The effect of repeated resveratrol administration on global ischemia-induced hippocampal neurodegeneration, neurochemical effects and functional alterations

Catrinel Girbovan

Thesis submitted to the
Faculty of Graduate and Postdoctoral Studies
in partial fulfillment of the requirements
for the Doctorate in Philosophy degree in Psychology

School of Psychology
Faculty of Social Sciences
University of Ottawa

ACKNOWLEDGMENTS

I would like to express my deepest gratitude to my husband, Mark, whose patience, support and constant encouragement throughout the years have made this all possible. You are my best friend and I cannot imagine sharing this accomplishment without you by my side. A big thank you to my children, Ana and Adrian, for bringing so much joy to my life and for countless colorful suggestions on manuscript drafts. I would also like to thank my parents, Dana and Sorin, for their endless love and support and whose leap of faith twenty years ago provided me with the opportunity to accomplish this personal goal.

The last few years would not have been the same had it not been for the many friendships created. I will cherish the endless memories created in the company of a great set of people and friends; Patricia, Megan, Idu, Irene, Lucie, Marie-Pier, Julie and Marc. You have all made this journey unforgettable and I will cherish the laughter and memories made on endless occasions.

I would also like to thank my committee members, Dr. Catherine Bielajew, Dr. Nafissa Ismail, Dr. Claude Messier and Dr. Jeffrey Stuart for their involvement, time and helpful comments.

Lastly, I would like to offer a heartfelt thank you to my supervisor, Hélène Plamondon. She has been an incredible mentor throughout the duration of my doctoral degree and I am extremely grateful for her friendship, support and expertise. Her patience, genuine caring and concern, and incredible faith in me during the dissertation process enabled me to attend to life and raise a family while earning a Ph.D.

Abstract

Global cerebral ischemia is an established animal model mimicking the effects of cardiac arrest in humans. It is characterized by selective neuronal damage in the hippocampus and significant behavioural and cognitive impairments. In this light, novel therapeutic compounds with numerous physiological targets as well as neuroprotective capabilities and the capacity to lessen residual cognitive deficits pose as great candidates in the treatment of ischemic pathology. The current thesis investigates the possible therapeutic properties of resveratrol (3, 4, 5´trihydroxystilbene), a naturally occurring phytoalexin present in the skin of grapes, against cerebral ischemia-induced neuronal degeneration and cognitive impairments, as well as elaborate on possible mechanisms of action of the compound in male Wistar rats. In Article 1, neuronal density assessment and behavioural testing following chronic pretreatment with resveratrol at two doses (1 and 10 mg/kg) revealed that the compound has important neuroprotective properties at short and long post-ischemic intervals. Despite comparable neuronal protection, the two resveratrol doses showed distinct behavioural effects, highlighting independent actions of the polyphenol on discrete physiological systems mediating cellular survival and behavioural recovery. Articles 2 and 3 investigated possible mechanisms of action of the polyphenol that have not yet been explored with regards to cerebral ischemia. Specifically, Article 2 demonstrated that resveratrol influences markers of plasticity in both ischemic and control animals as well as promotes angiogenesis in the hippocampal region postischemia. Further elaborating on documented effects attributing non-neuronal mechanisms of action of resveratrol in reducing glial activation postischemia, Article 3 highlighted important regulatory effects of resveratrol on mediating glial type-1 glutamate transporter expression at a short reperfusion interval. These findings support the

notion of multiple biological targets by resveratrol and highlight its potential role in attenuating forebrain ischemia-induced neuronal degeneration through multiple physiological targets, while cautioning against possible dose-related effects on behaviour and in healthy controls.

TABLE OF CONTENTS

LIST OF FIGURES

General Introduction

Article 1

Article 2

LIST OF TABLES

ABBREVIATIONS

4-VO	four-vessel occlusion model
AMPK	5' adenosine monophosphate-activated protein kinase
ANOVA	analysis of variance
BrdU	bromodeoxyuridine (5-bromo-2'-deoxyuridine)
CA1	Cornu Ammonis area 1
CA3	Cornu Ammonis area 3
CNS	central nervous system
CORT	corticosterone
CPR	cardiopulmonary resuscitation
CVD	cardiovascular disease
DG	dentate gyrus
DMTS	delayed matching-to-sample
DNMTS	delayed non-matching-to-sample
eNOS	endothelial nitric oxide synthase
GFAP	glial fibrillary acidic protein
GLAST	glutamate-aspartate transporter
GLT-1	type-1 glutamate transporter
h	hour (s)
HPA	hypothalamic-pituitary-adrenal
i.p	intraperitoneal
iNOS	inducible nitric oxide synthase
ir	immunoreactive/immunoreactivity
MCAO	middle cerebral artery occlusion
min	minute (s)
mRNA	messenger ribonucleic acid
MWM	morris water maze
NF-kB	nuclear factor kappa-light-chain-enhancer or activated b cells
NO	nitric oxide
NPCs	neural progenitor cells
ROS	reactive oxygen species
RSV	resveratrol
SGZ	subgranular zone
SIRT1	silent mating type information regulation 2 homolog 1/sirtuin 1
VAT	visual acuity test
VEGF	vascular endothelial growth factor

General Introduction

1. Cerebral ischemia, cardiac arrest and cognitive impairments

Cardiovascular disease remains a leading cause of death, disability and illness in Canada, despite a gradual drop in incidence in the last decade due to advances in surgical procedures, new pharmacological agents and prevention efforts (Statistics Canada – 2011). Furthermore, according to the World Health Organization (WHO), the incidence of cardiovascular disease (CVD) is increasing worldwide and an estimated 17.3 million people die from the disease each year, making CVD globally responsible for 30% of all deaths. Common risk factors include hypertension, obesity, oxidative stress, insulin resistance and aging (Petrovski, Gurusamy, & Das, 2011). CVDs are defined as diseases or injuries of the cardiovascular system, including the heart and its network of blood vessels as well as the peripheral network of blood vessels supplying the rest of the body and the brain. Among an array of consequences of CVDs, cerebral ischemia is characterized by prolonged interruption of blood flow and poor oxygen and nutrient supply to the brain and occurs when the cerebral metabolic demand exceeds the oxygen delivery or supply of systemic circulation (Hossmann, 2008). Cerebral ischemia can be classified as either global or focal in type; global cerebral ischemia occurs when cerebral blood flow is temporarily halted or drastically reduced throughout most or all the brain (cardiac arrest), whereas focal ischemia is characterized by occlusion of specific and selective cerebral blood vessels, producing damage to distinct regions of the brain (stroke) (Traystman, 2003). Both types lead to less than 10% of normative perfusion to forebrain tissue and significant nerve cell death along with ensuing behavioural and cognitive impairments that have been thoroughly documented in humans (Chiota, Freeman, & Barrett, 2011; Madl & Holzer, 2004; O'Reilly, Grubb, & O'Carroll,

2003; Roberts et al., 2013; Sandroni, Nolan, Cavallaro, & Antonelli, 2007; Schaapsmeerders et al., 2013; Varona, Bermejo, Guerra, & Molina, 2004).

Cardiopulmonary arrest is among the leading causes of death and disability, primarily occurring in the aged population due to ventricular fibrillation. Up to 40,000 cardiac arrests occur each year in Canada, making this one cardiac arrest every 12 minutes (Heart and Stroke Foundation of Canada, 2014). Cardiac arrest may be reversed if cardiopulmonary resuscitation (CPR) is performed and an automated external defibrillator is used to shock the heart and restore a normal heart rhythm within minutes. The availability of automated external defibrillators in various city locations, namely sport centers, has resulted in significantly increased resuscitation rates for out-of-hospital patients in recent years. Combined with CPR, the use of an automated external defibrillator can increase the likelihood of survival by 50% (Reynolds & Lawner, 2012). However, successful resuscitation does not necessarily translate into positive patient outcome in the long-term as only 2-15% of patients will be discharged alive from the hospital (Schneider, Bottiger, & Popp, 2009). Secondary brain damage leading to significant neurologic dysfunction, cognitive impairments and long-term disability have been documented as results of cardiac arrest (Chiota, et al., 2011; Madl & Holzer, 2004; J. T. Neumann, Cohan, Dave, Wright, & Perez-Pinzon, 2013; Wilder Schaaf et al., 2013; Young, 2009). Indeed, it has been reported that 26% of patients who survived an in-hospital cardiac arrest and 38% of patients resuscitated following an out-of-hospital cardiac arrest presented important memory impairments (O'Reilly, et al., 2003). Moreover, long-term survivors of cardiac arrest, with a median survival of 7.8 years, displayed significant impairments on measures of long-term memory and learning efficiency (Mateen et al., 2011). A systematic review looking at the

frequency and nature of cognitive impairments in survivors of out-of-hospital cardiac arrest highlighted that memory impairments are the most commonly reported cognitive impairments, followed by impairments in attention and executive functioning (Moulaert, Verbunt, van Heugten, & Wade, 2009). This is to say that there is a need to better understand recovery processes occurring over long periods following cardiac arrest as well as determine effects of natural and pharmacological tools, including the widely studied nutraceuticals, likely to exert prophylactic and/or therapeutic effects.

Most research aimed at preventing cerebral ischemia-mediated injury has relied on animal models of the disease. Global ischemia models, induced by transiently occluding blood vessels supplying the brain and which result in a widespread hypoxic episode, are analogous to cardiac arrest in humans (Small & Buchan, 2000). In rats, a transient ischemic episode can be induced through bilateral carotid artery occlusion coupled with systemic hypotension – 2-vessel occlusion model, which is sufficient in reducing collateral blood flow and inducing selective neuronal death in the CA1 and other vulnerable structures such as the caudoputamen and cortex (Nanri & Watanabe, 1999). The 4-vessel occlusion model (4-VO), implemented by permanent and temporary occlusion of both, the vertebral and carotid arteries, respectively, is a rodent model of global cerebral ischemia characterized by temporary, yet nearly complete, cessation of blood supply to the brain (Pulsinelli & Brierley, 1979). The brain receives one-fourth of cardiac output and metabolically has strict demands for oxygen and glucose delivery, making it extremely vulnerable to the cessation of systemic circulation and cerebral blood flow (Chiota, et al., 2011). Preventing adequate supply of oxygen and energy substrates to the brain during global cerebral ischemia leads to a selective and delayed degeneration of hippocampal CA1 pyramidal neurons in the rat. Other brain

regions may also be affected as ischemic duration is extended over 10 minutes (min) (Kirino, 1982; Pulsinelli, Brierley, & Plum, 1982). As such, occlusion periods exceeding 15 min can lead to documented extra-hippocampal damage to surrounding cortical and striatal regions, while short occlusion periods (~2-3 min) have commonly been used in preconditioning paradigms, priming the brain to better sustain a more prolonged ischemic episode (Pulsinelli & Brierley, 1979; Thompson, Dave, Saul, Narayanan, & Perez-Pinzon, 2013). Significant neuronal death can occur as early as 3 to 6 hours (h) post-insult with ischemic durations of 30 min (Pulsinelli, et al., 1982). Following 10 min global ischemia, a characteristic and specific degeneration of CA1 hippocampal neurons is observed beginning at 72 h post-insult and gradually reaching a maximum 7 days postischemia, a process that has been termed "delayed neuronal death" (Kirino, 1982). Furthermore, this type of brain insult is accompanied by locomotor hyperactivity and extensively documented impairments in working and spatial memory similar to those occurring following cardiac arrest in humans (Briones & Therrien, 2000; Kiyota, Miyamoto, & Nagaoka, 1991; Poignet, Beaughard, Lecoin, & Massingham, 1989; Volpe, Pulsinelli, Tribuna, & Davis, 1984). As such, the latter studies emphasize the need for novel treatment therapies for both acute neuronal damage and ensuing cognitive impairments associated with cerebral ischemia.

To date, no pharmacological agent allowing the blockade of neuronal death induced by cerebral ischemia has proven successful in clinical trials. The only available treatment for use following cardiac arrest is thrombolytic therapy (i.e., thrombolytic tissue plasminogen activator), which breaks up blood clots and provides rapid reperfusion to affected brain tissue but is inadvertently associated with a risk of cerebral hemorrhaging (Bednar, 2002). Moreover, to be effective, t-PA must be administered within 4.5 h of the onset of the insult

and can only be given to ischemic stroke patients, further limiting its use. Recent evidence suggests that a door-to-needle administration time of 60 min or less is associated with lower in-hospital mortality and intracranial hemorrhage, along with an increase in the percentage of patients discharged home (Fonarow et al., 2014). Antiplatelet agents (ex: acetylsalicylic acid and anticoagulants (ex: Warfarin) may also be given after an ischemic stroke to prevent clots from forming. Other pharmacological agents may be prescribed to regulate blood pressure, or treat diabetes or atherosclerosis with the aim of decreasing CVD risk. However, the latter prophylactic approaches may reduce CVD incidence but will not prevent the disease from affecting a certain number of patients developing sudden cardiac arrest, highlighting the need for parallel therapeutic approaches that can provide neuroprotection in the acute phase following ischemia and decrease ensuing functional impairments. Indeed, neurological damage remains a main obstacle in reducing sudden death induced by cardiac arrest. Moreover, survival to discharge is only 5-15% for a "witnessed" arrest, despite CPR and advanced cardiac life support and becomes almost absent if ischemia is prolonged due to delayed CPR in an "unwitnessed" arrest (Madl & Holzer, 2004). While the use of emergency percutaneous cardiopulmonary bypass has improved cardiac arrest survival to 30%, neuronal death remains unaffected (Allen & Buckberg, 2012). Lastly, 30-50% of patients who survive a witnessed arrest go on to develop significant neurological dysfunction which can range from subtle cognitive impairment, to coma, persistent vegetative state and brain death (Chiota, et al., 2011). Thus, prophylactic interventions in the form of supplemental pharmaceutical and dietary therapies have become great candidates in prolonging healthy life by decreasing the incidence of CVDs and also providing neuroprotection and/or functional recovery following cerebral ischemia.

2. Resveratrol: Biological activities and role in regulating metabolic health and disease

Many natural dietary compounds with promising biological activities against CVDs have been studied in recent years, and of those, resveratrol (RSV) is one of the most studied antioxidants. RSV belongs to the stilbene family of compounds and exists as two isoforms, the *cis* and the *trans* isomers, and bears a simple chemical structure that may interact with a variety of receptors and enzymes, making it both an activator and inhibitor in a number of pathways (Bhat, Kosmeder, & Pezzuto, 2001). Trans-resveratrol (Fig. 1) has the greatest biological activity and is the primary form found in plants (Xu & Si, 2012). It is found in abundance in the skin and seeds of grapes, cranberries, blueberries, mulberries and peanuts (Petrovski, et al., 2011). First isolated in the 1940's, RSV was classified as a phytoalexin; an antimicrobial agent synthesized by leaf tissues as a defence mechanism against pathogens. However, it is now known that RSV is synthesized in response to a number of environmental stressors, which include water deprivation, ultraviolet radiation as well as fungal infection (Petrovski, et al., 2011). Commercially, RSV is extracted from the dried roots of *Polygonum cuspidatum*, commonly called Japanese knotweed and which is mainly found in China and Japan (H. Wang et al., 2012). RSV did not attract much interest until 1992 when it was postulated that it might be responsible for some of the cardioprotective effects of red wine (Nakata, Takahashi, & Inoue, 2012; Siemann & Creasy, 1992; Xu & Si, 2012). Indeed, the renowned "French paradox" is a phenomenon predominantly observed in the south of France whereby low incidences of CVDs have been correlated to a diet rich in lipids and red wine consumption (Quincozes-Santos & Gottfried, 2011). A number of studies have shown moderate wine drinkers to be at lower risk than their heavy or non-drinking counterparts with respect to CVDs (Constant, 1997; Dudley et al., 2008). Additionally, it was discovered that

red wine was more beneficial than white wine, which was later attributed to the absence of RSV in white wine and further established polyphenols as the bioactive ingredients of red wine (Bhat, et al., 2001). Since then, a number of beneficial effects of RSV have been noted in mammals making it a prospective candidate in the prevention and treatment of cerebrovascular disease.

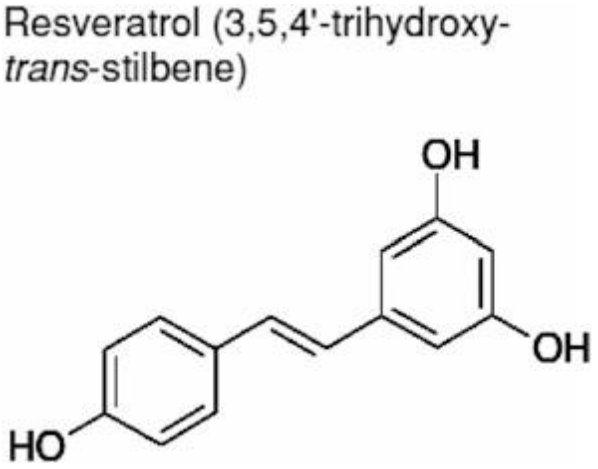


Figure 1. Chemical structure of trans-resveratrol. Resveratrol (3,5,4´-trihydroxystilbene) bears a simple chemical structure that may interact with a variety of receptors and enzymes, thus acting as an inhibitor or activator in a number of pathways. Adapted from "Resveratrol: from basic studies to bedside, " by A. Borriello et al. 2014, *Advances in Nutrition and Cancer,159*, p. 167-84.

2.1 Biological activity of resveratrol: bioavailability, pharmacokinetics and metabolism

The bioavailability of a nutrient is defined by the degree to which it becomes available in the target tissue following administration. Determining the bioavailability of a compound in vivo largely depends on knowledge of its absorption, distribution, and metabolism (Wenzel & Somoza, 2005). Early studies with RSV highlighted concerns over its bioavailability and rate of metabolism, mainly due to the fact that RSV has a short initial half-life and is metabolized extensively in the liver and gut (Nakata, et al., 2012). The most

abundant RSV metabolites identified are *trans-resveratrol-3-O-glucoronide* and *trans-resveratrol-3-sulfate* (Yu et al., 2002). The absorption of RSV in rats was first described by Bertelli's group in a series of experiments using Wistar rats that were administered red wine with a known RSV content (Bertelli, Giovannini, Stradi, Bertelli, & Tillement, 1996). It was discovered that RSV was quickly absorbed and reached peak concentrations 60 min following wine ingestion. Conversely, trans-resveratrol reaches peak concentrations in rat blood and serum very rapidly, 10-15 min following oral administration of a single dose of 2 mg/kg, and has a mean residential time of 33.83 min (Bradamante, Barenghi, & Villa, 2004). Moreover, less than 5% of free RSV has been detected in blood plasma following oral administration in both rats and humans (Wenzel, Soldo, Erbersdobler, & Somoza, 2005). Thus, it has been suggested that in vitro studies using unconjugated compounds lack physiological relevance and should be interpreted with caution. Instead, focus should be directed on studying the effect of RSV primarily using in vivo models or RSV's conjugates during in vitro investigations. Importantly, the polyphenol was first shown to cross the blood brain barrier when administered intraperitoneally (i.p.) prior to or during common carotid occlusion in gerbils at a concentration of 30 mg/kg (Q. Wang et al., 2002). The authors reported detectable RSV concentrations in serum, albeit in its glucuronide conjugate form, as early as 1 h following injection whereas peak concentrations were reached 4 h following administration in the brain and liver and steadily declined thereafter. Despite limited bioavailability of RSV and its conjugates following oral and intraperitoneal administration, the compound has been identified as an important mediator of neuroprotection in a number of disease models (Simao, Matte, Pagnussat, Netto, & Salbego, 2012b; Y. Wang, Xu, Fu, Ma, &

Xiang, 2011). In that light, the polyphenol remains a target of interest in the treatment and prevention of neurodegenerative disorders and pathologies inducing delayed neuronal injury.

2.2 Biological activities of resveratrol and noted effects in disease models

Documented pharmacological activities of RSV are varied and include: inhibition of lipid peroxidation (low-density lipoprotein, membranes), chelation of copper, free-radical scavenging, inhibition of platelet aggregation, anti-inflammatory, vasorelaxing, modulation of lipid metabolism, anticancer and estrogenic activities (Fremont, 2000; Y. M. Yang et al., 2008). RSV's free-radical scavenging properties are widely acknowledged and proposed as a main mechanism by which the polyphenol confers protection against myocardial ischemic injury (Ray et al., 1999). Thus, perfusion of isolated working rat hearts in the presence of RSV prior and for 2 h following 30 min of ischemia has been shown to induce myocardial protection. This was evidenced by improved recovery of post-ischemic ventricular function including developed pressure and aortic flow compared to the untreated group.

In recent years, the cancer preventative and therapeutic potential of RSV has been extensively studied as well, research yielding important contributions of the compound in reducing the incidence of tumorigenesis. Proposed mechanisms have targeted RSV`s selective actions at one or more stages of carcinogenesis involving induction of apoptosis in cancer cells and prevention of tumor cell promotion (Aluyen et al., 2012). Attenuation of oxidative stress through downregulation of the expression of inducible nitric oxide synthase (iNOS) and suppression of the inflammatory response has also been suggested as possible mechanisms of action in RSV's chemo-preventive effects (Bishayee, Barnes, Bhatia, Darvesh, & Carroll, 2010). In addition, RSV's ability to attenuate oxidative stress-mediated lipid peroxidation in liver sections has generated promising research venues. Hepatotoxicity

or chemically-driven liver damage is one of the major complications of methotrexate therapy, an antifolate drug used in the treatment of certain cancers. Intraperitoneal treatment with RSV at a dosage of 7 mg/kg for 7 days has been shown to significantly inhibit markers of lipid peroxidation and methotrexate-induced hepatotoxicity (Dalaklioglu, Genc, Aksoy, Akcit, & Gumuslu, 2013).

The structural similarities of trans-resveratrol and the synthetic estrogen diethylstilbestrol have also raised questions about possible estrogenic activity. However, RSV's biological effects on estrogenic activity remain highly controversial, since both estrogenic and anti-estrogenic effects have been noted (Bowers, Tyulmenkov, Jernigan, & Klinge, 2000). Importantly, the polyphenol has been categorized as a phytoestrogen due to its ability to compete with natural estrogens for binding to the estrogen receptor alpha with low affinity and thus modulating the biological response exerted by the receptor (Chakraborty, Levenson, & Biswas, 2013). Others have pointed towards selective activation of either alpha or beta estrogen receptor subtypes in RSV-mediated neuroprotection against cerebral ischemia (Saleh, Connell, & Saleh, 2013). Future studies using estrogen receptor alpha and beta knockout mice may provide a better understanding of RSV effects on each receptor subtype an elucidate possible disease-specific estrogenic activity of the compound.

Several other properties of RSV have recently been reported, which deserve some attention due to possible impact on ischemic injury. For instance, Dolinsky's group showed that RSV can reduce elevated blood pressure in spontaneously hypertensive rats, improve flow-mediated vasodilation and prevent cardiac hypertrophy through upregulation of endothelial nitric oxide synthase (Dolinsky et al., 2013). Moreover, Chen and Pace-Asciak (1996) observed that trans-resveratrol caused a nitric oxide (NO)-mediated relaxation of

precontracted endothelium-intact rat aorta. However, treatment with a NO inhibitor did not reverse RSV-induced relaxation of denuded aorta, indicating that the compound exerts both indirect and direct vasodilator effects on blood vessels through NO-mediated and non-NO-mediated mechanisms. The wide array of biological targets of RSV has contributed to intense research both in the cardiovascular system and the brain. Moreover, current studies suggest that the polyphenol may enhance prognosis of a number of neurological disorders. For instance, dietary supplementation with RSV in a mouse model of Alzheimer's disease for one year lead to significant decrease in amyloid-beta plaque density in the cortex, caudoputamen and hippocampus (Solberg et al., 2014). Resveratrol was also shown to regulate energy homeostasis through AMP-activated protein kinase (AMPK), sirtuin 1 (SIRT1) and peroxisome proliferator-activated receptor-gamma coactivator-1α resulting in increased mitochondrial oxidative function. This led the authors to suggest a putative role for RSV in the treatment of Parkinson's disease, a neurodegenerative disorder plagued by mitochondrial dysfunction and oxidative stress (Ferretta et al., 2014).

In recent years, RSV has been considered in the treatment of acute conditions such as ischemia-reperfusion injury with demonstrations of neuroprotective effects in a number of ischemic models. For instance, RSV administration for 21 days prior to middle cerebral artery occlusion in rats was shown to significantly decrease infarct volume compared with controls (Sinha, Chaudhary, & Gupta, 2002) whereas a 7-day RSV pretreatment significantly attenuated CA1 neuronal injury and glial cell activation after global ischemia (Simao et al., 2011; Q. Wang et al., 2009). Acute intravenous administration of 20 mg/kg RSV prior to bilateral carotid artery occlusion was further successful in decreasing neuronal loss through modulation of NO and free radical scavenging in rats (K. T. Lu et al., 2006). Through such

findings, RSV represents a novel agent promoting post-ischemic neuronal survival (Bradamante, et al., 2004), which could foster improved emotional, motor and/or cognitive recovery.

3. Neurogenesis: expression and suggested role following cerebral ischemia

The notion that the adult mammalian central nervous system is incapable of self-repair or regeneration was quite predominant until the discovery of self-renewing and multipotent neuronal stem cells in distinct regions of the brain, making us revisit the long-held belief (Altman & Das, 1965; Kaplan & Hinds, 1977; van Praag et al., 2002). We now know that neurogenesis in the adult mammalian brain persists throughout life and is predominantly localized in two regions: the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus and the subventricular zone of the lateral ventricles. Newly formed cells in the hippocampus migrate into the dentate granular cell layer, where most cells differentiate into neurons that assume a granule cell phenotype while the remainder transform into glial cells (astrocytes and oligodendrocytes) (Y. S. Choi et al., 2003; Winkelheide et al., 2008). The process takes approximately 2-4 weeks whereby newly generated neurons are functionally integrated and begin to modify active hippocampal circuits (Arsenijevic, Weiss, Schneider, & Aebischer, 2001). Previous studies have reported increased proliferation and differentiation to occur within these two regions following various kinds of environmental or pharmacological manipulations, including housing in an enriched environment (Kempermann, Kuhn, & Gage, 1997; Nilsson, Perfilieva, Johansson, Orwar, & Eriksson, 1999), antidepressant administration (Epp, Beasley, & Galea, 2013) or brain injury (Jiang, Gu, Brannstrom, Rosqvist, & Wester, 2001). In addition, pharmacological interventions such

as growth factor administration (K. Jin et al., 2002) or physical exercise (van Praag, Kempermann, & Gage, 1999), also enhance stroke-induced neurogenesis and behavioural recovery, whereas stress and aging have been shown to inhibit the neurogenic response and result in poor behavioural outcomes following stroke (J. H. Choi et al., 2012; Lichtenwalner & Parent, 2006).

3.1 Neurogenesis: expression and suggested role following cerebral ischemia

Neurogenesis, or the formation of new brain cells, has gathered increasing attention as an alternative way by which the brain could compensate for neuronal damage experienced following brain injury. Increased interest in cerebral ischemia induced neurogenesis in the hippocampus has prompted detailed characterization of post-ischemic neurogenic response profiles and suggested the process to play an active role in ischemic recovery, possibly acting as a compensatory mechanism against neuronal damage. Results have been conducive to an association between neurogenesis and improved behavioural outcome, which has paved the way for increased interest in compounds with neurogenic capabilities postischemia (Lagace, 2012).

In the last two decades, a growing number of laboratories have reported cerebral ischemia-induced neurogenesis in the hippocampus (J. H. Choi, et al., 2012; Y. S. Choi, et al., 2003; Engelhard et al., 2007; Kuge et al., 2009; Lasarzik et al., 2009; J. Liu, Solway, Messing, & Sharp, 1998; Schmidt & Reymann, 2002; R. Tanaka et al., 2004; Winkelheide, et al., 2008; Wojtowicz, Askew, & Winocur, 2008). For instance, gerbils exposed to 10 min bilateral common carotid artery occlusion showed a 12-fold increase in neurogenesis in the dentate subgranular zone 1-2 weeks post-insult while cells with a neuronal phenotype were present as early as 26 days following ischemia and survived for at least 7 months (J. Liu, et

al., 1998). In another report, rats were exposed to 10 min of bilateral carotid artery occlusion plus hemorrhagic hypotension for either 8 or 13 min, which lead to significant increase in neurogenesis in the DG at 28 days postischemia (Winkelheide, et al., 2008). Thus, forebrain ischemia stimulates neurogenesis in the adult DG and a significant volume of these cells mature into granule cell neurons, some of which are thought to extend axons to the CA3 region of the hippocampus (Y. S. Choi, et al., 2003; Markakis & Gage, 1999). However, apart from limited reports (Bendel et al., 2005; Nakatomi et al., 2002), there is no evidence of these cells migrating into the CA1 region, neither has an association between enhanced neurogenesis and improved behavioural outcome on spatial tasks been causally established, which prevents establishing a definite role of newly formed and migrating neurons in post-ischemic functional recovery. Nonetheless, irradiation of adult neurogenesis in gerbils coupled with subsequent global cerebral ischemia lead to significant impairments in the water maze, which was not observed in animals that received irradiation or ischemia alone, suggesting a possible role for these newly-formed cells in hippocampal-dependent functional recovery (Raber et al., 2004). The development and growing use of transgenic animal models that allow selective ablation of newly born cells will hopefully shed some light on the causal link between neurogenesis and recovery of discrete functions following cerebral ischemia.

3.2 Resveratrol effects on the regulation of neurogenesis

Different studies have supported RSV's neuroprotective effects against excitotoxic cell damage, studied in association with recovery of behavioural function postischemia in the current thesis. Stimulation of endogenous stem cell activity in the context of an ischemic insult has also been associated with functional recovery, but whether the polyphenol's ability

to confer neuroprotection and behavioural recovery is coupled with neurogenesis remains to be determined.

To date, the few studies that have assessed the effects of RSV administration on hippocampal neurogenesis in models of disease, both in vitro and in vivo, have reported mixed findings. For instance, Moriya et al. (2011) investigated the effect of RSV therapy on a model of chronic fatigue in mice known to cause significant hippocampal atrophy and decreased number of newborn cells in the hippocampus and is manifested as reduced daily running activity. Following 4-week RSV therapy (administered orally at a dose of 40 mg/kg), chronically fatigued mice exhibited greater levels of newborn cells and higher levels of brain-derived neurotrophic factor messenger ribonucleic acid (mRNA) in the hippocampus, which was concomitantly paired with reduced levels of neuronal apoptosis. This translated into chronically fatigued mice increasing their daily running activity by more than 20% and having reduced hippocampal atrophy compared to untreated mice. In another report, RSV prevented prenatal stress-induced inhibition of neurogenesis through enhancing the expression of precursor cells and newborn neurons and restoring decreased brain-derived neurotrophic factor levels in the DG (Madhyastha, Sekhar, & Rao, 2013). In a more recent report, dietary supplementation with RSV for 6 weeks in streptozotocin-induced diabetic mice lead to a normalization of the expression of genes implicated in neurogenesis and synaptic plasticity in the hippocampus, which is often defective in diabetic mice (Thomas, Garg, & Smith, 2013). As part of this study, the authors also noted that gene alterations led to significant inhibition of the Jak-Stat signaling pathway, which regulates a pro-inflammatory cascade of biochemical events. RSV effects on hippocampal expression of such array of genes were proposed as indirect evidence supporting a role of the polyphenol in promoting

neurogenesis in diabetic conditions. Of interest, while heightened expression of brain plasticity markers has been documented following RSV treatment in animal models of disease, inhibitory actions of RSV on neurogenesis in healthy young adult mice have recently been reported. For instance, a 14-day treatment with 1 or 10 mg/kg RSV lead to a decrease in primary mouse cortical neural progenitor cells (NPCs) and adult hippocampal progenitors, through direct activation of AMPK by RSV, albeit dose-dependent effects were noted (H. R. Park, Kong, Yu, Mattson, & Lee, 2012). In contrast, others have shown upregulation of both neurogenesis and angiogenesis in the DG of wild type mice following chronic pretreatment with RSV (Harada, Zhao, Kurihara, Nakagata, & Okajima, 2011). Indeed, angiogenesis has been shown to offer a favorable environment for neuronal stem cell proliferation via activation of a vascular endothelial growth factor (VEGF)-dependent mechanism (Palmer, Willhoite, & Gage, 2000), suggesting that angiogenesis may account, at least in part, for the RSV-induced increase in neurogenesis observed in wild type mice. A limited understanding of the role that post-ischemic neurogenesis plays in recovery as well as a scarce number of studies investigating brain cell proliferation following RSV administration make it increasingly difficult to interpret the polyphenol's intrinsic effects on such process. As such, studies assessing relationships between RSV-induced improvement of pathological symptoms following forebrain ischemia and hippocampal neurogenesis would help in bridging these events and fill this gap in knowledge.

4. Role of angiogenesis in the pathophysiological cascade of events following brain ischemia and regulation by resveratrol

4.1 Angiogenesis following cerebral ischemia

Angiogenesis, or the formation of blood vessels from a pre-existing vasculature network, is an event that occurs as part of embryonic vascular development, wound healing and organ regeneration as well as in a number of pathologic conditions such as diabetic retinopathy, cardiovascular disease and tumor growth and metastasis (Folkman & Shing, 1992; Hammes, Brownlee, Jonczyk, Sutter, & Preissner, 1996). Furthermore, its role in cerebral ischemia has been confirmed on numerous occasions (Beck & Plate, 2009; Hayashi et al., 2006; Hayashi, Noshita, Sugawara, & Chan, 2003; O. K. Park et al., 2010). The process begins with the sprouting of endothelial cells to the site of injury and its role is thought unique to discrete pathologies. For instance, inhibition of angiogenesis significantly prevents tumor growth, as neoplastic cells depend exclusively on newly formed tumor vessels (Folkman & Shing, 1992). Meanwhile, as part of ischemic recovery, angiogenesis has been interpreted as a self-defence mechanism that helps restore oxygen and energy substrates to affected brain tissue as well as provide neurotrophic support to newly generated neurons. Indeed, neuroblasts have been found in concentrated numbers around blood vessels following stroke (Yamashita et al., 2006). Furthermore, it has been shown that endothelial cells in the infarct area start to proliferate as early as 1 h post-occlusion leading to increased number of vessels in the peri-infarcted region 3-7 days following ischemic injury with maturation still continuing until day 21 (Hayashi, et al., 2003). This has been replicated in humans, with studies showing angiogenesis to take place 3-4 days following an ischemic insult (Krupinski, Kaluza, Kumar, Kumar, & Wang, 1994). However, long-term studies are lacking and

therefore, it is difficult to ascertain for how long the process continues following ischemic injury.

Angiogenesis is stimulated by cytokines and growth factors whose expression often correlates with neovascularization. Following cerebral ischemia, VEGF expression is induced and is a driving force behind angiogenesis in the injured tissue (K. L. Jin, Mao, Nagayama, Goldsmith, & Greenberg, 2000; Sun et al., 2003). Indeed, VEGF binding to receptor sites on the surface of endothelial cells activates intracellular tyrosine kinases, triggering multiple downstream signals that promote angiogenesis (Beck & Plate, 2009). Considering a vital role of revascularization of tissue for neuronal survival, the identification or development of pharmacological tools able to stimulate angiogenesis following cerebral ischemia represents a promising therapeutic venue.

4.2 Role of resveratrol in mediating angiogenesis

RSV has been shown to affect angiogenesis in a situation-specific manner, and pro-angiogenic effects of RSV have been primarily noted following myocardial infarcts (Fukuda et al., 2006; Robich et al., 2010) and in focal models of acute ischemic stroke (Dong et al., 2008). Thus, preconditioning with RSV has been shown to increase capillary density in rat and swine models of myocardial infarction (Fukuda, et al., 2006; Robich, et al., 2010). For instance, RSV induced a significant upregulation of the protein expression profile of VEGF and its tyrosine receptor Flk-1, 3 weeks post-infarct in the rat (Fukuda, et al., 2006). Additionally, pretreatment with RSV has been associated with upregulation of NOS (inducible and endothelial) along with increased antiapoptotic and proangiogenic factors (Robich, et al., 2010). RSV-induced upregulation of markers for angiogenesis associated with the VEGF signaling pathway thus appear tightly associated with improved myocardial

perfusion in the collateral-dependent region. In addition to reducing infarct size in vitro and in vivo, daily administration of 50 mg/kg RSV for 7 days following middle cerebral artery occlusion induced VEGF release, an effect associated with increased capillary density in the peri-infarct myocardium of mice (Dong, et al., 2008).

These aforementioned effects pave the way for possible actions of RSV administration on brain angiogenesis levels following global ischemia. While short-term effects are most likely to address metabolic demands linked to neuronal survival, maintenance of the vascular niche at long intervals following brain insults have never been determined. They will provide valuable information linked to the recovery process and association with neuronal survival at remote time intervals postischemia.

5. Effect of resveratrol on glial cells (*microglia and astrocytes*)' expression following global cerebral ischemia

5.1 Microglia

Microglia are specialized macrophages of the central nervous system (CNS) involved in immune regulation, tissue development, homeostasis and wound repair (Arnold & Betsholtz, 2013). Often described as sensors of pathological events, microglia make up 5-20% of the glial population in the mature brain and are present in greater numbers in grey than in white matter (Persson & Ronnback, 2012). They respond rapidly to changes in the microenvironment of the brain by modifying their morphology and expression of cell surface antigens, a process often referred to as *activation*. Microglia have been classified into three categories to describe their activation status: resting ramified microglia, activated/non-phagocytic and activated/phagocytic microglia (Graeber & Streit, 2010). As previously

stated, in the normal brain, resting microglia perform homeostatic activity; however, when activated as a result of brain injury, microglia's morphology changes to acquire an amoeboid appearance with phagocytic properties (D. Zhang, Hu, Qian, O'Callaghan, & Hong, 2010). Furthermore, their activation following a number of pathological insults has been associated with dual effects in the brain (Kim & de Vellis, 2005; Lee et al., 2010). For instance, in an activated state, microglia have the ability to destroy invading micro-organisms, remove deleterious debris, facilitate a return to homeostatic conditions and promote tissue repair, neuroprotection and neurogenesis (Imai et al., 2007; Kempermann & Neumann, 2003; Lee, et al., 2010; J. Neumann et al., 2006; Tikka, Fiebich, Goldsteins, Keinanen, & Koistinaho, 2001). Noteworthy, they are also capable of secreting a wide range of pro-inflammatory factors, including prostaglandins, chemokines, cytokines, reactive oxygen species (ROS) and reactive nitrogen species such as NO (Graeber & Streit, 2010). The accumulation of some of these factors in the injured brain is thought to contribute to the delayed neuronal death observed following brain ischemia and other neurodegenerative diseases while down-regulation of microglia activation has been associated with neuroprotection (Madinier et al., 2009; Tikka, et al., 2001; F. Zhang, Liu, & Shi, 2010). However, this dual role has impeded on the ability to fully interpret the significance of their presence under various conditions.

Following cerebral ischemia, microglia become positive for markers of activation in the hippocampus as early as 24 h post-insult, and are primarily localized within the CA1 region within 7 days (Anderova et al., 2011; Dos-Anjos et al., 2009; Langdon, Granter-Button, & Corbett, 2008; Madinier, et al., 2009; Moon et al., 2009). Microglial activation has been shown to persist up to one month after ischemia and to significantly decline by 3 months post-insult, although some have reported activation to persist for longer periods.

Indeed, microglia activation was shown to be greater in ischemic compared to sham rats up to 270 days following cerebral ischemia (Langdon, et al., 2008). However, the staining pattern changed from a uniform expression throughout the hippocampus to concentrated expression within the pyramidal layer of the CA1 with increasing survival periods. Thus, agreement exists as to early microglial activation following an ischemic insult peaking around 14 days, although consensus is not achieved as to how long activation persists for (Lee, et al., 2010). Furthermore, beneficial or detrimental effects of such process on adjacent neuronal populations following ischemia remain a controversial topic. Some believe that it leads to neurotoxicity, partly due to the release of chemokines and ROS, while others argue that microglia may promote neuroprotection by enhancing uptake of glutamate through upregulation of type-1 glutamate transporter (GLT-1) from the synaptic cleft (Nakajima, Yamamoto, Kohsaka, & Kurihara, 2008).

RSV's anti-inflammatory properties have been acknowledged and recent reports have documented RSV ability to downregulate microglial activation in vitro through inhibition of pro-inflammatory cytokines and key signaling molecules (Candelario-Jalil et al., 2007; X. Lu et al., 2010; F. Zhang et al., 2010; F. Zhang et al., 2013). In vivo, acute administration of RSV has been shown to reduce microglial activation in a number of brain regions following traumatic brain injury in mice through downregulation of inflammatory cytokines (Gatson et al., 2013). Some have suggested that RSV's anti-inflammatory actions and concomitant effects on microglial activation serve as mechanism of action in providing neuroprotection against ischemic injury (F. Zhang, J. Liu, et al., 2010). Indeed, RSV has been shown to inhibit the production of ROS, one of the many neurotoxic factors released by microglia in response to injury, through its scavenging properties (Candelario-Jalil, et al., 2007). In a

recent report, RSV administration for 7 days prior to global ischemia in rats led to significant decrease of glial activation and attenuated induction of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) and c-Jun N-terminal kinase signaling, which represent a class of proteins and protein kinases responsible for cytokine production (Simao, Matte, Pagnussat, Netto, & Salbego, 2012a). Taken together, RSV's inhibition of microglial activation and the ensuing inflammatory cascade in a number of disease models characterized by increased neuroinflammation suggests a therapeutic potential for the polyphenol in the clinical treatment of ischemic brain injury.

5.2 Astrocytes

Astrocytes are the most abundant cells of the CNS. As such, they represent an important type of glial cell, with multiple defined roles including scavengers of transmitters released during synaptic activity, control of fluid, ion and pH homeostasis, release of neurotrophic factors, shuttle metabolite and waste products and participate in the formation of the blood brain barrier (Swanson, Ying, & Kauppinen, 2004; Takano, Oberheim, Cotrina, & Nedergaard, 2009). In the CNS, astrocytes are divided into protoplasmic and fibrous astrocytes, based on their morphology and localization within gray and white matter, respectively (Nedergaard & Dirnagl, 2005). Traditionally, astrocytes were thought to be mere support cells in close morphological and functional relation with neurons. However, an active role in the pathological cascade following brain injury is considered (Maragakis & Rothstein, 2004; Ouyang, Voloboueva, Xu, & Giffard, 2007). Global ischemia is characterized by delayed neuronal death accompanied by features of programmed cell death selectively affecting CA1 neurons upon brief duration (Nitatori et al., 1995), whereby astrocyte viability has been harder to determine as affected cell bodies are not confined to discrete cell layers.

Following global ischemia for 3-10 min, Sugawara et al. (2002) characterized glial injury in the CA1 and found the number of astrocyte to gradually decline between 4-8 weeks following injury, although programmed cell death was not detected in astrocytes. In another report, Petito et al. (1998) analyzed glial death in several brain regions following 10 min global ischemia and while selective glial cell death was observed in the cortex and thalamus, this was primarily confined to oligodendrocytes. Scattered astrocytes with apoptotic bodies and deoxyribonucleic acid fragmentation were identified in those regions but the process preceded neuronal damage by several days.

In contrast, other researchers reported functional changes rather than a loss in viability in CA1 astrocytes following ischemia (Ouyang, et al., 2007). Thus, astrocytes have been documented to respond to brain injury by undergoing a process referred to as reactive astrogliosis, which is marked by changes in molecular, cellular and functional mechanisms (Sofroniew & Vinters, 2010). For instance, following ischemia, astrocytes' morphology is markedly altered as they become reactive and this is often noted by thickened and retracted processes and an enlarged soma (Panickar & Norenberg, 2005). Furthermore, increased proliferation of reactive astrocytes to the site of injury along with a sustained and progressive increase in levels of the cytoskeletal protein, glial fibrillary acidic protein (GFAP) in astrocytes have been reported (Panickar & Norenberg, 2005; Petito & Halaby, 1993). The tissue increase in GFAP immunoreactivity is currently taken as an index of reactive astrocytosis following brain insults, including ischemia (Cechetti, Pagnussat, et al., 2012; Eng, Ghirnikar, & Lee, 2000; Petito & Halaby, 1993). This process is associated with loss of the astrocytes' protective functions, which includes active removal of glutamate from the synaptic cleft via glutamate transporters. Indeed, glutamate uptake is the main mechanism by

which astrocytes alleviate neuronal injury and failure of uptake and/or glutamate release by impaired astrocytes has been proposed to contribute to delayed neuronal death following ischemia (Swanson, et al., 2004).

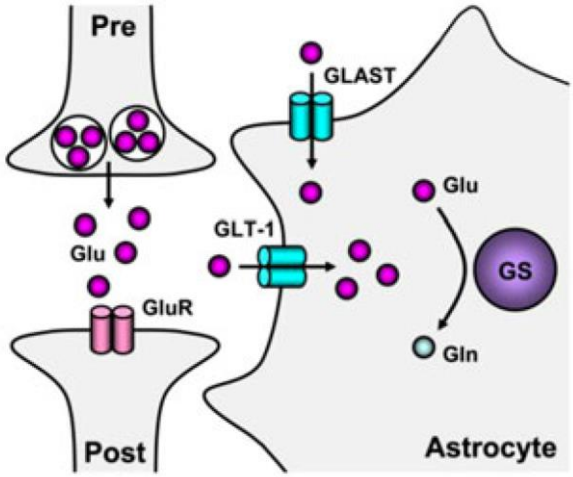


Figure 2. Schematic drawing of the synaptic cleft and surrounding cells. Glutamate (*Glu*) is stored in synaptic vesicles in presynaptic nerve terminals (*Pre*) and can be released into the synaptic cleft. Glutamate can activate glutamate receptors (*GluRs*) on postsynaptic cells such as postsynaptic neurons (*Post*) and transmit signals. During physiological conditions, the glutamate is cleared from the synaptic cleft mainly by the astroglial glutamate transporters GLT-1 and GLAST (glutamate-aspartate transporter) to prevent excitotoxicity due to prolonged activation of glutamate receptors. Astrocytes metabolize glutamate into glutamine (*Gln*) using glutamine synthetase (*GS*), which can then be safely transported back to neurons. Adapted from "Microglial self-defence mediated through GLT-1 and glutathione," by M. Persson and L. Ronnback, 2012, *Amino Acids, 42*, p. 207-19.

5.3 Astrocyte regulation of excess glutamate following cerebral ischemia

Glutamate accumulation within the synaptic cleft has been implicated in excitotoxic cell death in a number of pathologies and disorders including cerebral ischemia (Carbone, Duty, & Rattray, 2012). Upon reuptake by astrocytes, glutamate is converted by glutamine synthetase into glutamine (Fig.2), which is subsequently released into the synaptic cleft and uptaken by neurons as a precursor of newly synthesized glutamate; a process referred to as the "glutamate-glutamine cycle" (Quincozes-Santos & Gottfried, 2011). The surge in

extracellular glutamate following cerebral ischemia massively activates ionotropic glutamate receptors and initiates calcium overload in nearby nerve cells, leading to neuronal death through several pathways (Ketheeswaranathan et al., 2011). Astrocytic regulation of extracellular glutamate levels thus represents an important process, which can help minimize glutamate excitotoxicity (D. W. Choi, 1988; Swanson, et al., 2004).

In the healthy brain, efficient neurotransmission also involves high-affinity glutamate transporters located on both neurons and glia (Rothstein et al., 1996). In rodents, three high affinity glutamate transporter subtypes have been isolated: GLAST, expressed in astrocytes, GLT-1, predominantly expressed in astrocytes but also in microglia (Persson & Ronnback, 2012), and EAAC-1 (excitatory amino acid carrier-1), predominantly found in neurons (Ketheeswaranathan, et al., 2011). Akin to astrocytes, these transporters play an active role in clearing glutamate from the extracellular space and maintaining concentrations below neurotoxic levels, sustaining homeostasis (A. J. Liu, Hu, Li, Xu, & Zhang, 2011).

Glial GLT-1 is the principal subtype of glutamate transporters predominantly expressed on the pre- and post-synaptic glial cell membranes of the cerebral cortex and hippocampus (Yeh et al., 2005). GLT-1 plays a key role in terminating synaptic transmission of glutamate and mediates 90% of glutamate uptake (Danbolt, 2001). Chronic inhibition of glutamate transporters has been shown to reduce clearance of synaptic glutamate and promote excitotoxic neuronal damage (J. C. Chen et al., 2005). Moreover, knockout of the glial transporters GLT-1 and GLAST, but not the neuronal transporter excitatory amino acid carrier-1, leads to increased extracellular levels of glutamate and neuronal damage (K. Tanaka et al., 1997). Following forebrain ischemia, a number of reports have revealed a down regulation of GLT-1 mRNA expression and GLT-1 immunoreactivity in affected

regions. For instance, decreased GLT-1 protein and mRNA levels were observed in the CA1 region following global cerebral ischemia while GLAST protein levels remained unaffected (Yeh, et al., 2005). Similarly, GLT-1 mRNA expression is reduced in the ipsilateral hippocampus and cortex for at least 24 h following focal ischemia as compared to sham-operated mice (Ketheeswaranathan, et al., 2011). Downregulation of GLT-1 expression was also observed in the CA1 following perinatal hypoxic-ischemic injury in neonatal rats (Zhao, Cheng, Ou, Chen, & Ruan, 2012) as well as within 12 h of a 20 min of global cerebral ischemia in rats, while GLAST and EAAC1 levels remained unchanged in the latter condition (J. C. Chen, et al., 2005), suggesting reduced sensitivity of these two transporters to drastic glutamate surge. In support of such contention, EAAC1 knockout failed to affect neuronal survival over a 12-month period (Peghini, Janzen, & Stoffel, 1997) and infusion of an EAAC1 oligonucleotide antisense also had no effect on infarct size and neurological status following focal ischemia in rats (Rao et al., 2001). In a follow-up study the same group however reported decreased EAAC1 protein levels at both 24 h and 72 h postischemia, raising some questions as to possible involvement of EAAC1 in neuronal fate postischemia (Rao, Bowen, & Dempsey, 2001).

To date, no study has investigated the effect of RSV treatment on preventing ischemia-induced down-regulation of GLT-1 in vivo. However, in vitro, RSV has been shown to increase glutamate uptake by cortical astrocyte cultures at low doses, while inhibitory effects were noted with elevated levels of the compound, prompting the authors to attribute a protective role of RSV in pathologies marked by glutamate toxicity while cautioning against dose-related effects (de Almeida, Leite, et al., 2008; de Almeida et al., 2007). Thus, an essential role of glial cells and GLT-1 is suggested in attenuating excitotoxic

cell death. Moreover, region-specific changes could be indicative of a role of these endogenous factors in RSV- mediated neuroprotection.

6. Methodology, research questions, and series of thesis experiments

The current thesis selected a global cerebral ischemia model as it offers ecological validity in the study of the disease process using animals (Allen & Buckberg, 2012; Chiota, et al., 2011; Hossmann, 2008). This animal model also shares the same physiological pathways in inducing neuronal damage as other stroke models and excitotoxic conditions (such as epilepsy), making neuroprotective effects likely to generalize to other disease conditions (Plamondon, Blondeau, Heurteaux, & Lazdunski, 1999). To date, the search for neuroprotective agents with specific neuronal targets has proven unsuccessful in clinical trials. Efforts are now turning towards identification of therapeutic tools (pharmaceutical agents or dietary supplements) with wide-ranging biological activities, which could provide neuroprotection and behavioural/cognitive improvement, patients mentioning the latter as being most important. In this context, RSV's ability to regulate a number of physiological mechanisms makes it susceptible of improving neuronal and behavioural outcome following global ischemia.

6.1 Thesis research objectives

Through a series of experiments the current study addresses three main objectives, which evaluate prophylactic effects of RSV in the context of global cerebral ischemia.

The first thesis objective assessed neuroprotective effects of RSV in preventing hippocampal neuronal cell injury and determined contributions of RSV to behavioural outcome following cerebral ischemia (Article 1).

The second objective characterized possible mechanisms of action underlying RSV's effects on neuroprotection and/or behaviour using immunohistochemical detection of progenitor cell differentiation and angiogenesis at short and long reperfusion intervals postischemia (Article 2). Given the documented effect of global cerebral ischemia on the hypothalamic-pituitary-adrenal (HPA) axis sensitivity (de la Tremblaye, Raymond, Milot, Merali, & Plamondon, 2014; M. R. Milot & Plamondon, 2011), which could impact brain plasticity and behaviour, we investigated the effects of RSV on corticosterone (CORT) secretion 3 days postischemia and at various time intervals following exposure to an acute stressor. Lastly, neurogenesis being associated with hippocampus-dependent learning and memory, the effect of RSV on spatial learning in the Morris water maze (MWM) was determined.

The third objective (Article 3) aimed to investigate possible effects of RSV in mediation of glial cell and GLT-1 activation in the hippocampus 7 days postischemia, which could underlay its neuroprotective actions. Given the scarce information available on the polyphenol's actions in healthy populations, the inclusion of a control group treated with RSV at a concentration of 10 mg/kg enabled further describing RSV's effects on the regulations of GLT-1 under normal conditions.

The general hypothesis of the current thesis stipulates that in order for RSV to be considered a promising prophylactic therapy against global cerebral ischemia, the compound needs to provide significant neuronal protection and/or improvement of cognitive impairments. Existing literature on RSV supports numerous mechanisms of action for the polyphenol in a number of disease models including stroke, epilepsy, diabetes, Alzheimer's and Parkinson's disease (Ates et al., 2007; Y. K. Gupta, Briyal, & Chaudhary, 2002; Huber

& Superti-Furga, 2011; Sharma & Gupta, 2002; Tsai et al., 2007). In that light, we expect to observe a) reduced neuronal injury (that is maintained at long reperfusion intervals) and beneficial effects on behavioural impairments following cerebral ischemia b) dose- and time-related effects c) important effects of the polyphenol in mediating neuroplasticity following ischemia, as well as effects on non-neuronal populations of cells, possibly mediated through GLT-1 expression.

The following sections briefly describe the rationale and experimental procedures of the current thesis' sets of experiments.

6.2 Article 1: Repeated resveratrol administration confers lasting protection against neuronal damage but induces dose-related alterations of behavioural impairments following global ischemia

The first study was designed to investigate possible effects of RSV on providing neuroprotection and alterations of behavioural impairments. To date, few studies have assessed effects of RSV on behavioural recovery and it remains to be determined whether neuronal protection is paralleled by recovery of cognitive function following global ischemia. Moreover, recent reports suggest dose-dependent effects of the polyphenol, often characterized by bi-directional effects, leaving some researchers wondering whether RSV belongs to a class of hormetic compounds (Calabrese, Mattson, & Calabrese, 2010). Hormesis is an expression employed by toxicologists to describe a U-shaped (or J-shaped) dose-response characterized by a beneficial effect at low doses and toxic (or inhibitory) activity at high doses. Lastly, little is known about the effects of repeated RSV administration in healthy individuals, with measures limited to metabolic and biochemical assessments

(Simao, et al., 2011) while recent evidence points to possible consequences in healthy subjects that deserve further investigation (H. R. Park, et al., 2012).

In this initial experiment, rats were administered RSV daily for 21 days prior to global cerebral ischemia at two doses (1 or 10 mg/kg). Neuronal injury assessment was performed in the CA1 and CA3 regions of the hippocampus at 7 and 85 days post-reperfusion. Behavioural and cognitive effects of RSV treatment were determined using the open field, eight-arm radial arm maze and object recognition tests. Administration of the 10 mg/kg RSV dosage to a group of sham-operated rats allowed determination of intrinsic actions of the drug on behavioural and memory performance.

6.3 Article 2: Dose-related effects of chronic resveratrol administration on neurogenesis, angiogenesis and corticosterone secretion are associated with improved spatial memory retention following global cerebral ischemia

The second experiment was designed as a follow-up to the results of the first set of findings. RSV-induced neuroprotection against global cerebral ischemia as well as dose-related effects of the polyphenol on behavioural measures set the stage for possible compensatory mechanisms in the form of neuroplastic changes occurring following ischemia as well as behavioural recovery at a long post-ischemic interval. Neuroplastic changes post-insult have been documented in a number of ischemic models in the form of enhanced neurogenesis as well as changes in angiogenesis levels in the hippocampus (Beck & Plate, 2009; Lagace, 2012). Importantly, such alterations have recently been associated with partial behavioural recovery following ischemia (Bendel, et al., 2005). To date, few studies have investigated the effects of RSV pretreatment on plastic brain changes and concomitant behavioural changes following ischemia. As such, post-mortem immunohistochemical

assessment of neurogenesis and angiogenesis was performed 7 and 85 days following 10 min global cerebral ischemia. Given the documented effects of ischemic injury on HPA axis hypersensitivity and demonstrated contribution of such changes to behavioural impairments, the effect of RSV on CORT secretion was determined at multiple time intervals following exposure to an acute stressor 72 h postischemia. Lastly, neurogenesis having been associated with improved hippocampus-dependent learning and memory, the effect of RSV on spatial learning in the MWM was also determined.

6.4 Article 3: Chronic resveratrol pretreatment modulates glial cell activation and GLT-1 expression following global cerebral ischemia in rats

The last experiment aimed to breach the gap between RSV neuroprotective effects and propose actions of the polyphenol on inflammatory processes in part mediated by changes affecting non-neuronal populations of cells. As earlier noted, a well-documented inflammatory cascade is induced following cerebral ischemia, characterized by activation of the glial cell population, including microglia and astrocytes, and downregulation of GLT-1 thought to contribute to glutamate excitotoxicity and lead to the selective neuronal injury observed in the hippocampal CA1 region. In view of beneficial role of GLT-1 in preventing ischemia-induced neuronal death and evidence of increased glutamate uptake by astrocytes in vitro following treatment with RSV (de Almeida, et al., 2007), we hypothesized that upregulation of GLT-1 will accompany CA1 neuronal protection induced by RSV in ischemic animals. At present, there are no studies evaluating a possible relationship between RSV effects on gliosis and changes in GLT-1 expression following global ischemia. The current study therefore assessed the impact of in vivo chronic pretreatment with RSV on glial

cell activation 7 days following global cerebral ischemia and concomitant changes in GLT-1 expression within the CA1, CA3 and DG regions.

7. Scientific contribution of the proposed work

The contributions of the current studies lie in the characterization of RSV-mediated effects on important physiological regulators of neuronal and behavioural effects following global cerebral ischemia in the rat. These include assessment of long-lasting effects on neuronal viability and correlated dose-related effects on cognitive and behavioural tests. Furthermore, our work is the first to characterize novel biological targets as possible mechanisms of action of RSV, including effects on neurogenesis, angiogenesis, glial cell activation and GLT-1 expression, which will provide key findings helping better define future investigations. Finally, the characterization of dose-related effects and inclusion of short and long post-ischemic intervals help describe a comprehensive profile of RSV actions in control and ischemic animals, which represents an advancement in obtaining fundamental observations lending to the development of compelling treatment options for patients and care-providers.

In recent years, attempts to find prophylactic or clinically efficacious therapies against cerebral ischemia have remained unsuccessful, promising pharmacological treatments being restricted from clinical use by significant side effects and narrow therapeutic windows. The need for development of effective preventative therapies therefore remains a high priority. It has been suggested that in order for pharmacological compounds to be successful in clinical trials they need to provide significant neuroprotection paired with cognitive and behavioural recovery (J. T. Neumann, et al., 2013). One could debate that the latter is the

most important factor from a patient's perspective. At present, it is thought that for a compound to achieve such high standard of performance, its action need to affect multi-biological targets. The growing interest of the population in natural products as prophylactic therapy for various diseases has set RSV as an interesting candidate considering repeated demonstrations of the polyphenol's actions on numerous biological targets in a variety of disease models.

Article 1

Published as: Girbovan, C., & Plamondon, H. (2012). Repeated resveratrol administration confers lasting protection against neuronal damage but induces dose-related alterations of behavioural impairments following global ischemia. *Behav Pharmacol*, *23*(1), 1-13.

Abstract

Resveratrol, a naturally occurring polyphenol, has been shown to protect the heart and brain against ischemic injury. The current study investigated the effects of administration with either a 1 or 10 mg/kg dose of resveratrol on CA1 neuronal injury and behavioural/cognitive impairments following 10 min global ischemia in rats. The open field, 8-arm radial maze and object recognition tests served to evaluate effects of resveratrol treatment on ischemia-induced locomotor activity, and spatial and recognition memory impairments, respectively. CA1 and CA3 neuronal injury was assessed upon completion of behavioural testing 85 days following ischemia. A separate series of animal groups served to assess neuronal injury at 7 days postischemia. Ten minutes global ischemia led to ~50% CA1 cell injury, which was prevented at both short (7 days) and long (85 days) post-ischemic intervals by resveratrol treatment. Importantly, despite comparable neuronal protection, the two resveratrol doses showed distinct behavioural effects. Thus, the 10 mg/kg resveratrol dose led to enhanced locomotor activity in the open field 4 days postischemia and impaired spatial memory in the delayed non-matching-to-sample (DNMTS) and delayed matching-to-sample (DMTS) radial maze tasks initiated on day 13 postischemia. These findings suggest independent actions of resveratrol on distinct physiological systems mediating cellular survival and functional recovery and dose-related actions of the polyphenol on behavioural and memory processes.

Keywords: resveratrol; spatial memory; CA1 neuronal injury; cerebral ischemia; rat.

1. Introduction

The naturally occurring polyphenol phytoalexin resveratrol (RSV) has been shown to exert a number of biological benefits in various experimental paradigms including antioxidant, cardioprotective, antiaging, anti-inflammatory and anticarcinogenic actions (Fremont, 2000). In addition, in vivo and in vitro studies have demonstrated protective effects of RSV in multiple disease models, including stroke (Dong, et al., 2008; K. T. Lu, et al., 2006; Simao, et al.; Tsai, et al., 2007; Q. Wang, et al., 2009) epilepsy (Y. K. Gupta, et al., 2002; Wu et al., 2009), diabetes (Ates, et al., 2007), Alzheimer's (Sharma & Gupta, 2002; J. Wang et al., 2006) and Parkinson's disease (Huber & Superti-Furga, 2011; Y. Wang, et al., 2011; F. Zhang, J. S. Shi, et al., 2010). These effects have partly been attributed to RSV's antioxidant and anti-inflammatory activities in the injured brain (Q. Wang, et al., 2002). Similarly, inhibition of lipid peroxidation, upregulation of nitric oxide (NO) synthase and increased vasorelaxation have been associated to RSV cardioprotective effects (Bradamante, et al., 2004; Miatello et al., 2005).

Recently, different studies have demonstrated neuroprotective effects of RSV administration in a number of ischemic models. For example, RSV administration for 21 days prior to middle cerebral artery occlusion (MCAO) in rats was shown to significantly decrease infarct volume compared to controls (Sinha, et al., 2002) while a 7-day RSV pretreatment significantly attenuated CA1 neuronal injury and glial activation following global ischemia (Simao, et al., 2011; Q. Wang, et al., 2009). These findings suggest that RSV represents a novel agent promoting neuronal survival post-stroke (Bradamante, et al., 2004), which could have significant implications in cognitive recovery.

In rodents, global forebrain ischemia leads to selective and delayed degeneration of CA1 pyramidal neurons of the hippocampus, initiated ~48 h postischemia (Kirino, 1982; Pulsinelli, et al., 1982) and that appears maximal at 7 days (Nikonenko, Radenovic, Andjus, & Skibo, 2009; Taoufik & Probert, 2008). CA1 neuronal loss leads to well-documented behavioural impairments, notably affecting spatial memory as assessed by the Morris water maze (Sandstrom & Rowan, 2007) as well as standard and alternate versions of radial arm maze tasks (Nelson, Lebessi, Sowinski, & Hodges, 1997; Roberge, Messier, Staines, & Plamondon, 2008).

To date, there are few studies that have investigated the effect of RSV on behavioural recovery and it remains to be determined if neuronal protection is paralleled with recovery of cognitive function following global ischemia. Furthermore, little is known of the effect of repeated administration of RSV in healthy animals, and measures are limited to metabolic and biochemical assessments (Simao, et al., 2011). Finally, neuronal assessments have been limited to short reperfusion intervals, ranging from a few hours to 7 days postischemia (Simao, et al., 2011; Q. Wang, et al., 2002) and whether the protection can be maintained over weeks remains unknown.

Thus, the current study had two main goals. The first objective aimed to assess the effects of daily RSV administration for 21 days prior to global cerebral ischemia at two doses (1 or 10 mg/kg; (Mukherjee, Dudley, & Das, 2010) on neuronal injury in the CA1 and CA3 regions of the hippocampus observed at short and long reperfusion intervals (7 and 85 days, respectively) following 10 min global ischemia in rats. The second objective aimed, using the open field, 8-arm radial maze and object recognition tests, to determine behavioural and cognitive effects of RSV treatment in control animals as well as alterations of post-ischemic

impairments. Administration of the 10 mg/kg RSV dose to a group of sham animals allowed determining intrinsic actions of the drug on behavioural and memory performance.

2. Materials and Methods

2.1 Subjects

Male Wistar rats (N = 36 and 53 for the short and long reperfusion intervals, respectively) weighing between 100-125g at time of arrival to the animal facility (325-375g at time of surgery) were obtained from Charles River Laboratories (Rochefort, Quebec, Canada). Upon arrival, animals were individually housed and maintained on a 12 h light/dark cycle (lights on at 7:00 AM), with free access to water and standard (Purina) rat chow. Room temperature was maintained at 21-23°C with 60% relative humidity. One week following arrival, animals were randomly assigned to five experimental groups and chronic injections initiated: ischemia + saline (n=10), sham + saline (n=10), ischemia + 1 mg/kg RSV (n=12), ischemia + 10 mg/kg RSV (n=11) and sham + 10 mg/kg RSV (n=10). Four weeks following the rats' arrival and upon completion of drug treatment, sham or ischemic surgeries were performed. An additional 35 rats were randomly assigned to the same five experimental groups but killed 7 days following sham or ischemic surgery (short-term reperfusion interval): ischemia + saline (n=7), sham + saline (n=6), ischemia + 1 mg/kg RSV (n=8), ischemia + 10 mg/kg RSV (n=8) and sham + 10 mg/kg RSV (n=6). The surgery and injection schedule was identical to that of the long-term group with the exception of behavioural testing. Twenty-five rats out of 81 rats operated in the ischemic groups died during the ischemic surgery. The animal numbers indicated above represent rats remaining in each experimental group. All experimental procedures were in accordance with the guidelines set by the Canadian Council of Animal Care and approved by the University of Ottawa Animal

Care and Ethics Committee. Efforts were made to minimize the number of animals used and their suffering.

2.2 Resveratrol administration

RSV (Sigma, St. Louis, MO, USA) was dissolved in 50% ethanol and separated in 50 µg aliquots, which were lyophilized and then stored at -80^{o}C until use. Aliquots were freshly dissolved daily in a vehicle consisting of 0.9% saline solution containing 20% hydroxypropyl h-cyclodextrin (Sigma, St. Louis, MO, USA). Twenty-one days before the surgery, sham and ischemic animals received daily, between 9:00-11:00 h, an intraperitoneal injection of either saline or 1 or 10 mg/kg RSV (injection volume was 0.1 ml/100 g rat weight). The last saline or RSV injection was administered 1 h prior to ischemic or sham surgery. Rats were weighted daily and the injection volume adjusted to correspond to the animal's body weight. The RSV doses were selected based on earlier reports showing beneficial effects in different experimental paradigms (Mukherjee, et al., 2010; Sinha, et al., 2002).

2.3 Transient forebrain ischemia

Forebrain ischemia was performed using the four-vessel occlusion model as previously described (Pulsinelli & Brierley, 1979). Briefly, rats were deeply anesthetized by inhalation of 1.5% halothane in oxygen (1.5 to 2L/min). Vertebral arteries were irreversibly occluded by electrocoagulation, and a small-diameter silk thread looped around the carotid arteries to facilitate subsequent occlusion. Twenty-four hours later, common carotid arteries were occluded with microaneurysm clamps for 10 minutes (min) in spontaneously ventilating animals. Sham-operated animals underwent anaesthesia and received the same dorsal and ventral surgical incisions as the ischemic groups, with the exception of electrocoagulation of the vertebral arteries. Twenty-four hours later, carotid arteries were exposed, but not

clamped. Only rats that lost the righting reflex over the entire occlusion period were included in the study. Core temperature was kept at 37°C ± 0.5 throughout the surgery using a feedback-regulated heating blanket connected to a rectal thermometer (Homeothermic Blanket Control Unit, Harvard Instruments, Natick, MA). Rats' body temperature was further supported with a heating pad in the hours following surgery and reperfusion. One day prior to behavioural testing, the pupillary reflex was assessed in all animals to determine visual system/retinal functioning possibly impacting vision during behavioural testing. Animals were transported to a dark room and left to habituate for a minimum of 15 min. The pupillary reflex was examined by illuminating the rats' dark-adapted eyes with a mini-flashlight producing a focused light beam. Following examination of the first eye, chosen at random, an additional 60 seconds (s) in the dark was imposed prior to examining the second eye. The reflex was considered intact if constriction of both pupils in response to the light occurred under 10 s. All ischemic and sham-operated rats displayed a normal pupillary reflex, typically occurring within seconds of light stimulus.

2.4 Blood glucose measurements

Basal glycemic levels were assessed by the tail-bleeding method using Bayer's CONTOUR® meter (Bayer Canada). Glucose levels (in mmol/L) were assessed 1 minute prior to sham or ischemic occlusion and 3, 7 and 85 days following reperfusion. Assessments were performed between 9:00-10:00 h.

2.5 Apparatus and procedures for behavioural testing

Behavioural testing was initiated 4 days post-surgery (refer to Fig.1 for experimental timeline) and conducted in the light portion of the light/dark cycle.

Open field test

On days 4 and 56 following reperfusion, sham and ischemic subjects were placed in the open field and behaviour was monitored for a 15-min period. The observation arena was made of gray Plexiglas (LWH: 75×75×30 cm) with a clear Plexiglas floor. A painted grid divided the Plexiglas floor into 25 identical squares each measuring 10×10 cm. The entire arena was kept on a table 90 cm above the floor in the same room as the rats' housing. Subjects were monitored for frequency of line crossing, grooming and rearing behaviour by the experimenter using a portable PC computer (Toshiba 4250) and data logging software. To control for differences attributable to testing days, animals of all five groups were randomized so that rats of each group participated to each testing session.

Object recognition test

On the fifth and sixth day following reperfusion, rats were tested in the object recognition test, a memory test based on the natural propensity of animals to spend more time exploring a new rather than a formerly encountered object. Recognition memory was evaluated at two retention intervals (30 min and 24 h). Rats were transported from the animal vivarium to the testing laboratory and allowed to rest for at least 30 min before behavioural testing began. Testing was monitored by an overhead camera, and behavioural measures quantified online using data logging software (ODlog 2.0). The test was performed in the open field arena as previously described (Plamondon, Morin, & Charron, 2006). Briefly, each rat was exposed to three experimental conditions: in the initial trial (T1), one object-stimulus (O1) was placed in one corner of the open field and the rat positioned in the opposite corner of the arena. The time spent exploring the object (i.e., touching the object

with paws or exploring it by olfaction with direct contact of the snout) was measured. The session ended when the animal explored the object for 20 s or when 10 min had elapsed. During the second trial (T2), performed 30 min following T1, a second object (O2) was introduced in the adjacent corner to that of the reference object. The time spent exploring the familiar (O1) and the novel (O2) objects was measured for a period of 10 min. In the final trial (T3), performed 24 h following T1, O2 was replaced by a new object (O3) and the time the rat spent exploring the reference (O1) and novel (O3) objects was measured for 10 min. From rat to rat, the object presentation order was randomly permuted. The arena and objects were extensively cleaned before each trial in order to eliminate any olfactory cues. The objects consisted of plastic toys heavy enough to prevent the animals from moving them. Objects were carefully selected to elicit a similar level of exploration from animals.

Radial arm maze

Delayed-non-matching- and matching- to-sample (DNMTS; DMTS) radial arm maze.

Apparatus. The radial maze consisted of eight arms (60 × 12 cm with a 5 cm lip around each arm) extending radially from a central octagonal area (32 cm in diameter with a 30 cm high clear Plexiglas wall). Plexiglas sliding doors allowed entry into each arm. The floor of the arms and central area were covered with black rubber lining. The apparatus was elevated 50 cm above the floor and surrounded by extra-maze cues such as posters or calendars along the sidewalls. The experimenter sat behind a panel where he could observe and record behaviour unobtrusively and manipulate the overhead strings to open and close

the maze doors. At the end of each arm, a food cup (1 cm deep into the floor) contained a reward (a piece of Fruit Loop).

Training procedures. From day 6 to day 12 postischemia, rats began training in the radial maze. Daily food ration of animals was gradually reduced (over a 4-day period) and rats maintained at approximately 85% of their free-feeding rate during the testing period to ensure adequate motivation. Rats were familiarized with the radial arm maze during four daily sessions each lasting 10 min on successive days. Rewards were initially available throughout the maze to encourage exploration, but were gradually restricted to the food cups. Each animal was individually placed in the center of the maze with the doors to all arms closed. Upon opening the doors, the rat was permitted to enter any of the eight arms. Upon entry in any one arm, all other maze arms were closed. When the rat had consumed the reward at the end of one arm and returned to the center of the maze, all doors remained closed to confine the rat to the center zone of the maze for a 10 s delay. The doors were then reopened, and the procedure repeated. The test continued until all baits were consumed or until 10 min had elapsed**.** An arm entry was counted when the rat had its four paws within an arm. The orientation of the animal's head when placed into the central area was randomly permuted from trial to trial to minimize the development of a response pattern based on position. The floor of the maze's eight arms and central area was cleaned with a 70% ethanol solution after each trial to reduce olfactory cues.

DNMTS tasks: win-shift. The DNMTS task was initiated 13 days postischemia, and consisted in daily acquisition and retention trials separated by a 15-min delay. In the acquisition trials, four arms were blocked with transparent Plexiglas doors. Rats were free to

navigate to the four available arms, which were baited with a small piece of Fruit Loop. After consuming all food rewards (or when 10 min had elapsed), rats were removed from the maze and returned to their home cage for 15 min. After this delay, rats were placed back in the clean maze with a different body orientation for the retention trial. At this stage, all eight arms were available for entry, but only those previously blocked, contained a reward. At the completion of each day's retention trial, rats were given their daily food ration. The arms blocked in the acquisition trials were individually and randomly assigned to rats from a list of 30 possible patterns, and arm sequences were counterbalanced across groups. One sequence was assigned to each animal and maintained for the 15-day testing period. Following this period, rats were tested for an additional three days in a DNMTS task using the same arm sequence but a different inter-trial delay on each day (5, 30 or 240 min). Also, to determine if the rats had learned rules involved in the DNMTS task rather than simply memorized the arm sequences, we tested rats for an additional six days using the same methodology but novel arm sequences. Measures recorded in the acquisition trial (pre-delay) included total number of working memory errors and latencies to complete the task. In the retention trials, the error score was further divided into retroactive errors (entry into a non-rewarded arm), and proactive errors (re-entry into an arm within the test trial).

DMTS task: win-stay. Following the DNMTS tasks, animals underwent a DMTS task for an additional 8 days using a new arm sequence and a 15-min inter-trial delay. In this paradigm, animals were rewarded for selecting previously baited arms in the retention trials. Measures recorded in the acquisition and retention trials of the DMTS were identical as the DNMTS.

2.6 Analysis of neuronal density on thionin-stained sections

Eighty-five days following reperfusion, rats were deeply anesthetized using sodium pentobarbital and perfused using 0.9% saline followed by 4% paraformaldehyde solution. Brains were removed and stored at -80°C. Serial coronal sections (14 µm) of the hippocampal regions were subsequently obtained using a cryostat and stained for Nissl bodies with thionin. Neuronal density of the hippocampal CA1 and CA3 subfields was determined using the method of Kirino et al. (1991) and performed on coronal sections located between 3.14 and 4.16 mm posterior to bregma (Paxinos & Watson, 1986). The total linear length of the CA1 and CA3 sectors (as defined by Paxinos and Watson) was measured by means of a digitizer. The number of intact neurons in the stratum pyramidale within CA1 and CA3 subfields was counted using a Leica DAS microscope attached to a Sony digital camera and computer-assisted cell counting was performed using Norton Eclipse (v 6.0). Neurons that had shrunken cell bodies with surrounding empty spaces were excluded. The neuronal density of the CA1 and CA3 sectors**,** ***i.e.*** the number of intact pyramidal cells per 1 mm linear length of stratum pyramidale was quantified by a person that was blind to the treatment conditions. A mean value for each hippocampal substructure was obtained from six bilateral measurements per animal in each of the experimental groups. The neuronal density for a given animal represents the average of both the right and left hippocampal measures.

3. Data analysis

All statistical analyses were conducted using PASW Statistics 18.0 software package. A difference was considered significant when $p \leq .05$. On rare occasions, rats showing two standard deviations from the group average performance were considered outliers and were excluded from the related statistical analysis. The assumptions of homogeneity of variance

and of sphericity were verified. The Huynh-Feldt correction for violations to the assumption of sphericity was applied when appropriate and the degrees of freedom adjusted when the correction was used. Significant interactions were further analyzed using simple effects tests with Bonferroni modification of critical alpha level. Body weight data and blood glucose levels were analyzed using mixed a mixed analysis of variance (ANOVA) design, with two independent factors *surgery* and *treatment* and the repeated factor *time*. Behavioural activity in the open field (calculated from frequencies of line crossing and the time spent rearing and grooming) was assessed in three blocks of 5 min each (total = 15 min) using a mixed ANOVA design, with two independent factors *surgery* and *treatment* and the repeated factor *time*. One-way ANOVAs followed by LSD post hoc analyses were used to test for between-group activity level differences for each time interval. In the object recognition test, raw data obtained were transformed into a ratio, reflecting the preference of the animals for the novel versus the familiar object. The ratio formula was [tnovel/(tnovel + tfamiliar)], where tfamiliar is the time spent exploring the familiar object and tnovel is the time spent exploring the new object, in seconds (Gaskin et al., 2010). The closer this ratio gets to 1, the more the animal spent time exploring the novel object. Two factor (*surgery* and *treatment*) ANOVAs were performed on the dependent measure at each time interval to assess group differences. The data were analyzed as partially crossed design taking into account the two factors being assessed but also the administration of the 1mg/kg RSV dose to a unique experimental group (i.e., the ischemic animals). Radial arm maze data were analyzed using mixed ANOVA designs with two independent factors *surgery* and *treatment* and various levels of the repeated factor *time*. Data obtained from neuronal densities in the CA1 and CA3 regions of the hippocampus were analyzed using two factor (*surgery* and *treatment)* ANOVAs. Simple

effects tests were used to further analyze significant interactions. In the radial arm maze, data are expressed as total errors (±S.E.M.) for blocks of either 3 days (15-day DNMTS task) or 2 days (DNMTS; *novel sequence* and DMTS). Data from the different inter-trials delays in the DNMTS tasks were analyzed separately. Values are expressed as mean ±S.E.M. for all tests.

4. Results

4.1 Body weight

Body weight values from both short- and long-term studies were combined to assess possible effects of RSV treatment on the animals' body weight. Analysis revealed no effect of treatment ($F(2, 80) = 1.48$, $p > .05$) but a main effect of time ($F(3.53, 282.7) = 3535.57$, $p < .001$), attributable to increased body weight over time (data not shown).

4.2 Blood glucose level

Fig. 2 presents the mean blood glucose level of each rat group collected at baseline and 3, 7 and 80 days post-surgery. Values from the short- and long-term groups were combined for the initial three collection intervals. There was a main effect of time ($F(3, 135) = 9.94$, $p < .001$), attributable to slightly higher glucose levels at baseline than at any other time point ($p < .05$), yet baseline values remained within normal glycemic range.

4.3 Neuronal density assessment

Fig. 3 shows the effect of RSV treatment and 10 min global ischemia on hippocampal CA1 and CA3 neuronal densities 7 and 85 days following ischemia. In the short-term groups, there were main effects of surgery ($F(1, 30) = 49.59$, $p < .001$) and treatment ($F(2, 30) = 9.35$, $p < .005$) and a significant surgery × treatment interaction ($F(1, 30) = 10.32$, $p < .005$). Simple effects tests indicated that saline-treated ischemic rats had significantly less CA1

hippocampal neurons than their sham-counterparts, irrespective of treatment ($p = .001$) and that RSV-treated ischemic groups had significantly more CA1 neurons than ischemic rats treated with saline ($p < .005$, for both the short- and long-term experiments). In the long-term groups, there were main effects of surgery ($F(1, 46) = 24.83$, $p < .001$) and treatment ($F(2, 46) = 11.37$, $p < .001$) and a significant surgery × treatment interaction ($F(1, 46) = 10.67$, $p < .005$). Simple effect tests indicated that saline-treated ischemic rats had significantly less CA1 hippocampal neurons than saline-treated sham counterparts ($p = .001$), ischemic rats treated with 1 mg/kg ($p = .001$) and 10 mg/kg ($p = .001$) RSV and shams treated with 10 mg/kg RSV ($p = .001$). Simple effects also indicated that both RSV dosages significantly attenuated ischemia-induced CA1 neuronal damage ($p = .005$) compared to saline-treated animals. No significant differences in the number of CA3 neurons were observed among the groups.

Behavioural tests

4.4 Open field (4 days postischemia).

Fig. 4 (top row) highlights the total number of squares crossed by each experimental group during each of the three 5-min intervals. With regards to locomotion, there was a main effect of time ($F(1.8, 81.16) = 41.99$, $p < .001$), treatment ($F(2, 45) = 5.75$, $p < .01$) and a significant time × treatment interaction ($F(3.21, 81.16) = 3.18$, $p < .05$). Simple effects revealed that during the initial 5-min interval, animals treated with 10 mg/kg RSV explored significantly more than saline and 1 mg/kg-treated animals ($p = .001$), irrespective of surgical treatment. Exploration also diminished with time with all animals showing significantly decreased locomotion in the last 5 min of the test than at any other time interval ($p < .01$). With regards to rearing (Fig. 4, second row), mixed ANOVA revealed a main effect of time

($F(1.59, 3796.13) = 59.9$, $p < .001$), surgery ($F(1, 45) = 4.24$, $p < .05$) and treatment ($F(2, 45) = 12.86$, $p < .01$). This was attributable to ischemic animals spending significantly more time rearing than sham rats. Post hoc analyses also revealed that ischemic animals treated with 1mg/kg RSV spent significantly less time rearing than saline- and 10 mg/kg RSV-treated rats ($p < .01$ and $p < .001$, respectively). With regards to grooming, there was a main effect of time ($F(1.6, 72.04) = 21.78$, $p < .001$). In an inverse relationship with the rearing and locomotion data, post hoc analyses revealed that all animals gradually increased the time spent grooming from one interval to the next, with the most time spent grooming during the last 5 min of the test ($p < .05$).

4.5 Open field (56 days postischemia).

Table 1 shows the locomotion, rearing and grooming values for the three time intervals. Analysis of locomotion (total crossed squares) revealed no differences in exploratory activity among the groups but a main effect of time ($F(2, 90) = 98.33$, $p < .001$) attributable to heightened exploration of the arena in the first 5 min interval compared to the subsequent two ($p = .001$). Analyses of rearing time also revealed a main effect of time ($F(2, 90) = 38.05$, $p < .001$) with enhanced rearing behaviour present for all groups in the initial 5 min of the test ($p = .001$). Finally, analysis of grooming activity revealed a main effects of time ($F(1.31, 58.93) = 17.15$, $p < .001$), surgery ($F(1, 45) = 4.42$, $p < .05$) and a significant time × surgery interaction ($F(1.31, 58.93) = 3.97$, $p < .05$). Simple effects revealed that all animals spent the least amount of time grooming during the first 5 min of the test ($p = .05$) and that ischemic animals spent significantly less time grooming than sham animals at every time interval ($p = .05$).

4.6 Object recognition test

Fig. 5 shows the investigation ratios for all animal groups for the two retention intervals (30 min and 24 h). At the 30-min retention interval, statistical analysis revealed a main effect of surgery ($F(1, 48) = 4.95$, $p < .05$) and a surgery × treatment interaction ($F(1,48) = 5.84$, $p < .05$). Simple effects tests indicated that such difference was attributable to heightened exploration of the novel object in saline-treated sham compared to ischemic rats ($p = .01$). In addition, a significant reduction of the exploration of the novel object was observed in RSV-treated sham rats compared to saline-treated animals ($p = .032$). There were no significant differences between ischemic rat groups ($p > .05$). At the 24 h retention interval all groups showed comparable exploration ratios ($p > .05$). There was no between group differences in exploration time of O1 during familiarization, all rats reaching the 20 s exploration criteria within the allocated 10 min session.

4.7 Radial arm maze

Fig. 6 and 7 show the number of working memory errors during retention trials for the DNMTS and DMTS task, respectively. For the 1st DNMTS task, analyses of the performance in the acquisition trials revealed a main effect of time on the number of working memory errors ($F(3.4, 146.04) = 30.03$, $p < .001$) but no group differences, indicating that all animal groups learned to visit the four baited arms at the same rate (data not shown). However, analysis of the retention trial revealed main effects of time ($F(3.57, 153.62) = 77.92$, $p < .001$), surgery ($F(1, 43) = 16.65$, $p < .001$), treatment ($F(2, 43) = 4.92$, $p < .05$) and a time × surgery interaction ($F(3.57, 153.62) = 3.5$, $p < .05$). Simple effects analyses revealed that ischemic animals made significantly more errors than sham-operated animals on days 4-6 and 13-15. A trend was present on days 1-3, although it did not reach significance ($p =$

.062). The main effect of time was due to significant improvement of all animal groups over time ($p < .05$).

When presented with a 2nd DNMTS using different sequences of baited arms, analysis revealed no significant differences among the groups during both acquisition and retention trials. This suggests that once the animals have learned the win-shift rule from initial exposure to the DNMTS task, performance of the rats when presented with the same paradigm but using a new arm sequence is maintained, reducing between-group differences.

Analysis of the number or working memory errors when inter-trial intervals of 30, 120 and 240 min are applied revealed a main effect of time interval ($F(2, 90) = 9.49$, $p < .001$) and a time × surgery interaction ($F(2, 90) = 3.24$, $p < .05$). Simple effects tests revealed that ischemic animals made significantly more errors during the 240 min delay than sham-operated animals ($p = .025$). Furthermore, ischemic animals gradually made more errors as the inter-trial interval increased ($p < .05$), while sham rats were making just as many errors with delays of 120 and 240 min.

In the DMTS (win-stay) task, analysis of the number of errors during the acquisition trial revealed no significant differences between groups. However, analysis of errors during the retention trial, revealed main effects of time ($F(2.9, 130.57) = 43.3$, $p < .001$) associated with improved performance of all animals with increased trials, and of treatment ($F(2, 45) = 10.46$, $p < .001$) attributable to increased errors in animals treated with 10 mg/kg RSV as compared to 1 mg/kg RSV ($p = .001$) and saline-treated animals ($p = .029$).

5. Discussion

The current study had two main goals: to determine the effects of repeated administration of two RSV doses on cognition and behavioural impairments following global ischemia and to evaluate its effects on hippocampal neuronal injury at a remote post-ischemic interval.

Resveratrol leads to robust and long lasting neuronal protection following ischemia

To our knowledge, findings from this study represent the first demonstration that pretreatment with RSV confers lasting neuronal protection against ischemia-induced hippocampal damage (40% greater neuronal survival than observed in vehicle-treated ischemic animals), maintained up to 85 days postischemia. To date, preservation of CA1 hippocampal neurons in experimental models has consistently been reported at reperfusion intervals ranging from 6 h to 7 days following ischemic injury (Della-Morte et al., 2009; K. T. Lu, et al., 2006; Simao, et al., 2011; Q. Wang, et al., 2002). The robust and persistent neuronal preservation suggest that RSV treatment can induce long-term cellular effects. This is important as many therapeutic approaches have been shown to lose their beneficial impact as time following reperfusion is increased (see Corbett and Nurse (1998) for a review). Of note, repeated 21-day RSV administration prior to ischemic and sham surgery did not affect body weight and blood glucose measures at 3, 7 and 85 days post-surgery. This is consistent with a study in which 10 mg/kg RSV was administered to control animals by gavage chronically for 45 days and noted no significant differences in metabolic measures of body weight, glycemia and high density lipoprotein cholesterol levels compared to vehicle-treated animals (Miatello, et al., 2005).

Current hypotheses underlying RSV neuroprotective actions include its effect on reducing oxidative stress through scavenging of reactive oxygen species, releasing trophic factors important for angiogenesis (Dong, et al., 2008; Fukuda, et al., 2006; H. Wang et al., 2010) and reducing inflammation (Manna, Mukhopadhyay, & Aggarwal, 2000; Simao, et al.). In particular, RSV has been shown to enhance free radical scavenging and cerebral blood flow via upregulation of endothelial nitric oxide synthase release (Das et al., 2005; K. T. Lu, et al., 2006; Tsai, et al., 2007) and downregulation of inducible NOS (iNOS) (Bi et al., 2005). For instance, 21-day RSV pretreatment at a dose of 20 mg/kg led to significant inhibition of infarct volume following middle cerebral artery occlusion in rats, a phenomenon associated with alterations of discrete oxidative stress signals, including malondialdehyde and glutathione levels (Sinha, et al., 2002). The presence of similar physiological effects in our study remains to be determined but appears possible.

Resveratrol effects on behavioural performance are dose-related

At present, very few studies have characterized the impact of daily RSV administration on behavioural performance in normal animals or following global cerebral ischemia (Miatello, et al., 2005; Q. Wang, et al., 2009). We have used the open field, object recognition and radial arm maze tests to determine RSV effects on locomotor activity, visual and spatial memory. Our findings indicated dose-related effects of RSV on behaviour in ischemic animals and altered performance of sham animals treated with 10 mg/kg RSV in discrete behavioural tests, the latter suggesting long lasting intrinsic actions of the polyphenol to regulate locomotion and memory. Thus, in the open field, pretreatment with 10 mg/kg RSV led to enhanced locomotor activity in ischemic rats 4 days post-surgery as compared to 1 mg/kg- and saline-treated controls. Our study further indicated that locomotor

activity was enhanced in RSV-treated sham rats at the same time interval. At present, no study has examined the impact of repeated RSV administration on locomotor activity in control rats or animals exposed to global ischemia. In a recent study, Wang et al. (2009) have reported reduced open field activity in gerbils associated with oral administration of grape polyphenol extract (RSV content was estimated to be 0.7 µM) 4 days prior to and in the 48 h preceding post-ischemic open field testing. Together, these observations suggest does-related effects, although direct comparison of findings remains difficult considering a much shorter administration protocol and oral administration of a polyphenol extract. Of interest, saline-treated ischemic animals, whose global activity is commonly enhanced following ischemia (Green et al., 1995; M. Milot & Plamondon, 2008; M. R. Milot & Plamondon, 2009; Plamondon & Khan, 2005; Yan, Wang, Hou, Ji, & Zhou, 2007) showed comparable locomotion as sham animals. These observations further suggest that increased locomotion observed in 10 mg/kg-treated ischemic animals is linked to intrinsic effects of higher RSV dosages on neurochemical pathways mediating this response (Araki, Hino, Karasawa, Kawasaki, & Gomita, 1999; Poignet, et al., 1989). The reason for the absence of hyperactivity in saline-treated ischemic rats at this time interval is not fully understood but has been previously reported. Change in emotional reactivity following ischemia has been proposed to play a role in novelty-induced hyperactivity in ischemic animals (Plamondon & Khan, 2005; Walsh, Harley, Corbett, Skinner, & Martin, 2008). In the current study, animals were daily handled for 21 days prior to ischemia, while being injected with RSV or saline, a procedure which could have effects on emotional reactivity. This is supported by similar observations in cohorts of ischemic animals, which received extensive handling associated to discrete pre-ischemic feeding or injection protocols (De Butte-Smith, Gulinello, Zukin, &

Etgen, 2009; Plamondon & Roberge, 2008; Roberge, Hotte-Bernard, Messier, & Plamondon, 2008). Furthermore, healthy animals receiving daily handling prior to open field exposure have shown reduced open field activity (Izidio, Lopes, Spricigo, & Ramos, 2005; Schmitt & Hiemke, 1998). Upon re-exposure to the open field 56 days postischemia, all groups showed comparable open field activity. Other researchers have reported a similar decline in hyperactivity upon habituation to the testing environment (Dowden & Corbett, 1999; Plamondon & Khan, 2005; D. Wang & Corbett, 1990).

The presence of intrinsic effects of RSV on behaviour also stemmed from findings in the object recognition test where sham rats treated with 10 mg/kg RSV showed a recognition memory impairment comparable to that of ischemic animals at the 30 min retention interval. In addition, the 1 and 10 mg/kg RSV doses did not prevent ischemia-induced memory impairments. The presence of recognition impairments in RSV-treated sham as well as CA1 protected ischemic rats further support the dissociation of the performance in this test to CA1 neuronal injury (Aggleton, Albasser, Aggleton, Poirier, & Pearce, 2010; De Butte-Smith, et al., 2009; Mumby, 2001).

The participation of CA1 neurons in spatial memory is acknowledged and the radial arm maze is a reliable tool to measure spatial memory impairments. We selected delayed non matching- and matching-to sample tasks to assess spatial memory impairments, the latter paradigm (win-stay) being considered more challenging for rats due to their natural propensity to select alternate arms than those previously explored upon retention trials. In the DNMTS radial maze task, spatial memory impairments were apparent in both saline- and RSV-treated ischemic animals, with a tendency for the 10 mg/kg dose to worsen the rats' performance while ischemic animals treated with the lower 1 mg/kg dose showed a trend

towards improved performance. Ischemic rats displayed an increased number of errors in the DNMTS task when a 240 min inter-trial interval was used, particularly evident in ischemic animals treated with 10 mg/kg RSV. The DMTS led to increased errors for all groups, illustrating increased difficulty of the animals to apply a win-stay rule. Interestingly yet consistent with observations in the DNMTS task, 10 mg/kg RSV-treatment accentuated memory impairments in ischemic and sham rats, a difference maintained over days.

A number of studies have examined the relationship between hippocampal density and performance in various spatial memory tasks and consistently associated moderate to severe CA1 neuronal loss with significant spatial memory impairments (Block, 1999; Briones & Therrien, 2000; Hartman, Lee, Zipfel, & Wozniak, 2005; Hodges et al., 1996; Nelson, et al., 1997). Among the CA1 protected RSV ischemic rats, animals treated with the 10 mg/kg dose showed increased and longer lasting spatial memory impairments, suggesting that other factors than CA1 injury contributes to the memory deficits observed. Dissociations between CA1 neuronal injury and behavioural recovery following ischemia have been reported by earlier studies (De Butte-Smith, et al., 2009; Lyeth et al., 1990; Roberge, Hotte-Bernard, et al., 2008). De Butte-Smith et al. (2009) recently demonstrated that increased CA1 cell survival associated with chronic estradiol treatment in female rats failed to improve visual or spatial recognition memory impairment after global ischemia (De Butte-Smith, et al., 2009). Conversely, recovery of functional impairments post-stroke has been demonstrated in the absence of CA1 neuronal survival (Lyeth, et al., 1990; Ma et al., 2008; Olsen, Scheel-Kruger, Moller, & Jensen, 1994). To this effect, it has recently been suggested that pharmacologically manipulating norepinephrine release following ischemia by administering the alpha2-adrenoceptor agonist clonidine could attenuate working memory

deficits in the radial arm maze, despite significant CA1 cell loss (M. R. Milot & Plamondon, 2011). It is thus possible that higher RSV doses be associated with physiological changes affecting attentional and/or emotional processes interfering with memory performance.

Conclusion

Our findings demonstrate that CA1 neuronal protection in ischemic rats treated with either RSV doses is not predictive of improved behavioural recovery. Rather, RSV effects appeared dose-related and independent of CA1 injury in discrete behavioural tests. Our findings are consistent with those of different studies suggesting dose-related profile of RSV actions under basal or experimental conditions (Mukherjee, et al., 2010) and with recent findings highlighting disease specific physiological effects (Calabrese, et al., 2010). Although definite dosage-related effects remain difficult to confirm due to combined in vitro and in vivo evaluations and the range of RSV doses investigated, these observations do emphasize a continuum of physiological RSV actions dependent on dosage. Growing evidence also supports dose-dependent RSV effects in other pathological models, including osteoporosis (Dai et al., 2007) and Alzheimer's disease (Conte, Pellegrini, & Tagliazucchi, 2003).

Acknowledgements

This work was supported by a grant from the Natural Sciences and Engineering Research Council of Canada to H.P. We sincerely thank Sylvie Émond for excellent technical assistance and contribution to the research project.

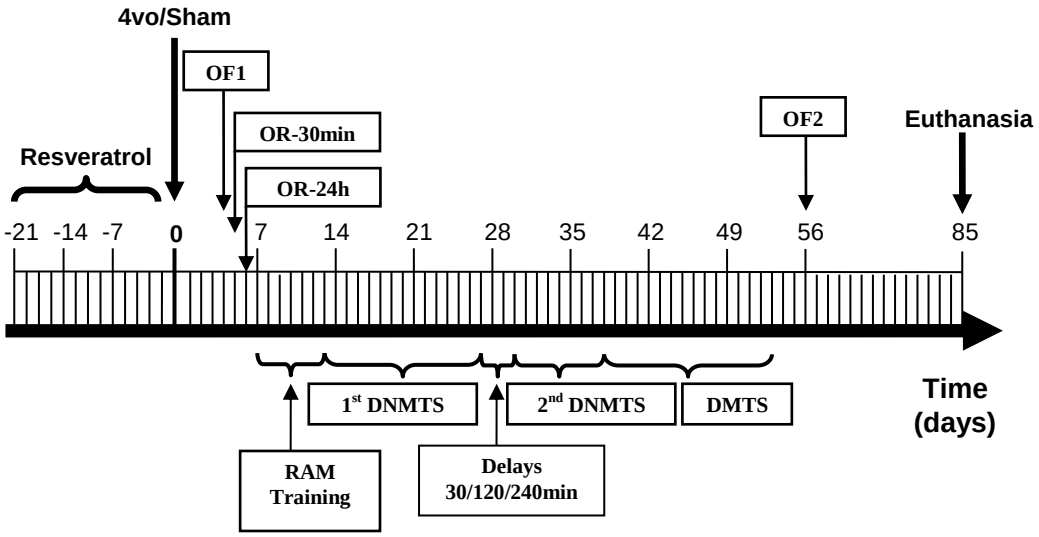


Figure 1. Experimental protocol. Schedule showing time intervals for all experimental procedures. 21-day pre-surgical period of RSV (1 mg/kg or 10 mg/kg;) or saline i.p. injections; 4vo: 4-vessel occlusion; OF1 and OF2: open field test 4 and 56 days following reperfusion; OR-30 and OR-24: object recognition test with 30 min and 24 h retention intervals; RAM Training: training in the radial arm maze; 1st DNMTS: first delayed non-matching to sample task; Delays 30, 120, 240 min: inter-trial delays of 30, 120 and 240 min in the 1st DNMTS; 2nd DNMTS: second delayed non-matching to sample task; DMTS: delayed matching to sample task. Day 0 refers to the day of the surgery.

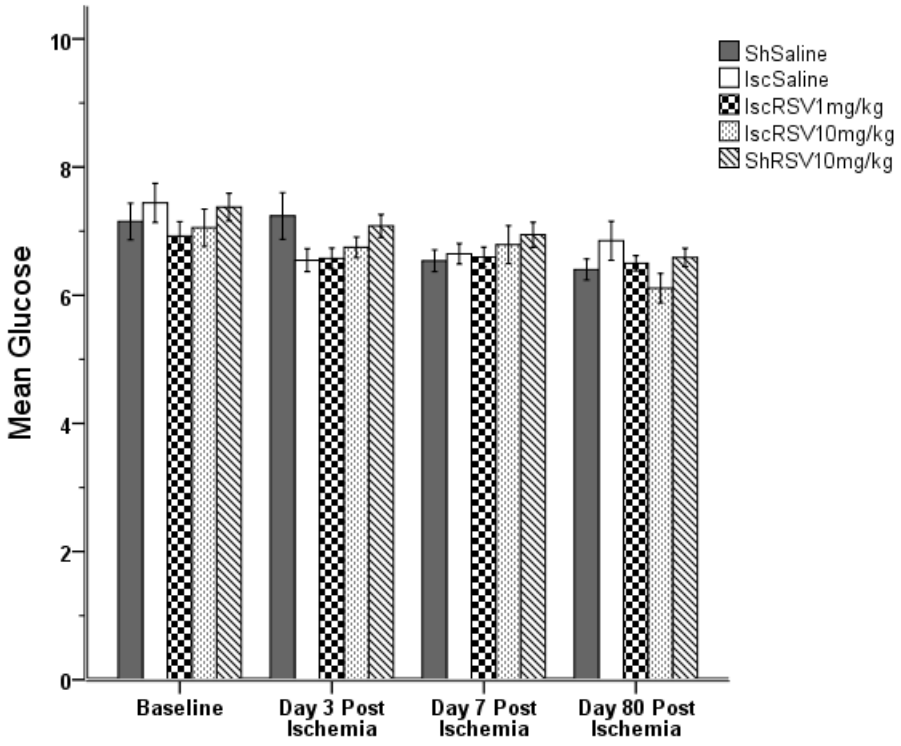
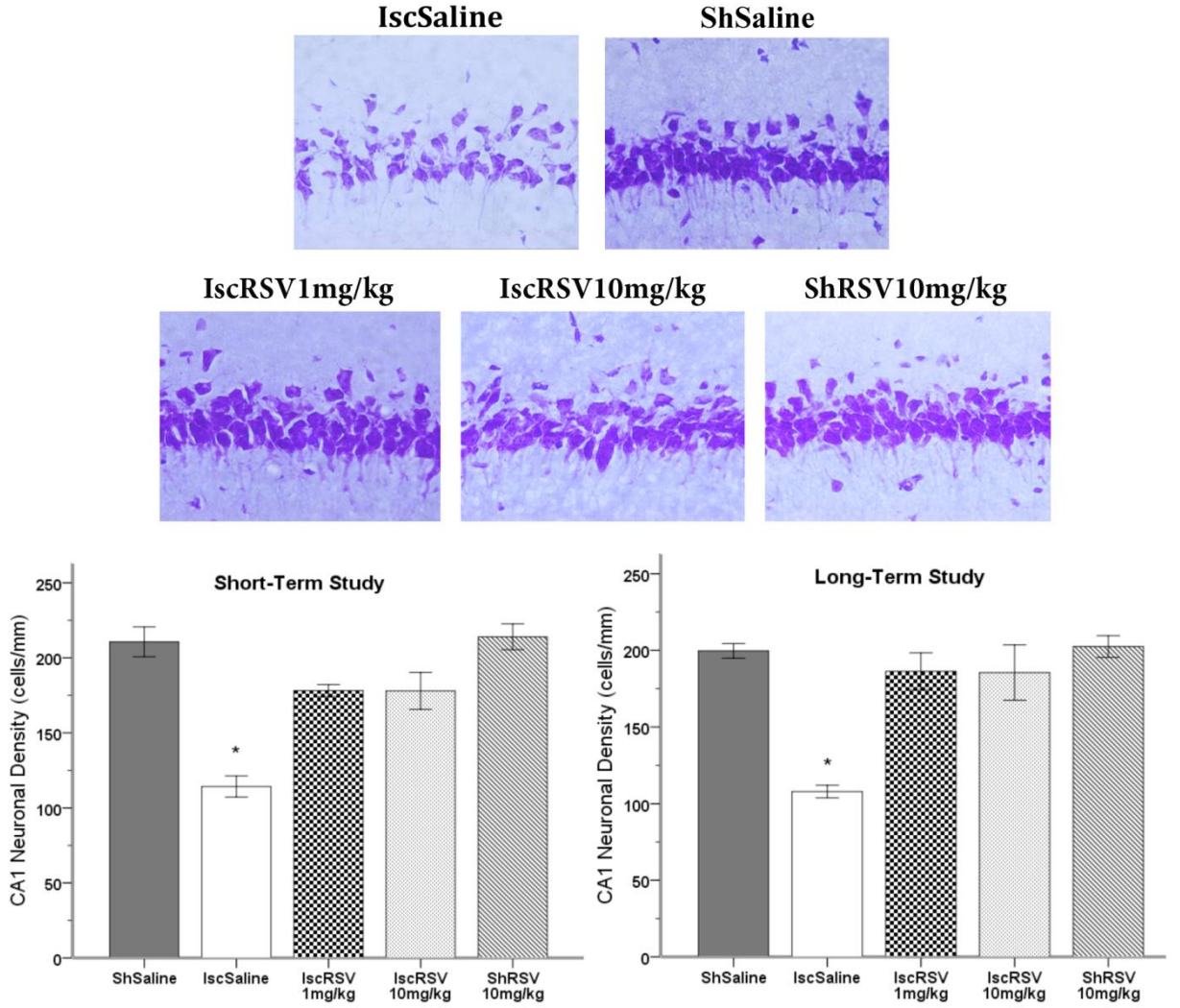


Figure 2. Mean blood glucose levels of saline and RSV-treated ischemic and sham animals at baseline (pre-surgery) and 3, 7 and 85 days postischemia or sham surgery. No significant differences were observed between groups ($p > .05$).



		ShSaline	IscSaline	IscRSV 1mg/kg	IscRSV 10mg/kg	ShRSV 10mg/kg
Long-term study	CA3	176.58±7.08	160.53±8.45	163.68±6.75	169.95±5.27	171.13±3.4
Short-term study	CA3	193.25±17.98	178.75±6.15	180.19±5.34	172.97±5.75	186.38±8.62

Figure 3. Top figure shows representative photomicrographs of thionin-stained neurons in the CA1 layer of sham and ischemic animals treated with either saline or RSV at 85 days postischemia. * indicates a significant reduction in number of surviving CA1 neurons (≈50%) by saline treated ischemic rats compared to all other groups at both short- and longer-term intervals ($p < .001$). 1 and 10 mg/kg RSV administration led to significant reduction in CA1 neuronal damage compared to saline-treated ischemic rats at both time intervals ($p < .001$) (middle panel - left and right graphs, respectively). Bottom table presents the cell

density (cells/mm) in the hippocampal CA3 region of sham and ischemic animals treated with either saline or RSV. There were no significant differences in CA3 cell density among the experimental groups ($p > .05$). Values represent mean investigation ratio ± S.E.M.

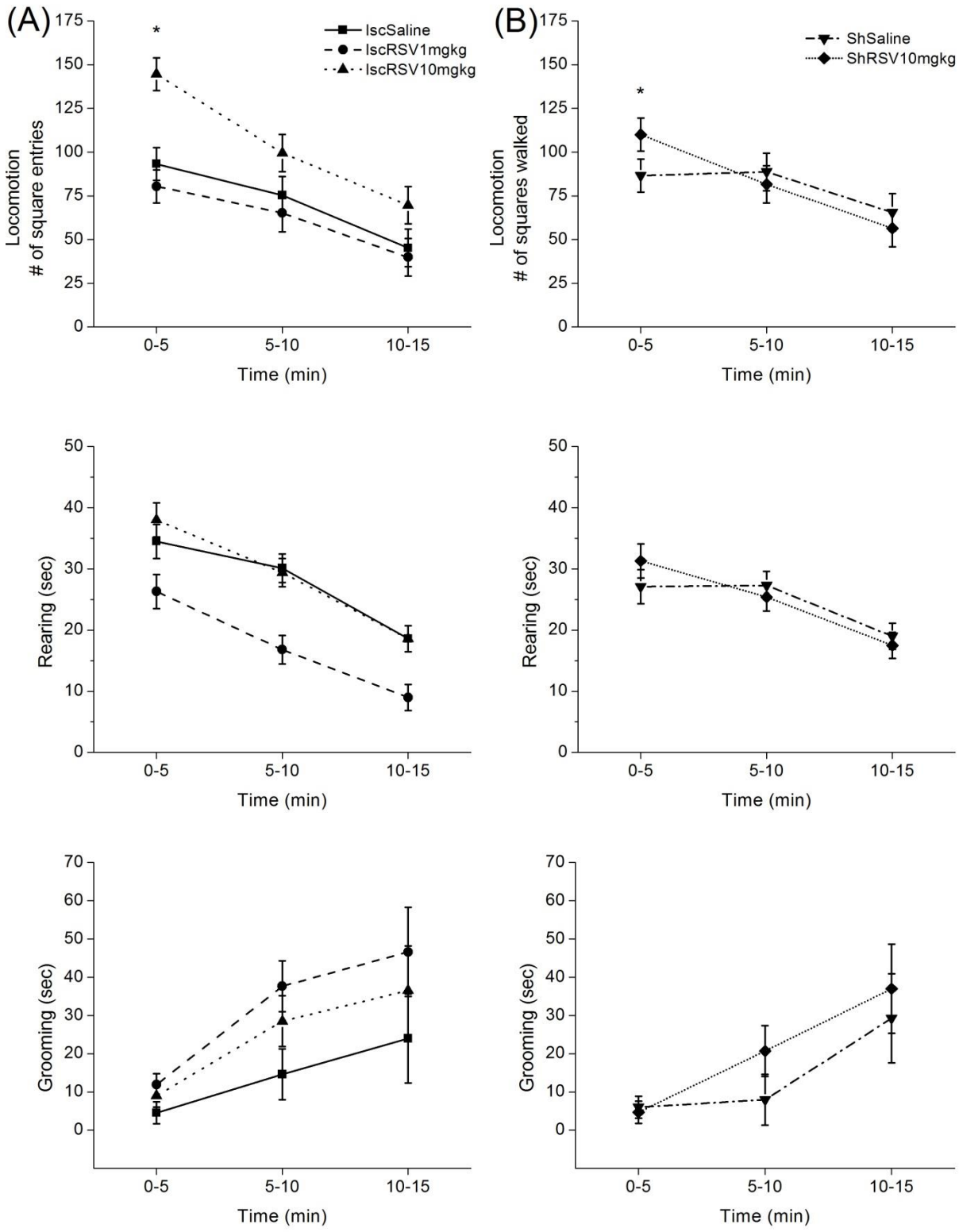


Figure 4. The effect of 10 min global ischemia and RSV treatment on locomotion, rearing and grooming activity during the 15-min open field test period on day 4 postischemia in ischemic (A) and sham (B) animals. During the first 5 min of the test, 10 mg/kg RSV-treated

rats explored the arena significantly more than all other groups. * indicates a significant difference between saline, 1 mg/kg and 10 mg/kg RSV-treated groups. Reported differences were significant at $p < .001$. Values represent mean score ± S.E.M.

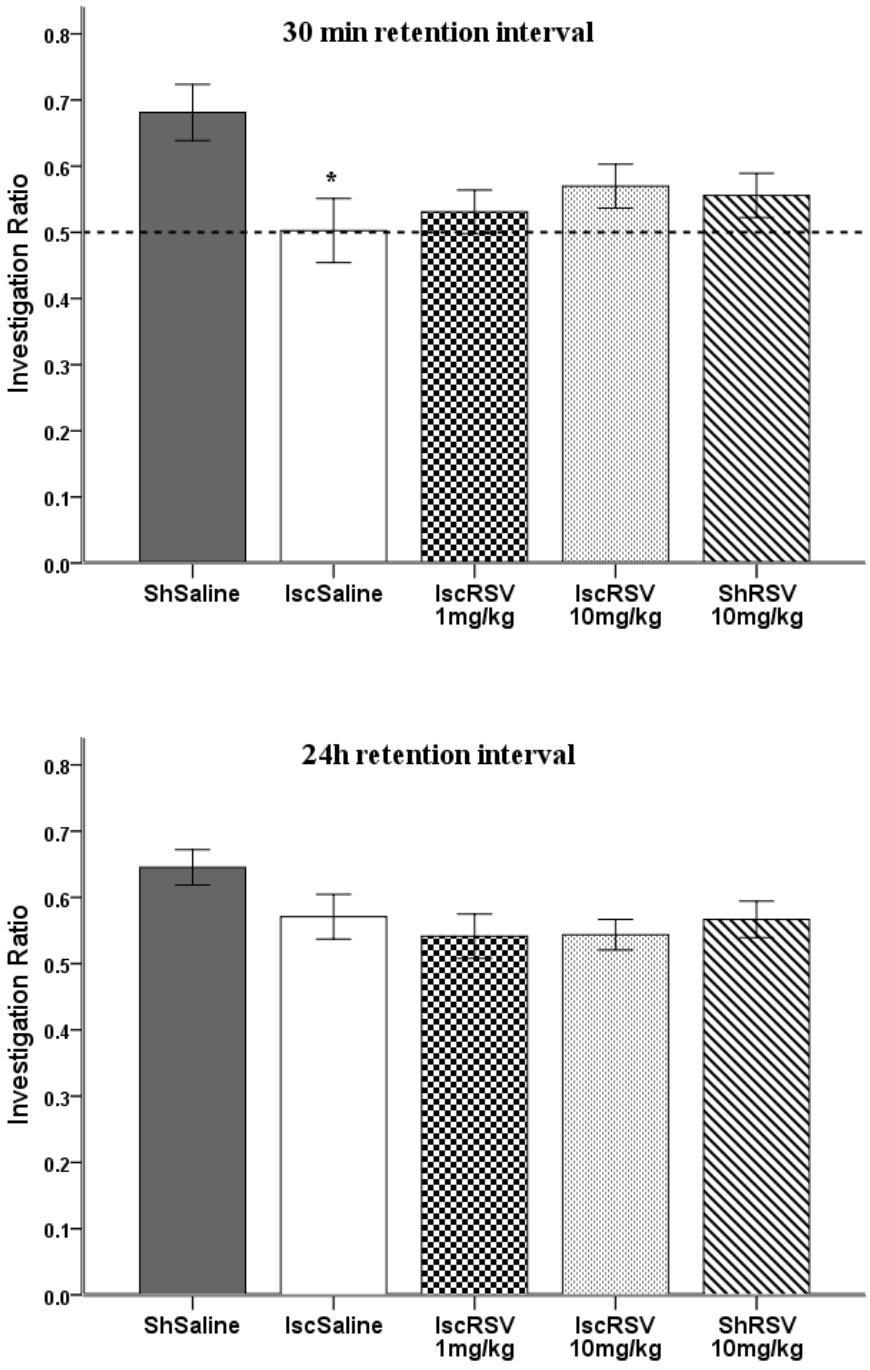


Figure 5. Mean investigation ratio of saline and RSV-treated ischemic and sham animals in the object recognition test at the 30 min and 24 h retention intervals. * indicates a significant reduction of the exploration ratio following 10 min of global ischemia in saline-treated animals ($p < .05$). Values represent mean investigation ratio ± S.E.M.

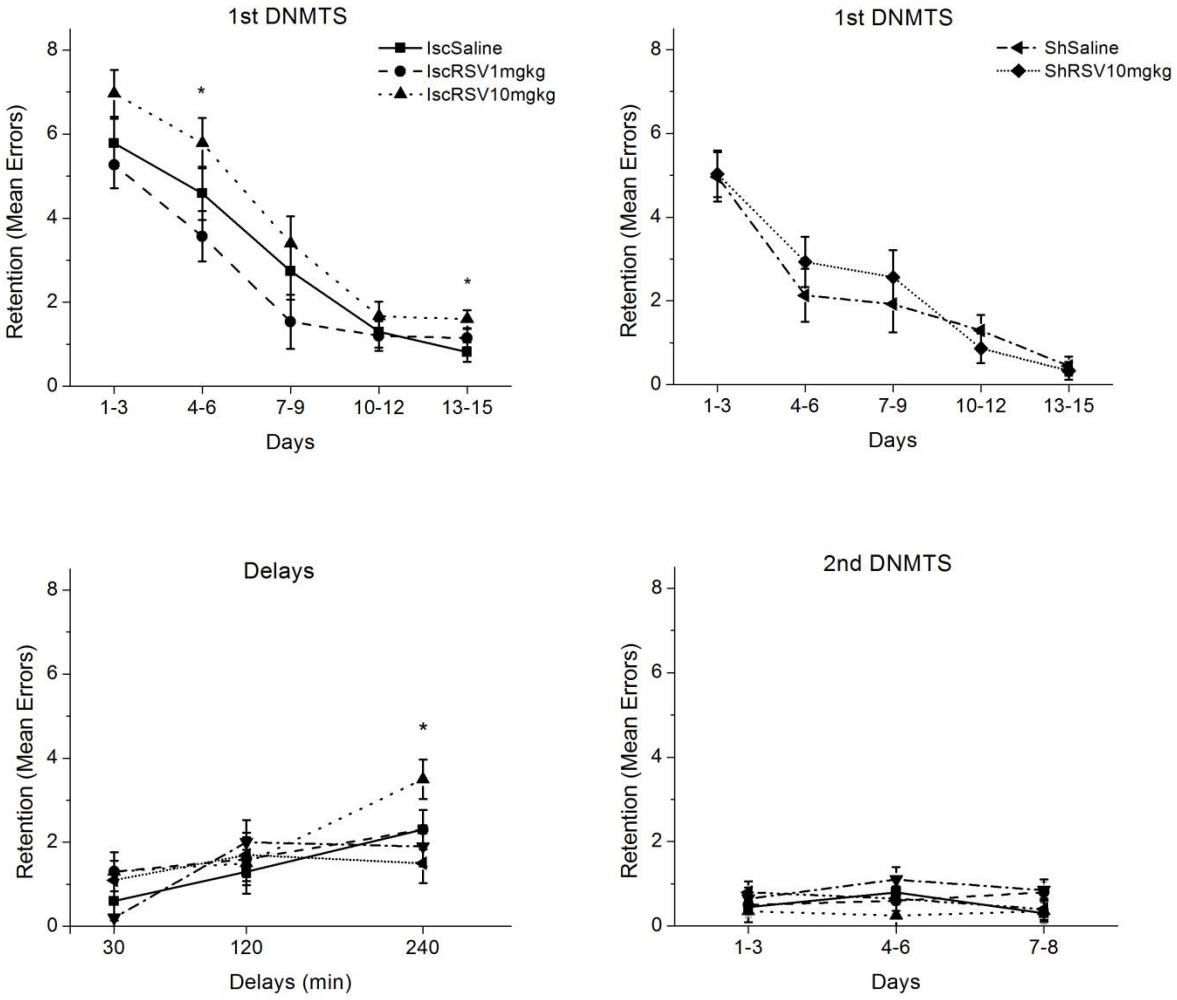


Figure 6. Effect of ischemia and RSV treatment on the total number of errors during retention trials in the first DNMTS (15 days), DNMTS (variable delays) and second DNMTS (6 days). * During the first DNMTS, ischemic animals made significantly more errors during days 3-5 and again on days 12-15, than sham-operated controls ($p < .001$). Upon acquisition of the first DNMTS task, extending retention delays did not have an impact on performance except for the 240 min inter-trial delay; * 10 mg/kg RSV-treated ischemic rats made significantly more errors than either 1 mg/kg RSV and saline-treated animals ($p < .05$). Values represent mean error ± S.E.M.

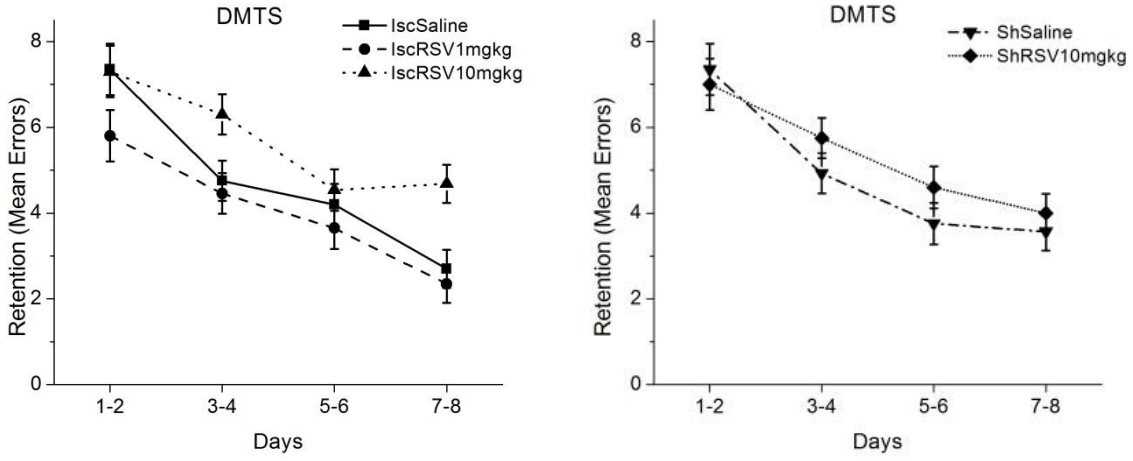


Figure 7. Effect of ischemia and RSV treatment on the total number of errors during retention trials in the DMTS task in the radial arm maze. Throughout the 8-day testing period, animals treated with 10 mg/kg RSV made significantly more errors than saline and 1 mg/kg RSV-treated animals ($p < .05$). Values represent errors ± S.E.M.

	Time (min)		
	0-5	**5-10**	**10-15**
Locomotion			
ShSaline	116.8±11.02	91.5±10.26	68.5±11.06
IscSaline	117.5±9.06	83.5±8.93	50.6±7.10
IscRSV1mg/kg	132.0±7.91	95.6±10.49	54.5±7.64
IscRSV10mg/kg	127.7±9.31	104.6±9.68	75.6±6.25
ShRSV10mg/kg	118.4±10.22	85.2±9.01	56.9±8.02
Rearing (sec)			
ShSaline	29.2±1.69	24.9±1.38	18.3±1.51
IscSaline	38.4±3.28	31.1±3.18	22.5±2.74
IscRSV1mg/kg	29.2±1.93	28.1±1.89	24.2±3.64
IscRSV10mg/kg	32.3±2.01	25.9±1.80	23.1±2.00
ShRSV10mg/kg	33.0±3.12	27.2±2.55	18.9±2.37
Grooming (sec)			
ShSaline	2.05±0.85	8.32±2.98	21.97±10.13
IscSaline	0.36±0.21	9.13±3.08	8.19±3.48
IscRSV1mg/kg	2.33±0.99	5.28±1.85	12.99±5.07
IscRSV10mg/kg	4.40±1.59	8.50±3.27	12.90±3.81
ShRSV10mg/kg	7.34±4.76	18.24±5.88	34.53±12.32

Table 1. Effect of 10 min global ischemia and RSV treatment on locomotion, rearing and grooming activity during the 15 min open field test performed on day 56 postischemia. Locomotion values represent # of square entries. Values represent mean score ± S.E.M.

Article 2

Published as: Girbovan, C., Kent, P., Merali, Z., & Plamondon, H. (2015). Dose-related effects of chronic resveratrol administration on neurogenesis, angiogenesis and corticosterone secretion are associated with improved spatial memory retention following global cerebral ischemia. *Nutritional Neuroscience. (Under Revision)*

Preface

In the previous experiment, we showed significant and long-lasting neuroprotection against global cerebral ischemia following 21-day pretreatment with RSV at two doses (1 and 10 mg/kg RSV). Additionally, we showed that CA1 neuronal protection by the polyphenol was not predictive of improved behavioural recovery and that dose-related effects were noted. Moreover 10 mg/kg RSV induced lasting effects on locomotion and memory in sham rats. Article 2 aimed to investigate possible mechanisms of action of RSV in mediating neuroprotection and behavioural recovery through stimulation of neurogenesis and angiogenesis processes in the hippocampus in the same cohort of rats at short- and long-reperfusion intervals. The documented HPA axis hyperactivity, which characterizes the post-ischemic period, can regulate these plastic changes as well as affect behaviour and thus encouraged characterization of RSV effects on the regulation of CORT secretion in sham and ischemic animals.

Abstract

The phytoalexin and natural polyphenol resveratrol has a variety of beneficial effects, including the ability to affect neurogenesis. According to the neurovascular niche hypothesis, neurogenesis likely involves stimulation of angiogenesis, both phenomena being influenced by brain injury. Time and dose-related effects of RSV and global ischemia on regulation of these plastic brain changes remain unknown. In rodents, global cerebral ischemia, which mimics cardiac arrest in humans, leads to selective CA1 neuronal damage and well-documented learning and memory deficits in numerous spatial tasks, and to increased hypothalamic-pituitary-adrenal (HPA) axis reactivity. The current study determined dose-related effects of 21-day RSV pretreatment on markers associated with neurogenesis (DCX, PSA-NCAM) and angiogenesis (CD31) in the hippocampus following global cerebral ischemia, at short (7-day) and longer-term (85-day) reperfusion intervals. Five groups of Wistar rats were included: sham/saline, ischemia/saline, ischemia/1 mg/kg RSV, ischemia/10 mg/kg RSV and sham/10 mg/kg RSV. Post-ischemic basal and stress-induced corticosterone (CORT) secretions and spatial memory performance in the Morris water maze (MWM) were determined and relationship to changes in hippocampal expression of plasticity markers evaluated. Our findings revealed significant attenuation of ischemia-induced expression of DCX/PSA-NCAM-immunoreactivity (ir) in RSV-treated rats, while RSV treatment increased angiogenesis in the injured CA1 region following reperfusion. These changes were not associated with improved spatial memory in the MWM. Furthermore, pretreatment with 10 mg/kg RSV attenuated CORT secretion 3 days following cerebral ischemia. These findings are discussed in terms of time- and dose-specific actions of RSV on behaviour and brain recovery.

Keywords: resveratrol; cerebral ischemia; neurogenesis; angiogenesis, spatial memory; corticosterone.

1. Introduction

Resveratrol (RSV), a naturally occurring phytoalexin present in high concentration in the skin and seeds of grapes, has shown cardioprotective and anti-inflammatory properties in a number of disease models (Bellaver, Souza, Souza, & Quincozes-Santos, 2014; Bradamante, et al., 2004). Neuroprotection and effects on functional recovery following cerebral ischemia have also been demonstrated (Girbovan, Morin, & Plamondon, 2012; Simao, et al., 2011; Simao, et al., 2012b; Q. Wang, et al., 2002). The possibility that these effects be accompanied by changes in endogenous stem cell activity and angiogenesis following ischemia remains unknown.

Neurogenesis is known to persist throughout life in the adult brain, being predominantly localized in two regions: the SGZ of the dentate gyrus (DG) in the hippocampus and the subventricular zone of the lateral ventricles (Gage, Kempermann, Palmer, Peterson, & Ray, 1998). The discovery of self-renewing and multipotent neuronal stem cells has uncovered the ability of the mammalian central nervous system to self-repair or regenerate. In the last two decades, numerous studies have reported ischemia-induced neurogenesis in the hippocampus (J. H. Choi, et al., 2012; Y. S. Choi, et al., 2003; Engelhard, et al., 2007; Kuge, et al., 2009; Lasarzik, et al., 2009; J. Liu, et al., 1998; Schmidt & Reymann, 2002; R. Tanaka, et al., 2004; Winkelheide, et al., 2008; Wojtowicz, et al., 2008). In an early report, gerbils exposed to 10 min bilateral common carotid artery occlusion showed a 12-fold increase in neurogenesis in the dentate subgranular zone 1-2 weeks post-insult (J. Liu, et al., 1998), a number of them maturing into granule cells with some extending axons to the CA3 region of the hippocampus and surviving for at least 7 months (Y. S. Choi, et al., 2003; Markakis & Gage, 1999). Observations that irradiation of adult

neurogenesis in gerbils lead to significant impairments in the water maze one month following global cerebral ischemia (Raber, et al., 2004) while enhanced neurogenesis improved post-ischemic behavioural outcome (Bendel, et al., 2005) have supported a role for these newly-formed cells in hippocampal-dependent functional recovery.

Angiogenesis, or the formation of blood vessels from a pre-existing vasculature network, is an event that occurs as part of embryonic vascular development, wound healing and organ regeneration as well as in a number of pathologic conditions (Folkman & Shing, 1992; Hammes, et al., 1996), including cerebral ischemia (Beck & Plate, 2009; Hayashi, et al., 2006; Hayashi, et al., 2003; O. K. Park, et al., 2010). As part of ischemic recovery, angiogenesis has been interpreted as a physiological response helping to restore oxygen and energy substrates to affected brain tissue as well as provide neurotrophic support to newly generated neurons found in concentrated numbers around blood vessels following stroke (Yamashita, et al., 2006).

To date, a limited number of studies have assessed RSV's effects on hippocampal neurogenesis and these have generated mixed findings. For instance, a 14-day treatment with 1 or 10 mg/kg RSV had dose-related inhibitory effects on cortical and hippocampal NPCs in healthy young adult mice through direct activation of AMPK (H. R. Park, et al., 2012). In contrast, RSV prevented impaired neurogenesis induced by prenatal stress exposure, enhancing expression of newborn cells and restoring brain-derived neurotrophic factor levels in the DG (Madhyastha, et al., 2013). Similarly, RSV has been shown to affect angiogenesis in a situation-specific manner, pro-angiogenic effects being primarily noted in myocardial infarction (Fukuda, et al., 2006; Robich, et al., 2010) and focal models of ischemic stroke

(Dong, et al., 2008). RSV effects on angiogenesis following global cerebral ischemia have not been determined.

Akin to potent stressors, global ischemia induces CORT hypersecretion and lasting changes in HPA axis activation/reactivity in rodents and humans (de la Tremblaye, et al., 2014; Neigh et al., 2009; Tavakoli, Bidari, & Shams Vahdati, 2012). Moreover, stress signalling has an acknowledged role on the regulation of brain plasticity (Czeh et al., 2007; Gould, McEwen, Tanapat, Galea, & Fuchs, 1997; Khorram et al., 2014). Elevated concentrations of glucocorticoids have been shown to negatively impact hippocampal neurogenesis, affecting proliferation and neuronal differentiation under stress conditions (Gould, Cameron, Daniels, Woolley, & McEwen, 1992; Mayer et al., 2006) through activation of glucocorticoid receptors signalling pathways (Anacker et al., 2013). Similarly, high levels of circulating maternal glucocorticoids have been implicated in the inhibition of offspring angiogenesis (Khorram, et al., 2014). In vitro, RSV has been shown to inhibit CORT secretion in rat adrenocortical cells (Supornsilchai, Svechnikov, Seidlova-Wuttke, Wuttke, & Soder, 2005) but little is known of RSV's actions on HPA axis activation or glucocorticoid secretion in vivo. In a recent study, RSV treatment for 7 days at 15 mg/kg per day led to attenuation of CORT secretion and depressive-like behaviour following exposure to a chronic unpredictable mild stress paradigm (Ge et al., 2013). To our knowledge, no study has determined effects of RSV on glucocorticoid secretion and HPA axis reactivity following brain ischemia.

The current study had three main goals. The first objective aimed to determine dosage- and delay-dependent effects of 21-day RSV pretreatment on progenitor cell differentiation and angiogenesis following 10 min global ischemia in rats.

Immunohistochemical co-labeling of doublecortin (DCX), a microtubule-associated protein, and the polysialylated-neural cell adhesion molecule (PSA-NCAM) in the CA1, CA3 and DG regions of the hippocampus was used to determine effects of 1 and 10 mg/kg RSV treatment on neuronal proliferation 7 and 85 days postischemia, whereas the vascular endothelial marker CD31 (PECAM-1) served to assess the formation of neogenetic blood vessels in ischemic and sham-control brains. A second goal aimed to characterize RSV effects on the regulation of CORT secretion pre- and postischemia and following exposure to an acute stressor. A third goal aimed to assess the possible interplay between these endogenous signals and association of these variables with spatial memory performance following global ischemia.

2. Materials and Methods

2.1 Subjects

Male Wistar rats (N = 35 and 53 for the 7 and 85-day reperfusion intervals, respectively) weighing 100-125g at time of arrival to the animal facility (325-375g at time of surgery) were obtained from Charles River Laboratories (Rochefort, Quebec, Canada). Upon arrival, animals were individually housed and maintained on a 12 h light/dark cycle (lights on at 7:00 AM), with free access to water and standard (Purina) rat chow. Room temperature was maintained at 21-23°C with 60% relative humidity. One week following arrival, animals were randomly assigned to five experimental groups and daily injections over a 21-day period initiated. Four weeks following the rats' arrival and upon completion of drug treatment, sham or ischemic surgeries were performed. The surgery and injection schedule was identical for the short and longer-term groups with the exception of the absence of behavioural testing in the 7-day interval (refer to Fig.1 for experimental timeline). Twenty-

five rats out of 81 rats operated in the ischemic groups died during the ischemic surgery. Final animal numbers and groups for the 7 and 85 day reperfusion intervals (i.e., first and second numbers in the brackets, respectively) were as follows: ischemia + saline (n=7, 10), sham + saline (n=6, 10), ischemia + 1 mg/kg RSV (n=8, 12), ischemia + 10 mg/kg RSV (n=8, 11) and sham + 10 mg/kg RSV (n=6, 10). All experimental procedures were in accordance with the guidelines set by the Canadian Council of Animal Care and approved by the University of Ottawa Animal Care and Ethics Committee. Efforts were made to minimize the number of animals used and their suffering.

2.2 Resveratrol administration

RSV (Sigma, St. Louis, MO, USA) was dissolved in 50% ethanol lyophilized and stored at -80^{o}C until use. Aliquots were freshly dissolved daily in a vehicle consisting of 0.9% saline solution containing 20% hydroxypropyl h-cyclodextrin (Sigma, St. Louis, MO, USA). Twenty-one days before the surgery, sham and ischemic animals received daily, between 9:00-11:00 h, intraperitoneal injections of either saline or 1 or 10 mg/kg RSV (injection volume of 0.1 ml/100 g body weight). The last saline or RSV injection was administered 1 h prior to ischemic or sham surgery. Rats were weighted daily and injection volume adjusted. The RSV dosages were selected based on earlier reports showing beneficial effects in different experimental paradigms (Mukherjee, et al., 2010; Sinha, et al., 2002).

2.3 Transient forebrain ischemia

Forebrain ischemia was performed using the four-vessel occlusion model as previously described (Pulsinelli & Brierley, 1979). Briefly, rats were deeply anesthetized by inhalation of 1.5% halothane in oxygen (1.5 to 2L/min). Vertebral arteries were irreversibly occluded by electrocoagulation, and a small-diameter silk thread looped around the carotid

arteries to facilitate subsequent occlusion. Twenty-four hours later, common carotid arteries were occluded with microaneurysm clamps for 10 minutes (min) in spontaneously ventilating animals. Sham-operated animals underwent anaesthesia and received the same dorsal and ventral surgical incisions as the ischemic groups, with the exception of electrocoagulation of the vertebral arteries. Twenty-four hours later, carotid arteries were exposed, but not clamped. Only rats that lost the righting reflex over the entire occlusion period were included in the study. Core temperature was kept at 37°C ± 0.5 throughout the surgery using a feedback-regulated heating blanket connected to a rectal thermometer (Homeothermic Blanket Control Unit, Harvard Instruments, Natick, MA). Rats' body temperature was further supported with a heating pad in the hours following surgery and reperfusion. One day prior to behavioural testing, the pupillary reflex was assessed in all animals to determine visual system/retinal functioning. Animals were transported to a dark room and left to habituate for a minimum of 15 min. The pupillary reflex was examined by illuminating the rats' dark-adapted eyes with a mini-flashlight producing a focused light beam. Following examination of the first eye, chosen at random, an additional 60 s in the dark was imposed prior to examining the second eye. The reflex was considered intact if constriction of both pupils in response to the light occurred under 10 s. All ischemic and sham-operated rats displayed a normal pupillary reflex, typically occurring within seconds of light stimulus.

2.4 Blood collection

The tail vein nick procedure was used to collect blood in unanesthetized rats. This procedure is not confounded by use of anesthetics and itself does not impact measurements of CORT if samples are collected under 3 min (Vahl et al., 2005). Animals were habituated to the procedure prior to surgery in order to minimize stress associated with blood collection.

Two blood drops (25–50 μL) per sample were collected on Schleicher and Schuell (Whatman International Ltd., Maidstone, UK) filter paper by lancing the scalpel-nicked tail close to its tip, a procedure taking less than 30 s to complete, in addition to the 30–60 s taken to transport the animal from its home cage to the blood sampling room. Collected samples were let to dry overnight at room temperature and stored at −80 °C. Blood samples were collected for all animals 7 days prior to surgery (baseline) and 3 days after ischemia or sham occlusion between 7:00 and 8:00 AM. To minimize the number of tail nicks a gauze soaked in sterile water was used to gently re-open fresh wounds during blood sampling. The filter paper collection provides reliable CORT measurements as well as values comparable to those measured in plasma, using minimal amount of blood (M. R. Milot, James, Merali, & Plamondon, 2012).

2.5 Restraint stress

On the 84th day following ischemia or sham surgery, animals were subjected to restraint stress by placing them in a plastic restrainer for 15 min during the light phase (between 9:00 AM and 11:00 AM). Blood samples were collected at baseline and 30, 60, and 120 min following onset of the stressor.

2.6 Quantification of blood corticosterone

CORT levels were assessed using commercially available radioimmunoassay kits (MP Biomedicals, USA) according to the manufacturer's instructions as previously described (M. R. Milot, et al., 2012). Briefly, 3.0 mm diameter circles were punched from Schleicher and Schuell specimen collection papers containing blood drop samples, using a Gem Hole Punch (McGill Inc., Marengo, IL) and placed in a tube containing 200 μl of Dulbecco's phosphate-buffered saline (DPBS; Sigma-Aldrich, St Louis, MO) containing 0.1% gelatin

(Avantor Performance Materials, Phillipsburg, NJ). The tubes were shaken in an orbital shaker at 90 rpm for 1 h at 24 °C and then refrigerated for 48 h at 4 °C prior to beginning the radioimmunoassay procedure. Samples and standards were subsequently assessed in triplicates according to the assay provided protocol. CORT concentrations in standard and sample tubes were determined using a HP Cobra II gamma counter (Canberra-Packard, Meriden, CT). Corticosterone concentrations were quantified in units of pg corticosterone per punch.

2.7 Apparatus and procedures for behavioural testing

Morris water maze

Animals were tested in the water maze 7-8 weeks following cerebral ischemia. Animals were brought to the testing room at least 30 min prior to test administration. The water maze is a white circular pool (183 cm diameter, 45 cm high) filled with water (35 cm depth) and divided into four quadrants. The pool was located in a dimly lit room and was surrounded by black curtains on which posters and calendars served as extra-maze cues. The water was rendered opaque by the addition of non-toxic black Tempera paint and was kept at a constant temperature of 21ºC. A movable circular escape platform (15 cm diameter, 35 cm high) was constructed of Plexiglas. Individual behaviour was monitored with a video camera suspended above the pool and a computerized tracking system (Ethovision 3.1, Noldus IT), enabling latency, distance traveled and swim speed to be later analyzed. The experimenter sat in the corner of the testing room facing a computer screen, which monitored a live feed of the pool, confirming latency to reach the platform using a stopwatch.

Place task

In this task, each rat was assigned a platform location in one of four quadrants ("Quadrant 1"). The platform remained in this location for the duration of the experiment. On a particular trial, a rat was released facing the wall of the pool from one of the starting locations according to a pseudorandom sequence that was different for each rat. Testing occurred over a period of four consecutive days and consisted of two blocks of two trials daily. The two blocks of trials were separated by a 2-h interval whereas each trial within a block was separated by a short 60 s inter-trial interval. Each trial lasted a maximum of 120 s or was terminated when the animal found the platform. If the rat failed to locate the platform within the allotted time, it was manually guided to it by the experimenter. Once the animal reached the platform it was allowed to stay on it for 15 s before being removed from the pool.

Probe trial

Twenty-four hours following the last testing trial, the animals were subjected to a probe trial that consisted of 120 s of free swim period in the absence of the platform. The starting position was in the quadrant opposite to the one containing the platform during testing.

Platform-switched task

Twenty-four hours following the probe trial, the animals were subjected to a "platform-switched" task for three consecutive days. In this task, the submerged platform was placed in the center of one of the four quadrants during the first training day, omitting the quadrant assigned in the place task, and was transferred to the center of the two remaining quadrants on the second and third day of testing. This configuration was given to train the subjects in finding the hidden platform that was repositioned at new locations on

any given day. Measures recorded in the learning-set task were identical to those of the place task.

Visual acuity task

The effects of cerebral ischemia on visual acuity were measured at the end of the experiment and animals were given a visual acuity task (VAT). Each rat was required to perform three consecutive trials in a training day, and the swimming starting position at the perimeter of the maze as well as the location of a visible platform (elevated 1.5 cm above the water surface and covered with a white rubber surface) were varied quasi-randomly from trial to trial. Measures recorded in the VAT were identical to those in the place and learning-set tasks.

2.8 Immunohistochemistry

Seven and eighty-five days following reperfusion, rats were deeply anesthetized using sodium pentobarbital and perfused using 0.9% saline followed by 4% paraformaldehyde solution. Brains were removed and stored at -80°C. Serial coronal sections (14 μm) of the hippocampal regions were subsequently obtained using a cryostat. Briefly, sections were hydrated in 0.01M Phosphate buffered saline (PBS) prior to blocking of nonspecific IgG binding using 0.01% bovine serum albumin in PBS (pH 7.0) for 30 min. Sections were then washed in PBS 5×3 min and incubated with primary antibodies overnight at room temperature (refer to Table 1 for antibody information). The next day, sections were rinsed in PBS 5×3 min and incubated with proper secondary antibodies for 2 h at room temperature. Following 5×3 min PBS rinses, slices were incubated for 10 min with the Hoechst 33342 stain (Invitrogen, Burlington, ON, Canada; product: V13244) enabling to visualize cell nuclei. The primary antibodies used were goat anti-platelet endothelial cell adhesion

molecule (PECAM-1)/CD31 (1:500; Santa Cruz, Santa Cruz, CA, USA; product: SC-1506), goat anti-DCX (1/400; Santa Cruz, Santa Cruz, CA, USA; product: SC-8066) and mouse anti-PSA-NCAM (1/500; Millipore, Billerica, MA, USA; product: MAB5324). DCX is a microtubule-associated protein required for normal neuron migration (Nacher, Crespo, & McEwen, 2001), while PSA-NCAM's expression facilitates structural remodeling and is involved in several neurodevelopmental events, such as neuronal migration, neurite outgrowth and synaptogenesis (Gomez-Climent et al., 2011). The vascular endothelial marker CD31 (PECAM-1) is a 130 kDA glycoprotein constitutively expressed on the cell surfaces of monocytes, neutrophils, platelets and a subpopulations of T cells (by endothelial cells) and is currently used as a marker of angiogenesis (Hu et al., 2010)(refer to Fig. 9 for staining profile). Secondary antibodies used were from Invitrogen Canada Inc. (Burlington, ON, Canada) and were applied for 2 h at room temperature to reveal immunopositive cells at the following concentrations: Alexa Fluor donkey anti-goat 594 (1/1000)(product: A-21203) and Alexa Fluor donkey anti-mouse 488 (1/1000))(product: A-21202). All antibodies were diluted in PBS containing 0.02% Triton-X100 (Sigma, Oakville, ON, Canada). After 5×3 min washes, an anti-fade medium containing 0.1% *p*-phenylenediamine in phosphate buffered glycerol was applied and the slides coverslipped and sealed with nail polish. The immunofluorescent signal detection was accomplished using an Olympus BX51 microscope (Center Valley, PA, USA). Digital images of immunofluorescence were obtained using the ProgRes Pro 2.7.6 software under 200× magnification unless otherwise specified. Brain regions showing positive CD31, DCX and PSA-NCAM tissue expression were corroborated using a rat brain atlas (Paxinos & Watson, 1986). Special controls were also included testing the specificity of the selected antibody. For instance, lack of visible staining when omitting

the primary antibody suggests specificity of the secondary antibody. Moreover, staining of sections through the DG, CA1 and CA3 regions produced a pattern of DCX, PSA-NACAM and CD31 immunoreactivity that was identical with previous descriptions (for immunoreactivity comparison with DCX refer to (Nacher, et al., 2001), PSA-NCAM (Nacher, Alonso-Llosa, Rosell, & McEwen, 2002) and CD31 (Robich, et al., 2010).

2.9 Quantification of double immunofluorescence staining for DCX and PSA-NCAM

In order to quantify DCX and PSA-NCAM immunoreactive cell numbers, 14 µm coronal hippocampal sections spaced 250 µm apart were used. This method led to the collection of three sections per subregion of the dentate gyrus (DG) at the level of -1.3 to -2.0 mm posterior to bregma. For DG quantification, only cells that were located in the granule cell layer (GCL) or the SGZ were counted, and cells that were located more than two cells away from the SGZ were classified as being part of the hilus (Y. S. Choi, et al., 2003). The number of DCX and PSA-NCAM-positive cells in the different subregions was expressed as means ±S.E.M of total counts from 6 hemispheres derived from 6-12 animals per group. The number of DCX and PSA-NCAM co-labelled cells was counted by an investigator blinded to the experimental groups at 400× magnification. Results were reported as the total number of co-labelled cells expressing DCX/PSA-NCAM. Quantification of co-labeled cells expressing DCX/PSA-NCAM was preferred as the total number of labelled cells with each antibody was not significantly different from one another ($P > .05$) (Fig. 6). Previous research also confirmed that within the subgranular zone of the DG, DCX-ir cells also express PSA-NCAM (Nacher, et al., 2001).

2.10 Quantification of CD31 expression

For all regions of interest and for CD31 expression, vascular processes were quantified using Image J software (Image J, National Institutes of Health, Bethesda, Maryland, USA) and the method described by Hayes and colleagues (Hayes, Knapp, Breese, & Thiele, 2005). Briefly, 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 initially requires subtracting the background and then measuring intensely labeled area. For analyses, great care was taken to match sections through the same region of the brain at the same level using anatomical landmarks. Six anatomically matched pictures of the left and right brain hemispheres were used to produce an average ir score for each brain region and animal. Data are presented as mean background corrected standardized image densities.

2.11 Data analysis

All statistical analyses were conducted using SPSS Statistics 20.0 software package. A difference was considered significant when $p \leq .05$. On rare occasions, rats showing two standard deviations from the group average performance were considered outliers and were excluded from the related statistical analysis. The assumptions of homogeneity of variance and of sphericity were verified. The Huynh-Feldt correction for violations to the assumption of sphericity was applied when appropriate and the degrees of freedom adjusted when the correction was used. Significant interactions were further analyzed using simple effects tests with Bonferroni modification of critical α level. CORT levels data were analyzed using a mixed ANOVA design with two independent factors *surgery* and *treatment* and various levels of the repeated factor *time* in order to explore changes in CORT levels for pre-

(Baseline = before surgeries and prior to occlusion) and following 10 min ischemia exposure (3 days postischemia) as well as for pre- and post- (30, 60 and 120 min) restraint time intervals (on post-ischemic day 84). Simple effects tests were used for further analysis of significant interactions. Planned pairwise contrasts followed omnibus ANOVA analysis and compared ischemia-saline vs. sham-saline; ischemia saline vs. ischemia 1 mg/kg RSV and ischemia saline vs. ischemia + 10 mg/kg RSV groups at 3 days postischemia. Spatial memory in the MWM was assessed by averaging performance across all four daily trials. Place task data were analyzed using a mixed three-way ANOVA design with two independent factors *surgery* and *treatment* and various levels of the repeated factor *time*. Probe trial data were analyzed using three-factor (*surgery, treatment* and *quadrant*) ANOVAs. Platform-switched task data were analyzed using three-factor (*surgery*, *treatment* and *day*) ANOVAs. Data from the VAT were analyzed using three-factor (*surgery*, *treatment* and *trial*) ANOVAs. Simple effects tests were used for further analysis of significant interactions. In the water maze, data are expressed as total time (s), distance (cm) and speed (cm/s) (± standard error of the mean) for blocks of four trials per day in the place and platform-switched tasks. Values are expressed as mean ± standard error of the mean for all other tests. Optical density measures obtained from signal to noise ratio of immunofluorescence were analyzed using two-factor (*surgery* and *treatment*) ANOVAs. Simple effects tests further analyzed significant interactions with Bonferroni modification of critical α level. Following omnibus analysis using ANOVA, three planned pairwise contrasts (separate one-way ANOVAS) compared ischemia-saline vs. sham-saline; ischemia-saline vs. ischemia + 1 mg/kg RSV and ischemia-saline vs. ischemia + 10 mg/kg RSV. Data are expressed as mean optical density in a given brain region. Values are expressed as mean ± standard error of the mean for all regions.

3. Results

3.1 Blood CORT levels prior to and following global ischemia

Statistical analysis revealed a main effect of surgery ($F(1, 35) = 4.59$, $p < .05$) was attributable to higher CORT values in ischemic compared to sham animals at the 3-day interval postischemia, irrespective of drug treatment. Planned contrasts indicated significantly higher CORT values in saline-treated ischemic compared to sham rats 3 days postischemia ($p < .05$) (Fig. 2). Moreover, ischemic rats treated with 10 mg/kg RSV displayed decreased post-ischemic CORT levels at this time interval compared to saline-treated rats ($p < .05$).

3.2 Effects of 15 min restraint stress on CORT secretion

CORT levels were assessed immediately before and 30, 60 and 120 min following restraint stress (Fig. 3). A three-way mixed ANOVA revealed a main effect of time ($F(2.49, 92.13) = 161.87$, $p < .001$), surgery ($F(1, 37) = 5.09$, $p < .05$) and a significant time × surgery interaction ($F(2.49, 92.13) = 5.52$, $p < .005$). Simple effects tests revealed that ischemic animals, irrespective of drug treatment, showed higher CORT levels 30 min: $p < .001$ and 60 min: $p < .05$ following restraint stress compared to sham controls. Sham animals also showed heightened CORT secretion in response to restraint stress (30 min: $p < .001$ & at 60 min: $p < .05$). CORT levels of the ischemic and shams groups were comparable to baseline values 120 min following restraint ($p > .05$).

3.3 Morris water maze performance

3.3.1 Place task

During the place task, analysis of the latencies revealed a main effect of days ($F(2.73, 131.21) = 72.49$, $p < .001$), attributable to reduced time to locate the hidden platform for all groups across the initial three testing days ($p < .001$). Analysis of the distance traveled (cm) revealed a main effect of days ($F(3, 144) = 87.19$, $p < .001$) and a days × surgery interaction ($F(3, 144) = 3.27$, $p < .05$). Simple effects tests revealed significantly greater distance traveled by all ischemic rats to reach the platform on testing day 2 compared to sham operated rats ($p < .05$) (Fig. 4). The main effect of days was linked to reduced distance traveled by all the groups across testing days ($p < .005$). Statistical analysis of the speed (cm/s) during the place task revealed a significant main effect of days ($F(2.77, 133.08) = 72.49$, $p < .001$). Post hoc analyses revealed that all rats increased their swimming speed while searching for the platform from day 1 to 2 ($p < .001$), perhaps indicative of increased task familiarity and/or motivation to escape water.

3.3.2 Probe trial

Analysis of swimming time in the target quadrant during the probe trial (Fig. 5) revealed a main effect of treatment ($F(1, 48) = 5.05$, $p < .05$) and a surgery × treatment interaction ($F(1, 48) = 4.2$, $p < .05$). Simple effects tests revealed that saline-treated ischemic rats spent significantly less time in the target quadrant compared to sham controls ($p = .007$). Additionally, ischemic rats treated with 1 mg/kg and 10 mg/kg RSV spent a greater portion of the trial swimming in the target quadrant than saline-treated rats ($p < .01$). Of note, despite the saline-treated ischemic rats showing reduced swim time in the target-coupled quadrant compared to all groups, they still showed a preference for swimming in the target-coupled quadrant compared to the uncoupled quadrant, spending ~35% of testing time swimming in the target zone (above the 25% chance level), which points to a motivated searching strategy.

3.3.3 Platform-switched task

Statistical analysis of the latency in the 'platform-switched' MWM task indicated a surgery × treatment × days interaction ($F(2, 144) = 3.61$, $p < .05$). Graphical representation of the data suggested this result to be due to differences among the groups on the first day of the task. Thus, separate two-way ANOVAs on each day revealed a surgery × treatment interaction on day 1 of testing ($F(1, 48) = 5.34$, $p < .05$). Simple effects tests revealed that 10 mg/kg RSV ischemic rats spent significantly more time to reach the platform than RSV-treated sham rats ($p = .006$). Unexpectedly, saline-treated ischemic rats showed reduced latency to find the hidden platform compared to 1 mg/kg RSV-treated ischemic rats ($p = .037$). Moreover, on the first testing day, 10 mg/kg RSV-treated sham rats displayed increased time to find the platform compared to saline- treated sham rats ($p < .05$). No between-group differences were noted on days 2 and 3, suggesting equivalent rapid learning of the task. As for the distance travelled, analysis revealed a significant surgery × treatment × days interaction ($F(2, 144) = 3.3$, $p < .05$). Graphical representation of the data suggested this result to be due to differences across days between saline and 10 mg/kg RSV-treated sham-operated rats. A separate two-way mixed ANOVA design with one independent factor *treatment* and various levels of the repeated factor *days* revealed a significant treatment × days interaction ($F(2, 36) = 3.54$, $p < .05$). Simple effects tests revealed a mild impairment on the part of RSV-treated rats on day 2 of the platform-switched task, evidenced by increased distance travelled in reaching the platform ($p = .02$). A similar pattern was observed on the last day of testing, although it did not reach significance ($p > .05$). No further between-group differences were detected ($p > .05$). Moreover, no significant differences among the groups were noted with regards to swim speed ($p > .05$).

3.3.4 Visual acuity task

As expected, no significant differences between the groups in swimming latency, distance travelled or speed were observed during the three trials with a visible platform.

3.4 DCX/PSA-NCAM expression in the CA1, CA3 and DG

7 days postischemia

In the DG (Fig. 7), statistical analysis revealed a main effect of surgery ($F(1, 30) = 26.27$, $p < .001$), treatment ($F(1, 30) = 4.55$), $p < .05$) and a surgery × treatment interaction ($F(1, 30) = 14.41$, $p < .005$). Simple effects tests showed increased numbers of DCX/PSA-NCAM co-labelled cells in saline-treated ischemic rats compared to sham controls ($p < .001$). Of interest, ischemic rats treated with the 1 and 10 mg/kg RSV dosage showed significantly reduced number of DCX/PSA-NCAM co-labelled cells compared to their saline-treated counterparts ($p = .002$ and .000, respectively) (Fig.8). Statistical analysis indicated no significant differences in the number of DCX and PSA-NCAM co-labelled cells in the CA1 and CA3 regions ($p > .05$) (Data not shown).

85 days postischemia

Fig. 8 shows the average number of DCX/PSA-NCAM co-labelled cells per hemisphere in the DG region of the hippocampus. Statistical analysis revealed a main effect of treatment ($F(1, 43) = 34.89$, $p < .001$), attributable to reduced DCX/PSA-NCAM co-labelled cells in rats treated with 10 mg/kg RSV compared to saline- and to 1 mg/kg RSV-treated rats ($p < .001$). Furthermore, planned contrasts indicated reduced neurogenesis in ischemic rats treated with 1 and 10 mg/kg RSV compared to saline-treated ischemic rats ($p <$

.05 and $p < .001$, respectively). No significant between-group differences were noted in the CA1 and CA3 regions ($p > .05$) (Data not shown).

3.5 CD31 expression in the CA1, CA3 and DG

7 days postischemia

Fig. 10 shows the effect of RSV treatment and 10 min global ischemia on CD31-ir levels as measured by CD31 immunofluorescence in the CA1 region of the hippocampus, 7 days postischemia. Statistical analysis revealed main effects of treatment ($F(2, 30) = 4.72$, $p < .05$) attributable to 1 mg/kg RSV-treated rats having greater CD31 expression compared to saline treated rats ($p < .01$), and surgery ($F(1, 30) = 7.57$, $p < .05$) attributable to increased CD31-ir expression in ischemic rats compared to sham controls. Planned contrasts revealed increased CA1 CD31-ir in saline-treated ischemic compared to saline-treated sham rats ($p < .05$). Lastly, ischemic rats treated with 1 mg/kg RSV showed heightened expression compared to saline-treated ischemic rats in this region ($p = .012$).

Fig. 11 shows the effect of RSV and 10 min global ischemia on CD31-ir in the CA3 region. Statistical analysis revealed a main effect of treatment ($F(2, 30) = 9.16$, $p < .005$), attributable to higher CD31-ir levels in RSV-treated (1 and 10 mg/kg doses) compared to saline-treated animals ($p < .05$). Planned contrasts indicated reduced angiogenesis in ischemic compared to sham rats at that reperfusion interval ($p = .004$). Additionally, ischemic rats treated with 1 and 10 mg/kg RSV showed enhanced CD31-ir compared to saline-treated ischemic rats ($p < .005$). In the DG region, analysis revealed a main effect of surgery ($F(1, 30) = 12.84$, $p < .005$), attributable to increased CD31-ir expression by ischemic animals, and treatment ($F(2, 30) = 9.78$, $p < .005$) due to decreased CD31-ir by ischemic rats treated with 1 mg/kg RSV compared to the other treatment groups in this region ($p < .005$). Planned

contrasts further revealed that ischemic rats treated with saline had significantly increased CD31-ir expression compared to that observed in sham controls ($p = .035$) and rats treated with 1 mg/kg RSV ($p = .005$).

85 days postischemia

Fig. 10 shows the effect of RSV treatment and 10 min global ischemia on CD31-ir in the CA1 region 85 days postischemia. Statistical analysis revealed a significant surgery × treatment interaction ($F(1, 39) = 5.16$, $p < .05$). Simple effects tests revealed increased CA1 CD31-ir in 10 mg/kg RSV-treated ischemic rats compared to their sham counterparts ($p < .05$). Both the 1 and 10 mg/kg RSV treatments significantly increased ischemia-induced CD31-ir expression in this region ($p = .03$ and $p = .003$, respectively). At the CA3 layer, analysis revealed a significant surgery × treatment interaction ($F(1, 39) = 9.93$, $p < .005$) (Fig. 11). Simple effects tests indicated that 10 mg/kg RSV significantly increased CD31-ir in ischemic rats compared to sham-operated rats receiving the same treatment ($p < .05$). At the DG, analysis revealed a significant main effect of treatment ($F(2, 39) = 11.12$, $p < .001$), attributable to significant increase in CD31-ir in the 10 mg/kg RSV-treated groups compared to saline-treated animals ($p < .001$). The 1 and 10 mg/kg RSV treatments also significantly increased CD31-ir expression postischemia in this region ($p < .03$).

4. Discussion

To our knowledge, this study provides the first evidence of time-related effects of RSV on neurogenesis and angiogenesis expression following brain ischemia. Our findings also provide a first report of the impact of RSV in the regulation of CORT secretion following ischemia and in response to acute stress. Importantly, RSV effects on neurogenesis and angiogenesis paired with reduced CORT secretion postischemia were not associated with

improved spatial memory performance in the MWM in RSV-treated ischemic rats, although they showed increased swim latency in the target zone during the probe trial compared to saline-treated ischemic rats.

Resveratrol reduces hippocampal DCX/PSA-NCAM co-labelling following global ischemia

Consistent with findings from various studies using different ischemic models (J. H. Choi, et al., 2012; Kee, Preston, & Wojtowicz, 2001; Schmidt & Reymann, 2002; Takagi et al., 1999; Tonchev, 2011; Tonchev & Yamashima, 2006; Yagita et al., 2001; Ziemka-Nalecz & Zalewska, 2012), increased expression of neural progenitor markers was observed in the DG 7 days following global ischemia, which had normalized at the 85-day interval. Noteworthy, both RSV dosages prevented ischemia-induced increased DCX/PSA-NCAM expression at the 7-day interval in a dose-related manner. Neurogenesis remained attenuated at the 85 day post-ischemic interval in RSV-treated rats. Increased neurogenesis following various brain insults has been interpreted as a self-repairing process, a proportion of new neurons migrating to injured brain tissue postischemia (Kernie & Parent, 2010; Lichtenwalner & Parent, 2006). In this context, inhibition of neurogenesis represents an interesting finding considering neuronal protection conferred by RSV treatment at the same dosages and post-ischemic time intervals (Girbovan, et al., 2012), which may suggest physiological actions of RSV in reducing neuronal injury also influence hippocampal expression of neurogenic markers. Supportive of this, hypothermia-induced CA1 neuronal protection prevented neurogenesis in the DG following global ischemia (Silasi et al., 2012). To our surprise, the 10 mg/kg RSV dosage also reduced DG DCX/PSA-NCAM expression in sham-operated rats. Although a few studies have characterized RSV effects on neurogenesis,

data is currently lacking on its neurogenic effects in control animals. Nonetheless, one study evaluated RSV administration in mice for a 14-day-period at identical 1 and 10 mg/kg dosages and found reduced proliferation of cultured multipotent NPCs as well as dentate gyrus NPCs in vivo 15 days following drug administration, in a dose-dependent manner (H. R. Park, et al., 2012). The authors also reported that RSV administration impaired spatial memory in the water maze. This is comparable to our findings at the 85-day interval, where the 10 mg/kg RSV-treated rats (ischemic and sham) showed a mild impairment on the platform-switched task compared to respective controls, a finding correlated with reduced DG neurogenesis. Reduced neurogenesis observed in sham rats 85 days following RSV administration suggests delayed dose-related adverse effects on brain processes and additional studies using remote time intervals are warranted to elaborate on these findings.

Resveratrol increases CD31 expression in the CA1 region following ischemia

This study is the first to characterize effects of RSV on remodelling of the vascular niche following global cerebral ischemia. Our findings revealed a significant increase in CD31-ir expression in the CA1 region 7 and 85 days postischemia in RSV-treated rats, although angiogenesis appeared delayed in animals treated with the 10 mg/kg dosage. This is consistent with significant elevations in the expression of VEGF and angiogenic marker CD31 and observed in rats fed a high fat diet and pretreated with RSV for a 15-day period prior to myocardial infarction, an effect associated with improved recovery of cardiac function (Penumathsa et al., 2007). Moreover, pretreatment with RSV increased capillary density and VEGF protein expression following myocardial infarction and has been associated with heightened expression of the anti-apoptotic nuclear factor kappa-light-chain-

enhancer of activated B cells (NF-kB) (Fukuda, et al., 2006), suggesting pro-angiogenic effects of RSV through VEGF signaling. Although detailed mechanisms await further clarification, RSV's ability to regulate angiogenic processes has further been linked to the natural polyphenol's ability to activate sirtuins, in particular SIRT1, which is highly expressed in the vascular endothelium during sprouting angiogenesis and plays a critical role in governing postnatal vascular growth (Hubbard & Sinclair, 2014; Potente et al., 2007; Y. Yang et al., 2013). Indeed, disruption of SIRT1 gene expression in zebrafish and mice results in dysregulated blood vessel formation and blunts ischemia-induced neovascularization while blockade of SIRT1 activation abolishes endothelial sprout formation and migration (Potente, et al., 2007). The involvement of similar mechanisms underlying RSV effects on angiogenesis following cerebral ischemia has not been determined. If involved, dose-related effects of RSV on regulations of these factors might be expected considering differential effects noted in the current study.

Resveratrol attenuated CORT secretion following global ischemia

Increased glucocorticoids has commonly been associated with reduced angiogenesis and neurogenesis in the hippocampus (Gould, et al., 1992; Khorram, et al., 2014; Mayer, et al., 2006) as well as shown to play a role in post-ischemic memory impairments (M. R. Milot & Plamondon, 2011) and neuronal injury (Sapolsky & Pulsinelli, 1985). The current study determined dose- and time-related effects of RSV on basal and stress-induced CORT secretion as well as the impact of the polyphenol on post-ischemic CORT secretion. Moreover, we examined the relationship of these changes to neuro- and angiogenic levels and spatial memory impairments. Consistent with previous observations, 10 min global

ischemia-induced increased CORT secretion 3 days following reperfusion and hypersensitivity of the HPA axis in response to restraint stress (de la Tremblaye, et al., 2014). At the same time interval, CORT levels were reduced in ischemic rats treated with 10 mg/kg RSV. Moreover, RSV showed a trend toward attenuating CORT secretion in response to 15 min restraint stress. To our knowledge, only one study assessed effects of RSV on plasma CORT secretion, reporting inhibitory effects of RSV on CORT levels while hypothalamic CRH mRNA expression remained unchanged, suggesting that RSV exerts its regulative effect on CORT independently of HPA axis activation (Ge, et al., 2013). It is possible that the selected post-ischemic intervals prevented observing significant inhibitory effects of the lower RSV dosage on CORT secretion in our study. Indeed CORT secretion has been shown maximal within minutes to hours on the first day following ischemia and gradually declining thereafter (de la Tremblaye, et al., 2014; M. R. Milot & Plamondon, 2011). A role for glucocorticoids in mediating angiogenesis and neurogenesis in the hippocampus has also been suggested, although the direction and magnitude of the effect remains elusive. While some have reported an association between enhanced glucocorticoids and downregulation of angiogenesis and neurogenesis in the hippocampus (Gould, et al., 1992; Khorram, et al., 2014; Mayer, et al., 2006), others have shown increased CORT levels in conjunction with reduced angiogenesis although having no effect on neurogenesis (Ekstrand, Hellsten, Wennstrom, & Tingstrom, 2008). It is difficult to ascertain the direction of such an effect in the current study as CORT levels were most notably influenced by ischemia 3 days post-insult. Despite this, we saw notable differences in both angiogenesis and neurogenesis levels in the DG following RSV treatment and ischemic surgery 7 days post-surgery. Region variability with regards to both angiogenesis and neurogenesis were also noted in the current

study, making the association between these events and CORT results harder to interpret. It thus remains probable that significant inhibition of CORT secretion by RSV occurred at an earlier time interval, which could have influenced angiogenesis in the CA1 region, and neurogenesis in the DG as well as previously reported neuronal protection (Sapolsky & Pulsinelli, 1985). Studies manipulating CORT levels in control and RSV–treated ischemic animals are necessary to confirm this proposition.

Resveratrol had no effects on spatial memory performance in the MWM

Impaired spatial memory represents a hallmark of global cerebral ischemia (Hartman, et al., 2005; Roberge, Messier, et al., 2008). We selected a demanding MWM task, with reduced number of daily trials, increased inter-block interval and the use of a platform-switched task as findings using standard protocols with a stationary platform have varied considerably (Briones & Therrien, 2000; D'Hooge & De Deyn, 2001; Nelson, et al., 1997). Despite a more challenging paradigm, spatial memory deficits were mild in saline-treated ischemic rats, which achieved comparable performance as that of sham rats within a few trials. Noteworthy however, saline-treated ischemic rats showed reduced swim latencies in the target quadrant during the probe trial, compared to sham controls and RSV-treated counterparts, with swim duration in the target zone approaching chance level. The short-lasting impairments of ischemic animals on the MWM task are in line with other reports, which also support a modest impairment following ischemic injury limited to the first training sessions (Auer, Jensen, & Whishaw, 1989; Block, 1999; Corbett & Nurse, 1998; Hagan & Beaughard, 1990; Nakatomi, et al., 2002; Nunn et al., 1994). Of interest, sustained functional deficits in ischemic animals have been shown in the radial arm maze with normal

performance in the MWM (Langdon, et al., 2008). It has been suggested that unlike other spatial tasks, including the 8-arm radial maze, which are motivated by food reward, the water maze employs aversive escape motivation, which is considered more stressful but can lead to faster learning (Nelson, et al., 1997). Neurogenesis within the CA1 field of the hippocampus between 21 to 90 days following global ischemia has also been associated with improved learning and memory in a water maze task, raising the possibility that neuronal compensation occurring within the CA1 at long intervals postischemia may underlay functional recovery (Bendel, et al., 2005). However, these newly regenerated neurons had a relatively short-life and most underwent apoptosis three months following global ischemia (Bueters, von Euler, Bendel, & von Euler, 2008). Our findings did not show significant differences in the number of co-labelled DCX/PSA-NCAM cells within the CA1 region at either time interval. Interestingly, decreased neurogenesis in sham rats treated with 10 mg/kg RSV was accompanied by a mild impairment limited to the MWM platform-switched task. This is an intriguing finding, which might suggest that neurogenesis could contribute to memory processes that more exclusively depend on hippocampal function such as visually guided spatial memory, a hypothesis that may partly explain inconsistent findings in studies assessing relationship of neurogenesis to behaviour using different paradigms. The use of tasks that selectively target hippocampal-dependent memory processes may raise more consistent findings.

Conclusion

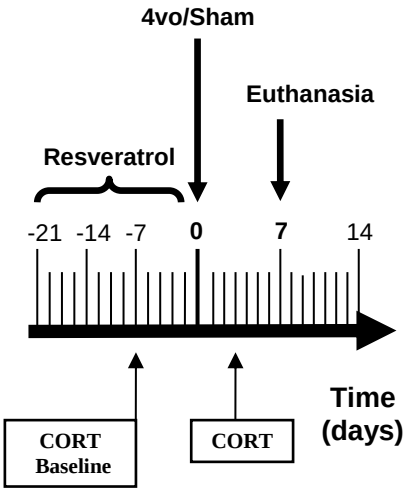
Our study is the first to examine time and dose-related effects of RSV on the expression of neuro- and angiogenesis markers as well as to examine the relationship of post-ischemic CORT levels with hippocampal plasticity postischemia. We also demonstrated

region-specific effects of RSV on neurogenesis and angiogenesis following brain ischemia, which are coherent with enhanced CA1 neuronal survival conferred by the polyphenol following global ischemia. RSV's neuroprotective actions and ability to upregulate angiogenesis paired with dose-related effects on glucocorticoids make it an interesting prophylactic candidate displaying an ability to act on multiple biological targets. Nonetheless, RSV's broad biological targets commands further investigation into possible dose-related effects and actions in healthy controls as well as in-depth characterization of performance on behavioural and cognitive measures.

Acknowledgements

The authors sincerely thank Sylvie Émond for her excellent technical assistance in the animal facility.

Short-term group



Long-term group

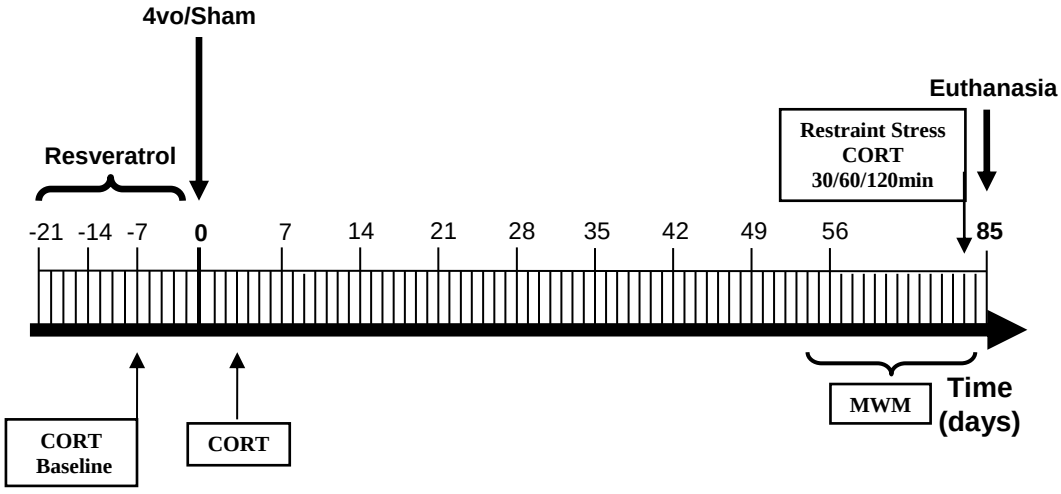


Figure 1. Experimental protocol. Schedule showing time intervals for all experimental procedures. 21-day pre-surgical period of RSV (1 mg/kg or 10 mg/kg;) or saline i.p. injections; 4vo: 4-vessel occlusion; MWM: Morris water maze. Day 0 refers to the day of the surgery.

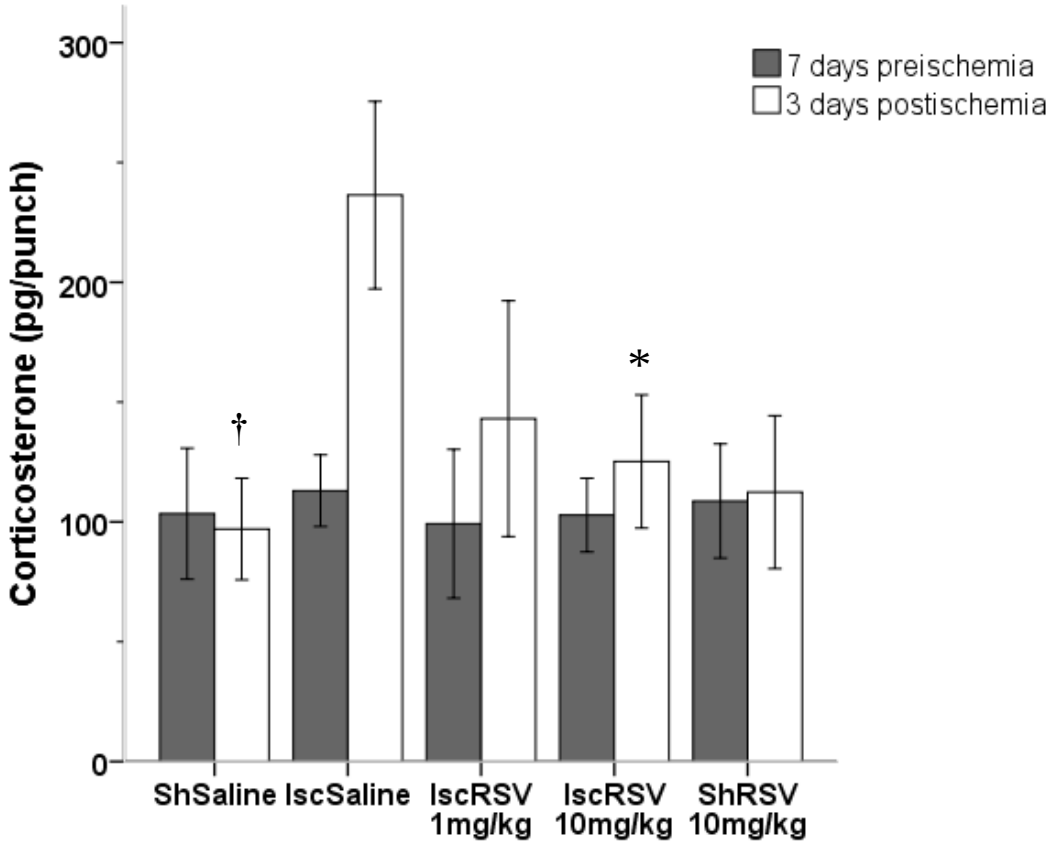


Figure 2. Effect of 10 min global ischemia or sham occlusion on blood CORT secretions measured 7 days prior to and 3 days postischemia. Symbols indicate a significant difference between saline-treated ischemic rats, their sham counterparts († $p < .05$) and 10 mg/kg RSV-treated ischemic rats (* $p < .05$). Values are expressed as mean score ± S.E.M.

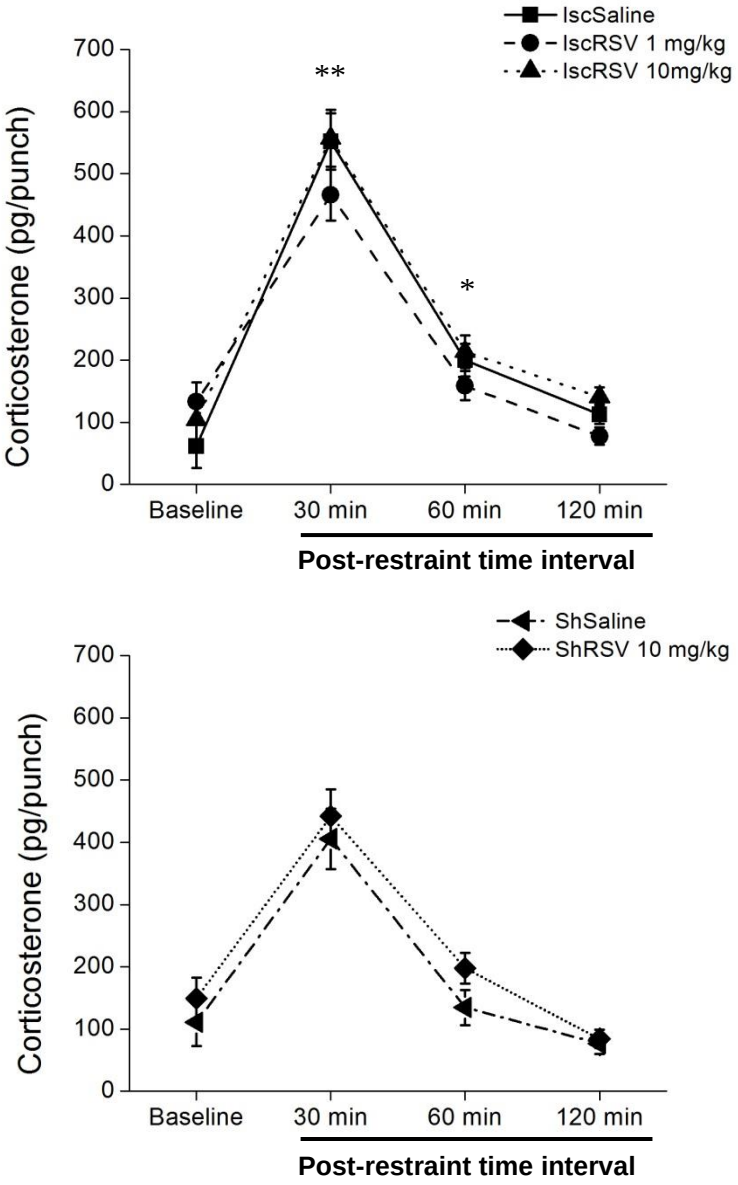


Figure 3. Effect of 15 min restraint stress introduced at 84 days following reperfusion on blood CORT concentrations (pg/punch). Acute restraint stress significantly elevated CORT secretion in ischemic compared to sham rat groups, irrespective of treatment at 30 (* $p < .05$) and 60 min (** $p < .001$) following restraint stress. Values are expressed as mean ± S.E.M.

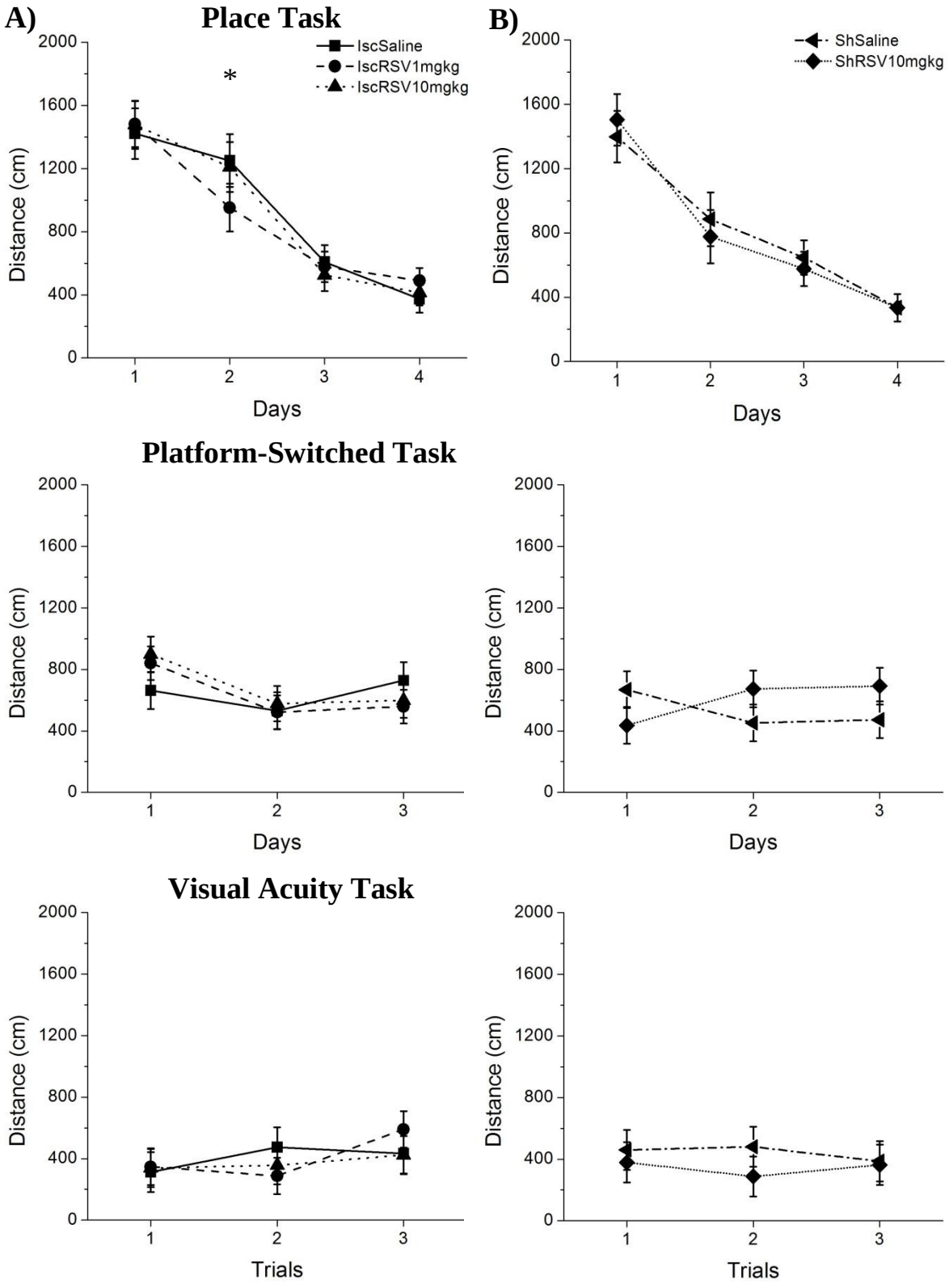

A)
Place Task
IscSaline
IscRSV1mgkg
IscRSV10mgkg
*
Distance (cm)
Days
B)
ShSaline
ShRSV10mgkg
Distance (cm)
Days
Platform-Switched Task
Distance (cm)
Days
Distance (cm)
Days
Visual Acuity Task
Distance (cm)
Trials
Distance (cm)
Trials

Figure 4. Effect of 10 min global ischemia and RSV treatment on distance travelled the place task, platform-switched task and visual acuity task (VAT) in the MWM on day 75 postischemia in ischemic (A) and sham (B) animals. On day 2 of the place task, ischemic animals, irrespective of treatment, travelled significantly greater distance in search of the platform than sham-operated animals (* $p < .05$). Values represent mean score ± S.E.M.

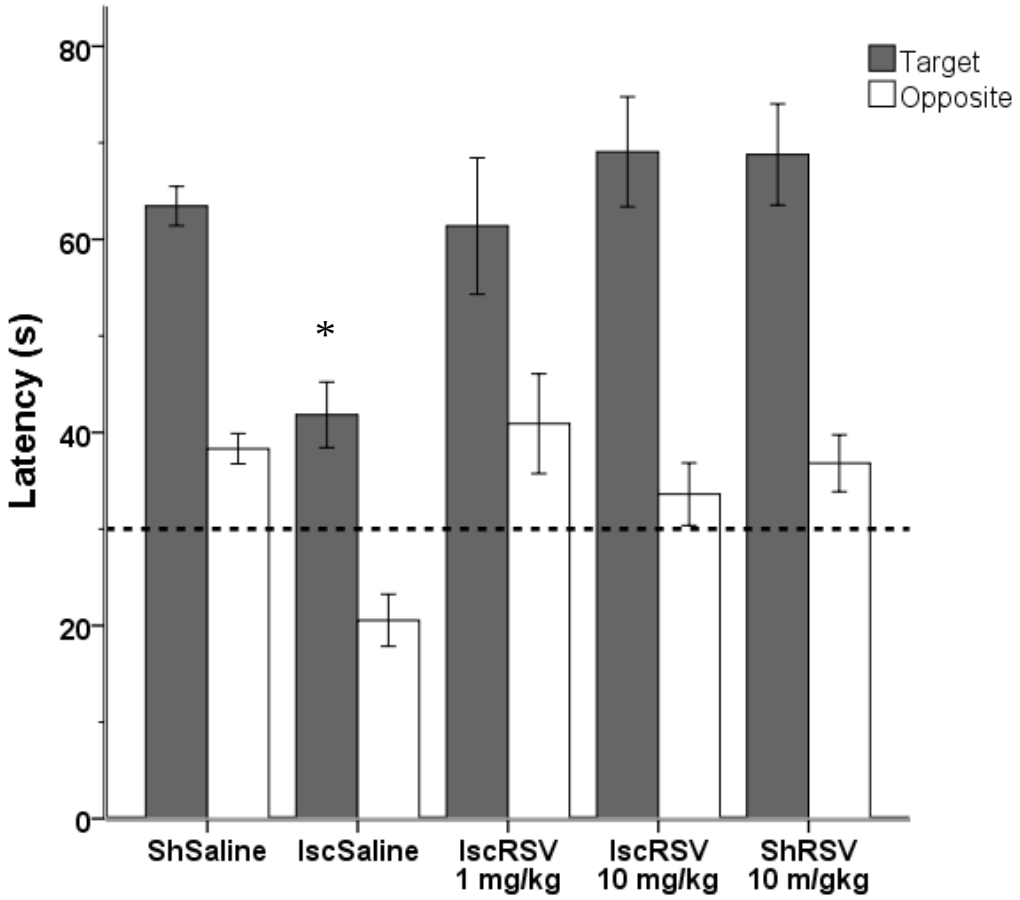


Figure 5. Histogram showing mean latency (s) of saline and RSV-treated ischemic and sham animals during the probe trial of the MWM. Saline-treated ischemic animals spent significantly less time swimming in the target quadrant compared to 1 and 10 mg/kg RSV-treated ischemic rats (* $p < .01$). Values represent mean latency ±S.E.M.

Primary Antibodies

Antigen	Type	Isotype	Host	Immunogen	Dilution	Source
PECAM-1	polyclonal	IgG	Goat	Platelets	1:500	Santa Cruz
DCX	polyclonal	IgG	Goat	Immature neurons	1:400	Santa Cruz
PSA-NCAM	monoclonal	IgG	Mouse	Immature/migrating neurons	1:500	Millipore

Secondary Antibodies

Host	Reactivity	Target Isotype	Label/Dye	Dilution	Source
Donkey	Mouse	IgG	Alexa Fluor 488	1:1000	Invitrogen
Donkey	Goat	IgG	Alexa Fluor 594	1:1000	Invitrogen

Table 1. Summary of antibodies used during immunohistochemistry procedures. Abbreviations; Doublecortin (DCX); Platelet/Endothelial Cell Adhesion Molecule 1 (PECAM-1); Polysialic Acid – Neural Cell Adhesion Molecule (PSA-NCAM).

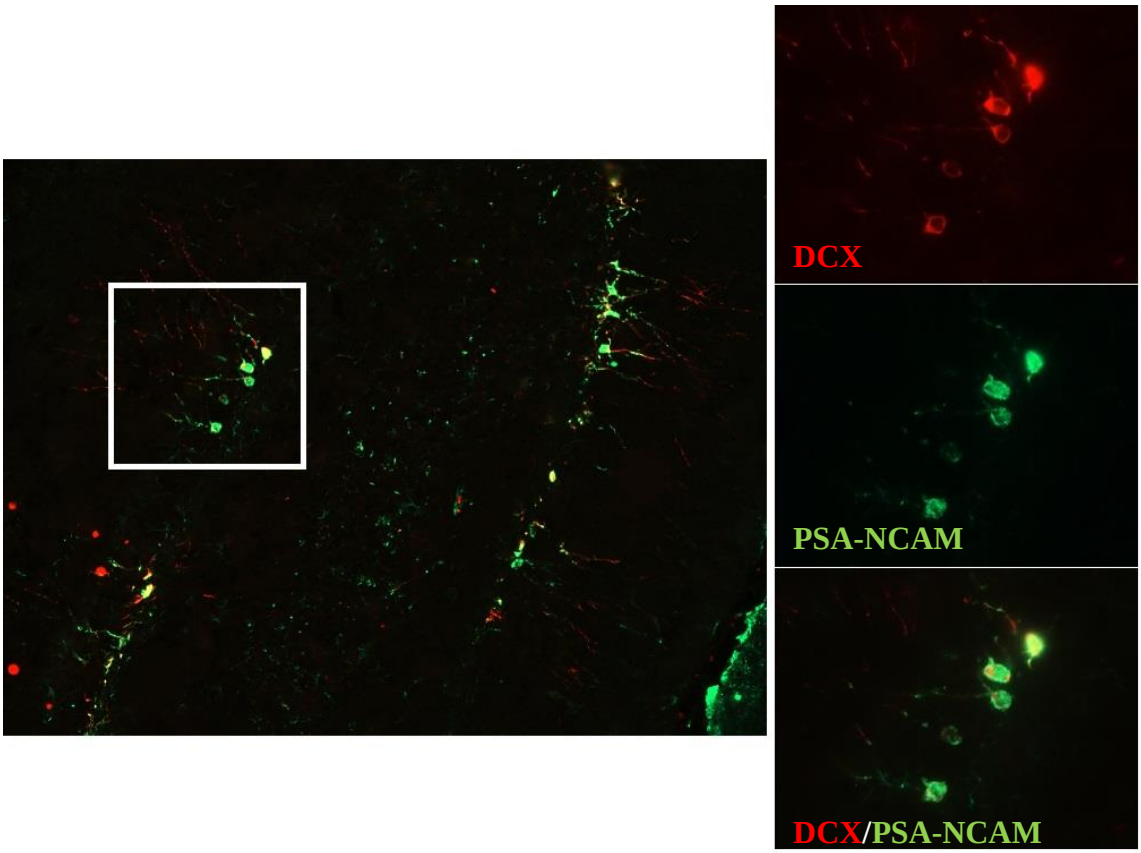


Figure 6. DCX and PSA-NCAM immunoreactivity in the DG of the hippocampus at 100× and 400× magnification.

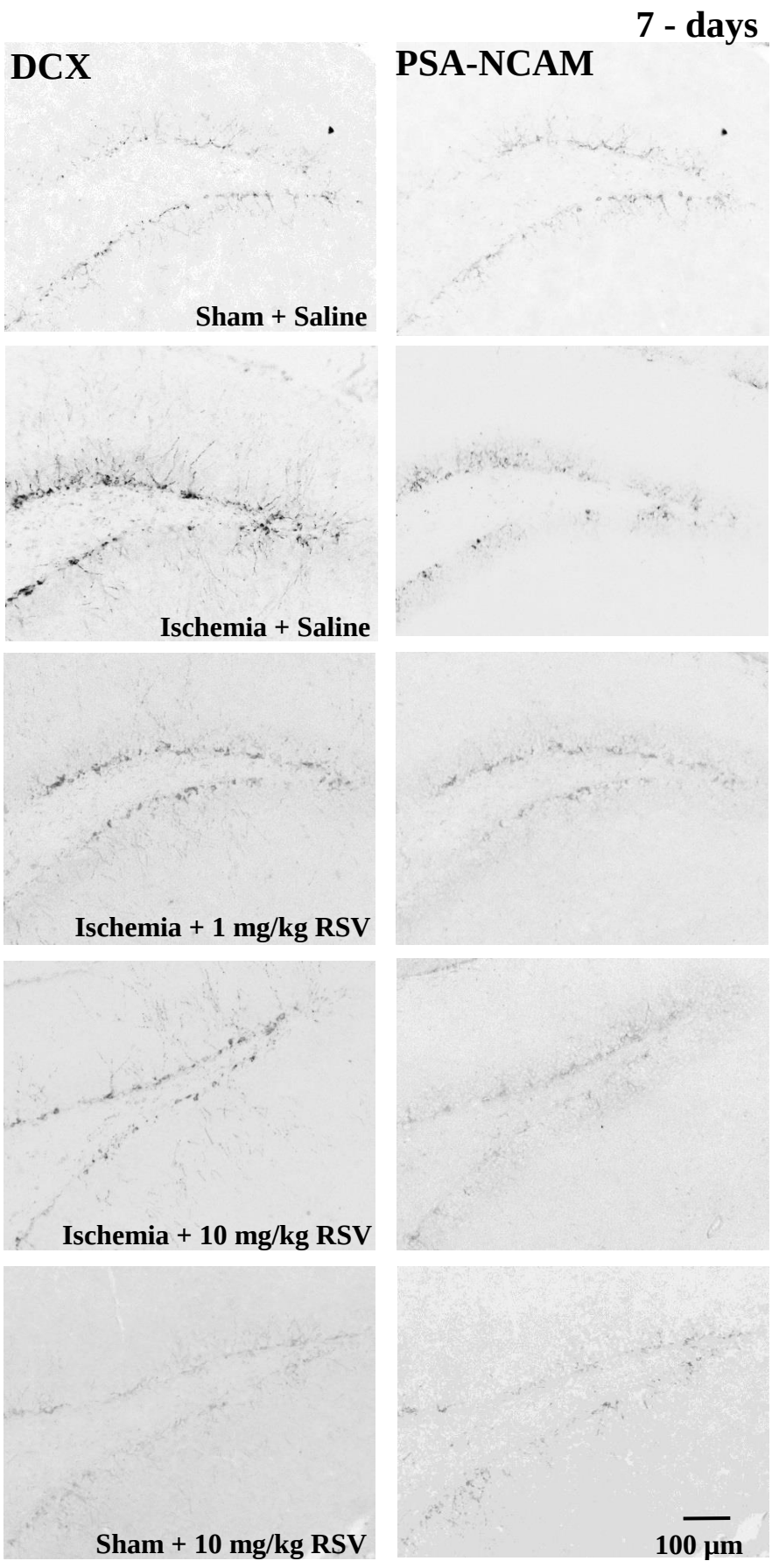

7 - days
DCX
PSA-NCAM
Sham + Saline
Ischemia + Saline
Ischemia + 1 mg/kg RSV
Ischemia + 10 mg/kg RSV
Sham + 10 mg/kg RSV
100 µm

Figure 7. Representative photomicrographs of DCX and PSA-NCAM labelling in the DG region of the hippocampus at 7 days following ischemic and sham surgery at 100× magnification.

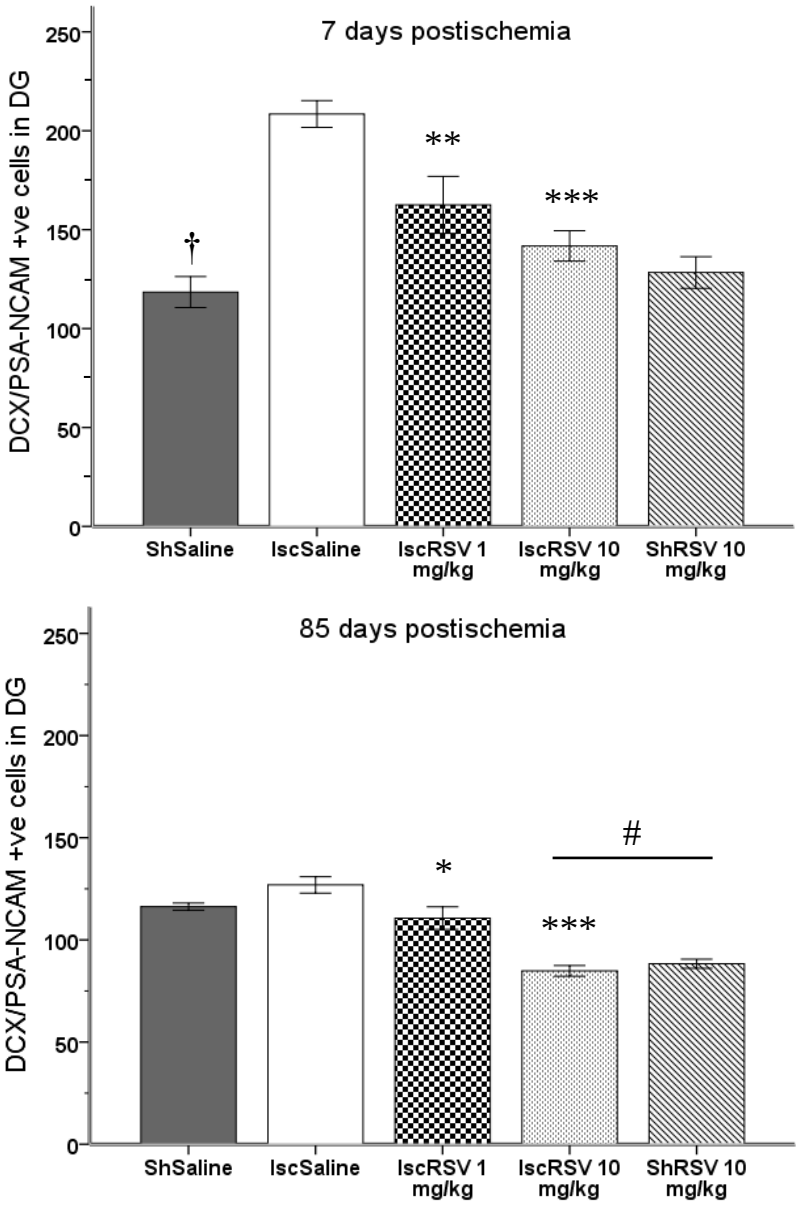


Figure 8. The effect of 10 min global ischemia and RSV treatment on the expression of DCX and PSA-NCAM in the DG region of the hippocampus at 7 and 85 days postischemia. Ischemic animals treated with saline had significantly greater number of DCX/PSA-NCAM co-labelled cells in the DG at 7 days postischemia than both 1 and 10 mg/kg RSV-treated animals. At 85 days postischemia, pretreatment with 10 mg/kg RSV prior to ischemic surgery resulted in decreased numbers of DCX/PSA-NCAM co-labelled cells compares to saline-treated rats. Symbols indicate a significant difference between saline-treated ischemic rats their sham counterparts († P<.001) and RSV-treated ischemic rats (* $p < .05$, ** $p < .005$, $p < .001$). # indicates a significant difference between 10 mg/kg RSV-treated rats and all

other treatment groups ($p < .001$). Values represent mean optical density ± S.E.M.

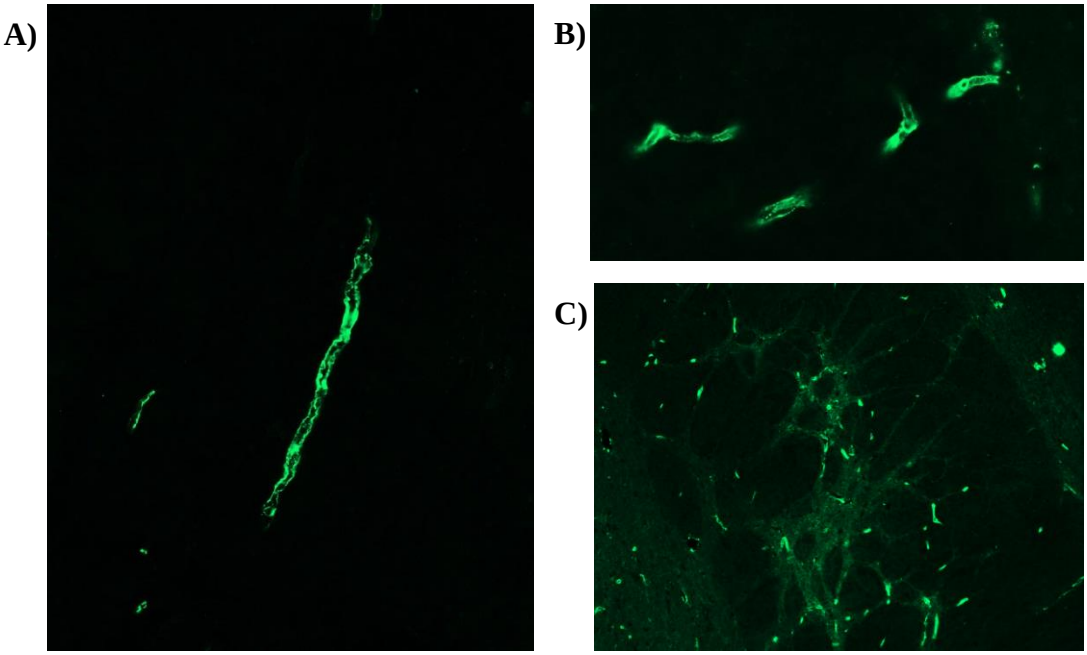


Figure 9. Characteristic CD31 expression in the hippocampus (A & B) at 400 × magnification and in the internal capsule (C) at 100 × magnification. PECAM-1 (CD31) is a protein normally found on endothelial cells, platelets, macrophages and is also expressed in certain tumor cells. Here, we illustrate how CD31 expression highlights vessel morphology in the hippocampus and internal capsule, making it a widely used marker for angiogenesis.

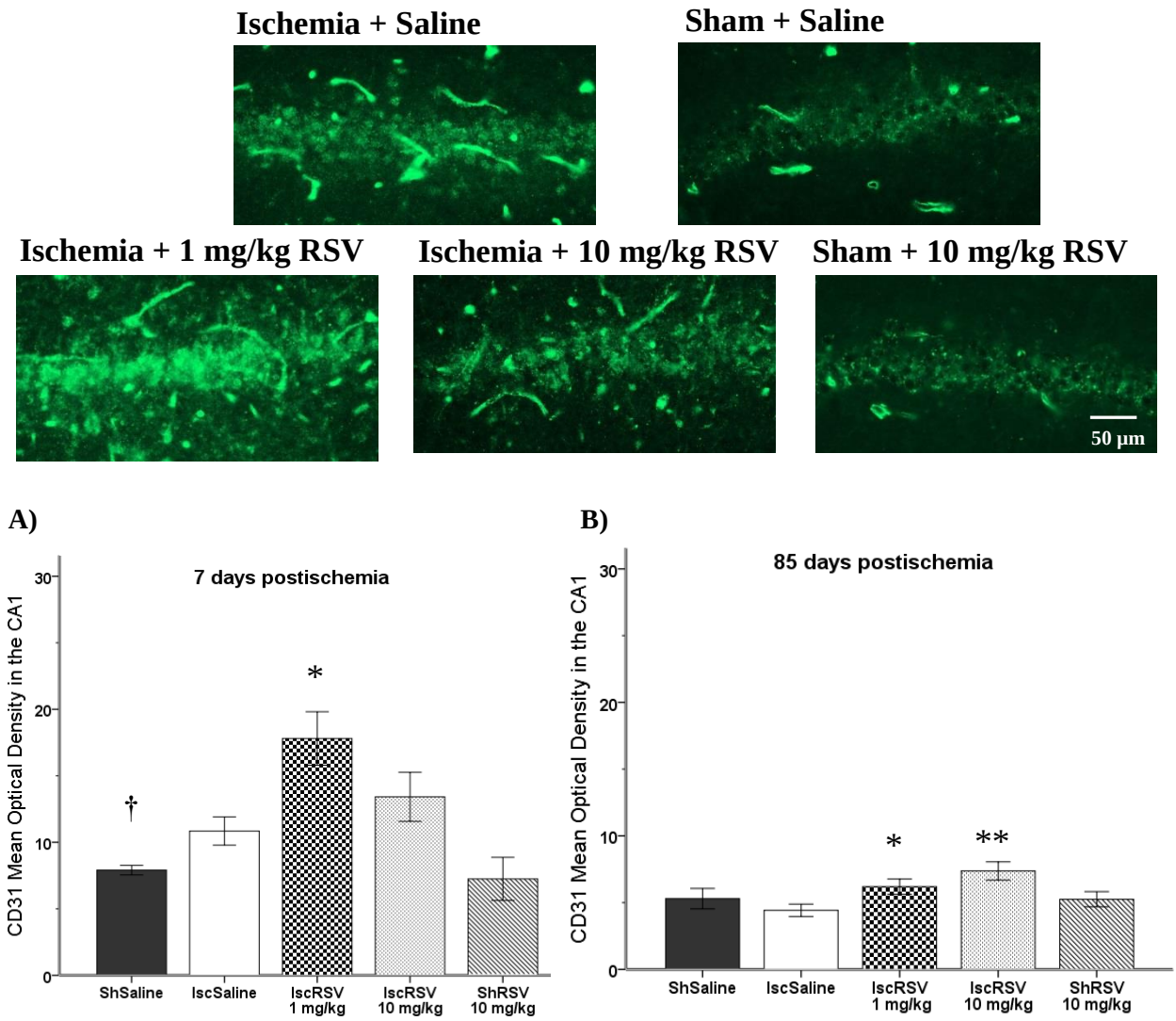


Figure 10. The effect of 10 min global ischemia and RSV treatment on mean optical density of CD31 expression in the CA1 region of the hippocampus at 7 (A) and 85 (A) days postischemia. Ischemic rats treated with 1 mg/kg RSV showed heightened CD31 expression compared to saline-treated ischemic rats at 7 days, whereas pretreatment with both RSV doses increased CD31 expression in ischemic rats at 85 days. Symbols indicate a significant difference between saline-treated ischemic rats, their sham counterparts († $p < .05$) and RSV-treated ischemic rats (* $p < .05$, ** $p < .005$). Values represent mean optical density ± S.E.M.

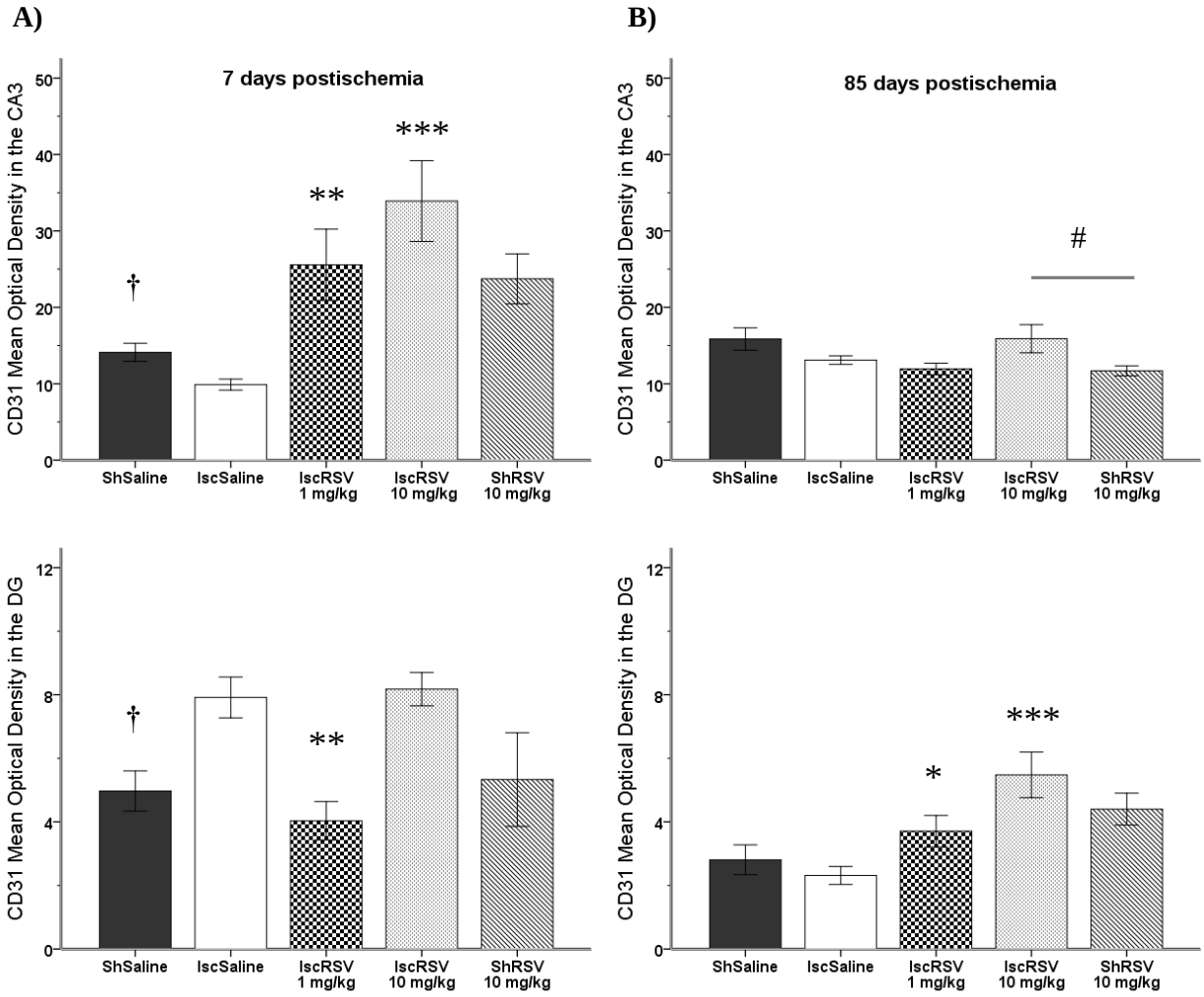


Figure 11. The effect of 10 min global ischemia and RSV treatment on mean optical density of CD31 expression in the CA3 and DG regions of the hippocampus at 7 (A) and 85 (B) days postischemia. At 7 days postischemia, 1 and 10 mg/kg RSV treated rats showed heightened CD31 expression in the CA3 while in the DG, 1 mg/kg RSV-treated ischemic rats showed decreased expression compared to saline-treated rats. Symbols indicate a significant difference between saline-treated ischemic rats, their sham counterparts († P<.05) and RSV-treated ischemic rats (* $p < .05$, ** $p < .01$, *** $p < 0.005$). # indicates a significant difference between 10mg/kg RSV-treated rats and all other treatment groups ($p < 0.05$). Values represent mean optical density ± S.E.M.

Article 3

Published as: Girbovan, C., & Plamondon, H. (2015). Chronic resveratrol pretreatment modulates glial cell activation and GLT-1 expression following global cerebral ischemia in rats. *Brain Research. (In Press).*

Preface

The second experiment determined that RSV effects on neuronal protection are accompanied by regulation of neurogenesis and angiogenesis markers following global ischemia and sham surgery. The third experiment of the thesis aimed at investigating RSV effects on glial cell populations, which are known to fluctuate with the degree of neuronal injury as well as help regulate extracellular levels of glutamate via cell membrane glutamate transporters. The current study thus investigated RSV effects on glial cell activation and astrocytic type-1 glutamate transporter in the short-reperfusion (7 days postischemia) cohort of rats. This study will shed light on non-neuronal mechanisms of action of the polyphenol in providing neuroprotection against ischemia.

Abstract

Cell surface glutamate transporters on perisynaptic astrocytes regulate extracellular glutamate levels and synaptic activation. Accumulating evidence suggests down-regulation of astroglial type-1 glutamate transporter (GLT-1) expression in the CA1 pyramidal layers of the hippocampus contributes to neurotoxic levels of glutamate postischemia. The naturally occurring polyphenol phytoalexin resveratrol (RSV) has been shown to protect the brain against ischemic injury. While a number of RSV's actions on neuronal populations have been related to prevention of neuronal damage, regulatory effects of the polyphenol on astroglial GLT-1 expression following an ischemic insult remain unknown. The current study examined the effects of 21-day RSV pretreatment (1 or 10 mg/kg dose; i.p.) on microglia and astrocyte activation and characterized GLT-1 expression in the DG, CA1 and CA3 layers of the hippocampus 7 days following 10 min global ischemia. Male Wistar rats were divided into five groups; sham/saline, ischemia/saline, ischemia/1 mg/kg RSV, ischemia/10 mg/kg RSV and sham/10 mg/kg RSV. Immunohistochemical detection of OX-42, glial fibrillary acidic protein (GFAP) and GLT-1 was used to assess microglial/glial cell activation and GLT-1 7 days following sham surgery or global ischemia. Our findings revealed significant increases in OX-42 and GFAP expression in ischemic compared to sham-operated animals and a reduction in glial cell activation by RSV-treated rats in CA1 neurons. This was accompanied by reduced GLT-1 expression in the CA1 region postischemia, a phenomenon prevented by RSV treatment.

Keywords: resveratrol; microglia; astrocytes; cerebral ischemia; GLT-1

1. Introduction

The pathogenesis of cerebral ischemia involves a complex sequence of physiological events that includes a well-documented inflammatory cascade, characterized by activation of glial cells, including microglia and astrocytes, and pro-inflammatory cytokines thought to contribute to the selective neuronal injury observed in the hippocampal CA1 region postischemia (Madinier, et al., 2009; F. Zhang, J. Liu, et al., 2010). Often described as sensors of pathological events, microglia make up 5-20% of the glial population in the mature brain (Persson & Ronnback, 2012). Following cerebral ischemia, microglia become positive for markers of activation in the hippocampus as early as 24 h post-insult, and are primarily localized within the CA1 region 7 days post-insult (Anderova, et al., 2011; Dos-Anjos, et al., 2009; Langdon, et al., 2008; Madinier, et al., 2009; Moon, et al., 2009). Likewise, astrocytes are the most abundant cells of the CNS and their roles are diverse, including release of neurotrophic factors, control of fluid, ion and pH homeostasis, neurotransmitter scavenging and management of metabolite and waste products (Swanson, et al., 2004; Takano, et al., 2009). Astrocytes' morphology is markedly altered as they become reactive following ischemia, a phenomenon characterized by thickened and retracted processes and an enlarged soma (Panickar & Norenberg, 2005). Increased proliferation of reactive astrocytes to the site of injury along with a sustained and progressive increase in levels of the cytoskeletal protein, GFAP in astrocytes have been reported to occur in tandem (Panickar & Norenberg, 2005; Petito & Halaby, 1993). Notwithstanding a close association with the degree of brain injury, the role of glial activation on adjacent neuronal populations postischemia remains a matter of debate. While release of chemokines and reactive oxygen

species has conferred a neurotoxic role, enhanced glutamate uptake and upregulation of GLT-1 following glial activation promote neuroprotection (Nakajima, et al., 2008).

GLT-1 is the predominant subtype of glutamate transporters expressed in astrocytes of the cerebral cortex and hippocampus (Yeh, et al., 2005), and plays a key role in terminating synaptic glutamate transmission by mediating 90% of glutamate uptake (Danbolt, 2001). Chronic inhibition of glutamate transporters has been shown to significantly reduce clearance of synaptic glutamate and increase excitotoxic neuronal damage following ischemia (J. C. Chen, et al., 2005). Reduced GLT-1 mRNA expression has been measured for 24 h in the ipsilateral hippocampus and cortex following focal ischemia in mice (Ketheeswaranathan, et al., 2011), and GLT-1 protein expression is attenuated in hippocampal CA1 neurons following perinatal hypoxic-ischemic injury in rats (Zhao, et al., 2012). Thus, increasing glutamate uptake following brain ischemia may prove beneficial in attenuating neuronal injury.

Among nutraceuticals, RSV demonstrates a variety of pharmacological actions including antioxidant, anti-inflammatory, neuroprotective as well as improves learning and memory impairments in a number of disease models (Bhat, et al., 2001; Bradamante, et al., 2004; Girbovan, et al., 2012). In vitro, recent reports have demonstrated its ability to downregulate glial cell activation through inhibition of pro-inflammatory cytokines and key signalling molecules (Candelario-Jalil, et al., 2007; X. Lu, et al., 2010; F. Zhang, J. S. Shi, et al., 2010; F. Zhang, et al., 2013). In this context, RSV's inhibitory actions on microglia and astrocytes' activation have been proposed as mechanisms fostering neuroprotection against ischemic injury (F. Zhang, J. Liu, et al., 2010).

1.1 Objective of the study

An essential role of glial cells and GLT-1 is suggested in attenuating excess synaptic glutamate and excitotoxic cell death. Moreover, in vitro, RSV has been shown to enhance glutamate uptake from astrocytes. Based on the above, it is reasonable to hypothesize that GLT-1 may play an important role in the acquisition of brain ischemic tolerance induced by RSV pretreatment. Thus, the current study aims to examine effects of 21-day pretreatment with RSV on glial cell activation 7 days following global cerebral ischemia and asses concomitant changes in the expression of GLT-1 within the hippocampus. Region-dependent changes could be indicative of a role of these endogenous factors in RSV- mediated neuroprotection postischemia (Girbovan, et al., 2012).

2. Materials and methods

2.1 Animals

Male Wistar rats (N = 35) weighing between 100-125g at time of arrival to the animal facility (325-375g at time of surgery) were obtained from Charles River Laboratories (Rochefort, Quebec, Canada). Upon arrival, animals were individually housed and maintained on a 12 h light/dark cycle (lights on at 7:00 AM), with free access to water and standard (Purina) rat chow. Room temperature was maintained at 21-23°C with 60% relative humidity. One week following arrival, animals were randomly assigned to five experimental groups and chronic injections initiated: ischemia + saline (n=7), sham + saline (n=6), ischemia + 1 mg/kg RSV (n=8), ischemia + 10 mg/kg RSV (n=8) and sham + 10 mg/kg RSV (n=6). Four weeks following the rats' arrival and upon completion of drug treatment, sham or ischemic surgeries were performed (refer to Fig. 1 for experimental timeline). Nine out of 32 rats operated in the ischemic groups died during the ischemic surgery. The animal numbers

indicated above represent rats remaining in each experimental group. All experimental procedures were in accordance with the guidelines set by the Canadian Council of Animal Care and approved by the University of Ottawa Animal Care and Ethics Committee. Efforts were made to minimize the number of animals used and their suffering.

2.2 Resveratrol administration

RSV (Sigma, St. Louis, MO, USA) was dissolved in 50% ethanol and separated in 50 µg aliquots, which were lyophilized and then stored at -80^{o}C until use. Aliquots were freshly dissolved daily in a vehicle consisting of 0.9% saline solution containing 20% hydroxypropyl h-cyclodextrin (Sigma, St. Louis, MO, USA). Twenty-one days before the surgery, sham and ischemic animals received daily, between 9:00-11:00 h, intraperitoneal injections of either saline or 1 or 10 mg/kg RSV (injection volume was 0.1 ml/100 g rat weight). The last saline or RSV injection was administered 1 h prior to ischemic or sham surgery. Rats were weighted daily and the injection volume adjusted to correspond to the animal's body weight. The RSV doses were selected based on earlier reports showing beneficial effects in different experimental paradigms (Mukherjee, et al., 2010; Sinha, et al., 2002).

2.3 Transient forebrain ischemia

Forebrain ischemia was performed using the four-vessel occlusion model as previously described (Pulsinelli & Brierley, 1979). Briefly, rats were deeply anesthetized by inhalation of 1.5% halothane in oxygen (1.5 to 2L/min). Vertebral arteries were irreversibly occluded by electrocoagulation, and a small-diameter silk thread looped around the carotid arteries to facilitate subsequent occlusion. Twenty-four hours later, common carotid arteries were occluded with microaneurysm clamps for 10 minutes in spontaneously ventilating animals. Sham-operated animals underwent anaesthesia and received the same dorsal and

ventral surgical incisions as the ischemic groups, with the exception of electrocoagulation of the vertebral arteries. Twenty-four hours later, carotid arteries were exposed, but not clamped. Only rats that lost the righting reflex over the entire occlusion period were included in the study. Core temperature was kept at 37°C ± 0.5 throughout the surgery using a feedback-regulated heating blanket connected to a rectal thermometer (Homeothermic Blanket Control Unit, Harvard Instruments, Natick, MA). Rats' body temperature was further supported with a heating pad in the hours following surgery and reperfusion.

2.4 Analysis of neuronal density on thionin-stained sections

Seven days following reperfusion, rats were deeply anesthetized using sodium pentobarbital and perfused using 0.9% saline followed by 4% paraformaldehyde solution. Brains were removed and stored at -80°C. Serial coronal sections (14 µm) of the hippocampal regions were subsequently obtained using a cryostat and stained for Nissl bodies with thionin. Neuronal density of the hippocampal CA1 subfield was determined using the method of Kirino et al. (1991) and performed on coronal sections located between 3.14 and 4.16 mm posterior to bregma (Paxinos & Watson, 1986). The total linear length of the CA1 sector (as defined by Paxinos and Watson) was measured by means of a digitizer. The number of intact neurons in the stratum pyramidale within the CA1 subfield was counted using a Leica DAS microscope attached to a Sony digital camera and computer-assisted cell counting was performed using Norton Eclipse (v 6.0). Neurons that had shrunken cell bodies with surrounding empty spaces were excluded. The neuronal density of the CA1 sector**,** *i.e.* the number of intact pyramidal cells per 1 mm linear length of stratum pyramidale was quantified by a person that was blind to the treatment conditions. A mean value for the CA1 hippocampal subfield was obtained from six bilateral measurements per animal in each of the

experimental groups. The neuronal density for a given animal represents the average of both the right and left hippocampal measures.

2.5 Immunohistochemistry

Activated microglia were detected through immunohistochemistry by the presence of the protein CD11B (clone OX-42). This protein is expressed on the surface of microglia and forms part of the complement receptor 3. Tissue levels have been shown to markedly increase upon activation of microglia (Kettenmann, Hanisch, Noda, & Verkhratsky, 2011; Tsuda et al., 2003). Immunohistochemical detection of astrocytes was performed using an antibody against the cytoskeletal protein and class-III intermediate filament, GFAP (Anderova, et al., 2011; Middeldorp & Hol, 2011). GFAP is an astrocyte-specific intermediate filament protein whose expression is required for fibrous astrocyte normal functioning, including maintenance of CNS white matter and blood-brain barrier integrity (Liedtke et al., 1996). The tissue increases in GFAP immunoreactivity is currently used as an index of reactive astrocytosis following various brain insults, including ischemia (Cechetti, Pagnussat, et al., 2012; Eng, et al., 2000; Petito & Halaby, 1993). Briefly, brain sections were washed in 0.01M phosphate-buffer saline (PBS) 5×3 min and then exposed to a pretreatment solution (97% PBS, 1% BSA, 0.02% Triton X-100; Sigma, Oakville, ON, Canada) for 30 min at room temperature. Sections were then incubated for three hours at room temperature using mouse monoclonal for CD11b/c to OX-42 (1/500; Abcam, Cambridge, MA, USA; product: ab1211), rabbit polyclonal for GFAP (1/1000; Abcam, Cambridge, MA, USA; product: ab7260) and guinea-pig polyclonal for GLT-1 (1/500; Millipore, Billerica, MA, USA; product: ab1783) (refer to Table 1). Following incubation, slides were rinsed in PBS (5×3 min) and Alexa Fluor donkey anti-mouse 488 (1/500; Invitrogen, Burlington, ON,

Canada; product: 21202), Alexa Fluor donkey anti-rabbit 594 (1/500; Invitrogen, Burlington, ON, Canada; product: 21207) or Alexa Fluor goat anti-guinea-pig 488 (1/500; Invitrogen, Burlington, ON, Canada; product: A-11073) were applied and incubated at 37°C for 30 min to reveal immunopositive cells. All the primary and Alexa-Fluor-conjugated secondary antibodies were diluted in the blocking solution previously described. Special controls were performed using omission of the primary antibodies to assess background staining associated to non-selective binding of the secondary antibody. Moreover, the pattern of immunoreactive staining through the hippocampus obtained using OX-42, GFAP and GLT-1 primary antibodies was comparable to previous descriptions (Carbone, et al., 2012; Langdon, et al., 2008; Simao, et al., 2012a). Following 5×3 min rinses, slides were incubated with 1µg/ml Hoescht 33342 stain (Invitrogen, Burlington,ON, Canada) for 10 min at room temperature to label cell nuclei. After three washes, an anti-fade medium containing 0.1% *p*-phenylenediamine in phosphate buffered glycerol was then applied and the slides coverslipped and sealed with nail polish. The immunofluorescent signal detection was accomplished using an Olympus DX51 microscope (Center Valley, PA, USA). Digital images of immunofluorescence were obtained using ProgRes Pro 2.7.6 software under 400× (CA1 and CA3) and 200× (DG) magnification.

2.6 Data analysis

All statistical analyses were conducted using SPSS Statistics 20.0 software package. A difference was considered significant when $p \leq .05$. On rare occasions, rats showing two standard deviations from the group average performance were considered outliers and were excluded from the related statistical analysis. The assumption of homogeneity of variance was verified. Data obtained from neuronal densities in the CA1 subfield of the hippocampus

and signal to noise ratio of immunofluorescence were analyzed using two-factor (*surgery* and *treatment*) ANOVAs. Simple effects tests were used for further analysis of significant interactions with Bonferroni modification of critical α level. Planned pairwise contrasts followed omnibus ANOVA analysis and compared ischemia-saline vs. sham-saline; ischemia-saline vs. ischemia + 1 mg/kg RSV and ischemia-saline vs. ischemia + 10 mg/kg RSV. A Pearson's product correlation was also run to determine the relationship between neuronal density and GLT-1 immunoreactivity in the CA1 subfield of the hippocampus. Data are expressed as mean optical density in a given brain region. Values are expressed as mean ± standard error of the mean for all regions.

2.7 Quantification of OX-42, GFAP and GLT-1 expression

For all regions of interest and for OX-42, GFAP and GLT-1 expression, immunoreactive (ir) cell bodies, processes or transporters were quantified using Image J software (Image J, National Institutes of Health, Bethesda, Maryland, USA) and the method described by Hayes and colleagues (Hayes, et al., 2005). Briefly, 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 initially requires subtracting the background and then measuring intensely labeled area. For analyses, great care was taken to match sections through the same region of the brain at the same level using anatomical landmarks. Six anatomically matched pictures of the left and right hemispheres of the brain were used to produce an average ir score for each brain region in each animal. Data are presented as mean background corrected standardized image densities for each brain.

3. Results

3.1 OX-42 expression assessment in the CA1, CA3 and DG

Fig. 3 shows the effect of RSV treatment and 10 min global ischemia on microglial activation as measured by OX-42 expression in the CA1 region of the hippocampus, 7 days postischemia. Statistical analysis revealed a significant surgery × treatment interaction ($F(1, 29) = 10.93$, $p < .005$). Simple effects tests indicated that ischemic rats treated with saline had significantly more activated microglia in the CA1 than sham-operated controls ($p < .005$) and ischemic rats treated with 10 mg/kg RSV ($p < .05$). This was evident by the presence of amoeboid-like cells with plump cell bodies and short thick processes which are characteristic features of activated microglia (Fig. 2). The decrease in CD11b/c (OX-42) immunoreactivity in ischemic animals treated with the 10 mg/kg RSV dose is indicative of a dose-related reduction in post-ischemic inflammation. Interestingly, simple effects also indicated that sham rats treated with 10 mg/kg resveratrol displayed an upregulation of activated microglia in the CA1 compared to their saline-treated sham counterparts ($p < .05$). Fig. 4 shows the effect of RSV treatment and 10 min global ischemia on microglial activation in the CA3 and DG regions of the hippocampus, 7 days postischemia. No significant differences were observed in microglial activation in the CA3 region of the hippocampus among the groups. In the DG, analysis of variance revealed a significant main effect of surgery ($F(1, 29) = 5.67$, $p < .05$) attributable to ischemic animals having significantly more activated microglia in this region than sham-operated animals, irrespective of treatment.

3.2 GFAP expression assessment in the CA1, CA3 and DG

Fig. 5 shows the effect of RSV treatment and 10 min global ischemia on GFAP expression in the CA1 region of the hippocampus 7 days postischemia. In the CA1, statistical analysis revealed a significant main effect of surgery ($F(1, 30) = 14.48$, $p < .005$) and treatment ($F(2, 30) = 6.44$, $p < .01$). Ischemic rats had significantly greater numbers of

activated astrocytes as demonstrated by increased expression of cells with hypertrophic cellular morphology than sham-operated rats, which displayed cells with thin and long processes. Furthermore, post hoc analysis revealed that rats treated with 10 mg/kg RSV had significantly less activated astrocytes in the CA1 compared to saline-treated rats ($p < .05$), irrespective of surgery. Moreover, in the CA1, planned comparisons revealed greater GFAP expression in the CA1 by ischemic rats treated with saline than their sham counterparts ($p = .011$). Planned contrasts also revealed attenuated GFAP expression in the CA1 subfield of rats treated with both 1 and 10 mg/kg RSV doses ($p = .019$ and $p = .011$, respectively) compared to saline-treated ischemic rats. In the CA3, analysis of variance revealed a significant main effect of surgery ($F(1, 30) = 4.42$, $p < .05$) with ischemic rat showing greater activation of astrocytes compared to sham controls (Fig. 6). No significant differences in astrocyte activation within the DG were observed among the groups.

3.3 GLT-1 expression assessment in the CA1, CA3 and DG

Fig. 7 and 8 show the effect of RSV treatment and global cerebral ischemia on GLT-1 expression in the CA1, CA3 and DG regions of the hippocampus 7 days postischemia. Analysis of variance revealed a significant main effect of surgery ($F(1, 30) = 15.82$, $p < .001$), treatment ($F(2, 30) = 13.01$, $p < .001$) and a surgery × treatment interaction ($F(1, 30) = 4.66$, $p < .05$). Simple effects tests showed that ischemic rats treated with saline had significantly less GLT-1 expression in the CA1 compared to saline-treated sham rats ($p = .001$). Furthermore, ischemic rats treated with 1 mg/kg and 10 mg/kg RSV had significantly greater GLT-1 expression compared to saline-treated ischemic rats ($p = .001$ and $p = .002$, respectively) suggesting that RSV prevented GLT-1 depletion in the synaptic cleft. No

significant differences were noted in the CA3 and the DG with regards to GLT-1 expression ($p > .05$).

3.4 Neuronal density assessment

Fig. 9 shows the effect of RSV treatment and 10 min global ischemia on hippocampal CA1 neuronal density 7 days postischemia. Statistical analysis revealed a significant main effect of surgery ($F(1, 30) = 49.59$, $p < .001$) and treatment ($F(2, 30) = 9.35$, $p < .005$) and a significant surgery × treatment interaction ($F(1, 30) = 10.32$, $p < .005$). Simple effect tests indicated that saline-treated ischemic rats had significantly less CA1 hippocampal neurons than their sham-counterparts ($p = .001$) and that RSV-treated ischemic groups had significantly more CA1 neurons than ischemic rats treated with saline ($p < .005$).

A Pearson's product correlation was also run to determine the relationship between neuronal density and GLT-1 staining intensity in the hippocampus. Fig. 9 highlights a strong and positive correlation between GLT-1 immunoreactivity and neuronal density in the CA1 ($r(33)= .809$, $p < .001$).

4. Discussion

Ischemic injury has traditionally been evaluated using neuronal loss as the main hallmark, the selective vulnerability of CA1 pyramidal neurons in the hippocampus following global ischemia being widely acknowledged. We have previously shown that the 4-vessel occlusion model leads to severe neuronal death in the CA1 region of the hippocampus at 7 days postischemia which persists up to 85 days post-reperfusion, and demonstrated significant effects of RSV in reducing ischemia-induced neuronal injury (Girbovan, et al., 2012). The current study further examined RSV's ability to regulate the expression of endogenous markers associated with glial cell activation and glutamate uptake

following global cerebral ischemia. Importantly, RSV showed dose-related effects on ischemia-induced microglial activation, the 10 mg/kg dosage significantly decreasing OX-42-ir within the CA1 layer, while the lower 1 mg/kg dosage showed no effect. Moreover, RSV treatment markedly attenuated ischemia-induced GFAP-ir expression at both dosages and normalized GLT-1 expression to control levels in the CA1 region.

As anticipated, density of OX-42-ir staining in the hippocampal CA1 region was markedly increased in ischemic animals 7 days following reperfusion, while only a few scattered ramified microglia, reminiscent of resting glia, were observed in saline-treated sham rats. Furthermore, ischemic tissue predominantly expressed amoeboid-like cells with plump cell bodies and short thick processes characteristic of activated microglia. These findings are consistent with previous reports of increased microglial activation following ischemia (Anderova, et al., 2011; Bueters, et al., 2008; Dos-Anjos, et al., 2009; Langdon, et al., 2008; Lee, et al., 2010; Madinier, et al., 2009; Moon, et al., 2009; Simao, et al., 2012a; Sugawara, et al., 2002). Moreover, pretreatment with the 10 mg/kg RSV dosage significantly reduced OX-42-ir expression in the hippocampal CA1 region at 7 days postischemia, highlighting that dose-related effects of the polyphenol are highly probable (Girbovan, et al., 2012; H. R. Park, et al., 2012). These findings further support a neuroprotective role of RSV against ischemic brain injury through attenuation of ischemia-induced neuroinflammation (Simao, et al., 2012a; F. Zhang, J. Liu, et al., 2010).

The effectiveness of RSV in reducing microglial activation has first been demonstrated in primary microglial cultures and brain-derived cell lines, and more recently described following varying types of brain insults in vivo (F. Zhang, J. Liu, et al., 2010). For instance, RSV treatment dose-dependently conferred neuroprotection following induction of

acute bacterial meningitis through inhibition of calcium-mediated microglial activation and pro-inflammatory cytokines tumor necrosis factor-alpha, interlukin-1 beta (IL-1β) and interlukin-6 (IL-6) in the hippocampus (Sheu et al., 2013). It has been proposed that limiting inflammatory cytokine production by activated microglia should be beneficial for prevention of neuroinflammation and neurodegeneration (X. Lu, et al., 2010). Although we did not assess inflammatory cytokine release postischemia, the current literature attributes an important suppressive effect of RSV on the production of pro-inflammatory molecules, possibly through direct effects on SIRT1 (L. M. Wang et al., 2013). Indeed, RSV has been termed an activator of SIRT1 (Chung et al., 2010), which has been reported to inhibit nuclear factor-kappa-light-chain-enhancer of activated B cells (NF-kβ) production (Fu et al., 2013), a protein responsible for cytokine release. Studies assessing whether activation of SIRT1 signaling also contributes to the inhibitory effect of RSV on microglial activation postischemia are thus warranted.

Noteworthy, increased OX-42-ir was present in the DG following ischemia, irrespective of treatment, although expression was not affected in the CA3 region. This observation is consistent with greater microglial activation observed in the DG 7 days following 2-vessel occlusion in gerbils (Moon, et al., 2009). The authors reported significant neuronal damage in the DG following 5 min ischemia, which we could not find in our cohorts of ischemic rats (unreported results). Given that microglial activation has also been implicated in favorable process under some conditions, including ischemia, such as growth factor release, it is possible that heightened expression may contribute to the inherent resistance of the DG against ischemia-induced neuronal damage, although future studies need to assess this further. Conversely, microglial activation in the DG may be related to different

processes occurring as a result of ischemia in this hippocampal subregion. As such, the SGZ of the DG is one of two known neurogenic niches documented to undergo significant neurogenic processes following an ischemic insult (Lagace, 2012), and microglial activation has been associated with neurotrophic factor support in certain events, including neurogenesis, through release of growth factors (Battista, Ferrari, Gage, & Pitossi, 2006). To date, few studies have reported on microglial activation in regions outside the CA1 following global ischemia, making microglial cell activation in the dentate gyrus following forebrain ischemia harder to interpret. It is possible that the process is unique and independent of the role that glial cell activation appears to play in the CA1. Such proposition is partly supported by differential effects of RSV at the different brain loci.

Recent evidence supports that RSV's effect on neuroprotection and microglial activation may in part be regulated through modulation of transcription factors responsible for cytokine release (Simao, et al., 2012a). In this context, the observation of increased CA1 OX-42-ir in sham animals treated with10 mg/kg RSV was an unexpected finding. To date, evidence supports the use of high doses in animals (up to 750 mg/kg bw/day) and in humans (up to 2000 mg/day) with no adverse effects noted (la Porte et al., 2010; Williams, Burdock, Edwards, Beck, & Bausch, 2009). However, our findings, along with other reports, suggest the need for further investigation into RSV's effects on discrete biological systems and behaviour in healthy controls at pharmacologically relevant doses to ensure no adverse effects occur following chronic treatment (H. R. Park, et al., 2012).

Consistent with different studies supporting an involvement of astrogliosis following ischemia, we observed significant increase in astrocyte activation following 10 min global ischemia (Anderova, et al., 2011; Bueters, et al., 2008; Langdon, et al., 2008; Panickar &

Norenberg, 2005; Simao, et al., 2012a; Sugawara, et al., 2002; Tulsulkar & Shah, 2013). A robust increase in GFAP-ir and hypertrophic cellular morphology of astrocytes was observed in ischemic rats treated with saline. RSV treatment markedly attenuated ischemia-induced GFAP-ir expression in a dose-dependent manner. Tissue GFAP-ir expression in sham-operated animals contrasted by a uniform expression within the CA1 with thin and long processes of the labeled astrocytes. Although generally viewed as negative, the role of astrogliosis postischemia remains a matter of debate (Barreto, White, Ouyang, Xu, & Giffard, 2011). Positive correlations between astrogliosis and infarct size shown by a number of studies have argued for the involvement of such process in ischemic injury through release and over-expression of pro-inflammatory cytokines (Buchhold et al., 2007; Chu et al., 2012; Mao et al., 2012; Xuan et al., 2012). However, under some circumstances, astrocyte proliferation has been observed concurrent with neuronal protection and improved post-ischemic recovery. Thus, exposure to low-frequency magnetic field (ELF-50Hz) following 10 min global ischemia in gerbils led to decreased CA1 neuronal injury in the presence of increased activation of both astrocytes and microglia (Raus et al., 2013). The authors suggested that increased glial cell activation may result in greater microvesicle motility of astrocytes, enabling prompt responses to metabolic demand and distribution of growth factors to injured neurons. Noteworthy, time-dependent alterations of glial cell and cytokine expression following ischemia has also been proposed: an initial glial activation phase rapidly occurring within 48h of the ischemic insult and a delayed phase coincidental with neuronal death occurring over a period of several days postischemia (Yasuda et al., 2011). It has been suggested that the balance between pro- and anti-inflammatory factors released post-injury is what determines the ensuing direction of glial activation (Battista, et al., 2006).

The present study reveals that chronic pretreatment with 1 and 10 mg/kg RSV reduced global ischemia-induced activation of astrocytes within the CA1. The latter results are in line with other studies attributing anti-inflammatory actions of the polyphenol through downregulation of astrogliosis following ischemia (Quincozes-Santos & Gottfried, 2011; Simao, et al., 2012a). In vitro demonstrations of the effect of RSV in modulating astroglial functions have relied on data accumulated from primary cultures, cell lines and acute hippocampal slices whereas in vivo studies remain scarce. In one of the first reports of its kind, Wang et al. (2002) found that administration of a single i.p. injection of 30 mg/kg RSV during or shortly after global cerebral ischemia in gerbils significantly downregulated the inflammatory response of both microglia and astrocytes at 4 days post-insult. In a more methodologically similar study to our own, Simao's (2012a) group found significant attenuation of neuronal injury and of the astrocytic response postischemia by preconditioning with RSV. This was coupled with decreased activation of transcription factors NF-kβ and c-Jung N terminal-kinase and release of inflammatory cytokines cyclooxygenase-2 and iNOS (Simao, et al., 2012a). Their study does differ from our own in that RSV was administered for a shorter period of time prior to ischemia and at higher dose than ours; for 7 days at a dose of 30 mg/kg, respectively. However, the latter findings highlight a potential mechanism of action of RSV in decreasing ischemia-induced neuroinflammation through modulation of signal transduction pathways and neuroprotection.

The present investigation also provides the first evidence that chronic pre-treatment with RSV, at two doses, prevents ischemia-induced depletion of astrocytic GLT-1 in the CA1 region. This highlights a possible role of RSV on regulating glutamate activity within the most vulnerable region following brain ischemia. Our findings demonstrate that both the 1

and 10 mg/kg RSV dosages normalized GLT-1 expression to control levels in the CA1 region. The strong positive correlation between GLT-1 expression and neuronal density in the CA1 further supports these changes likely contribute to RSV's neuroprotective effects (Girbovan, et al., 2012). RSV had no impact on GLT-1-ir in the CA3 and DG subfields of the hippocampus. Although evidence of RSV's actions of glutamate uptake is scarce, in vitro studies using astrocyte-like cell lines derived from rat brain tumors support this hypothesis. Thus, exposing C6 glioma cells to increasing RSV doses ranging from 1 to 100μM led to a linear increase in glutamate uptake (dos Santos et al., 2006). RSV was also shown to increase glutamate uptake and glutathione content in rat cortical astrocyte cultures and hippocampal slices, albeit some dose-dependent effects were noted (de Almeida, Leite, et al., 2008; de Almeida, Pineiro, et al., 2008; de Almeida, et al., 2007). RSV's actions on regulating GLT-1 activity following ischemia likely contribute to its neuroprotective effects independent of its antioxidative properties, although enhanced glutamate uptake will lead to reduced extracellular glutamate concentration and excitotoxicity. Future studies are necessary to elucidate physiological mechanisms involved in such changes.

Conclusion

Given that neuronal survival is dependent on an environment with strict metabolic demands, it is unlikely that pharmacological agents that strictly target neuronal viability be successful (Takano, et al., 2009). In this context, there is a general agreement that alterations of glial cell populations are inherently tied to neuronal fate postischemia. Recently, a "gliocentric view" of stroke injury has emerged, which sustains that new therapeutic tools need to target glial cell integrity, with an emphasis on preservation of astrocytes' functions. Among pharmacological agents, RSV's wide range of biological activities including effects

documented in the current study, such as its ability to downregulate ischemia-induced neuroinflammation through direct actions on glial activation and preventing GLT-1 depletion in the CA1 region poses it as a great candidate in investigations of novel therapeutic targets.

A**cknowledgements**

The authors sincerely thank Sylvie Émond for excellent technical assistance in the animal facility.

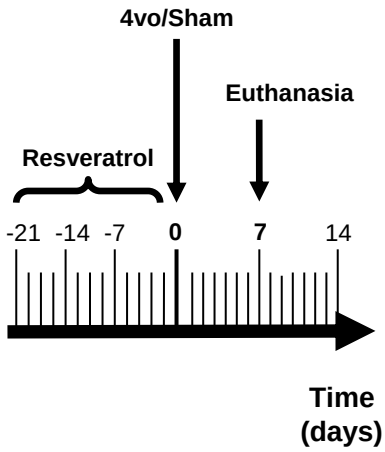


Figure 1. Experimental timeline of the study. Daily 21-day pre-surgical period of RSV (1 mg/kg or 10 mg/kg;) or saline i.p. injections; 4-vessel occlusion (4vo) or sham occlusion. Day 0 refers to the day of the surgery.

Primary Antibodies

Antigen	Type	Isotype	Host	Immunogen	Dilution	Source
GFAP	Polyclonal	IgG	Rabbit	Astrocytes	1:1000	Abcam
CD11b/c	Monoclonal	IgG	Mouse	Resident/activated macrophages/microglia	1:500	Abcam
GLT-1	Polyclonal	IgG	Guinea-pig	Glial type-1 glutamate transporter	1:500	Millipore

Table 1. Summary of antibodies used during immunohistochemistry procedures. Abbreviations; CD11B (clone to OX-42); Glial Fibrillary Acidic Protein (GFAP); Type-1 Glutamate Transporter (GLT-1).

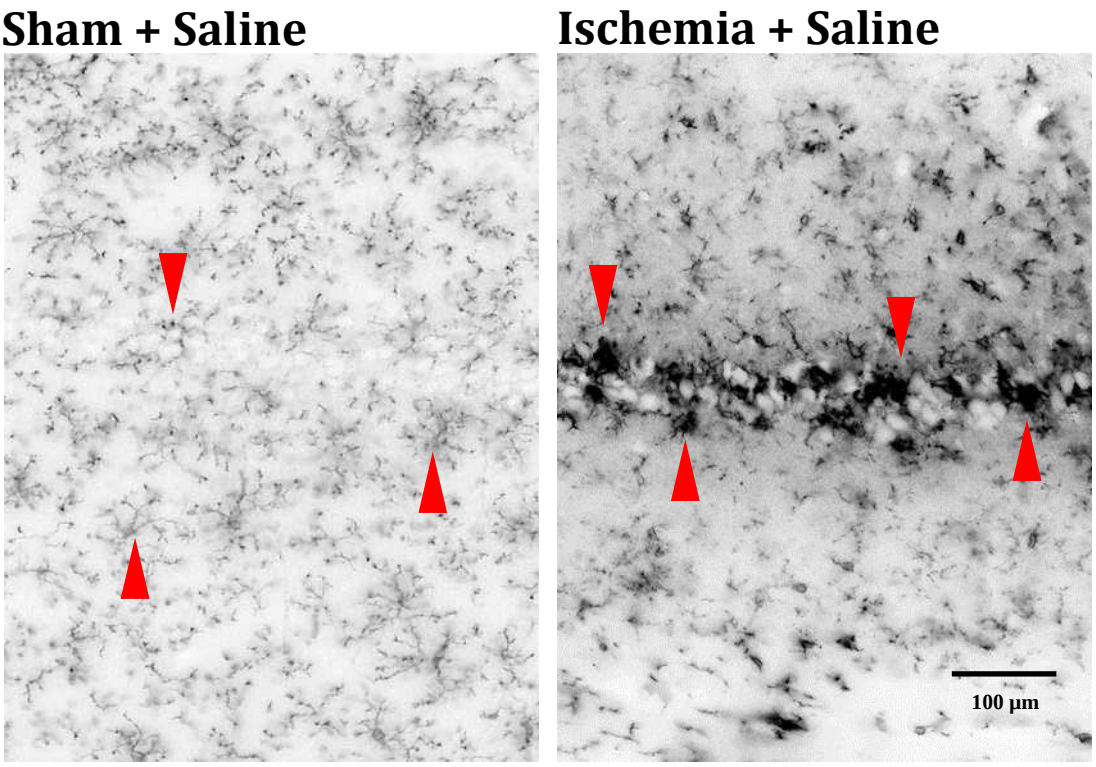
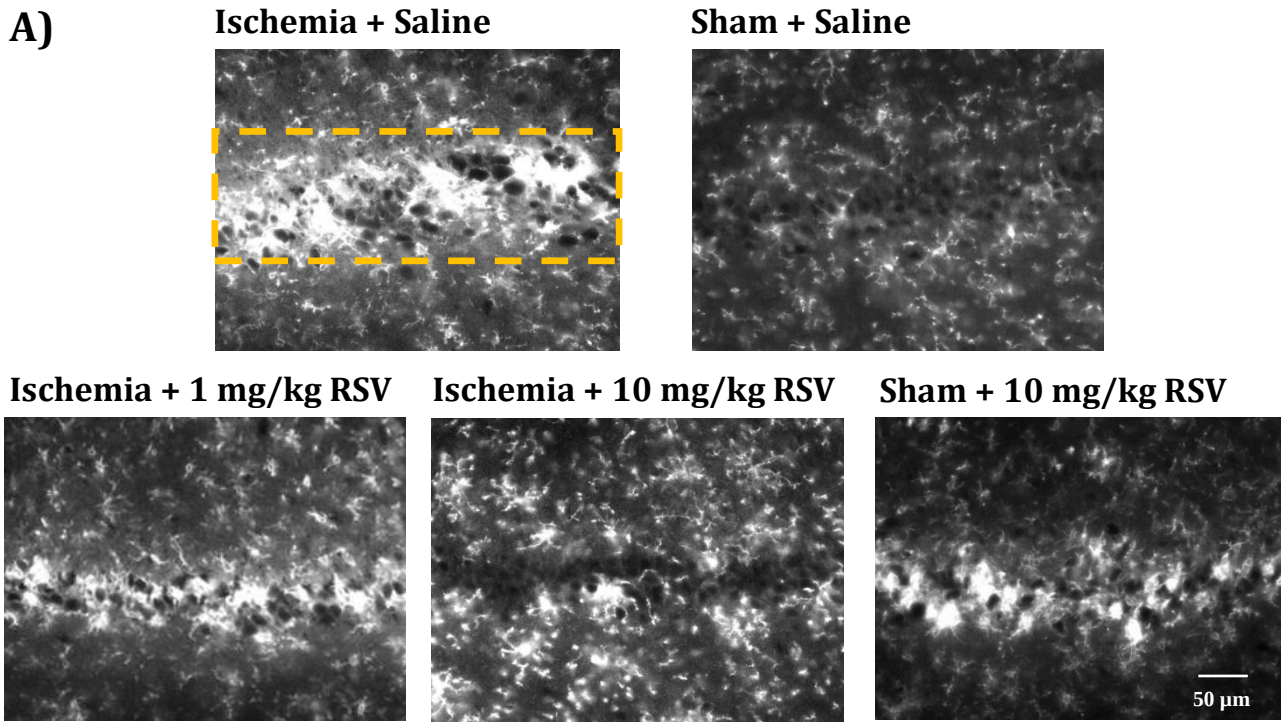


Figure 2. Representative photomicrographs of OX-42 immunoreactivity in the CA1 subfield of the hippocampus of sham and ischemic animals treated with saline. Arrows allow comparisons of resting ramified microglia in sham animals while activated microglia manifest as small round cells lacking processes in the ischemic brain.

A)


Ischemia + Saline
Sham + Saline
Ischemia + 1 mg/kg RSV
Ischemia + 10 mg/kg RSV
Sham + 10 mg/kg RSV
50 µm

B)

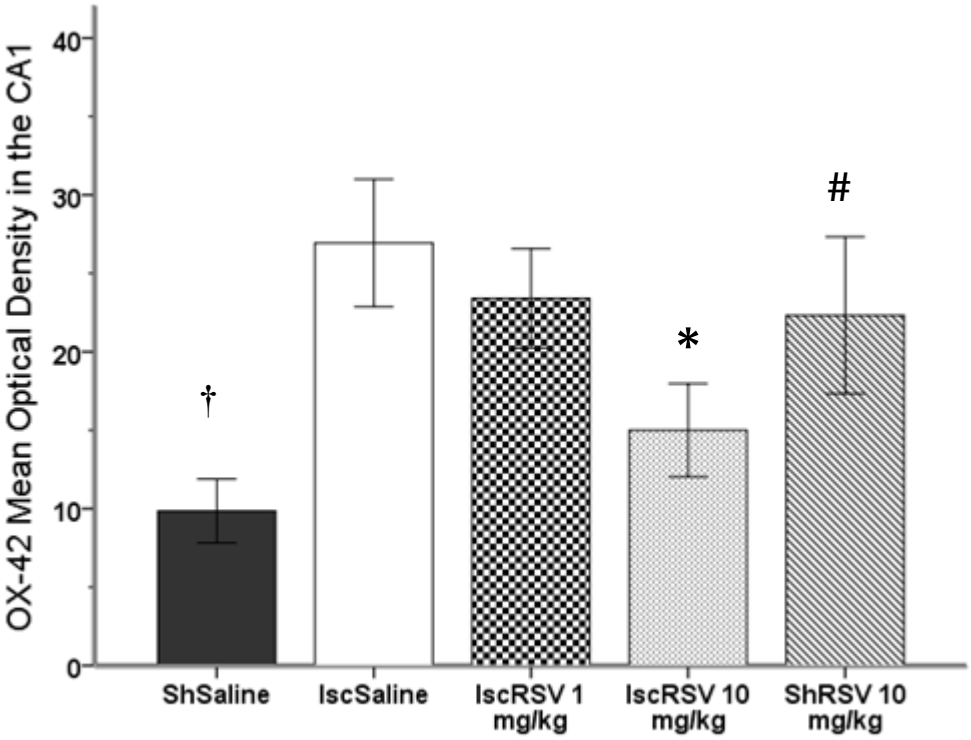
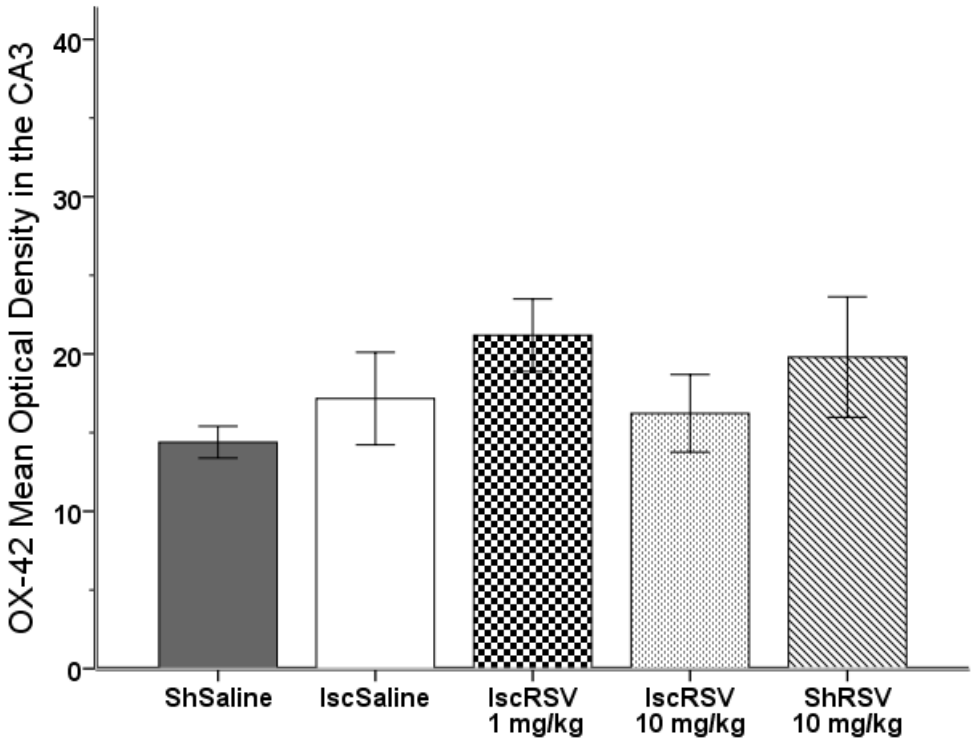

OX-42 Mean Optical Density in the CA1
0
10
20
30
40
†
*
#
ShSaline
IscSaline
IscRSV 1 mg/kg
IscRSV 10 mg/kg
ShRSV 10 mg/kg

Figure 3. Representative photomicrographs of OX-42 immunoreactivity in the CA1 layer of sham and ischemic animals treated with either saline or RSV 7 days following reperfusion (A). Histogram illustrating mean optical density values for each experimental group (B). Symbols indicate a significant upregulation in OX-42 optical density surrounding CA1 neurons by saline treated ischemic rats compared to sham rats treated with saline († $p < .005$) and ischemic rats treated with 10 mg/kg RSV (* $p < .05$). # indicates a significant increase in OX-42 expression within the CA1 subfield by sham rats treated with 10 mg/kg RSV compared to saline-treated sham rats 7 days postischemia ($p < .05$). Values are expressed as mean optical density ± S.E.M.


OX-42 Mean Optical Density in the CA3
40
30
20
10
0
ShSaline
IscSaline
IscRSV 1 mg/kg
IscRSV 10 mg/kg
ShRSV 10 mg/kg

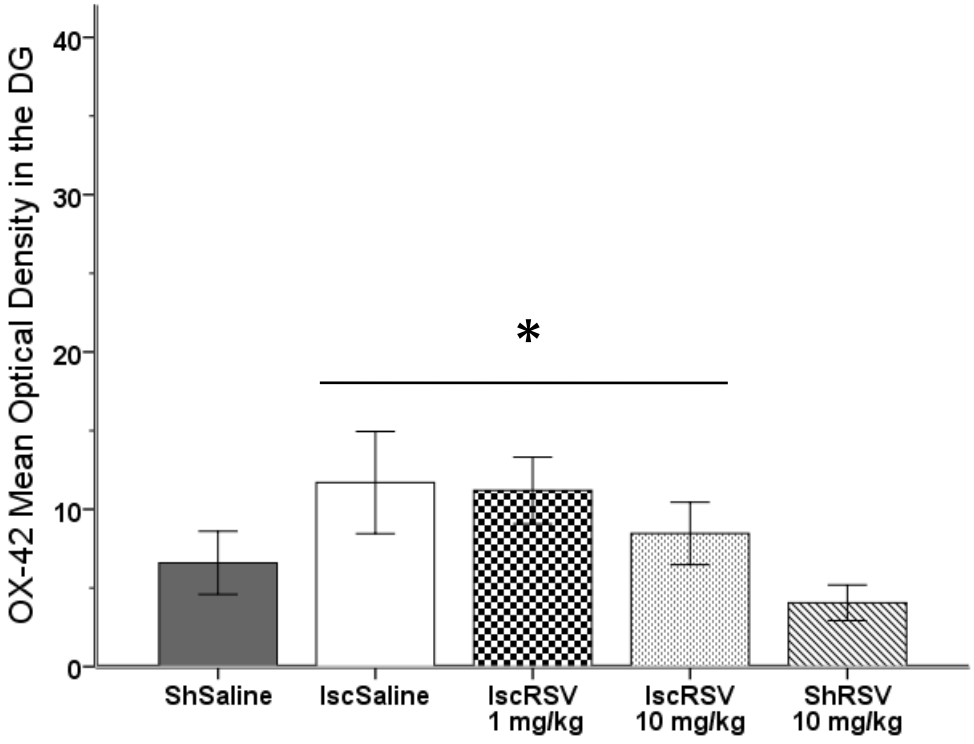

OX-42 Mean Optical Density in the DG
40
30
20
10
0
*
ShSaline
IscSaline
IscRSV 1 mg/kg
IscRSV 10 mg/kg
ShRSV 10 mg/kg

Figure 4. Effect of RSV treatment and 10 min global ischemia on OX-42 expression in the CA3 and DG regions of the hippocampus 7 days postischemia (top and bottom figures, respectively). OX-42 expression in the CA3 layer was comparable among all experimental groups ($p > .05$). In the DG, OX-42-ir mean values were significantly higher in ischemic than sham rats, irrespective of drug treatment ($^*p < .05$). Values are expressed as mean optical density ± S.E.M.

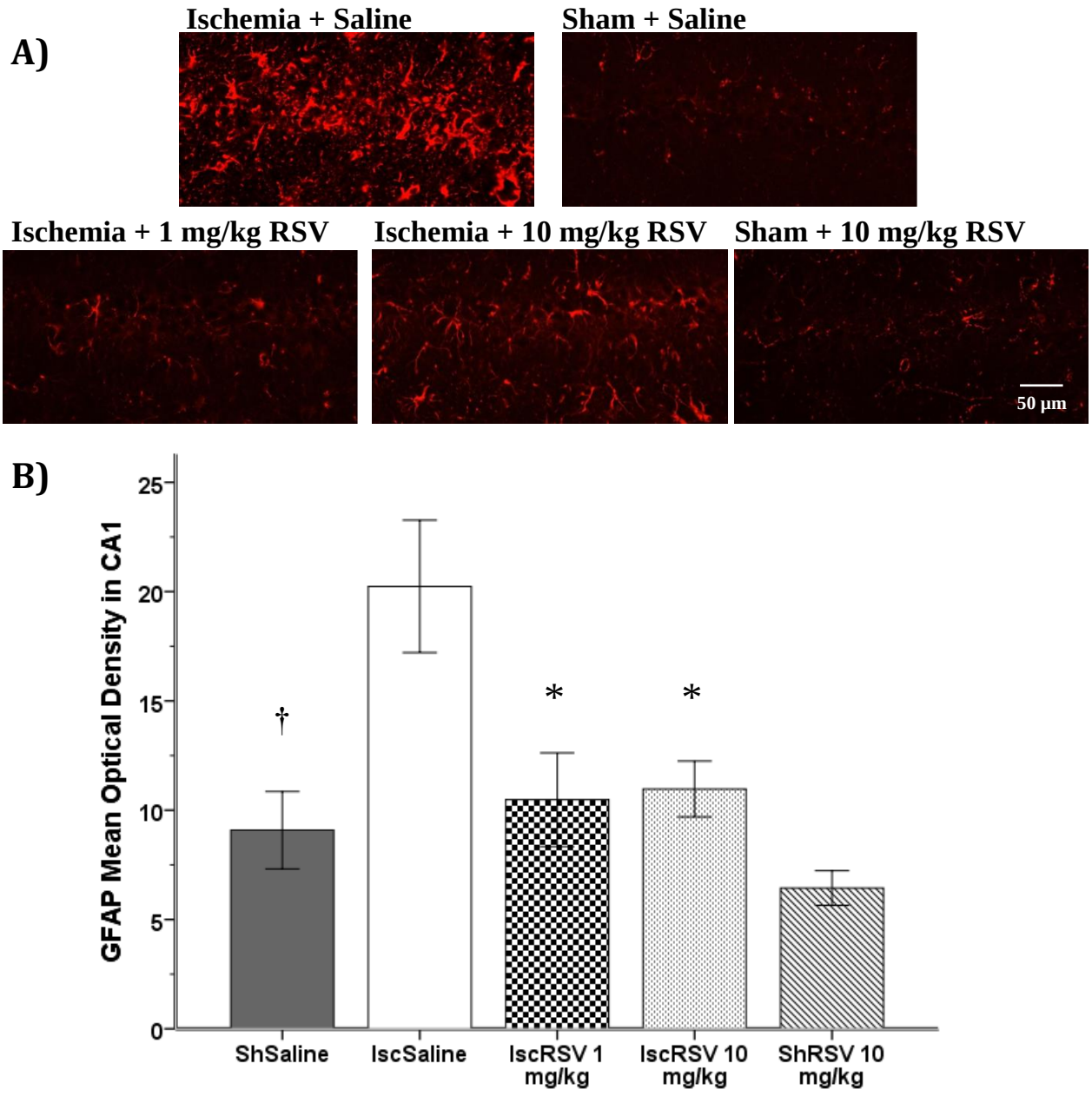
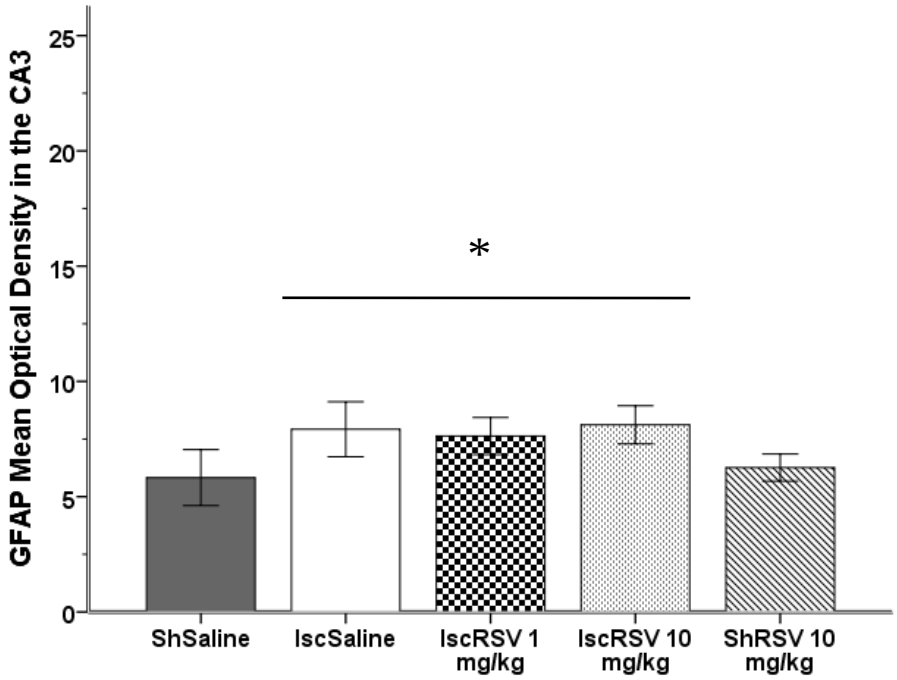


Figure 5. Representative photomicrographs of GFAP-ir in the CA1 hippocampal subfield of saline or RSV-treated sham and ischemic animals 7 days following reperfusion (A). Histogram illustrating mean optical density values for each experimental group (B). Symbols indicate a significant difference between saline-treated ischemic rats their sham counterparts († $p < .05$) and both 1 and 10 mg/kg RSV-treated ischemic rats (* $p < .05$). Values are expressed as mean optical density ± S.E.M.


GFAP Mean Optical Density in the CA3
25
20
15
10
5
0
*
ShSaline
IscSaline
IscRSV 1 mg/kg
IscRSV 10 mg/kg
ShRSV 10 mg/kg

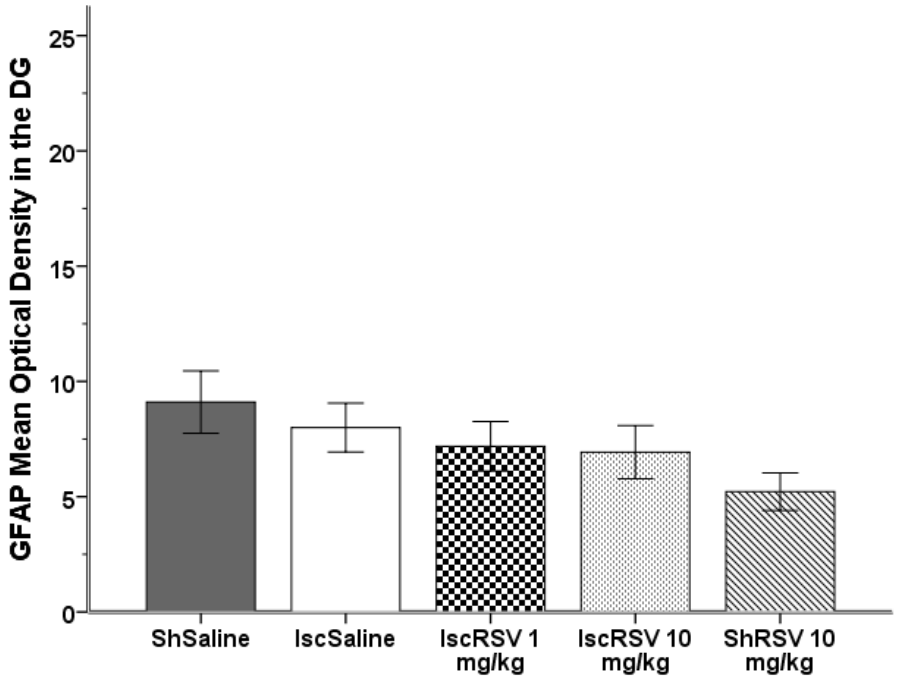
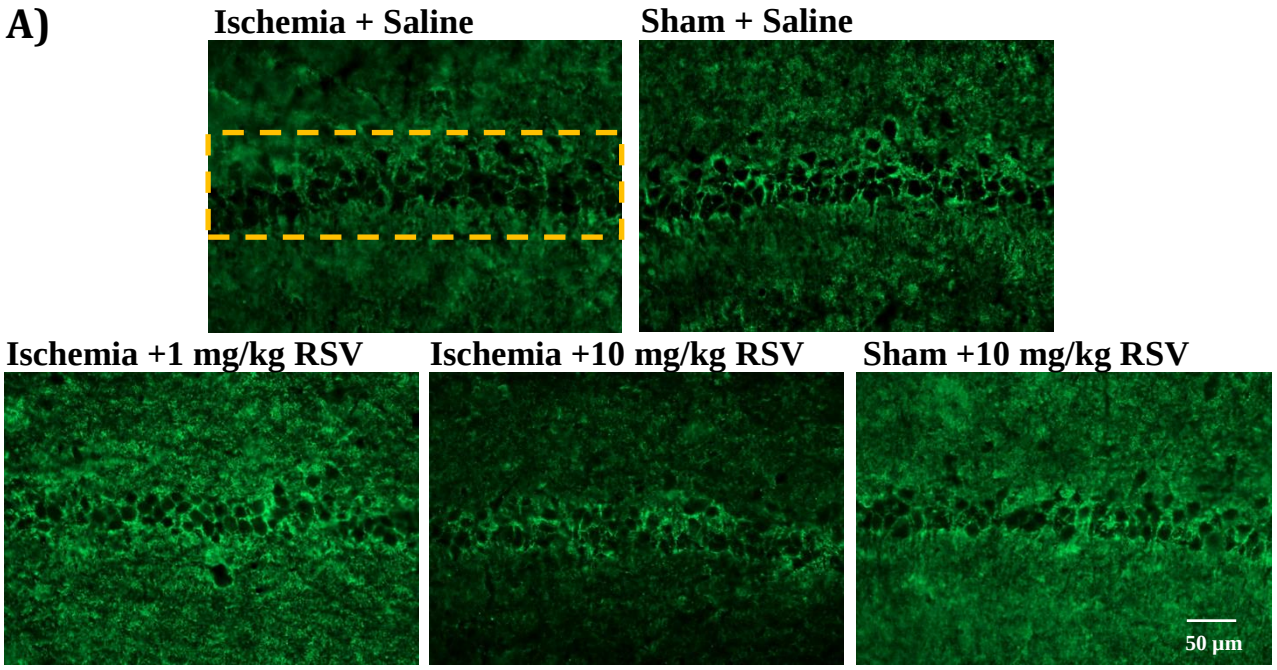

GFAP Mean Optical Density in the DG
25
20
15
10
5
0
ShSaline
IscSaline
IscRSV 1 mg/kg
IscRSV 10 mg/kg
ShRSV 10 mg/kg

Figure 6. Effect of RSV treatment and 10 min global ischemia on GFAP-ir expression in the CA3 and DG regions of the hippocampus (top and bottom figures, respectively), 7 days postischemia. In the CA3, GFAP-ir was enhanced in ischemic compared to sham rats, irrespective of treatment (*$p < .05$). No between-group differences were observed in the DG ($p > .05$). Values are expressed as mean optical density ± S.E.M.


A)
Ischemia + Saline
Sham + Saline
Ischemia +1 mg/kg RSV
Ischemia +10 mg/kg RSV
Sham +10 mg/kg RSV
50 µm

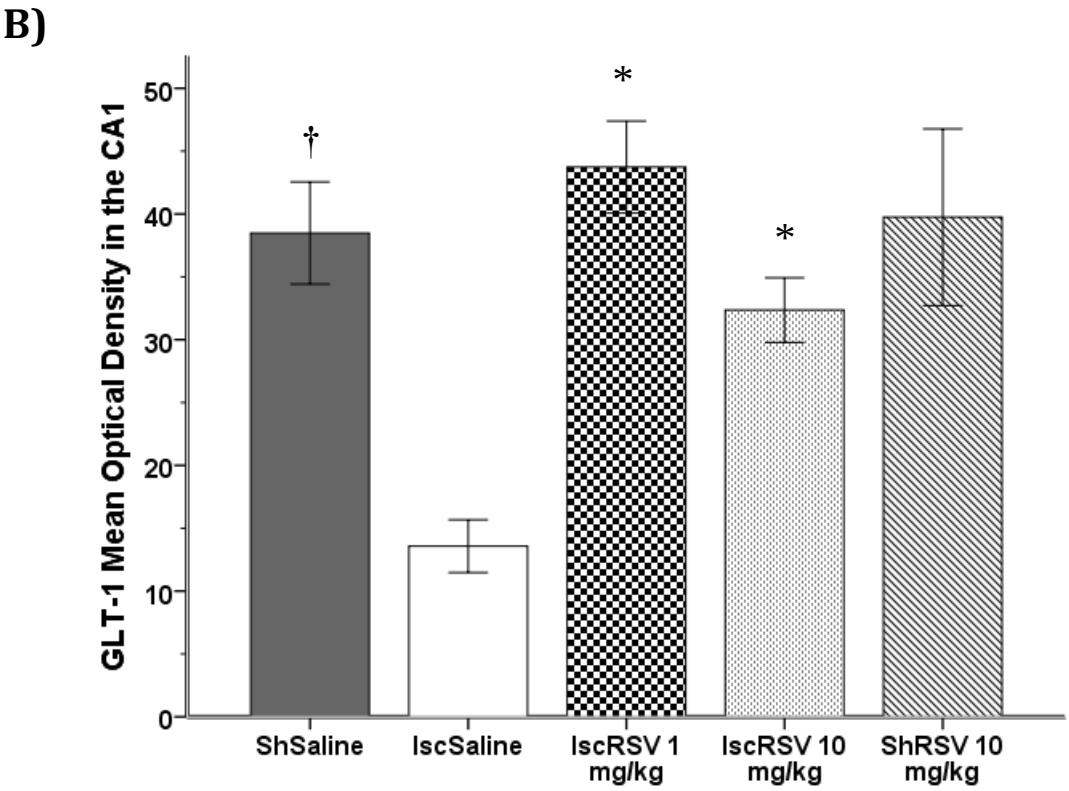

B)
GLT-1 Mean Optical Density in the CA1
0
10
20
30
40
50
†
*
*
ShSaline
IscSaline
IscRSV 1 mg/kg
IscRSV 10 mg/kg
ShRSV 10 mg/kg

Figure 7. Representative photomicrographs of GLT-1-ir expression in the CA1 region for saline- or RSV-treated sham and ischemic animals 7 days postischemia (A). Histogram of the mean optical densities for the different groups (B). Symbols indicate a significant difference in GLT-1 expression 7 days following reperfusion in saline-treated ischemic compared to saline-treated sham- rats († $p < .005$) and both 1 and 10 mg/kg RSV-treated ischemic rats (* $p < .005$). Values are expressed as mean optical density ± S.E.M.

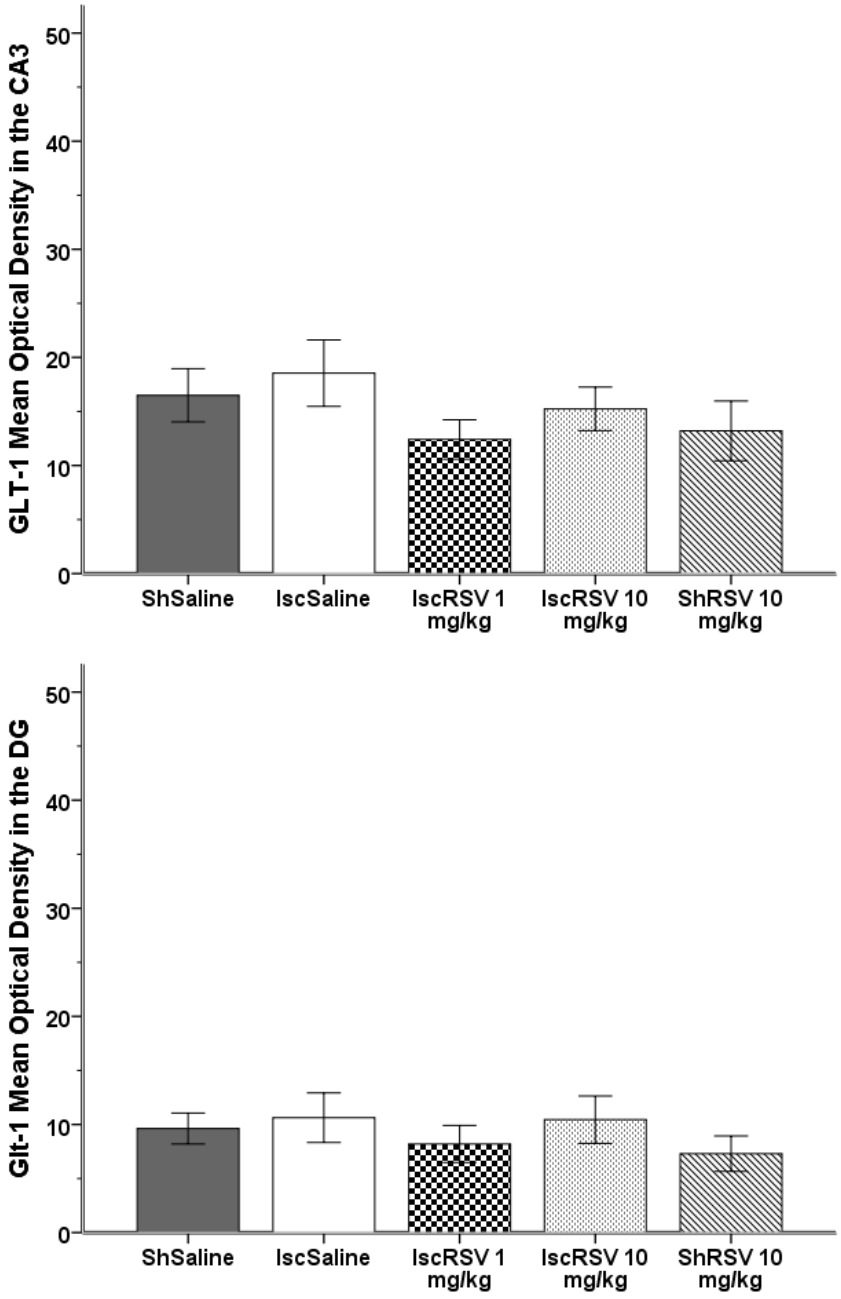


Figure 8. Effect of RSV treatment and 10 min global ischemia on GLT-1 expression in the CA3 and DG subfields of the hippocampus 7 days postischemia (top and bottom figures, respectively). No between-group differences in GLT-1 expression were detected in either region ($p > .05$). Values are expressed as mean optical density ± S.E.M.

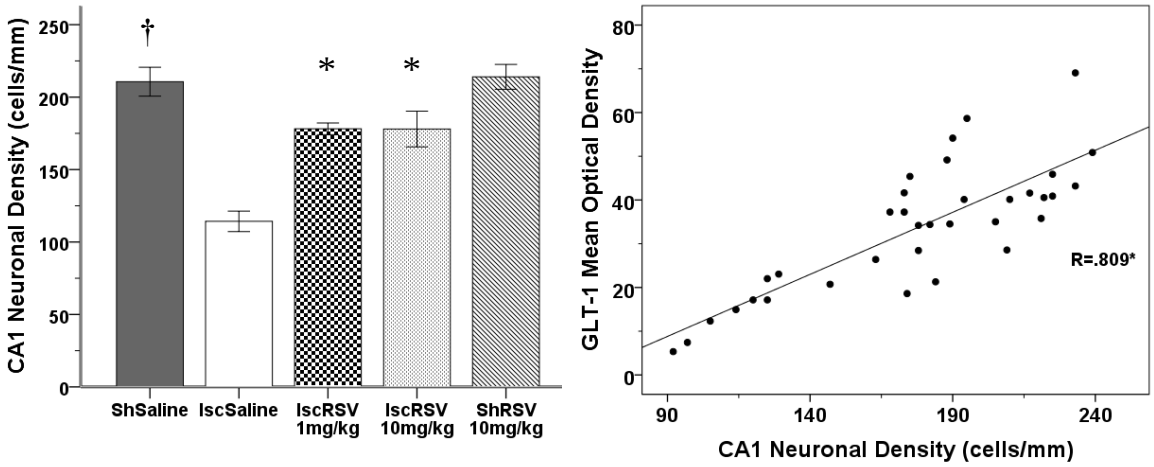


Figure 9. Neuronal density in the CA1 hippocampal subfield for the different experimental groups of animals at 7 days following 10 min global ischemia and correlation plot between GLT-1-ir expression and neuronal density in the hippocampus. Saline-treated ischemic rats show a reduced number of surviving neurons (≈50%) compared to saline-treated sham rats († $p < .005$) in the CA1. Moreover, rats treated with 1 or 10 mg/kg RSV show a significant reduction in CA1 neuronal damage compared to saline-treated ischemic rats (* $p < .005$). Neuronal density was positively correlated with GLT-1 expression in the CA1 ($r = .809$) ($p < .001$).

General Discussion

The present thesis evaluated prophylactic effects of RSV in the context of global cerebral ischemia through a series of experiments that addressed three main objectives.

The first thesis objective assessed neuroprotective effects of RSV in preventing hippocampal neuronal cell death and determined contributions of RSV to behavioural outcome following cerebral ischemia (Article 1). The second objective characterized possible mechanisms of action underlying RSV's effects on neuroprotection and/or behaviour using immunohistochemical detection of progenitor cell differentiation and angiogenesis at short and long reperfusion intervals postischemia (Article 2). Additionally, given the documented effect of ischemic injury on HPA-axis reactivity and its possible influence on neural plasticity processes, we further investigated the effect of RSV on CORT secretion 72 h postischemia and at multiple time intervals following exposure to an acute stressor. Lastly, considering a proposed role for neurogenesis in mediating aspects of hippocampus-dependent learning and memory, we evaluated the effect of RSV on spatial learning in the water maze. The third objective aimed to investigate possible effects of RSV in regulating glial cell and GLT-1 expression in the hippocampus 7 days postischemia (Article 3). Given the scarce information available on the polyphenol's actions in healthy populations, the inclusion of a control group treated with RSV at a concentration of 10 mg/kg enabled further describing RSV's effects under normal conditions.

The general hypothesis of the current thesis stipulates that in order for RSV to be considered a promising prophylactic therapy for global cerebral ischemia, the compound needs to confer neuronal protection and/or improvement of behavioural impairments, the former not being sufficient. Existing literature on RSV supports numerous mechanisms of

action for the polyphenol in a number of disease models including stroke, epilepsy, diabetes, Alzheimer's and Parkinson's disease (Ates, et al., 2007; Y. K. Gupta, et al., 2002; Huber & Superti-Furga, 2011; Sharma & Gupta, 2002; Tsai, et al., 2007). Considering these observations, we expected to observe a) reduced neuronal injury (that is maintained at long reperfusion intervals) and beneficial effects on behavioural impairments following cerebral ischemia b) dose- and time-related effects c) important effects of the polyphenol in mediating neuroplasticity following ischemia, as well as effects on non-neuronal populations of cells, possibly mediated through glial GLT-1 expression.

Findings from the current thesis demonstrated significant neuroprotection conferred by RSV on ischemia-induced hippocampal damage, which was maintained at 85 days postischemia. Moreover, investigation into possible mechanisms of action elaborating on the polyphenol's neuroprotective properties revealed downregulation of ischemia-induced CORT levels paired with upregulation of angiogenesis in the CA1 subfield of the hippocampus, albeit dose-related effects were noted. To our surprise, RSV dose-dependently inhibited cerebral ischemia-induced expression of neural progenitor cells in the DG of the hippocampus, suggesting that actions of RSV on neuroplasticity in this region may be independent of RSV-conferred neuroprotection in the CA1. Findings from the last experiment supported a role of RSV in attenuating post-ischemic inflammation through downregulation of glial cell activation in the hippocampus. Furthermore, our results highlight a possible role of RSV on regulating glutamate activity within the most vulnerable region following brain ischemia by preventing ischemia-induced depletion of glial GLT-1 in the CA1 region. Lastly, behavioural test results revealed dose-related effects of RSV on functional recovery postischemia and altered performance of sham animals treated with RSV

on discrete behavioural measures, suggesting independent actions of the compound on distinct physiological systems mediating cellular survival and functional recovery, as well as dose-related actions of RSV on behavioural and memory processes.

1. Resveratrol and neuroprotection

The current thesis demonstrates that the beneficial effects of RSV on ischemia-induced hippocampal damage are maintained at long intervals, and are still of significant magnitude 85 days postischemia (40% greater neuronal survival in RSV- than vehicle-treated ischemic animals). This is consistent with RSV neuroprotective effects demonstrated at short intervals in various disease models, including epilepsy (Y. K. Gupta, et al., 2002; Wu, et al., 2009), diabetes (Ates, et al., 2007; Kumar, Negi, & Sharma, 2013), Alzheimer's (Sharma & Gupta, 2002; J. Wang, et al., 2006), Parkinson's disease (Huber & Superti-Furga, 2011; Lofrumento et al., 2014; Y. Wang, et al., 2011; F. Zhang, J. S. Shi, et al., 2010) and stroke (Dong, et al., 2008; K. T. Lu, et al., 2006; Saleh et al., 2014; Simao, et al.; Tsai, et al., 2007; L. M. Wang, et al., 2013; Q. Wang, et al., 2009).

Our findings were insightful and have added to existing studies that have focussed on the cellular and molecular pathways of RSV protection, and have assessed short intervals post-lesion. Of great interest, we found that although both RSV dosages conferred comparable protection of CA1 neurons, RSV induced effects on behaviour in ischemic animals that were dose-related. In agreement, over the years, it has been noted that there is no direct relationship between neuronal protection and functional recovery (De Butte-Smith, et al., 2009; Ma, et al., 2008; Plamondon & Roberge, 2008; Roberge, Hotte-Bernard, et al., 2008). Moreover, in finding promising agents to improve neurological and cognitive

outcome following cardiac arrest, there is an imperative for functional outcomes to accompany effects of the polyphenol on cellular processes. Following global cerebral ischemia, these would include locomotor, emotional and cognitive (memory) impairments.

RSV neuroprotective effects have been consistent in various ischemic models in rodents (Della-Morte, et al., 2009; Saleh, et al., 2014; Sinha, et al., 2002; Q. Wang, et al., 2009), and supporting evidence have been described throughout the thesis. Currently, hypotheses as to how RSV provides neuroprotection include effects on reducing oxidative stress through scavenging of ROS (Bi, et al., 2005; Das, et al., 2005; K. T. Lu, et al., 2006; Tsai, et al., 2007) and decreasing inflammation (Manna, et al., 2000; Simao, et al., 2012a), increasing trophic factors release, including those important for angiogenesis (Dong, et al., 2008; Fukuda, et al., 2006; H. Wang, et al., 2010), and direct actions on activating SIRT1 (Albani, Polito, Signorini, & Forloni, 2010; Hubbard & Sinclair, 2014).

Interest in the field of RSV and neuroprotection has considerably risen following the description of RSV's activating action on the enzyme SIRT1 in various disease models (Albani, et al., 2010; Chung, et al., 2010; Hubbard & Sinclair, 2014), shown to foster increased mitochondrial oxidative function (Ferretta, et al., 2014). This suggested a possible role for RSV in the treatment of stroke, Parkinson's disease and Alzheimer's disease, all plagued by mitochondrial dysfunction and oxidative stress (Sims & Muyderman, 2010). In a recent study, long-term RSV treatment counteracted amyloid-beta (Aβ) toxicity in a mouse model of Alzheimer's disease, through increased activation of SIRT1 and AMPK pathways (Porquet et al., 2014). The neuroprotective effect of SIRT1 in cerebral ischemia was first reported by Raval et al. (2006) in an in vitro model using organotypic hippocampal slice cultures, which showed that RSV pretreatment mimics ischemic preconditioning through

direct activation of SIRT1. Similar results were reported using in vivo models of the disease, whereby RSV treatment prior to global cerebral ischemia in rats led to significant protection in hippocampal pyramidal neurons through increased SIRT1 activity and decrease of mitochondrial uncoupling protein 2 (UPC2) levels (Della-Morte, et al., 2009). Despite growing interest in RSV's actions on SIRT1, some have reported RSV-induced neuroprotection in SIRT1-independent ways as well (Alvira et al., 2007) while the role of SIRT1 in many other RSV pathways has not been identified (Y. Yang, et al., 2013) further highlighting the complexity surrounding RSV's many targeted pathways.

2. Resveratrol and brain plasticity

Resveratrol-induced neuroprotection against global cerebral ischemia as well as dose-dependent effects of the polyphenol on behavioural measures set the stage for possible compensatory mechanisms in the form of neuroplastic changes occurring postischemia and possibly regulating behavioural recovery observed at long post-ischemic intervals. Article 2 highlighted attenuation of ischemia-induced expression of neural progenitor markers in the DG region by both RSV dosages, while the polyphenol's effects on angiogenesis levels were dose-related at this time interval, the 1 mg/kg RSV dose enhancing CD31 expression in the CA1 region. Moreover, our findings describing attenuation of CORT levels with the 10 mg/kg RSV dosage 3 days postischemia provided the first demonstration of the polyphenol 'actions on decreasing cerebral ischemia-induced CORT secretion. HPA axis hypersensitivity following brain ischemia (de la Tremblaye, et al., 2014; M. R. Milot & Plamondon, 2011) highlights a supporting role for neuroendocrine dysregulation as a major contributor to emotional and cognitive impairments observed in survivors of cardiac arrest. Indeed,

important data has described that survivors of cardiac arrest report a greater number of symptoms associated with anxiety, depression and post-traumatic stress disorder (Wilder Schaaf, et al., 2013) which have also been documented in animals (Bah, Kaloustian, Rousseau, & Godbout, 2011; Bantsiele et al., 2009) and RSV's possible actions on the HPA axis make it a possible candidate in the regulation of emotional impairments following brain ischemia/cardiac arrest.

2.1 Resveratrol effects on neuro-and angiogenic expression in the hippocampus

As was previously discussed in Article 2, several lines of evidence point towards an indisputable link between angiogenesis and neurogenesis in repair following brain injury (Sawada, Matsumoto, & Sawamoto, 2014) and the induction of angiogenesis has been deemed necessary to the survival and differentiation of neuroblasts post-stroke (Brea, Sobrino, Ramos-Cabrer, & Castillo, 2009; Louissaint, Rao, Leventhal, & Goldman, 2002; Nih et al., 2012). Indeed, clusters of NPCs, expressing various neuronal markers (DCX), are found in close proximity to proliferating endothelial cells (Ekstrand, et al., 2008). In support of this, our findings show that upregulation of DCX/PSA-NCAM-positive cells in the DG postischemia is associated with concomitant increase in CD31 in the same region. However, the role of angiogenesis in this region is less well-understood in the context of cerebral ischemia, the process often being associated with improved neurological score in the injured CA1 region instead. In that regard, RSV effects are unclear and dose-related in this region, with the 1 mg/kg RSV dosage downregulating both neurogenesis and angiogenesis markers in the DG, while the 10 mg/kg RSV dosage showing opposing actions on these processes. It remains possible that RSV-induced upregulation of angiogenesis occurring in the injured CA1 postischemia is responsible for the polyphenol's protection of CA1 neurons. In support

of this, animal studies have shown that local delivery of pro-angiogenic growth factors such as VEGF to the site of injury significantly enhanced angiogenesis and neuronal survival (Sun, et al., 2003; Z. G. Zhang et al., 2000). Likewise, RSV has been documented to increase VEGF expression in the penumbral region postischemia (Dong, et al., 2008), further suggesting a role for RSV-mediated angiogenesis in the polyphenol's actions on protecting CA1 neurons against ischemic injury. Our results are also in line with others demonstrating upregulation of angiogenesis markers in the infarct region postischemia, with no change in expression in the DG (Sun, et al., 2003). Hence, the knowledge of molecular mechanisms that regulate angiogenesis following forebrain ischemia could contribute to the development of new therapeutic alternatives based on angiogenesis as a vehicle to promote neural repair and functional recovery. In contrast, the decrease of neurogenesis observed in the DG by both RSV dosages may suggest a neurogenesis-independent mechanism for neuroprotection. Indeed, it is possible that RSV-conferred neuroprotection restrained the necessity for enhanced neurogenesis following the insult. Similar to our observations, administration of the antiepileptic drug lacosamide in a rat electrical status epilepticus model lead to significant neuroprotection in the CA1 region whereas dose-dependent attenuation of newborn granule cell numbers was observed in the DG (Licko et al., 2013). Moreover, hypothermia-induced CA1 neuroprotection against global ischemia was not associated with any changes in DG neurogenesis in treated animals, suggesting regional specificity on plasticity markers in response to neuroprotective treatment (Silasi, et al., 2012). Future studies using cranial irradiation directed at neurogenic zones (Hellstrom, Bjork-Eriksson, Blomgren, & Kuhn, 2009; Naylor et al., 2008) along with genetically engineered rodent models allowing tracking

and alteration of specific neurogenic cell populations (Dhaliwal & Lagace, 2011) are needed in order to directly address the functional relevance of RSV on this endogenous process.

2.2 Lack of neurogenesis in the CA1 and CA3 following ischemia

Findings from Article 2 add to the already growing literature documenting an increase in dentate gyrus neurogenesis following cerebral ischemia (J. H. Choi, et al., 2012; Y. S. Choi, et al., 2003; Engelhard, et al., 2007; Kuge, et al., 2009; Lasarzik, et al., 2009; J. Liu, et al., 1998; Schmidt & Reymann, 2002; R. Tanaka, et al., 2004; Winkelheide, et al., 2008; Wojtowicz, et al., 2008), which is in contrast with the significant neuronal cell loss seen in the CA1 and lack of neurogenesis in this region (Tonchev & Yamashima, 2006; Ziemka-Nalecz & Zalewska, 2012). There have been a few reports of neuronal regeneration in the CA1 following global ischemia and these studies warrant some consideration (Bendel, et al., 2005; Nakatomi, et al., 2002; Schmidt & Reymann, 2002). Nakatomi et al. (2002) infused fibroblast growth factor-2 and epidermal growth factor, known progenitor cell mitogens, into the lateral ventricles of rats following cerebral ischemia and while the treatment did not prevent CA1 neuronal degeneration, increased number of the neuronal nuclei marker, NeuN-positive cells within the CA1 was detected by day 28 postischemia, suggestive of neuronal regeneration. Specifically, the authors showed a 40% neuronal restoration within the CA1 and these cells displayed morphological characteristics reminiscent of intact pyramidal neurons. Additionally, the cells were integrated within the neural circuitry by forming functional synapses determined using electron microscopic examination coupled with electrophysiological measures and survived for at least 6 months following the ischemic insult. These neurons were proposed to have originated from the periventricular region near the hippocampus and to have contributed to amelioration of spatial memory deficits in the

water maze. The authors however failed to detect changes indicative of neurogenesis in vehicle-treated ischemic rats. In contrast, Bendel and colleagues (2005) reported neurogenesis within the CA1 following 11 min of global ischemia in rats, which by 90 days postischemia reached 40% of the number of viable neurons of sham-operated animals. Again, the newly formed neurons in this region were proposed to originate from progenitor neuronal stem cells residing in the adjacent lateral periventricular region. This phenomenon was associated with recovery of ischemia-induced spatial memory impairments in in the water maze 90 days postischemia. In a later publication by the same group (Bueters, et al., 2008), degeneration of newly formed neurons was observed 250 days following ischemia, accompanied by an increase in glial reactivity and mineralized calcium deposits within the CA1 field. Of note, despite the extensive CA1 damage observed at this remote time interval performance in the water maze was not altered, an effect that the authors attributed to an altered searching strategy by ischemic rats. This is in agreement with our observation of minimal impairments in the water maze tasks 80 days postischemia, in the absence of changes in the expression of neurogenic markers within the CA1 region assessed 5 days later. This raises the possibility that rehabilitation of cognitive abilities is related to other mechanisms possibly linked to compensatory regulation of spatial learning and memory processes by adjacent brain regions. Nonetheless, it remains possible that the extensive behavioural training protocol in our study may have affected PSA-NCAM levels in the DG of sham-operated rats, thus masking the effect of ischemia on neurogenesis at that time interval. One study has shown a significant increase in PSA-NCAM expression in the DG of control rats soon after water maze training (Van der Borght, Wallinga, Luiten, Eggen, & Van der Zee, 2005).

Although these studies have shown coincidental changes in neurogenesis and memory function, future studies using sophisticated genetic tools to manipulate neurogenesis are now required to examine causal relationships between neurogenesis levels and spatial learning and memory and determine memory processes to which such phenomenon may contribute. Despite the lack of active neurogenesis observed in the CA1 region by ischemic animals in our study, the region has been shown to undergo an upregulation of gliogenesis following ischemia. In a primate model of global ischemia, analysis of the distribution of newborn cells in the hippocampus of macaque monkeys confirmed increased proliferation of progenitor cells in the DG by the second week postischemia similar to that observed in rodents, while the CA1 region, devoid of neurogenesis, showed glial cell renewal (Tonchev & Yamashima, 2006). Over 80% of the 5-bromo-2'-deoxyuridine (BrdU)-positive cells within the CA1 had a microglial immunophenotype, co-expressing the microglia and macrophage marker Iba-1 (ionized calcium binding adaptor molecule 1), and lacking co-expression with the neuronal nuclei marker (NeuN), the remainder of the cells expressed astrocyte markers and a few labeled by the oligodendrocyte marker CNP. Thus, it is possible that neurogenesis detected through BrdU immunohistochemistry within the CA1 may indicate a different cell phenotype when co-labelling techniques are used.

3. Resveratrol effects on non-neuronal populations of cells

Glial cells have been shown to act as scavengers following brain injury as well as play a role in discrete immune function, while their activated state is inherently tied to neuronal injury postischemia. In further review of the neuronal protection conferred by RSV,

a final experiment (Article 3) was undertaken to investigate the impact of RSV on factors coincidental to post-injury inflammation, and that affect glial cell populations.

3.1 Effect of resveratrol on ischemia-induced microglial (OX-42), GFAP and GLT-1 expression in the hippocampus.

Immunostaining for OX-42 revealed dose-related effects of RSV on ischemia-induced microglial activation, the 10 mg/kg dosage significantly decreasing OX-42-ir within the CA1 layer. This finding is in line with reports of neuroprotection against hypoxic-ischemic injury with simultaneous reduction of inflammatory microglia as assessed by OX-42 immunohistochemistry in the injured hippocampus (Shin et al., 2006). Moreover, RSV treatment at either dosage markedly attenuated ischemia-induced GFAP-ir and hypertrophic cellular morphology of astrocytes within the CA1 region. It has been proposed that limiting inflammatory cytokine production by activated glial should be beneficial for prevention of neuroinflammation and neurodegeneration (X. Lu, et al., 2010; Simao, et al., 2012a; F. Zhang, J. Liu, et al., 2010). In line with these results and as discussed in greater detail elsewhere (Article 3), the current findings support the notion that long-lasting neuroprotection conferred by RSV may in part be mediated through attenuation of glial cell activation postischemia.

Increased activation of glial cells following ischemia has also been associated with impairments in glutamate transporter function (Ouyang, et al., 2007) and elevated extracellular glutamate levels are thought to aggravate post-ischemic neuronal injury. Specifically, the early increase in mitochondrial ROS in CA1 astrocytes following ischemia has been proposed to contribute to the loss of GLT-1 transport activity in this region

(Ouyang, et al., 2007). Strongly reactive astrocytes have been associated with reduced glutamate transport activity while moderately reactive astrocytes in the lesion periphery maintain overall glutamate transporter expression and function (Schreiner, Berlinger, Langer, Kafitz, & Rose, 2013). In this context, knockout studies have provided support for the possible therapeutic role of compounds regulating GLT-1 expression following injury. Thus, antisense knockout of GLT-1 prior to focal ischemia has been shown to worsen neurological status and increase infarct volume and mortality rate (Rao, Dogan, et al., 2001). Moreover, a recent study showed that neuronal protection postischemia provided by ischemic preconditioning was paired with attenuated glutamate concentrations in the CA1, effects that were abolished following administration of dihydrokainate, a selective inhibitor of GLT-1 (Gong et al., 2014). Together, these findings highlight an important role of GLT-1 in the neuronal fate postischemia. While evidence of RSV's actions of glutamate uptake is scarce and limited to in vitro manipulations (reviewed in detail in Article 3), RSV has been hypothesized to affect glutamate transporter expression following brain insults through its direct actions on astrocyte activation (de Almeida, et al., 2007; Quincozes-Santos & Gottfried, 2011), a hypothesis that our study aimed to validate. Consistently, the results of the last experiment demonstrate that both 1 and 10 mg/kg RSV dosages normalized GLT-1 expression to control levels in the CA1 region postischemia while a strong positive correlation with neuronal density in this region further supports these effects likely contribute to RSV's neuroprotective actions (Girbovan, et al., 2012). However, future studies using GLT-1 antisense therapies prior to ischemia are necessary to assess the importance of this transporter in RSV protective effects. Indeed, it remains possible that RSV's actions on GLT-1 post-ischemic regulation may induce neuroprotection independently or in coordination with

the well-validated antioxidative and angiogenic activities of the compound. Considering diverse beneficial actions of RSV reported thus far, a number of mechanisms likely act in concert to provide such effects under various pathological conditions.

The role of RSV's actions on non-neuronal populations of cells may be important in the context of newly emerging hypotheses which sustain that glial cells play a fundamental role in the pathogenesis of ischemic neuronal death. In recent years, glial cells have been involved in a number of active processes. In this context, the "gliocentric view" of stroke injury sustains that new therapeutic agents need to target glia integrity as a main component of neuronal recovery processes, with an emphasis on astrocyte integrity, and that pharmacological agents that strictly target neurons are unlikely to be successful, given that neuronal viability is dependent on an environment with strict metabolic demands (Takano, et al., 2009). Indeed, the neurocentric view of the nervous system has considerably evolved with recent understanding of the many essential functions played by astrocytes and microglia in normal brain functioning and as a result of brain pathology (Barreto, et al., 2011). As such, our findings support an important role of RSV in mediating astrocytic GLT-1 transmission in the hippocampus postischemia, which strengthens the involvement of glial cell scavenging properties in selective neuronal death following ischemia.

3.2 GLT-1 immunostaining in the CA3

Glutamate excitotoxicity is a hallmark of the neuronal death cascade which ensues following global ischemia. Moreover, GLT-1 expression is significantly altered at delays coinciding with CA1 cell loss, a phenomenon that questions its exact role in the process (Bruhn et al., 2000). Supportive of this, our findings highlight an important downregulation of GLT-1 expression in the CA1 subfield of the hippocampus postischemia, which was

positively correlated with neuronal density in this region. In contrast, GLT-1 expression and protein levels in the CA3 region have been shown to progressively increase following ischemia, which was confirmed through both in situ hybridization and immunohistochemistry, and was taken as indication of a possible link between GLT-1 upregulation and inherent resistance of CA3 neurons following ischemia (Bruhn, et al., 2000). In line with this, Zhang and colleagues (2011) reported an upregulation of GLT-1 in the CA3 and DG of male Wistar rats at 7 days postischemia (8 min of 4-VO) compared to CA1 levels and suggested that the increase may be linked to the inherent resistance of these regions against ischemic injury protein levels being significantly upregulated in these regions as early as 2 days postischemia (M. Zhang, et al., 2011). However, when occlusion time was increased to 25 min, CA3 and DG neurons were severely affected and GLT-1 expression was comparable to that observed in the CA1 subfield. In contrast with Zhang et al. `s findings, we observed no alterations of GLT-1 expression in the CA3 or DG regions postischemia. However, unaltered expression levels may still contribute to the inherent resistance of these regions against ischemia. Thus, future investigations require comparative studies using immunohistochemical techniques paired with protein analysis techniques to better assess the relevance of region specificity and neuronal resistance postischemia (Jing, He, Zhang, & Li, 2013).

3.3 GFAP immunostaining in the CA3

Our findings further indicated increased astrocyte activation in the CA3 region, which was not attenuated by RSV treatment. Pyramidal neurons of the CA3 region are less vulnerable to ischemia with occlusion times of 10 min or less (Pulsinelli, et al., 1982; Yoo et al., 2010; M. Zhang, et al., 2011). However, less is known about the temporal profile of glial

cell activation, its correlation with occlusion times following ischemia and expression beyond the vulnerable CA1 region. In a recent report, Zhang et al. (2011) found that 8 min global ischemia produced neuronal damage and astrocyte activation restricted to the CA1 subfield, while astrocytes within the CA3 region showed normal somas with slender processes in close proximity to, and uniformly surrounding, pyramidal neurons. In contrast, 25 min global ischemia produced severe necrosis in all hippocampal fields along with an astrocyte profile characteristic of activation; hypertrophied somas with shortened processes. In another study, increased glial cell activation expanded beyond the CA1 within the first 3 days postischemia following 10 min global ischemia (Yasuda, et al., 2011), a phenomenon gradually selectively restricted to the CA1 by 7 days as permanent neuronal death selectively established within this region. Such observation suggests that increased astrocyte activation in the CA3 could indicate a temporary response to the ischemic event, akin to what is observed in penumbral regions after focal ischemic insults.

4. Resveratrol and cognition in various disease models and normal conditions

The current thesis revealed that despite significant and long-lasting neuronal protection conferred by RSV, dose-related effects of the polyphenol on functional recovery postischemia as well as altered performance of sham animals treated with RSV on discrete behavioural measures were noted. Specifically, Articles 1 and 2 highlight important behavioural alterations following pretreatment with 10 mg/kg RSV as demonstrated by increased locomotor activity in the open field test as well as recognition and spatial memory impairments, assessed in the object recognition test, radial arm and Morris water mazes, respectively. Moreover, ischemic rats treated with the 1 mg/kg RSV dosage showed a trend

toward improved performance on the radial arm maze whereas mild and transient impairments similar to saline-treated ischemic rats' performance were noted in the water maze. It must be noted, that the negligible deficits displayed by ischemic rats treated with saline on a number of behavioural measures make it increasingly difficult to assess possible RSV-mediated effects. In contrast with our results, the current literature supports a role for RSV-mediated improvement of cognitive function following brain ischemia (Anastacio et al., 2014) and in various animal models of disease (Harada, et al., 2011; D. Liu et al., 2014; Meng, Wang, & Li, 2014; Sharma & Gupta, 2002), granting methodologies and testing paradigms vary greatly in these studies.

For instance, Anastacio et al. (2014) found that post-ischemic i.p. administration of 20 mg/kg RSV for 7 days significantly attenuated pyramidal cell death in the rat CA1 subfield and prevented both spatial and working memory impairments. Similarly, in a mouse model of Alzheimer's disease, daily RSV consumption (16 mg/kg/day) for ten months lead to concomitant neuroprotection and significant improvement in short-term memory performance assessed in the object recognition test (Porquet, et al., 2014). Likewise, Tiwari et al. (2011) showed that RSV dose-dependently improved alcohol-induced cognitive impairment in rats following daily administration, at doses of 5, 10 or 20 mg/kg, for 10 weeks. Despite a trend for improvement of cognitive function following RSV treatment in models of disease, a lack of significant improvement has also been reported. As such, 21-day pretreatment with 10 or 20 mg/kg RSV failed to reveal any behavioural effects on scopolamine-induced cognitive impairment in mice as assessed by water maze performance (R. Gupta, Gupta, Mediratta, & Bhattacharya, 2012). The latter studies highlight a wide range or drug administration protocols, species used, and disease models, which could all

greatly impact the results observed. Future studies assessing cognitive and behavioural impairments following 4-VO, with an emphasis on possible dose-related effects of the polyphenol, are required to better characterize possible dissociations between neuronal protection and cognitive effects of RSV.

Studies assessing RSV effects in clinical populations are only gradually beginning to surface following the growing animal literature supporting RSV's wide range of biological actions. Moreover, the former remain limited and inconclusive at this time, while evidence of cognitive effects are currently lacking and rely on correlational evidence instead. In that regard, it has been suggested that improvement in systemic vasodilator function with RSV may be extended to cerebral vasculature, and it is hypothesized that improved cerebral endothelial function may enhance cognitive function (Sinn, Milte, & Howe, 2010). This hypothesis was partially tested in the human population, whereby a single-dose of trans-resveratrol (30, 90 or 270 mg) lead to dose-dependent improvements in endothelial function in overweight/obese mildly hypertensive adults (Wong et al., 2011). In a subsequent study, the authors show these effects to persist following daily RSV supplementation for six weeks (75 mg/day), leading them to suggest possible beneficial actions on cognitive performance, although the latter was not evaluated (Wong et al., 2013). Thus, at the moment, little is known of RSV's effect on cognitive and behavioural measures in clinical cohorts and effects remain speculative at best.

The current thesis further highlighted that the 10 mg/kg RSV dosage altered the performance of sham animals in discrete behavioural tests. Of note, we found no dose-related effects of RSV on neuronal density following ischemia. The latter observations suggested that intrinsic actions of the polyphenol have a lasting impact on locomotion and memory.

These results are also supportive of increasing evidence of hormesis (e.g., biphasic drug response phenomenon) displayed by RSV in a number of biologic models while behavioural demonstrations remain limited (Calabrese, et al., 2010). In vitro, hormesis has been documented in tumor cell lines, whereby low-doses of the polyphenol have been shown to enhance cell proliferation whereas higher doses suppressed cell proliferation relative to the control group (Li et al., 2006). These are important observation since RSV has emerged as an agent with complex biological activity in recent years, which attracted numerous researchers and widespread consumer interest. Different products containing RSV are now commercially available and advertised for their health benefits. However, dose-related effects have been reported in vitro in the case of tumor cell lines and in animal models of disease, in which behavioural assessment is either absent or limited. To date, interventional studies investigating potential effects of long-term RSV supplements on learning and memory formation in both at risk and healthy populations are lacking and whether the dose-related effects noted in this thesis would be associated with similar results in humans remains unknown. The few studies that have assessed such measures in healthy cohorts have reported mixed findings. For instance, Kennedy et al. (2010) found that consumption of a single dose (250 mg or 500 mg) of RSV by healthy young adults resulted in a dose-dependent overall increase in cerebral perfusion of active brain regions (measures by near-infrared spectroscopy) during a 9-min cognitive test battery. Despite this, the authors did not find enhancement in cognitive performance, which may in part be due to the acute administration protocol. Moreover, a recent study evaluated the effects of supplementary RSV treatment, albeit a single dose (200 mg per day) was used, for 26 weeks on memory performance in a cohort of healthy older adults (Witte, Kerti, Margulies, & Floel, 2014). The authors reported

improved retention of words over a 30 min delay which was correlated with an increase in resting-state functional connectivity of the hippocampus, as assessed by fMRI and suggestive of improvements in the integrity and functionality of the hippocampus. While a few clinical trials assessing cognitive effects of RSV are currently ongoing (see www.clinicaltrials.gov website), epidemiologic studies are required to assess the nature of its dose-response on cognitive and behavioural tasks in healthy and clinical populations.

5. Considerations related to the present work and future research directions

Our studies have used immunohistochemical detection in assessing the impact of RSV on different endogenous molecules. This technique has become a hallmark in the field of neuroscience providing important information on cell typing and colocalization. However no technique is perfect, and the influx of information attained with immunohistochemistry has not been without issues. Thus, the method is plagued by concerns regarding specificity of antibodies and replicability of results. Moreover, inability to replicate staining with the same antibody from different lot/batches or manufacturers has accentuated scepticism. It has been suggested that in order for immunohistochemistry results to hold a strong degree of validity, complete information regarding the antibody (source of antibody and preparation information e.g. what was the antibody raised against), knowledge of specificity characterization and cross-reactivity as well thorough disclosure of controls used are unprecedented (Saper, 2005). In that regard, information regarding the antibodies used in this thesis' set of experiments has been thoroughly detailed in the methods section of each experiment. Furthermore, antibodies were selected based on thorough examination of literature supporting their use for the purpose of the planned investigations. In general, profile of

protein and mRNA levels of the antibodies used is similar to patterns of immunohistochemical changes noted in other reports (J. H. Choi, et al., 2012; Hein, Horvath, & Kugler, 2001). Careful measures were also taken to ensure that appropriate controls were devised and staining was uniform across subjects and matched previously documented staining profiles. Nonetheless, despite great care and attention to detail during both slice preparation and quantification, our immunohistochemistry results need to be interpreted with caution and a general awareness of generalizability limitations.

5.1 Detection of neurogenic processes

Stimulation of neurogenesis is a common response to a variety of brain insults characterized by neuronal death and tissue injury. In the current thesis, we selected two markers, DCX and PSA-NCAM, prominently used in the general characterization of neurogenesis based on their inherent ability to localize neuronal precursor cells and immature neurons. PSA-NCAM expression is documented in neuronal precursor cells and its expression in the postnatal neurogenic niche seems to correlate to the developmental stage at which newborn neurons start to extend processes, detach from the proliferative niche and migrate (Gascon, Vutskits, & Kiss, 2010). However, caution is warranted when using PSA-NCAM detection coupled with certain behavioural tasks as the marker has been shown to be up-regulated in the hippocampus during hippocampal-dependent learning tasks (Venero et al., 2006). DCX is a brain-specific microtubule-associated protein that promotes microtubule polymerization (von Bohlen Und Halbach, 2007). It is present in migrating neuroblasts and young neurons and its expression is temporally in-frame with PSA-NCAM expression (Nacher, et al., 2001; R. Tanaka, et al., 2004). Moreover, DCX expression is limited to late mitotic neuronal precursors and early postmitotic neurons, thus making it impossible to

group differently marked cells to different stages of neurogenesis (von Bohlen und Halbach, 2011). In that light, we attempted to supplement the study findings with the addition of experimental groups exposed to ischemic injury in the absence of behavioural tasks and treated with BrdU. BrdU is an S-phase-specific marker whose incorporation into deoxyribonucleic acid allows the detection of all newly formed cells and whose expression could have further corroborated our neurogenesis findings (Kuhn & Cooper-Kuhn, 2007). Unfortunately, immunohistochemical detection with this marker was not successful following a number of attempts at optimizing expression in our samples, suggesting possible issues with tissue preparation/fixation or the antibody itself and the task was therefore abandoned.

In the pathogenesis of cerebral ischemia, increased neurogenesis is generally regarded as an endogenous attempt of the brain to foster enhanced functional recovery through replacement of lost neuronal connections. Indeed, newly generated granular neurons extend dendrites into the molecular layer of the DG establishing synapses with mature neurons born during development (R. Tanaka, et al., 2004). At present, studies are focussing on assessing a causal relationship between neurogenesis and functional recovery in disease models (Raber, et al., 2004) using cranial irradiation directed at neurogenic zones (Hellstrom, et al., 2009; Naylor, et al., 2008) along with genetically engineered rodent models (Dhaliwal & Lagace, 2011) in order to directly address the functional relevance of this endogenous process.

5.2 Microglial detection of activation state

The antibody specific for cluster of differentiation 11b (CD11b) molecules (clone OX-42) is widely used in characterizing activated microglia through immunohistochemistry. The protein is expressed on the surface of microglia and forms part of the complement

receptor 3 and its levels have been shown to markedly increase upon activation of microglia (Kettenmann, et al., 2011; Tsuda, et al., 2003). Quantification of the signal was achieved through the use of the thresholding technique of optical density using the ImageJ software. Typically, when a difference (most commonly an increase) in the overall level of thresholded staining is observed is it described as reflecting 'microglial activation' (Hinwood et al., 2013). While the technique is commonly used to semi-quantitatively label density, it has important limitations. In the case of microglial staining these are related to the fact that this technique does not account for microglial morphological changes in response to pathological and non-pathological conditions, thus oversimplifying a complex set of processes (Beynon & Walker, 2012). Importantly, the terms 'hyper-ramification' and 'process extension and reorientation' have been coined to describe microglial process branching, extension (increased process length) and reorientation in response to a stimulus (Fontainhas et al., 2011; Tremblay, Lowery, & Majewska, 2010). The 'hyper-ramification' state in microglia has been documented in non-pathological conditions whereas rapid process extension and reorientation towards the site of injury seems to occur as a result of brain injury (Davalos et al., 2005). It is then suggested that microglia transition to a reactive phagocytic amoeboid stage, which can further be divided in a series of phases (transitional, motile and locomotor stages) most notably characterized by different mobility rates of microglia processes (Stence, Waite, & Dailey, 2001). Lastly, in response to neuronal injury, the rate of motility of phagocytic microglia seems heterogeneous and may depend largely on time after cell death, mode of cell death, direct cell-to-cell contact and release of substances from nearby dead cells (Petersen & Dailey, 2004). Thus, it is important to highlight that OX-42 labelling will label all stages of microglial morphological remodelling. Caution is thus warranted in the

interpretation of these results as the diffuse labelling is an oversimplification of true microglial activity.

To date, the preferred quantification technique in identifying activated microglia has been manual quantification of morphological characteristics as it remains the most accurate technique in defining distinct temporal and functional stages of activation, enabling the quantification of activation response occurring on timescales of minutes on a per cell basis (Stence, et al., 2001). This however represents a tedious task that requires multiple investigator analysis for increased reliability and accuracy, which limits its use in analyzing large data sets. For this reason, others have elaborated on this method through the development of automated image analysis methods enabling the quantitative assessment of microglia activation states within tissue based solely on cell morphology (Kozlowski & Weimer, 2012). Kozlowski and Weimer (2012) implemented a thresholding method to segment the cell shape of individual microglia with ~90% accuracy compared to manual methods, a method they deem much faster, involves no human intervention and relies on a priori knowledge of a single characteristic of microglia; cell size. Due to variations in the morphology of resting microglia, which can vary across brain regions, the method does require a high degree of calibration for enhanced performance. Complementary to this technique, another method involves digitally reconstructing microglia from high magnification images, allowing for detailed morphological information to be obtained (Hinwood, et al., 2013). While manual reconstruction is possible, difficulty arises when trying to accurately trace the movement of microglia processes in the z-axis. Thus, this method is best implemented while collecting z-stacks of individual cells and importing them into a semi-automatic reconstruction package (Beynon & Walker, 2012). This technique

allows an outflow of information to be obtained, including primary process number, branch number, process volume, process length, soma size, as well as the total area occupied by the cell, enabling detailed characterization of cell alterations following injury or other interventions. However, this method represents a time-consuming process which requires advanced training in digital reconstruction software and access to a confocal microscope. While digital reconstruction was attempted in the present experiment following extensive training in the technique, a number of hurdles made it impossible to continue and had to be abandoned. Most notably, the task was abandoned due to extreme difficulty in accurately tracing the movement of microglia processes in the z-axis, as it required visual determination of which portion of a microglia process is most in focus at a given z-coordinate. Normally, collecting a z-stack of individual cells is proposed to circumvent this problem but an inability to eliminate blurriness from visual examination coupled with time restraints made this task impossible at the time. Despite a focus on microglia quantification in the current segment, these methods are increasingly being utilized in the morphological assessment of other cell types and in time will provide more thorough characterization of changes in cell morphology.

Thus, the method of quantification used over-simplifies the diverse processes suggested to occur in response to neuronal death as a result of ischemia and impact of RSV treatment. Only detailed microscopic analysis of time-dependent morphological changes could provide a comprehensive account of disease and RSV impact on microglial activation. Our observations however point to the relevance of further exploring such effects, an initiative that would also help further elucidate the role of activation of microglia (beneficial and deleterious effects being documented).

5.3 General considerations

5.3.1 Age-dependent changes

The current set of experiments has used cohorts of young rats. Although this is really common in the field of ischemic research, such choice likely impairs conclusions that can be drawn from these studies considering that populations afflicted by stroke and brain ischemia are significantly older. Thus, unlike young healthy rodents, the aged population is plagued by disease comorbidities such as hypertension and diabetes (Anton, Vitetta, Cortizo, & Sali, 2005) and a general yet significant age-related cognitive decline, affecting both learning and memory (Dennis, Kim, & Cabeza, 2007). While aging per se is not a direct cause of cardiac arrest in humans, it is associated with increased risk of coronary or ischemic heart disease, which is the leading cause of sudden cardiac arrest.

The use of younger subjects in most studies is preferred due to the ease of implementation of the different ischemic models as it relates to replicability of the insult and increased survival rates. However, important cognitive functions and biological processes/parameters are affected by age. Indeed, cognitive decline has been characterized in aged rodents and neurogenesis while still present in the SGZ, is of smaller magnitude than that observed in younger subjects (Yagita, et al., 2001). The expression of markers for cell proliferation BrdU and neuroblast differentiation (DCX) has been shown to be significantly reduced in aged compared to young gerbils following global ischemia (J. H. Choi, et al., 2012).

In this light, consideration should be taken in the interpretation of the current results and their generalizability to the aging population, as the aged brain seems to contain factors inhibiting survival of newborn cells and lacks factors necessary for neuronal differentiation

and survival. Moreover, future studies investigating therapeutic interventions to enhance endogenous neurogenesis following brain ischemia in aging rodents could provide an interesting research venue.

5.3.2 Sex differences

To date, the use of male subjects in vascular research represents the norm. Documented cardioprotective effects of female reproductive hormones such as estrogen (Hale, Birnbaum, & Kloner, 1997; Lagranha, Deschamps, Aponte, Steenbergen, & Murphy, 2010; M. Wang et al., 2009) and their cyclical nature is often mentioned as a factor behind experimental variability that is not present when using male subjects. However, a recent report was unable to support a link between circulating hormones during various estrous cycle stages and cardiac-ischemia reperfusion injury in rats (Frasier et al., 2013).

The human literature highlights that the incidence of sudden cardiac arrest remains higher in young men than women (aged 1-40 years) despite CVD being a leading cause of death in both men and women (Vaartjes et al., 2009). Moreover, women have been shown to have a higher rate of survival until hospital admission and a more favorable one month survival and neurological outcome compared to men (Hasan et al., 2014). Despite a slightly higher incidence of CVD in aged women (Stecker et al., 2014), the female gender offers a survival and outcome advantage following an out-of-hospital cardiac arrest. In another recent report, females had lower mortality rates following cardiac arrest when researchers controlled for differences in age, obesity and other variables (Greenberg et al., 2014).

Although it is appropriate to begin investigating effects using male cohorts, preferably aged animals, considering difficulties inherent to vascular surgeries and

experiments, upon identification of promising venues, investigation of such phenomenon using female subjects should follow.

5.3.3 Rodent four-vessel occlusion model and cardiac arrest in humans

The 4-VO animal model and cardiac arrest in humans share a lot of communalities in terms of physiological processes involved and behavioural impairments observed. However, they also present some differences. Thus, while human cardiac arrest leads to complete cessation of blood and oxygen supply in the brain and body, cerebral blood flow, although significantly compromised in discrete brain regions, continues to supply certain brain regions such as the lower brainstem ensuring viability of the animals following extended 4-VO durations (Small & Buchan, 2000). Moreover, the cauterization of the vertebral arteries during 4-VO is achieved blindly by the experimenter and unintentional failure to occlude a vertebral artery can lead to collateral circulation and incomplete brain ischemia (Furlow, 1982).

In light of these limitations, rodent models have been devised that can produce cardiac arrest, which coupled with CPR techniques, likely better represent human cardiac arrest pathology (Kofler et al., 2004). In rats, initial models induced cardiac arrest using trans-oesophageal electrical fibrillation, a procedure associated with high mortality rates mainly due an inability to restore blood perfusion following cardiac arrest, an issue that is not present in the 4-VO model (Popp, Vogel, Teschendorf, & Bottiger, 2007). Recently, a new model has been developed in mice by Deng and colleagues (2014) with promising/similar results also obtained in rats. In this model, cardiac arrest is induced through jugular injection of potassium chloride to stop the heart and the procedure is completed with CPR procedures (i.e., chest compressions, ventilation, administration of epinephrine and physiological

monitoring) (Deng, et al., 2014). In mice, this model demonstrates consistent injury (with 6-8 min of cardiac arrest) in the CA1 layer of the hippocampus and in striatal neurons. However, mortality rates remain high and the procedure requires intense care to animals for the first 24 to 48 h after cardiac arrest. Thus, although there are limitations to consider in using animal models of cerebral ischemia related to an inability to mimic all aspects of the disease, these models provide a great degree of physiological control in that the resulting injury is reproducible and variability is as limited as possible (Traystman, 2003). They are also associated with selective neuronal damage and memory deficits upon short duration akin to those experienced following cardiac arrest in humans.

6. Final word

Clinically, neuroprotective drugs might be used either prophylactically in patients at known risk of ischemic brain injury (e.g., patients undergoing procedures such as cardiac surgery, endarterectomy, or endovascular therapy, which have a risk of cerebral ischemic events during a defined period). These clinical populations may thus be considered for short-term or as post-insult ‘rescue’ therapy (after acute perinatal asphyxia). Such people, as well as those at risk for ischemic recurrence after minor strokes, are readily identifiable and perhaps appropriate for long-term prophylactic neuroprotection. Drug pretreatment could offer distinct advantages in patient groups identified at high risk for ischemic stroke if side effects are minimal. Pharmacological advances based on enhanced comprehension of the pathophysiology of ischemic brain injury have led to the development of therapies that can potentially ameliorate the consequence of these events (Rodrigo et al., 2013; Simao, et al., 2012a).

Despite an overwhelming amount of research into treatment strategies for protecting the brain against ischemia-induced neuronal degeneration, there is currently no robust option available. The approach to treatment through assessment of neuroprotective effects needs to reprioritize treatments aiming to reduce cognitive impairments be it concomitant or not with providing neuroprotection. This is especially important as one of the main clinical consequences of hypoxic brain injury with regards to long-term functioning is cognitive impairment (Moulaert, et al., 2009) and patients' participation in daily activities and overall quality of life are inadvertently affected (Lundgren-Nilsson, Rosen, Hofgren, & Sunnerhagen, 2005; Middelkamp et al., 2007). In light of this, rehabilitative approaches such as exercise (Shimada et al., 2013), exposure to an enriched environment (Cechetti, Worm, et al., 2012) and caloric restriction (Roberge, Messier, et al., 2008) have been associated with significant improvements in function and a trend toward better learning in rodent models despite not always providing a change in infarct volume (Roberge, Hotte-Bernard, et al., 2008; Xie et al., 2013).

It is possible that prophylactic dietary therapies could play a role to prevent or minimize such impairments acting on mechanisms related to neuronal and/or glial functions but also likely on neuroplastic changes post-injury such as those related to angiogenesis or glial expression of glutamate transporters. Importantly, the latter changes may represent better indicators of functional outcome following brain ischemia than effects on neuronal injury.

References

Aggleton, J. P., Albasser, M. M., Aggleton, D. J., Poirier, G. L., & Pearce, J. M. (2010). Lesions of the rat perirhinal cortex spare the acquisition of a complex configural visual discrimination yet impair object recognition. *Behav Neurosci, 124*(1), 55-68. doi: 2010-01915-015 [pii] 10.1037/a0018320

Albani, D., Polito, L., Signorini, A., & Forloni, G. (2010). Neuroprotective properties of resveratrol in different neurodegenerative disorders. *Biofactors, 36*(5), 370-376. doi: 10.1002/biof.118

Allen, B. S., & Buckberg, G. D. (2012). Studies of isolated global brain ischaemia: I. Overview of irreversible brain injury and evolution of a new concept - redefining the time of brain death. *Eur J Cardiothorac Surg, 41*(5), 1132-1137. doi: 10.1093/ejcts/ezr315

Altman, J., & Das, G. D. (1965). Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. *J Comp Neurol, 124*(3), 319-335.

Aluyen, J. K., Ton, Q. N., Tran, T., Yang, A. E., Gottlieb, H. B., & Bellanger, R. A. (2012). Resveratrol: potential as anticancer agent. *J Diet Suppl, 9*(1), 45-56. doi: 10.3109/19390211.2011.650842

Alvira, D., Yeste-Velasco, M., Folch, J., Verdaguer, E., Canudas, A. M., Pallas, M., & Camins, A. (2007). Comparative analysis of the effects of resveratrol in two apoptotic models: inhibition of complex I and potassium deprivation in cerebellar neurons. *Neuroscience, 147*(3), 746-756. doi: 10.1016/j.neuroscience.2007.04.029

Anacker, C., Cattaneo, A., Luoni, A., Musaelyan, K., Zunszain, P. A., Milanesi, E., . . . Pariante, C. M. (2013). Glucocorticoid-related molecular signaling pathways regulating hippocampal neurogenesis. *Neuropsychopharmacology, 38*(5), 872-883. doi: 10.1038/npp.2012.253

Anastacio, J. R., Netto, C. A., Castro, C. C., Sanches, E. F., Ferreira, D. C., Noschang, C., . . . Pagnussat, A. (2014). Resveratrol treatment has neuroprotective effects and prevents cognitive impairment after chronic cerebral hypoperfusion. *Neurol Res, 36*(7), 627-633. doi: 10.1179/1743132813y.0000000293

Anderova, M., Vorisek, I., Pivonkova, H., Benesova, J., Vargova, L., Cicanic, M., . . . Sykova, E. (2011). Cell death/proliferation and alterations in glial morphology contribute to changes in diffusivity in the rat hippocampus after hypoxia-ischemia. *J Cereb Blood Flow Metab, 31*(3), 894-907. doi: 10.1038/jcbfm.2010.168

Anton, B., Vitetta, L., Cortizo, F., & Sali, A. (2005). Can we delay aging? The biology and science of aging. *Ann N Y Acad Sci, 1057*, 525-535. doi: 10.1196/annals.1356.040

Araki, H., Hino, N., Karasawa, Y., Kawasaki, H., & Gomita, Y. (1999). Effect of Dopamine Blockers on Cerebral Ischemia-Induced Hyperactivity in Gerbils. *Physiology & Behavior, 66*(2), 263-268. doi: 10.1016/s0031-9384(98)00293-5

Arnold, T., & Betsholtz, C. (2013). Correction: The importance of microglia in the development of the vasculature in the central nervous system. *Vasc Cell, 5*(1), 12. doi: 10.1186/2045-824x-5-12

Arsenijevic, Y., Weiss, S., Schneider, B., & Aebischer, P. (2001). Insulin-like growth factor-I is necessary for neural stem cell proliferation and demonstrates distinct actions of epidermal growth factor and fibroblast growth factor-2. *J Neurosci, 21*(18), 7194-7202.

Ates, O., Cayli, S. R., Yucel, N., Altinoz, E., Kocak, A., Durak, M. A., . . . Yologlu, S. (2007). Central nervous system protection by resveratrol in streptozotocin-induced diabetic rats. *J Clin Neurosci, 14*(3), 256-260. doi: S0967-5868(05)00463-7 [pii] 10.1016/j.jocn.2005.12.010

Auer, R. N., Jensen, M. L., & Whishaw, I. Q. (1989). Neurobehavioral deficit due to ischemic brain damage limited to half of the CA1 sector of the hippocampus. *J Neurosci, 9*(5), 1641-1647.

Bah, T. M., Kaloustian, S., Rousseau, G., & Godbout, R. (2011). Pretreatment with pentoxifylline has antidepressant-like effects in a rat model of acute myocardial infarction. *Behav Pharmacol, 22*(8), 779-784. doi: 10.1097/FBP.0b013e32834d1385

Bantsiele, G. B., Bentue-Ferrer, D., Saikali, S., Laviolle, B., Bourin, M., & Reymann, J. M. (2009). Behavioral effects of four antidepressants on an ischemic rat model of emotional disturbances. *Behav Brain Res, 201*(2), 265-271. doi: 10.1016/j.bbr.2009.02.022

Barreto, G., White, R. E., Ouyang, Y., Xu, L., & Giffard, R. G. (2011). Astrocytes: targets for neuroprotection in stroke. *Cent Nerv Syst Agents Med Chem, 11*(2), 164-173.

Battista, D., Ferrari, C. C., Gage, F. H., & Pitossi, F. J. (2006). Neurogenic niche modulation by activated microglia: transforming growth factor beta increases neurogenesis in the adult dentate gyrus. *Eur J Neurosci, 23*(1), 83-93. doi: 10.1111/j.1460-9568.2005.04539.x

Beck, H., & Plate, K. H. (2009). Angiogenesis after cerebral ischemia. *Acta Neuropathol, 117*(5), 481-496. doi: 10.1007/s00401-009-0483-6

Bednar, M. M. (2002). Combining antiplatelet and thrombolytic therapies for stroke. *Expert Opin Pharmacother, 3*(4), 401-410. doi: 10.1517/14656566.3.4.401

Bellaver, B., Souza, D. G., Souza, D. O., & Quincozes-Santos, A. (2014). Resveratrol increases antioxidant defenses and decreases proinflammatory cytokines in hippocampal astrocyte cultures from newborn, adult and aged Wistar rats. *Toxicol In Vitro, 28*(4), 479-484. doi: 10.1016/j.tiv.2014.01.006

Bendel, O., Bueters, T., von Euler, M., Ove Ogren, S., Sandin, J., & von Euler, G. (2005). Reappearance of hippocampal CA1 neurons after ischemia is associated with recovery of learning and memory. *J Cereb Blood Flow Metab, 25*(12), 1586-1595. doi: 9600153 [pii] 10.1038/sj.jcbfm.9600153

Bertelli, A. A., Giovannini, L., Stradi, R., Bertelli, A., & Tillement, J. P. (1996). Plasma, urine and tissue levels of trans- and cis-resveratrol (3,4',5-trihydroxystilbene) after short-term or prolonged administration of red wine to rats. *Int J Tissue React, 18*(2-3), 67-71.

Beynon, S. B., & Walker, F. R. (2012). Microglial activation in the injured and healthy brain: what are we really talking about? Practical and theoretical issues associated with the measurement of changes in microglial morphology. *Neuroscience, 225*, 162-171. doi: 10.1016/j.neuroscience.2012.07.029

Bhat, K. P. L., Kosmeder, J. W., 2nd, & Pezzuto, J. M. (2001). Biological effects of resveratrol. *Antioxid Redox Signal, 3*(6), 1041-1064. doi: 10.1089/152308601317203567

Bi, X. L., Yang, J. Y., Dong, Y. X., Wang, J. M., Cui, Y. H., Ikeshima, T., . . . Wu, C. F. (2005). Resveratrol inhibits nitric oxide and TNF-alpha production by

lipopolysaccharide-activated microglia. *Int Immunopharmacol, 5*(1), 185-193. doi: S1567-5769(04)00276-0 [pii] 10.1016/j.intimp.2004.08.008

Bishayee, A., Barnes, K. F., Bhatia, D., Darvesh, A. S., & Carroll, R. T. (2010). Resveratrol suppresses oxidative stress and inflammatory response in diethylnitrosamine-initiated rat hepatocarcinogenesis. *Cancer Prev Res (Phila), 3*(6), 753-763. doi: 1940-6207.CAPR-09-0171 [pii] 10.1158/1940-6207.CAPR-09-0171

Block, F. (1999). Global ischemia and behavioural deficits. *Prog Neurobiol, 58*(3), 279-295. doi: S0301-0082(98)00085-9 [pii]

Borriello, A., Bencivenga, D., Caldarelli, I., Tramontano, A., Borgia, A., Zappia, V., & Della Ragione, F. (2014). Resveratrol: from basic studies to bedside. *Cancer Treat Res, 159*, 167-184. doi: 10.1007/978-3-642-38007-5_10

Bowers, J. L., Tyulmenkov, V. V., Jernigan, S. C., & Klinge, C. M. (2000). Resveratrol acts as a mixed agonist/antagonist for estrogen receptors alpha and beta. *Endocrinology, 141*(10), 3657-3667.

Bradamante, S., Barenghi, L., & Villa, A. (2004). Cardiovascular protective effects of resveratrol. *Cardiovasc Drug Rev, 22*(3), 169-188.

Brea, D., Sobrino, T., Ramos-Cabrer, P., & Castillo, J. (2009). [Reorganisation of the cerebral vasculature following ischaemia]. *Rev Neurol, 49*(12), 645-654.

Briones, T. L., & Therrien, B. (2000). Behavioral effects of transient cerebral ischemia. *Biol Res Nurs, 1*(4), 276-286.

Bruhn, T., Levy, L. M., Nielsen, M., Christensen, T., Johansen, F. F., & Diemer, N. H. (2000). Ischemia induced changes in expression of the astrocyte glutamate transporter GLT1 in hippocampus of the rat. *Neurochem Int, 37*(2-3), 277-285.

Buchhold, B., Mogoanta, L., Suofu, Y., Hamm, A., Walker, L., Kessler, C., & Popa-Wagner, A. (2007). Environmental enrichment improves functional and neuropathological indices following stroke in young and aged rats. *Restor Neurol Neurosci, 25*(5-6), 467-484.

Bueters, T., von Euler, M., Bendel, O., & von Euler, G. (2008). Degeneration of newly formed CA1 neurons following global ischemia in the rat. *Exp Neurol, 209*(1), 114-124. doi: S0014-4886(07)00339-1 [pii] 10.1016/j.expneurol.2007.09.005

Calabrese, E. J., Mattson, M. P., & Calabrese, V. (2010). Resveratrol commonly displays hormesis: occurrence and biomedical significance. *Hum Exp Toxicol, 29*(12), 980-1015. doi: 10.1177/0960327110383625

Candelario-Jalil, E., de Oliveira, A. C., Graf, S., Bhatia, H. S., Hull, M., Munoz, E., & Fiebich, B. L. (2007). Resveratrol potently reduces prostaglandin E2 production and free radical formation in lipopolysaccharide-activated primary rat microglia. *J Neuroinflammation, 4*, 25. doi: 10.1186/1742-2094-4-25

Carbone, M., Duty, S., & Rattray, M. (2012). Riluzole neuroprotection in a Parkinson's disease model involves suppression of reactive astrocytosis but not GLT-1 regulation. *BMC Neurosci, 13*, 38. doi: 10.1186/1471-2202-13-38

Cechetti, F., Pagnussat, A. S., Worm, P. V., Elsner, V. R., Ben, J., da Costa, M. S., . . . Netto, C. A. (2012). Chronic brain hypoperfusion causes early glial activation and neuronal death, and subsequent long-term memory impairment. *Brain Res Bull, 87*(1), 109-116. doi: S0361-9230(11)00301-7 [pii] 10.1016/j.brainresbull.2011.10.006

Cechetti, F., Worm, P. V., Lovatel, G., Moyses, F., Siqueira, I. R., & Netto, C. A. (2012). Environmental enrichment prevents behavioral deficits and oxidative stress caused by

chronic cerebral hypoperfusion in the rat. *Life Sci, 91*(1-2), 29-36. doi: 10.1016/j.lfs.2012.05.013

Chakraborty, S., Levenson, A. S., & Biswas, P. K. (2013). Structural insights into Resveratrol's antagonist and partial agonist actions on estrogen receptor alpha. *BMC Struct Biol, 13*(1), 27. doi: 10.1186/1472-6807-13-27

Chen, C. K., & Pace-Asciak, C. R. (1996). Vasorelaxing activity of resveratrol and quercetin in isolated rat aorta. *Gen Pharmacol, 27*(2), 363-366.

Chen, J. C., Hsu-Chou, H., Lu, J. L., Chiang, Y. C., Huang, H. M., Wang, H. L., . . . Yeh, T. S. (2005). Down-regulation of the glial glutamate transporter GLT-1 in rat hippocampus and striatum and its modulation by a group III metabotropic glutamate receptor antagonist following transient global forebrain ischemia. *Neuropharmacology, 49*(5), 703-714. doi: S0028-3908(05)00183-8 [pii] 10.1016/j.neuropharm.2005.05.002

Chiota, N. A., Freeman, W. D., & Barrett, K. M. (2011). Hypoxic-ischemic brain injury and prognosis after cardiac arrest. *Continuum (Minneap Minn), 17*(5 Neurologic Consultation in the Hospital), 1094-1118. doi: 0.1212/01.CON.0000407062.25284.f3

Choi, D. W. (1988). Calcium-mediated neurotoxicity: relationship to specific channel types and role in ischemic damage. *Trends Neurosci, 11*(10), 465-469.

Choi, J. H., Yoo, K. Y., Lee, C. H., Park, J. H., Yan, B. C., Kwon, S. H., . . . Won, M. H. (2012). Comparison of neurogenesis in the dentate gyrus between the adult and aged gerbil following transient global cerebral ischemia. *Neurochem Res, 37*(4), 802-810. doi: 10.1007/s11064-011-0675-z

Choi, Y. S., Lee, M. Y., Sung, K. W., Jeong, S. W., Choi, J. S., Park, H. J., . . . Kim, S. Y. (2003). Regional differences in enhanced neurogenesis in the dentate gyrus of adult rats after transient forebrain ischemia. *Mol Cells, 16*(2), 232-238.

Chu, K., Yin, B., Wang, J., Peng, G., Liang, H., Xu, Z., . . . Luo, B. (2012). Inhibition of P2X7 receptor ameliorates transient global cerebral ischemia/reperfusion injury via modulating inflammatory responses in the rat hippocampus. *J Neuroinflammation, 9*, 69. doi: 10.1186/1742-2094-9-69

Chung, S., Yao, H., Caito, S., Hwang, J. W., Arunachalam, G., & Rahman, I. (2010). Regulation of SIRT1 in cellular functions: role of polyphenols. *Arch Biochem Biophys, 501*(1), 79-90. doi: S0003-9861(10)00170-0 [pii] 10.1016/j.abb.2010.05.003

Constant, J. (1997). Alcohol, ischemic heart disease, and the French paradox. *Clin Cardiol, 20*(5), 420-424.

Conte, A., Pellegrini, S., & Tagliazucchi, D. (2003). Synergistic protection of PC12 cells from [beta]-amyloid toxicity by resveratrol and catechin. *Brain Research Bulletin, 62*(1), 29-38. doi: DOI: 10.1016/j.brainresbull.2003.08.001

Corbett, D., & Nurse, S. (1998). The problem of assessing effective neuroprotection in experimental cerebral ischemia. *Progress in Neurobiology, 54*(5), 531-548. doi: Doi: 10.1016/s0301-0082(97)00078-6

Czeh, B., Muller-Keuker, J. I., Rygula, R., Abumaria, N., Hiemke, C., Domenici, E., & Fuchs, E. (2007). Chronic social stress inhibits cell proliferation in the adult medial prefrontal cortex: hemispheric asymmetry and reversal by fluoxetine treatment. *Neuropsychopharmacology, 32*(7), 1490-1503. doi: 10.1038/sj.npp.1301275

D'Hooge, R., & De Deyn, P. P. (2001). Applications of the Morris water maze in the study of learning and memory. *Brain Res Brain Res Rev, 36*(1), 60-90. doi: S0165017301000674 [pii]

Dai, Z., Li, Y., Quarles, L. D., Song, T., Pan, W., Zhou, H., & Xiao, Z. (2007). Resveratrol enhances proliferation and osteoblastic differentiation in human mesenchymal stem cells via ER-dependent ERK1/2 activation. *Phytomedicine, 14*(12), 806-814. doi: DOI: 10.1016/j.phymed.2007.04.003

Dalaklioglu, S., Genc, G. E., Aksoy, N. H., Akcit, F., & Gumuslu, S. (2013). Resveratrol ameliorates methotrexate-induced hepatotoxicity in rats via inhibition of lipid peroxidation. *Hum Exp Toxicol, 32*(6), 662-671. doi: 10.1177/0960327112468178

Danbolt, N. C. (2001). Glutamate uptake. *Prog Neurobiol, 65*(1), 1-105.

Das, S., Alagappan, V. K., Bagchi, D., Sharma, H. S., Maulik, N., & Das, D. K. (2005). Coordinated induction of iNOS-VEGF-KDR-eNOS after resveratrol consumption: a potential mechanism for resveratrol preconditioning of the heart. *Vascul Pharmacol, 42*(5-6), 281-289. doi: S1537-1891(05)00068-6 [pii] 10.1016/j.vph.2005.02.013

Davalos, D., Grutzendler, J., Yang, G., Kim, J. V., Zuo, Y., Jung, S., . . . Gan, W. B. (2005). ATP mediates rapid microglial response to local brain injury in vivo. *Nat Neurosci, 8*(6), 752-758. doi: 10.1038/nn1472

de Almeida, L. M., Leite, M. C., Thomazi, A. P., Battu, C., Nardin, P., Tortorelli, L. S., . . . Gottfried, C. (2008). Resveratrol protects against oxidative injury induced by H2O2 in acute hippocampal slice preparations from Wistar rats. *Arch Biochem Biophys, 480*(1), 27-32. doi: S0003-9861(08)00443-8 [pii] 10.1016/j.abb.2008.09.006

de Almeida, L. M., Pineiro, C. C., Leite, M. C., Brolese, G., Leal, R. B., Gottfried, C., & Goncalves, C. A. (2008). Protective effects of resveratrol on hydrogen peroxide induced toxicity in primary cortical astrocyte cultures. *Neurochem Res, 33*(1), 8-15. doi: 10.1007/s11064-007-9399-5

de Almeida, L. M., Pineiro, C. C., Leite, M. C., Brolese, G., Tramontina, F., Feoli, A. M., . . . Goncalves, C. A. (2007). Resveratrol increases glutamate uptake, glutathione content, and S100B secretion in cortical astrocyte cultures. *Cell Mol Neurobiol, 27*(5), 661-668. doi: 10.1007/s10571-007-9152-2

De Butte-Smith, M., Gulinello, M., Zukin, R. S., & Etgen, A. M. (2009). Chronic estradiol treatment increases CA1 cell survival but does not improve visual or spatial recognition memory after global ischemia in middle-aged female rats. *Horm Behav, 55*(3), 442-453. doi: S0018-506X(08)00319-X [pii] 10.1016/j.yhbeh.2008.11.011

de la Tremblaye, P. B., Raymond, J., Milot, M. R., Merali, Z., & Plamondon, H. (2014). Evidence of lasting dysregulation of neuroendocrine and HPA axis function following global cerebral ischemia in male rats and the effect of Antalarmin on plasma corticosterone level. *Horm Behav*. doi: 10.1016/j.yhbeh.2014.01.003

Della-Morte, D., Dave, K. R., DeFazio, R. A., Bao, Y. C., Raval, A. P., & Perez-Pinzon, M. A. (2009). Resveratrol pretreatment protects rat brain from cerebral ischemic damage via a sirtuin 1-uncoupling protein 2 pathway. *Neuroscience, 159*(3), 993-1002. doi: DOI: 10.1016/j.neuroscience.2009.01.017

Deng, G., Yonchek, J. C., Quillinan, N., Strnad, F. A., Exo, J., Herson, P. S., & Traystman, R. J. (2014). A novel mouse model of pediatric cardiac arrest and cardiopulmonary resuscitation reveals age-dependent neuronal sensitivities to ischemic injury. *J Neurosci Methods, 222*, 34-41. doi: 10.1016/j.jneumeth.2013.10.015

Dennis, N. A., Kim, H., & Cabeza, R. (2007). Effects of aging on true and false memory formation: an fMRI study. *Neuropsychologia, 45*(14), 3157-3166. doi: 10.1016/j.neuropsychologia.2007.07.003

Dhaliwal, J., & Lagace, D. C. (2011). Visualization and genetic manipulation of adult neurogenesis using transgenic mice. *Eur J Neurosci, 33*(6), 1025-1036. doi: 10.1111/j.1460-9568.2011.07600.x

Dolinsky, V. W., Chakrabarti, S., Pereira, T. J., Oka, T., Levasseur, J., Beker, D., . . . Dyck, J. R. (2013). Resveratrol prevents hypertension and cardiac hypertrophy in hypertensive rats and mice. *Biochim Biophys Acta, 1832*(10), 1723-1733. doi: 10.1016/j.bbadis.2013.05.018

Dong, W., Li, N., Gao, D., Zhen, H., Zhang, X., & Li, F. (2008). Resveratrol attenuates ischemic brain damage in the delayed phase after stroke and induces messenger RNA and protein express for angiogenic factors. *J Vasc Surg, 48*(3), 709-714. doi: S0741-5214(08)00584-3 [pii] 10.1016/j.jvs.2008.04.007

Dos-Anjos, S., Martinez-Villayandre, B., Montori, S., Regueiro-Purrinos, M. M., Gonzalo-Orden, J. M., & Fernandez-Lopez, A. (2009). Global ischemia-induced modifications in the expression of AMPA receptors and inflammation in rat brain. *Brain Res, 1287*, 20-27. doi: S0006-8993(09)01283-9 [pii] 10.1016/j.brainres.2009.06.065

dos Santos, A. Q., Nardin, P., Funchal, C., de Almeida, L. M., Jacques-Silva, M. C., Wofchuk, S. T., . . . Gottfried, C. (2006). Resveratrol increases glutamate uptake and glutamine synthetase activity in C6 glioma cells. *Arch Biochem Biophys, 453*(2), 161-167. doi: 10.1016/j.abb.2006.06.025

Dowden, J., & Corbett, D. (1999). Ischemic preconditioning in 18- to 20-month-old gerbils: long-term survival with functional outcome measures. *Stroke, 30*(6), 1240-1246.

Dudley, J. I., Lekli, I., Mukherjee, S., Das, M., Bertelli, A. A., & Das, D. K. (2008). Does white wine qualify for French paradox? Comparison of the cardioprotective effects of red and white wines and their constituents: resveratrol, tyrosol, and hydroxytyrosol. *J Agric Food Chem, 56*(20), 9362-9373. doi: 10.1021/jf801791d

Ekstrand, J., Hellsten, J., Wennstrom, M., & Tingstrom, A. (2008). Differential inhibition of neurogenesis and angiogenesis by corticosterone in rats stimulated with electroconvulsive seizures. *Prog Neuropsychopharmacol Biol Psychiatry, 32*(6), 1466-1472. doi: 10.1016/j.pnpbp.2008.05.012

Eng, L. F., Ghirnikar, R. S., & Lee, Y. L. (2000). Glial fibrillary acidic protein: GFAP-thirty-one years (1969-2000). *Neurochem Res, 25*(9-10), 1439-1451.

Engelhard, K., Winkelheide, U., Werner, C., Kluge, J., Eberspacher, E., Hollweck, R., . . . Kochs, E. (2007). Sevoflurane affects neurogenesis after forebrain ischemia in rats. *Anesth Analg, 104*(4), 898-903. doi: 10.1213/01.ane.0000255730.73018.31

Epp, J. R., Beasley, C. L., & Galea, L. A. (2013). Increased Hippocampal Neurogenesis and p21 Expression in Depression: Dependent on Antidepressants, Sex, Age, and Antipsychotic Exposure. *Neuropsychopharmacology*. doi: 10.1038/npp.2013.132

Ferretta, A., Gaballo, A., Tanzarella, P., Piccoli, C., Capitanio, N., Nico, B., . . . Cocco, T. (2014). Effect of resveratrol on mitochondrial function: Implications in parkin-associated familiar Parkinson's disease. *Biochim Biophys Acta*. doi: 10.1016/j.bbadis.2014.02.010

Folkman, J., & Shing, Y. (1992). Angiogenesis. *J Biol Chem, 267*(16), 10931-10934.

Fonarow, G. C., Zhao, X., Smith, E. E., Saver, J. L., Reeves, M. J., Bhatt, D. L., . . . Schwamm, L. H. (2014). Door-to-needle times for tissue plasminogen activator administration and clinical outcomes in acute ischemic stroke before and after a quality improvement initiative. *JAMA, 311*(16), 1632-1640. doi: 10.1001/jama.2014.3203

Fontainhas, A. M., Wang, M., Liang, K. J., Chen, S., Mettu, P., Damani, M., . . . Wong, W. T. (2011). Microglial morphology and dynamic behavior is regulated by ionotropic glutamatergic and GABAergic neurotransmission. *PLoS One, 6*(1), e15973. doi: 10.1371/journal.pone.0015973

Frasier, C. R., Brown, D. A., Sloan, R. C., Hayes, B., Stewart, L. M., Patel, H. D., . . . Rosenbaum, M. D. (2013). Stage of the estrous cycle does not influence myocardial ischemia-reperfusion injury in rats (Rattus norvegicus). *Comp Med, 63*(5), 416-421.

Fremont, L. (2000). Biological effects of resveratrol. *Life Sci, 66*(8), 663-673. doi: S0024320599004105 [pii]

Fu, Y., Wang, Y., Du, L., Xu, C., Cao, J., Fan, T., . . . Fan, F. (2013). Resveratrol inhibits ionising irradiation-induced inflammation in MSCs by activating SIRT1 and limiting NLRP-3 inflammasome activation. *Int J Mol Sci, 14*(7), 14105-14118. doi: 10.3390/ijms140714105

Fukuda, S., Kaga, S., Zhan, L., Bagchi, D., Das, D. K., Bertelli, A., & Maulik, N. (2006). Resveratrol ameliorates myocardial damage by inducing vascular endothelial growth factor-angiogenesis and tyrosine kinase receptor Flk-1. *Cell Biochem Biophys, 44*(1), 43-49. doi: CBB:44:1:043 [pii] 10.1385/CBB:44:1:043

Furlow, T. W., Jr. (1982). Cerebral ischemia produced by four-vessel occlusion in the rat: a quantitative evaluation of cerebral blood flow. *Stroke, 13*(6), 852-855.

Gage, F. H., Kempermann, G., Palmer, T. D., Peterson, D. A., & Ray, J. (1998). Multipotent progenitor cells in the adult dentate gyrus. *J Neurobiol, 36*(2), 249-266.

Gascon, E., Vutskits, L., & Kiss, J. Z. (2010). The role of PSA-NCAM in adult neurogenesis. *Adv Exp Med Biol, 663*, 127-136. doi: 10.1007/978-1-4419-1170-4_8

Gaskin, S., Tardif, M., Cole, E., Piterkin, P., Kayello, L., & Mumby, D. G. (2010). Object familiarization and novel-object preference in rats. *Behav Processes, 83*(1), 61-71. doi: S0376-6357(09)00229-0 [pii] 10.1016/j.beproc.2009.10.003

Gatson, J. W., Liu, M. M., Abdelfattah, K., Wigginton, J. G., Smith, S., Wolf, S., & Minei, J. P. (2013). Resveratrol decreases inflammation in the brain of mice with mild traumatic brain injury. *J Trauma Acute Care Surg, 74*(2), 470-474; discussion 474-475. doi: 10.1097/TA.0b013e31827e1f51

Ge, J. F., Peng, L., Cheng, J. Q., Pan, C. X., Tang, J., Chen, F. H., & Li, J. (2013). Antidepressant-like effect of resveratrol: involvement of antioxidant effect and peripheral regulation on HPA axis. *Pharmacol Biochem Behav, 114-115*, 64-69. doi: 10.1016/j.pbb.2013.10.028

Girbovan, C., Morin, L., & Plamondon, H. (2012). Repeated resveratrol administration confers lasting protection against neuronal damage but induces dose-related alterations of behavioral impairments after global ischemia. *Behav Pharmacol, 23*(1), 1-13. doi: 10.1097/FBP.0b013e32834eafa3

Gomez-Climent, M. A., Guirado, R., Castillo-Gomez, E., Varea, E., Gutierrez-Mecinas, M., Gilabert-Juan, J., . . . Nacher, J. (2011). The polysialylated form of the neural cell adhesion molecule (PSA-NCAM) is expressed in a subpopulation of mature cortical

interneurons characterized by reduced structural features and connectivity. *Cereb Cortex, 21*(5), 1028-1041. doi: 10.1093/cercor/bhq177

Gong, J., Gong, S., Zhang, M., Zhang, L., Hu, Y., Liu, Y., & Li, W. (2014). Cerebral ischemic preconditioning reduces glutamate excitotoxicity by up-regulating the uptake activity of GLT-1 in rats. *Amino Acids, 46*(6), 1537-1545. doi: 10.1007/s00726-014-1723-1

Gould, E., Cameron, H. A., Daniels, D. C., Woolley, C. S., & McEwen, B. S. (1992). Adrenal hormones suppress cell division in the adult rat dentate gyrus. *J Neurosci, 12*(9), 3642-3650.

Gould, E., McEwen, B. S., Tanapat, P., Galea, L. A., & Fuchs, E. (1997). Neurogenesis in the dentate gyrus of the adult tree shrew is regulated by psychosocial stress and NMDA receptor activation. *J Neurosci, 17*(7), 2492-2498.

Graeber, M. B., & Streit, W. J. (2010). Microglia: biology and pathology. *Acta Neuropathol, 119*(1), 89-105. doi: 10.1007/s00401-009-0622-0

Green, E. J., Pazos, A. J., Dietrich, W. D., McCabe, P. M., Schneiderman, N., Lin, B., . . . Ginsberg, M. D. (1995). Combined postischemic hypothermia and delayed MK-801 treatment attenuates neurobehavioral deficits associated with transient global ischemia in rats. *Brain Research, 702*(1-2), 145-152. doi: Doi: 10.1016/0006-8993(95)01034-1

Greenberg, M. R., Ahnert, A. M., Patel, N. C., Bennett, C. E., Elliott, N., Lundquist, M., . . . Burmeister, D. B. (2014). Sex differences in cardiac arrest survivors who receive therapeutic hypothermia. *Am J Emerg Med, 32*(6), 545-548. doi: 10.1016/j.ajem.2014.02.004

Gupta, R., Gupta, L. K., Mediratta, P. K., & Bhattacharya, S. K. (2012). Effect of resveratrol on scopolamine-induced cognitive impairment in mice. *Pharmacol Rep, 64*(2), 438-444.

Gupta, Y. K., Briyal, S., & Chaudhary, G. (2002). Protective effect of trans-resveratrol against kainic acid-induced seizures and oxidative stress in rats. *Pharmacol Biochem Behav, 71*(1-2), 245-249. doi: S0091305701006633 [pii]

Hagan, J. J., & Beaughard, M. (1990). The effects of forebrain ischaemia on spatial learning. *Behav Brain Res, 41*(2), 151-160.

Hale, S. L., Birnbaum, Y., & Kloner, R. A. (1997). Estradiol, Administered Acutely, Protects Ischemic Myocardium in Both Female and Male Rabbits. *J Cardiovasc Pharmacol Ther, 2*(1), 47-52. doi: 10.1054/jcpt.1997.0047

Hammes, H. P., Brownlee, M., Jonczyk, A., Sutter, A., & Preissner, K. T. (1996). Subcutaneous injection of a cyclic peptide antagonist of vitronectin receptor-type integrins inhibits retinal neovascularization. *Nat Med, 2*(5), 529-533.

Harada, N., Zhao, J., Kurihara, H., Nakagata, N., & Okajima, K. (2011). Resveratrol improves cognitive function in mice by increasing production of insulin-like growth factor-I in the hippocampus. *J Nutr Biochem, 22*(12), 1150-1159. doi: 10.1016/j.jnutbio.2010.09.016

Hartman, R. E., Lee, J. M., Zipfel, G. J., & Wozniak, D. F. (2005). Characterizing learning deficits and hippocampal neuron loss following transient global cerebral ischemia in rats. *Brain Res, 1043*(1-2), 48-56. doi: S0006-8993(05)00298-2 [pii] 10.1016/j.brainres.2005.02.030

Hasan, A. F., Al Suwaidi, J., Omer, A. A., Ghadban, W., Alkilani, H., Gehani, A., & Salam, A. M. (2014). The Influence of female Gender on Cardiac Arrest Outcomes: a Systematic Review of the literature. *Curr Med Res Opin*, 1-30. doi: 10.1185/03007995.2014.936552

Hayashi, T., Deguchi, K., Nagotani, S., Zhang, H., Sehara, Y., Tsuchiya, A., & Abe, K. (2006). Cerebral ischemia and angiogenesis. *Curr Neurovasc Res, 3*(2), 119-129.

Hayashi, T., Noshita, N., Sugawara, T., & Chan, P. H. (2003). Temporal profile of angiogenesis and expression of related genes in the brain after ischemia. *J Cereb Blood Flow Metab, 23*(2), 166-180.

Hayes, D. M., Knapp, D. J., Breese, G. R., & Thiele, T. E. (2005). Comparison of basal neuropeptide Y and corticotropin releasing factor levels between the high ethanol drinking C57BL/6J and low ethanol drinking DBA/2J inbred mouse strains. *Alcohol Clin Exp Res, 29*(5), 721-729. doi: 00000374-200505000-00004 [pii]

Hein, C., Horvath, E., & Kugler, P. (2001). Glutamate transporter expression in astrocytes of the rat dentate gyrus following lesion of the entorhinal cortex. *Eur J Neurosci, 13*(10), 1839-1848.

Hellstrom, N. A., Bjork-Eriksson, T., Blomgren, K., & Kuhn, H. G. (2009). Differential recovery of neural stem cells in the subventricular zone and dentate gyrus after ionizing radiation. *Stem Cells, 27*(3), 634-641. doi: 10.1634/stemcells.2008-0732

Hinwood, M., Tynan, R. J., Charnley, J. L., Beynon, S. B., Day, T. A., & Walker, F. R. (2013). Chronic stress induced remodeling of the prefrontal cortex: structural re-organization of microglia and the inhibitory effect of minocycline. *Cereb Cortex, 23*(8), 1784-1797. doi: 10.1093/cercor/bhs151

Hodges, H., Sowinski, P., Fleming, P., Kershaw, T. R., Sinden, J. D., Meldrum, B. S., & Gray, J. A. (1996). Contrasting effects of fetal CA1 and CA3 hippocampal grafts on deficits in spatial learning and working memory induced by global cerebral ischaemia in rats. *Neuroscience, 72*(4), 959-988. doi: 0306452296000048 [pii]

Hossmann, K. A. (2008). Cerebral ischemia: models, methods and outcomes. *Neuropharmacology, 55*(3), 257-270. doi: S0028-3908(07)00391-7 [pii] 10.1016/j.neuropharm.2007.12.004

Hu, X., Zheng, H., Yan, T., Pan, S., Fang, J., Jiang, R., & Ma, S. (2010). Physical exercise induces expression of CD31 and facilitates neural function recovery in rats with focal cerebral infarction. *Neurol Res, 32*(4), 397-402. doi: 10.1179/016164110x12670144526309

Hubbard, B. P., & Sinclair, D. A. (2014). Small molecule SIRT1 activators for the treatment of aging and age-related diseases. *Trends Pharmacol Sci, 35*(3), 146-154. doi: 10.1016/j.tips.2013.12.004

Huber, K., & Superti-Furga, G. (2011). After the grape rush: Sirtuins as epigenetic drug targets in neurodegenerative disorders. *Bioorg Med Chem*. doi: S0968-0896(11)00034-4 [pii] 10.1016/j.bmc.2011.01.018

Imai, F., Suzuki, H., Oda, J., Ninomiya, T., Ono, K., Sano, H., & Sawada, M. (2007). Neuroprotective effect of exogenous microglia in global brain ischemia. *J Cereb Blood Flow Metab, 27*(3), 488-500. doi: 10.1038/sj.jcbfm.9600362

Izidio, G. S., Lopes, D. M., Spricigo, L., Jr., & Ramos, A. (2005). Common variations in the pretest environment influence genotypic comparisons in models of anxiety. *Genes Brain Behav, 4*(7), 412-419. doi: GBB121 [pii] 10.1111/j.1601-183X.2005.00121.x

Jiang, W., Gu, W., Brannstrom, T., Rosqvist, R., & Wester, P. (2001). Cortical neurogenesis in adult rats after transient middle cerebral artery occlusion. *Stroke, 32*(5), 1201-1207.

Jin, K., Zhu, Y., Sun, Y., Mao, X. O., Xie, L., & Greenberg, D. A. (2002). Vascular endothelial growth factor (VEGF) stimulates neurogenesis in vitro and in vivo. *Proc Natl Acad Sci U S A, 99*(18), 11946-11950. doi: 10.1073/pnas.182296499

Jin, K. L., Mao, X. O., Nagayama, T., Goldsmith, P. C., & Greenberg, D. A. (2000). Induction of vascular endothelial growth factor and hypoxia-inducible factor-1alpha by global ischemia in rat brain. *Neuroscience, 99*(3), 577-585.

Jing, L., He, Q., Zhang, J. Z., & Li, P. A. (2013). Temporal profile of astrocytes and changes of oligodendrocyte-based myelin following middle cerebral artery occlusion in diabetic and non-diabetic rats. *Int J Biol Sci, 9*(2), 190-199. doi: 10.7150/ijbs.5844

Kaplan, M. S., & Hinds, J. W. (1977). Neurogenesis in the adult rat: electron microscopic analysis of light radioautographs. *Science, 197*(4308), 1092-1094.

Kee, N. J., Preston, E., & Wojtowicz, J. M. (2001). Enhanced neurogenesis after transient global ischemia in the dentate gyrus of the rat. *Exp Brain Res, 136*(3), 313-320.

Kempermann, G., Kuhn, H. G., & Gage, F. H. (1997). More hippocampal neurons in adult mice living in an enriched environment. *Nature, 386*(6624), 493-495. doi: 10.1038/386493a0

Kempermann, G., & Neumann, H. (2003). Neuroscience. Microglia: the enemy within? *Science, 302*(5651), 1689-1690. doi: 10.1126/science.1092864

Kennedy, D. O., Wightman, E. L., Reay, J. L., Lietz, G., Okello, E. J., Wilde, A., & Haskell, C. F. (2010). Effects of resveratrol on cerebral blood flow variables and cognitive performance in humans: a double-blind, placebo-controlled, crossover investigation. *Am J Clin Nutr, 91*(6), 1590-1597. doi: ajcn.2009.28641 [pii] 10.3945/ajcn.2009.28641

Kernie, S. G., & Parent, J. M. (2010). Forebrain neurogenesis after focal Ischemic and traumatic brain injury. *Neurobiol Dis, 37*(2), 267-274. doi: S0969-9961(09)00313-1 [pii] 10.1016/j.nbd.2009.11.002

Ketheeswaranathan, P., Turner, N. A., Spary, E. J., Batten, T. F., McColl, B. W., & Saha, S. (2011). Changes in glutamate transporter expression in mouse forebrain areas following focal ischemia. *Brain Res, 1418*, 93-103. doi: 10.1016/j.brainres.2011.08.029

Kettenmann, H., Hanisch, U. K., Noda, M., & Verkhratsky, A. (2011). Physiology of microglia. *Physiol Rev, 91*(2), 461-553. doi: 10.1152/physrev.00011.2010

Khorram, O., Ghazi, R., Chuang, T. D., Han, G., Naghi, J., Ni, Y., & Pearce, W. J. (2014). Excess maternal glucocorticoids in response to in utero undernutrition inhibit offspring angiogenesis. *Reprod Sci, 21*(5), 601-611. doi: 10.1177/1933719113508819

Kim, S. U., & de Vellis, J. (2005). Microglia in health and disease. *J Neurosci Res, 81*(3), 302-313. doi: 10.1002/jnr.20562

Kirino, T. (1982). Delayed neuronal death in the gerbil hippocampus following ischemia. *Brain Res, 239*(1), 57-69.

Kirino, T., Tsujita, Y., & Tamura, A. (1991). Induced tolerance to ischemia in gerbil hippocampal neurons. *J Cereb Blood Flow Metab, 11*(2), 18.

Kiyota, Y., Miyamoto, M., & Nagaoka, A. (1991). Relationship between brain damage and memory impairment in rats exposed to transient forebrain ischemia. *Brain Res, 538*(2), 295-302. doi: 0006-8993(91)90443-Y [pii]

Kofler, J., Hattori, K., Sawada, M., DeVries, A. C., Martin, L. J., Hurn, P. D., & Traystman, R. J. (2004). Histopathological and behavioral characterization of a novel model of cardiac arrest and cardiopulmonary resuscitation in mice. *J Neurosci Methods, 136*(1), 33-44. doi: 10.1016/j.jneumeth.2003.12.024

Kozlowski, C., & Weimer, R. M. (2012). An automated method to quantify microglia morphology and application to monitor activation state longitudinally in vivo. *PLoS One, 7*(2), e31814. doi: 10.1371/journal.pone.0031814

Krupinski, J., Kaluza, J., Kumar, P., Kumar, S., & Wang, J. M. (1994). Role of angiogenesis in patients with cerebral ischemic stroke. *Stroke, 25*(9), 1794-1798.

Kuge, A., Takemura, S., Kokubo, Y., Sato, S., Goto, K., & Kayama, T. (2009). Temporal profile of neurogenesis in the subventricular zone, dentate gyrus and cerebral cortex following transient focal cerebral ischemia. *Neurol Res, 31*(9), 969-976. doi: ner1753 [pii] 10.1179/174313209X383312

Kuhn, H. G., & Cooper-Kuhn, C. M. (2007). Bromodeoxyuridine and the detection of neurogenesis. *Curr Pharm Biotechnol, 8*(3), 127-131.

Kumar, A., Negi, G., & Sharma, S. S. (2013). Neuroprotection by resveratrol in diabetic neuropathy: concepts & mechanisms. *Curr Med Chem, 20*(36), 4640-4645.

la Porte, C., Voduc, N., Zhang, G., Seguin, I., Tardiff, D., Singhal, N., & Cameron, D. W. (2010). Steady-State pharmacokinetics and tolerability of trans-resveratrol 2000 mg twice daily with food, quercetin and alcohol (ethanol) in healthy human subjects. *Clin Pharmacokinet, 49*(7), 449-454. doi: 10.2165/11531820-000000000-00000

Lagace, D. C. (2012). Does the endogenous neurogenic response alter behavioral recovery following stroke? *Behav Brain Res, 227*(2), 426-432. doi: 10.1016/j.bbr.2011.08.045

Lagranha, C. J., Deschamps, A., Aponte, A., Steenbergen, C., & Murphy, E. (2010). Sex differences in the phosphorylation of mitochondrial proteins result in reduced production of reactive oxygen species and cardioprotection in females. *Circ Res, 106*(11), 1681-1691. doi: 10.1161/circresaha.109.213645

Langdon, K. D., Granter-Button, S., & Corbett, D. (2008). Persistent behavioral impairments and neuroinflammation following global ischemia in the rat. *Eur J Neurosci, 28*(11), 2310-2318. doi: EJN6513 [pii] 10.1111/j.1460-9568.2008.06513.x

Lasarzik, I., Winkelheide, U., Stallmann, S., Orth, C., Schneider, A., Tresch, A., . . . Engelhard, K. (2009). Assessment of postischemic neurogenesis in rats with cerebral ischemia and propofol anesthesia. *Anesthesiology, 110*(3), 529-537. doi: 10.1097/ALN.0b013e318195b4fe

Lee, C. H., Moon, S. M., Yoo, K. Y., Choi, J. H., Park, O. K., Hwang, I. K., . . . Won, M. H. (2010). Long-term changes in neuronal degeneration and microglial activation in the hippocampal CA1 region after experimental transient cerebral ischemic damage. *Brain Res, 1342*, 138-149. doi: S0006-8993(10)00943-1 [pii] 10.1016/j.brainres.2010.04.046

Li, Y., Liu, J., Liu, X., Xing, K., Wang, Y., Li, F., & Yao, L. (2006). Resveratrol-induced cell inhibition of growth and apoptosis in MCF7 human breast cancer cells are associated with modulation of phosphorylated Akt and caspase-9. *Appl Biochem Biotechnol, 135*(3), 181-192.

Lichtenwalner, R. J., & Parent, J. M. (2006). Adult neurogenesis and the ischemic forebrain. *J Cereb Blood Flow Metab, 26*(1), 1-20. doi: 9600170 [pii] 10.1038/sj.jcbfm.9600170

Licko, T., Seeger, N., Zellinger, C., Russmann, V., Matagne, A., & Potschka, H. (2013). Lacosamide treatment following status epilepticus attenuates neuronal cell loss and alterations in hippocampal neurogenesis in a rat electrical status epilepticus model. *Epilepsia, 54*(7), 1176-1185. doi: 10.1111/epi.12196

Liedtke, W., Edelmann, W., Bieri, P. L., Chiu, F. C., Cowan, N. J., Kucherlapati, R., & Raine, C. S. (1996). GFAP is necessary for the integrity of CNS white matter architecture and long-term maintenance of myelination. *Neuron, 17*(4), 607-615.

Liu, A. J., Hu, Y. Y., Li, W. B., Xu, J., & Zhang, M. (2011). Cerebral ischemic pre-conditioning enhances the binding characteristics and glutamate uptake of glial glutamate transporter-1 in hippocampal CA1 subfield of rats. *J Neurochem, 119*(1), 202-209. doi: 10.1111/j.1471-4159.2011.07396.x

Liu, D., Zhang, Q., Gu, J., Wang, X., Xie, K., Xian, X., . . . Wang, Z. (2014). Resveratrol prevents impaired cognition induced by chronic unpredictable mild stress in rats. *Prog Neuropsychopharmacol Biol Psychiatry, 49*, 21-29. doi: 10.1016/j.pnpbp.2013.10.017

Liu, J., Solway, K., Messing, R. O., & Sharp, F. R. (1998). Increased neurogenesis in the dentate gyrus after transient global ischemia in gerbils. *J Neurosci, 18*(19), 7768-7778.

Lofrumento, D. D., Nicolardi, G., Cianciulli, A., De Nuccio, F., La Pesa, V., Carofiglio, V., . . . Panaro, M. A. (2014). Neuroprotective effects of resveratrol in an MPTP mouse model of Parkinson's-like disease: possible role of SOCS-1 in reducing pro-inflammatory responses. *Innate Immun, 20*(3), 249-260. doi: 10.1177/1753425913488429

Louissaint, A., Jr., Rao, S., Leventhal, C., & Goldman, S. A. (2002). Coordinated interaction of neurogenesis and angiogenesis in the adult songbird brain. *Neuron, 34*(6), 945-960.

Lu, K. T., Chiou, R. Y., Chen, L. G., Chen, M. H., Tseng, W. T., Hsieh, H. T., & Yang, Y. L. (2006). Neuroprotective effects of resveratrol on cerebral ischemia-induced neuron loss mediated by free radical scavenging and cerebral blood flow elevation. *J Agric Food Chem, 54*(8), 3126-3131. doi: 10.1021/jf053011q

Lu, X., Ma, L., Ruan, L., Kong, Y., Mou, H., Zhang, Z., . . . Le, Y. (2010). Resveratrol differentially modulates inflammatory responses of microglia and astrocytes. *J Neuroinflammation, 7*, 46. doi: 10.1186/1742-2094-7-46

Lundgren-Nilsson, A., Rosen, H., Hofgren, C., & Sunnerhagen, K. S. (2005). The first year after successful cardiac resuscitation: function, activity, participation and quality of life. *Resuscitation, 66*(3), 285-289. doi: 10.1016/j.resuscitation.2005.04.001

Lyeth, B. G., Jenkins, L. W., Hamm, R. J., Dixon, C. E., Phillips, L. L., Clifton, G. L., . . . Hayes, R. L. (1990). Prolonged memory impairment in the absence of hippocampal cell death following traumatic brain injury in the rat. *Brain Res, 526*(2), 249-258. doi: 0006-8993(90)91229-A [pii]

Ma, B., Li, M., Nong, H., Shi, J., Liu, G., & Zhang, J. (2008). Protective effects of extract of Coeloglossum viride var. bracteatum on ischemia-induced neuronal death and cognitive impairment in rats. *Behav Pharmacol, 19*(4), 325-333. doi: 10.1097/FBP.0b013e3282feb0ac 00008877-200807000-00007 [pii]

Madhyastha, S., Sekhar, S., & Rao, G. (2013). Resveratrol improves postnatal hippocampal neurogenesis and brain derived neurotrophic factor in prenatally stressed rats. *Int J Dev Neurosci*. doi: 10.1016/j.ijdevneu.2013.06.010

Madinier, A., Bertrand, N., Mossiat, C., Prigent-Tessier, A., Beley, A., Marie, C., & Garnier, P. (2009). Microglial involvement in neuroplastic changes following focal brain ischemia in rats. *PLoS One, 4*(12), e8101. doi: 10.1371/journal.pone.0008101

Madl, C., & Holzer, M. (2004). Brain function after resuscitation from cardiac arrest. *Curr Opin Crit Care, 10*(3), 213-217.

Manna, S. K., Mukhopadhyay, A., & Aggarwal, B. B. (2000). Resveratrol suppresses TNF-induced activation of nuclear transcription factors NF-kappa B, activator protein-1, and apoptosis: potential role of reactive oxygen intermediates and lipid peroxidation. *J Immunol, 164*(12), 6509-6519. doi: ji_v164n12p6509 [pii]

Mao, X., Ji, C., Sun, C., Cao, D., Ma, P., Ji, Z., . . . Cao, Y. (2012). Topiramate attenuates cerebral ischemia/reperfusion injury in gerbils via activating GABAergic signaling and inhibiting astrogliosis. *Neurochem Int, 60*(1), 39-46. doi: 10.1016/j.neuint.2011.10.015

Maragakis, N. J., & Rothstein, J. D. (2004). Glutamate transporters: animal models to neurologic disease. *Neurobiol Dis, 15*(3), 461-473. doi: 10.1016/j.nbd.2003.12.007

Markakis, E. A., & Gage, F. H. (1999). Adult-generated neurons in the dentate gyrus send axonal projections to field CA3 and are surrounded by synaptic vesicles. *J Comp Neurol, 406*(4), 449-460.

Mateen, F. J., Josephs, K. A., Trenerry, M. R., Felmlee-Devine, M. D., Weaver, A. L., Carone, M., & White, R. D. (2011). Long-term cognitive outcomes following out-of-hospital cardiac arrest: a population-based study. *Neurology, 77*(15), 1438-1445. doi: 10.1212/WNL.0b013e318232ab33

Mayer, J. L., Klumpers, L., Maslam, S., de Kloet, E. R., Joels, M., & Lucassen, P. J. (2006). Brief treatment with the glucocorticoid receptor antagonist mifepristone normalises the corticosterone-induced reduction of adult hippocampal neurogenesis. *J Neuroendocrinol, 18*(8), 629-631. doi: 10.1111/j.1365-2826.2006.01455.x

Meng, X. J., Wang, F., & Li, C. K. (2014). Resveratrol is Neuroprotective and Improves Cognition in Pentylenetetrazole-kindling Model of Epilepsy in Rats. *Indian J Pharm Sci, 76*(2), 125-131.

Miatello, R., Vazquez, M., Renna, N., Cruzado, M., Zumino, A. P., & Risler, N. (2005). Chronic administration of resveratrol prevents biochemical cardiovascular changes in fructose-fed rats. *Am J Hypertens, 18*(6), 864-870. doi: S0895-7061(05)00013-0 [pii] 10.1016/j.amjhyper.2004.12.012

Middeldorp, J., & Hol, E. M. (2011). GFAP in health and disease. *Prog Neurobiol, 93*(3), 421-443. doi: 10.1016/j.pneurobio.2011.01.005

Middelkamp, W., Moulaert, V. R., Verbunt, J. A., van Heugten, C. M., Bakx, W. G., & Wade, D. T. (2007). Life after survival: long-term daily life functioning and quality of life of patients with hypoxic brain injury as a result of a cardiac arrest. *Clin Rehabil, 21*(5), 425-431. doi: 10.1177/0269215507075307

Milot, M., & Plamondon, H. (2008). Ischemia-induced hyperactivity: effects of dim versus bright illumination on open-field exploration and habituation following global ischemia in rats. *Behav Brain Res, 192*(2), 166-172. doi: S0166-4328(08)00185-X [pii] 10.1016/j.bbr.2008.03.044

Milot, M. R., James, J. S., Merali, Z., & Plamondon, H. (2012). A refined blood collection method for quantifying corticosterone. *Lab Anim (NY), 41*(3), 77-83. doi: 10.1038/laban0312-77

Milot, M. R., & Plamondon, H. (2009). Time-dependent effects of global cerebral ischemia on anxiety, locomotion, and habituation in rats. *Behav Brain Res, 200*(1), 173-180.

Milot, M. R., & Plamondon, H. (2011). Changes in HPA reactivity and noradrenergic functions regulate spatial memory impairments at delayed time intervals following cerebral ischemia. *Hormones and Behavior, 59*(4), 594-604. doi: 10.1016/j.yhbeh.2011.02.017

Moon, J. B., Lee, C. H., Park, C. W., Cho, J. H., Hwang, I. K., Yoo, K. Y., . . . Won, M. H. (2009). Neuronal degeneration and microglial activation in the ischemic dentate gyrus of the gerbil. *J Vet Med Sci, 71*(10), 1381-1386. doi: JST.JSTAGE/jvms/001381 [pii]

Moriya, J., Chen, R., Yamakawa, J., Sasaki, K., Ishigaki, Y., & Takahashi, T. (2011). Resveratrol improves hippocampal atrophy in chronic fatigue mice by enhancing neurogenesis and inhibiting apoptosis of granular cells. *Biol Pharm Bull, 34*(3), 354-359.

Moulaert, V. R., Verbunt, J. A., van Heugten, C. M., & Wade, D. T. (2009). Cognitive impairments in survivors of out-of-hospital cardiac arrest: a systematic review. *Resuscitation, 80*(3), 297-305. doi: 10.1016/j.resuscitation.2008.10.034

Mukherjee, S., Dudley, J. I., & Das, D. K. (2010). Dose-dependency of resveratrol in providing health benefits. *Dose Response, 8*(4), 478-500. doi: 10.2203/dose-response.09-015.Mukherjee

Mumby, D. G. (2001). Perspectives on object-recognition memory following hippocampal damage: lessons from studies in rats. *Behav Brain Res, 127*(1-2), 159-181. doi: S0166432801003679 [pii]

Nacher, J., Alonso-Llosa, G., Rosell, D., & McEwen, B. (2002). PSA-NCAM expression in the piriform cortex of the adult rat. Modulation by NMDA receptor antagonist administration. *Brain Res, 927*(2), 111-121.

Nacher, J., Crespo, C., & McEwen, B. S. (2001). Doublecortin expression in the adult rat telencephalon. *Eur J Neurosci, 14*(4), 629-644.

Nakajima, K., Yamamoto, S., Kohsaka, S., & Kurihara, T. (2008). Neuronal stimulation leading to upregulation of glutamate transporter-1 (GLT-1) in rat microglia in vitro. *Neurosci Lett, 436*(3), 331-334. doi: 10.1016/j.neulet.2008.03.058

Nakata, R., Takahashi, S., & Inoue, H. (2012). Recent advances in the study on resveratrol. *Biol Pharm Bull, 35*(3), 273-279.

Nakatomi, H., Kuriu, T., Okabe, S., Yamamoto, S., Hatano, O., Kawahara, N., . . . Nakafuku, M. (2002). Regeneration of hippocampal pyramidal neurons after ischemic brain injury by recruitment of endogenous neural progenitors. *Cell, 110*(4), 429-441. doi: S0092867402008620 [pii]

Nanri, M., & Watanabe, H. (1999). [Availability of 2VO rats as a model for chronic cerebrovascular disease]. *Nihon Yakurigaku Zasshi, 113*(2), 85-95.

Naylor, A. S., Bull, C., Nilsson, M. K., Zhu, C., Bjork-Eriksson, T., Eriksson, P. S., . . . Kuhn, H. G. (2008). Voluntary running rescues adult hippocampal neurogenesis after irradiation of the young mouse brain. *Proc Natl Acad Sci U S A, 105*(38), 14632-14637. doi: 10.1073/pnas.0711128105

Nedergaard, M., & Dirnagl, U. (2005). Role of glial cells in cerebral ischemia. *Glia, 50*(4), 281-286. doi: 10.1002/glia.20205

Neigh, G. N., Karelina, K., Zhang, N., Glasper, E. R., Owens, M. J., Plotsky, P. M., . . . Devries, A. C. (2009). Cardiac arrest and cardiopulmonary resuscitation dysregulates

the hypothalamic-pituitary-adrenal axis. *J Cereb Blood Flow Metab, 29*(10), 1673-1682. doi: 10.1038/jcbfm.2009.85

Nelson, A., Lebessi, A., Sowinski, P., & Hodges, H. (1997). Comparison of effects of global cerebral ischaemia on spatial learning in the standard and radial water maze: relationship of hippocampal damage to performance. *Behav Brain Res, 85*(1), 93-115. doi: S0166432896001672 [pii]

Neumann, J., Gunzer, M., Gutzeit, H. O., Ullrich, O., Reymann, K. G., & Dinkel, K. (2006). Microglia provide neuroprotection after ischemia. *FASEB J, 20*(6), 714-716. doi: 10.1096/fj.05-4882fje

Neumann, J. T., Cohan, C. H., Dave, K. R., Wright, C. B., & Perez-Pinzon, M. A. (2013). Global cerebral ischemia: synaptic and cognitive dysfunction. *Curr Drug Targets, 14*(1), 20-35.

Nih, L. R., Deroide, N., Lere-Dean, C., Lerouet, D., Soustrat, M., Levy, B. I., . . . Kubis, N. (2012). Neuroblast survival depends on mature vascular network formation after mouse stroke: role of endothelial and smooth muscle progenitor cell co-administration. *Eur J Neurosci, 35*(8), 1208-1217. doi: 10.1111/j.1460-9568.2012.08041.x

Nikonenko, A. G., Radenovic, L., Andjus, P. R., & Skibo, G. G. (2009). Structural features of ischemic damage in the hippocampus. *Anat Rec (Hoboken), 292*(12), 1914-1921. doi: 10.1002/ar.20969

Nilsson, M., Perfilieva, E., Johansson, U., Orwar, O., & Eriksson, P. S. (1999). Enriched environment increases neurogenesis in the adult rat dentate gyrus and improves spatial memory. *J Neurobiol, 39*(4), 569-578. doi: 10.1002/(SICI)1097-4695(19990615)39:4<569::AID-NEU10>3.0.CO;2-F [pii]

Nitatori, T., Sato, N., Waguri, S., Karasawa, Y., Araki, H., Shibanai, K., . . . Uchiyama, Y. (1995). Delayed neuronal death in the CA1 pyramidal cell layer of the gerbil hippocampus following transient ischemia is apoptosis. *J Neurosci, 15*(2), 1001-1011.

Nunn, J. A., LePeillet, E., Netto, C. A., Hodges, H., Gray, J. A., & Meldrum, B. S. (1994). Global ischaemia: hippocampal pathology and spatial deficits in the water maze. *Behav Brain Res, 62*(1), 41-54.

O'Reilly, S. M., Grubb, N. R., & O'Carroll, R. E. (2003). In-hospital cardiac arrest leads to chronic memory impairment. *Resuscitation, 58*(1), 73-79.

Olsen, G. M., Scheel-Kruger, J., Moller, A., & Jensen, L. H. (1994). Relation of spatial learning of rats in the Morris water maze task to the number of viable CA1 neurons following four-vessel occlusion. *Behav Neurosci, 108*(4), 681-690.

Ouyang, Y. B., Voloboueva, L. A., Xu, L. J., & Giffard, R. G. (2007). Selective dysfunction of hippocampal CA1 astrocytes contributes to delayed neuronal damage after transient forebrain ischemia. *J Neurosci, 27*(16), 4253-4260. doi: 27/16/4253 [pii] 10.1523/JNEUROSCI.0211-07.2007

Palmer, T. D., Willhoite, A. R., & Gage, F. H. (2000). Vascular niche for adult hippocampal neurogenesis. *J Comp Neurol, 425*(4), 479-494. doi: 10.1002/1096-9861(20001002)425:4<479::AID-CNE2>3.0.CO;2-3 [pii]

Panickar, K. S., & Norenberg, M. D. (2005). Astrocytes in cerebral ischemic injury: morphological and general considerations. *Glia, 50*(4), 287-298. doi: 10.1002/glia.20181

Park, H. R., Kong, K. H., Yu, B. P., Mattson, M. P., & Lee, J. (2012). Resveratrol inhibits the proliferation of neural progenitor cells and hippocampal neurogenesis. *J Biol Chem, 287*(51), 42588-42600. doi: 10.1074/jbc.M112.406413

Park, O. K., Lee, C. H., Hwang, I. K., Yoo, K. Y., Choi, J. H., & Won, M. H. (2010). Effects of repeated restraint stress on platelet endothelial cell adhesion molecule-1 immunoreactivity and protein levels in the gerbil hippocampus after transient cerebral ischemia. *Anat Cell Biol, 43*(1), 54-63. doi: 10.5115/acb.2010.43.1.54

Paxinos, G., & Watson, C. (1986). *The rat brain in stereotaxic coordinates.* San Diego: Academic Press.

Peghini, P., Janzen, J., & Stoffel, W. (1997). Glutamate transporter EAAC-1-deficient mice develop dicarboxylic aminoaciduria and behavioral abnormalities but no neurodegeneration. *EMBO J, 16*(13), 3822-3832. doi: 10.1093/emboj/16.13.3822

Penumathsa, S. V., Thirunavukkarasu, M., Koneru, S., Juhasz, B., Zhan, L., Pant, R., . . . Maulik, N. (2007). Statin and resveratrol in combination induces cardioprotection against myocardial infarction in hypercholesterolemic rat. *J Mol Cell Cardiol, 42*(3), 508-516. doi: 10.1016/j.yjmcc.2006.10.018

Persson, M., & Ronnback, L. (2012). Microglial self-defence mediated through GLT-1 and glutathione. *Amino Acids, 42*(1), 207-219. doi: 10.1007/s00726-011-0865-7

Petersen, M. A., & Dailey, M. E. (2004). Diverse microglial motility behaviors during clearance of dead cells in hippocampal slices. *Glia, 46*(2), 195-206. doi: 10.1002/glia.10362

Petito, C. K., & Halaby, I. A. (1993). Relationship between ischemia and ischemic neuronal necrosis to astrocyte expression of glial fibrillary acidic protein. *Int J Dev Neurosci, 11*(2), 239-247.

Petito, C. K., Olarte, J. P., Roberts, B., Nowak, T. S., Jr., & Pulsinelli, W. A. (1998). Selective glial vulnerability following transient global ischemia in rat brain. *J Neuropathol Exp Neurol, 57*(3), 231-238.

Petrovski, G., Gurusamy, N., & Das, D. K. (2011). Resveratrol in cardiovascular health and disease. *Ann N Y Acad Sci, 1215*, 22-33. doi: 10.1111/j.1749-6632.2010.05843.x

Plamondon, H., Blondeau, N., Heurteaux, C., & Lazdunski, M. (1999). Mutually protective actions of kainic acid epileptic preconditioning and sublethal global ischemia on hippocampal neuronal death: involvement of adenosine A1 receptors and K(ATP) channels. *J Cereb Blood Flow Metab, 19*(12), 1296-1308. doi: 10.1097/00004647-199912000-00002

Plamondon, H., & Khan, S. (2005). Characterization of anxiety and habituation profile following global ischemia in rats. *Physiology & Behavior, 84*(4), 543-552. doi: DOI: 10.1016/j.physbeh.2005.02.001

Plamondon, H., Morin, A., & Charron, C. (2006). Chronic 17beta-estradiol pretreatment and ischemia-induced hippocampal degeneration and memory impairments: a 6-month survival study. *Horm Behav, 50*(3), 361-369. doi: S0018-506X(06)00096-1 [pii] 10.1016/j.yhbeh.2006.04.006

Plamondon, H., & Roberge, M. C. (2008). Dietary PUFA supplements reduce memory deficits but not CA1 ischemic injury in rats. *Physiol Behav, 95*(3), 492-500. doi: S0031-9384(08)00229-1 [pii] 10.1016/j.physbeh.2008.07.020

Poignet, H., Beaughard, M., Lecoin, G., & Massingham, R. (1989). Functional, behavioral, and histological changes induced by transient global cerebral ischemia in rats: effects of cinnarizine and flunarizine. *J Cereb Blood Flow Metab, 9*(5), 646-654.

Popp, E., Vogel, P., Teschendorf, P., & Bottiger, B. W. (2007). Effects of the application of erythropoietin on cerebral recovery after cardiac arrest in rats. *Resuscitation, 74*(2), 344-351. doi: 10.1016/j.resuscitation.2007.01.019

Porquet, D., Grinan-Ferre, C., Ferrer, I., Camins, A., Sanfeliu, C., Del Valle, J., & Pallas, M. (2014). Neuroprotective Role of Trans-Resveratrol in a Murine Model of Familial Alzheimer's Disease. *J Alzheimers Dis*. doi: 10.3233/jad-140444

Potente, M., Ghaeni, L., Baldessari, D., Mostoslavsky, R., Rossig, L., Dequiedt, F., . . . Dimmeler, S. (2007). SIRT1 controls endothelial angiogenic functions during vascular growth. *Genes Dev, 21*(20), 2644-2658. doi: 10.1101/gad.435107

Pulsinelli, W. A., & Brierley, J. B. (1979). A new model of bilateral hemispheric ischemia in the unanesthetized rat. *Stroke, 10*(3), 267-272.

Pulsinelli, W. A., Brierley, J. B., & Plum, F. (1982). Temporal profile of neuronal damage in a model of transient forebrain ischemia. *Ann Neurol, 11*(5), 491-498. doi: 10.1002/ana.410110509

Quincozes-Santos, A., & Gottfried, C. (2011). Resveratrol modulates astroglial functions: neuroprotective hypothesis. *Ann N Y Acad Sci, 1215*, 72-78. doi: 10.1111/j.1749-6632.2010.05857.x

Raber, J., Fan, Y., Matsumori, Y., Liu, Z., Weinstein, P. R., Fike, J. R., & Liu, J. (2004). Irradiation attenuates neurogenesis and exacerbates ischemia-induced deficits. *Ann Neurol, 55*(3), 381-389. doi: 10.1002/ana.10853

Rao, V. L., Bowen, K. K., & Dempsey, R. J. (2001). Transient focal cerebral ischemia down-regulates glutamate transporters GLT-1 and EAAC1 expression in rat brain. *Neurochem Res, 26*(5), 497-502.

Rao, V. L., Dogan, A., Todd, K. G., Bowen, K. K., Kim, B. T., Rothstein, J. D., & Dempsey, R. J. (2001). Antisense knockdown of the glial glutamate transporter GLT-1, but not the neuronal glutamate transporter EAAC1, exacerbates transient focal cerebral ischemia-induced neuronal damage in rat brain. *J Neurosci, 21*(6), 1876-1883.

Raus, S., Selakovic, V., Manojlovic-Stojanoski, M., Radenovic, L., Prolic, Z., & Janac, B. (2013). Response of hippocampal neurons and glial cells to alternating magnetic field in gerbils submitted to global cerebral ischemia. *Neurotox Res, 23*(1), 79-91. doi: 10.1007/s12640-012-9333-8

Raval, A. P., Dave, K. R., & Perez-Pinzon, M. A. (2006). Resveratrol mimics ischemic preconditioning in the brain. *J Cereb Blood Flow Metab, 26*(9), 1141-1147. doi: 9600262 [pii] 10.1038/sj.jcbfm.9600262

Ray, P. S., Maulik, G., Cordis, G. A., Bertelli, A. A., Bertelli, A., & Das, D. K. (1999). The red wine antioxidant resveratrol protects isolated rat hearts from ischemia reperfusion injury. *Free Radic Biol Med, 27*(1-2), 160-169. doi: S0891-5849(99)00063-5 [pii]

Reynolds, J. C., & Lawner, B. J. (2012). Management of the post-cardiac arrest syndrome. *J Emerg Med, 42*(4), 440-449. doi: 10.1016/j.jemermed.2011.09.026

Roberge, M. C., Hotte-Bernard, J., Messier, C., & Plamondon, H. (2008). Food restriction attenuates ischemia-induced spatial learning and memory deficits despite extensive CA1 ischemic injury. *Behav Brain Res, 187*(1), 123-132. doi: S0166-4328(07)00464-0 [pii] 10.1016/j.bbr.2007.09.002

Roberge, M. C., Messier, C., Staines, W. A., & Plamondon, H. (2008). Food restriction induces long-lasting recovery of spatial memory deficits following global ischemia in delayed matching and non-matching-to-sample radial arm maze tasks. *Neuroscience, 156*(1), 11-29. doi: S0306-4522(08)01082-8 [pii] 10.1016/j.neuroscience.2008.05.062

Roberts, B. W., Kilgannon, J. H., Chansky, M. E., Mittal, N., Wooden, J., Parrillo, J. E., & Trzeciak, S. (2013). Multiple organ dysfunction after return of spontaneous circulation in postcardiac arrest syndrome. *Crit Care Med, 41*(6), 1492-1501. doi: 10.1097/CCM.0b013e31828a39e9

Robich, M. P., Osipov, R. M., Nezafat, R., Feng, J., Clements, R. T., Bianchi, C., . . . Sellke, F. W. (2010). Resveratrol improves myocardial perfusion in a swine model of hypercholesterolemia and chronic myocardial ischemia. *Circulation, 122*(11 Suppl), S142-149. doi: 10.1161/circulationaha.109.920132

Rodrigo, R., Fernandez-Gajardo, R., Gutierrez, R., Matamala, J. M., Carrasco, R., Miranda-Merchak, A., & Feuerhake, W. (2013). Oxidative stress and pathophysiology of ischemic stroke: novel therapeutic opportunities. *CNS Neurol Disord Drug Targets, 12*(5), 698-714.

Rothstein, J. D., Dykes-Hoberg, M., Pardo, C. A., Bristol, L. A., Jin, L., Kuncl, R. W., . . . Welty, D. F. (1996). Knockout of glutamate transporters reveals a major role for astroglial transport in excitotoxicity and clearance of glutamate. *Neuron, 16*(3), 675-686.

Saleh, M. C., Connell, B. J., Rajagopal, D., Khan, B. V., Abd-El-Aziz, A. S., Kucukkaya, I., & Saleh, T. M. (2014). Co-administration of resveratrol and lipoic acid, or their synthetic combination, enhances neuroprotection in a rat model of ischemia/reperfusion. *PLoS One, 9*(1), e87865. doi: 10.1371/journal.pone.0087865

Saleh, M. C., Connell, B. J., & Saleh, T. M. (2013). Resveratrol induced neuroprotection is mediated via both estrogen receptor subtypes, ER(alpha) and ER(beta). *Neurosci Lett, 548*, 217-221. doi: 10.1016/j.neulet.2013.05.057

Sandroni, C., Nolan, J., Cavallaro, F., & Antonelli, M. (2007). In-hospital cardiac arrest: incidence, prognosis and possible measures to improve survival. *Intensive Care Med, 33*(2), 237-245. doi: 10.1007/s00134-006-0326-z

Sandstrom, N. J., & Rowan, M. H. (2007). Acute pretreatment with estradiol protects against CA1 cell loss and spatial learning impairments resulting from transient global ischemia. *Horm Behav, 51*(3), 335-345. doi: S0018-506X(06)00335-7 [pii] 10.1016/j.yhbeh.2006.12.002

Saper, C. B. (2005). An open letter to our readers on the use of antibodies. *J Comp Neurol, 493*(4), 477-478. doi: 10.1002/cne.20839

Sapolsky, R. M., & Pulsinelli, W. A. (1985). Glucocorticoids potentiate ischemic injury to neurons: therapeutic implications. *Science, 229*(4720), 1397-1400.

Sawada, M., Matsumoto, M., & Sawamoto, K. (2014). Vascular regulation of adult neurogenesis under physiological and pathological conditions. *Front Neurosci, 8*, 53. doi: 10.3389/fnins.2014.00053

Schaapsmeerders, P., Maaijwee, N. A., van Dijk, E. J., Rutten-Jacobs, L. C., Arntz, R. M., Schoonderwaldt, H. C., . . . de Leeuw, F. E. (2013). Long-term cognitive impairment after first-ever ischemic stroke in young adults. *Stroke, 44*(6), 1621-1628. doi: 10.1161/strokeaha.111.000792

Schmidt, W., & Reymann, K. G. (2002). Proliferating cells differentiate into neurons in the hippocampal CA1 region of gerbils after global cerebral ischemia. *Neurosci Lett, 334*(3), 153-156. doi: S0304394002010728 [pii]

Schmitt, U., & Hiemke, C. (1998). Strain differences in open-field and elevated plus-maze behavior of rats without and with pretest handling. *Pharmacol Biochem Behav, 59*(4), 807-811. doi: S0091305797005029 [pii]

Schneider, A., Bottiger, B. W., & Popp, E. (2009). Cerebral resuscitation after cardiocirculatory arrest. *Anesth Analg, 108*(3), 971-979. doi: 10.1213/ane.0b013e318193ca99

Schreiner, A. E., Berlinger, E., Langer, J., Kafitz, K. W., & Rose, C. R. (2013). Lesion-induced alterations in astrocyte glutamate transporter expression and function in the hippocampus. *ISRN Neurol, 2013*, 893605. doi: 10.1155/2013/893605

Sharma, M., & Gupta, Y. K. (2002). Chronic treatment with trans resveratrol prevents intracerebroventricular streptozotocin induced cognitive impairment and oxidative stress in rats. *Life Sci, 71*(21), 2489-2498. doi: S0024320502020830 [pii]

Sheu, J. N., Liao, W. C., Wu, U. I., Shyu, L. Y., Mai, F. D., Chen, L. Y., . . . Chang, H. M. (2013). Resveratrol suppresses calcium-mediated microglial activation and rescues hippocampal neurons of adult rats following acute bacterial meningitis. *Comp Immunol Microbiol Infect Dis, 36*(2), 137-148. doi: 10.1016/j.cimid.2012.11.002

Shimada, H., Hamakawa, M., Ishida, A., Tamakoshi, K., Nakashima, H., & Ishida, K. (2013). Low-speed treadmill running exercise improves memory function after transient middle cerebral artery occlusion in rats. *Behav Brain Res, 243*, 21-27. doi: 10.1016/j.bbr.2012.12.018

Shin, D. H., Bae, Y. C., Kim-Han, J. S., Lee, J. H., Choi, I. Y., Son, K. H., . . . Han, B. H. (2006). Polyphenol amentoflavone affords neuroprotection against neonatal hypoxic-ischemic brain damage via multiple mechanisms. *J Neurochem, 96*(2), 561-572. doi: JNC3582 [pii] 10.1111/j.1471-4159.2005.03582.x

Siemann, E. H., & Creasy, L. L. (1992). Concentration of the phytoalexin resveratrol in wine. *Am J Enol Vitic, 43*(1), 49-52.

Silasi, G., Klahr, A. C., Hackett, M. J., Auriat, A. M., Nichol, H., & Colbourne, F. (2012). Prolonged therapeutic hypothermia does not adversely impact neuroplasticity after global ischemia in rats. *J Cereb Blood Flow Metab*. doi: 10.1038/jcbfm.2012.38 jcbfm201238 [pii]

Simao, F., Matte, A., Matte, C., Soares, F. M., Wyse, A. T., Netto, C. A., & Salbego, C. G. (2011). Resveratrol prevents oxidative stress and inhibition of Na(+)K(+)-ATPase activity induced by transient global cerebral ischemia in rats. *J Nutr Biochem*. doi: S0955-2863(10)00193-2 [pii] 10.1016/j.jnutbio.2010.07.013

Simao, F., Matte, A., Pagnussat, A. S., Netto, C. A., & Salbego, C. G. (2012a). Resveratrol preconditioning modulates inflammatory response in the rat hippocampus following global cerebral ischemia. *Neurochem Int*. doi: 10.1016/j.neuint.2012.06.009

Simao, F., Matte, A., Pagnussat, A. S., Netto, C. A., & Salbego, C. G. (2012b). Resveratrol prevents CA1 neurons against ischemic injury by parallel modulation of both GSK-3beta and CREB through PI3-K/Akt pathways. *Eur J Neurosci, 22*(10), 1460-9568.

Sims, N. R., & Muyderman, H. (2010). Mitochondria, oxidative metabolism and cell death in stroke. *Biochim Biophys Acta, 1802*(1), 80-91. doi: 10.1016/j.bbadis.2009.09.003

Sinha, K., Chaudhary, G., & Gupta, Y. K. (2002). Protective effect of resveratrol against oxidative stress in middle cerebral artery occlusion model of stroke in rats. *Life Sci, 71*(6), 655-665. doi: S0024320502016910 [pii]

Sinn, N., Milte, C., & Howe, P. R. (2010). Oiling the brain: a review of randomized controlled trials of omega-3 fatty acids in psychopathology across the lifespan. *Nutrients, 2*(2), 128-170. doi: 10.3390/nu2020128

Small, D. L., & Buchan, A. M. (2000). Animal models. *Br Med Bull, 56*(2), 307-317.

Sofroniew, M. V., & Vinters, H. V. (2010). Astrocytes: biology and pathology. *Acta Neuropathol, 119*(1), 7-35. doi: 10.1007/s00401-009-0619-8

Solberg, N. O., Chamberlin, R., Vigil, J. R., Deck, L. M., Heidrich, J. E., Brown, D. C., . . . Sillerud, L. O. (2014). Optical and SPION-Enhanced MR Imaging Shows that trans-Stilbene Inhibitors of NF-kappaB Concomitantly Lower Alzheimer's Disease Plaque Formation and Microglial Activation in AbetaPP/PS-1 Transgenic Mouse Brain. *J Alzheimers Dis, 40*(1), 191-212. doi: 10.3233/jad-131031

Stecker, E. C., Reinier, K., Marijon, E., Narayanan, K., Teodorescu, C., Uy-Evanado, A., . . . Chugh, S. S. (2014). Public health burden of sudden cardiac death in the United States. *Circ Arrhythm Electrophysiol, 7*(2), 212-217. doi: 10.1161/circep.113.001034

Stence, N., Waite, M., & Dailey, M. E. (2001). Dynamics of microglial activation: a confocal time-lapse analysis in hippocampal slices. *Glia, 33*(3), 256-266.

Sugawara, T., Lewen, A., Noshita, N., Gasche, Y., & Chan, P. H. (2002). Effects of global ischemia duration on neuronal, astroglial, oligodendroglial, and microglial reactions in the vulnerable hippocampal CA1 subregion in rats. *J Neurotrauma, 19*(1), 85-98. doi: 10.1089/089771502753460268

Sun, Y., Jin, K., Xie, L., Childs, J., Mao, X. O., Logvinova, A., & Greenberg, D. A. (2003). VEGF-induced neuroprotection, neurogenesis, and angiogenesis after focal cerebral ischemia. *J Clin Invest, 111*(12), 1843-1851. doi: 10.1172/jci17977

Supornsilchai, V., Svechnikov, K., Seidlova-Wuttke, D., Wuttke, W., & Soder, O. (2005). Phytoestrogen resveratrol suppresses steroidogenesis by rat adrenocortical cells by inhibiting cytochrome P450 c21-hydroxylase. *Horm Res, 64*(6), 280-286. doi: 10.1159/000089487

Swanson, R. A., Ying, W., & Kauppinen, T. M. (2004). Astrocyte influences on ischemic neuronal death. *Curr Mol Med, 4*(2), 193-205.

Takagi, Y., Nozaki, K., Takahashi, J., Yodoi, J., Ishikawa, M., & Hashimoto, N. (1999). Proliferation of neuronal precursor cells in the dentate gyrus is accelerated after transient forebrain ischemia in mice. *Brain Res, 831*(1-2), 283-287.

Takano, T., Oberheim, N., Cotrina, M. L., & Nedergaard, M. (2009). Astrocytes and ischemic injury. *Stroke, 40*(3 Suppl), S8-12. doi: STROKEAHA.108.533166 [pii] 10.1161/STROKEAHA.108.533166

Tanaka, K., Watase, K., Manabe, T., Yamada, K., Watanabe, M., Takahashi, K., . . . Wada, K. (1997). Epilepsy and exacerbation of brain injury in mice lacking the glutamate transporter GLT-1. *Science, 276*(5319), 1699-1702.

Tanaka, R., Yamashiro, K., Mochizuki, H., Cho, N., Onodera, M., Mizuno, Y., & Urabe, T. (2004). Neurogenesis after transient global ischemia in the adult hippocampus visualized by improved retroviral vector. *Stroke, 35*(6), 1454-1459. doi: 10.1161/01.STR.0000126480.40967.b3

Taoufik, E., & Probert, L. (2008). Ischemic neuronal damage. *Curr Pharm Des, 14*(33), 3565-3573.

Tavakoli, N., Bidari, A., & Shams Vahdati, S. (2012). Serum Cortisol Levels as a Predictor of Neurologic Survival inSuccessfully Resuscitated Victims of Cardiopulmonary Arrest. *J Cardiovasc Thorac Res, 4*(4), 107-111. doi: 10.5681/jcvtr.2012.026

Thomas, J., Garg, M. L., & Smith, D. W. (2013). Dietary resveratrol supplementation normalizes gene expression in the hippocampus of streptozotocin-induced diabetic C57Bl/6 mice. *J Nutr Biochem*. doi: 10.1016/j.jnutbio.2013.11.005

Thompson, J. W., Dave, K. R., Saul, I., Narayanan, S. V., & Perez-Pinzon, M. A. (2013). Epsilon PKC increases brain mitochondrial SIRT1 protein levels via heat shock protein 90 following ischemic preconditioning in rats. *PLoS One, 8*(9), e75753. doi: 10.1371/journal.pone.0075753

Tikka, T., Fiebich, B. L., Goldsteins, G., Keinanen, R., & Koistinaho, J. (2001). Minocycline, a tetracycline derivative, is neuroprotective against excitotoxicity by inhibiting activation and proliferation of microglia. *J Neurosci, 21*(8), 2580-2588.

Tiwari, V., & Chopra, K. (2011). Resveratrol prevents alcohol-induced cognitive deficits and brain damage by blocking inflammatory signaling and cell death cascade in neonatal rat brain. *J Neurochem, 117*(4), 678-690. doi: 10.1111/j.1471-4159.2011.07236.x

Tonchev, A. B. (2011). Brain ischemia, neurogenesis, and neurotrophic receptor expression in primates. *Arch Ital Biol, 149*(2), 225-231.

Tonchev, A. B., & Yamashima, T. (2006). Differential neurogenic potential of progenitor cells in dentate gyrus and CA1 sector of the postischemic adult monkey hippocampus. *Exp Neurol, 198*(1), 101-113. doi: 10.1016/j.expneurol.2005.11.022

Traystman, R. J. (2003). Animal models of focal and global cerebral ischemia. *ILAR J, 44*(2), 85-95.

Tremblay, M. E., Lowery, R. L., & Majewska, A. K. (2010). Microglial interactions with synapses are modulated by visual experience. *PLoS Biol, 8*(11), e1000527. doi: 10.1371/journal.pbio.1000527

Tsai, S. K., Hung, L. M., Fu, Y. T., Cheng, H., Nien, M. W., Liu, H. Y., . . . Huang, S. S. (2007). Resveratrol neuroprotective effects during focal cerebral ischemia injury via nitric oxide mechanism in rats. *J Vasc Surg, 46*(2), 346-353. doi: S0741-5214(07)00728-8 [pii] 10.1016/j.jvs.2007.04.044

Tsuda, M., Shigemoto-Mogami, Y., Koizumi, S., Mizokoshi, A., Kohsaka, S., Salter, M. W., & Inoue, K. (2003). P2X4 receptors induced in spinal microglia gate tactile allodynia after nerve injury. *Nature, 424*(6950), 778-783. doi: 10.1038/nature01786

Tulsulkar, J., & Shah, Z. A. (2013). Ginkgo biloba prevents transient global ischemia-induced delayed hippocampal neuronal death through antioxidant and anti-inflammatory mechanism. *Neurochem Int, 62*(2), 189-197. doi: 10.1016/j.neuint.2012.11.017

Vaartjes, I., Hendrix, A., Hertogh, E. M., Grobbee, D. E., Doevendans, P. A., Mosterd, A., & Bots, M. L. (2009). Sudden death in persons younger than 40 years of age: incidence and causes. *Eur J Cardiovasc Prev Rehabil, 16*(5), 592-596. doi: 10.1097/HJR.0b013e32832d555b

Van der Borght, K., Wallinga, A. E., Luiten, P. G., Eggen, B. J., & Van der Zee, E. A. (2005). Morris water maze learning in two rat strains increases the expression of the polysialylated form of the neural cell adhesion molecule in the dentate gyrus but has

no effect on hippocampal neurogenesis. *Behav Neurosci, 119*(4), 926-932. doi: 10.1037/0735-7044.119.4.926

van Praag, H., Kempermann, G., & Gage, F. H. (1999). Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus. *Nat Neurosci, 2*(3), 266-270. doi: 10.1038/6368

van Praag, H., Schinder, A. F., Christie, B. R., Toni, N., Palmer, T. D., & Gage, F. H. (2002). Functional neurogenesis in the adult hippocampus. *Nature, 415*(6875), 1030-1034. doi: 10.1038/4151030a

Varona, J. F., Bermejo, F., Guerra, J. M., & Molina, J. A. (2004). Long-term prognosis of ischemic stroke in young adults. Study of 272 cases. *J Neurol, 251*(12), 1507-1514. doi: 10.1007/s00415-004-0583-0

Venero, C., Herrero, A. I., Touyarot, K., Cambon, K., Lopez-Fernandez, M. A., Berezin, V., . . . Sandi, C. (2006). Hippocampal up-regulation of NCAM expression and polysialylation plays a key role on spatial memory. *Eur J Neurosci, 23*(6), 1585-1595. doi: 10.1111/j.1460-9568.2006.04663.x

Volpe, B. T., Pulsinelli, W. A., Tribuna, J., & Davis, H. P. (1984). Behavioral performance of rats following transient forebrain ischemia. *Stroke, 15*(3), 558-562.

von Bohlen Und Halbach, O. (2007). Immunohistological markers for staging neurogenesis in adult hippocampus. *Cell Tissue Res, 329*(3), 409-420. doi: 10.1007/s00441-007-0432-4

von Bohlen und Halbach, O. (2011). Immunohistological markers for proliferative events, gliogenesis, and neurogenesis within the adult hippocampus. *Cell Tissue Res, 345*(1), 1-19. doi: 10.1007/s00441-011-1196-4

Walsh, S. J., Harley, C. W., Corbett, D., Skinner, D. M., & Martin, G. M. (2008). CA1 ischemic injury does not affect the ability of Mongolian gerbils to solve response, direction, or place problems. *Brain Res, 1187*, 194-200. doi: S0006-8993(07)02493-6 [pii] 10.1016/j.brainres.2007.10.050

Wang, D., & Corbett, D. (1990). Cerebral ischemia, locomotor activity and spatial mapping. *Brain Research, 533*(1), 78-82. doi: Doi: 10.1016/0006-8993(90)91798-l

Wang, H., Yang, Y. J., Qian, H. Y., Zhang, Q., Xu, H., & Li, J. J. (2012). Resveratrol in cardiovascular disease: what is known from current research? *Heart Fail Rev, 17*(3), 437-448. doi: 10.1007/s10741-011-9260-4

Wang, H., Zhou, H., Zou, Y., Liu, Q., Guo, C., Gao, G., . . . Gong, Y. (2010). Resveratrol modulates angiogenesis through the GSK3beta/beta-catenin/TCF-dependent pathway in human endothelial cells. *Biochem Pharmacol, 80*(9), 1386-1395. doi: S0006-2952(10)00572-1 [pii] 10.1016/j.bcp.2010.07.034

Wang, J., Ho, L., Zhao, Z., Seror, I., Humala, N., Dickstein, D. L., . . . Pasinetti, G. M. (2006). Moderate consumption of Cabernet Sauvignon attenuates Abeta neuropathology in a mouse model of Alzheimer's disease. *FASEB J, 20*(13), 2313-2320. doi: 20/13/2313 [pii] 10.1096/fj.06-6281com

Wang, L. M., Wang, Y. J., Cui, M., Luo, W. J., Wang, X. J., Barber, P. A., & Chen, Z. Y. (2013). A dietary polyphenol resveratrol acts to provide neuroprotection in recurrent stroke models by regulating AMPK and SIRT1 signaling, thereby reducing energy requirements during ischemia. *Eur J Neurosci, 37*(10), 1669-1681. doi: 10.1111/ejn.12162

Wang, M., Wang, Y., Weil, B., Abarbanell, A., Herrmann, J., Tan, J., . . . Meldrum, D. R. (2009). Estrogen receptor beta mediates increased activation of PI3K/Akt signaling and improved myocardial function in female hearts following acute ischemia. *Am J Physiol Regul Integr Comp Physiol, 296*(4), R972-978. doi: 10.1152/ajpregu.00045.2009

Wang, Q., Sun, A. Y., Simonyi, A., Miller, D. K., Smith, R. E., Luchtefeld, R. G., . . . Sun, G. Y. (2009). Oral administration of grape polyphenol extract ameliorates cerebral ischemia/reperfusion-induced neuronal damage and behavioral deficits in gerbils: comparison of pre- and post-ischemic administration. *J Nutr Biochem, 20*(5), 369-377. doi: S0955-2863(08)00090-9 [pii] 10.1016/j.jnutbio.2008.04.007

Wang, Q., Xu, J., Rottinghaus, G. E., Simonyi, A., Lubahn, D., Sun, G. Y., & Sun, A. Y. (2002). Resveratrol protects against global cerebral ischemic injury in gerbils. *Brain Res, 958*(2), 439-447. doi: S0006899302035436 [pii]

Wang, Y., Xu, H., Fu, Q., Ma, R., & Xiang, J. (2011). Protective effect of resveratrol derived from Polygonum cuspidatum and its liposomal form on nigral cells in Parkinsonian rats. *J Neurol Sci, 304*(1-2), 29-34. doi: S0022-510X(11)00101-8 [pii] 10.1016/j.jns.2011.02.025

Wenzel, E., Soldo, T., Erbersdobler, H., & Somoza, V. (2005). Bioactivity and metabolism of trans-resveratrol orally administered to Wistar rats. *Mol Nutr Food Res, 49*(5), 482-494. doi: 10.1002/mnfr.200500003

Wenzel, E., & Somoza, V. (2005). Metabolism and bioavailability of trans-resveratrol. *Mol Nutr Food Res, 49*(5), 472-481. doi: 10.1002/mnfr.200500010

Wilder Schaaf, K. P., Artman, L. K., Peberdy, M. A., Walker, W. C., Ornato, J. P., Gossip, M. R., & Kreutzer, J. S. (2013). Anxiety, depression, and PTSD following cardiac arrest: a systematic review of the literature. *Resuscitation, 84*(7), 873-877. doi: 10.1016/j.resuscitation.2012.11.021

Williams, L. D., Burdock, G. A., Edwards, J. A., Beck, M., & Bausch, J. (2009). Safety studies conducted on high-purity trans-resveratrol in experimental animals. *Food Chem Toxicol, 47*(9), 2170-2182. doi: 10.1016/j.fct.2009.06.002

Winkelheide, U., Engelhard, K., Kaeppel, B., Winkler, J., Hutzler, P., Werner, C., & Kochs, E. (2008). Cerebral ischemia and neurogenesis: a two-time comparison. *Neurocrit Care, 9*(3), 387-393. doi: 10.1007/s12028-008-9121-8

Witte, A. V., Kerti, L., Margulies, D. S., & Floel, A. (2014). Effects of resveratrol on memory performance, hippocampal functional connectivity, and glucose metabolism in healthy older adults. *J Neurosci, 34*(23), 7862-7870. doi: 10.1523/jneurosci.0385-14.2014

Wojtowicz, J. M., Askew, M. L., & Winocur, G. (2008). The effects of running and of inhibiting adult neurogenesis on learning and memory in rats. *Eur J Neurosci, 27*(6), 1494-1502. doi: EJN6128 [pii] 10.1111/j.1460-9568.2008.06128.x

Wong, R. H., Berry, N. M., Coates, A. M., Buckley, J. D., Bryan, J., Kunz, I., & Howe, P. R. (2013). Chronic resveratrol consumption improves brachial flow-mediated dilatation in healthy obese adults. *J Hypertens, 31*(9), 1819-1827. doi: 10.1097/HJH.0b013e328362b9d6

Wong, R. H., Howe, P. R., Buckley, J. D., Coates, A. M., Kunz, I., & Berry, N. M. (2011). Acute resveratrol supplementation improves flow-mediated dilatation in

overweight/obese individuals with mildly elevated blood pressure. *Nutr Metab Cardiovasc Dis, 21*(11), 851-856. doi: 10.1016/j.numecd.2010.03.003

Wu, Z., Xu, Q., Zhang, L., Kong, D., Ma, R., & Wang, L. (2009). Protective effect of resveratrol against kainate-induced temporal lobe epilepsy in rats. *Neurochem Res, 34*(8), 1393-1400. doi: 10.1007/s11064-009-9920-0

Xie, H., Wu, Y., Jia, J., Liu, G., Zhang, F., Zhang, Q., . . . Hu, R. (2013). Enriched environment preconditioning induced brain ischemic tolerance without reducing infarct volume and edema: the possible role of enrichment-related physical activity increase. *Brain Res, 1508*, 63-72. doi: 10.1016/j.brainres.2013.02.052

Xu, Q., & Si, L. Y. (2012). Resveratrol role in cardiovascular and metabolic health and potential mechanisms of action. *Nutr Res, 32*(9), 648-658. doi: 10.1016/j.nutres.2012.07.002

Xuan, A., Long, D., Li, J., Ji, W., Hong, L., Zhang, M., & Zhang, W. (2012). Neuroprotective effects of valproic acid following transient global ischemia in rats. *Life Sci, 90*(11-12), 463-468. doi: S0024-3205(12)00014-8 [pii] 10.1016/j.lfs.2012.01.001

Yagita, Y., Kitagawa, K., Ohtsuki, T., Takasawa, K., Miyata, T., Okano, H., . . . Matsumoto, M. (2001). Neurogenesis by progenitor cells in the ischemic adult rat hippocampus. *Stroke, 32*(8), 1890-1896.

Yamashita, T., Ninomiya, M., Hernandez Acosta, P., Garcia-Verdugo, J. M., Sunabori, T., Sakaguchi, M., . . . Sawamoto, K. (2006). Subventricular zone-derived neuroblasts migrate and differentiate into mature neurons in the post-stroke adult striatum. *J Neurosci, 26*(24), 6627-6636. doi: 10.1523/jneurosci.0149-06.2006

Yan, X.-B., Wang, S.-S., Hou, H.-L., Ji, R., & Zhou, J.-N. (2007). Lithium improves the behavioral disorder in rats subjected to transient global cerebral ischemia. *Behavioural Brain Research, 177*(2), 282-289. doi: DOI: 10.1016/j.bbr.2006.11.021

Yang, Y., Duan, W., Li, Y., Yan, J., Yi, W., Liang, Z., . . . Jin, Z. (2013). New role of silent information regulator 1 in cerebral ischemia. *Neurobiol Aging*. doi: 10.1016/j.neurobiolaging.2013.06.008

Yang, Y. M., Wang, X. X., Chen, J. Z., Wang, S. J., Hu, H., & Wang, H. Q. (2008). Resveratrol attenuates adenosine diphosphate-induced platelet activation by reducing protein kinase C activity. *Am J Chin Med, 36*(3), 603-613. doi: 10.1142/s0192415x08006016

Yasuda, Y., Shimoda, T., Uno, K., Tateishi, N., Furuya, S., Tsuchihashi, Y., . . . Fujita, S. (2011). Temporal and sequential changes of glial cells and cytokine expression during neuronal degeneration after transient global ischemia in rats. *J Neuroinflammation, 8*, 70. doi: 10.1186/1742-2094-8-70

Yeh, T. H., Hwang, H. M., Chen, J. J., Wu, T., Li, A. H., & Wang, H. L. (2005). Glutamate transporter function of rat hippocampal astrocytes is impaired following the global ischemia. *Neurobiol Dis, 18*(3), 476-483. doi: S0969-9961(04)00322-5 [pii] 10.1016/j.nbd.2004.12.011

Yoo, K. Y., Hwang, I. K., Lee, C. H., Choi, J. H., Kwon, S. H., Kang, I. J., . . . Won, M. H. (2010). Difference of fibroblast growth factor receptor 1 expression among CA1-3 regions of the gerbil hippocampus after transient cerebral ischemia. *J Neurol Sci, 296*(1-2), 13-21. doi: 10.1016/j.jns.2010.06.018

Young, G. B. (2009). Clinical practice. Neurologic prognosis after cardiac arrest. *N Engl J Med, 361*(6), 605-611. doi: 10.1056/NEJMcp0903466

Yu, C., Shin, Y. G., Chow, A., Li, Y., Kosmeder, J. W., Lee, Y. S., . . . van Breemen, R. B. (2002). Human, rat, and mouse metabolism of resveratrol. *Pharm Res, 19*(12), 1907-1914.

Zhang, D., Hu, X., Qian, L., O'Callaghan, J. P., & Hong, J. S. (2010). Astrogliosis in CNS pathologies: is there a role for microglia? *Mol Neurobiol, 41*(2-3), 232-241. doi: 10.1007/s12035-010-8098-4

Zhang, F., Liu, J., & Shi, J. S. (2010). Anti-inflammatory activities of resveratrol in the brain: role of resveratrol in microglial activation. *Eur J Pharmacol, 636*(1-3), 1-7. doi: S0014-2999(10)00240-2 [pii] 10.1016/j.ejphar.2010.03.043

Zhang, F., Shi, J. S., Zhou, H., Wilson, B., Hong, J. S., & Gao, H. M. (2010). Resveratrol protects dopamine neurons against lipopolysaccharide-induced neurotoxicity through its anti-inflammatory actions. *Mol Pharmacol, 78*(3), 466-477. doi: mol.110.064535 [pii] 10.1124/mol.110.064535

Zhang, F., Wang, H., Wu, Q., Lu, Y., Nie, J., Xie, X., & Shi, J. (2013). Resveratrol protects cortical neurons against microglia-mediated neuroinflammation. *Phytother Res, 27*(3), 344-349. doi: 10.1002/ptr.4734

Zhang, M., Li, W. B., Liu, Y. X., Liang, C. J., Liu, L. Z., Cui, X., . . . Xian, X. H. (2011). High expression of GLT-1 in hippocampal CA3 and dentate gyrus subfields contributes to their inherent resistance to ischemia in rats. *Neurochem Int, 59*(7), 1019-1028. doi: 10.1016/j.neuint.2011.08.023

Zhang, Z. G., Zhang, L., Jiang, Q., Zhang, R., Davies, K., Powers, C., . . . Chopp, M. (2000). VEGF enhances angiogenesis and promotes blood-brain barrier leakage in the ischemic brain. *J Clin Invest, 106*(7), 829-838. doi: 10.1172/jci9369

Zhao, Y. D., Cheng, S. Y., Ou, S., Chen, P. H., & Ruan, H. Z. (2012). Functional response of hippocampal CA1 pyramidal cells to neonatal hypoxic-ischemic brain damage. *Neurosci Lett, 516*(1), 5-8. doi: 10.1016/j.neulet.2012.02.067

Ziemka-Nalecz, M., & Zalewska, T. (2012). Endogenous neurogenesis induced by ischemic brain injury or neurodegenerative diseases in adults. *Acta Neurobiol Exp (Wars), 72*(4), 309-324.