis, 2007; Pistis et al, 2002; Pralong, Magistretti & Stoop, 2002). More specifically, the mechanism at play with the agonist results in eCB acting to inhibit GABAergic interneurons causing increased dopamine release as well as a surge in the GABA to glutamate ratio (Gerdeman & Fernandez-Ruiz, 2008; Katona et al, 1999; Khoury et al, 2012; Patel, Rademacher, & Hillard, 2003). Inhibition of this pathways by CB1 antagonist lead to decreased dopamine and glutamate transmission (Colombo et al, 2005; Corbille et al, 2007 De Vries et al, 2001; Khoury et al, 2012; Randall et al, 2012; Sidhpura & Parsons, 2011).

The effect of AM251 treatment, administered prior to vessel occlusion, on reducing dopamine and glutamate release is believed to be a contributing step in improving ischemic outcome. This is supported by various studies that have shown that dopamine and glutamate antagonists improve outcome when administered prior to ischemia (Berman & Hastings, 1997; Filloux & Wamsley, 1997 Globus et al, 1987; Meldrum, 1985; Park et al, 2004; Ren, Li, & Xu, 1997; Yammoto et al 1994). This contention also pairs nicely with data showing that blockade of CB₁ receptor protects against NMDA-induced excitotoxicity (Hansen et al, 2002). It is believed

that by blocking excess dopamine and glutamate release, AM251 is able to attenuate the excitotoxic events, including: surge in calcium, depletion of energy stores and breakdown of cells resulting in cell death (Farber, Chien & Mittnacht, 1981; Globus et al, 1987; Globus et al, 1988; Martin et al, 1998; Niquet et al 2003; Paschen, 1996; Zhang et al, 2008).

As expected, assessment of neuronal injury within the hippocampus revealed a vulnerability of the pyramidal neurons of the CA1 layer to global ischemia. In contrast, the pyramidal cells of the CA3 layer appeared resistant consistent with previous literature suggesting that vulnerability in the CA3 layer is restricted to a subpopulation of interneurons in the stratum lucidum (Hsu & Buzsaki, 1993; Nunn & Hodges, 1994). This is interesting in particular as we found between group differences in TH-ir in the CA3, while all the groups showed comparable CA3 neuronal density. The ability of AM251 to attenuate excitotoxicity of pyramidal neurons was exemplified by increased neuronal survival within the CA1 of the hippocampus in the absence of significant pyramidal cell injury in the CA3. Noteworthy, a recent study demonstrated that DA via D1 receptor signaling, but not adrenergic signaling, is important for successful expression of spike timing-dependent plasticity (STDP) at CA3-CA1 synapses and that DA effect on STDP is paralleled by changes in spike firing properties, thereby changing intrinsic excitability of postsynaptic CA1 neurons. This gating is selective to DA in the CA1 layer (Edelmann and Lessmann, 2013). Whether such mechanism is at play following ischemia along with the ischemia-induced initial surge of DA altering CA1 ischemic injury is not known.

Interestingly, DA also regulates conventional LTP in the hippocampus (reviewed in e.g., Lisman and Grace, 2005; Lisman et al., 2011). The main DAergic input to the hippocampus is delivered by VTA pathways (Gasbarri et al., 1994). DA via D1 receptor activation can facilitate LTP induction by lowering the threshold to induce LTP (Li et al., 2003, 2011; Gao et al., 2006; Lemon and Manahan-Vaughan, 2006). Furthermore, endogenously released (by applying DA transporter antagonists) or exogenously applied DA, both increase LTP in hippocampal slices (Swant and Wagner, 2006). Therefore, changes observed in the current study could have significant impact on recovery of memory deficits related to CA1 functions, notably ischemia-induced spatial memory impairment. The observed reduction of CA1 neuronal injury by AM251 treatment pairs nicely with previous findings showing that eCB antagonist treatment prior to ischemia increased neuronal survival in the area of infarct (Hansen et al, 2002; Muthian et al, 2004). Moreover, AM251 administration has been implicated in protection of hippocampal neurons during oxygen-glucose deprivation (Landucci et al, 2011). Thus, our results show consistency with previous data demonstrating neuroprotective effects of eCB antagonists when administered prior to cerebral ischemia.

Although the main interest of this thesis is the characterization of the impact of AM251 treatment on dopamine function, it should be noted that the antagonist also works directly at reducing anandamide which itself is known to have toxic properties at high concentrations (Amantea et al, 2007; Berger et al, 2004; Muthian et al, 2004). Similarly to dopamine and glutamate, the endocannabinoids anandamide and 2-archidonoylglycerol both significantly increase during traumatic events in the brain and in particular during ischemia (Amantea et al,

2007, Degn et al, 2007, Hill et al, 2010). This increase in AEA and 2-AG induces a rapid up-regulation of the catalyzing enzymes, FAAH and NAPE-PDL, which causes a breakdown in integrity of surrounding cells, resulting in neuronal degeneration (Amantea et al, 2007). It is proposed that treatment with the eCB antagonist, AM251, would then prevent this rapid increase in AEA and 2-AG therefore reducing the damaging impact of the ischemic insult. This is consistent with studies showing that other eCB antagonists, SR141716 and LY32135, similarly reduced infarct volume (Muthian et al, 2004). Despite these findings, there remains contention and conflicting evidence on whether eCBs antagonists are protective or neurodegenerative during injury (Fernandez-Ruiz, Hernandez & Ramos, 2010; Hill et al 2010; Khoury et al, 2012; Pellegrini-Giampietro, Mannaioni & Bagetta, 2009). As mentioned, state-dependent effect of eCBs and their related antagonists should be fully acknowledged. It has been stipulated that under normal physiological levels eCBs act as regulatory messengers keeping the system in balance (Hill et al, 2010). Once this system is disrupted eCB lose their regulatory function and can in fact worsen outcome (Hill et al, 2010; Khoury et al, 2012). The current findings confirm that eCBs may in fact play a role in neurodegeneration in non-steady-state conditions, such as ischemia, and blocking such action may in fact be beneficial.

The state-dependent ability of eCBs and their antagonists is further supported by the current results, as it was in specific comparisons in the context of ischemia and in no other circumstances that AM251 pretreatment altered dopamine transmission, behaviour, or neuronal survival in sham animals. That is to say, under normal steady-state conditions AM251 did not affect outcome significantly, however had an impact in the context of ischemia.

The current dichotic view on possible neuroprotective/neurodegenerative roles of endocannabinoids needs to be put in perspective as endogenous modulators seldom act in isolation. As previously discussed the function of eCBs largely depends on the physiological state of the subject at hand. The current findings are not intended to suggest that eCBs are toxic and eCB antagonists are protective all of the time. There may be a beneficial role for both the eCBs and eCBs antagonists. For instance, AM251 is protective when administered *prior* to ischemia due to its role excessive dopamine, glutamate and eCBs release causing neuronal degeneration. Since it was confirmed that neurotransmitter function is indeed disrupted by ischemia, the administration of eCBs during the recovery period, for instance in the hours or days following cerebral ischemia, may in fact exert beneficial effects in activating these altered systems. Given the proper dosage, eCB treatment following ischemia may help the dopamine reward pathway, for instance, return to normal function. Indeed this seems to be case as there is sufficient evidence suggesting that eCB treatment after brain injury and ischemia improves outcome (Arevalo-Martin et al, 2012; Fernandez-Ruiz, Hernandez & Ramos, 2010; Landucci et al, 2011). Furthermore, the application of AM251, or a related antagonist, may hinder recovery if given after ischemic outcome (Arevalo-Martin et al, 2012; Landucci et al, 2011). According to the proposed mechanisms of action under more stable physiological conditions this appears plausible as post ischemic AM251 administration might then further impair recovery of neurochemical function.

Some limitations remain given the context of the present research. Firstly, eCBs and eCB antagonists do not act directly on dopamine neurons. This poses a challenge as the resulting effect of AM251 on altering dopamine transmission when given prior to ischemia can only be inferred by the underlying action on GABA and glutamate neurons. This leads to an indirect pathway and mechanism of action, consequently cannot be considered a direct causal relationship by itself. Recently, there has been some interesting research suggesting CB₁ receptor existence on dopamine cells within the nucleus accumbens, ventral tegmental area, and striatum (Wenger, Moldrich & Furst, 2003). This would imply a more direct mechanism of action and may help clarify the role of eCBs in the brain, although substantial research need to be done in order to ascertain these results. Ideally, with the current data at hand, a next step would be to more clearly define the role of AM251 effects on GABA and glutamate expression during ischemia. Future research, could consider compromising GABA or glutamatergic transmission prior to AM251 administration to further understand its effect. If the results show that the effect is changed when either GABA or glutamate secretion is blocked then it would help confirm that this mechanism is the primary one affected by the AM251. Additionally, other eCB antagonists, such as Rimonabant (SR141716) should be tested using the present experimental paradigm to ensure the drug effect is a result of its action on eCBs. AM251 has been found to act as a partial agonist of the G protein couple receptor GPR55, thus confirming the findings using other endocannabinoid antagonists would be essential for deducting any key findings (Pertwee, 2010).

In conclusion, the results of the current study implicate AM251 as a potential therapeutic tool for global ischemia. We demonstrated that it could play a role in attenuating dopamine

dysfunction in brain regions associated with mediation of reward signals as well as its ability to confer protection to hippocampal neurons most susceptible to ischemia, the CA1 neurons in particular. The proposed mechanism of action is related to excitotoxic events from rapid increases in glutamate, dopamine, AEA and 2-AG. Our findings in this study were however restricted to AM251 administration prior to global cerebral ischemia. Within most clinical application this seriously limits AM251's use, as it would require predicting ischemic onset. Furthermore, most preventive strategies would require repetitive and long-term pharmaceutical administration, and more extensive research would need to be performed to account for long term dose effects. Considering that a single acute dose of AM251 has been implicated in effecting emotionality, there is a strong probability that long term administration would have profound effects. Some research thus far has suggested chronic administration of AM251 can effect food consumption, weight and induced some behavioural disturbances in rats, supporting the need for further investigation (Tallet, Blundell, & Rodgers, 2007). Moreover, unlike many other preventative therapeutics used for ischemia, the use of AM251 administered repeatedly on a long-term interval cannot be suggested from the data presented. Chronic blockade of cannabinoid receptors may have some negative effects on the brain function and behaviour (Landucci et al, 2011; Tambaro, Tomasi, & Bortolado, 2012). From the current findings, we can only infer benefit from an acute single dose given immediately prior to ischemia. However, there are cases when cardiac arrest must be induced during surgery as a preventative measure. Within such cases AM251 may be used a clinical therapy given before induced cardiac arrest as a means of neurological protection (Chambers, 2003). That being said, AM251's capacity as a therapeutic should not be overlooked for ischemia and related diseases. Additionally, the present research

provides more insight into the mechanisms at play during ischemia, fostering a better understanding of the disturbances to the dopamine system as well as the role of endocannabinoids and their antagonists as potential mediators.

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Appendix

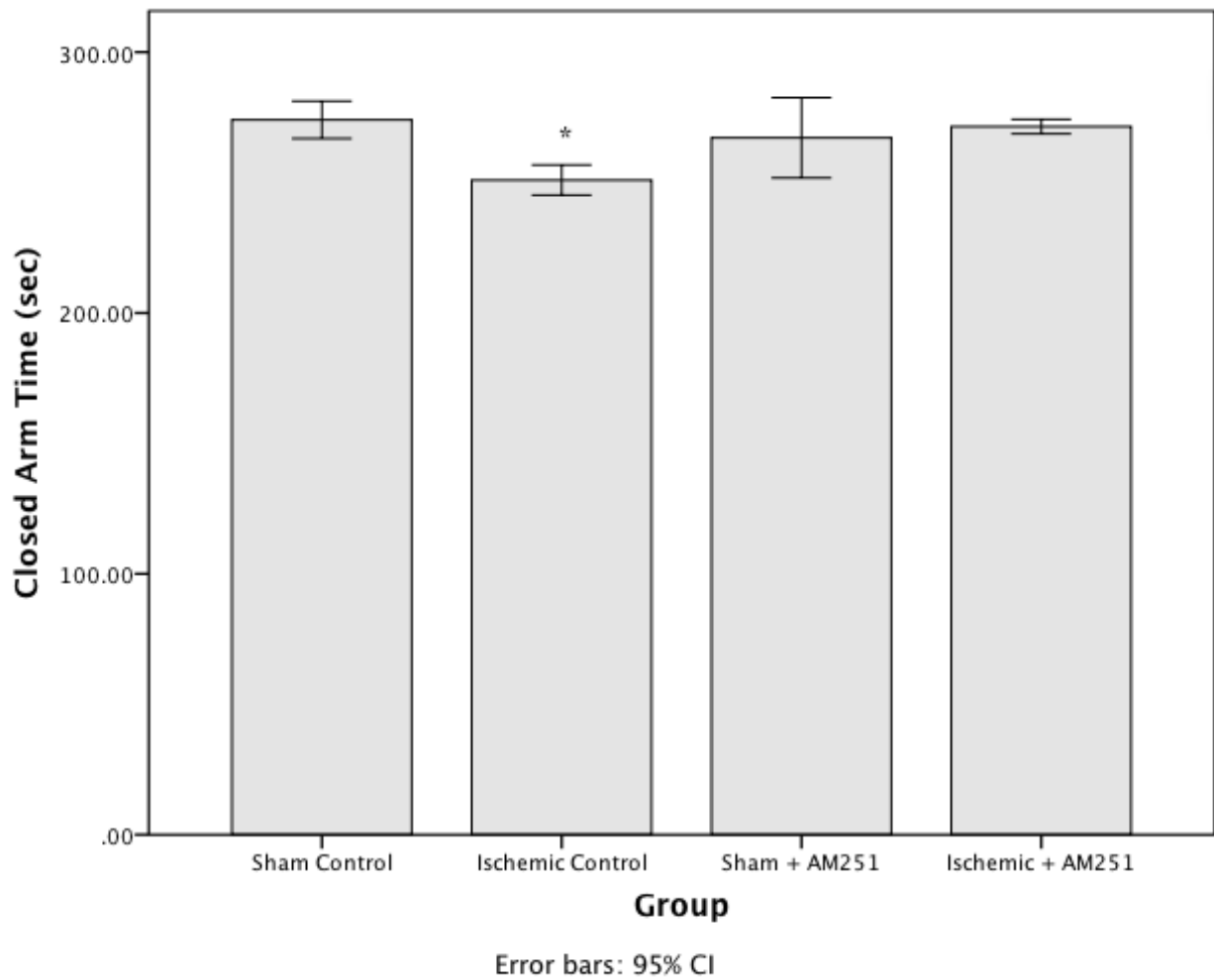


Figure i. This graph illustrates the mean closed arm time for each of the experimental groups, Ischemic Controls spent significantly less time in the open arm than all other groups based on a pairwise comparison ($P < 0.002$).

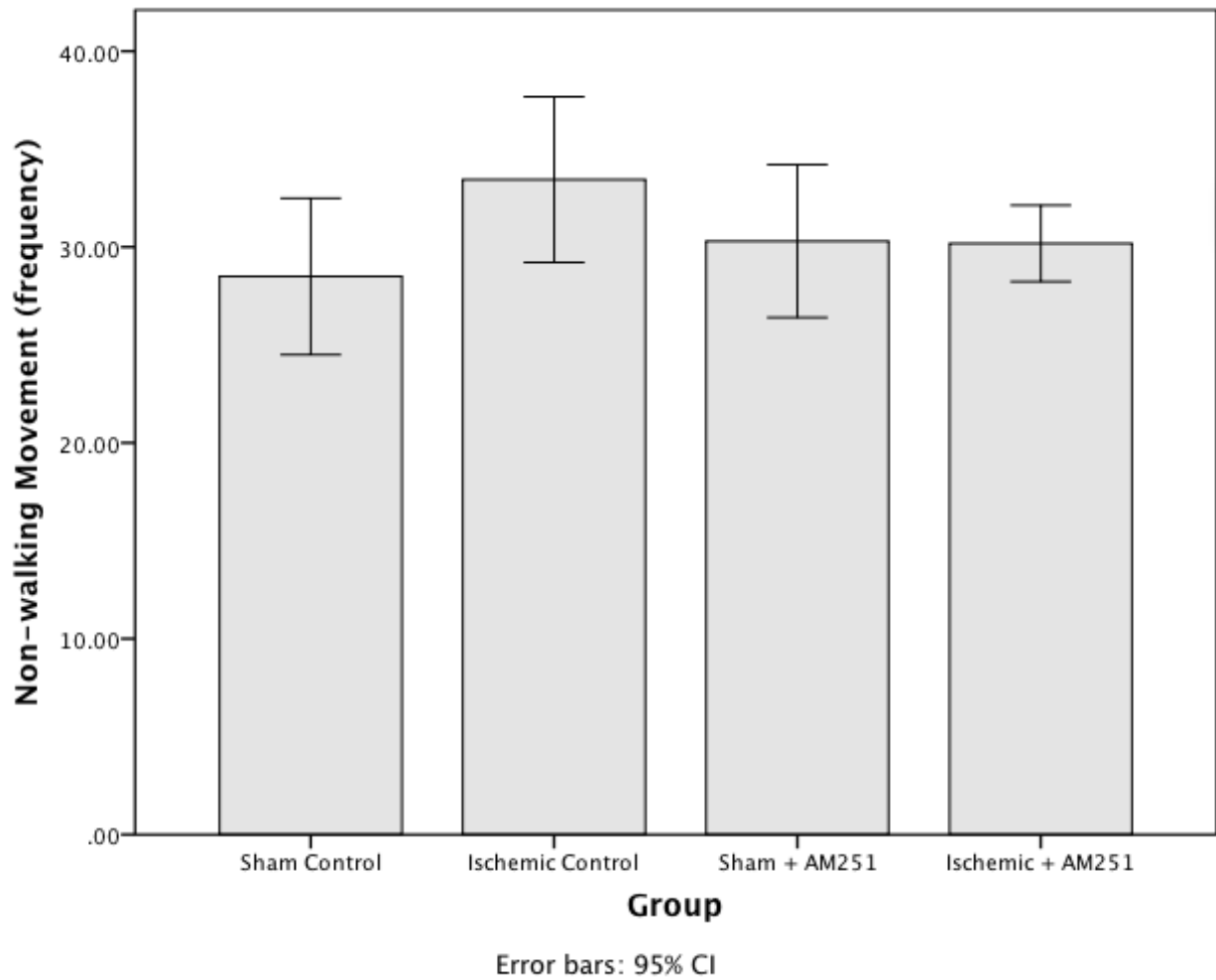


Figure ii. This graph illustrates frequency of non-walking movements (referred to as risk assessment), including head dips into the open arm and rearing. No significant differences were found ($p>0.05$).

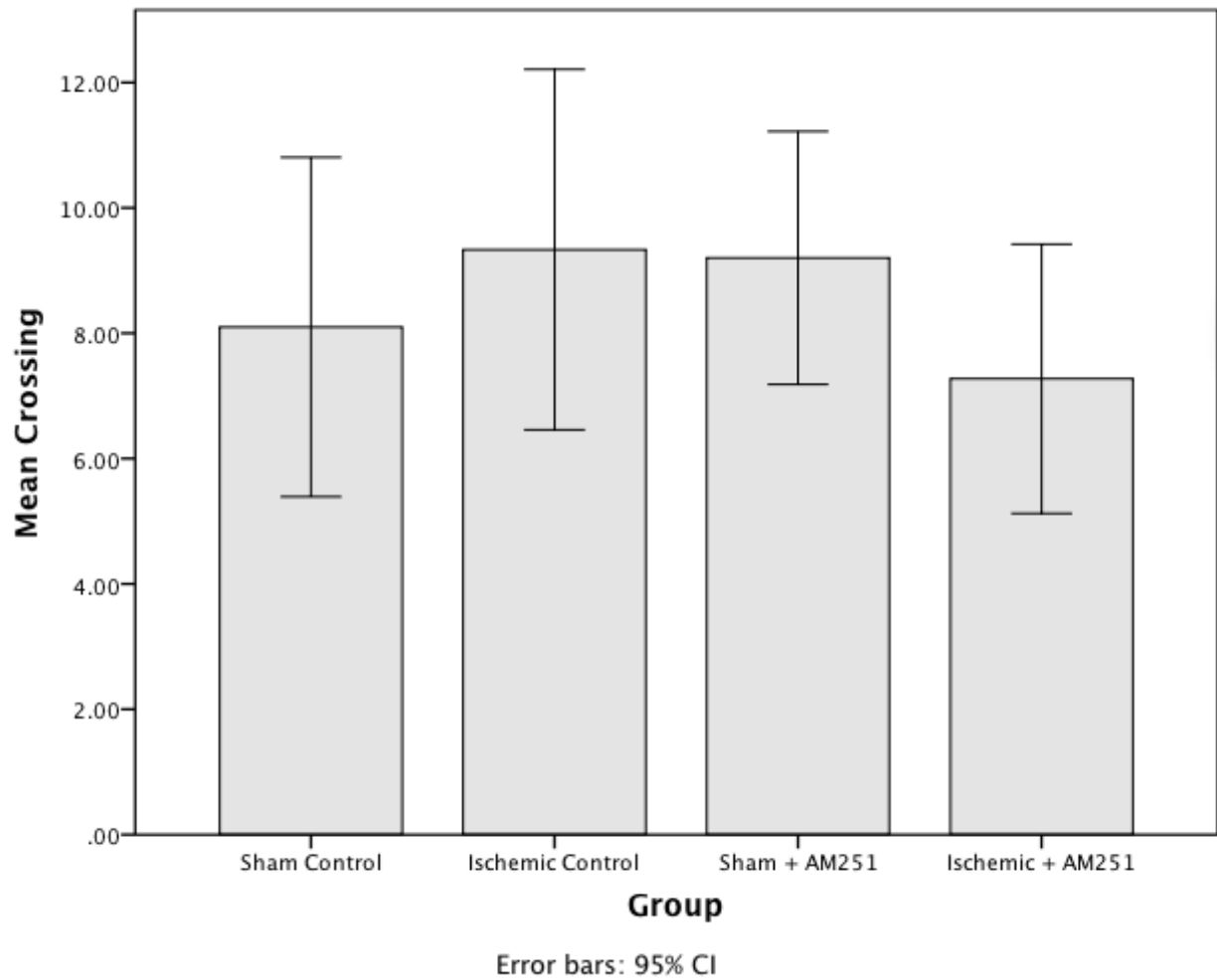


Figure iii. This figure illustrates the mean crossing frequency for all four experimental groups. There were no significant differences among groups based on a one-way ANOVA analyses.

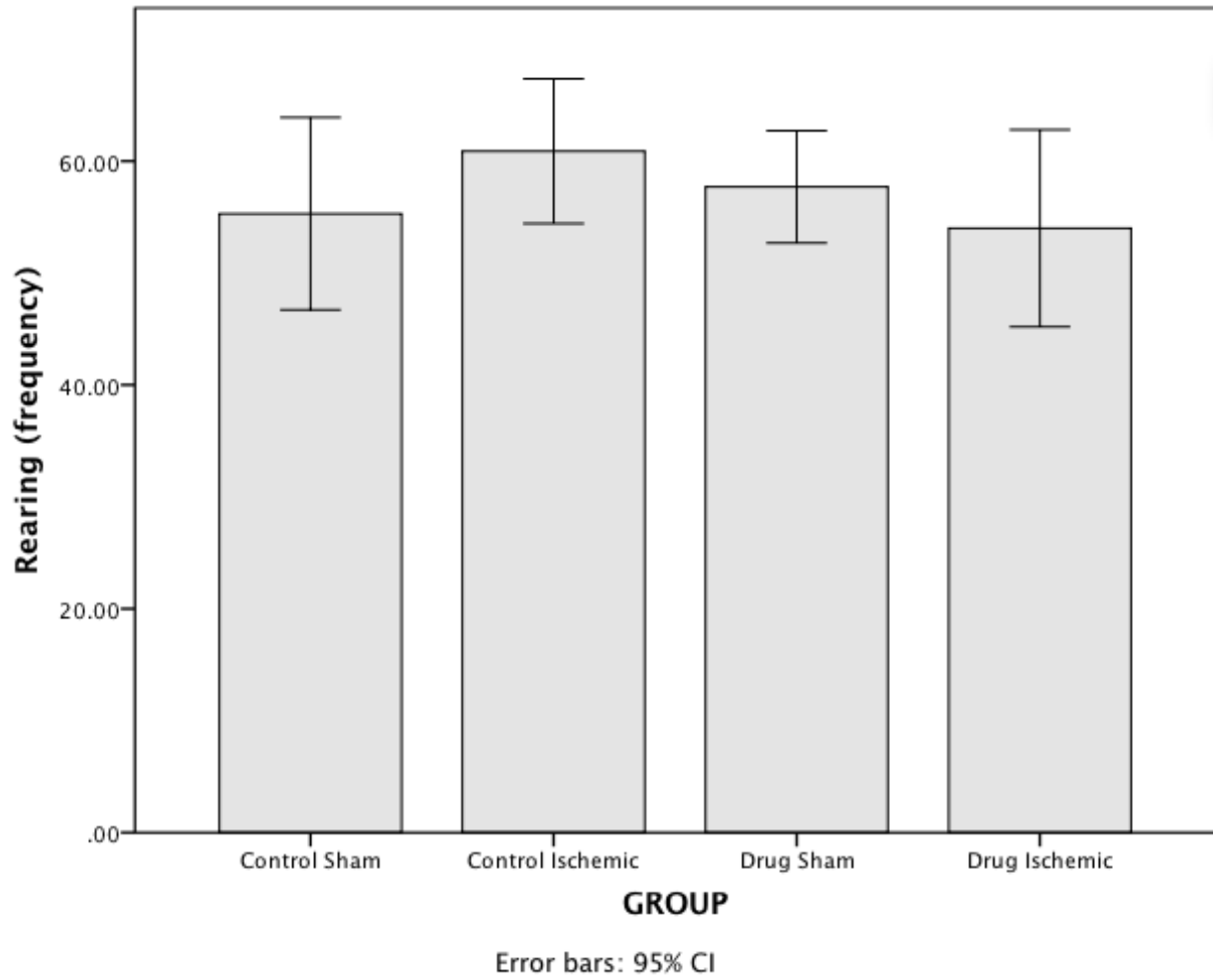


Figure iv. The mean frequency of rearing was examined between all four experimental groups, no significant differences were found ($p>0.05$).

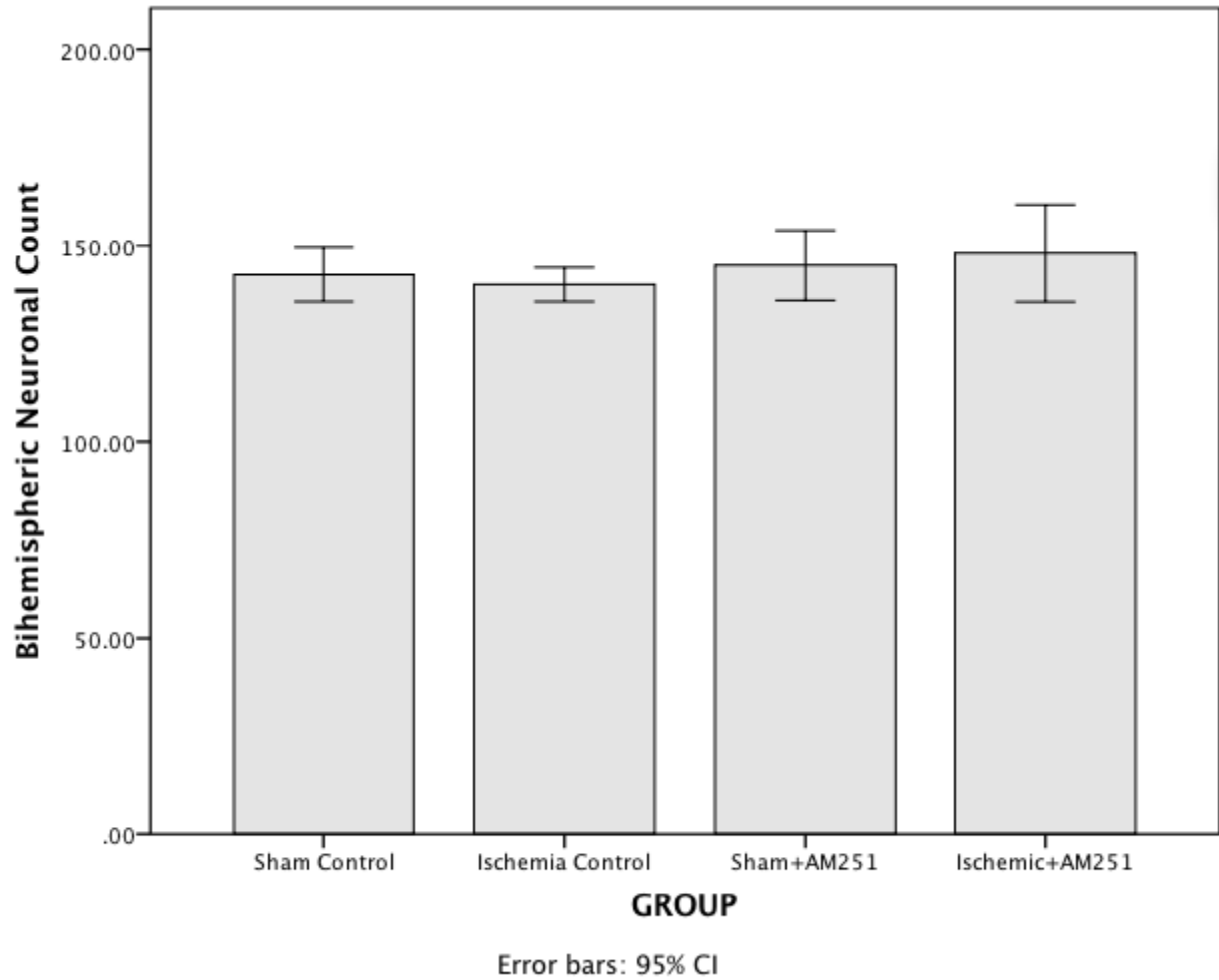


Figure v. Histogram representation of bihemispheric neuronal survival in the CA3 of the hippocampus. No significant main effects or interactions were found ($p>0.05$).