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**Cognitive-Behavioural Impairments After Forebrain Ischemia in Rats : Relationship to Disruption in  
Emotionality and Neuroendocrine Regulation**

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Cognitive-behavioural impairments after forebrain ischemia in rats:  
Relationship to disruptions in emotionality and neuroendocrine regulation

**Marc R. Milot**

Thesis submitted to the  
Faculty of Graduate and Postdoctoral Studies  
in partial fulfillment of the requirements  
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Don't let schooling interfere with your education.

--Oscar Wilde

Proliferation of theories is beneficial for science, while uniformity impairs its critical  
power

--Paul Feyerabend

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## Abstract

Cognitive deficits observed in rodents subjected to forebrain ischemia (mimicking the cerebral ischemia observed following cardiac arrest) are generally interpreted as produced by neuronal degeneration to discrete regions of the brain, principally the hippocampus. Challenging this view, the presence of ischemia-induced neuronal damage is not always associated with manifestations of cognitive impairment, or conversely, impairments post-reperfusion can originate at times when neuronal damage has yet to occur. The current thesis investigated whether the impairing effects of ischemia on cognitive functioning might in part be attributable to endogenous alterations of emotional systems regulating anxiety, stress, and/or arousal, thus not exclusively to discrete neuronal damage. The behavioural findings of Experiments 1, 2 and 3 suggested that hyperactivity in response to novel testing contexts in ischemic animals (as measured in the open field) is modulated by changes in emotional reactivity, and demonstrated time-dependent alterations of anxiety and behavioural activation at discrete reperfusion delays following a 10 minute forebrain ischemia. In Experiment 4, we demonstrated that cerebral ischemia is a significant stressor having lasting impact on HPA axis reactivity, effects associated to spatial memory impairments at delayed post-reperfusion intervals. Specifically, the testing-induced elevations in plasma corticosterone observed in ischemic rats (to a greater extent than sham-operated rats) was elicited after a return to pre-surgery resting levels, suggesting that increased neuroendocrine response was the result of behavioural testing and closely associated with cognitive deficits. Finally, Experiment 5 showed that excessive central noradrenergic reactivity in ischemic rats mediated some of the observed cognitive impairments or behavioural alterations. Overall, the thesis

experiments suggest that cognitive-behavioural outcome after forebrain ischemia in rats is associated with impaired emotional regulation and/or arousal. These findings are not compatible with the common hippocampal-based interpretation of cognitive deficits after ischemia, and question the ecological validity (and generalizability) of functional outcome measurements of rats subjected to cerebral ischemia given that cognitive impairments in human survivors of ischemic episodes are not considered the result of arousal deficits.

## **General Introduction**

### **1. Cardiac arrest, cerebral ischemia, and cognitive impairment**

Cerebral ischemia refers to a deficiency in blood supply to the brain, reducing amounts of oxygen and essential nutrients to neurons and leading to a cascade of neurophysiological changes implicated in cellular degeneration (Lipton, 1999). In humans, this condition can result from a disruption in blood circulation within a focused area of the brain (stroke) or to the entire brain and periphery (cardiac arrest; CA). Human survivors of CA often experience a variety of cognitive impairments (Bergner, Hallstrom, Bergner, Eisenberg, & Cobb, 1985; Bertini et al., 1990; Grubb, O'Carroll, Cobbe, Sirel, & Fox, 1996; O'Reilly, Grubb, & O'Carroll, 2003; Parnia, Spearpoint, & Fenwick, 2007; Roine, Kajaste, & Kaste, 1993; Sauve, 1995; Sauve, Doolittle, Walker, Paul, & Scheinman, 1996) and a reduction in their quality of life (Bergner, Hallstrom, Bergner, Eisenberg, & Cobb, 1985; Middelkamp et al., 2007). A recent systematic review of 286 articles concluded that approximately 50% of CA survivors suffer from cognitive impairments significantly impacting their daily lives and overall functioning, and that delayed recall capabilities (versus recognition) are the most impaired (Moulaert, Verbunt, van Heugten, & Wade, 2009).

Over the years, animal models of cerebral ischemia have been the main tool of investigation of the physiological mechanisms regulating functional impairments and cell death post injury. They can be classified as either global or focal model of cerebral ischemia. Focal ischemia is considered an approximation of the most commonly experienced human stroke and is induced in animals using the middle cerebral arterial occlusion (MCAO) model producing heterogeneous neuropathology principally affecting

cortical and caudate-putamen (striatal) neurons and characterized by inflammation of the penumbral tissue. Global ischemia reproduces the severe brain hypoperfusion observed during CA, and consists of temporary (e.g., 5-30 min) but almost complete cessation of blood flow to forebrain structures (A. R. Green & Cross, 1997; Small & Buchan, 2000), with only partial reductions to hindbrain structures (Pulsinelli & Brierley, 1979). The most commonly selected models to produce global ischemia in rats involve either occlusion of both the vertebral and carotid arteries – the four-vessel occlusion model (4VO) (Pulsinelli & Brierley, 1979; Pulsinelli, Brierley, & Plum, 1982; Pulsinelli, Levy, & Duffy, 1982) or occlusion of the common carotid arteries combined with arterial hypotension, called the two-vessel occlusion model (2VO) (Hartman, Lee, Zipfel, & Wozniak, 2005; McBean & Kelly, 1998; O'Neill & Clemens, 2001; Small & Buchan, 2000). Cessation of blood flow from the two pairs of major arteries (carotid and vertebral) leads to less than 10% of normative perfusion to forebrain tissues (Small & Buchan, 2000). A 2VO combined with hypotension is also sufficient to similarly compromise cerebral blood flow. The 4VO model utilized in the present thesis involves permanent occlusion of the anterior vertebral arteries followed by transient carotid artery occlusion lasting 10 min, using pupillary dilation and the righting reflex as exclusion criteria. In comparison to 2VO, this model involving no hypotension or anesthetic during occlusion “provides a more realistic, physiologically relevant model of global forebrain ischemia” (Small & Buchan, 2000).

The cellular damage appearing in rodents following a global ischemic insult is similar to that observed in the human brain following CA (Petito, Feldmann, Pulsinelli, & Plum, 1987). The process termed “delayed neuronal death” refers to the observation that

ischemia-induced morphological changes leading to cell death become apparent in hippocampal neurons only following a period of at least 48 h (Kirino, 1982; Kirino, Tamura, & Sano, 1984). This depends on the duration of occlusion, and damage will occur earlier the longer the global ischemic insult (Pulsinelli, Brierley, & Plum, 1982). For example, although a 10 min forebrain ischemia in rats (employed in the present thesis) will lead to delayed neuronal death beginning 3 days post-ischemia (and largely complete at 7 days) (Deshpande, Siesjo, & Wieloch, 1987), a 30 min ischemia can lead to significant damage visible at 3 to 6 h post insult (Pulsinelli, Brierley, & Plum, 1982). Therefore, the rate of cell death is accelerated by increased insult duration, and the hippocampus appears to be the most vulnerable structure. Specifically, cerebral ischemia in rodents produces nearly selective neuronal degeneration in hippocampal subfield CA1, especially if ischemia duration is brief (e.g., 5-10 min) (Nunn et al., 1994). A variety of additional brain regions are impacted by forebrain ischemia, particularly as the occlusion period is extended (e.g., >10-15 min), both in intra-hippocampal (e.g., CA2, CA3, hilus) and extra-hippocampal regions (e.g., striatum, neocortex)(Chan et al., 1998; Letechipia-Vallejo et al., 2007; McBean et al., 1995). However, given that humans subjected to CA (thus forebrain ischemia) lasting more than 7 min cannot be resuscitated (Herlitz et al., 1994), a 5-10 min ischemia has more ecological validity in this respect.

Animal models of cerebral ischemia have primarily been designed in the hope of understanding the physiological cascade involved in neuronal death and of finding ways to remediate brain injury post insult. At present, research on cerebral ischemia remains primarily concerned with the prevention of neuronal damage (O'Neill & Clemens, 2001) with only a small proportion of animal ischemia experiments actually investigating

memory/cognitive performance, compared to human studies of ischemic survivors typically including mnemonic assessment without systematic assessment of related brain damage. Due to their shape and size and their vulnerability to global ischemic damage, hippocampal neurons have rapidly become the main focus of examination and a perfect target to understand cellular processes regulating neuronal death post injury. The primary focus being on understanding mechanisms involved in cell injury per se, the assessment of functional recovery has been used as a tool to verify that improved neuronal and behavioural outcomes were concomitant.

Hippocampal cellular degeneration in the CA1 of rodents is generally believed to be the chief cause of memory impairment observed post-reperfusion (oftentimes in spatial tasks). This is a common sense interpretation given the cells in this structure have been considered as “place cells”, neurons which fire correspondingly to a rats’ location in a spatial environment (McNaughton, Barnes, & O’Keefe, 1983; O’Keefe, 1976). Consequently, in most studies assessing the effects of a particular treatment on neuronal protection and functional recovery following global ischemia, histopathological examination still commonly remains confined to the CA1 subfield of the hippocampus.

## **2. An unclear relationship between hippocampal cell death and functional impairments**

Although now debated by different research groups, cognitive deficits produced by cerebral ischemia are largely considered memory-based and the result of hippocampal cell loss. Spatial memory impairments in the radial maze (RM) and/or Morris water maze (MWM) after global ischemia have been associated with hippocampal cell loss (H. P.

Davis, Baranowski, Pulsinelli, & Volpe, 1987; Gionet et al., 1991; Hagan & Beaughard, 1990), and positive correlations between CA1 neuronal injury and post-ischemic impairments in spatial tasks seem to confirm a functional relationship between the two events (Gionet et al., 1991; Nelson, Lebessi, Sowinski, & Hodges, 1997; Olsen, Scheel-Kruger, Moller, & Jensen, 1994b; Rod, Whishaw, & Auer, 1990; Wood, Mumby, Pinel, & Phillips, 1993). Similar functional relationships have been proposed for post-ischemic hyperactivity following exposure to a novel environment (Andersen, Zimmer, & Sams-Dodd, 1997; Katsuta, Umemura, Ueyama, & Matsuoka, 2003). Consistent with these observations, neuroprotection is often reported concomitant with attenuations of ischemia-induced behavioural impairment. For example, pre-ischemia administration of MK-801, a glutamate receptor antagonist, simultaneously reduced neuronal death in the CA1 and spatial memory impairment (Corbett, Evans, Thomas, Wang, & Jonas, 1990), as did administration of lithium (X. B. Yan, Hou, Wu, Liu, & Zhou, 2007), opioid agonists (Charron, Messier, & Plamondon, 2008a) and flupirtine (4-Aminopyridine)(Block, Pergande, & Schwarz, 1997), to name only a few experiments. Together, these findings present evidence of a strong relationship between standard histopathological measures of degeneration and behavioural performance supporting an association between hippocampal cell death and cognitive impairments.

In recent years, however, different studies assessing the effects of discrete brain lesions in various experimental paradigms have stimulated questions and debates about the relationship between ischemia-induced hippocampal cell loss and behavioral impairment (Bachevalier & Meunier, 1996; Squire & Zola, 1996). Correlations between the extent of CA1 hippocampal cell loss and the degree of behavioural impairment are

not always reported in spatial memory tasks (Nunn et al., 1994; Olsen, Scheel-Kruger, Moller, & Jensen, 1994b; Paganelli et al., 2004). Many of the aforementioned correlations (Olsen, Scheel-Kruger, Moller, & Jensen, 1994b; Rod, Whishaw, & Auer, 1990; Wood, Mumby, Pinel, & Phillips, 1993) compromises clear interpretation of the findings as a strong correlation is expected from analysis including bi-directional distribution (i.e., ischemic rats showing important neuronal damage and behavioral impairments and sham animals with no neuronal damage nor impairment). Importantly, ischemia-induced spatial memory impairment have been observed prior to CA1 neuronal degeneration (Iwasaki, Kitamura, Ohgami, Mishima, & Fujiwara, 1996; Kuroiwa, Bonnekoh, & Hossmann, 1991), while functional recovery was demonstrated despite extensive CA1 injury (Bueters, von Euler, Bendel, & von Euler, 2008; Gobbo & O'Mara, 2004; Milot & Plamondon, 2005; Roberge, Hotte-Bernard, Messier, & Plamondon, 2008). Further, no spatial memory deficits reported despite significant CA1 ischemic damage (Iwasaki et al., 2006). In one interesting study, rats subjected to 10 min forebrain ischemia displayed impaired spatial memory in a MWM and deficits in an odour and object discrimination task, impairments that were not observed in ischemic rats reared in an enriched environment despite no reductions in hippocampal damage (Gobbo & O'Mara, 2004). Further, global ischemia impairs nonspatial object recognition (Mumby et al., 1996; Plamondon, Davignon, Khan, & Charron, 2008; Plamondon, Morin, & Charron, 2006; Wood, Mumby, Pinel, & Phillips, 1993) while complete hippocampal ablation (including the entire CA1 subfield) has no effect (Ainge et al., 2006; Mumby, 2001; Mumby et al., 1996). Finally, one research group (Jaspers, Block, Heim, & Sontag, 1990) investigated the effect of transient (20 min) 2VO alone (carotid occlusion, without

hypotension or vertebral occlusion) which produces approximately 50-60% reduction in blood flow, and reported spatial memory impairments in the MWM despite no evidence of hippocampal neuronal degeneration. In the open field, ischemia-induced hyperactivity (often interpreted as a habituation memory deficit) is not necessarily attenuated by recovery of hippocampal CA1 neurons. For example, ischemic preconditioning failed to alter hyperactivity despite improved neuronal recovery (Corbett & Crooks, 1997; Dowden & Corbett, 1999; Plamondon, Davignon, Khan, & Charron, 2008). Conversely, pre-treatment with the CRH-R1 antagonist CP154,526 significantly reduced hyperactivity post-ischemia without effects on CA1 neuronal injury (Plamondon & Khan, 2006). These findings support the proposition that other factors besides the commonly assessed hippocampal neuronal degeneration represent important endogenous mediators of behavioural performance post-reperfusion.

### **3. Extra-hippocampal cell death, synaptic dysfunction, or neurogenesis as alternative mediators of post-ischemic behaviour**

Bachevalier & Meunier (1996) hypothesized that extra-hippocampal damage might mediate post-reperfusion behaviour. There was however no direct evidence provided for such a relationship, and the authors referred to studies using 20-30 min of ischemia (Volpe, Davis, Towle, & Dunlap, 1992; Volpe, Pulsinelli, Tribuna, & Davis, 1984). Extra-hippocampal damage certainly might impact behaviour, but it is difficult to determine the specific contribution of such damage, particularly with ischemic durations over 15 min given expected significant extra-hippocampal damage in a variety of regions. However, after 10 forebrain ischemia extra-hippocampal damage is presumed minimal,

and not always observable using standard histological techniques such as Nissl staining (cresyl violet) and silver impregnation (Nunn et al., 1994). Specifically, the hippocampal subfield CA3 important in spatial memory was not damaged after 10 min global ischemia (Chan et al., 1998; X. H. Liu, Kato, Nakata, Kogure, & Kato, 1993; Martins, Inamura, Themner, Malmqvist, & Siesjo, 1988; Nelson, Sowinski, & Hodges, 1997; Petito & Halaby, 1993), and reports of damage show only a minimal number of neurons affected (Araki, Kato, Shuto, & Itoyama, 1998; Briones & Therrien, 2000). In the DG, also important in learning and memory, neuronal death does not occur after 10 min ischemia (Araki, Kato, Shuto, & Itoyama, 1998; Chan et al., 1998; Jorgensen, Jensen, & Diemer, 1991). Finally, degeneration in neocortex is not necessary reported after 10 min ischemia (Globus et al., 1991; Kawai et al., 1992; Nunn et al., 1994), and when it is, damage to this structure is minimal (Fortuna et al., 1997; Nasu et al., 2006) compared to the 60-90% reduction in CA1 neurons typically observed. Consequently, many of the brain structures implicated in cognitive functions typically shown to be impaired after ischemia (e.g., spatial memory, working memory, object recognition) are not damaged, and if so, very minimally. In this context, the 10 min model of ischemia employed for the present thesis does indeed produce significant spatial memory (working and non-working) deficits in the radial maze (Gionet et al., 1991; Okada et al., 1996; Plamondon & Khan, 2006; Plamondon, Morin, & Charron, 2006) and MWM (Nunn, Gray, & Hodges, 1998; Nunn et al., 1994), and object recognition (Mumby, 2001; Plamondon, Morin, & Charron, 2006).

Besides discrete hippocampal cell death, post-reperfusion alterations in behaviour may be the result of other modifications in the brain. One such post-reperfusion

modification is synaptic dysfunction. Although whole cell membrane resting potentials are not altered 24 h after ischemia (Ogiso et al., 1998), long-term potentiation (LTP) is not present in hippocampal neurons 24 h after brain ischemia (Hori et al., 2002), indicating that synaptic dysfunction precedes neuronal degeneration (Dave et al., 2004). In fact, ischemia-induced changes in synaptic function appear to precede neuronal death per se, and have been observed to occur only within the few hours and days post-ischemia (depending on degree of insult)(Schurr et al., 1995; Urban, Neill, Crain, Nadler, & Somjen, 1989). As a result, the degree to which alterations in hippocampal synaptic functioning can account for post-ischemic behavior remains to be determined. It is possible that early changes in behavioral activity observed within days post-ischemia could be related to such changes in hippocampal or extra-hippocampal neurons. However, given the lack of relationship between behavioural outcome and neuronal death at delayed post-ischemic time intervals when neuronal degeneration is largely complete, other factors appear to participate in functional impairments.

The neurogenesis observed after cerebral ischemia in the DG (Briones, Suh, Hattar, & Wadowska, 2005; Kee, Preston, & Wojtowicz, 2001) and more recently in the hippocampal CA1 subfield (Schmidt & Reymann, 2002) might impact post-reperfusion behaviour. Indeed, the reappearance of CA1 neurons 90 days after ischemia (neurogenesis was confirmed with BrdU staining) was associated to recovery of spatial memory capabilities in the MWM (Bendel et al., 2005). Despite such findings appearing to indicate a relationship between CA1 cell death and functional outcome, a subsequent study by this same research group revealed that following the disappearance of these newly created neurons months later (and in spite of the extensive CA1 damage) ischemic

rats showed no deficiencies in spatial learning and memory in the same MWM task (although a somewhat less efficient overall search strategy) (Bueters, von Euler, Bendel, & von Euler, 2008). Consequently, factors other than CA1 neurogenesis might have directly been associated to functional recovery in their early study. Studies such as these represent important findings as past experiments have largely determined the relationship between ischemic injury and impairments at early post-ischemic intervals (within a 15 day of the insult) prior to the time at which neurogenesis is induced in injured brain tissue. However, neurogenesis cannot explain the lack of relationship between cell death and behavioural outcome observed in a growing number of reports that have assessed long-term functional recovery (as previously detailed), given that the standard histological techniques used could not differentiate between newly created cells and already existing ones, ensuring the inclusion of both types of cells in the analysis. So far, no studies have demonstrated a role in functional outcome for neurogenesis observed in the subgranular zone of DG of the hippocampus after 10 min forebrain ischemia. Further, despite the involvement of the DG in different types of memory function (Gilbert, Kesner, & Lee, 2001), significant cell death in this structure has not been reported after 10 min ischemia, suggesting that learning and memory impairments are not likely related to loss of neurons in this area. Overall, these findings suggest that the occurrence of neurogenesis cannot itself account for the many discrepant findings present at delayed post-ischemic intervals between histopathological and behavioural outcomes.

#### **4. A possible role for discrete physiological alterations of the neuroendocrine system and emotional dysfunction in ischemia-induced behavioural impairment**

In addition to aforementioned cellular events and neuroanatomical changes post-ischemia, the interaction between cognition/behaviour and neurochemical/neuroendocrine regulation makes it likely that neurophysiological alterations represent a potent modulator of post-reperfusion performance. To date, the study of the biochemical/neuroendocrine alterations produced by global ischemia has been mainly geared towards an understanding of the mechanisms involved in neuronal degeneration (Lipton, 1999; Plamondon & Khan, 2006; Sapolsky & Pulsinelli, 1985), thereby little is actually known about the participation of discrete neurochemical signals outside the excitotoxic cascade occurring in the minutes to hours following ischemia, let alone at extended post-reperfusion intervals when cognitive assessment occurs.

In addition to the glutamatergic system known to play a decisive role in excitotoxicity post-ischemia but also to contribute to discrete memory processes and long-term potentiation (Granger et al., 1996; Vianna et al., 2000), the hypothalamic-pituitary-adrenal (HPA) axis and noradrenergic system represent two major mediators of the neuroendocrine response to stress which role to modulate cognition and behavior is well documented (McEwen & Sapolsky, 1995; Myhrer, 2003; Sapolsky, 2003; Sara, 2009). The HPA axis controls stress and immune responses, regulates mood and emotions, as well as learning and memory processes (see below). In response to physical or psychological stress, the hypothalamus releases corticotropin-releasing hormone (CRH) which (via the portal blood vessel system of the hypophyseal stalk) stimulates the pituitary gland to secrete adrenocorticotrophic hormone (ACTH) which in turn (via blood

transport) acts to stimulate the biosynthesis of cortisol (in humans) or corticosterone (CORT; in rodents) from the adrenal glands. CORT is then transported to all major body tissues, including the brain where it acts via mineralocorticoid and glucocorticoid receptors expressed by many different types of neurons in a variety of brain regions.

The noradrenergic system is responsible for the synthesis, storage, and release of the neurotransmitter norepinephrine (NE). Release of the catecholamine NE is initiated by the sympathetic nervous system, and the neurotransmitter mediates the “fight or flight” reaction by affecting cardiovascular function (to name one peripheral effect) and has many effects on numerous brain functions related to sleep, emotions, and learning and memory (more details to come). The majority of central NE is produced in the locus coeruleus (LC) which projects to the prefrontal cortex, hippocampus and amygdala, and many other brain areas. There is an interaction between NE producing limbic structures and HPA axis stimulation. Another such structure is the amygdala, which has stimulatory effects on the HPA axis. Electrical stimulation of the basolateral amygdala can increase CORT release (Feldman, Conforti, & Siegel, 1982), and lesions to the structure can diminish ACTH and CORT release both in resting levels (Feldman, Conforti, Itzik, & Weidenfeld, 1995) and in response to a stressor (Van de Kar, Piechowski, Rittenhouse, & Gray, 1991) Amygdaloid norepinephrine release can directly stimulate the hypothalamus to release CRH and depletion of NE in this structure with 6-hydroxydopamine prevents its facilitatory effect on HPA axis activity (Feldman & Weidenfeld, 1996), which is mediated via alpha 1-adrenergic receptors in the PVN of the hypothalamus (Itoi et al., 1994; Plotsky, Cunningham, & Widmaier, 1989). Importantly, NE release in the amygdala is increased by stress (both physical and psychological) (Pacak et al., 1993; M.

Tanaka et al., 1991; T. Tanaka et al., 1991). Neurons in the LC show increased firing in response to various novel/stressful/emotional stimuli (Aston-Jones, Rajkowski, Kubiak, Valentino, & Shipley, 1996; Cullinan, Herman, Battaglia, Akil, & Watson, 1995; Kollack-Walker, Watson, & Akil, 1997) and this structure also has stimulatory effects on the HPA axis; rats subjected to bilateral LC lesions displayed reduced CORT and ACTH in response to restraint stress (Ziegler, Cass, & Herman, 1999). The HPA axis and noradrenergic system are consequently interlinked and are important to consider in the study of emotionality and neuroendocrine alterations in response to stress.

#### *4.1. Alterations of the HPA axis and noradrenergic system following forebrain ischemia*

Components of the neuroendocrine system (engaging the HPA axis and noradrenergic functioning) have been studied in relation to ischemia-induced neuronal degeneration but their contribution to short or longer-term cognitive and behavioural outcomes has not been identified. In the context of the effects of these systems on neuronal outcome, increased circulating levels of CORT are thought to compromise the ability of hippocampal neurons to survive neurological insults (Stein-Behrens, Mattson, Chang, Yeh, & Sapolsky, 1994) and increased circulating CORT is associated to aggravation of ischemic injury (Sapolsky & Pulsinelli, 1985). Inhibition of CORT synthesis with metyrapone is neuroprotective at short post-reperfusion intervals (24 h, 3 and 7 days) following global or focal ischemia in rodents (Adachi, Chen, Liu, Nagaro, & Arai, 1999; Krugers, Kemper, Korf, Ter Horst, & Knollema, 1998; Smith-Swintosky et al., 1996). With respect to noradrenergic alterations, there are massive but short lasting elevations of extracellular NE in the hippocampus following 30 min (Perego, Gatti,

Vetrugno, Marzatico, & Algeri, 1992), 20 min (Globus et al., 1989), and 10 min cerebral ischemia (Miura et al., 1999), as well as in the neocortex (Gustafson, Westerberg, & Wieloch, 1991), effects which have been associated to ischemia-induced neuronal degeneration (Busto, Harik, Yoshida, Scheinberg, & Ginsberg, 1985; Engelhard et al., 1999; Hoffman et al., 1992; J. A. Schultz, Hoffman, & Albrecht, 1993). It is interesting to note that enhanced noradrenergic neurotransmission (in this case, increased NE synthesis in mPFC) is observed after traumatic brain injury (TBI), another cerebral injury model associated cognitive deficits such as working memory (Kobori, Clifton, & Dash, 2006). The role of the noradrenergic system and HPA axis is consequently well documented with respect to neuronal outcome, but these observations do not address the possibility of long lasting sensitization of these systems post-ischemia possibly impacting functional outcome.

Notwithstanding the demonstrated relationship with functional impairments, there is however evidence (both direct and indirect) for altered neuroendocrine and behavioural arousal/reactivity in ischemic rats at delayed post-reperfusion intervals. Cerebral ischemia can be considered a strong acute stressor shown to enhance CRH release/expression in multiple brain regions, including the central nucleus of the amygdala and the hypothalamic lateral and paraventricular nuclei (Chen et al.; Rothwell et al., Khan, Milot, Lecompte & Plamondon, 2004) as well as to increase circulating levels of CORT in the hours and days following reperfusion (Hwang et al., 2006). An ischemic stressor might potentiate behavioural and neuroendocrine alterations even at relatively longer delay. Indeed, “it is now well established that a single exposure to some stressors is able induce behavioral and physiological changes that last for days to weeks,

although the precise mechanisms are poorly known” (Belda et al., 2008). For example, a single dose of CORT can exert effects on emotional reactivity even 12 days later (Mitra & Sapolsky, 2008) and single exposure (5 min) to a water stressor can alter the serotonergic system even seven days later (S. Davis, Heal, & Stanford, 1995), showing that acute stressors can have long lasting impact of behaviour and neurophysiology. Specifically related to memory, a single predator exposure in mice led to learning difficulties in the radial maze 16 to 22 days post-stressor and in object recognition test (26 to 28 days) (El Hage, Griebel, & Belzung, 2006), two capabilities that are similarly impacted by cerebral ischemia (as aforementioned). A single exposure to immobilization stressor increased HPA axis reactivity up to two weeks later in response to open-field exposure (Belda et al., 2008). It is also important to consider that 10 min ischemia is perhaps more than an acute stressor, and acting somewhat as a subchronic stressor (defined as less than seven days of increased CORT/stressor exposure). This phenomenon is associated to increased HPA axis sensitivity and spatial memory impairment (Moosavi, Naghdi, Maghsoudi, & Zahedi Asl, 2007; Radecki, Brown, Martinez, & Teyler, 2005), suggesting that even longer lasting effects of an ischemic stressor on behaviour might be expected compared to other acute stressors. Similar long-term effects on behaviour or neuroendocrine reactivity have been observed following all types of stressors, including CORT administration, emotional/physical, and immune stressors (Belda et al., 2008). One particularly important effect described with an acute stressor (in this case a single session of tail shocks) was a significant increase of neuroendocrine reactivity to a novel environment observed at least 10 days post-stressor (J. D. Johnson et al., 2002). Overall, these findings show that a prior acute stressor can prime the HPA axis. Consequently, the

acute and subchronic stressor of ischemia previously reported (Hwang et al., 2004) may be significant enough to potentiate the neuroendocrine response (CORT and ACTH) during subsequent exposure to a heterotypic stressor such as behavioural testing in a novel environment, having a negative impact on cognitive functions.

Although no studies have reported the effect of ischemia on neuroendocrine response to a stressor at times when cognitive testing occurs, alterations to the noradrenergic system have been reported and suggest increased reactivity and arousal in ischemic animals. The first study to report relatively delayed noradrenergic dysfunction after ischemia showed increased NE immunoreactivity in hippocampal and frontal neurons up to 7 days post-reperfusion, but no studies have examined this effect at later intervals (Iwasaki, Kitamura, Ohgami, Mishima, & Fujiwara, 1996; Miyauchi, Wieloch, & Lindvall, 1989) when functional outcome in ischemic animals is commonly assessed. However, a relatively more recent experiment demonstrated increased central noradrenergic sensitivity in ischemic rats at remote intervals (Pich et al., 1993). In this study, ischemic rats displayed increased analgesia in the weeks following reperfusion, effects which were potentiated by yohimbine administration (an alpha2 adrenoceptor antagonist acting on presynaptic autoreceptors), and re-instated even 60 days post-reperfusion. These observations strongly suggested that forebrain ischemia increased activity of norepinephrine-containing neurons, which when similarly upregulated in normal rats also induced analgesic effects (Kepler & Bodnar, 1988). This demonstration in ischemic animals is also consistent with observation that significant stressors can induce analgesia, due to adaptive processes (Amit & Galina, 1986). Consequently, cerebral ischemia represent a physiological stressor which can upregulate the

noradrenergic system and affect its sensitivity at remote post-reperfusion intervals in ways which could impact cognitive performance.

In this context, the HPA axis and noradrenergic system might be important to consider in post-reperfusion behaviour, because they regulate the main endocrine response following exposure to physical or psychological stressors, effects which are known to affect memory processes. Of interest, rodents placed in a simple behavioural apparatus such as the open-field displayed significant post-testing elevations in CORT (Denenberg et al., 1981; Gentsch, Lichtsteiner, & Feer, 1981; Plyusnina & Oskina, 1997; Suarez, Perassi, & Dal Zotto, 1996), despite no food deprivation, water or shock exposure, highlighting the stressful nature of testing animals in novel contexts, which characterizes all behavioural paradigms. Even relatively less stressful behavioural paradigms such as the Y-maze (which does not include open-space and has high walls) can lead to substantial increases in CORT secretion (unpublished observation). Thus, many if not all animal tests of cognitive functioning might be impacted by individual/group differences in neuroendocrine reactivity to testing conditions. Consequently, the aforementioned stimulatory effects of an ischemic episode on the neuroendocrine and noradrenergic systems may likely contribute to manifestations of emotional and cognitive impairments observed post-ischemia.

#### *4.2. Open-field alterations observed post-ischemia: manifestations of noradrenergic and HPA axis hyper-reactivity?*

Given that very little research has examined the specific impact of forebrain ischemia on the neuroendocrine response at delayed post-reperfusion intervals or in

association with behavioural testing, this section will discuss a very common ischemia-induced behaviour (open-field hyperactivity) that supports the idea of increased neuroendocrine sensitivity in ischemic rodents. The open-field is a large box, typically placed in a novel environment and under some degree of illumination. Rodents are placed inside the open-field and observed for their exploration, generally locomotor activity. During open-field testing involving rodents, observers can be said to be measuring treatment effects on the reactivity of the subjects to a stressful event rather than limited effects on exploration (Prut & Belzung, 2003). In this context, a simple but consistent effect of cerebral ischemia in rodents on post-reperfusion behaviour is open-field hyperactivity in gerbils (E. J. Green et al., 1995; Plamondon & Khan, 2005; X. B. Yan, Wang, Hou, Ji, & Zhou, 2007) and rats (E. J. Green et al., 1995; Plamondon & Khan, 2005; X. B. Yan, Wang, Hou, Ji, & Zhou, 2007). This hyperactivity is relatively robust and has been observed at a variety of post-ischemic intervals, ranging from hours after ischemia to weeks later.

Importantly, increased exploration in a novel open-field exploration is associated to neuromodulatory systems implicated in stress and arousal. One possibility might be that the behavioural activation observed in ischemic rats exposed to brightly lit open-field arenas is a manifestation of increased HPA activation, similar to the effects of CRH administration (i.c.v.) on open-field behaviour (Britton, Hoffman, Lederis, & Rivier, 1984; Britton, Koob, Rivier, & Vale, 1982), which are considered as activational (Koob & Bloom, 1985). Specifically, in terms of locomotion, tested under bright illumination (1000 lux) low doses of CRH (i.c.v.) increased central exploration and locomotion in an open-field (Sutton, Koob, Le Moal, Rivier, & Vale, 1982), similar to another study

employing a low dose of CRH and reporting increased open-field locomotion (Veldhuis & De Wied, 1984), thereby comparable to the effects of ischemia on open-field activity. Conversely, CRH deficiency reduced open-field activity (Swain & Maric, 1995). While these studies examined the acute effects of CRH on open-field activity, they may not represent what occurs after sustained CRH increases or alterations in cellular content or expression, as observed in after 10 min ischemia (Khan, Milot, Lecompte-Collin, & Plamondon, 2004). Repeated CRH administration (for 5 days) at a number of doses also increased open-field activity (Song, Earley, & Leonard, 1995), suggesting that both the acute and subchronic effects of HPA axis upregulation can be activational. Interestingly, administration of a single dose of CRH antagonist pre-ischemia prevents the appearance of open-field hyperactivity 4 to 7 days later (Plamondon & Khan, 2006), suggesting long-term effects of the initial ischemia-induced HPA upregulation on behavioural activation at distant test intervals. Consequently, the neuroendocrine effects of cerebral ischemia might similarly regulate open field hyperactivity.

Relatively less is known about the effects of cerebral ischemia on the noradrenergic system. However, like CRH administration, behavioural activation following central norepinephrine administration has been observed in a novel open-field, with low doses increasing locomotor activity and high doses suppressing activity (Smee, Weston, Skinner, & Day, 1975). Intrahippocampal NE administration also increased open-field locomotor activity (Plaznik, Danysz, & Kostowski, 1983) as did intra-amygdalar administration (Plaznik, Danysz, & Kostowski, 1985a). Conversely, central depletion of NE (by DSP-4)(van den Buuse, Lambert, Fluttert, & Eikelis, 2001) or LC lesions (Heybach, Coover, & Lints, 1978) can suppress open-field locomotor activity.

Consequently, similar to the effects of CRH on locomotor activity, NE appears to exert effects on activity level, most of which are stimulatory, effects which have great similitude to behavioural changes observed in ischemic animals upon open-field exposure.

Overall, these findings suggest that behavioural arousal /activation observed in the open-field in ischemic animals might in part be mediated by discrete changes in neurochemical turnover and/or altered neuronal sensitivity post ischemia leading to increased neuroendocrine responses to stressors and novel contexts, such as behavioural testing. To our knowledge, increased reactivity or behavioural activation following forebrain ischemia has not been reported in other behavioural paradigms.

#### *4.3. Are noradrenergic and HPA axis alterations related to post-ischemia cognitive-behavioural impairment? Relationship between emotionality, arousal and cognition*

The last section highlighted the importance of the neuroendocrine response in open-field activity/behavioural activation. In the current section, associations between cognitive performance (namely, spatial memory) and changes in HPA axis responses and the noradrenergic system will be proposed. Indeed, alterations of these systems' responses have been shown to impact memory retrieval, working memory, as well as brain circuits implicated in memory consolidation. These findings suggest that modulations of neurochemical systems regulating emotional responses (and/or arousal) post-ischemia may impact behavioural performance under a variety of paradigms measuring memory capabilities.

Different studies have demonstrated that increased neuroendocrine response is implicated in the enhancement of memory consolidation. Thus, post-training CORT administration has been shown to enhance retention in contextual fear conditioning (Thompson, Erickson, Schulkin, & Rosen, 2004), spatial memory in a MWM (Roosendaal, 2002; Roosendaal, Okuda, Van der Zee, & McGaugh, 2006) and object recognition (Okuda, Roosendaal, & McGaugh, 2004). Similarly, post-training administration of NE into the central nucleus of the amygdala enhanced retention in a defensive-burying paradigm (Roosendaal, Koolhaas, & Bohus, 1993), and the central administration of CRH is associated to improved memory consolidation in a spatial MWM task via a noradrenergic mechanism (Row & Dohanich, 2008). Consistent with this, post-training administration of a CRH receptor antagonist impaired consolidation in an inhibitory avoidance task (Roosendaal, Brunson, Holloway, McGaugh, & Baram, 2002). Importantly, NE is a co-factor for the facilitative effects of CORT on memory consolidation (Roosendaal, Griffith, Buranday, De Quervain, & McGaugh, 2003; Roosendaal, Hahn, Nathan, de Quervain, & McGaugh, 2004). Conversely, blockade of noradrenergic beta receptors with propranolol after training in a step-down avoidance task impaired long-term retention (e.g., when tested 1, 3 or 7 days later) (Cohen & Hamburg, 1975). These findings show that increased activation of the HPA or NE systems can improve memory consolidation in numerous paradigms, and that these neurochemical signals are intrinsically linked.

On the other hand, increased neuroendocrine response has been shown to impair memory retrieval. For example, pre-training administration of CORT can impair memory retrieval (Roosendaal, 2002; Roosendaal, Griffith, Buranday, De Quervain, & McGaugh,

2003; Roozendaal, Hahn, Nathan, de Quervain, & McGaugh, 2004). With regards to NE, optimal levels are required for retrieval, likely due to its effects on selective attention (Sara, 1985), and administration of NE agonists can have positive impact on memory retrieval (Murchison et al., 2004). Excessive levels of NE however can impair memory retrieval, as demonstrated following pre-training intra-hippocampal (Aghajanian, 1982) and basolateral amygdalar (Barros et al., 2001) administrations. Relatively little is known about the impact of CRH on memory retrieval. Consequently, while arousal is necessary to retrieve memory, excessive arousal may impair retrieval processes.

Similarly to memory retrieval, working memory can be impaired by exacerbated neuroendocrine secretions. In a delayed alternation task in a Y-maze, CORT administration induced working memory impairment, but only in animals with an intact basolateral amygdala (Okuda, Roozendaal, & McGaugh, 2004). Spatial memory impairments in a MWM task were induced by pre-testing CORT administration (Woodson, Macintosh, Fleshner, & Diamond, 2003), but only when rats were exposed to a predator (cat) prior to testing, indicating that CORT impaired working memory only when paired with a fear-provoking stimuli. With regards to NE, working memory deficits have been observed following lesion to the locus coeruleus, disrupting the major source of frontal and hippocampal NE (Compton, Dietrich, Smith, & Davis, 1995) as well as following administration of a NE agonist in the PFC (Arnsten, Mathew, Ubriani, Taylor, & Li, 1999), indicating that excessive or unbalanced NE availability can impair working memory (similar to retrieval). These findings suggest that increased arousal can impair working memory, but perhaps not as reliably as memory retrieval.

Consequently, the effects of ischemia on the noradrenergic system and arousal (Hickey, Akino, Strausbaugh, & De Courten-Myers, 1996; Pich et al., 1993) and its plausible effects on HPA axis sensitivity or regulation predict increased consolidation but impaired working memory and retrieval in these animals. While increased consolidation in ischemic animals has yet to be reported and may appear counter-intuitive, one possibility is that any increased consolidation related to arousal might mask underlying impairments in reference memory (this is tested in Experiment 5). This is relatively commonsensical, particular given this type of memory (e.g., reliant on consolidation, such as reference memory) appears less impacted by ischemia (versus working memory)(Volpe, Davis, Towle, & Dunlap, 1992; Volpe, Pulsinelli, Tribuna, & Davis, 1984). In terms of memory retrieval and working memory, excessive arousal in ischemic rats might be negatively correlated to performance. Further, the observation of improved spatial working memory performances in ischemic rats in spatial memory tests over days (Charron, Messier, & Plamondon, 2008a, , 2008b; Plamondon, Davignon, Khan, & Charron, 2008; Plamondon & Khan, 2006; Roberge, Messier, Staines, & Plamondon, 2008) suggest that endogenous changes (possibly involved in alterations of arousal or emotional responses to increasing familiarity to testing conditions) may play a role in learning capabilities of these animals.

## **5. Methodology, research questions, and series of thesis experiments**

Cerebral ischemia represents a potent acute physiological stressor leading to increased HPA axis activity, a phenomenon shown to significantly impact behavioural performance in various animal studies/behavioural paradigms. In addition, cerebral

ischemia is associated with increased activation of NE-containing neurons for at least two months post-reperfusion (Pich et al., 1993), and to open-field hyperactivity linked to increased neuroendocrine reactivity, which in turn is implicated in learning and memory. Despite the relationship between ischemia-induced neuroendocrine dysfunction and behavioural performance being unexplored, these findings suggest that forebrain ischemia might have important effects on biochemical system affecting responses to testing, negatively impacting cognitive functioning independent of ischemia-induced hippocampal damage.

Therefore, the current thesis had three main objectives that formed the basis for the course of the experiments. The first thesis objective (Experiments 1 and 2) aimed to determine whether ischemia-induced behavioural reactivity deficits might be related to illumination level (bright versus dim) which could modulate the emotional tone during testing. If so, common post-ischemic behavioural impairment might be attenuated by testing under dim illumination (thereby reducing behavioural reactivity). The second objective (Experiment 3) aimed to characterize time-dependent changes in emotional reactivity and behavioural activation (namely, in the open-field and EPM) in test-naïve ischemic animals 1, 5, 15 and 30 days following reperfusion, thus to assess post-ischemic behavioural reactivity at longer post-ischemic time intervals when more extensive and complex memory tests typically occur (e.g., spatial memory tests). Finally, the third main objective of the current thesis aimed to provide a more thorough investigation of the effects of ischemia on short and longer term regulation of the HPA axis (Experiment 4) and noradrenergic system (Experiment 5), and determine the possible relationship of these changes to cognitive impairments observed at delayed intervals when neuronal

degeneration is complete. More specifically, the thesis studies aimed to answer the following questions: 1) Can ischemia-induced alterations in behaviour be modified by manipulating the emotional context during testing (Experiments 1 & 2)? 2) What are the effects of ischemia on measures of emotionality and behavioural arousal at short and delayed post-ischemia time intervals (Experiment 3)? Do impairments (e.g., hyper-reactivity) in HPA axis activation and noradrenergic functioning contribute to the observed behavioural deficits in spatial memory tasks? (Experiments 4 and 5).

The general hypothesis of the current thesis stipulates that since emotionality represents a variable that contributes to behavioural performance, any effects of ischemia to alter arousal or emotionality or systems regulating these states might be associated to behavioural changes post-ischemia. If emotionality does have an impact on post ischemic behaviours, we should expect: a) evidence for emotional change (e.g., behavioural and neurophysiological) at times when cognitive (memory) impairments appear and b) that pharmacological and/or environmental manipulations that affect emotional reactivity/arousal will differentially impact behaviour of ischemic rats compared to control animals. We expect ischemia to increase behavioural and neuroendocrine activation relative to sham-operated controls during testing, effects having important repercussions on measures of cognitive performance.

The following sections briefly describe the rationale and experimental procedures which characterize the five specific experiments of the current thesis.

### *5.1. Experiment 1: The effect of illumination on ischemia-induced open-field alterations*

The first experiment was designed to test the possibility that manipulation of environmental conditions having effects on emotionality and arousal may represent a factor that influence manifestations of open-field activity after forebrain ischemia. To date, studies that have examined the effects of manipulating emotional contingencies on behavioural performance in normal animals have shown that one of the simplest manipulation producing effects on the emotional/behavioural reactivity of normal animals is alteration of the level of illumination. Illumination level has been observed to mediate the level of anxiety and exploration in the open-field and elevated-plus maze (EPM). Bright open-field illumination in normal rats suppresses exploration compared to dimmer light illumination (Bouwknicht et al., 2007; Hale, Bouwknicht, Spiga, Shekhar, & Lowry, 2006; Mar, Spreekmeester, & Rochford, 2002). Illumination levels might exert these effects by mediating an animal's appraisal of the "stressful" nature of a novel environment, with bright illumination being more anxiogenic and activational/arousing (Cardenas, Lamprea, & Morato, 2001; Chapillon & Debouzie, 2000; Pereira, da Cunha, Neto, Paschoalini, & Faria, 2005).

In this initial experiment, the emotional context was consequently manipulated via illumination (bright – 450 lux versus dim – 40 lux) and animals tested on within- and between-session measures of exploration and habituation in an open-field between days 3-10 post-reperfusion. At present, the impact of manipulating emotional contingencies, including illumination level, on behavioural performance following forebrain ischemia remains unknown. If ischemia impacts open-field behaviour due to changes in emotional reactivity (and not solely to habituation deficits related to memory impairments), we

should expect manipulation of illumination level to affect the ischemia/sham discrimination in exploration. More specifically, one general hypothesis for Experiment 1 was that testing under dim illumination might reduce the open-field hyperactivity impairment by attenuating stress-induced arousal and/or behavioural disinhibition. This hypothesis was motivated in part by the finding that CRH can increase locomotor activity in a novel open-field, but not when tested in a familiar and less stressful environment. Consequently, testing ischemic animals under less stressful conditions (dim illumination) may similarly attenuate any disruptive effects of increased arousal, or attenuate the elicitation of arousal.

*5.2. Experiment 2: The effect of illumination level during testing on ischemia-induced spatial memory and object recognition memory (spontaneous measures)*

The second experiment represented a direct follow-up to the first study and examined the effects of manipulating emotional tone (bright versus dim illumination) on ischemia-induced behavioural impairments in common (and spontaneous) measures of object recognition and working/reference spatial memory. Indeed, Chapillon and Debouzie (2000) reported effects of illumination level on spatial memory performance in the MWM. Specifically, they found that spatial memory acquisition, defined as the latency to find the platform across the testing days, was significantly improved when tested under a halogen lamp providing diffuse lighting compared to testing under direct and brighter neon tubes. This effect was hypothesized to be due to attenuation of arousal/anxiety-induced memory “impairment” under diffuse lighting. Considering these observations, the main hypothesis of this study was that reduced behavioural activation

and arousal in ischemic animals tested under dim illumination might reduce memory impairment.

*5.3. Experiment 3: The effect of forebrain ischemia on behavioural measures of emotional reactivity at short and long post-reperfusion time intervals.*

The effects of cerebral ischemia on behavioural measures of emotional reactivity (e.g., such as measured in the EPM and open-field) at different post-ischemic intervals have received little attention, and existing reports are inconsistent. Experiments 1 and 2 characterized the effects of manipulation of emotional context during testing on ischemia-induced behavioural and memory deficits at short time intervals (between 3-10 days post ischemia). As previously discussed, open-field hyperactivity after ischemia in a novel open-field has been consistently (but not always) observed. Importantly, most testing of post-ischemic open-field behaviour at longer delays (Colbourne & Corbett, 1994; Wang & Corbett, 1990) have however not used test-naïve animals, thus activity levels across the testing days might not represent what might occur when the animals are exposed to a novel testing paradigm. Similarly, although ischemic rodents have been shown to exhibit changes in anxiety in the elevated-plus maze (EPM), the results have not been consistent, with decreased anxiety (Nelson, Lebessi, Sowinski, & Hodges, 1997; Plamondon & Khan, 2005; B. Yan et al., 2007), increased anxiety (Dhooper, Young, & Reid, 1997; Nakashima, Ajiki, Nakashima, & Takahashi, 2003) or no changes (Bantsiele et al., 2004; Roberge, Hotte-Bernard, Messier, & Plamondon, 2008). These mixed findings render difficult the establishment of the effect of global ischemia on measures of emotional reactivity and behavioural activation at different reperfusion time-intervals.

Therefore, in this third experiment, the main objective was to characterize the manifestations of behavioural activation/locomotion and changes in anxiety levels following cerebral ischemia at both short and longer post-reperfusion intervals in test-naïve animals. Specifically, this study tested ischemic and sham-operated animals in the EPM immediately followed by open-field assessment (in a distinct/novel spatial context) on days 1, 5, 15, or 30 post-reperfusion. Experiment 3 fulfilled the second main goal of this thesis, which aimed to determine whether alterations in emotional and behavioural reactivity are observed at delayed post-ischemic intervals when more extensive and complex memory assessment typically occurs. The findings of this experiment will also help determine whether ischemia-induced changes in behavioural activation (e.g., open-field alterations) show a constant relationship to EPM activity at the distinct time intervals

#### *5.4. Experiment 4: The effect of cerebral ischemia and metyrapone pre-treatment on HPA axis reactivity: relationship to neuronal and behavioural outcome*

As mentioned earlier, significant physiological or psychogenic stressors have shown the ability to prime the neuroendocrine response to subsequent heterotypic stressors (including behavioural testing). The fourth experiment of this thesis examined the effect of cerebral ischemia on the neuroendocrine response profile (plasma CORT and ACTH levels) at rest (1 h, and 1, 3, and 7 days post-reperfusion) and in response to spatial memory assessment between days 9-14, and evaluated their relationship to cognitive impairments and ischemic neuronal damage.

In this fourth study, we hypothesized that cerebral ischemia would lead to significant up-regulation of the HPA axis lasting for a few days as compared to basal/resting levels, which might exert a remote influence on neuroendocrine reactivity to behavioural testing occurring later when neuronal degeneration is relative complete and circulating CORT levels have returned to baseline. Consequently, in addition to the above-mentioned post-ischemic assessment of neuroendocrine activation, blood samples were collected 30 min post-testing. We predicted increased CORT levels in ischemic rats as compared to sham animals after behavioural testing, and a relationship between CORT concentration and impairments in spatial working memory (as assessed in Y-maze; spontaneous alternation) and retrieval deficits (as assessed in the spatial Barnes maze tasks, considered a “land” version of the MWM).

As described earlier, inhibition of CORT synthesis with metyrapone can reduce neuronal degeneration in the CA1 subfield at short-post ischemic intervals. As part of the current study, we used metyrapone administration to further examine the relationship between ischemia-induced CORT secretion to neuronal injury after a long (four weeks) post-reperfusion interval as well as subsequent behavioural outcome. This was of interest considering that many of the physiological effects of metyrapone (hyperglycemia, sustained upregulation of the HPA axis) (Rotllant & Armario, 2005; Rotllant, Ons, Carrasco, & Armario, 2002) actually predict aggravated neuronal outcome. One hypothesis was that administration of metyrapone would lead to accentuated and longer lasting alterations of HPA axis reactivity in ischemic as compared to sham animals, possibly aggravating ischemic neuronal injury.

*5.5. Experiment 5: Alteration of noradrenergic activity following ischemia and related effects on behavioural/cognitive performance*

The notion of a NE dysfunction in relation to cognitive impairment following ischemia has been proposed but only tested at short post-ischemic intervals when neuronal degeneration was incomplete (Iwasaki, Kitamura, Ohgami, Mishima, & Fujiwara, 1996). However, the study by Pich et al. (1993) demonstrated increased activation of NE-containing neurons in ischemic rats up to two months after reperfusion. Yohimbine (an alpha 2-adrenoceptor antagonist increasing synaptic levels of NE) administration increased the ischemia-induced analgesic effect, while clonidine (an alpha 2-adrenoceptor agonist decreasing synaptic levels of NE) had the opposite effect and attenuated the effects of ischemia. Therefore, the main objective of Experiment 5 was to determine the effects of regulation of synaptic NE availability on post-ischemic working and reference memory impairments. Noradrenergic levels were altered by the administration of yohimbine and clonidine prior to training (and once neuronal degeneration is considered largely complete) in the working memory version of the radial maze (days 10-25), and following training in the reference memory version (days 40-48).

This study represented the first to test acute impact of pharmacological treatments on ischemia-induced cognitive impairments at delayed time intervals. The predictions, based on observations by Pich et al. (1993) and demonstrated effects of NE on memory capabilities, were of attenuated working memory impairment by pre-training clonidine administration (leading to attenuated NE release), and aggravated memory capabilities after yohimbine administration (leading to excessive NE release).

## **6. Scientific contribution and relevance of thesis findings**

The major contribution of the present thesis lies in the characterization of the contribution of endogenous factors related to changes in emotional regulation or arousal post-ischemia that could play a role in expression of impairments beyond the commonly examined hippocampal neuronal death. At present, studies in the field of cerebral ischemia, even the ones focusing on examination of the role of synaptic dysfunction, neurogenesis, or extra-hippocampal cell death in post-reperfusion functional outcome remain largely confined to a hippocampal-based (or neuronal damage-based) interpretation, and do not address the potential effect that an ischemic stressor might have on performance via neuroendocrine alterations. For example, the finding that 2VO alone (without vertebral occlusion or hypotension) can produce spatial memory impairment in MWM in the absence of neuronal degeneration (Jaspers, Block, Heim, & Sontag, 1990) was interpreted by others as due to “functional changes and reorganization within the hippocampus” (Nelson, 1997). Similarly, neurogenesis studies attempt to make an association between recovery of CA1 neurons and functional recovery despite consideration of past observations failing to report a relationship between neuronal and behavioural outcome at remote post ischemic intervals when histological measurements would be including these neurons. Further, the notion of significant extra-hippocampal damage as mediating post-reperfusion performance, while certainly plausible, might be restricted to ischemic durations lasting between 15-30 min, and not to those of 5-10 min and more representative of situations with human survivors of CA whom do not survive ischemic insults longer than 7 min duration (Herlitz et al., 1994).

It is believed that further examining the role of distinct endogenous changes outside brain damage per se will lead to increased understanding of post-ischemic cognitive impairments and that such knowledge might directly impact how behavioural alterations after ischemia (in rodents) can be interpreted and appraised, particularly with respect to neuroprotective studies. For example, ascertaining that protection against ischemic-induced neuronal degeneration (e.g., by a potential neuroprotective treatment) is actually related to the observed functional recovery can be considered crucial, particularly for the generation of treatments to be used for clinical trials. In addition, given that the relationship between cognitive impairments and the size of the brain lesion post-injury is not a linear one, it is possible that the general observations of the current thesis may help foster novel research questions for human studies further addressing the relationship between emotional changes and cognitive impairments post-injury. It is also hoped that the research presented in this thesis will help improve and better understand animal models of cerebral ischemia, their effects on behavioural and neuronal outcome, and their generalizability to humans.

## Experiment 1

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*Abstract*

The current study reports the impact of different illumination conditions on exploratory activity following global ischemia in rats. Exploratory activity was tested at different post-ischemic intervals under bright (450 lux) or dim (40 lux) light exposure. A 30 min testing period performed 5 days post-reperfusion examined within-session open-field habituation in ischemic and sham-operated animals. Additional animals were tested in the open-field under the two illumination conditions for shorter 10 min tests on days 3, 6, and 9 following reperfusion. Our findings demonstrated illumination-related activity profile in the open-field in ischemic animals. While ischemic rats showed increased activity when tested under bright open-field illumination, reduced activity was observed under dim illumination as compared to sham-operated controls. Further, habituation deficits were not apparent in animals subjected to global ischemia under any illumination condition. Similar behavioral profiles and habituation were observed in ischemic animals when exposed to repetitive open-field tests at days 3, 6, and 9 following reperfusion. CA1 neuronal injury ( $\approx 75\%$  as compared to sham rats) was comparable in all ischemic groups at day 12 following reperfusion. The present findings suggest that differences in initial behavioral reactivity of sham and ischemic rats to bright versus dimly lighted environments may contribute to differences in open-field exploration reported between these groups. They also challenge the notion that deficits in exploration in ischemic animals are mainly attributable to processes related to habituation, that hyperactivity represents a reliable predictor of CA1 neuronal injury. These observations may help explain discrepant findings present in the literature.

## 1. Introduction

Global cerebral ischemia in rodents not only produces neuronal degeneration in hippocampal subfield CA1, a brain area exquisitely sensitive to hypoperfusion, but its effects are also manifested behaviorally under a variety of tasks, both spatial (Gionet et al., 1991; Hagan & Beaughard, 1990; Kiyota, Miyamoto, & Nagaoka, 1991) and nonspatial (Mumby et al., 1996; Wood, Mumby, Pinel, & Phillips, 1993). A relatively simple and cost-effective paradigm found to discriminate between ischemic and sham-operated rodents is the novel open-field. The open-field consists of a walled and open-roofed arena (a “field”) situated in a novel testing environment. Exploratory behavior can be observed for a pre-determined amount of time, ranging from a few min (Mello e Souza, Rohden, Meinhardt, Goncalves, & Quillfeldt, 2000) to more extended periods of time (Plamondon & Khan, 2005). Measures of locomotion (i.e., amount of distance traveled) and rearing together quantify horizontal and vertical exploration, respectively. Exploration rates gradually decline given a test session of sufficient duration, as the testing environment becomes more familiar (Plamondon & Khan, 2005). The observation of decreased exploration of an increasingly familiar environment is referred to as open-field habituation. Between-session habituation can also be displayed by decreased exploration in subsequent open-field exposures (Vianna et al., 2000).

The open-field has been found to reliably discriminate ischemic and sham-operated gerbils on the basis of locomotor activity. Generally, ischemic animals show increased locomotor activity relative to sham-operated controls as early as 1-2 hours and as late as 60 days post-reperfusion (Chaulk, Wells, Evans, Jackson, & Corbett, 2003; X. B. Yan, Wang, Hou, Ji, & Zhou, 2007). The increase in locomotor activity has been

interpreted as a deficit in habituation to a novel environment (Colbourne, Auer, & Sutherland, 1998; Colbourne & Corbett, 1994), with ischemic animals failing to display normal reductions in exploration of an increasingly familiar location. Locomotor hyperactivity following ischemia in gerbils has been shown to be correlated to CA1 cell death, suggesting that such neuronal damage contributes to the observed habituation deficits (Andersen, Zimmer, & Sams-Dodd, 1997; Katsuta, Umemura, Ueyama, & Matsuoka, 2003). However, CA1 histological protection does not guarantee functional preservation in the open-field. Pharmacological treatments (Chaulk, Wells, Evans, Jackson, & Corbett, 2003) or ischemic preconditioning (Corbett, Nurse, & Colbourne, 1997) providing CA1 neuroprotection failed to attenuate hyperactivity in the open-field. Further, the presence of hyperlocomotion as early as 2 or 30 h post-ischemia (Corbett, Nurse, & Colbourne, 1997; Kuroiwa, Bonnekoh, & Hossmann, 1991) when no hippocampal damage is present further indicates that CA1 cell viability is not the sole mediator of exploratory changes following global cerebral ischemia in rodents. Nonetheless, it has been argued that hyperlocomotion at these time intervals can predict hippocampal cell death (Mileson & Schwartz, 1991).

Although patterns of hyperlocomotion in the open-field following global ischemia have been consistently reported in gerbils, there have been reports of increases (E. J. Green et al., 1995; Plamondon & Khan, 2005; X. B. Yan, Wang, Hou, Ji, & Zhou, 2007), no changes (Gionet et al., 1991; Gulinello, Lebesgue, Jover-Mengual, Zukin, & Etgen, 2006), and reductions (Hori et al., 2002) in locomotion following global cerebral ischemia in rats, despite similar reperfusion testing intervals and CA1 neuronal damage. In a recent study, we observed that pharmacological blockade of CRH receptors prior to

occlusion reversed ischemia-induced hyperactivity in the open-field while having no effects on hippocampal cellular damage or spatial memory impairments in ischemic animals (Plamondon, Morin, & Charron, 2006), supporting the potential involvement of discrete neurochemical factors in this behavior.

Differences in the duration of ischemia, the day of testing following reperfusion, and/or the extent of neuronal damage in different brain areas represent variables possibly mediating conflicting results in rat studies. In addition, the level of open-field illumination represents one typically unreported parameter which might affect behavioral differentiation of sham and ischemic animals. Bright open-field illumination in normal rats suppresses exploration compared to dimmer light illumination (Bouwknicht et al., 2007; Hale, Bouwknicht, Spiga, Shekhar, & Lowry, 2006; Mar, Spreekmeester, & Rochford, 2002). Illumination levels might exert these effects by mediating an animals' appraisal of the "stressful" nature of a novel environment, with bright illumination being more anxiogenic (Pereira, da Cunha, Neto, Paschoalini, & Faria, 2005), effects which could inhibit exploration. Indeed, the open-field has been used to assess emotionality in rats, and different studies examining anxiolytic properties of drugs have used open-field exploration as a measure of anxiety, with decreased anxiety being manifested through enhanced open-field exploration (Prut & Belzung, 2003). To this effect, Prut & Belzung (2003) commented that during open-field testing involving rodents, observers are measuring treatment effects on the reaction of the subjects to a stressful event rather than limited effects on exploration.

The effects of cerebral ischemia on emotionality in animals, including the reaction to a stressful event or novel environment, remain largely unknown. In non-ischemic rats,

increased time spent in the closed less “anxiogenic” arms of the elevated-plus maze (EPM) has been associated with decreased exploratory activity in the open-field. In contrast, hypertensive rats simultaneously demonstrate reduced anxiety in the EPM and increased exploratory activity in the open-field (Gentsch, Lichtsteiner, & Feer, 1987). Although limited, existing studies have reported reduced anxiety in the EPM in rats following forebrain ischemia, manifested through increased time spent in the more “anxiogenic” open arms (Nelson, Lebessi, Sowinski, & Hodges, 1997; Plamondon & Khan, 2005; X. B. Yan, Wang, Hou, Ji, & Zhou, 2007). Thus, it appears possible that cerebral ischemia could promote increased open-field exploration by mediating the reactivity of the animals to stressful (or non stressful) events. More specifically, neuronal alterations and/or systemic changes in ischemic animals might stimulate exploration of novel and/or stressful environments via alteration of stress-induced (e.g., high levels of illumination) inhibition of exploratory behavior normally observed in non-ischemic rodents.

In light of the difficulties in reconciling findings related to ischemia-induced alterations in open-field exploration, the principal objective of the current study aimed to determine whether exploratory activity following global ischemia in rats could be affected by illumination levels. One possibility lies in differences between ischemic and sham-operated animals in their behavioral reactivity to open-field illumination conditions, affecting the animals’ response to environmental novelty independent of habituation deficits or acute hyperactivity. The present study tested animals in the open-field under bright (450 lux; high stress) and dim (40 lux; low stress) illumination conditions. Animals were tested for 30 min on day 5 post-ischemia, and for 10 min on

days 3, 6, 9 to determine within and between session exploration and habituation, respectively. The present study represents the first attempt to examine the effect of illumination on exploratory tendencies following cerebral ischemia in rodents, thus the results are difficult to predict. We hypothesize that under conditions of bright illumination ischemic animals might display increased exploratory activity related to behavioral inhibition in sham-operated controls (relative to controls tested under dim illumination). Conversely, under conditions of dim illumination when stress is minimized, control animals might display increased activity as compared to those tested under bright conditions, possibly minimizing differences in open-field exploration relative to ischemic animals.

## **2. Materials and methods**

### **2.1. Animals**

Male Wistar rats (n=59) weighing between 250–320 g at time of surgery were obtained from Charles River Laboratories (Rochefort, Quebec, Canada). They 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. The room temperature was maintained at 21–23 °C with 60% relative humidity. In Experiment 1, different sets of animals were used in the bright and dim open field conditions [sham (n= 7 in each group) and ischemic (n=7 in each group)], and tested 5 days following ischemia. Similarly, different sets of animals were used in the bright and dim open field conditions [sham (n= 9 & 8, respectively) and ischemic (n=7 in each group)] in Experiment 2, and tested on

days 3, 6, and 9 post-ischemia. All animals were handled daily for about 2 min by the experimenter 3 days preceding surgery.

## 2.2. Surgical procedure

Forebrain ischemia was performed using the four-vessel occlusion model as previously described by Pulsinelli & Brierley (Pulsinelli & Brierley, 1979). Briefly, rats were anesthetized by inhalation of 1.5% halothane in oxygen. The core temperature was kept at  $37\pm 0.5$  °C throughout the surgeries, including during global ischemia, by means of a feedback regulated heating blanket connected to a rectal thermometer (Homeothermic Blanket Control Unit, Harvard Instruments, Natick, MA). The vertebral arteries were irreversibly occluded by electrocoagulation, and a small-diameter silk thread looped around the carotid arteries to facilitate subsequent occlusion. Sham-operated animals underwent anesthesia and received the same dorsal and ventral surgical incisions as the ischemic group, without electrocoagulation of the vertebral arteries. Twenty-four hours later, rats were briefly exposed to halothane, removed from it, and the common carotid arteries were clamped with microvascular clamps for 10 min. Rats spontaneously ventilated on their backs, and only those that lost the righting reflex over the entire occlusion period were used in the study. Under these conditions, and without clamping of the carotid arteries, sham-operated rats regain the righting reflex  $< 90$  s following halothane removal. Body temperature was supported with a heating pad placed under their home cage in the hours following reperfusion. On day 2 post occlusion, the pupillary reflex was assessed in all animals to determine visual system/retinal damage possibly affecting the animals' visual response to light conditions. To do so, 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.

### 2.3. Experimental apparatus and behavioral procedures

#### 2.3.1. *Novel open-field test*

The observation arena was made of gray Plexiglas (LWH: 75×75×30 cm). For animals tested in Experiment 2, the open-field diameters were identical except for the wall height being 15 cm. A painted grid divided the Plexiglas floor into 36 identical squares. The arena was located on a table 90 cm above the floor and was surrounded by black curtains. For animals tested under bright conditions (450 lux), illumination was provided by fluorescent lights situated 8 feet above the open-field. For the dim condition (40 lux), the lights were covered with black opaque material. Lux measurements were taken with a lightmeter (ProsKit MT-4007) placed at the center of the open-field. Groups of sham and ischemic subjects were transported from their vivarium to the testing laboratory and allowed to habituate at least one hour before testing began. Animals were placed in a random corner of the open-field and behavior was monitored for 30 min on day 5 (Experiment 1), or for 10 min on days 3, 6 and 9 (Experiment 2). Subjects were

monitored by a camera, and the frequency of square entries and rearing behaviors quantified online using a datalogging software (ODlog 2.0, USA). The open-field was wiped clean (10% ethanol) between each test.

### *2.3.2. Analysis of neuronal density on thionin stained sections*

Upon completion of the behavioral tests, on day 12 post-ischemia rats were euthanized and the brains removed, frozen on dry ice, and stored at  $-80^{\circ}\text{C}$ . Serial coronal sections ( $14\ \mu\text{m}$ ) of the hippocampal regions were subsequently obtained using a cryostat (Leica Instruments) and stained for Nissl bodies with thionin. Neuronal degeneration was determined using computer-assisted light microscopy. Analysis of neuronal density of the hippocampal CA1 subfield was performed on coronal sections between 3.14 and 4.16 mm posterior to bregma. The total linear length of the CA1 pyramidal cell layer was measured by means of a digitizer. The number of living neurons in the stratum pyramidale within CA1 subfields was counted using a LEICA DAS microscope attached to a SONY digital camera and computer-assisted cell counting performed using Norton Eclipse software (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 the CA1 stratum pyramidale observed in each  $14\ \mu\text{m}$  section, was quantified. A mean value for each hippocampal CA1 substructure was obtained from 6 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 neuronal cell densities. Neuronal density values are expressed as mean  $\pm$  SEM.

#### *2.3.4. Statistical analysis*

The frequency of square entries and rearing behavior in the open-field was calculated every 10 min over the monitoring period in Experiment 1, and on each of the three daily sessions in Experiment 2. A global activity score was calculated by adding these behaviors at each 10 min interval. Two-way repeated measures ANOVAs were conducted with Surgery, Illumination, Time (within-session) or Day (between-session) set as the independent variables. Following a significant interaction between Surgery and Illumination, or Surgery, Illumination, and Time/Day, pairwise comparisons (controlling for multiple comparisons using Bonferroni corrections) were performed to compare the means of the different groups at each time interval. Experiment 1 also includes analysis of the first 2 min of global exploratory activity to determine initial behavioral reactivities of the animals in response to open-field exposure. The a priori interest was only in comparing the two groups within each illumination condition, thus two separate one-way ANOVAs comparing ischemic and sham-operated animals tested under bright illumination, and ischemic and sham-operated tested under dim illumination were performed.

Habituation profiles were calculated with global activity scores from Experiment 1 and 2. Since habituation is dependent on baseline levels of performance (considered here as the first 10 min of exploration), habituation profiles for individual animals during the single 30 min open-field exposure (Experiment 1) were calculated as percent changes from baseline using the following formula:  $\text{global activity (i.e., total square entries and rearing frequencies) in the second 10 min interval} / \text{global activity in the first 10 min interval (baseline)} \times 100$ , and again,  $\text{global activity in the third 10 min interval} / \text{global}$

activity in the first 10 min interval (baseline) x 100. For Experiment 2, habituation profiles for global exploratory activity for individual animals were calculated using the following formula: global activity at day 6 / global activity at day 3 (baseline) x 100, and again, global activity at day 9 / global activity at day 3 (baseline) x 100. These calculations yield time-dependent percent changes from baseline exploratory activity scores in the two last 10 min testing periods (H1 = 10-20 min interval, H2 = 20-30 min interval for Experiment 1; H1 = day 6, H2 = day 9 for Experiment 2). To determine habituation rates within each group, Surgery and Illumination were collapsed into 4 groups (isc/sham dark, and isc/sham light), and individual one-way ANOVAs for each group were conducted comparing baseline, H1 and H2. Following significant differences between means, Tukey's post-hoc tests were used to examine whether H1 and H2 were significantly different than baseline. Habituation was considered as manifested by a group when H1 and/or H2 were significantly lower than baseline. If habituation relative to baseline was observed under a particular illumination period by both groups, a one-way ANOVA comparing the two groups was used to determine whether individual groups had habituated more or less than another. Therefore, a habituation deficit in ischemic animals would be observed if they failed to habituate relative to baseline at times when sham-operated animals did, or if they habituated less than sham-operated animals, either at H1 and/or H2.

Analysis of neuronal density (number of pyramidal cells/1 mm CA1 tissue) was performed using one-way ANOVAs comparing sham and ischemic animals in bright and dim illumination conditions. One-way ANOVAs were also conducted for both Experiment 1 and 2, comparing the neuronal density of ischemic animals tested under the

different illumination conditions to ensure equivalent neuropathology. Pearson's correlation analyses were conducted with the number of viable cells at day 12 and each behavioral measure (global activity, square entries, and rearing behavior) for each group of ischemic animals. In all statistical tests, differences were considered significant when  $p < 0.05$ . Values are expressed as mean  $\pm$  SEM.

### **3. Results**

3.1. Experiment 1: Activity level and habituation to a single 30 min open-field exposure on day 5 following global ischemia

#### *3.1.1. Global exploratory activity*

Fig. 1 shows the effect of illumination on open-field global activity following cerebral ischemia. Repeated-measures two-way ANOVA on global activity at each 10 min interval revealed a main effect of Time ( $F(2,48) = 130.09$ ,  $p < .001$ ), and an interaction of Surgery x Illumination ( $F(1,24) = 8.42$ ,  $p = .008$ ), and Time x Surgery x Illumination ( $F(2,48) = 11.17$ ,  $p < .001$ ). Post-hoc comparisons revealed that differences in global activity were limited to the initial 10 min period. Within this time interval, under bright illumination ischemic animals explored more than sham-operated animals ( $p = .001$ ). Conversely, under dim illumination ischemic animals showed less exploratory activity than sham-operated animals ( $p < .001$ ). Ischemic animals also displayed significantly more exploratory behavior when tested under bright as compared to dim illumination ( $p < .001$ ). This was the opposite for sham-operated animals which showed less exploration under bright relative dim illumination ( $p < .001$ ).

### 3.1.2. Habituation profile for global exploratory activity

Fig. 2 shows the habituation profile under the different illumination conditions for global exploratory activity following global cerebral ischemia. All animals displayed habituation to the open-field. Results of the independent one-way ANOVAs revealed significant differences between baseline, H1, and H2, in all groups including ischemics tested under bright illumination ( $F(2,18)= 58.74, p<.001$ ), sham-operated animals tested under bright illumination ( $F(2,18)= 12.94, p<.001$ ), ischemic rats tested under dim illumination, ( $F(2,18)= 16.79, p<.001$ ), and sham-operated animals ( $F(2,18)= 117.62, p<.001$ ). Post-hoc analysis revealed that H1 and H2 were both significantly different than baseline in ischemic rats tested in brightness ( $p<.001, p<.001$ ), in sham-operated animals tested in brightness ( $p=.01, p<.001$ ), in ischemic rats tested in dimness ( $p=.002, p<.001$ ), and in sham-operated animals tested in dimness ( $p<.001, p<.001$ ). No significant differences in habituation rates at both H1 and H2 were observed between any of the groups under the two different illumination conditions.

### 3.1.3. Initial behavioral reactivity (first 2 min of open-field testing)

Fig. 3 demonstrates the initial behavioral reactivity of animals tested under both bright and dim illumination. A one-way ANOVA on global activity revealed that ischemic rats explored significantly more than sham-operated animals during the first two min of testing ( $F(1,12)= 6.29, p=.028$ ). A second one-way ANOVA revealed that ischemic rats explored significantly less than sham-operated animals during the first two min of testing ( $F(1,12)= 13.95, p=.003$ ).

3.2. Experiment 2: Activity level and habituation to repeated 10 min open-field exposures on day 3, 6, and 9, following global ischemia.

### 3.2.1. Global exploratory activity

Fig. 4 shows the effect of illumination on open-field global activity following cerebral ischemia. Repeated-measures two-way ANOVA revealed a main effect of Illumination ( $F(1,27)= 5.52, p=.026$ ), and an interaction of Surgery x Illumination ( $F(1,27)= 44.32, p<.001$ ), and Day x Illumination ( $F(2,54)= 21.25, p<.001$ ). On day 3, under bright illumination ischemic animals explored more than sham-operated animals ( $p=.001$ ), but under dim illumination ischemia animals explored less than sham-operated animals ( $p<.001$ ). On day 6, under bright illumination ischemic animals showed only a trend in exploring more than sham-operated animals ( $p=.059$ ), but under dim illumination explored less than sham-operated animals ( $p<.001$ ). On day 9, under bright illumination ischemic animals explored more than sham-operated animals ( $p=.04$ ), but under dim illumination explored less than sham-operated animals ( $p=.001$ ). On day 3, under bright illumination explored less than sham-operated animals ( $p=.001$ ). On day 3, under bright illumination ischemic animals explored more than those tested under dim illumination ( $p<.001$ ), and sham-operated animals tested under bright illumination explored more than those tested under dim illumination ( $p<.001$ ). At day 6, while only a trend was observed for ischemic animals tested under bright illumination to explore more than those tested under dim illumination ( $p=.053$ ), under bright illumination sham-operated animals explored less than those tested under dim conditions ( $p<.001$ ). On day 9, under bright illumination sham-operated animals explored less than those tested under dim illumination ( $p<.001$ ).

### 3.2.2. Habituation profile for global exploratory activity

Fig. 5 displays the habituation profile under the different illumination conditions for global exploratory activity following global cerebral ischemia. Only animals (both ischemic and sham-operated) tested under bright illumination displayed habituation by H2. Results of independent one-way ANOVAs revealed significant differences between baseline, H1, and/or H2 in ischemic rats tested under bright illumination ( $F(2,18)= 3.57$ ,  $p=.049$ ), and in sham-operated animals tested under bright illumination ( $F(2,24)= 6.41$ ,  $p=.006$ ). By H2, both ischemic and sham-operated animals tested under bright illumination displayed a significant percentage change from baseline levels ( $p=.041$ ,  $p=.02$ ). Habituation rates at H2 were comparable between these animals tested under bright illumination revealed that habituation rates at H2 were comparable. Under dim illumination, independent one-way ANOVAs revealed significant differences from baseline activity in ischemic ( $F(2,18)= 5.08$ ,  $p=.018$ ), but not in sham-operated animals. This effect was attributable to increased exploratory activity of ischemic rats relative to baseline values when tested under dim illumination ( $p=.016$ ), a trend that was observed, not significant in sham-operated animals. Thus, sham-operated and ischemic animals tested under dim illumination failed to display habituation, in that H1 and/or H2 means for both groups were not lower than baseline.

### 3.3. CA1 neuronal injury

Fig. 6 displays representative photomicrographs of CA1 hippocampal subfields from ischemic and sham-operated animals, and the neuronal density in this region at day

12 post-reperfusion. Individual one-way ANOVAs performed on CA1 neuronal counts for Experiment 1 and 2 revealed significant differences between sham and ischemic groups [ $F(3,24)=93.31$ ,  $p<.001$  and  $F(3,27)=197.44$ ,  $p<.001$ , respectively] attributable to reduced CA1 neuronal density in ischemic [bright ( $53\pm 9.89$ ) and dim ( $57.5\pm 6.1$ ) - Experiment 1; bright ( $56.71\pm 6.88$ ) and dim ( $49.28\pm 8.72$ ) - Experiment 2] as compared sham [bright ( $214.4\pm 6.41$ ) and dim ( $210.14\pm 6.07$ ) - Experiment 1; bright ( $217\pm 5.8$ ) and dim ( $208.5\pm 4.63$ ) - Experiment 2] animals (see Table 1). There were no group differences in neuronal density between ischemic animals tested under bright or dim illumination in both experiments. Correlational analysis revealed no significant relationship between CA1 pyramidal cell loss in the ischemic groups and activity measures at each time interval.

#### **4. Discussion**

The findings from the present study indicate that activity level in a novel open-field following global cerebral ischemia in rats is sensitive to simple variations in environmental conditions, such as the level of illumination. The alteration of open-field testing environment in the current study was motivated by inconsistent findings reported by various studies, including those of our laboratory, reporting increased, decreased, or unaltered post-ischemic exploratory activity. Global ischemia produced hyperactivity relative to sham-operated animals under bright (450 lux) open-field illumination in both Experiments 1 and 2, but activity level was reduced in these animals when testing occurred under dim (40 lux) illumination. Further, ischemic animals tested in bright illumination explored more than those tested in dim illumination, and conversely, sham-

operated animals tested under bright illumination explored less than those tested under dim conditions, as would be expected in normal animals (Bouwknicht et al., 2007; Hale, Bouwknicht, Spiga, Shekhar, & Lowry, 2006). Global cerebral ischemia in rodents therefore does not necessarily lead to hyperactivity. Even with elevated or decreased levels of exploratory activity, effects dependent on illumination, ischemic animals still displayed normal habituation to the testing context. Further, no correlations were found between hippocampal cellular viability at day 12 post-reperfusion and exploratory activity in ischemic animals, whether the testing occurred under dim or bright illumination indicating the lack of a relationship between final histopathological outcome and behavioral performance in the open-field. Lack of correlation between the degree of CA1 cell loss following global ischemia and behavioral performance have also been observed in spatial memory tasks (Paganelli et al., 2004). However, since our correlational analysis was conducted with small groups (~7 ischemic animals) and with hippocampal damage relatively homogenous within these groups, it is difficult to confidently ascertain the nature of the relationship between open-field performance and hippocampal damage.

The current study also demonstrated that global ischemia in rats does not necessarily produce habituation deficits in a novel environment or acute hyperactivity. Instead, initial behavioral reactivity to the open-field may underlie ischemic/sham differences in exploration. This is supported by Experiment 1 showing comparable reduction in overall behavioral activity in sham and ischemic animals relative to baseline levels in the last two 10 min periods of open-field exposure, irrespective of illumination conditions, and by the observation that differences in behavioral activity between the

groups were restricted to the first 10 min period, including the first 2 min of testing. If hyperactivity represented an intrinsic and pervasive effect of cerebral ischemia on exploratory tendencies, we might have expected heightened levels of activity under both bright and dim illumination conditions. With respect to between-session habituation in Experiment 2, ischemic and sham-operated animals tested under bright illumination displayed identical habituation levels over the testing days, with a reduction in global exploratory activity relative to baseline levels at day 9. This suggests that the increased frequency of exploratory behavior in ischemic animals (relative to sham animals) observed over days when tested under bright illumination is not due to a between-days habituation deficit, but perhaps to a tendency for ischemic animals to continue to explore a brightly lit environment more so than sham-operated animals independent of memory-based processes such as habituation. Of interest, the between-session data demonstrated an illumination-dependent effect on exploratory habituation. Specifically, unlike those tested under bright illumination, neither sham-operated nor ischemic animals tested under dim illumination had yet to display habituation relative to day 3 performance on the final testing day. Consequently, it is difficult to determine whether a habituation deficit in ischemic animals was present under dim illumination. Together these findings suggest that initial behavioral reactivity to novel environments underlie differences between sham-operated animals in open-field exploration, effects which are altered by differential illumination conditions, and appear independent of deficits in overall habituation or final hippocampal outcome. Had cerebral ischemia solely produced a habituation deficit or acute hyperactivity, we might have expected ischemia-induced increases in exploratory activity independent of illumination, as well as consistent differences in habituation

profiles between surgical groups over the testing periods, either within or between testing sessions.

Our hypothesis correctly predicted that ischemic animals would display increased exploratory activity only under conditions of bright illumination, an effect which appears related to the inhibition of sham-operated behavior. While cerebral ischemia (relative to controls) produced hyperexploration under bright illumination, normal exploratory activity was not apparent under dim illumination, but rather a significant tendency for hypoexploration was observed. As previously reported in untreated animals (Bouwknicht et al., 2007; Hale, Bouwknicht, Spiga, Shekhar, & Lowry, 2006; Mar, Spreekmeester, & Rochford, 2002), bright illumination suppressed locomotor activity in sham-operated animals relative to those tested under dim conditions. In contrast, bright and dim illumination had opposite effects on open-field activity levels in ischemic animals. These opposing effects observed between the two surgical groups appear to have produced the consistent differences between ischemic and sham-operated animals tested under similar illumination conditions. Interestingly, the absence of a decrease in exploration over the testing days under dim illumination suggests that habituation is not necessarily a naturally occurring phenomenon. Indeed, it is not uncommon for rats tested under low illumination (3-71 lux) to increase their exploration over days of testing in an open-field (Harro, Tonissaar, & Eller, 2001; Mallo et al., 2007). This could represent an interaction between decreased anxiety and increased familiarity, potentiating exploration over each subsequent exposure.

Since habituation deficits in exploratory activity could not be ascertained in the current study, even despite significant and comparable hippocampal cell loss in all

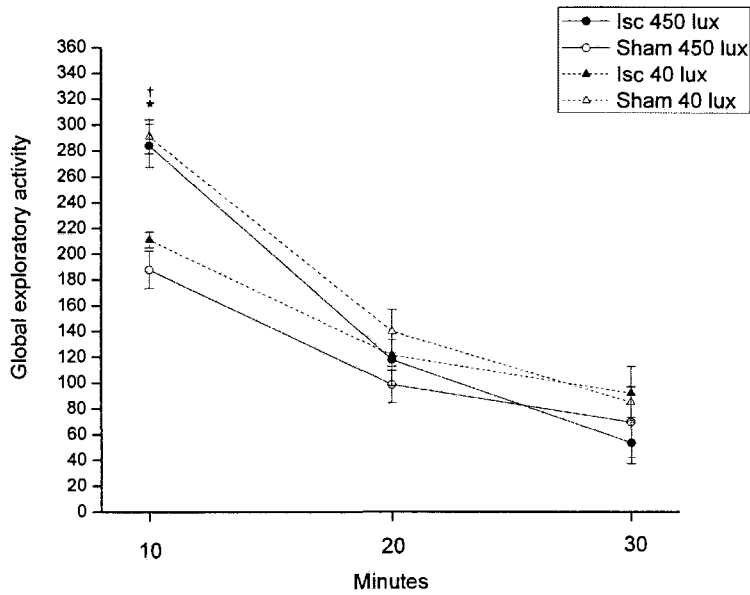
ischemic animals, it remains to be determined which system mediated these differences in exploration both under bright and dim illumination. Although the suppressing effects of bright illumination have previously been reported in non-ischemic animals, and associated with an increase in anxiogenesis, it is not known how illumination levels might exert their effects on animals subjected to cerebral ischemia. In the present study, ischemic animals appeared behaviorally disinhibited (more exploration) when tested under the more demanding situation (bright light) and behaviorally inhibited (less exploration) when tested under dim illumination. Although the long-term effects of cerebral ischemia on neurocognitive systems implicated in anxiety or motivational states have yet to be investigated, one possibility is that modulation of such a system post-ischemia mediated the observed behavioral responses to novelty under different conditions of illumination. As suggested by its impact on EPM performance (Nelson, Lebessi, Sowinski, & Hodges, 1997; Plamondon & Khan, 2005; X. B. Yan, Wang, Hou, Ji, & Zhou, 2007), cerebral ischemia may produce effects on affect and/or emotional regulation. Thus, the emotional tone of a particular situation might modulate the motivation of ischemic animals to differentially explore less stressful dimly lighted environments, while disinhibiting a naturally occurring inhibition reaction under more stressful conditions. In this context, illumination may represent an emotionally-toned condition that differentially mediates behavioral exploration of a novel environment in sham-operated and ischemic animals.

In conclusion, the current findings suggest that illumination appears to be an important parameter to consider in assessments of open-field activity, and may be helpful in the consolidation of behavioral differences reported with respect to ischemia-induced

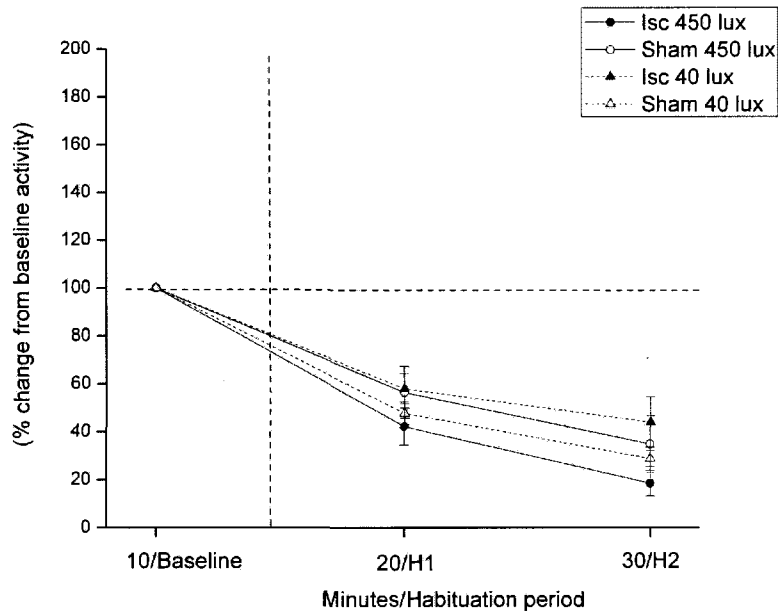
exploratory changes in this paradigm. Further studies addressing differences in emotionality, affect and/or arousal levels between sham and ischemic animals may help identify neural substrates involved in the behavioral differences observed in this study.

*Acknowledgements*

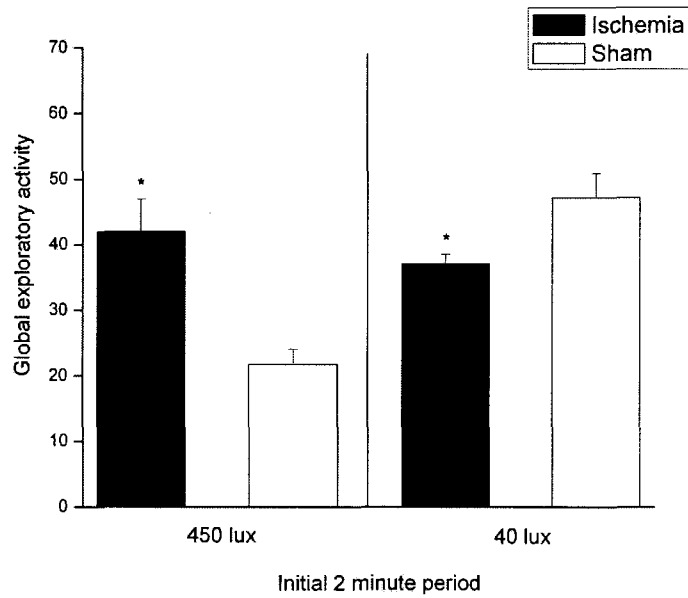
We sincerely thank Sylvie Émond for excellent technical assistance. This work was supported by a grant from the Natural Sciences and Engineering Research Council of Canada to H.P.



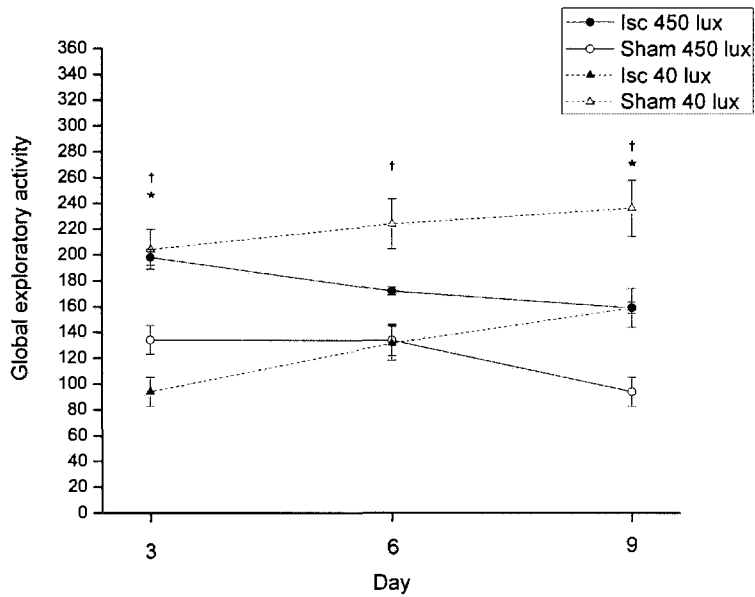
**Figure 1.** Illumination-dependent global exploratory activity (# of square entries + rearing) in the open-field during a 30 min session following cerebral ischemia. Differences were restricted to the first 10 min of testing. Global ischemia produced hyperexploration under 450 lux, but hypoexploration under 40 lux. Symbols indicate a significant difference between ischemic and sham-operated animals tested under bright illumination (\*) and dim illumination (†). Reported differences were significant at  $p < 0.05$ . Values are expressed as mean  $\pm$  SEM.



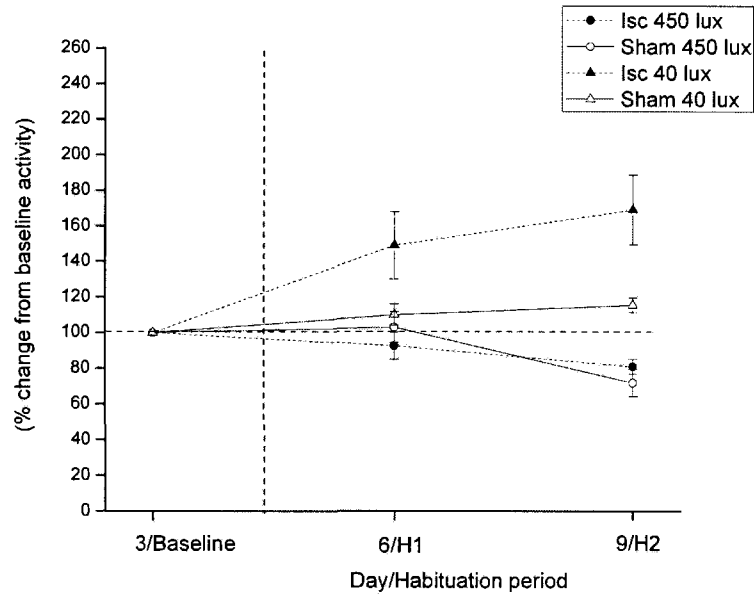
**Figure 2.** Habituation profile over a 30 min open-field test 5 days following reperfusion under bright or dim conditions. Global ischemia failed to produce habituation deficits, as indicated by comparable and significant percent change reductions in exploratory activity at H1 and H2 relative to baseline activity (first 10 min) in all animals. Reported differences were significant at  $p < 0.05$ . Values are expressed as mean  $\pm$  SEM.



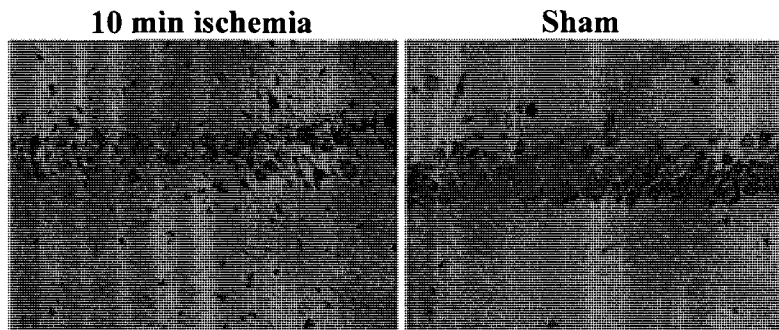
**Figure 3.** Illumination-dependent behavioral reactivity during the first 2 min of novel open-field exposure. Ischemia-induced differences in exploration were maintained during this initial time period. \*Indicates a significant difference compared to sham-operated animals. Reported differences were significant at  $p < 0.05$ . Values are expressed as mean  $\pm$  SEM.



**Figure 4.** Illumination-dependent global exploratory activity (# of square entries + rearing) in the open-field 3, 6, and 9 days following cerebral ischemia. Global ischemia produced consistent increases in exploratory activity upon repeated testing when performed under 450 lux, and hypoexploration under 40 lux. Symbols indicate a significant difference between ischemic and sham-operated animals tested under bright illumination (\*) and dim illumination (+). Reported differences were significant at  $p < 0.05$ . Values are expressed as mean  $\pm$  SEM.



**Figure 5.** Habituation profile over repeated exposure to bright or dim open-field environment. Habituation was observable in ischemic and sham animals tested under 450 lux illumination, as highlighted by significant percent change decreases in activity at day 9 (H2) relative to baseline activity (i.e., initial 10 min on day 3). In contrast, dim illumination was not associated with reduced activity level over days in either ischemic or sham-operated animals. Reported differences were significant at  $p < 0.05$ . Values are expressed as mean  $\pm$  SEM.



*Neuronal degeneration*

Surgical group	CA1 cell count
Experiment 1	
450 lux – Sham	214.4±6.41
450 lux – Ischemia	53±9.89 *
40 lux – Sham	210.14±6.07
40 lux – Ischemia	57.5±6.1 *
Experiment 2	
450 lux – Sham	217±5.8
450 lux – Ischemia	56.71±6.88 *
40 lux – Sham	208.5±4.63
40 lux – Ischemia	49.28±8.72 *

**Figure 6.** Representative photomicrographs and table (top and bottom panel, respectively) of the neuronal density in CA1 hippocampal subfields for the different experimental groups. Ten min of global ischemia produced significant neuronal death compared to sham-operated animals. Ischemic animals tested in Experiments 1 and 2 showed CA1 neuronal injury ( $\approx 75\%$ ) compared to sham-operated animals. There were no significant differences in neuronal density between ischemic animals tested under bright and dim illumination in both experiments. \*Indicates a significant difference compared to sham-operated animals. Reported differences were significant at  $p < 0.05$ . Values are expressed as mean  $\pm$  SEM.

## Experiment 2

The effect of illumination level during testing on ischemia-induced cognitive-behavioural impairment in rats

### Preface

The opposite open-field behavioural pattern observed in ischemics relative to sham animals suggests that these animals had unique appraisal and behavioural reactivity to the two illumination conditions. Experiment 2 aimed to determine whether similar effects of illumination could be elicited in other behavioural paradigms assessing more directly spatial and recognition memory capabilities.

*Abstract*

Previous experiments in our laboratory revealed illumination-dependent (bright versus dim) changes in open-field exploration in rats subjected to forebrain ischemia appearing largely independent of memory deficits, suggesting context-dependent behavioural activation in ischemic animals and attenuated behavioural reactivity under less stressful conditions (dim illumination). The present study aimed to determine whether testing under dim illumination level might improve the performance of rats subjected to ischemia on additional behavioural measures of commonly observed impairment after ischemia. Rats subjected to 10 min global ischemia or sham-operation were assessed for open-field exploration, object recognition memory, and spatial working/reference memory (e.g., spontaneous alternation) under bright (450 lux) or dim (40 lux) illumination at post-reperfusion days 7, 8, 9 and 10, respectively. Although dim illumination reversed the ischemia-induced hyperactivity in the open-field (to hypoexploration), impairments in recognition and spatial memory were not attenuated. These findings suggest that testing conditions (bright versus dim illumination) has no impact on measures of cognitive performance at the tested post-ischemic time intervals, and reveal illumination-dependent deficits in behavioural reactivity/activation in ischemic rats as demonstrated in the open-field.

## 1. Introduction

Behavioural changes observed following forebrain ischemia in rodents are generally interpreted as the result of neuronal degeneration, particularly within the hippocampus. The relationship between ischemia-induced hippocampal cell loss and functional deficits is however not that clear (Bachevalier & Meunier, 1996; Nunn et al., 1994; Olsen, Scheel-Kruger, Moller, & Jensen, 1994b; Paganelli et al., 2004), suggesting there may be additional mediators of post-ischemic behavioural change.

One possibility is that behavioural impairment after cerebral ischemia is a manifestation of alterations in emotional reactivity. Emotional and cognitive systems are intertwined (Khakpour-Taleghani, Lashgari, Motamedi, & Naghdi, 2009; Mair, Zhang, Bailey, Toupin, & Mair, 2005; Roozendaal, Okuda, de Quervain, & McGaugh, 2006), and global ischemia in rats has an impact on behavioural measures of emotional reactivity, although findings have been inconsistent. In the elevated-plus maze (EPM), the most frequently used test for unconditioned anxiety, global ischemia has no impact (Bantsiele et al., 2004) or exerts anxiolytic (Plamondon & Khan, 2005; X. B. Yan, Wang, Hou, Ji, & Zhou, 2007) or anxiogenic (Dhooper, Young, & Reid, 1997) effects. Time-dependent effects of ischemia on anxiety might explain the aforementioned discrepancies in EPM behaviour (Milot & Plamondon, 2009).

The most robust effect of forebrain ischemia on behavioural arousal in rodents is manifested by hyperactivity in the open-field (Corbett, Nurse, & Colbourne, 1997; E. J. Green et al., 1995; Herman, Adams, & Prewitt, 1995; Kuroiwa, Bonnekoh, & Hossmann, 1991; Plamondon & Khan, 2005; X. B. Yan, Wang, Hou, Ji, & Zhou, 2007). Although commonly interpreted as the result of a habituation deficit (Calabresi et al., 2000;

Colbourne, Auer, & Sutherland, 1998; Colbourne & Corbett, 1994), ischemia-induced hyperactivity might be a manifestation of alterations in behavioural/emotional arousal (Milot & Plamondon, 2008a). In non-ischemic rats, open-field exploratory activity is increased under dim illumination (versus bright) (Bouwknicht et al., 2007; Hale, Bouwknicht, Spiga, Shekhar, & Lowry, 2006; Mar, Spreekmeester, & Rochford, 2002), and possibly a manifestation of a reduction in anxiety/behavioural arousal. Global ischemia however has the opposite effect on open-field exploration, with decreased activity under dim illumination and increased activity under bright illumination (Milot & Plamondon, 2008a), suggesting that ischemia-induced hyperactivity is not inherent and/or solely attributable to a deficit in habituation memory.

One alternative explanation for open-field hyperactivity after global ischemia under bright illumination is that it is related to stress-induced behavioural disinhibition or activation. Illumination level has the effect of altering performance possibly by mediating an animals' appraisal of a testing environment, with bright illumination being more anxiogenic and stressful (Bert, Fink, Huston, & Voits, 2002; Bertoglio & Carobrez, 2002; Bouwknicht et al., 2007; Cardenas, Lamprea, & Morato, 2001; Hale, Bouwknicht, Spiga, Shekhar, & Lowry, 2006), even impairing spatial memory performance in the water maze (Chapillon & Debouzie, 2000). Other conditions increasing stress (e.g. the handling, pre-training and novelty of testing) can negatively impact cognitive performance in the water maze (Holscher, 1999). Hyperactivity (as observed in ischemic rats) is a common symptom in animal models of attentional deficit hyperactivity disorder (ADHD) in which animals (spontaneously hypertensive rats; SHR) also show cognitive/memory impairments (Ueno et al., 2002) and a reduced capacity to cope with

stressors (McDougall, Paull, Widdop, & Lawrence, 2000). In this context, cognitive impairment after ischemia might be associated to increased behavioural arousal/activation under stressful conditions such as behavioural testing.

The current experiment therefore investigated the effect of illumination level (bright versus dim) on object recognition and spontaneous alternation/spatial memory deficits in sham and ischemic animals, and whether dim illumination might positively impact performance. Discrepancies in the literature with respect to the effects of ischemia on behaviour (e.g., none versus some) could be related to unreported differences in methodology such as illumination level during testing (Milot & Plamondon, 2008a). One advantage of this manipulation is to assess the intrinsic response of ischemic animals to environments with different emotional tones, preserving some degree of ethological significance. If behavioural impairments in ischemic animals are not maintained under both illumination conditions, this could imply that underlying differences in emotional reactivity play a role in mediation of ischemia-induced cognitive impairments. Understanding the effects of testing conditions related to arousal and emotionality on ischemia-induced behavioural impairment is important, given that emotionality and arousal are usually not considered as participating factors in evaluation of functional outcome in human survivors of cerebral ischemia.

## **2. Materials and Methods**

### **2.1. Subjects**

Male Wistar rats (n=38) weighing between 250–320 g at time of surgery were obtained from Charles River Laboratories (Rochefort, Quebec, Canada). 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 rat chow. Room temperature was maintained at 21–23 °C with 60% relative humidity. Animals were handled by the experimenter for two min each on the three days preceding surgery. Animal groups were made of sham (n=7) and ischemic rats (n=7) tested under bright illumination (450 lux), and sham (n=7) and ischemic rats (n=7) tested under dim illumination (40 lux).

## 2.2. Surgical procedure

Forebrain ischemia was performed using the four-vessel occlusion model as previously described (Pulsinelli & Brierley, 1979). Briefly, rats were anesthetized by inhalation of 1.5% halothane in oxygen. The core temperature was kept at  $37\pm 0.5$  °C throughout the surgeries, including during global ischemia, by means of a feedback regulated heating blanket connected to a rectal thermometer. The vertebral arteries were irreversibly occluded by electrocoagulation, and a small-diameter silk thread looped around the carotid arteries to facilitate subsequent occlusion. Sham-operated animals underwent anesthesia and received the same dorsal and ventral surgical incisions as the ischemic group, without electrocoagulation of the vertebral arteries. Then, 24 h later, rats were briefly exposed to 1.5% halothane in oxygen, and carotid arteries re-exposed for clamping. Animals were then removed from the anaesthetic, and the common carotid arteries occluded with microvascular clamps for a 10 min period in freely ventilating rats. Only rats that lost the righting reflex over the entire occlusion period were used in the study. Under these conditions and without clamping of the carotid arteries, sham-operated rats regain the righting reflex  $< 90$  s following halothane removal. Out of the 22

animals subjected to global ischemia 8 regained the righting reflex during the 10 min period and were excluded from the study.

On day 3 post-occlusion the pupillary reflex was assessed in all animals to determine visual system/retinal damage possibly affecting the visual response to light conditions. To do so, animals were transported to a dark room and left to habituate for at least 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.

### 2.3. Experimental apparatus and behavioral procedures

Open-field locomotor activity and object recognition were assessed in the same room while the spatial recognition tests in the Y-maze were performed in a different room. Subjects were transported from the animal vivarium to the testing laboratories and allowed to rest for at least 1 h before behavioural testing began in the open-field and for object recognition. Subjects were monitored by an overhead camera, and the behaviours quantified online using datalogging software (ODlog 2.0). The open-field and Y-maze were cleaned (10% ethanol) between each test. Lux measurements were taken with a lightmeter (Proskit MT-4007) placed at the center of the open-field and Y-maze. For animals tested under bright conditions (450 lux), illumination was provided by fluorescent lights situated ~8 feet above the floor of the testing apparatus. For the dim

condition (40 lux) the lights were covered with black opaque material. The experimenter was blind to the group conditions during testing.

### *2.3.1. Novel open-field*

Animals were tested in the open-field for 10 min on day 7 post-reperfusion. The observation arena was made of gray Plexiglas (LWH: 75×75×15 cm). A painted grid divided the floor into 36 identical squares (20 peripheral, 16 center), and the entire arena was on a table 90 cm above the floor. Animals were placed in a random corner of the open-field and behavior monitored for 10 min during which the frequency of square entries (in periphery and center) was recorded for the initial and final 5 min testing period. Since habituation is dependent on baseline levels of performance within each animal (considered here as the first 5 min of exploration), habituation profiles for individual animals during the single 10 min open-field exposure were calculated as percent changes from baseline using the following formula: number of square entries in the second 5 min interval / number of square entries in the first 5 min interval (baseline) x 100. These calculations yield a percentage of activity score (H1) relative to baseline locomotor activity levels, and account for within-subject changes in activity. Lower scores indicate a greater decrease in locomotor activity in the second 5 min period relative to baseline, thus increased habituation. A habituation deficit in ischemic animals would be observed if they failed to habituate or showed decreased habituation relative to sham-operated animals.

### 2.3.2. Object recognition test

Animals were tested for object recognition on day 8 post-reperfusion in the same room and open-field arena. The object recognition memory test is based on the natural propensity of animals to spend more time exploring a novel rather than a formerly encountered (termed “familiar”) object. During testing, each rat was exposed to two trials in the open field. The objects consisted of plastic objects heavy enough to prevent a displacement by the animals. In the initial test (T1), two identical objects (O1) were placed in two corners of the open field. The time spent exploring individual objects (e.g., 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 either of the O1 objects for 20 s or when 10 min had elapsed. During the second trial (T2) performed 1 h following T1, a novel object (O2) replaced one of the O1 objects. The time spent exploring the familiar (O1) and the novel (O2) object was measured for a period of 2.5 min. The object presentation order and location was randomly permuted between animals. The arena and objects were cleaned before each trial to eliminate olfactory cues. The data was transformed into a ratio score, reflecting the preference of the animals for the novel versus the familiar object. The ratio formula is  $B - A/B+A$ , where  $A$  is the time spent exploring the familiar object and  $B$ , the time spent exploring the new object, in seconds (Ennaceur & Delacour, 1988). The higher the ratio, the more time the animal spent exploring the novel object. In order to be included in the statistical analysis, individual animals had to enter the object zone, defined as the three squares surrounding the corner square/object, a minimum of two times for each of the two objects during the test trial. All tested animals met this criterion.

### 2.3.3. *Spontaneous spatial working/reference memory*

Working memory was measured by spontaneous alternation behaviour (SAB)(Lalonde, 2002) on days 9 and 10 post-reperfusion. Similar to the object recognition test, SAB in the Y-maze is based on the propensity of rats to explore novel rather than already encountered and more familiar arms/spatial locations. Rats were tested for continuous SAB in the Y-maze, considered here as a measure of spatial recognition/working memory, in a testing environment containing salient cues (e.g., posters, paper cutouts) visible to the rat. Animals were placed into a random arm and observed for 8 min, and the sequence of arm entries recorded. An arm entry was recorded when the rat entered an arm with its four paws. A SAB was defined as any entry sequence combining the three different arms (ex: BCACBABACB), thus when the rat chose to explore the arm that had not just previously been entered. The percentage of continuous SAB was calculated using the following formula: # of spontaneous alternations/(total # of entries – 2). The higher (over 50%) the percentage of SAB, the better the spatial working memory performance is considered. The total number of arm entries quantified locomotion.

The following day, animals were tested in a delayed SAB task, considered here a measure of spatial reference memory (Coburn-Litvak, Pothakos, Tata, McCloskey, & Anderson, 2003). Rats were placed in the Y-maze surrounded by differently arranged visual cues (with respect to their location during the continuous SAB trials) but entry into a randomly chosen arm was blocked by an opaque panel. Animals explored the two available arms for 5 min then were returned to their home cage. Following a 1 h delay rats were returned to the maze for 2.5 min with all three arms now open, and the time

spent exploring the previously closed (and now relatively more novel) arm was calculated. Exploration of the middle portion of the maze (when one or more paws were not in any one arm) was not measured. Increased time spent exploring the novel arm indicates greater spatial reference memory.

#### 2.4. Analysis of neuronal survival in thionin-stained brain sections

On day 11 post-ischemia, rats were euthanized and the brains removed, frozen on dry ice, and stored at  $-80^{\circ}\text{C}$ . Serial coronal sections ( $14\ \mu\text{m}$ ) of specific brain regions were obtained using a cryostat and stained for Nissl bodies with thionin. Analysis of neuronal density was performed in hippocampal subfield CA1. The number of neurons were counted using a LEICA DAS microscope attached to a SONY digital camera and image analysis software Norton Eclipse (v 6.0), UTHSCSA ImageTool (v 3.00), and GIMP (v 2.4.4). Only neurons with normal morphology with distinct cytoplasmic and nuclear outlines and a visible nucleolus were measured. Analysis of neuronal density was performed between  $-3.14$  and  $-4.16$  mm to bregma for pyramidal neurons in hippocampal CA1 sections. The number of intact neurons per 1 mm linear length of the CA1 in each  $14\ \mu\text{m}$  section was measured. A mean value was obtained from four bilateral measurements per animal in each of the experimental groups at each brain area. The neuronal density for a given animal represents the average of both the right and left neuronal cell densities. Neuronal density values are expressed as mean  $\pm$  SEM.

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## 2. 5. Statistical analysis

Two-way ANOVA was performed on the number of open-field square entries, object recognition ratio scores, spontaneous alternation percentages/total number of arm entries (Y-maze/day 9), and time spent in the novel arm (Y-maze/day 10). Two-way repeated-measures ANOVAs were conducted with Surgery, Illumination, Time (within-session) and/or Day (between-session) set as the independent variables. Following significant interaction between Surgery and Illumination, indicating an impact of illumination on certain groups, pairwise comparisons were performed controlling for multiple comparisons using Bonferroni corrections. Following a main effect of surgery in the absence of an interaction, two contrasts compared the means of the ischemic and sham-operated animals in either of the two illumination conditions to establish the effect of ischemia on the behavioural measures. Pearson's correlation coefficients were calculated to determine the relationship between CA1 neuronal counts and all behavioural measures. One analysis included ischemic animals tested under dim and bright illumination, and two sub-analyses determined the correlation existing within each ischemic group. All tests were considered significant when  $p < .05$ . Values are expressed as mean  $\pm$  SEM.

## 3. Results

### 3.1. Open-field locomotor activity

Fig. 1 shows the effect of illumination on open-field locomotor activity following global cerebral ischemia. Two-way ANOVA revealed an interaction between surgery and illumination ( $F(3,27) = 33.39$ ,  $p < .001$ ). Post-hoc comparisons revealed that under bright

illumination ischemic animals explored more than sham-operated animals ( $p < .001$ ). Conversely, under dim illumination ischemic animals showed less exploratory activity than sham-operated animals ( $p = .005$ ). Ischemic animals also displayed significantly more exploratory behavior when tested under bright as compared to dim illumination ( $p < .001$ ). This was the opposite for sham-operated animals which showed less open-field locomotion under bright relative to dim illumination ( $p = .04$ ). Ischemic rats tested under bright illumination explored more than sham-operated animals tested in dim illumination ( $p = .008$ ), and sham-operated animals tested under bright illumination were indistinguishable from ischemic rats tested under dim illumination ( $p = .4$ ). Fig. 1 shows the effect of illumination on open-field habituation. Two-way ANOVA failed to demonstrate an interaction between surgery and illumination, indicating that illumination had no selective effects on any of the groups in habituation ( $p = .7$ ). There was also no main effect of surgery ( $p = .98$ ), indicating that ischemia produced no deficits in habituation compared to sham-operated animals (e.g., H1 scores were similar).

### 3. 2. Object recognition memory

Fig. 2 shows the effect of global ischemia on object recognition memory under bright and dim illumination. Two-way ANOVA failed to demonstrate an interaction between surgery and illumination ( $p = .65$ ). There was however a main effect of surgery ( $F(1,24) = 48.12$ ,  $p < .001$ ). Post-hoc comparisons revealed that under both bright and dim illumination ischemic animals had impaired recognition memory, as indicated by a significant decreased preference ratio compared to sham-operated animals ( $p < .001$ ,  $p < .001$ , respectively).

### 3. 3. Spatial working/reference memory

Fig. 3 (top panel) shows the effect of global ischemia on spontaneous spatial working memory under bright and dim illumination. Two-way ANOVA showed a main effect of surgery ( $F(1,24)=51.56$ ,  $p<.001$ ) but failed to demonstrate an interaction between surgery and illumination ( $p=.83$ ) or a main effect of illumination ( $p=.69$ ). Pairwise comparisons revealed that under both bright and dim illumination, ischemic animals had impaired spatial working memory, as indicated by a significant decrease in spontaneous alternation compared to sham-operated animals ( $p<.001$ ,  $p<.001$ , respectively). Fig. 3 (bottom panel) shows the effect of ischemia on the locomotion in the Y-maze under the two-illumination conditions. Two-way ANOVA revealed an interaction between surgery and illumination ( $F(3,27)= 6.67$ ,  $p=.016$ ). Post-hoc comparisons revealed that ischemic rats tested under bright entered more arms than those tested under dim illumination ( $p=.02$ ). There were no significant differences between ischemic and sham-operated animals in locomotor activity under bright ( $p=.095$ ) and dim ( $p=.067$ ) illumination.

Fig. 4 shows the effect of global ischemia on spatial reference memory under bright and dim illumination. Two-way ANOVA indicated a main effect of surgery ( $F(3,27)=25.9$ ,  $p<.001$ ) although no interaction between surgery and illumination ( $F(3,27)=1.6$ ,  $p=.31$ ) was present. Post-hoc comparisons revealed that under both bright and dim illumination ischemic animals had impaired spatial reference memory, as indicated by a significant decrease in time spent exploring the novel arm compared to sham-operated animals ( $p<.01$ ,  $p<.001$ , respectively).

### 3. 4. Neuronal degeneration

Fig. 5 shows the effect of global ischemia on neuronal degeneration in the CA1 subfield. Two-way ANOVA failed to demonstrate an interaction between surgery and illumination ( $F(3,27)=1.6, p=.31$ ). There was however a main effect of surgery ( $F(3,27)=2111, p<.001$ ). Post-hoc comparisons revealed that under both bright and dim illumination ischemic animals had suffered significant neuronal degeneration in the CA1 subfield, as indicated by a significant decrease in number of pyramidal neurons compared to sham-operated animals ( $p<.01, p<.001$ , respectively). No significant correlations were observed between the number of surviving CA1 neurons and any of the behavioural measures when including all ischemic animals tested under both illumination conditions or when analyzed separately.

## 4. Discussion

The present study investigated the impact of illumination condition on spontaneous measures of exploration and memory after forebrain ischemia. Although testing under dim illumination prevented the ischemia-induced open-field hyperactivity and reduced the locomotor activity of ischemic rats in the Y-maze, it failed to attenuate ischemia-induced impairments in object recognition and spatial memory. Contrary to a previous report showing an impairing effect of bright illumination on spatial memory performance in mice (Chapillon & Debouzie, 2000), testing under bright or dim illumination had no effect on spatial memory nor object recognition memory in both sham or ischemic groups in our study. In the open-field, the impact of illumination level was opposite in sham-operated groups, which as expected showed suppressed locomotor

activity in the more anxiogenic (bright illumination) context (Milot & Plamondon, 2008a). These findings are consistent with previously described illumination-dependent changes in open-field exploratory profiles of sham and ischemic rats, showing no habituation deficits related to the ischemic status (Milot & Plamondon, 2008). Overall, our findings suggest that testing ischemic rats under bright illumination, thus under more stressful conditions found to elicit behavioural disinhibition/hyperactivity in these animals, is not a pre-requisite for the observation of cognitive impairment in the assessed tasks, and that testing under dim illumination does not attenuate impairment.

This study was largely prompted by our interest in investigating alternative mediators of cognitive impairment following cerebral ischemia in rodents beyond those related to discrete neuronal degeneration. As aforementioned, the relationship between neuronal damage and cognitive impairment is not clear. Behavioral impairment can occur even at times when CA1 neuronal degeneration has yet to occur (Iwasaki, Kitamura, Ohgami, Mishima, & Fujiwara, 1996; Kuroiwa, Bonnekoh, & Hossmann, 1991). Further, the ischemia-induced object recognition deficit (Mumby et al., 1996; Plamondon, Morin, & Charron, 2006; Wood, Mumby, Pinel, & Phillips, 1993) is difficult to explain by hippocampal neuronal degeneration, since unlike ischemic rats tested here with selective CA1 damage those receiving complete chemical ablation of the hippocampus do not show impaired recognition memory (Lehmann, Glenn, & Mumby, 2007; Mumby, Glenn, Nesbitt, & Kyriazis, 2002). In the present study there was no direct support for an association between the observed hippocampal cell death and behavioural impairment, as no significant correlations between CA1 neuronal count and any of the behavioural measures were observed. However, since both the variability in neuronal death and the

number of animals in the ischemic groups were low, a significant correlation coefficient may be difficult to achieve. Comparing the effect of different occlusion lengths would provide greater variability in neuronal death and provide a better context for the analysis of correlation between ischemic cell death and behavioural impairment.

One possibility is that the behavioural tests used were not sensitive enough to elicit an effect of illumination due to their relative simplicity. For example, bright illumination aggravated spatial memory performance in a water maze task which included a learning component (e.g., acquisition), thus was not entirely based on spontaneous/unconditioned behaviour as assessed in the current study. The object recognition and spatial memory tasks (e.g. spontaneous alternation behaviour) are based on novelty-seeking tendencies of animals and are independent of a learning component. Thus, the effects of illumination might be elicited in more complex behavioural tasks requiring numerous trials and encompassing a learning component, or administered at later post-ischemic time intervals. Our findings however replicate the usefulness of these specific tasks in discriminating ischemic and sham behavioural performances (Mumby, 2001; X. B. Yan, Wang, Hou, Ji, & Zhou, 2007).

The open-field behaviour is difficult to interpret. Both increased and decreased locomotor activity has previously been observed following treatments which are considered anxiolytic in the EPM (Prut & Belzung, 2003), thus it is not easy to determine based on our behavioural findings the nature of the ischemia-induced reactivity deficit. What is clear however is that testing under dim illumination did not prevent the occurrence of a behavioural reactivity deficit. Indeed, ischemic rats displayed abnormal behavioural arousal in the open-field when tested under both illumination conditions not

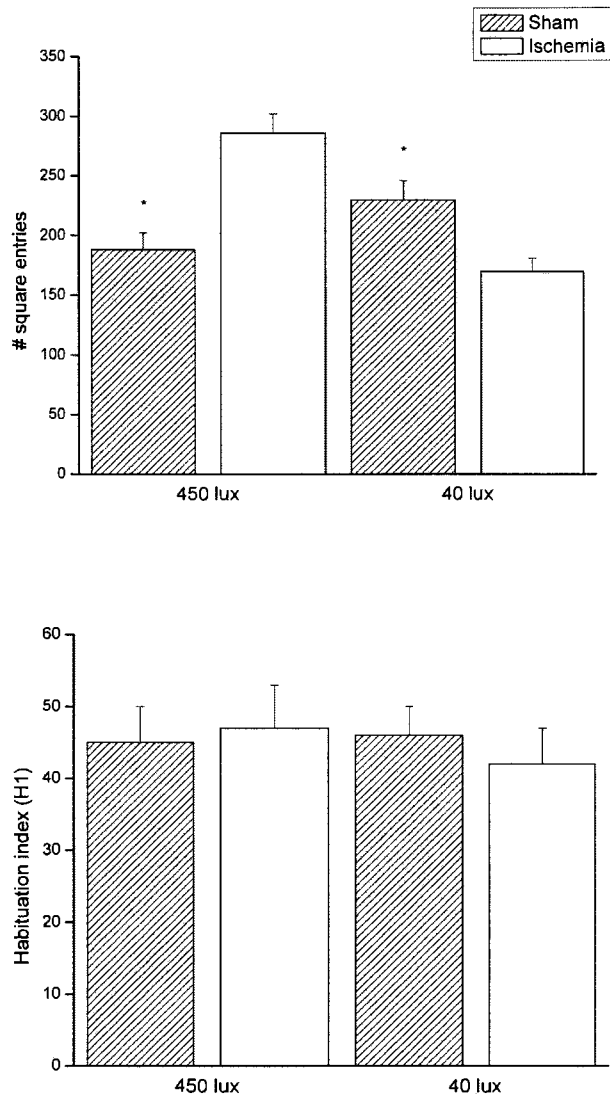
explainable by a deficit in habituation memory. Consequently, the behavioural deficits observed in recognition and spatial memory tests under dim illumination might still have been manifestations of an underlying deficit in behavioural/emotional reactivity related to illumination condition, although this is very speculative. It would be interesting to test animals under an illumination condition (ex: 200-300 lux) that would yield no differences in behavioural activation (neither hypo nor hyperactivity) to better determine the impact of emotional tone on post-ischemic behaviour.

To elucidate the impact of cerebral ischemia on emotional reactivity, future studies could measure the effect of ischemia on neurophysiological measures of stress and arousal. Indeed, very little is known about the impact of cerebral ischemia on long-term neurophysiological alterations related to emotional reactivity, despite its effects resembling those of an acute stressor enhancing CRH release (Khan, Milot, Leconte, & Plamondon) and increasing plasma levels of corticosterone in the hours and days following reperfusion (Hwang et al., 2006). Open-field hyperactivity under bright illumination has also often been associated to increased neuroendocrine reactivity (Plaznik, Danysz, & Kostowski, 1983, , 1985a, , 1985b; Sutton, Koob, Le Moal, Rivier, & Vale, 1982; Veldhuis & De Wied, 1984). It could be that long-term alterations in the HPA axis or neuroendocrine functioning after the stressor of ischemia, as commonly observed following other acute stressors (Belda et al., 2008), has significant effects on behavioural performance (e.g., indirect to the neuronal cell death) at delayed time intervals by affecting emotional reactivity to the testing conditions (ex: apparatus, novelty, and handling). Indeed, cerebral ischemia has long-lasting effects on the noradrenergic system, appearing to increase the sensitivity (e.g., outflow) of NE-

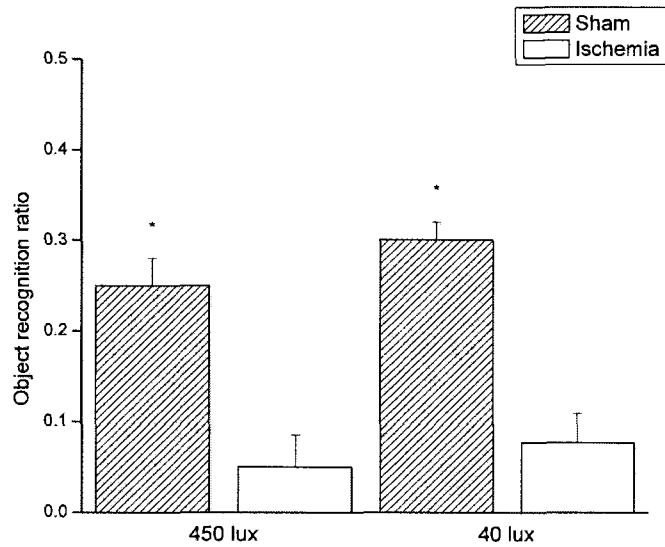
containing neurons (Pich et al., 1993). The present study was an attempt to elicit behavioural manifestations of underlying ischemia-induced neurophysiological alterations related to stress and arousal via manipulation of emotional tone during testing. In this context, other studies investigating the impact of global ischemia on common neurophysiological measures of emotional and neuroendocrine reactivity (ex: the HPA axis and neuroadrenergic system) under a variety of tasks and time intervals might further help determine the neurobiological underpinnings of behavioural impairment post-ischemia.

#### *Acknowledgements*

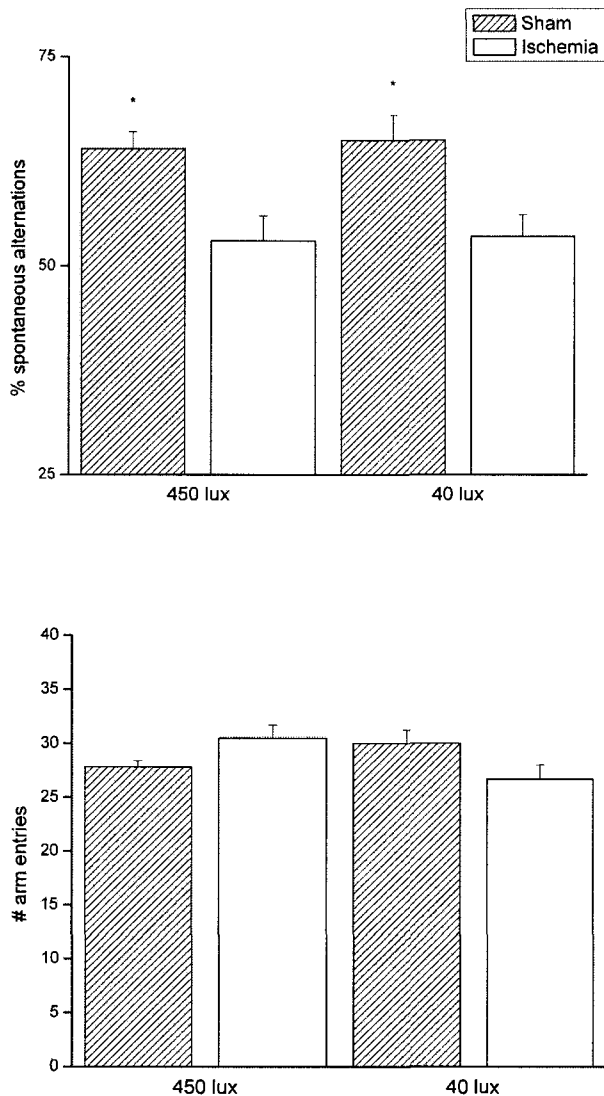
We sincerely thank Sylvie Émond for excellent technical assistance. This work was supported by a grant from the Natural Sciences and Engineering Research Council of Canada to H.P.



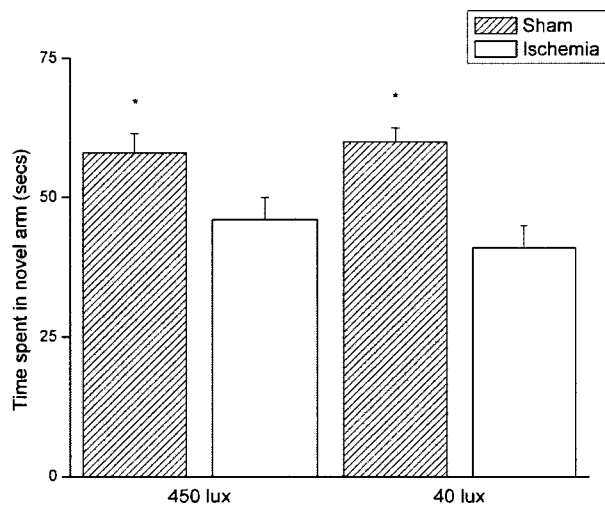
**Figure 1.** The effect of global ischemia on locomotor activity in a brightly (450 lux) or dimly lit (40 lux) open-field. Global ischemia increased locomotor activity compared to sham-operated controls when tested at 450 lux, but decreased locomotion when tested at 40 lux. \* indicates significant differences relative to ischemics tested under same illumination. Differences were significant at  $p < .05$ . Values are expressed as mean  $\pm$  SEM.



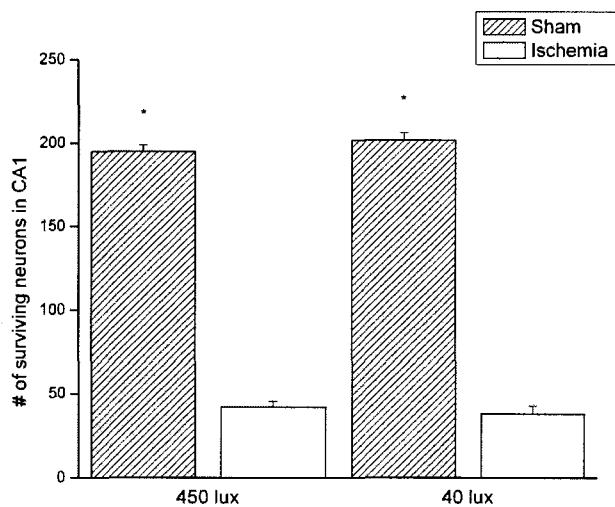
**Figure 2.** The effect of global ischemia treatment on object recognition in a brightly (450 lux) or dimly lit (40 lux) open-field. Global ischemia produced deficits in object recognition, as indicated by a reduced preference for the novel object compared to sham-operated controls at both 450 and 40 lux. Illumination had no impact on object recognition in any group. \* indicates significant differences relative to ischemics tested under same illumination. Differences were significant at  $p < .05$ . Values are expressed as mean  $\pm$  SEM.



**Figure 3.** The effect of global ischemia on spatial working memory and number of arm entries in a brightly (450 lux) or dimly (40 lux) lit Y-maze. Global ischemia impaired working memory, as indicated by reduced spontaneous alternation rates compared to sham-operated rats at both 450 and 40 lux. \* indicates significant differences relative to ischemics tested under same illumination. Illumination had no impact in any group on any measure. Differences were significant at  $p < .05$ . Values are expressed as mean  $\pm$  SEM.



**Figure 4.** The effect of global ischemia on spatial reference memory in a brightly (450 lux) or dimly (40 lux) lit Y-maze. Global ischemia produced deficits in spatial recognition/reference memory, as indicated by a significantly decreased amount of time spent in the novel arm by ischemic rats following a 1 h delay, under both 450 and 40 lux illumination. \* indicates significant differences relative to ischemics tested under same illumination. Illumination had no impact in any group on any measure. Differences were significant at  $p < .05$ . Values are expressed as mean  $\pm$  SEM.



**Figure 5.** Assessment of CA1 hippocampal injury in rats subjected to 10 min global ischemia or sham procedures. Ischemia resulted in a severe reduction of CA1 pyramidal neurons compared to controls in rats tested under both bright and dim illumination. Reported differences were significant at  $p < .05$ . Values are expressed as mean  $\pm$  SEM.

### Experiment 3

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#### Preface

Experiment 2 determined that the effects of illumination were confined to open-field exploration, but nonetheless suggested underlying ischemia-induced behavioural reactivity deficits. Given that most animal behavioural testing occurs in bright (not dim) illumination, and that ischemics display functional impairments at delayed post-reperfusion delays (Experiment 2 only examined behaviour up to day 10), Experiment 3 investigated the effect of forebrain ischemia on open-field and EPM measures of emotional/behaviour reactivity under normal illumination at delayed post-reperfusion delays (1, 5, 15, and 30 days). Doing so allowed the determination of whether ischemia-induced alterations in behavioural reactivity are longer lasting.

*Abstract*

Although changes in emotionality represent common features of post-ischemic recovery in humans, little is known about the effects of global cerebral ischemia on standard behavioral measures of emotionality in rodents. The present study investigated anxiety, locomotor activity, and habituation in test-naïve ischemic (subjected to 10 min global ischemia) and sham-operated rats tested 1, 5, 15, and 30 days post-reperfusion in the elevated plus-maze and the open-field. Although rats tested on day 1 post-reperfusion showed increased anxiety relative to sham-operated controls, they demonstrated decreased anxiety on day 5. Anxiety levels were normal on days 15 and 30 following ischemia. Similarly, time-dependent changes in locomotor activity were observed with ischemic rats showing increased activity level on days 1, 5, and 30 post-reperfusion. Surprisingly, locomotor activity was suppressed at day 15. Habituation deficits in the open-field were apparent only on day 1 despite the lack of CA1 neuronal degeneration at this time interval. These findings suggest that both the nature and extent of the effects of global ischemia on behavioral measures of emotionality, locomotion, and habituation in rats are time-dependent.

## 1. Introduction

Cardiac arrest (CA) in humans not only provokes cognitive deficits (Bergner, Hallstrom, Bergner, Eisenberg, & Cobb, 1985; Bertini et al., 1990; Grubb, O'Carroll, Cobbe, Sirel, & Fox, 1996; O'Reilly, Grubb, & O'Carroll, 2003; Parnia, Spearpoint, & Fenwick, 2007; Roine, Kajaste, & Kaste, 1993; Sauve, Doolittle, Walker, Paul, & Scheinman, 1996), it can also lead to emotional impairment manifested by increased anxiety and/or depression (Bertini et al., 1990; Grubb, O'Carroll, Cobbe, Sirel, & Fox, 1996; Ladwig et al., 1999; Roine, Kajaste, & Kaste, 1993; Sauve, 1995). On the other hand, although much is known about the effects of transient global cerebral ischemia in rodents (mimicking the severe forebrain ischemia occurring during CA) on learning and memory, its effects on behavioral measures of emotionality are not very well documented.

Like human survivors of CA, rats subjected to global ischemia exhibit changes in emotionality in the elevated plus-maze (EPM) (Bantsiele et al., 2004; Dhooper, Young, & Reid, 1997; Nelson, Lebessi, Sowinski, & Hodges, 1997; Plamondon & Khan, 2005; X. B. Yan, Wang, Hou, Ji, & Zhou, 2007). Unexpectedly, ischemic animals have been found to spend more time exploring the open arms of the EPM (Nelson, Lebessi, Sowinski, & Hodges, 1997; Plamondon & Khan, 2005; X. B. Yan, Wang, Hou, Ji, & Zhou, 2007), a behavior indicative of a decrease in anxiety. The effects of global ischemia on EPM performance are inconsistent however, and there have been reports of increased anxiety (Dhooper, Young, & Reid, 1997) or no changes in anxiety (Bantsiele et al., 2004; Roberge, Hotte-Bernard, Messier, & Plamondon, 2008) post-reperfusion. In mice, global ischemia similarly produced the contradictory effects of anxiolysis (B. Yan et al., 2007)

and angiogenesis (Nakashima, Ajiki, Nakashima, & Takahashi, 2003). Open-field exploration, which can also be considered a measure of emotional reactivity (Prut & Belzung, 2003), is strongly impacted by global ischemia. Similar to the conflicting reports with the EPM, rats subjected to global ischemia have demonstrated hyperactivity (E. J. Green et al., 1995; Plamondon & Khan, 2005; X. B. Yan, Wang, Hou, Ji, & Zhou, 2007), normal activity (Gionet et al., 1991; Gulinello, Lebesgue, Jover-Mengual, Zukin, & Etgen, 2006), or hypoactivity (Hori et al., 2002; Milot & Plamondon, 2008a) in the open-field.

These mixed findings render difficult clear establishment of the impact of global ischemia on standard measures of emotionality in rats. Many uncontrolled and/or unreported parameters affecting the reactivity of animals during EPM and open-field testing could lead to divergent results, such as illumination level (Milot & Plamondon, 2008a), duration of occlusion, day of testing post-reperfusion, or any previous experience an animal might have with testing. One trend however in EPM behavior following cerebral ischemia, without controlling for the aforementioned variables, is that of increased anxiety shortly after reperfusion (2 days)(Dhooper, Young, & Reid, 1997), decreased anxiety a few days later (e.g., 4-7 days)(Plamondon & Khan, 2005; X. B. Yan, Wang, Hou, Ji, & Zhou, 2007), and normative emotionality three weeks post-reperfusion (20 days)(Bantsiele et al., 2004).

To date, no single study has investigated the possibility of time-dependent effects of cerebral ischemia on anxiety and locomotor activity in test-naïve animals. Test naivety is an important factor to consider when assessing emotionality in the EPM. Behavioral responses in subsequent exposures to the EPM might represent processes related to

memory and habituation with respect to the testing apparatus and spatial context rather than anxiety level per se. Thus, although global cerebral ischemia by CA in rats produced increased anxiety for a period of many days following reperfusion (Dhooper, Young, & Reid, 1997), contradicting the results of decreased anxiety between days 4 and 7, the same group of rats was repeatedly tested in the EPM. Perhaps ischemic rats were more disinterested in exploring the open arms because of differences in how they habituated to/reacted to the testing apparatus over the repeated exposures compared to sham-operated animals. In this context, the measurement of EPM behavior representing more closely changes in anxiety following global ischemia can be accomplished by the utilization of test-naïve animals at each test interval.

The current study therefore investigates the short- and long-term effects of global ischemia (e.g., days 1, 5, 15, 30 post-reperfusion) on behavioral measures of anxiety (EPM) and exploration/habituation (open-field) in test-naïve rats tested under normal illumination conditions (400 lux). An additional objective was to determine whether the open-field hyperactivity observed following cerebral ischemia is mediated by ischemia-induced changes in anxiety, as previously hypothesized (Milot & Plamondon, 2008a; Plamondon & Khan, 2005).

## **2. Materials and methods**

### **2.1. Subjects**

Male Wistar rats (n=77) weighing between 250 and 320 g at time of surgery were obtained from Charles River Laboratories (Rochefort, Quebec, Canada). Animals were individually housed and maintained on a 12 h light/dark cycle (lights on at 7:00 a.m.), with free

access to water and standard rat chow. Room temperature was maintained at 21–23 °C with 60% relative humidity. Animals tested on days 5, 15, and 30 were handled for 2-3 min on the 2 days prior to surgery and on the 2 days preceding behavioral testing, and those tested on day 1 were handled on the 2 days prior to surgery and on the day they were subjected to occlusion.

## 2.2. Surgical procedure

Forebrain ischemia was performed using the four-vessel occlusion model as previously described (Pulsinelli & Brierley, 1979). Briefly, rats were anesthetized by inhalation of 1.5% halothane in oxygen. The core temperature was kept at  $37\pm 0.5$  °C throughout the surgeries, including during global ischemia, by means of a feedback regulated heating blanket connected to a rectal thermometer. The vertebral arteries were irreversibly occluded by electrocoagulation, and a small-diameter silk thread looped around the carotid arteries to facilitate subsequent occlusion. Sham-operated animals underwent anesthesia and received the same dorsal and ventral surgical incisions as the ischemic group, without electrocoagulation of the vertebral arteries. Then, 24 h later rats were briefly exposed to 1.5% halothane in oxygen, and carotid arteries re-exposed for clamping. Animals were then removed from the anaesthetic, and the common carotid arteries occluded with microvascular clamps for a 10 min period in freely ventilating rats. Only rats that lost the righting reflex over the entire occlusion period were used in the study. Under these conditions and without clamping of the carotid arteries, sham-operated rats regain the righting reflex < 90 s following halothane removal. Out of the 46 animals subjected to global ischemia, 17 regained the righting reflex during the 10 min

period and were excluded from the study. One animal died during global ischemia and two during the electrocauterization procedure.

### 2.3. Animal groups

The groups of animals were divided as follows: ischemic and sham-operated animals tested at day 1 (n=7, n=6, respectively), day 5 (n=8 in each group), and days 15 and 30 (n=7 in each group and for each of the testing days) following reperfusion.

### 2.4. Apparatus and experimental procedures

Rats were transported from the animal vivarium to the testing laboratory and allowed to rest for at least 1 h before behavioral testing began. Testing was monitored by an overhead camera, and behavioral measures quantified online using datalogging software (ODlog 2.0). The EPM and open-field were separated by a divider panel, and surrounded by visual cues present in the testing room (e.g., equipment, posters). Rats were first tested in the EPM then immediately placed in the open-field for assessment of locomotor activity. The open-field and EPM were wiped clean (15% ethanol) between each test. Lux measurements were taken with a lightmeter (Proskit MT-4007) placed in the open arms and in the middle of the open-field. Illumination was provided by overhead fluorescent lights. The experimenter was blind to the group conditions during testing.

#### 2.4.1. *Elevated plus-maze*

The EPM consisted of two opposing open arms (50 cm × 10 cm with a 5 mm clear Plexiglas lip), an open 10 cm × 10 cm area in the center and two opposing closed

arms (50 cm × 10 cm with 40 cm high walls). The entire maze was elevated 60 cm from the floor. The rat was placed in the center of the elevated plus-maze facing one of the closed arms. The time spent and the number of entries in the open and closed arms was recorded during a 6 min test period. A closed or open arm entry was defined as all four paws entering an arm.

#### *2.4.2. Open-field*

The observation arena was made of gray Plexiglas (LWH: 75 cm × 75 cm × 15 cm). A painted grid divided the floor into 36 identical squares (20 peripheral, 16 center), and the entire arena was on a table 90 cm above the floor. Animals were placed in a random corner of the open-field and behavior monitored for 10 min during which the frequency of square entries (in periphery and center) was recorded for the initial and final 5 min testing period.

Since habituation is dependent on baseline levels of performance within each animal (considered here as the first 5 min of exploration), habituation profiles for individual animals during the single 10 min open-field exposure were calculated as percent changes from baseline using the following formula: number of square entries in the second 5 min interval / number of square entries in the first 5 min interval (baseline) × 100. These calculations yield a percentage of activity score (H1) relative to baseline locomotor activity levels, and account only for within-subject changes in activity. Lower scores indicate a greater decrease in locomotor activity in the second 5 min period relative to baseline, thus increased habituation.

#### *2.4.3. Analysis of neuronal survival in thionin stained brain sections*

Following completion of the testing session, rats were immediately euthanized and the brains removed, frozen on dry ice, and stored at  $-80^{\circ}\text{C}$ . Serial coronal sections ( $14\ \mu\text{m}$ ) of specific brain regions were obtained using a cryostat and stained for Nissl bodies with thionin. Analysis of neuronal density was performed in hippocampal sectors CA1. The number of neurons was counted manually using a LEICA DAS microscope attached to a SONY digital camera, and image analysis software Norton Eclipse (v 6.0), UTHSCSA ImageTool (v 3.00) and GIMP (v 2.4.4). Only neurons with normal morphology with distinct cytoplasmic and nuclear outlines and a visible nucleolus were counted. Analysis of density of pyramidal neurons in hippocampal areas CA1 was performed on sections between  $-3.14$  and  $-4.16$  mm relative to bregma. The number of intact neurons per 1 mm linear length of the CA1 in each  $14\ \mu\text{m}$  section was quantified. A mean value was obtained from three 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 neuronal cell densities. Neuronal density values are expressed as mean  $\pm$  SEM.

#### *2.4.4. Statistical analysis*

Separate one-way ANOVA were performed at each time interval and compared ischemic to sham-operated animals on the time spent in open arms, the number of open arm entries, and the total number of arm entries in the EPM. For the open-field measures, one-way ANOVA were performed on the number of central square entries and the percent of central square entries (total number of central entries/total entries  $\times$  100). One-way repeated measures ANOVA compared ischemic to sham-operated rats at each post-reperfusion test interval in their number of square entries during the first and second 5

min test period, and significant effects of group or an interaction between group and test block were followed by ischemia/sham comparisons at each of the two 5 min testing bins using one-way ANOVA. To determine habituation rates one sample t-tests comparing H1 to a score of 100 were conducted for each surgical group at each post-reperfusion time interval. If habituation relative to baseline was observed by both groups, a one-way ANOVA comparing H1 between the two groups determined whether individual groups had habituated more or less than another. A habituation deficit in ischemic animals would be observed if they failed to habituate relative to baseline at times when sham-operated animals did, or if they habituated less than sham-operated animals. Finally, one-way ANOVA determined the between-group effects of global ischemia on the number of surviving CA1 neurons at each time interval.

To determine the impact of post-reperfusion delay on the dependent variables assessed within each surgical group, separate one-way ANOVA were conducted comparing the total number of square entries in the 10 min open-field period, the time spent in the EPM open arms, and the CA1 neuronal degeneration recorded at post-ischemic days 1, 5 15, and 30 within the ischemic or sham-operated groups. Following a significant effect of testing day Tukey's post-hoc tests compared each of these measures across the testing days. One exception was the use of the Kruskal-Wallis test to determine the effect of testing day on the time spent in open arms by ischemics (followed by Games-Howell post-hoc comparisons) since the assumption of homogeneity of variance was violated for this particular analysis.

### 3. Results

#### 3.1. Between-group comparisons

##### 3.1.1. Day 1

Fig. 1a and 2a show the effect of global ischemia on EPM and open-field behavior on day 1 post-reperfusion. In the EPM global ischemia produced a significant decrease in the time spent in open arms ( $F(1,11) = 15.18, p = .002$ ), and in the number of open arm entries ( $F(1,11) = 39.44, p < .001$ ). No group differences were observed in the total number of arm entries ( $p = .89$ ). In the open-field global ischemia increased locomotor activity during the 10 min test period ( $F(1,11) = 24.72, p < .001$ ). Specifically, global ischemia produced hyperactivity during both the initial and final 5 min of testing ( $p = .001, p = .001$ , respectively). Global ischemia reduced the percentage of square entries made into central squares ( $F(1,11) = 5.56, p = .038$ ), although the total number was not different ( $p = .16$ ). Although both ischemic and sham-operated rats habituated relative to baseline [ $t(6) = -7.13, p < .001, t(5) = -10.79, p < .001$ , respectively], ischemic animals displayed a habituation deficit as indicated by a higher H1 score compared to sham-operated animals ( $F(1, 11) = 5.19, p = .044$ ).

##### 3.1.2. Day 5

Fig. 1b and 2b show the effect of global ischemia on EPM and open-field behavior on day 5 post-reperfusion. In the EPM global ischemia produced a significant increase in the time spent in open arms ( $F(1,14) = 7.22, p = .018$ ), and in the number of open arm entries ( $F(1,14) = 9.35, p = .009$ ). No group differences were observed in the total number of arm entries ( $p = 0.16$ ). In the open-field global ischemia increased

locomotor activity during the 10 min test period ( $F(1,14)= 7.98, p=.014$ ). Specifically, global ischemia produced hyperactivity during both the initial and final 5 min of testing ( $p=.018, p=.021$ , respectively). Global ischemia increased the percentage of square entries made into central squares ( $F(1,14)= 5.72, p=.031$ ), as well as the total number of entries ( $F(1,14)= 6.85, p=.02$ ). Both ischemic and sham-operated animals habituated relative to baseline [ $t(7)= -14.98, p<.001$  ,  $t(7)= -17.58, p<.001$ , respectively)], and habituation (H1) was comparable between the two groups ( $p=.429$ ).

### 3.1.3. Day 15

Fig. 1c and 2c show the effect of global ischemia on EPM and open-field behavior on day 15 post-reperfusion. In the EPM global ischemia did not significantly modify the time spent in open arms ( $p=.37$ ), nor the number of open arm entries ( $p=.22$ ). No group differences were observed in the total number of arm entries ( $p=0.72$ ). In the open-field global ischemia produced significantly decreased locomotor activity during the 10 min test period ( $F(1,12)= 46.44, p<.001$ ). Global ischemia produced hyperactivity during both the initial and final 5 min of testing ( $p<.001, p=.002$ , respectively). Global ischemia reduced the percentage of square entries made into central squares ( $F(1,12)= 5.86, p=.032$ ), as well as the total number of entries ( $F(1,14)= 7.19, p=.02$ ). Both ischemic and sham-operated ( $t(6)= -4.36, p=.005$ ) habituated relative to baseline, although a t-test was not possible for ischemics since every animal had a score of zero for the final 5 min period. Although ischemic animals appeared to have increased habituation (a lower H1 score) compared to sham-operated animals ( $F(1,12)= 12.98, p<.001$ ), this was likely due to their overall severe reductions in locomotor activity.

### 3.1.4. Day 30

Fig. 1d and 2d show the effect of global ischemia on EPM and open-field behavior on day 30 post-reperfusion. In the EPM global ischemia had no effect on the time spent in open arms ( $p=.89$ ), nor in the number of open arm entries ( $p=.82$ ). No group differences were observed in the total number of arm entries in the EPM ( $p=.81$ ). In the open-field global ischemia produced significantly increased locomotor activity during the 10 min test period ( $F(1,12)= 5.04$ ,  $p=.044$ ). Global ischemia did not produce significant change in locomotor activity at the initial and final 5 min of testing ( $p=.081$ ,  $p=.14$ , respectively). Global ischemia did not modify the percentage of entries made into central squares ( $F(1,12)=.02$ ,  $p=.89$ ), as well as the total number of entries ( $F(1,12)= .52$ ,  $p=.48$ ). Both ischemic and sham-operated animals habituated relative to baseline [ $t(6)= -5.08$ ,  $p=.002$ ,  $t(6)= -9.99$ ,  $p<.001$ , respectively), and habituation (H1) was comparable ( $p=.34$ )

### 3.5. Neuronal degeneration

Fig. 5 shows the time-dependent effects of global ischemia on neuronal degeneration in the CA1. Global ischemia produced no significant CA1 cell loss on day 1 following reperfusion ( $F(1,11)= .026$ ,  $p=.87$ ), but led to significant neuronal degeneration at days 5, 15, and 30 [ $F(1,14)= 122.63$ ,  $p<.001$ ,  $F(1,12)= 293.45$ ,  $p<.001$ ,  $F(1,12)= 107.12$ ,  $p<.001$ , respectively].

### 3.6. Within-group effects over the testing delays

Figs. 3-4 show the within-group effects over the testing delays in the ischemic or sham-operated groups in the time spent in open arms and total number of square entries

during the 10 min open-field session, and CA1 neuronal degeneration, respectively. In ischemic rats there was a significant effect of testing delay on the time spent in open arms ( $\chi^2(3, N = 29) = 15.87, p < .001$ ), the number of square entries ( $F(3,25) = 42.25, p < .001$ ), and neuronal degeneration ( $F(3, 25) = 64.53, p < .001$ ). Following sham-operation there was a significant effect of testing delay on the number of square entries ( $F(3,24) = 3.51, p = .031$ ), but not on the time spent in the open arms ( $F(3,24) = .17, p = .92$ ) or neuronal degeneration ( $F(3,24) = .46, p = .72$ ). Results of post-hoc tests are reported in Tables 1-3 for open arm time, locomotion, and CA1 neuronal count, respectively.

#### **4. Discussion**

Our findings revealed time-dependent effects of global ischemia on behavioral measures of emotionality, exploration, and habituation in rats. In the EPM and open-field global ischemia produced anxiogenic-like behavior 24 h following reperfusion. The reverse, anxiolytic-like effects of ischemia were observed after 5 days. No differences between ischemic and sham-operated animals on measures of anxiety were apparent after 15 and 30 days. In parallel, locomotor activity was significantly increased after 1 and 5 days, but was unexpectedly reduced after 15 days compared to sham controls. Hyperactivity re-appeared after 30 days, although to a lesser degree than that observed on day 1, and comparable to activity on day 5. Cerebral ischemia produced a habituation memory deficit only 24 h post-reperfusion when neuronal degeneration was absent. The effects of global ischemia on measures of anxiety and habituation were therefore transient, while alterations in locomotion apparent at all intervals although their nature was time-dependent.

The observed effects of global ischemia on EPM behavior are in agreement with previous findings of decreased anxiety 4-7 days following reperfusion (Plamondon & Khan, 2005; X. B. Yan, Wang, Hou, Ji, & Zhou, 2007), with normal levels observed a few weeks after ischemia (Bantsiele et al., 2004). Our study is the first to report increased anxiety 24 h following cerebral ischemia in rats, although reduced anxiety was observed 2 days following forebrain ischemia by CA (Dhooper, Young, & Reid, 1997). The hyperactivity observed in the open-field was largely concordant with previous findings with ischemic rats and gerbils (Colbourne, Auer, & Sutherland, 1998; Colbourne & Corbett, 1994; E. J. Green et al., 1995; Plamondon & Khan, 2005; X. B. Yan, Hou, Wu, Liu, & Zhou, 2007). The significant reduction in open-field activity on day 15 is unusual given that normal locomotor activity was observed in the EPM. To our knowledge, no studies have examined novel open-field exploration at (or near) 15 days post-reperfusion in test-naïve rats, thus the hypoactivity we observed at this time interval remains to be corroborated. Hypoactivity in the open-field has been reported at other time intervals (Hori et al., 2002) or under different conditions of illumination following ischemia (Milot & Plamondon, 2008a), suggesting that exploratory deficits following global ischemia can be bidirectional. After 24 h ischemic animals displayed impaired habituation to the open-field, a phenomenon which can be interpreted as a type of nonassociative memory failure, and/or an inability to form spatial maps (Wang & Corbett, 1990). The findings of unimpaired habituation at all other testing intervals are in agreement with previous observations in our laboratory which showed normal within and between open-field session habituation in ischemic animals tested between 3 and 9 days post-reperfusion (Milot & Plamondon, 2008a). Further, it is likely that the lower H1 score by ischemic

animals on day 5 represents an overall deficit in exploration rather than enhanced habituation relative to sham-operated controls. Finally, although the open-field test was novel (both the apparatus and the spatial environment), the locomotor activity displayed by the ischemic and sham-operated rats might have been different without initial exposure to the EPM.

We previously hypothesized that changes in anxiety might contribute to the hyperactivity observed in ischemic rats, with reductions in anxiety preventing the normally occurring inhibition of exploration under stressful situations (Milot & Plamondon, 2008a; Plamondon & Khan, 2005). However, having assessed both anxiety and open-field activity in the present study we observed the co-occurrence of highest anxiety and hyperactivity levels at 24 h post-reperfusion. Also, while locomotor activity was comparable after 5 and 30 days, ischemic rats displayed different EPM behavioral profiles at these intervals. Finally, while anxiety levels were comparable between ischemic animals tested after 15 and 30 days, their locomotor activity changes were in opposite directions. These observations indicate that anxiety level does not consistently predict open-field activity at the different time intervals following global ischemia.

The comparable locomotor activity displayed by sham and ischemic rats in the EPM indicates that ischemia-induced exploratory deficits are not pervasive across all testing contexts, and are more prone to be elicited in situations where rats are forced to explore a bright open environment (e.g., open-field) presenting no possible escape into darker and presumably safer compartment. One possibility is that locomotor changes observed after cerebral ischemia represent a behavioral reactivity/arousal deficit (e.g., hypereactivity/hyporeactivity), given that anxiety and habituation deficits can be and

large be ruled out of their mediation. This is supported by the observation that ischemics display deficits in exploration (hyper or hypoexploration depending on the illumination condition) even in the initial 2 min of open-field testing (Milot & Plamondon, 2008a). These immediately elicited exploratory deficits may represent impaired behavioral reactivity to stressful testing contexts.

The absence of overt neuronal death 24 h following ischemia and the gradual degeneration during the initial weeks are similar to previous findings (Bendel et al., 2005; Colbourne, Li, Buchan, & Clemens, 1999). The observation of ischemia-induced habituation, locomotor and emotional differences as early as 24 h suggests that the presence of discrete CA1 neuronal death was not necessary for such behavioral change. The locomotor activity profile was similar at post-ischemic day 5 and 30 when CA1 neuronal assessments were comparable, but was suppressed on day 15 (compared to day 5) when there was significantly more neuronal degeneration (than day 5). This latter observation is contrary to what would be expected if exploratory changes after ischemia (e.g., hyperactivity) were solely related to habituation/spatial mapping deficits associated to hippocampal cell death (Wang & Corbett, 1990). However, the extent to which neuronal degeneration was associated to locomotor activity (and other observed behaviors) at remote time intervals when neuronal degeneration has occurred remains to be determined. Comparing the effect of different occlusion lengths (e.g., 10 min versus 20 min) would provide a clearer picture with respect to the relationship between cell loss and changes in behavioral activity.

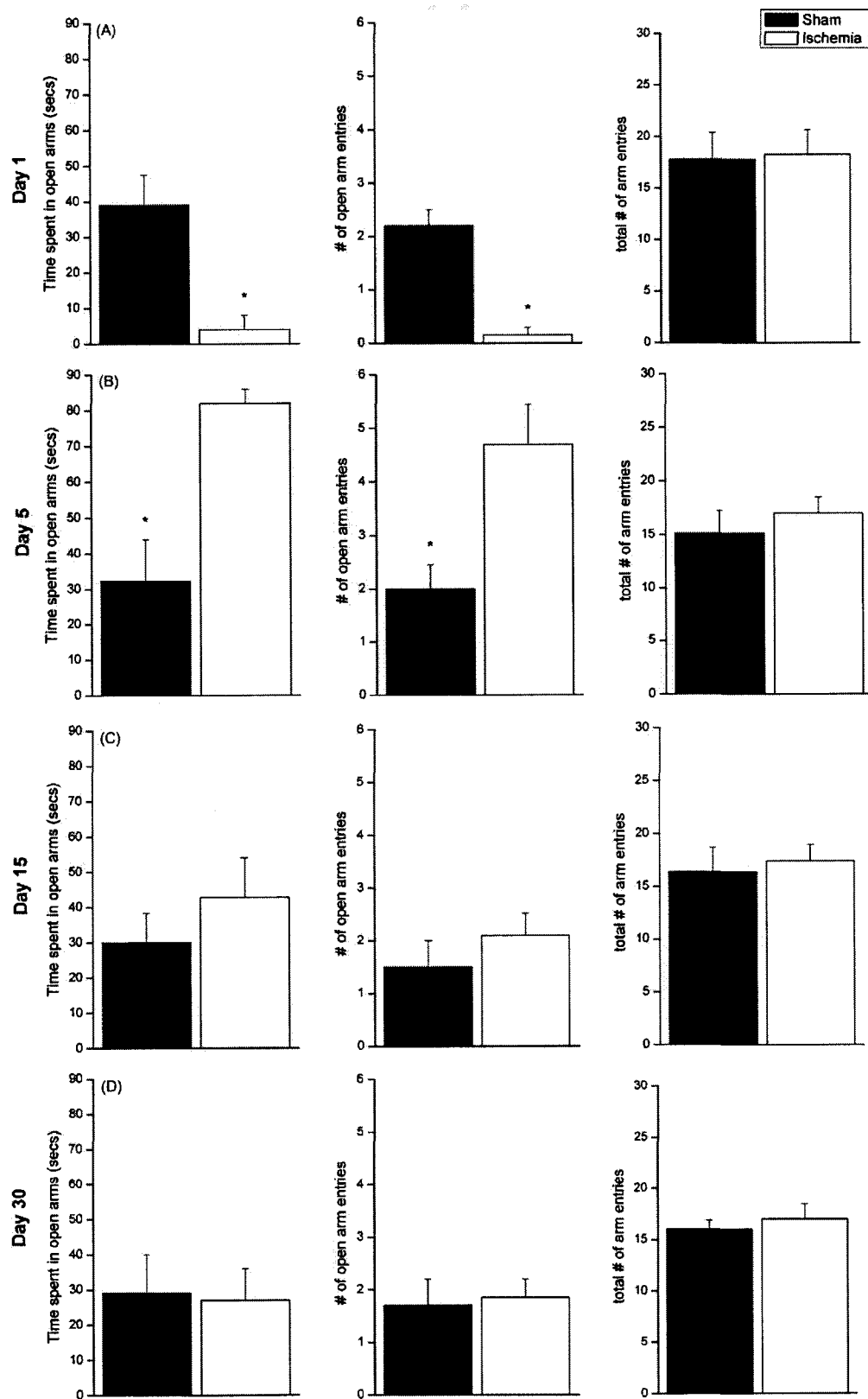
One possibility is that transient neurophysiological changes induced by cerebral ischemia produce some of the effects on emotionality and locomotor activity, explaining

the time-dependent effects. Opposing behavioral changes observed at distinct time-intervals in the EPM (e.g., day 1 versus day 5) and open-field (e.g., day 1 versus day 15) suggest the initiation of compensatory mechanisms, which could be the result of ischemia-induced physiological dysregulation. An interesting finding supporting the potential involvement of discrete neurochemical factors in ischemia-induced behavioral change was the prevention of ischemia-induced hyperactivity in the open-field by pharmacological blockade of CRH receptors prior to global ischemia despite having no neuroprotective effects (Plamondon & Khan, 2006). One interesting avenue may be the investigation of the effects of cerebral ischemia on neurobiological systems implicated in behavioral reactivity and arousal (e.g., HPA and noradrenergic functioning) at times when behavioral testing occurs.

Whether emotional change following cerebral ischemia represents an important factor to consider in the assessment of post-ischemic cognitive and functional recovery remains to be determined. Indeed, emotional changes in humans subjected to cerebral ischemia are not simply related to discrete neurochemical effects or brain damage, but to complex variables related to disruptions in functional capabilities, quality of life (Middelkamp et al., 2007), sense of control (etc.), which are not accessible in animal studies. Future clinical studies combining investigation of the biological and psychological conditions that regulate emotional change after stroke and CA could address these issues.

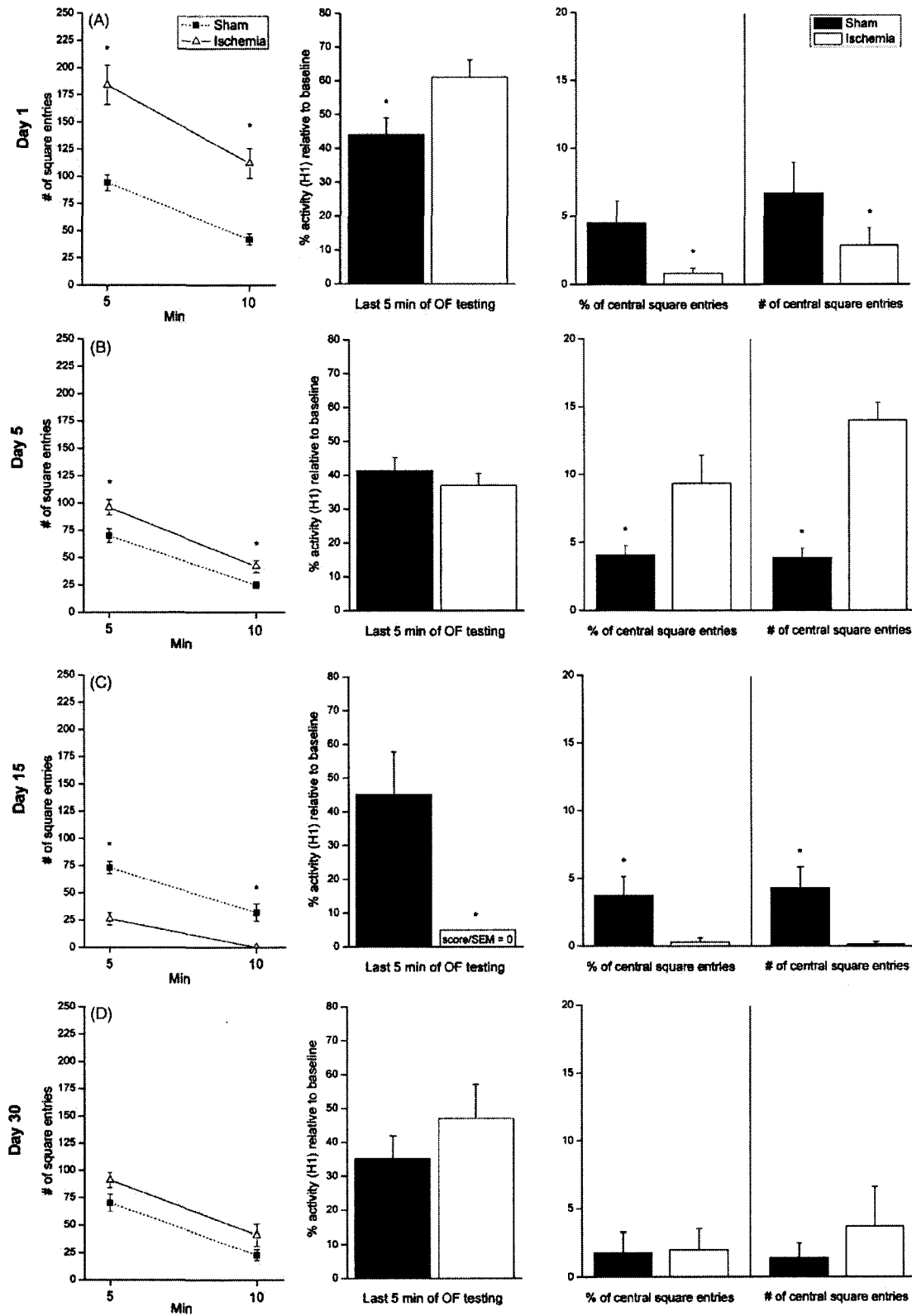
*Acknowledgements*

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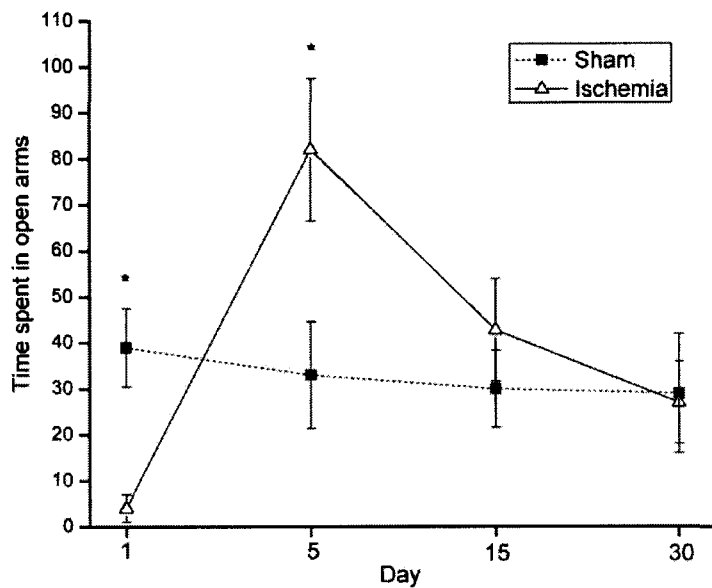
**Figure 1.** The effect of 10 min global ischemia on measures of anxiety in the EPM on days 1, 5, 15, and 30 post-reperfusion. Global ischemia produced increased anxiety (less

time in open arms/open arm entries) on day 1, but decreased anxiety on day 5 relative to sham-operated controls. No effects of ischemia on measures of anxiety were observed at days 15 and day 30. Locomotor activity (total number of entries) in the plus-maze was unaffected by global ischemia at any post-reperfusion delay. Symbols indicate a significant difference between ischemic and sham-operated (\*). Reported differences were significant at  $p < .05$ . Values are expressed as mean  $\pm$  SEM.

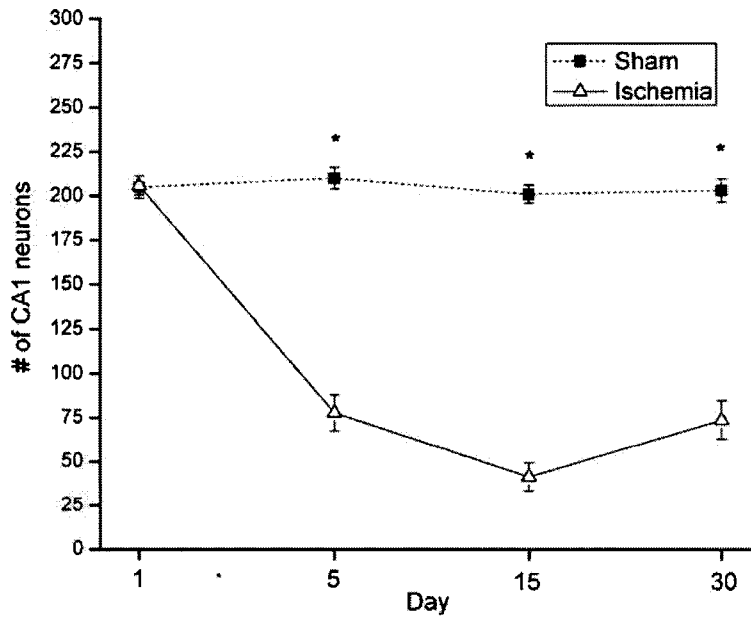


**Figure 2.** The effect of 10 min global ischemia on locomotor activity and habituation in the open-field (OF) on days 1, 5, 15, and 30. Global ischemia produced increased

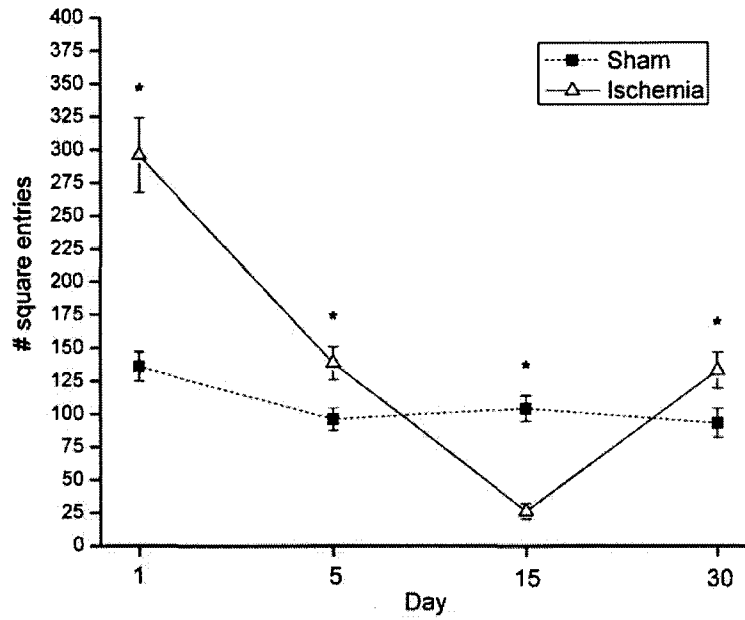
locomotor on days 1, 5, and 30 post-reperfusion, but decreased locomotion on day 15. Impaired habituation by ischemics was observed only on day 1. Ischemic animals explored the center of the open-field less on days 1 and 15, but explored the center more on day 5. Symbols indicate a significant difference between ischemic and sham-operated animals (\*). Reported differences were significant at  $p < .05$ . Values are expressed as mean  $\pm$  SEM.



**Figure 3.** The effects of 10 min global ischemia on anxiety at each test interval. Symbols indicate a significant difference between ischemic and sham-operated animals (\*). Table 1 compares anxiety levels between days within the ischemic or sham-operated groups. Global ischemia significantly decreased the time spent in open-arms on day 1 compared to other ischemic rats tested on days 5 and 15. Animals subject to global ischemia spend more time in the open arms on day 5 compared to day 30. No between-day differences were observed in the sham-operated animals on measures of anxiety. Reported differences were significant at  $p < .05$ . Values are expressed as mean  $\pm$  SEM.



**Figure 4.** The effects of 10 min global ischemia on locomotor activity during the 10 min open-field test period at each test interval. Symbols indicate a significant difference between ischemic and sham-operated animals (\*). Table 2 compares locomotor activity between days within the ischemic or sham-operated groups. Global ischemia significantly increased locomotor activity on day 1 compared to other ischemics tested on days 5, 15, and 30. Animals subject to global ischemia displayed more locomotor activity on day 5 compared to day 15, and on day 15 compared to day 30. Sham-operated animals tested at day 1 displayed more locomotor activity on day 1 compared to days 5 and 30. Reported differences were significant at  $p < .05$ . Values are expressed as mean  $\pm$  SEM.



**Figure 5.** The effects of 10 min global ischemia on neuronal survival in CA1 subfield at each test interval. Symbols indicate a significant difference between ischemic and sham-operated animals (\*). Table 3 compares neuronal counts between days within the ischemic or sham-operated groups. Global ischemia led to significant neuronal degeneration on days 5, 15, and 30 compared to day 1. Neuronal degeneration on day 15 was increased relative to day 5, and similar to day 30. No between-day differences were observed in the sham-operated animals on neuronal counts. Reported differences were significant at  $p < .05$ . Values are expressed as mean  $\pm$  SEM.

Table 1.

*Between-day multiple comparisons: time spent in open arms*

Group/day	30	Day 15	5
<i>Ischemia</i>			
Day 1	p=.17	*p=.049	*p=.004
Day 5	*p=.03	p=.19	N/A
Day 15	p=.68	N/A	p=.19
<i>Sham</i>			
No differences to report			

Table 2.

*Between-day multiple comparisons: number of square entries in 10 min period*

Group/day	30	Day 15	5
<i>Ischemia</i>			
Day 1	*p<.001	*p<.001	*p<.001
Day 5	p=.99	*p<.001	N/A
Day 15	*p<.001	N/A	*p<.001
<i>Sham</i>			
Day 1	*p=.036	p=.16	*p=.046
Day 5	p=.99	p=.94	N/A
Day 15	p=.87	N/A	P=.93

Table 3.

*Between-day multiple comparisons: CA1 neuronal degeneration*

Group/day	30	Day 15	5
<i>Ischemia</i>			
Day 1	*p<.001	*p<.001	*p<.001
Day 5	p=.98	*p=.034	N/A
Day 15	p=.082	N/A	*p=.034
<i>Sham</i>			
No differences to report			

## Experiment 4

HPA axis dysregulation after forebrain ischemia: Relationship to long-term neuronal and behavioural outcome, and effect of metyrapone administration

### Preface

Overall, Experiments 1, 2 and 3 together showed that forebrain ischemia can impact behavioural and emotional reactivity, effects which were relatively long lasting (e.g., open-field hyperactivity persisting at 30 days post-ischemia). Given that alterations in open-field activity are associated to neuroendocrine reactivity, and this system implicated in cognitive functioning (e.g., in spatial memory tasks), Experiment 4 aimed to determine the impact of forebrain ischemia on HPA axis regulation, both in the short-term and at delayed intervals when behavioural testing occurs (e.g., in normal/bright illumination). This could establish whether a relationship exists between the effects of ischemia as a stressor and functional and neuronal outcome, a research endeavor further advanced by determining the impact of inhibiting corticosterone synthesis (by metyrapone administration) pre-occlusion on these same measures.

*Abstract*

Cerebral ischemia is a significant stressor associated to HPA axis dysregulation. Investigation of the effects of ischemia on the HPA axis and neuronal injury has nonetheless been restricted to short-term evaluation. Further, as observed following other acute and subchronic stressors, ischemia could potentially produce long-term neuroendocrine dysfunction associated to cognitive impairment, a possibility which has yet to be considered. The present study investigated the impact of forebrain ischemia on plasma glucose, corticosterone (CORT), and ACTH at a variety of post-reperfusion intervals (1 h, and 1, 3 and 7 days). Wistar rats were administered metyrapone, a glucocorticoid synthesis inhibitor, or the vehicle prior to 10 min forebrain ischemia induced by four-vessel occlusion. Spatial working memory was assessed 9 days post-reperfusion in the Y-maze (spontaneous alternation), and spatial reference memory assessed in the Barnes maze between days 10 and 14. Neuronal survival in the hippocampal subfield CA1 was assessed 30 days post-reperfusion. The results showed ischemia-induced upregulation of the HPA axis lasting for at least 3 days (significant elevations in CORT), as well as in response to behavioural testing (significantly increased post-testing CORT levels) despite normal resting levels (e.g., as observed on day 7), effects associated to impairments in memory. Metyrapone treatment reduced ischemia-induced CORT elevations at all time intervals (including in response to testing), but significantly elevated early HPA axis activity (e.g., ACTH) up to 24 h post-reperfusion, and produced hyperglycemia in treated animals observed 1 h after ischemia. Importantly, metyrapone treated ischemics displayed no deficits in spatial memory despite showing increased CA1 neuronal death compared to vehicle-treated animals.

These findings reconsider the role of the HPA axis in post-ischemia neuronal and functional outcome, and suggest that ischemia-induced cognitive impairments were significantly associated to neuroendocrine dysregulation, not hippocampal pathology per se. Finally, our results showed that inhibition of glucocorticoid synthesis can potentiate, rather than attenuate ischemia-induced neuronal degeneration, possibly by increasing glucose availability and by stimulating early HPA axis activity (due to impaired negative feedback).

## 1. Introduction

As observed in human survivors of cardiac arrest (CA), forebrain ischemia in rodents produces brain injury and cognitive impairment. Cerebral ischemia in rodents also acts as an acute/subchronic stressor enhancing CRH release and expression (Khan, Milot, Lecompte-Collin, & Plamondon, 2004) and circulating levels of glucocorticoids in the hours and days following reperfusion (Hwang et al., 2006). The role of the HPA axis in ischemia has largely been studied in the context of neuronal outcome, as increased circulating levels of corticosterone (CORT) are thought to compromise the ability of hippocampal neurons to survive neurological insults (Stein-Behrens, Mattson, Chang, Yeh, & Sapolsky, 1994) and associated to aggravation of ischemic injury (Sapolsky & Pulsinelli, 1985).

Inhibition of CORT synthesis with metyrapone is neuroprotective following global or focal ischemia in rodents at short post-reperfusion intervals (24 h, 3 and 7 days) (Adachi, Chen, Liu, Nagaro, & Arai, 1999; Krugers, Kemper, Korf, Ter Horst, & Knollema, 1998; Smith-Swintosky et al., 1996). However, post-ischemic metyrapone administration significantly increased infarct volume measured four weeks after focal ischemia (Risedal et al., 1999), demonstrating that the drug can have negative effects on cell survival, perhaps following long post-ischemic intervals. Interestingly, despite inhibiting the conversion of 11-deoxycorticosterone to CORT, the depletion of glucocorticoids by metyrapone actually leads to stimulation of the early components of the HPA axis (namely, hypothalamic CRH and pituitary ACTH) due to impaired negative feedback (normally provided by CORT itself)(Alexander, Irvine, Livesey, & Donald, 1993; Rotllant & Armario, 2005; Rotllant, Ons, Carrasco, & Armario, 2002). A single

dose of metyrapone can significantly elevate not only plasma ACTH levels, but following the drugs' metabolism, CORT secretion can be elevated for many days post-administration, with increased CRH mRNA expression in the hypothalamus demonstrated one week later (Rotllant & Armario, 2005; Rotllant, Ons, Carrasco, & Armario, 2002). These metyrapone-induced changes in HPA axis reactivity in normal rats suggest that the drug acts as a physiological stressor.

The mechanisms by which metyrapone exerts effects on neuronal survival after ischemia largely remain undetermined. Although glucocorticoids are regarded as playing a deleterious role, increased glucocorticoid exposure after metyrapone administration (Rotllant & Armario, 2005; Rotllant, Ons, Carrasco, & Armario, 2002) might predict potentiation of neuronal injury (Sapolsky, Krey, & McEwen, 1985; Sapolsky & Pulsinelli, 1985). So would the effect of metyrapone administration to produce acute hyperglycemia (Rotllant, Ons, Carrasco, & Armario, 2002), a phenomenon (e.g., increased plasma glucose) associated to aggravation of ischemic damage (Lin, Ginsberg, & Busto, 1998; L. Liu et al., 2007; Schurr, Payne, Miller, & Tseng, 2001) and another salient marker of stress likely reflecting meduloadrenal activation (Armario, Marti, & Gil, 1990; De Boer, Koopmans, Slangen, & Van der Gugten, 1990). Despite these findings, the effects of metyrapone on HPA axis secretions in ischemic animals have been examined only at short post-reperfusion intervals (30-60 min), leaving protracted effects undetermined.

In addition to an association to neuronal outcome, ischemia-induced neuroendocrine alterations may have effects on cognitive performance during the post-reperfusion period. Indeed, "it is now well established that a single exposure to some

stressors is able induce behavioral and physiological changes that last for days to weeks” (Belda et al., 2008). For example, a single predator exposure in mice led to learning disruptions in the radial maze 16 to 22 days post-stressor and in object recognition performance (26 to 28 days)(El Hage, Griebel, & Belzung, 2006), two capabilities that are similarly negatively impacted by cerebral ischemia (Kiyota, Miyamoto, & Nagaoka, 1991; Mumby et al., 1996; Plamondon, Morin, & Charron, 2006). Such long-term effects on behaviour or neuroendocrine reactivity have been observed following all types of stressors, including CORT administration, emotional/physical, and immune stressors (Belda et al., 2008). Consequently, an ischemic stressor could potentially prime the neuroendocrine response and alter behaviour at relatively long post-ischemic delays. Indeed, the open-field hyperactivity observed in ischemic rats at different time intervals appears elicited independent of memory, anxiety, and motoric deficits (Experiments 1, 2, and 3), and is associated to stimulation of the neuroendocrine response in normal animals (Plaznik, Danysz, & Kostowski, 1985a; Sutton, Koob, Le Moal, Rivier, & Vale, 1982; Veldhuis & De Wied, 1984). In this context, cerebral ischemia also produces long-lasting (up to 60 days) increased activation of NE-containing neurons (Pich et al., 1993), a phenomenon (excessive NE availability) associated to impairments in working memory (Arnsten, Mathew, Ubriani, Taylor, & Li, 1999; Roozendaal, Hahn, Nathan, de Quervain, & McGaugh, 2004) and memory retrieval (Aghajanian, 1982; Barros et al., 2001; Roozendaal, 2002) in spatial tasks in normal animals. Whether forebrain ischemia may be a significant enough stressor to exert lasting impact on the neuroendocrine response potentiating HPA axis activation upon exposure to a heterotypic stressor such as behavioural testing remains to be determined.

The current experiment investigated time-dependent alterations of ACTH and CORT synthesis following 10 min forebrain ischemia and administration of the glucocorticoid synthesis inhibitor metyrapone, and determined the relationship of these neuroendocrine alterations to post-ischemic functional outcome and neuronal survival. The first goal of this study aimed to determine the effect of ischemia and metyrapone on neuronal degeneration at a delayed post-ischemic interval (4 weeks), yet to be investigated in a model of global (versus focal) ischemia. Alterations in plasma glucose, ACTH (providing a measure of early HPA axis activation) and CORT (providing a measure of late HPA axis activation) will be assessed at different intervals following ischemia. A second goal of the experiment aimed to determine the effects of ischemia on neuroendocrine reactivity in *response* to behavioural testing in a Y-maze (spatial working memory) and a Barnes maze (spatial reference memory) by measuring post-testing CORT secretions. The rats' spatial memory capabilities will be assessed at times when neuronal degeneration is expected to be largely complete and resting HPA axis stabilized (e.g., after 7 days post-reperfusion). We predict higher post-testing CORT levels in ischemics, an effect possible negatively correlated with performance.

## **2. Methods and Material**

### **2.1. Animals**

Male Wistar rats (n=45) weighing between 250–320g at time of surgery were obtained from Charles River Laboratories (Rochefort, Quebec, Canada). 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. Animals were handled daily by the experimenter for 2 min 3 days before the first day of surgery.

## 2.2. Surgical procedure

Forebrain ischemia was performed using the four-vessel occlusion model as previously described (Pulsinelli & Brierley, 1979). Briefly, rats were anesthetized by inhalation of 1.5% halothane in oxygen. The core temperature was kept at  $37\pm 0.5$  °C throughout the surgeries, including during global ischemia, by means of a feedback regulated heating blanket connected to a rectal thermometer. The vertebral arteries were irreversibly occluded by electrocoagulation, and a small-diameter silk thread looped around the carotid arteries to facilitate subsequent occlusion. Sham-operated animals underwent anesthesia and received the same dorsal and ventral surgical incisions as the ischemic group, without electrocoagulation of the vertebral arteries. Then, 24 h later, rats were briefly exposed to 1.5% halothane in oxygen, and carotid arteries re-exposed for clamping. Animals were then removed from the anesthetic, and the common carotid arteries occluded with microvascular clamps for a 10 min period in freely ventilating rats. Only rats that lost the righting reflex over the entire occlusion period and which displayed dilated pupils (in response to a beam of light) and unresponsiveness prior to removal of the clamps were used in the study. Of the 31 animals subjected to global ischemia 14 regained the righting reflex or displayed constricted pupils/responsiveness in response to light stimulus, and 3 additional animals died during surgery.

On day 4 post-occlusion the pupillary reflex was assessed in all animals to determine visual system/retinal damage possibly affecting the visual response to light

conditions. To do so, animals were transported to a dark room and left to habituate for at least 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 before 10 s had elapsed. All ischemic and sham-operated rats displayed a normal pupillary reflex.

### 2.3. Drug treatment and groups after exclusion

Metyrapone, a glucocorticoid synthesis inhibitor, was dissolved in a 0.9% saline solution and administered subcutaneously (175 mg/kg) to rats 30 min prior to induction of forebrain ischemia (n=7) or sham surgery (n=6). Vehicle-treated ischemic (n=8) and sham-operated (n=7) rats were administered the saline solution.

### 2.4. Blood collection

Animals were habituated to the blood collection procedure by being transported to the blood collection room located in another room in the vivarium, covered with a huck towel, and tail gently stroked for 1 min. This procedure was performed daily in the three days preceding the first day of surgery.

The tail vein nick procedure was used to collect blood (500  $\mu$ l) in Eppendorf tubes coated with ethylenediaminetetraacetic acid (EDTA), as well as drops of blood on Schleicher and Schuell filter paper (dried overnight, and stored at -80°C). During blood collection, rats were transported to the same room and the same procedure as previously

described employed. Tail blood samples were collected by lancing the tail close to its tip, and took no more than 2.5 min to complete. Filter paper blood samples were taken both between 7:00-8:00 (AM sample) and 18:00-19:00 (PM sample), while blood collection in Eppendorf tubes (500  $\mu$ l) occurred only in the AM followed by centrifugation at 5000 RPM for 8 min and plasma extracted and stored at  $-80^{\circ}\text{C}$ . Blood samples were collected for all animals one day before surgery between 7:00-8:00 AM (baseline), and 1 h after ischemia or sham occlusion, and on days 1, 3, and 7 post-reperfusion (only filter paper blood collection occurred on day 7). Food was removed at least 1 h prior to 4VO and returned after blood collection taken 1 h later. Blood was also collected on filter paper 30 min after initial exposure to any behavioural test (described below). Throughout the experiment any given rat received a total 2-3 nicks; previously existing nicks were re-opened with sterile water/cotton swabs.

## 2.5. Quantification of CORT, ACTH, and glucose

Plasma CORT and ACTH were quantified using commercially available radioimmunoassay kits (MP Biomedical). While plasma ACTH analysis used a standard protocol, CORT determinations based on filter paper blood drops used a modified protocol (Konkle et al., 2003). 2.5mm punches of filter paper blood spots were placed in tubes containing 200 $\mu$ L phosphate buffered saline (containing 0.1% gelatin). These tubes were then shaken at 60 rpm for 1 h at room temperature, and refrigerated/incubated for 48 h at  $4^{\circ}\text{C}$  prior to beginning the RIA procedure. This method yields values highly correlated ( $r=0.91$ ,  $p<.001$ ; as previously validated in our laboratory; unpublished data) to plasma CORT values quantified via the normal RIA procedure and blood collection.

Tubes were analyzed in a HP Cobra II gamma counter. The CORT and ACTH values are based on averages of duplicates and triplicates, respectively. Glucose measurements were taken at baseline, and 1 and 24 h post-reperfusion with a handheld glucose meter (Glucometer Elite)(Messier & Kent, 1995).

## 2.6. Behavioural tests

For the Y-maze and Barnes maze tests, animals were individually carried to the testing laboratory (between 10am-3pm) located 15 s away from homeroom and were tested 5 min after arrival. Subjects were monitored by an overhead camera which recorded performances on videotape. Lux measurements were taken with a lightmeter (Proskit MT-4007) placed at the center of the Y-maze and the Barnes maze. Illumination for the Y-maze (450 lux) was provided by fluorescent lights. For the Barnes maze two flood lights (150 watts each), provided uniform and bright illumination in addition to the testing rooms' regular fluorescent lighting (for a total of 800 lux). All animal handling during the behavioural tests and coding was performed by a blind experimenter who was not involved in the blood collection.

### 2.6.1. *Working memory: Y-maze*

Working memory was measured by spontaneous alternation behavior (SAB)(Lalonde, 2002) on day 9 post-reperfusion. Similar to the object recognition test (Mumby, 2001), SAB in the Y-maze is based on the propensity for rats to explore novel rather than recently encountered and more familiar arms/spatial locations. Rats were tested for continuous SAB in the Y-maze, considered here as a measure of spatial

recognition/working memory, in a testing environment containing salient cues (e.g., posters, paper cutouts) visible to the rat. Animals were placed into a randomly selected arm and observed for 8 min, and the sequence of arm entries recorded. An arm entry was recorded when the rat entered an arm with its four paws. A SAB was defined as any entry sequence combining the three different arms (ex: BCACBABACB), thus when the rat chose to explore the arm that had not just previously been entered. The percentage of continuous SAB was calculated using the following formula: # of spontaneous alternations/(total # of entries – 2). The higher (over 50%) the percentage of SAB, the better the spatial working memory performance is considered. The total number of arm entries quantified locomotion. The apparatus was wiped clean (15% ethanol) between each test.

#### *2.6.2. Spatial reference memory: Barnes maze*

Testing in the Barnes maze began 10 days following reperfusion. The maze was a thick plastic circular slab (d = 4'; C = 12.56') located 4.5' above floor level, with 18 holes equidistant from one another near its border. Several spatial cues were placed on the black curtains surrounding the maze. The center of the maze, where each training trial began was 1.5' away from the peripheral holes. During any given trial one of these holes contained a black escape box held in place by a drawer-style system. A black skirt hung from the edge of the maze to the ground, to reduce the visibility of the escape box through the other holes.

The testing included one pre-training session (day 10) followed by testing sessions conducted over a period of four days (day 11-14) which included two trials per

day separated by 5 min. The pre-training session introduced rats to the escape box and its location, which was randomly determined for each animal and remained in the same location for the rest of the testing sessions. At the beginning of each trial, rats were placed facing a random direction under the start box (an opaque plastic bucket) located in the middle of the maze. After 30 s, the bucket was lifted via a pulley system and the rats explored the maze. On the pre-training day, once the escape box was entered, the hole was covered, and the rat remained in the box for 90 s. A second pre-training trial repeated these conditions immediately after the first trial. The same procedure was used during the subsequent four days of training with the exception that rats remained in the escape box for 30 s after entry. If the escape box was not located after 3 min, the animal was manually guided towards it. After each of the initial daily training trials rats were returned to their home cage, the Barnes maze and escape box cleaned (15% ethanol), and after 5 min, the second daily trial initiated. Precisely 30 min after being placed in the Barnes maze (e.g., the first trial) a drop of blood was collected on filter paper using the previously described procedure. The distance traveled by the animal during each trial was quantified by a blind experimenter by encoding the VHS data to MPEG format and analyzing the path distance. Distance calculations began once the start box was lifted (up and out of the camera's field) and ended once a rat located the escape hole. Because of the necessity for bright lighting directly situated above the Barnes maze in this task, reflected illumination made it difficult for our tracking software (Ethovision 3.0) to accurately track the animal (some data would be lost). Instead, Universal Desktop Ruler 3.2 (combined with AutoMouseClicker 2.10) was used to manually track via a 1600 dpi laser mouse the rat's head during forward momentum in slow motion. This program

combination allowed for the quantification of traveled distance in each trial. Time to reach the escape box was also recorded.

## 2.7. Analysis of neuronal survival in thionin stained brain sections.

Animals were euthanized 30 days following ischemia, and the brains removed, frozen on dry ice, and stored at  $-80^{\circ}\text{C}$ . Serial coronal sections ( $14\ \mu\text{m}$ ) of specific brain regions were obtained using a cryostat and stained for Nissl bodies with thionin. Analysis of neuronal density was performed between  $-3.14$  and  $-4.16$  mm relative to bregma for pyramidal neurons in the dorsal portion of the hippocampal CA1 layer. The number of neurons were counted using a LEICA DAS microscope attached to a SONY digital camera, and image analysis software Norton Eclipse (v 6.0), UTHSCSA ImageTool (v 3.00), and GIMP (v 2.4.4). Only neurons with normal morphology with distinct cytoplasmic and nuclear outlines and a visible nucleolus were measured. The number of intact neurons per 1 mm linear length of the CA1 in each  $14\ \mu\text{m}$  section was quantified. A mean value was obtained from four 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 neuronal cell densities. Neuronal density values are expressed as mean  $\pm$  SEM.

## 2.8. Statistical Analysis

For behavioural tasks, two-way ANOVA determined overall omnibus effects, followed by three planned pairwise contrasts (isch-vehicle vs. sham-vehicle, isch-met vs. isch-vehicle, sham-met vs. sham-vehicle). For the Barnes maze data, group comparisons

were made on the average distance traveled of both daily trials on each test day, as well as on distance traveled on each of the individual trials (first and second) on each test day. Such within-day analysis was performed given that altered arousal in ischemic rats might be more readily manifested on the trial subsequent to the first daily trial. Additional analyses compared the total distance traveled across all test days. The values for plasma ACTH and CORT were log-transformed when necessary to achieve homogeneity of variances, and analyzed using repeated-measures two-way ANOVA. Pearson's correlation coefficients were calculated between post-training CORT values and distance traveled in the Barnes maze on the first and second daily trials, and between post-training CORT values in the Y-maze and spontaneous alternation rates (all animals and all test data included in these analyses).

An additional analysis of the Barnes maze data examined within-subject change in distance traveled on the second trial relative to the first trial, and calculated as percent change from baseline. One sample t tests compared the percent change score from each group to a test value of 100 (e.g., representing baseline for all animals), to determine whether performance significantly improved or worsened on the second daily trial relative to the first trial. All values represent mean scores and S.E.M., and significance was set at  $p < .05$ .

### **3. Results**

#### **3.1. Baseline levels (CORT, ACTH, and glucose) and temperature**

Table 1. shows baseline levels in CORT, ACTH, glucose values for all groups. No differences were observed between any of the groups. The morning values of CORT

(pg/punch) is equivalent to plasma levels of 13 ng/ml (determined using standard RIA protocol/blood collection; unpublished data), which corresponds to morning CORT levels in undisturbed animals (Akana, Jacobson, Cascio, Shinsako, & Dallman, 1988; Dal-Zotto, Marti, & Armario, 2003). CORT, ACTH, and glucose levels returned to pre-surgery baseline levels in all groups by post-reperfusion days 7, 3, and 1, respectively.

### 3.2. Impact of forebrain ischemia and metyrapone on plasma ACTH

Fig. 1 and 2 show the impact of forebrain ischemia and metyrapone administration on post-reperfusion ACTH plasma levels. Two-way repeated-measures ANOVA on ACTH values at baseline, 1 h post-ischemia, and days 1 and 3 (AM) revealed an interaction between surgery and treatment ( $F(1,24)=6.43$ ,  $p=.018$ ), as well a main effect of surgery ( $F(1,24)=4.6$ ,  $p=.042$ ) and treatment ( $F(1,24)=70.72$ ,  $p<.001$ ). A significant effect of time was observed ( $F(2,48)=251.02$ ,  $p<.001$ ), as well as significant interactions between time and surgery ( $F(2,48)=3.81$ ,  $p=.029$ ), time and treatment ( $F(2,48)=35.64$ ,  $p<.001$ ). No differences were observed between any of the compared groups in baseline ACTH levels ( $p>.66$  for all comparisons). One hour post-reperfusion, global ischemia increased ACTH levels compared to sham-operated animals ( $p<.001$ )(Fig. 1), although these groups were indistinguishable after 24 h and 3 days ( $p=.2$ ,  $p=.22$ , respectively)(Fig. 2). Both animals subjected to sham or ischemic surgery and receiving metyrapone showed increased ACTH secretion compared to vehicle-treated animals 1 and 24 h ( $p<.001$  for both groups and comparisons) but not 3 days post-reperfusion ( $p=.6$  and  $p=.86$  for sham and ischemic animals, respectively).

### 3.3. Impact of forebrain ischemia and metyrapone on CORT

Fig. 1 and 3 show the effect of global ischemia and metyrapone administration on post-reperfusion CORT levels. Two-way repeated-measures ANOVA on CORT values 1 h post-reperfusion revealed a significant between-group interaction of surgery and treatment ( $F(1,24)=29.77$ ,  $p<.001$ ) and main effect of surgery ( $F(1,24)=111.59$ ,  $p<.001$ ) and drug ( $F(1,24)=21.74$ ,  $p<.001$ ). Within-subject, there was a significant main effect of time ( $F(1,24)=73.6$ ,  $p<.001$ ) and interaction between time and surgery ( $F(1,24)=41.07$ ,  $p=.004$ ), time and treatment ( $F(1,24)=107$ ,  $p<.001$ ), and time, surgery and treatment ( $F(1,24)=17.49$ ,  $p<.001$ ). One hour post-reperfusion, global ischemia increased CORT levels compared to sham-operated animals ( $p<.001$ )(Fig. 1), while animals subjected to ischemia and receiving metyrapone displayed decreased CORT ( $p<.001$ ), as did sham-operated animals compared to vehicle-treated controls ( $p=.046$ ).

Fig. 3 shows the effect of global ischemia and metyrapone administration on CORT levels 1, 3 and 7 days following reperfusion. Two-way repeated-measures ANOVA on CORT values one day following reperfusion in the AM and PM revealed a significant interaction of surgery and treatment ( $F(1,24)=5.56$ ,  $p=.027$ ) and main effect of surgery ( $F(1,24)=7.14$ ,  $p=.013$ ). Within-subject, there was a significant interaction between time and surgery ( $F(1,24)=6.98$ ,  $p=.014$ ) and time, surgery and treatment ( $F(1,24)=26.72$ ,  $p<.001$ ). One day post-reperfusion when blood collection was performed in the morning, global ischemia increased CORT levels compared to sham-operated animals ( $p=.002$ ), and metyrapone-treated ischemic animals showed decreased CORT ( $p=.001$ ). Metyrapone-treated sham animals were comparable to vehicle-treated controls

( $p=.73$ ). When blood was collected in the PM, ischemic animals showed comparable levels as sham-operated animals ( $p=.23$ ) and metyrapone-treated ischemic rats ( $p=.81$ ). However, metyrapone-treated sham animals had increased CORT compared to vehicle-treated controls ( $p=.004$ ).

On day 3 post-reperfusion, two-way repeated-measures ANOVA on CORT values in the AM and PM revealed a significant interaction between surgery and treatment ( $F(1,24)=5.25$ ,  $p=.031$ ) and a main effect of surgery ( $F(1,24)=5.57$ ,  $p=.027$ ). Within-subject there was a main effect of time ( $F(1,24)=71.1$ ,  $p<.001$ ) and interaction between surgery, treatment and time ( $F(1,24)=6.43$ ,  $p=.018$ ). In the morning, global ischemia increased CORT levels compared to sham-operated animals ( $p=.001$ ), and animals subjected to ischemia and receiving metyrapone displayed decreased CORT ( $p=.046$ ). Sham-operated animals treated with metyrapone compared to vehicle-treated controls had increased CORT ( $p=.043$ ). In the PM, all animals had similar CORT levels ( $p>.4$  for all comparisons). On day 7 post-reperfusion, repeated-measures ANOVA on CORT values in the AM and PM revealed a main effect of time ( $F(1,24)=124.82$ ,  $p<.001$ ), but no between-group differences ( $F(1,24)=.01$ ,  $p=.92$ ).

### 3.3. Impact of forebrain ischemia and metyrapone administration on glucose

Fig. 1 shows the impact of ischemia and metyrapone administration on post-reperfusion glucose levels at 1 h post-reperfusion. Two-way repeated measures ANOVA on glucose levels at baseline, and 1 h and day 1 (AM) post-reperfusion revealed an interaction between surgery and treatment ( $F(1,24)=13.85$ ,  $p=.001$ ), time and surgery ( $F(2,48)=15.01$ ,  $p<.001$ ), time and treatment ( $F(2,48)=61.2$ ,  $p<.001$ ), time, surgery and

treatment ( $F(2,48)=11.4$ ,  $p<.001$ ), and a main effect of time ( $F(2,48)=88.73$ ,  $p<.001$ ), treatment ( $F(1,24)=66.02$ ,  $p<.001$ ), and surgery ( $F(1,24)=20.37$ ,  $p<.001$ ). Ischemia had no impact on glucose levels measured at 1 or 24 h, and both sham and ischemic rats treated with metyrapone displayed increased glucose 1 h post-reperfusion ( $p=.003$  and  $p<.001$ , respectively), with no impact at 24 h.

#### 3.4. Spatial working memory: Y-maze

Fig. 4 shows the effect of global ischemia and metyrapone administration on spontaneous alternation (working memory) behaviour and post-testing CORT levels. For the percentage of spontaneous alternation, two-way ANOVA revealed a significant interaction between surgery and treatment ( $F(1,24)=10.93$ ,  $p=.003$ ). Global ischemia reduced spontaneous alternation behaviour compared to sham-operated animals ( $p=.002$ ), and animals subjected to ischemia and receiving metyrapone displayed increased spontaneous alternations ( $p=.042$ ). Repeated-measure two-way ANOVA revealed no significant interaction of surgery and treatment ( $p=.98$ ) in the number of arm entries, and no group differences ( $p>.7$  for all comparisons). For post-testing CORT levels, two-way repeated-measures ANOVA revealed a main effect of surgery ( $F(1,24)=20.67$ ,  $p<.001$ ) and drug ( $F(1,24)=6.02$ ,  $p=.022$ ). Ischemic rats displayed increased CORT levels compared to sham-operated animals ( $p=.001$ ), and metyrapone-treated ischemic animals showed reduced CORT compared to vehicle-treated rats ( $p=.039$ ). There was a non-significant negative (moderate) correlation between post-training CORT values and spontaneous alternation behaviour ( $r=-.29$ ,  $p=.09$ ).

### 3.5. Spatial reference memory: Barnes maze

Fig. 5 shows the effect of global ischemia and metyrapone administration on spatial reference memory performance (traveled distance) in the Barnes maze and CORT levels assessed 30 min following initial maze exposure. In terms of average (in both daily trials) traveled distance across the test days, two-way repeated-measures ANOVA revealed a significant interaction of surgery and treatment ( $F(1,24)=4.45$ ,  $p=.046$ ), and main effect of treatment ( $F(1,24)=10.21$ ,  $p=.004$ ) and surgery ( $F(1,24)=5.22$ ,  $p=.031$ ). Within-subject a main effect of time ( $F(3,72)=15.87$ ,  $p<.001$ ) and interaction between time and treatment ( $F(3,72)=5.02$ ,  $p=.003$ ) was observed. Global ischemia increased the distance traveled to reach the escape box compared to sham-operated animals at test day 1 ( $p=.007$ ), 2 ( $p=.03$ ) and 3 ( $p=.011$ )(Fig. 5a). Metyrapone-treated ischemic rats displayed reduced traveled distance compared to vehicle-treated ischemic animals at day 1 ( $p=.001$ ), 2 ( $p=.02$ ) and 3 ( $p=.006$ ). Metyrapone- and vehicle-treated sham animals showed comparable traveled distance for all testing days. Compiling all the test trials, vehicle-treated ischemics showed increased total traveled distance compared to shams ( $p=.004$ ), and metyrapone-treated ischemics showed reduced travel compared to vehicle-treated ischemics ( $p<.001$ ). Considering only the initial daily trials (Fig 5b), ischemic rats showed spatial memory deficits on test day 1 ( $p=.033$ ), and metyrapone reduced traveled distance at this time interval in these animals ( $p=.011$ ). No significant differences were observed between any of the groups on any other test day, and when compiling across all the test trials, metyrapone-treated ischemics displayed reduced traveled distance compared to vehicle-treated rats ( $p=.015$ ). Considering only the second daily trials (Fig 5c), global ischemia led to increased traveled distance to reach the escape box compared

to sham-surgery on test days 1 ( $p=.007$ ), 2 ( $p=.009$ ) and 3 ( $p=.002$ ). Metyrapone-treatment reduced traveled distance in ischemic rats on all these days ( $p<.001$ ,  $p=.02$  and  $p=.004$ , respectively). Metyrapone treatment failed to alter traveled distance of sham-operated animals on any testing day. Compiling all trials over the entire testing period, vehicle-treated ischemic rats showed increased total traveled distance compared to sham-operated animals ( $p<.001$ ), and metyrapone-treated ischemic rats traveled less distance to find the hidden box ( $p=.001$ ). Metyrapone- and vehicle-treated sham-operated rats were indistinguishable ( $p=.47$ ).

Two-way repeated-measures ANOVA performed on CORT levels after testing sessions revealed significant interaction between surgery, treatment and time ( $F(3,72)=2.81$ ,  $p=.045$ ), and a main effect of surgery ( $F(1,24)=7.79$ ,  $p=.01$ ), treatment ( $F(1,24)=5.51$ ,  $p=.027$ ), and test day ( $F(3,72)=23.37$ ,  $p<.001$ ). Vehicle-treated ischemic rats displayed significantly increased CORT secretion on test day 1 ( $p=.018$ ) and 3 ( $p=.001$ )(Fig. 5d). Metyrapone-treated ischemic rats had reduced post-training CORT levels on day 1 ( $p=.043$ ) and 3 ( $p=.002$ ), while drug-treated sham rats were not different than vehicle-treated controls. Compiling all days, ischemic rats had increased CORT ( $p=.031$ ) and metyrapone treated ischemic rats had reduced levels ( $p=.047$ ). A significant positive correlation between CORT and distance traveled was observed on the second trial ( $r=.36$ ,  $p=.001$ ), but not the first trial ( $r=.0165$ ,  $p=.13$ ).

Fig. 6 shows the percent change in traveled distance on the second trial relative to the first trial in the Barnes maze. Vehicle-treated ischemic rats displayed increased percentage of traveled distance on second trial relative to the value of 100 (representing the baseline)( $t_7=2.67$ ,  $p=.033$ ) while sham rats showed the expected decreased percentage

in traveled distance ( $t_6$ , -3.01,  $p=.02$ ). Metyrapone-treated sham rats showed a significant reduction in traveled distance from trial 1 to 2 ( $t_5$ , -3.04,  $p=.029$ ), and significant changes were not observed in metyrapone-treated ischemic rats ( $p=.42$ ).

Two-way repeated measures ANOVA on the average time taken to complete the trials across the test days (data not shown) revealed a main effect of time ( $F(3,72)=27.76$ ,  $p<.001$ ) but no differences were observed between any of the groups at any test day, nor when compiling values from all the test days. The observation that ischemics required increased traveled distance to find the escape box yet were not temporally slower in the completion of the task suggests these animals had increased velocity and/or hyperactivity (although this was not quantified), concordant with previous observations in the open-field under bright illumination (Milot & Plamondon, 2008a).

### 3.6. Neuronal degeneration in CA1 hippocampal subfield

Fig. 7 shows the impact of global ischemia and metyrapone treatment on CA1 neuronal survival 30 days post-reperfusion. Two-way ANOVA revealed a main effect of surgery ( $F(1,24)= 291.26$ ,  $p<.001$ ) and an interaction between treatment and surgery ( $F(1,24) = 4.92$ ,  $p=.036$ ). Global ischemia produced significant neuronal death ( $p<.001$ ), and metyrapone had no effect on neuronal counts in sham-operated animals ( $p=.52$ ). Ischemic animals receiving metyrapone had significantly fewer surviving CA1 neurons than those administered the vehicle ( $p=.003$ ).

#### 4. Discussion

Our findings revealed important effects of the glucocorticoid synthesis inhibitor metyrapone on both neuronal and behavioural outcome after 10 min forebrain ischemia. Administration of metyrapone aggravated neuronal degeneration compared to vehicle-treated ischemic rats, despite attenuating ischemia-induced CORT elevations and spatial memory impairment. Forebrain ischemia increased neuroendocrine reactivity in response to testing in the Y-maze and Barnes maze, effects associated to impaired spatial memory capabilities and attenuated by metyrapone administration pre-ischemia. These findings present novel information that reconsiders the contribution of the HPA axis to post-ischemic neuronal and functional outcome.

In the present study we observed long-lasting ischemia-induced elevations in resting plasma CORT particularly in the morning sampling periods, suggesting that ischemia acted as a significant stressor. The highest elevation in CORT secretion was observed 1 h after ischemia, followed by a gradual return to pre-surgery baseline levels by 7 days post-reperfusion. Concordant with previous findings in normal rats (Rotllant & Armario, 2005; Rotllant, Ons, Carrasco, & Armario, 2002), metyrapone stimulated ACTH release in both sham and ischemic animals for at least 24 h, a response consistent with impaired negative feedback of the HPA axis. A stimulatory effect of metyrapone on CORT secretion in sham controls was observed at day 3/AM when CORT levels in saline treated animals had returned to pre-surgical levels, as previous reported at this post-administration time interval in non-surgered rats (Rotllant, Ons, Carrasco, & Armario, 2002). The stimulatory effect of metyrapone on CORT secretion at 24 h post-administration (Rotllant & Armario, 2005) was likely undetected/masked because of the

significant stressor of sham-operation. Metyrapone administration was not stimulatory in ischemics, and rather, it attenuated the ischemia-induced CORT elevations at all times where they were observed in saline treated rats. This suggests that the stimulatory effects of metyrapone (Rotllant & Armario, 2005) are more readily observable in normal animals not exposed to a significant stressor.

The heightened CORT levels observed in all groups except vehicle-treated sham animals at day 3/AM post-surgery in the absence of concomitant increased ACTH secretion suggest a temporal dissociation of HPA axis secretion, a phenomenon previously reported by others (Akana, Jacobson, Cascio, Shinsako, & Dallman, 1988; Dal-Zotto, Marti, & Armario, 2003). Although the dissociation between ACTH and CORT observed at 24 h after metyrapone administration suggests long-lasting inhibitory effects of the compound on glucocorticoid synthesis, this is unlikely given it increased CORT levels at day 1/PM in treated sham animals, indicating short lasting effects of the compound on inhibition of CORT synthesis, as previously reported (Rotllant & Armario, 2005).

These findings broaden the scope of metyrapone action in ischemic animals, showing that inhibition of CORT secretion is linked to the daily period of assessment (e.g., CORT inhibition is observed over days in AM samplings periods although secretion appears comparable in PM collected samples). Moreover, the effect of metyrapone to suppress ischemia-induced CORT levels at delayed time intervals (e.g., day 3), suggests that the longer lasting elevations of CORT secretion observed in ischemics over the days were potentiated by the initial elevations (e.g., those observed at 1 or 24 h post-reperfusion) of CORT.

*Cerebral ischemia and neuronal degeneration*

The finding of aggravated neuronal damage (43% decrease in CA1 pyramidal cells) in metyrapone-treated ischemic rats despite inhibition of CORT output might appear counterintuitive considering the demonstrations of CORT-induced aggravation of ischemic neuronal death (Sapolsky & Pulsinelli, 1985) and the neuroprotective of metyrapone observed up to 7 days after cerebral ischemia (Adachi, Chen, Liu, Nagaro, & Arai, 1999; Krugers, Kemper, Korf, Ter Horst, & Knollema, 1998; Krugers, Maslam, Van Vuuren, Korf, & Joels, 1999; Payne, Tseng, & Schurr, 2003; Smith-Swintosky et al., 1996). However, our findings are consistent with aggravating effects of metyrapone on neuronal damage assessed four weeks post-reperfusion in a focal ischemia model (Risedal, Nordborg, & Johansson, 1999).

The mechanism potentiating the aggravation of hippocampal neuronal injury we observed might be associated to the stimulatory effect of metyrapone on early HPA axis activity (e.g., as demonstrated by elevated plasma ACTH up to 24 h post-reperfusion). Although the non-fragmented ACTH molecule has yet to be studied in the context of ischemia-induced cell death, the role of CRH is more documented. Despite not measured in the present study it is likely the increased plasma ACTH observed after metyrapone administration (or ischemia) was reflective of increased hypothalamic CRH output (Khan, Milot, Lecompte-Collin, & Plamondon, 2004; Rotllant & Armario, 2005). Acute doses of CRH can be neuroprotective against ischemic insults both in vivo (Charron, Frechette, Proulx, & Plamondon, 2008) and in vitro (Charron et al., 2009; Fox, Anderson, & Meyer, 1993) and not considered as inherently toxic under basal conditions (Craighead et al., 2000). On the other hand, CRH-R1 receptor deficiency can provide resistance to

ischemic damage (Stevens et al., 2003). This latter finding suggests that contrary to the effects of acute CRH administration, sustained upregulation of the peptide (or of stimulation of its receptor) might be associated to the potentiation of ischemic injury. The increased neuronal generation observed in metyrapone-treated ischemic rats might consequently have been the result of increased early HPA axis upregulation (e.g., CRH/ACTH) observed for a period of at least 24 h, potentiating the neurotoxic effects of cerebral ischemia.

Our study replicated previous reports of metyrapone-induced hyperglycemia (Rotllant & Armario, 2005; Rotllant, Ons, Carrasco, & Armario, 2002), which has been associated to aggravation of ischemic neuronal injury (Lin, Ginsberg, & Busto, 1998; L. Liu et al., 2007; Martin et al., 2006; Schurr, Payne, Miller, & Tseng, 2001; Welsh, Ginsberg, Rieder, & Budd, 1980), thereby might represent another mechanism associated to the increased cell death in metyrapone-treated animals in our study. The “glucose paradox” of ischemia (in brief) refers to the notion that neurons sustaining ischemic injury require energy to initiate and maintain processes related to cellular death (Payne, Tseng, & Schurr, 2003). Of interest, the documented hyperglycemic effect of metyrapone was not observed in any of the previous experiments reporting a neuroprotective effect of the compound, despite similar doses and blood sampling periods shortly after ischemia (Adachi, Chen, Liu, Nagaro, & Arai, 1999; Krugers, Kemper, Korf, Ter Horst, & Knollema, 1998; Krugers, Maslam, Korf, Joels, & Holsboer, 2000; Krugers, Maslam, Van Vuuren, Korf, & Joels, 1999; Smith-Swintosky et al., 1996). The most notable methodological difference was that contrary to these studies our forebrain ischemia model does not require prolonged use of anesthetics (namely, halothane) during the

occlusion period. Our study is the first to investigate the effect of metyrapone in animals not anaesthetized during the ischemic period, and subjected only to very brief duration of halothane exposure (2-3 min), just long enough to clamp the carotid arteries and induce forebrain ischemia. The findings of reduced glucose metabolism by sustained halothane exposure (Ferreira, Palmer, & Fournier, 1998; Heath, Frayn, & Rose, 1978; G. V. Johnson & Hartzell, 1985) might explain why the normally observed hyperglycemic effect of metyrapone was not observed in the aforementioned studies given the reliance of their models (2VO+hypotension) on prolonged halothane exposure prior to and during the actual ischemia. Interestingly, and in support of such conclusions, post-reperfusion administration of metyrapone (when rats were no longer exposed to halothane) but not pre-ischemic administration (followed by halothane exposure) aggravated neuronal outcome four weeks later (Risedal, Nordborg, & Johansson, 1999). Future experiments using the 2VO + hypotension model should take into consideration the impact of extended halothane (or other anesthetics) exposure on the effects of metyrapone (and other administered compounds) on physiological variables (such as glucose) important to neuronal outcome.

*Neuroendocrine reactivity to behavioural testing and memory impairments post ischemia*

Cerebral ischemia produced spatial working and reference memory impairments. Consistent with other assessments of post-ischemia spatial memory impairments in the Morris water maze (MWM)(Nelson, Lebessi, Sowinski, & Hodges, 1997; Nunn et al., 1994; Olsen, Scheel-Kruger, Moller, & Jensen, 1994a, , 1994b) (the Barnes maze can be considered a “land” version of the MWM), ischemic rats traveled greater distances to find

the escape box when combining both daily trials, normally interpreted as impaired spatial reference memory for locations across the testing days. However, performance on any trials subsequent to the initial trial (within the same day) would be expected to improve given the short between-trial delay (5 min). Indeed, significantly reduced percentage in traveled distance was observed in sham-operated animals on the second daily trials. In contrast, the performance of ischemic rats worsened on the second trial relative the first. These behavioural manifestations support the notion of ischemia-induced differential reactivity or arousal to testing, given that memory impairment in the Barnes maze was observed on test days 2 and 3 (on the second daily trial) despite intact performance on the first trial (e.g., impairments appeared potentiated/elicited by test exposure). Metyrapone prevented the increased traveled distance on the second trial, suggesting attenuated behavioural reactivity to testing in these animals. In the Y-maze ischemic animals displayed reduced spontaneous alternation rates compared to sham-operated animals, and metyrapone similarly attenuated these working memory impairments.

The higher post-testing levels of CORT in ischemics support the idea that ischemic animals had impaired reactivity to the testing, associated to disrupted spatial memory capabilities. Indeed, ischemics displayed elevated neuroendocrine reactivity to testing in both the Y-maze and Barnes maze, as demonstrated by significant elevations in post-testing levels of CORT. Our analysis revealed a significant (and moderate) positive correlation between post-training CORT levels and the degree of impairment (e.g., traveled distance) in the second trial, but not the first trial in the Barnes maze, further supporting the notion that testing itself (the first trial) potentiated arousal-related memory impairments (in the second trial). To this effect, functional recovery in both overall and

first/second trials in metyrapone-treated ischemic rats in the Barnes maze occurred simultaneous with reduced CORT secretion as compared to vehicle-treated ischemic animals, despite aggravated neuronal degeneration in these animals. The negative correlation between CORT and working memory capabilities (e.g., spontaneous alternation) was moderate and not significant ( $p=.11$ ), perhaps attributable to the small data set. These findings seem to confirm that an ischemic stressor can potentiate cognitive impairments at delayed intervals, as observed following other significant acute stressors (Belda et al., 2008). In the context of animal models of ischemia, the increased neuroendocrine and behavioural reactivity to testing conditions in ischemic rats might be expected in other similar tasks, such as the MWM, which similarly requires multiple daily trials and considered an even greater stressor than the Barnes maze (e.g., water exposure) and could be used to further explore and validate our findings.

The mechanism through which increased post-training CORT in ischemic rats contributed to working memory or memory retrieval impairment remains to be determined. Given the resting levels of CORT were similar between ischemic and sham-operated animals prior to testing (e.g., day 7), it is unlikely that memory impairments were mediated by differences in circulating levels of CORT, particularly given that a delay of 15-30 min is typically required to elicit effects (both genomic and non-genomic) on cognitive performance (Roosendaal, 2002; Sajadi, Samaei, & Rashidy-Pour, 2006). One possible mediator of the impaired memory retrieval (and working memory) might have been underlying excessive noradrenergic activity previously observed after ischemia (Pich et al., 1993), given its rapid immediately elicited effects (McEwen & Sapolsky, 1995) and the documented relationship between noradrenergic systems and HPA axis

activation (Feldman, Conforti, & Siegel, 1982; Ziegler, Cass, & Herman, 1999).

Excessive noradrenergic activity previously shown to impair working memory (Arnsten, Mathew, Ubriani, Taylor, & Li, 1999) and memory retrieval (Aghajanian, 1982; Barros et al., 2001) might have similarly mediated the ischemia-induced impairments, although this hypothesis remains to be directly tested.

The behavioural and histological findings of the current study provide additional evidence that cognitive impairments post-ischemia do not necessarily predict the degree of CA1 neuronal degeneration. Behavioural impairments observed following 10 min forebrain ischemia in rats can be dependent on illumination level during testing (Milot & Plamondon, 2008a) or attenuated by diet or pharmacological compounds administered pre-ischemia despite failing to provide protection against neuronal degeneration (Plamondon & Khan, 2006; Plamondon & Roberge, 2008; Roberge, Hotte-Bernard, Messier, & Plamondon, 2008; Roberge, Messier, Staines, & Plamondon, 2008). Rearing in an enriched environment does not provide neuroprotection against forebrain ischemia, but prevents spatial memory or object discrimination impairments (Gobbo & O'Mara, 2004), and spatial memory deficits are not necessarily observed following forebrain ischemia leading to significant neuronal degeneration (Iwasaki et al., 2006). Such findings, like those reported in the present study (i.e., metyrapone-treated ischemics displaying functional recovery despite reduced neuronal survival), show that post-reperfusion behavioural alterations might be mediated by factors unrelated to hippocampal cell death, particularly given our 10 min ischemia does not produce significant (or very little) damage in extra-CA1 brain areas such as the CA3, neocortex and dentate gyrus (Araki, Kato, Shuto, & Itoyama, 1998; Chan et al., 1998; Globus et al.,

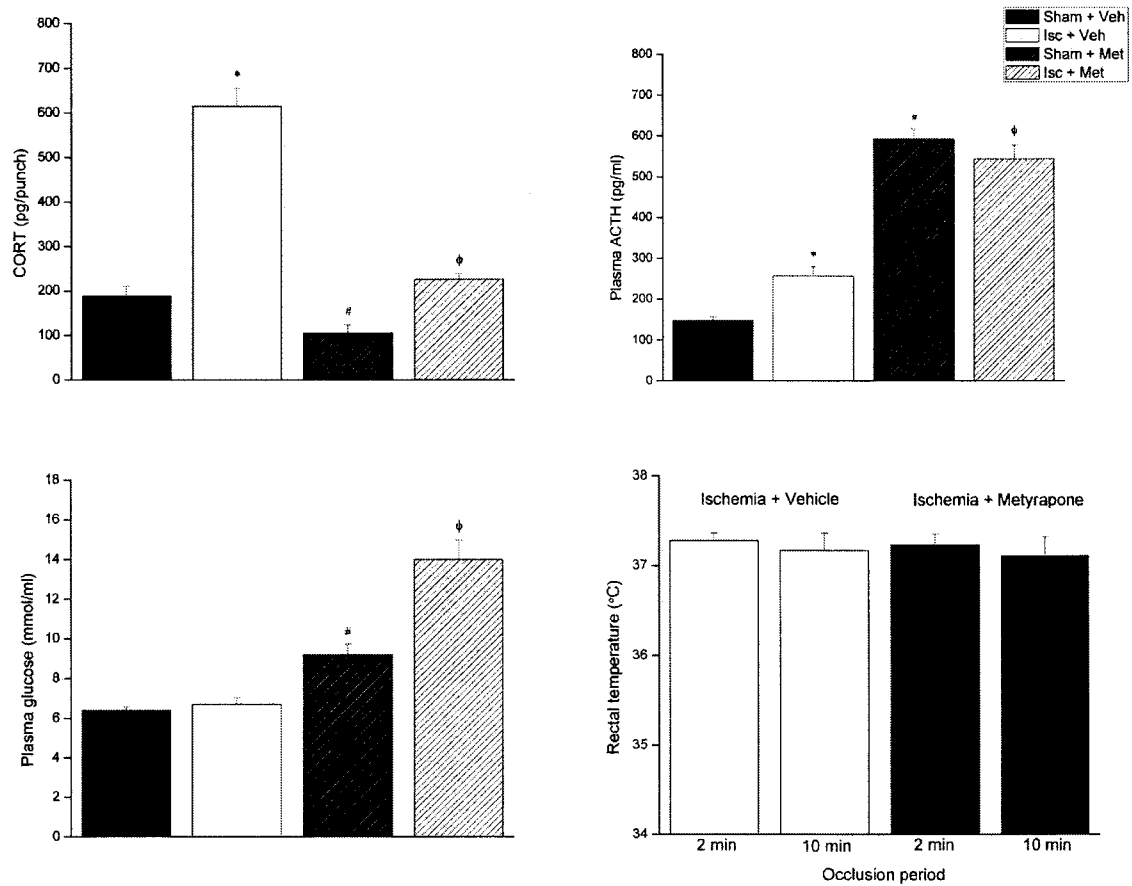
1991; Jorgensen, Jensen, & Diemer, 1991; Kawai et al., 1992; Nunn et al., 1994; Petito & Halaby, 1993), structures that are implicated in learning and (spatial/working) memory and which could have supported the intact functional outcome observed in metyrapone-treated ischemics.

### *Conclusion*

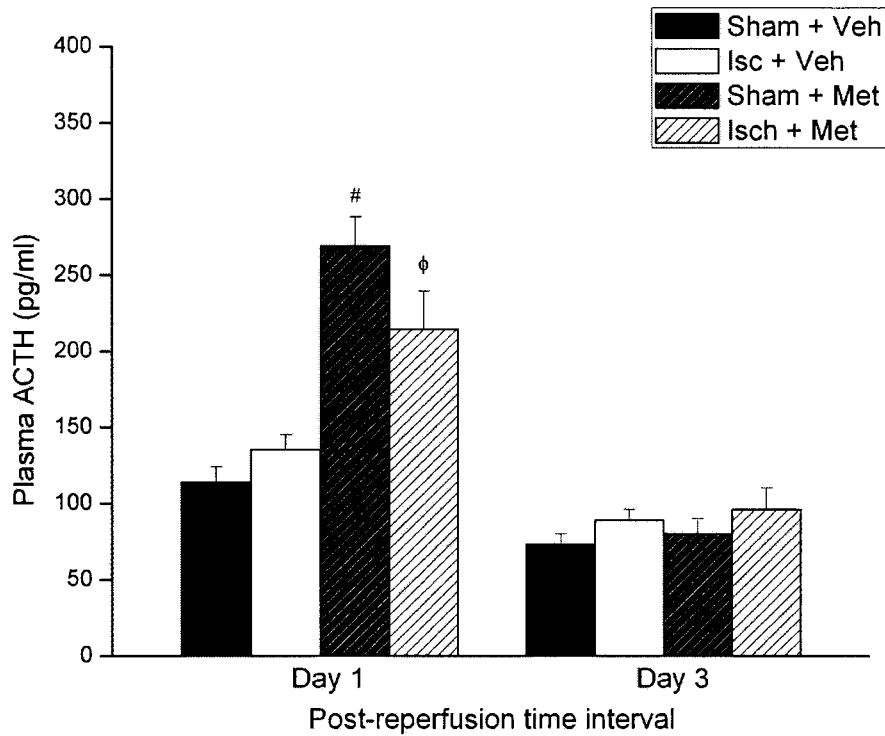
In sum, the present study demonstrated long-lasting effects of metyrapone on ischemia-induced glucocorticoid secretion potentially associated to aggravation of ischemic injury, and documented differences in HPA axis reactivity between vehicle- and metyrapone-treated ischemic rats which suggest that altered reactivity to testing post-ischemia contributes to cognitive impairment. From a clinical perspective, the results of the present experiment are important to consider. First, blockade of glucocorticoid synthesis with metyrapone in the event of CA or stroke in humans might not prove beneficial due to possible detrimental effects of ensuing acute hyperglycemia and relatively long lasting HPA axis dysregulation. The observation of increased recovery by CA survivors displaying elevated plasma cortisol during the first 24 hr after return of spontaneous circulation compared to those eventually succumbing after resuscitation displaying reduced levels (C. H. Schultz et al., 1993) supports the notion that increased circulating glucocorticoids after CA are not necessarily harmful. In neonatal rats, CORT is protective against hypoxic-ischemic damage (Tuor, 1997; Tuor & Del Bigio, 1996), an effect which is also inconsistent with the notion that glucocorticoids are universally lethal after brain injury.

*Acknowledgements*

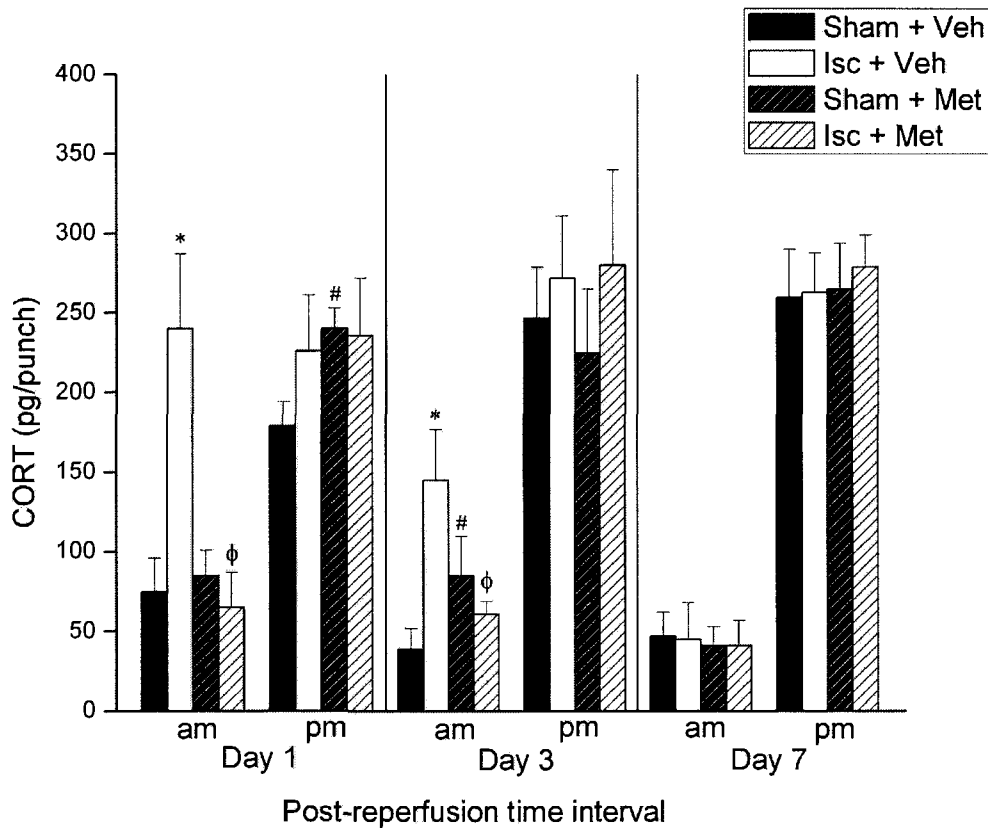
We sincerely thank Sylvie Émond and Jon James for excellent technical assistance. This work was supported by a grant from the Natural Sciences and Engineering Research Council of Canada to H.P.



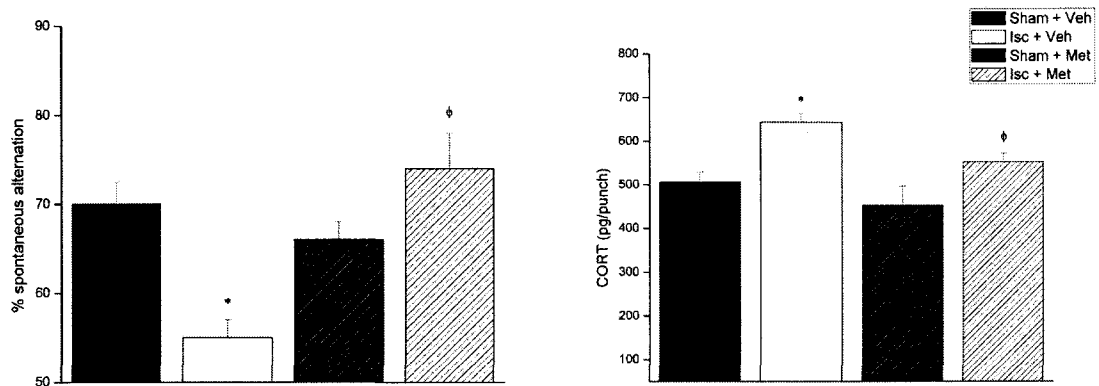
**Figure 1.** The impact of forebrain ischemia and metyrapone administration on CORT, ACTH, and glucose levels 1 h post-reperfusion, and rectal temperature during occlusion. Symbols indicate significant differences between vehicle-treated ischemic and sham-operated groups (\*), between vehicle and metyrapone treated ischemic rats ( $\phi$ ), and between vehicle and metyrapone treated sham-operated animals (#). Reported differences were significant at  $p < .05$ . Values are expressed as mean  $\pm$  SEM.



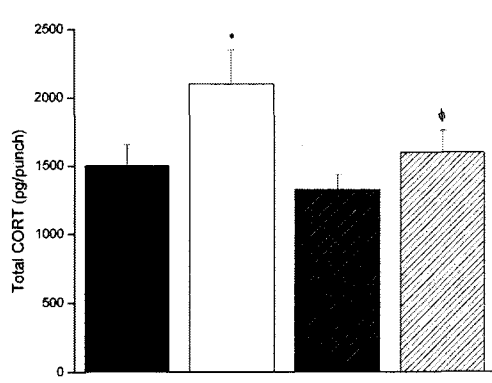
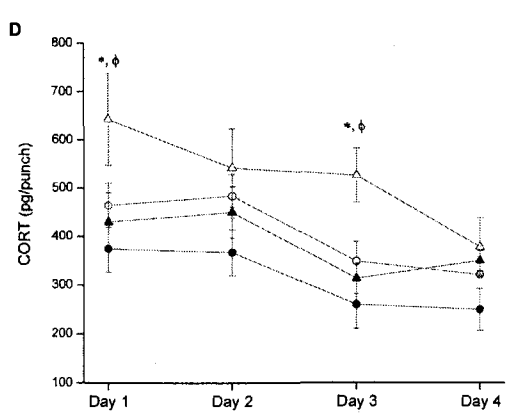
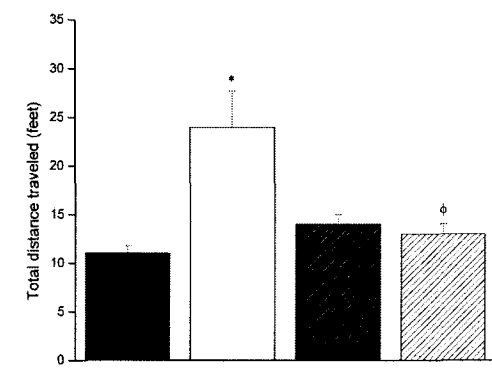
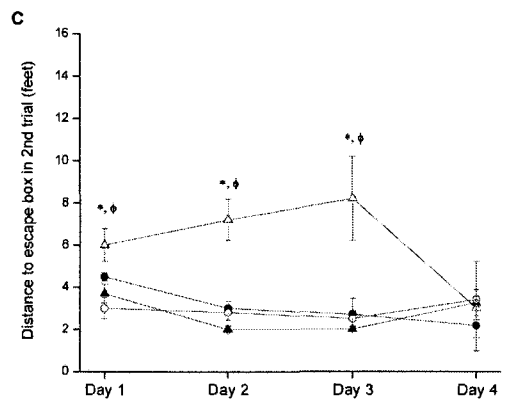
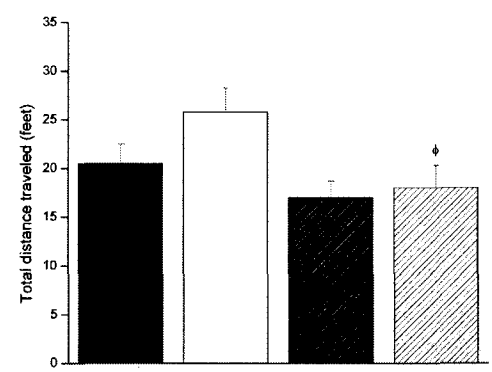
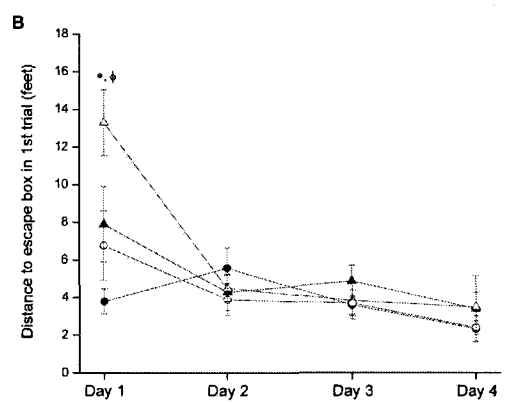
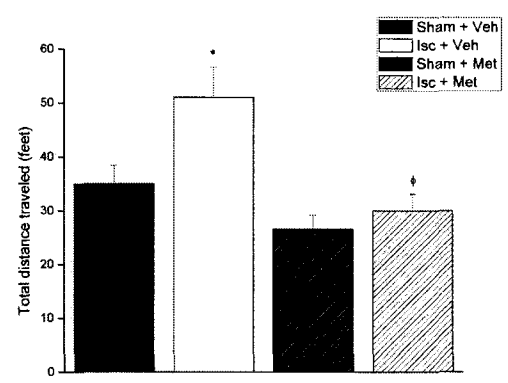
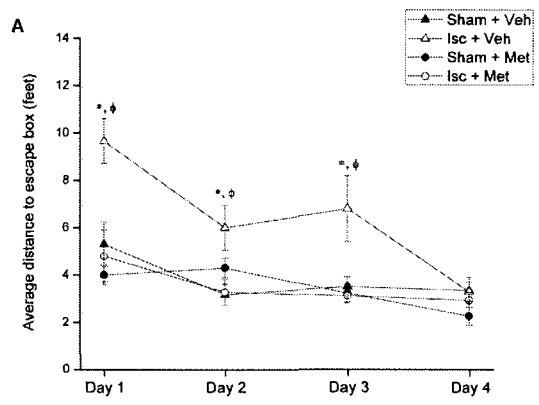
**Figure 2.** The impact of forebrain ischemia and metyrapone administration on plasma ACTH at 1 and 3 days post-reperfusion. Symbols indicate significant differences between vehicle-treated ischemic and sham-operated groups (\*), between vehicle and metyrapone treated ischemic rats ( $\phi$ ), and between vehicle and metyrapone treated sham-operated animals ( $\#$ ). Reported differences were significant at  $p < .05$ . Values are expressed as mean  $\pm$  SEM.



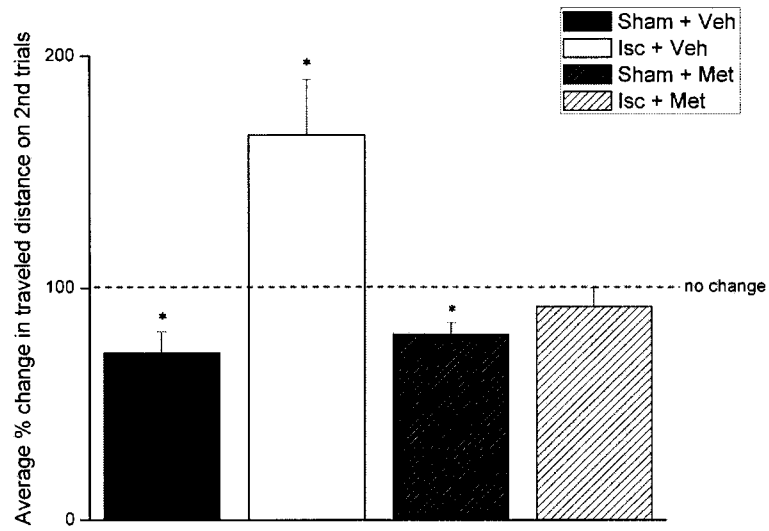
**Figure 3.** The impact of forebrain ischemia and metyrapone administration on CORT levels at 1, 3, and 7 days post-reperfusion (AM and PM). Symbols indicate significant differences between vehicle-treated ischemic and sham-operated groups (\*), between vehicle and metyrapone treated ischemic rats ( $\phi$ ), and between vehicle and metyrapone treated sham-operated animals (#). Reported differences were significant at  $p < .05$ . Values are expressed as mean  $\pm$  SEM.



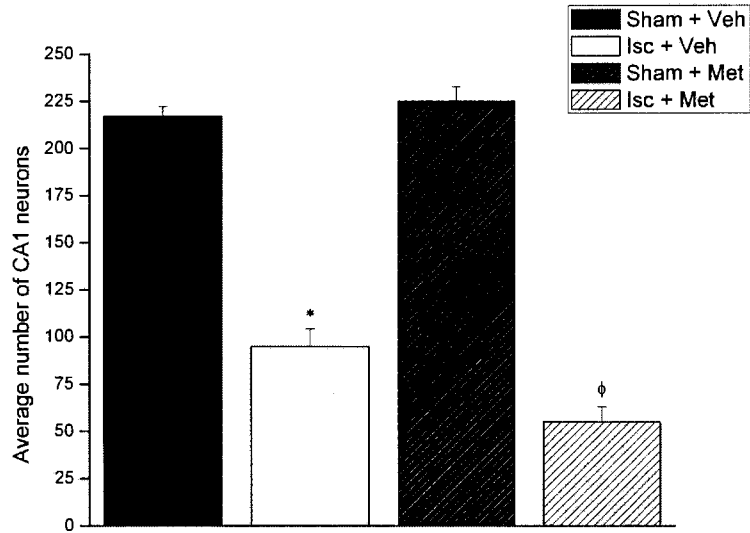
**Figure 4.** The impact of forebrain ischemia and metyrapone administration on spontaneous alternation behaviour (working memory) and post-testing CORT levels. Symbols indicate significant differences between vehicle-treated ischemic and sham-operated groups (\*), between vehicle and metyrapone treated ischemic rats ( $\phi$ ), and between vehicle and metyrapone treated sham-operated animals (#). Reported differences were significant at  $p < .05$ . Values are expressed as mean  $\pm$  SEM.



**Figure 5.** The impact of forebrain ischemia and metyrapone administration on spatial reference memory in the Barnes maze (decreased traveled distance indicating better memory), and post-testing CORT levels. Symbols indicate significant differences between vehicle-treated ischemic and sham-operated groups (\*), between vehicle and metyrapone treated ischemic rats ( $\phi$ ), and between vehicle and metyrapone treated sham-operated animals (#). Reported differences were significant at  $p < .05$ . Values are expressed as mean  $\pm$  SEM.



**Figure 6.** The impact of forebrain ischemia and metyrapone administration on the percent change in distance traveled on the second Barnes maze daily trials relative to first trial. Symbols indicate significant differences compared to the value of 100 representing baseline/trial 1 (\*). Reported differences were significant at  $p < .05$ . Values are expressed as mean  $\pm$  SEM.



**Figure 7.** The impact of forebrain ischemia and metyrapone administration on the number of surviving neurons in hippocampal subfield CA1 four weeks post-reperfusion as measured in thionin stained brain sections. Symbols indicate significant differences between vehicle-treated ischemic and sham-operated groups (\*), between vehicle and metyrapone treated ischemic rats ( $\phi$ ), and between vehicle and metyrapone treated sham-operated animals (#). Reported differences were significant at  $p < 0.05$ . Values are expressed as mean  $\pm$  SEM.

Table 1. Baseline values (and S.E.M.) of ACTH, CORT and glucose

Group	CORT (pg/punch)		ACTH (pg/ml)	Glucose (mmol/l)
	AM	PM	AM	AM
Ischemia + Veh	35 (12)	303 (35)	74 (4.6)	6.1 (.34)
Sham + Veh	43 (15)	330 (41)	71 (3.8)	6.2 (.29)
Ischemia + Met	37 (16)	327 (50)	75 (3.9)	6.2 (.31)
Sham + Met	38 (11)	290 (23)	69 (5.5)	6.1 (.39)

## Experiment 5

Long lasting alterations in noradrenergic regulation mediate working and reference spatial memory capabilities after forebrain ischemia in rats.

### Preface

Experiment 4 showed that 10 min cerebral ischemia can act as a significant potentiator of the HPA axis. Given the relationship between noradrenergic systems and the HPA axis, and the much more rapid effects of NE on cognitive functioning, Experiment 5 aimed to determine whether cognitive-behavioural outcome after ischemia might be associated to alterations in noradrenergic regulation.

*Abstract*

Recent findings from our laboratory indicated that forebrain ischemia produces alterations in behavioural and neuroendocrine reactivity, effects which might mediate cognitive impairment independently of or in addition to cell death. The present study examined the possibility of lasting alterations of the noradrenergic system in rats subjected to 10 min forebrain ischemia as a contributing factor to behavioural impairment. Clonidine (0.04 mg/kg, S.C.), an alpha2-adrenoceptor agonist, or yohimbine (0.3 mg/kg, S.C.), an alpha2-adrenoceptor antagonist, were administered 30 min prior to training in a working memory version of the radial maze or immediately following training in a reference memory version. Our findings revealed transient impairment in spatial working memory in ischemic rats assessed between day 12-23 post-reperfusion, which were attenuated by clonidine and aggravated by yohimbine administrations. Post-training clonidine administration worsened reference memory in ischemic rats, while yohimbine had no impact, suggesting that increased arousal in ischemic rats may have masked reference memory impairment by increasing consolidation. These effects were not observed in sham-operated animals. Interestingly, our within-trial analysis revealed that although ischemic rats made significant working memory errors in the initial 2-5 arms radial maze arm entries, fewer errors were made compared to sham-operated controls in the final 6-9 entries. Our findings suggest that increased activation (e.g., outflow) of norepinephrine-containing neurons may contribute to alterations in spatial memory capabilities after cerebral ischemia.

## 1. Introduction

Understanding the cause of behavioural impairment after forebrain ischemia in animal models is necessary for the development of treatments aiming to reduce the negative impact of cardiac arrest (and to a lesser extent, stroke) on cognitive functioning. Despite the commonsensical association between discrete ischemia-induced neuronal degeneration and behavioural impairment, many studies have demonstrated inconsistencies in this relationship. In brief, impairments in spatial memory performance were not observed following forebrain ischemia leading to significant neuronal degeneration (Iwasaki et al., 2006), or conversely, have been reported following transient carotid artery occlusion (i.e., without arterial hypoperfusion or vertebral artery occlusion) despite no measurable neuronal hippocampal degeneration (Jaspers, Block, Heim, & Sontag, 1990). Recent findings from our laboratory and others showed that behavioural impairments observed following forebrain ischemia in rats can be influenced by illumination level during testing (Milot & Plamondon, 2008a) or attenuated by diet, environmental enrichment, or pharmacological compounds administered pre-ischemia despite failing to provide protection against neuronal degeneration (Gobbo & O'Mara, 2004; Plamondon & Khan, 2006; Plamondon & Roberge, 2008; Roberge, Hotte-Bernard, Messier, & Plamondon, 2008; Roberge, Messier, Staines, & Plamondon, 2008). These findings suggest the possibility of alternative mediators of post-ischemic behaviour.

There is support for a neuroendocrine or neurochemical basis for some of the behavioural changes observed after cerebral ischemia in rodents. Forebrain ischemia can be considered an acute and short-term stressor (e.g., subchronic) as it produces significant increases in corticosterone (CORT) for days after reperfusion (Hwang et al.,

2006)(Experiment 4) and increased CRH release from numerous brain areas (Khan, Milot, Lecompte-Collin, & Plamondon, 2004). Exposure to a single severe stressor can prime the neuroendocrine response (Belda et al., 2008), an effect we recently observed in ischemic rats which demonstrated increased CORT elevations in response to cognitive assessment (in Barnes maze and Y-maze) which were associated to impairments in spatial memory (Experiment 4).

Although additional neuromodulatory systems are acutely affected by cerebral ischemia, the present study investigated the noradrenergic system as it impacts arousal, attention, and learning and memory (Harley, 1987, , 2007; Lapid & Morilak, 2006; Roozendaal, Okuda, de Quervain, & McGaugh, 2006). Forebrain ischemia leads to decreased cellular content of NE in the cerebral cortex, an effect observed at short post-reperfusion time intervals (e.g., 60 min) (Harik, Yoshida, Busto, & Ginsberg, 1986; Iijima, Hara, Suga, Nakamura, & Kameyama, 1986) and likely due to massive release of intracellular stores (Bentue-Ferrer et al., 1986) and enhanced NE turnover in cortex (Gustafson, Liden, & Wieloch, 1992). Indeed, direct measurements of extracellular NE demonstrated massive release in the hippocampus following 10 (Miura et al., 1999), 20 (Globus et al., 1989) and 30 min (Perego, Gatti, Vetrugno, Marzatico, & Algeri, 1992) cerebral ischemia, as well as in the neocortex (Gustafson, Westerberg, & Wieloch, 1991). These studies showed a rapid return (<1 hour post-reperfusion) to pre-surgery levels, indicating short-lasting elevations of extracellular NE, effects proposed to play a role in ischemia-induced neuronal degeneration (Busto, Harik, Yoshida, Scheinberg, & Ginsberg, 1985; Engelhard et al., 1999; Hoffman et al., 1992; J. A. Schultz, Hoffman, & Albrecht, 1993).

In spite of an association between the noradrenergic system and histopathological outcome after cerebral ischemia, the possibility of a relationship between NE dysfunction and post-ischemic functional outcome has received very little attention. The noradrenergic system is one of the first to produce immediate effects on cognitive functions upon exposure to a stressor due to its rapid non-genomic effects (McEwen & Sapolsky, 1995) and its effects on arousal and attention (Lapiz & Morilak, 2006; Mair, Zhang, Bailey, Toupin, & Mair, 2005; Sara, 1985; Smee, Weston, Skinner, & Day, 1975). Optimal levels of NE are consequently required for good spatial working memory performance, and significantly reducing NE availability by lesions to the locus coeruleus can impair spatial memory in the MWM (Compton, Dietrich, Smith, & Davis, 1995). Conversely, excessive stimulation of NE can impair spatial working memory (Arnsten, Mathew, Ubriani, Taylor, & Li, 1999) as do high doses of yohimbine, an alpha2-adrenoceptor antagonist (stimulating NE release) which impair spatial working memory in the radial maze (McAllister, 2001; Zhang & Cai, 2005).

An association between noradrenergic dysfunction and spatial working memory impairments after cerebral ischemia has previously been proposed, but only examined at relatively short post-ischemic intervals when neuronal degeneration was incomplete (Iwasaki, Kitamura, Ohgami, Mishima, & Fujiwara, 1996). There is however evidence for a disrupted noradrenergic system at longer post-ischemic intervals. Rats subjected to forebrain ischemia displayed increased latencies in tail withdrawal (persisting for 2 weeks) in response to a hotplate test, thereby demonstrating ischemia-induced analgesia. This effect- was blocked by administration of clonidine and augmented by yohimbine (Pich et al., 1993). The authors concluded that reduced pain sensitivity in ischemic rats

was produced by increased activity (e.g., outflow) of central norepinephrine-containing neurons. Even at 60 days post-reperfusion when ischemia-induced effects on analgesia were no longer observable, yohimbine administration reinstated prolonged tail withdrawal latencies, indicating long-lasting effects of ischemia on central noradrenergic reactivity. Consequently, it is possible that the increased NE cellular content in prefrontal cortex observed 7 days after ischemia (Iwasaki, Kitamura, Ohgami, Mishima, & Fujiwara, 1996) might indicate a tendency for increased release and arousal in ischemic animals in response to a stressor (e.g., such as behavioural testing). Indeed, 7 min asphyxial arrest in rats (reproducing forebrain ischemia) resulted in higher acoustic startle one week after ischemia, suggesting increased arousal in these animals (Hickey, Akino, Strausbaugh, & De Courten-Myers, 1996). The findings are also concordant with our demonstration of ischemia-induced increased HPA axis reactivity in response to behavioural testing at delayed post-reperfusion intervals (Experiment 4).

One possibility is that long-term disruption of the noradrenergic system might mediate cognitive performance after cerebral ischemia. Interestingly, increased NE synthesis (in mPFC) has been reported following traumatic brain injury (TBI) in rodents and associated to impaired working memory (Kobori, Clifton, & Dash, 2006). Therefore, the main goal of the present study was to determine whether the spatial memory deficits in the radial maze reported following 10 min forebrain ischemia (Gionet et al., 1991; Hartman, Lee, Zipfel, & Wozniak, 2005; Plamondon & Khan, 2006; Plamondon, Morin, & Charron, 2006; Plamondon & Roberge, 2008) can be impacted by administration of pharmacological compounds yohimbine and clonidine, increasing and decreasing synaptic NE availability, respectively. Assuming enhanced noradrenergic reactivity in

ischemic rats, we expect attenuations in working memory impairment following pre-training administration of clonidine, and aggravated working memory performance following yohimbine administration, given that excessive stimulation of noradrenergic receptors can disrupt frontal functions related to attentional processes and working memory. There is also a substantial contribution of the NE system to spatial reference memory and activation of this system appears important in consolidation of learning (Roozendaal, Okuda, de Quervain, & McGaugh, 2006; Roozendaal, Okuda, Van der Zee, & McGaugh, 2006; Row & Dohanich, 2008). Reference memory (reliant on consolidation) after ischemia is sometimes found to be less impaired than working memory (Volpe, Pulsinelli, Tribuna, & Davis, 1984). Thus, we propose that post-training clonidine administration might worsen reference spatial memory in ischemic rats by preventing consolidation related to increased arousal.

## **2. Materials and methods**

### **2.1. Animals**

Male Wistar rats (n= 70) weighing between 250–320 g at time of surgery were obtained from Charles River Laboratories (Rocheffort, Quebec, Canada). They 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 rat chow. The room temperature was maintained at 21–23 °C with 60% relative humidity. All animals were handled daily for two min by the experimenter on the four days preceding surgery, and for two min two days preceding the first radial maze pre-training day.

## 2.2. Surgical procedure

Forebrain ischemia was performed using the four-vessel occlusion model as previously described (Pulsinelli & Brierley, 1979). Briefly, rats were anesthetized by inhalation of 1.5% halothane in oxygen. The core temperature was kept at  $37\pm 0.5$  °C throughout the surgeries and during global ischemia by means of a feedback regulated heating blanket connected to a rectal thermometer. The vertebral arteries were irreversibly occluded by electrocoagulation, and a small-diameter silk thread looped around the carotid arteries to facilitate subsequent occlusion. Sham-operated animals underwent anesthesia and received the same dorsal and ventral surgical incisions as the ischemic group, without electrocoagulation of the vertebral arteries. Then, 24 h later rats were briefly exposed to 1.5% halothane in oxygen, and carotid arteries re-exposed for clamping. Animals were then removed from the anesthetic, and the common carotid arteries occluded with microvascular clamps for a 10 min period in freely ventilating rats. Only rats that lost the righting reflex over the entire occlusion period and displayed unresponsiveness to touch prior to removal of the clamps were used in the study. Of the 40 animals subjected to global ischemia 15 met the exclusion criteria. Three other animals died during surgery.

## 2.3. Experimental apparatus and behavioural procedures

Groups of sham and ischemic subjects were transported from their vivarium to the radial maze laboratory and allowed to rest at least 30 min before injection. The radial maze was evenly illuminated at 300 lux and surrounded by distinct visuo-spatial cues. During all testing the experimenter was blind to the groups.

#### 2.4. Drug treatments and animal groups

The drugs clonidine (0.04 mg/kg, s.c.) and yohimbine (0.3 mg/kg, s.c.) were suspended in saline (0.9% sodium chloride in distilled water) and administered 30 min before the working memory task, or immediately following testing in the reference memory task. These doses were selected because they had no significant impact on working memory in normal animals in other radial maze tasks (McCann, Rabin, & Winter, 1987; Ohta, Matsumoto, Watanabe, & Shimizu, 1993), thus selective sensitivity in ischemic rats to their administration could be more easy to detect. Sham-operated and ischemic controls received the vehicle.

Animals administered clonidine for the working memory task were administered yohimbine for the reference memory task, and vice versa. Seven groups of animals were tested in the present study: ischemia-vehicle (n=9), sham-vehicle (n=8), ischemia-drug group 1 (clonidine/WM, then yohimbine/RM)(n=7), sham-drug group 1 (clonidine/WM, then yohimbine/RM)(n=7), ischemia-drug group 2 (yohimbine RM, then clonidine WM)(n=9), and sham-drug group 2 (yohimbine RM, then clonidine WM)(n=7). A group of sham-operated animals not receiving S.C. injection (n=5) was tested to determine if injection itself had an impact on performance.

#### 2.5. Radial maze – spatial working memory

Pre-training in the radial maze began on day 8 and lasted for four days. In order to maximize motivation, rats were maintained at 90% of their body weight via a restricted diet initiated on day 7 post-reperfusion, assuring that pre-surgery weights had been surpassed at this time point. Animals were weighted daily following behavioural testing and received between 15-20 grams of standard rat chow. Every seven days 5 grams was

added to their target weight to maintain a normal growth curve. During the pre-training sessions, Froot Loop (FL) pieces were scattered over the entire radial maze (both in the arms and center), to gradually be restricted to the small wells located at the end of the arms (and not visible to the rats from the center of the maze) on the fourth pre-training training. During this pre-training phase, rats were placed in the center of the maze facing a random direction, and allowed to explore the maze and eat the food for 15 min. After returning to the central area following an arm entry, the radial maze arms were closed and re-opened after a 10 s interval. On the fifth day, the training phase was initiated and conducted for 12 days (one trial per day), using the same procedure as pre-training with one FL piece placed in the well at the end of each of the eight arms. A working memory error was recorded when a rat re-entered a previously visited arm. A trial ended when a total of 9 arm entries had been made. The maze was cleaned between each trial with a 15% ethanol solution.

In order to determine whether pre-training administration of yohimbine or clonidine had any effect on locomotor activity, the total number of arms visited was divided by the total amount of time spent in the maze across all trials. This number reflects the overall rate of arm entries per min.

## 2.6. Radial maze – spatial reference memory

Two weeks separated the end of the last working memory trial from the first reference memory trial. Each animal was randomly assigned four arms consistently baited with a FL piece. On the first trial (not included in analysis) rats were exposed to the four FL locations which remained constant across the subsequent 8 trials, conducted

on the following days. The same procedure as the working memory task was employed except that a reference memory error was recorded when a rat entered one of the four unbaited arms, and a trial ended following entry into each of the baited arms.

## 2.7. Statistical analysis

Two-way repeated (or non-repeated) ANOVA analyzed the number of working memory errors, arm entry rates, and reference memory errors. Following omnibus analysis, planned pairwise contrasts (isch+veh vs sham+veh; isch+drug versus isch+sal; sham+drug versus sham+veh) were conducted. For the working memory task, the numbers of errors during the 12 trials were collapsed into blocks of 2 testing days, and a block of errors made across all trials. The number of errors made in entries 2-5, 6-9, and 2-9 were examined for each trial given the hypothesis of ischemia-induced arousal deficits which might predict time-dependent within-trial performance. For the reference memory version of the radial maze task, the number of reference and working memory errors made in the 8 trials of testing were similarly collapsed in 4 blocks of 2 trials, and a block of errors made across all trials. No within-trial analysis was performed for reference memory performance since the total number of entries could not be controlled. Finally, one-way ANOVA compared sham-operated animals receiving vehicle injection to those receiving no injection on all behavioural measures. Differences were considered significant when  $p < .05$ . Reported are the group averages and S.E.M.

## 3. Results

### 3.1. Working memory: effect of forebrain ischemia and yohimbine

### 3.1.1. Entries 2-5

Fig. 1a shows the effect of ischemia and pre-training administration of yohimbine on working memory errors during within-trial entries 2-5. Analysis of the 6 blocks of 12 trials with two-way repeated measures revealed a main effect of surgery ( $F(1,29)=14.53$ ,  $p<.001$ ) and test block ( $F(5,145)=11.5$ ,  $p<.001$ ). Global ischemia increased the number of working memory errors at the test block 1 ( $p=.046$ ) and 2 ( $p=.01$ ). Yohimbine administration to ischemic rats increased the number of working memory errors at the test block 5 ( $p<.001$ ) and 6 ( $p=.001$ ) relative to saline treated rats. Analysis of the total number of errors across the 12 trials revealed that ischemic animals made more errors than sham-operated rats ( $p=.005$ ).

### 3.1.2. Entries 6-9

Fig. 1b shows the effect of ischemia and pre-training administration of yohimbine on working memory errors during within-trial entries 6-9. Analysis of the 6 blocks of 2 trials with two-way repeated measures revealed an interaction between treatment and group ( $F(1,29)=4.91$ ,  $p=.035$ ), test block and treatment ( $F(5,145)=4.76$ ,  $p<.001$ ), and a main effect of treatment ( $F(1,29)=5.33$ ,  $p=.029$ ) and test block ( $F(5,145)=16.71$ ,  $p<.001$ ). Ischemic rats made fewer re-entries at block 3 ( $p=.031$ ) compared to sham-operated animals. Yohimbine administration increased the number of working errors at block 3 ( $p=.028$ ), 4 ( $p=.049$ ), 5 ( $p=.018$ ), and 6 ( $p=.002$ ) compared to saline-treated ischemic rats. Analysis of the total number of errors across the 12 trials revealed that saline-treated ischemic made less errors than saline-treated sham rats ( $p=.039$ ). Yohimbine-treated made more errors than saline-treated ischemic rats ( $p=.001$ ).

### 3.1.3. Entries 2-9

Fig. 1c shows the effect of ischemia and pre-training administration of yohimbine on working memory errors during the entire trial (entries 2-9). Two-way repeated measures analysis of the 6 blocks of 2 trials revealed an interaction between treatment and surgery ( $F(1,29)=10.04$ ,  $p=.004$ ), test block and treatment ( $F(5,145)=8.66$ ,  $p<.001$ ), and a main effect of test block ( $F(5,145)=27.19$ ,  $p<.001$ ). Yohimbine administration to ischemic rats significantly increased the number of working errors at block 4 ( $p=.009$ ) 5 ( $p=.001$ ) and 6 ( $p<.001$ ). Yohimbine improved performance in sham-operated animals at block 1 ( $p=.026$ ) and 3 ( $p=.039$ ). Analysis of the total number of errors across the 12 trials revealed that yohimbine-treated ischemic rats made more errors than saline-treated controls ( $p=.002$ ).

## 3.2. Working memory: effect of clonidine administration

### 3.2.1. Entries 2-5

Fig. 2a shows the effect of ischemia and pre-training administration of clonidine on working memory errors during within-trial entries 2-5. Two-way repeated measures analysis of the 6 blocks of 2 trials revealed an interaction between treatment and surgery ( $F(1,27)=8.53$ ),  $p=.007$ ) and test block and treatment ( $F(5,135)=2.55$ ,  $p=.031$ ), and a main effect of surgery ( $F(1,27)=4.41$ ,  $p=.045$ ) and test block ( $F(5,135)=15.4$ ,  $p<.001$ ). Clonidine reduced the number of errors in ischemic rats at block 2 ( $p<.001$ ) and 3 ( $p=.03$ ). Analysis of the total number of errors across the 12 trials indicated that clonidine-treated sham animals made more errors compared to vehicle-treated controls ( $p=.007$ ).

### 3.2.2. Entries 6-9

Fig. 2b shows the effect of ischemia and pre-training administration of clonidine on working memory errors during within-trial entries 6-9. Two-way repeated measures analysis of the 6 blocks of 2 trials revealed a main effect of time ( $F(5,135)=19.21$ ,  $p<.001$ ). No effects of clonidine administration were observed in any group, including the analysis of the total number of errors across the 12 trials.

### 3.2.3. Entries 2-9

Fig. 2c shows the effect of ischemia and pre-training administration of clonidine on working memory errors during the entire trial (entries 2-9). Two-way repeated measures analysis the 6 blocks of 2 trials revealed an interaction between test block and treatment ( $F(5,135)=3.87$ ,  $p=.003$ ), and main effect of test block ( $F(5,135)=30.59$ ,  $p<.001$ ). Clonidine administration to ischemic rats reduced the number of working errors at block 2 ( $p=.018$ ). Analysis of the total number of errors across the 12 trials revealed no differences between any of the groups.

### 3.3. Arm entry rate

Fig. 3 shows the effect of ischemia and drug administration of the average arm entry rates per min across the 12 trials. Two-way ANOVA analyzing the impact of clonidine and ischemia on locomotion revealed no interaction between treatment and surgery ( $p=.43$ ), and no main effect of surgery ( $p=.13$ ) and treatment ( $p=.26$ ). Similarly, analysis of the effects of yohimbine and ischemia demonstrated no interaction between

treatment and surgery ( $p=.21$ ), and no main effect of surgery ( $p=.36$ ) and treatment ( $p=.25$ ).

#### 3.4. Reference memory: effect of forebrain ischemia and post-training clonidine administration

Fig. 4 shows the effect of ischemia and post-training administration of clonidine on reference memory errors. Two-way repeated measures ANOVA revealed an interaction between surgery and treatment ( $F(1,29)=6.79$ ,  $p=.015$ ), test block and surgery ( $F(3,87)=2.15$ ,  $p<.028$ ), and a main effect of test block ( $F(3,87)=9.06$ ,  $p<.001$ ). Ischemia had no significant effects on reference memory performance on any of the trials. Clonidine-treated ischemic rats displayed more reference memory errors compared to saline-treated controls at block 1 ( $p=.042$ ) and 2 ( $p=.014$ ). Combining all the trials, clonidine-treated ischemic rats made more errors than saline-treated controls ( $p=.044$ ). No group differences were observed in working memory, with no interaction between test block and surgery ( $p=.57$ ) or main effect of surgery ( $p=.61$ ), even when compiling all groups (data not shown).

#### 3.5. Reference memory: effect of forebrain ischemia and post-training yohimbine administration

Fig. 5 shows the effect of ischemia and post-training administration of yohimbine on reference memory errors. Two-way repeated measures ANOVA revealed a main effect of time ( $F(3,81)=8.38$ ,  $p<.001$ ) but no interaction between surgery and treatment ( $p=.33$ ), and no main effects of surgery ( $p=.85$ ) or treatment ( $p=.4$ ). No significant

differences were observed between any of the groups in their total number of errors. No group differences were observed in working memory, with no interaction between test block and surgery ( $p=.43$ ) or main effect of surgery ( $p=.71$ ), even when compiling all groups (data not shown).

### 3.5. Effect of S.C. injection

No significant differences were observed between sham-operated animals receiving the vehicle and those receiving no injection on any behavioural measures ( $p>.4$  for all comparisons; data not shown). This indicated that injection prior to testing (or after) did not impact performance.

## 4. Discussion

To our knowledge, this study represents the first attempt to improve functional recovery after cerebral ischemia by administering drugs prior to behavioural testing at remote post- ischemic time intervals (e.g., not prior, during or within hours post-ischemia in a context of neuroprotection). Our findings support long lasting effects of 10 min forebrain ischemia on central noradrenergic reactivity, effects associated to the observed impairments in spatial working memory in the radial maze. Ischemia produced short-lasting working memory errors observed in the early arm entries (2-5) within a test trial, and had no impact on reference memory assessed two weeks later in the same radial maze. The results suggested that increased central activity of noradrenergic-containing neurons were associated to ischemia-induced working memory deficits, given attenuation or accentuation of impairments following clonidine and yohimbine administration,

respectively. Specifically, blockade of alpha2-adrenoceptor by yohimbine worsened working memory in ischemic rats, both in overall and within-trial performance (entries 6-9 and 2-9), while administration of the alpha2-adrenoceptor agonist clonidine blocked ischemia-induced working memory impairment manifested in initial entries (2-5) of the first test blocks. Yohimbine did not impair performance in sham-operated animals, as previously reported in rats receiving equivalent doses and tested in a similar radial maze task (Ohta, Matsumoto, Watanabe, & Shimizu, 1993), demonstrating selective sensitivity of ischemic rats to its administration. The effect of clonidine to selectively impair working memory in shams (entries 2-5) could be attributable to lower than optimal NE availability shown to worsen spatial working memory (Compton, Dietrich, Smith, & Davis, 1995). Conversely, it is possible that a negative impact of clonidine administration on working memory was not observed in ischemic rats due to an overactive noradrenergic system counterbalancing its effects. Cerebral ischemia consequently appears to exert relatively long lasting effects on the noradrenergic system that go beyond transient NE release reported at short reperfusion intervals (Gustafson, Westerberg, & Wieloch, 1991; Miura et al., 1999) and may impact reactivity to noradrenergic stimulation and/or testing.

The present data suggest that arousal deficits/noradrenergic dysfunction represent a plausible contributing factor to the behavioural alterations observed following cerebral ischemia. Concordant with our findings, excessive NE levels and working memory impairments have been reported in animal models of traumatic brain injury (TBI)(Kobori, Clifton, & Dash, 2006). Our findings are also corroborated by studies showing the influence of this neurochemical system on various prefrontal tasks and spatial memory

(Arnsten, Mathew, Ubriani, Taylor, & Li, 1999; Compton, Dietrich, Smith, & Davis, 1995), with impaired working memory associated to excessive noradrenergic stimulation (Arnsten, Mathew, Ubriani, Taylor, & Li, 1999; McAllister, 2001; Zhang & Cai, 2005). Increased noradrenergic stimulation by yohimbine in humans is associated to increased impulsivity and distractibility (Swann, Birnbaum, Jagar, Dougherty, & Moeller, 2005), effects which have been shown (e.g., high doses of yohimbine) to correlate to impairments in spatial working memory (Zhang & Cai, 2005) and short-term brain function consistent with increased impulsivity (Arnsten, Mathew, Ubriani, Taylor, & Li, 1999) in rodents.

The effect observed of yohimbine administration on working memory in ischemic rats was similar to the impairing effects of high yohimbine doses (much higher than those used in the current study) on spatial working memory in delayed non-matching to sample (McAllister, 2001) and delayed alternation tasks in the radial maze (Zhang & Cai, 2005) observed in normal animals. Consequently, the effect of the low dose of yohimbine on ischemic rats resembled the effect of higher doses in normal animals, supporting the lack of effect (the low dose) in sham-operated animals (as observed in the current study) or as reported by others in non-surgered rats (Ohta, Matsumoto, Watanabe, & Shimizu, 1993). Interestingly, super-sensitivity to yohimbine effects and improving effects of clonidine on arousal-induced working memory impairments have been demonstrated in different animal models of neuropathology. For example, PCP-induced working memory deficits in the radial maze were attenuated by pre-treatment with clonidine (Bardgett et al., 2008; McCann, Rabin, & Winter, 1987). Conversely, increased sensitivity to yohimbine was observed in chronically stressed rats (Park, Campbell, & Diamond, 2001). The effect of

ischemia to increase HPA axis reactivity (Experiment 4) may consequently have been a primer for the increased sensitivity to yohimbine administration.

Although we cannot comment on noradrenergic release (e.g., ascertainable by *in vivo* measurements of testing-induced extracellular levels or plasma level determination) based on the present results, our behavioural observations upon pharmacological treatments are concordant with ischemia-induced noradrenergic hypersensitivity observed at a similar delayed reperfusion intervals (e.g., up to 2 months) in a study of ischemia-induced analgesia (Pich et al., 1993), and may explain (at least partially) the working memory impairments we observed after ischemia. The impairing effects of yohimbine in ischemics might represent increased outflow of noradrenergic terminal areas involved in spatial/working memory due to ischemia-induced elevations in synthesis of NE and/or increased distribution/density (e.g., upregulation) of alpha2-adrenoceptors. As aforementioned, increased NE synthesis (in mPFC) and working memory impairments have been reported in an animal model traumatic brain injury (TBI)(Kobori, Clifton, & Dash, 2006), and therefore might represent a plausible outcome of cerebral ischemia.

Our findings in the second radial maze assessment revealed no differences in the number of reference memory errors between sham and ischemic animals, similar to our previous observations at longer post-ischemic delays (Plamondon, Morin, & Charron, 2006). Post-training clonidine administration to ischemic rats (but not sham controls) was associated with impaired retention of spatial locations of food items. The impairing effect of clonidine on reference memory suggests that the impact of ischemia on increased noradrenergic reactivity was still in effect, and may have acted to facilitate arousal-related memory consolidation as observed in other memory paradigms (McGaugh &

Roozendaal, 2002; Roozendaal, Okuda, de Quervain, & McGaugh, 2006; Roozendaal, Okuda, Van der Zee, & McGaugh, 2006), thereby possibly explaining the absence of effects of ischemia on reference memory. This phenomenon could help clarify why reference memory deficits after cerebral ischemia are much less apparent compared to working memory deficits (H. P. Davis, Baranowski, Pulsinelli, & Volpe, 1987; Plamondon, Morin, & Charron, 2006; Volpe, Pulsinelli, Tribuna, & Davis, 1984). Although spatial cues and task parameters were familiar at this testing interval given previous experience with the maze, impairments in ischemic rats might still have been expected (e.g., in the context of hippocampal damage) since animals had to remember selective spatial locations (e.g., a skill not acquired during the working memory version).

This study is the first to analyze radial maze working memory performance from a within-trial perspective, an approach that provides contrasting findings compared to previous assessments of ischemic working memory impairments in this paradigm (Gionet et al., 1991; Okada et al., 1996; Plamondon & Khan, 2006). Within-trial analysis of the working memory performance revealed that while ischemic rats made more errors in initial entries (2-5), they displayed improved working memory in the last four entries (6-9) compared to sham-operated controls, minimizing overall differences between the two groups. The effects were not related to S.C. injection (e.g., stress leading to masking of differences between groups) since non-injected sham rats demonstrated the same behavioural performance as the vehicle-treated group. It may be important to consider that previous reports of more severe working memory impairment after 10 min ischemia did not control for the total number of arm entries made between the surgical groups, given that daily trials ended after a certain time interval (or following completion of entry

into all arms). Any group differences in the total number of arm entries in rats not completing the task within the prescribed time could impact the results by increasing or decreasing the probability of committing a working memory error. The only study (aside from the current one) having examined the effect of 10 min ischemia on working memory in the radial maze and controlling for equivalent entries between groups (e.g., in this case analyzing only the first 8 choices) similarly reported no overall working memory impairments at comparable post-reperfusion time intervals (Iwasaki et al., 2006). This same group also described extremely short-lasting working memory impairment (only observed on the first testing day) in the radial maze after 10 min ischemia (Iwasaki, Kitamura, Ohgami, Mishima, & Fujiwara, 1996). Consequently, the overall lack of working memory impairment in the radial after 10 min ischemia is not without precedent.

Although the observation of improved performance in ischemic rats as they progressed within a trial might not appear supportive of the proposition of noradrenergic hyperactivity in these animals since working memory impairments might have been expected across all entries, this finding may be reconcilable. Previous reports showing impairing effect of excessive noradrenergic stimulation have obtained these effects in contexts where drugs are administered and protracted effects expected. This may not represent what occurs endogenously in response to the stressor of behavioural testing. Given that excessive levels of NE impair and optimal levels facilitate working memory performance, it may be postulated that when arousal was greatest following immediate exposure to the maze (and including the stress of handling, injection, etc.), excessive levels of NE might have been released, contributing to immediately elicited impairments in working memory in ischemic animals. However, after increased exposure to the maze

(trials could last 10-15 min, particularly in the first blocks), arousal and noradrenergic hyperactivity might have attained optimal stimulation levels which improved performance (and not present at that time in sham-operated animals). In the case of ischemic rats receiving yohimbine, the NE stimulation was likely more protracted and thus would explain the greater (and overall) impairments. In a recent study examining performance in 5 min radial maze trials (thus presumably involving very few arm entries) working memory impairments in ischemic rats were similarly observed (Langdon, Granter-Button, & Corbett, 2008), supporting the idea that ischemia-induced effects on working memory are rapidly elicited following exposure to testing. Further, the reduced working memory errors demonstrated by ischemics at entries 6-9 cannot be explained by an increased probability to enter an arm previously visited given that ischemics made less errors despite not making more initial errors (e.g., at test block 3).

One distinct possibility is that spatial memory capabilities per se are not disrupted by cerebral ischemia. Studies investigating the effect of selective CA1 lesion in rats, the most impacted structure after 10 min ischemia (Nunn et al., 1994), do not report impaired spatial memory (Goodrich-Hunsaker, Hunsaker, & Kesner, 2008; Hunsaker, Fieldsted, Rosenberg, & Kesner, 2008; Hunsaker & Kesner, 2008; Hunsaker, Lee, & Kesner, 2008), including working spatial memory (Gilbert, Kesner, & Lee, 2001; Lee, Jerman, & Kesner, 2005), but rather lesioned rats display impaired processing of temporal order (Gilbert, Kesner, & Lee, 2001; Hoge & Kesner, 2007; Hunsaker, Fieldsted, Rosenberg, & Kesner, 2008; Hunsaker & Kesner, 2008; Lee, Jerman, & Kesner, 2005). Other studies have similarly concluded that spatial working memory is not CA1 dependent (Kikusui, Aoyagi, & Kaneko, 2000). Therefore, despite CA1 neurons considered as “place” cells

(O'Keefe, 1976), an ischemic lesion to this structure does not predict spatial memory alterations in the tasks we used. In this context, CA1 ischemic injury in gerbils did not impair performance on a place task conducted in the open-field (Walsh, Harley, Corbett, Skinner, & Martin, 2008).

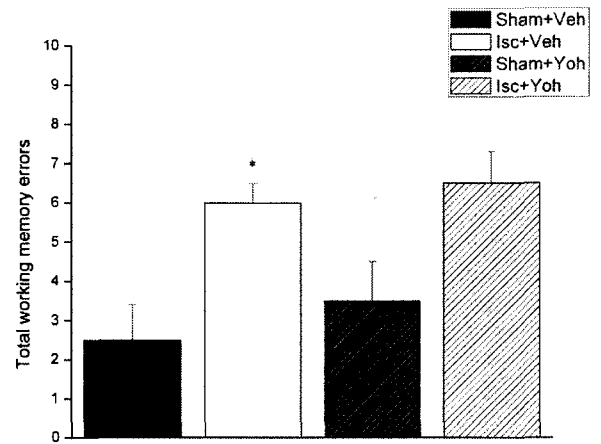
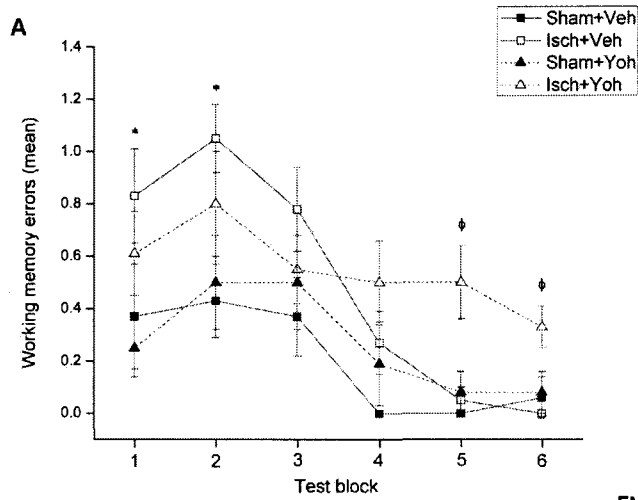
The notion of ischemia-induced neuroendocrine alterations as mediating post-reperfusion (Experiment 4) performance is just as (if not more) plausible than a hippocampal-based interpretation, given the impact of such systems (e.g., HPA axis, noradrenergic) on spatial working memory, reference memory, and object recognition memory (also not reliant on the CA1). It is important to note that these findings might be more applicable to 10 min models of ischemia which do not generally produce significant extra-hippocampal damage (as aforementioned). Longer durations of ischemia would produce significant extra-hippocampal damage (H. P. Davis, Baranowski, Pulsinelli, & Volpe, 1987; Volpe, Pulsinelli, Tribuna, & Davis, 1984) which might impact behavioural performance to a greater degree, but would have limited ecological validity given that humans subjected to CA do not survive ischemic durations longer than 7 min (Herlitz et al., 1994).

In conclusion, given the recovery of ischemics (saline- and clonidine-treated) in spatial working memory, it is likely that extra-CA1 structures supported learning and memory in these animals. Intact structures such as the hippocampal subfield CA3 (Walsh, Harley, Corbett, Skinner, & Martin, 2008) and the frontal cortex (Dalley, Cardinal, & Robbins, 2004) are both good candidates for the mediation of the functional recovery observed in saline and clonidine-treated animals. Finally, given that cognitive impairments after cardiac arrest in humans (Moulaert, Verbunt, van Heugten, & Wade,

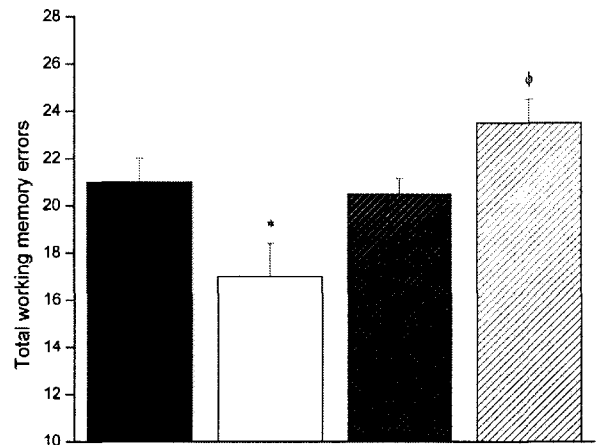
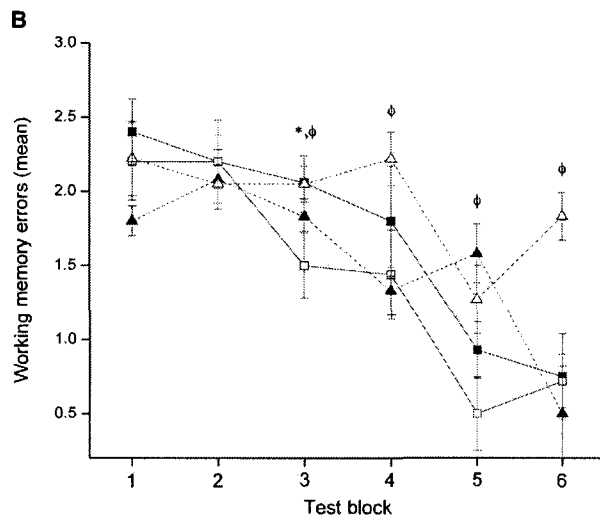
2009) are not interpreted as related to alterations in arousal, and assuming more experimental support for a role of neuroendocrine/arousal deficits in post-ischemic functional outcome, recontextualization of animal models and their generalizability to findings with humans will need to be considered. Without a firm understanding of the causes of cognitive impairment after forebrain ischemia in animal models, it may not be possible to efficiently develop potential treatments aimed to reduce the negative impact of cardiac arrest (and perhaps even stroke) on cognitive outcome.

#### *Acknowledgements*

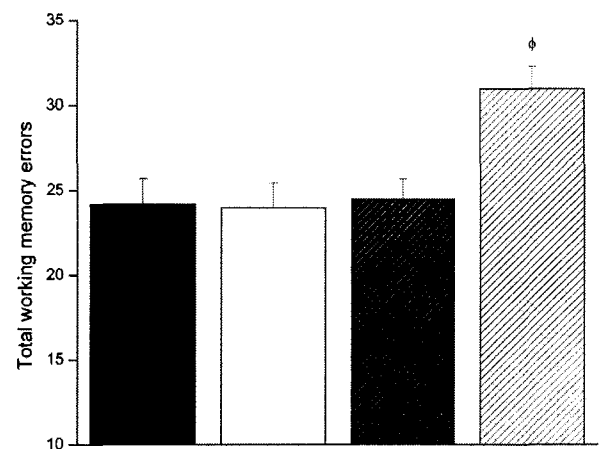
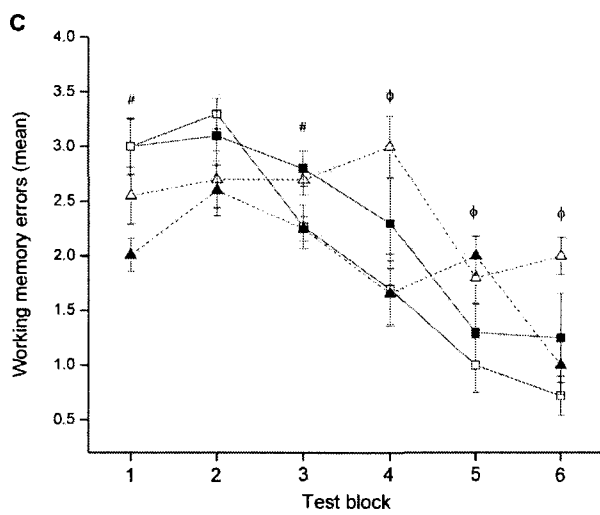
We sincerely thank Sylvie Émond, Vivianne Tailler, Catrinel Girbovan, and Mélanie Lambrou for excellent technical assistance. This work was supported by a grant from the Natural Sciences and Engineering Research Council of Canada to H.P.



ENTRIES 2-5

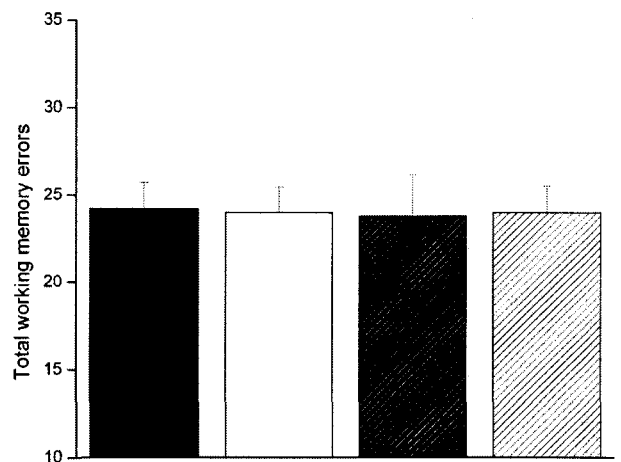
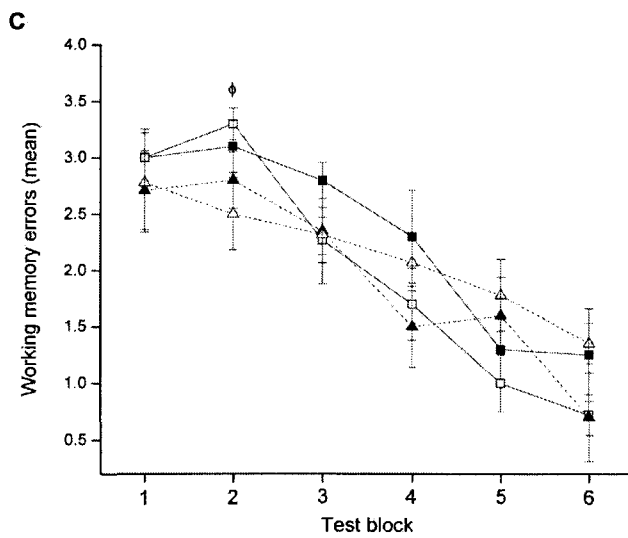
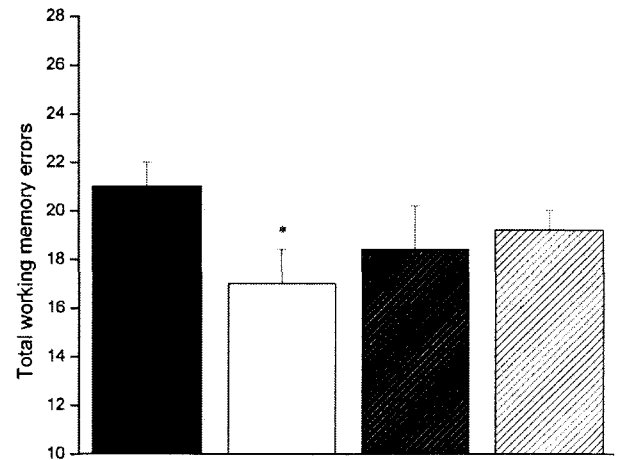
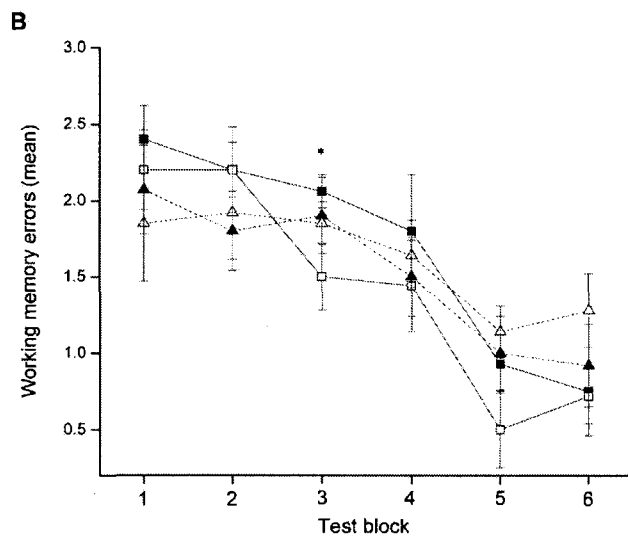
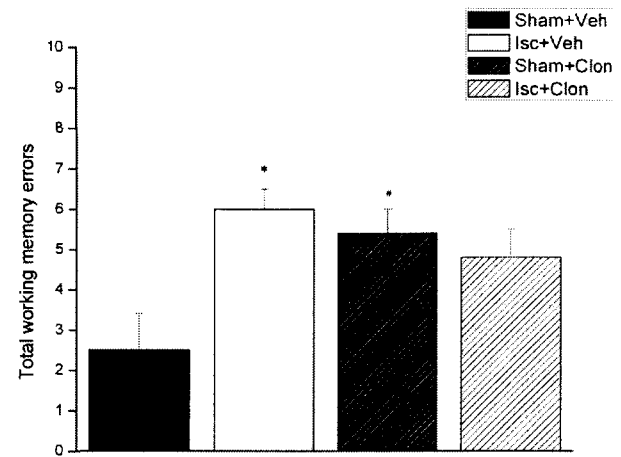
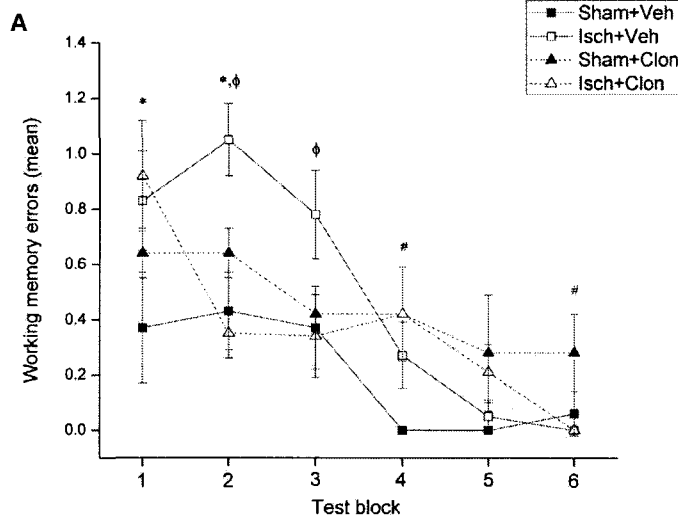


ENTRIES 6-9

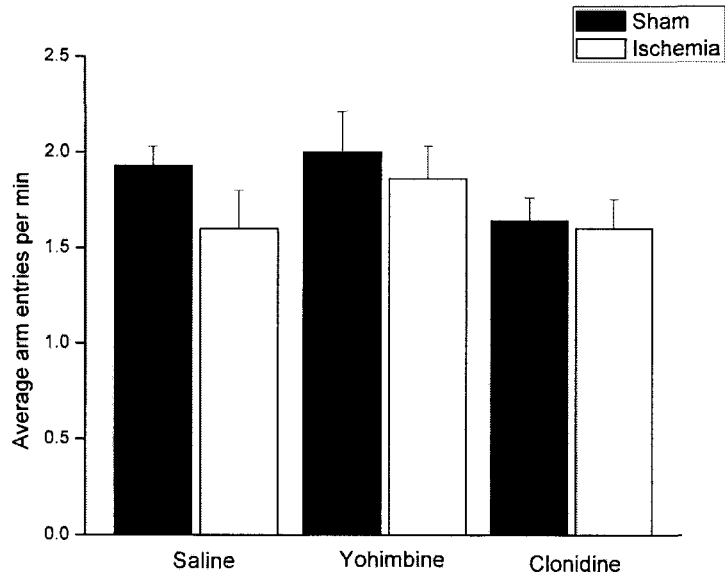


ENTRIES 2-9

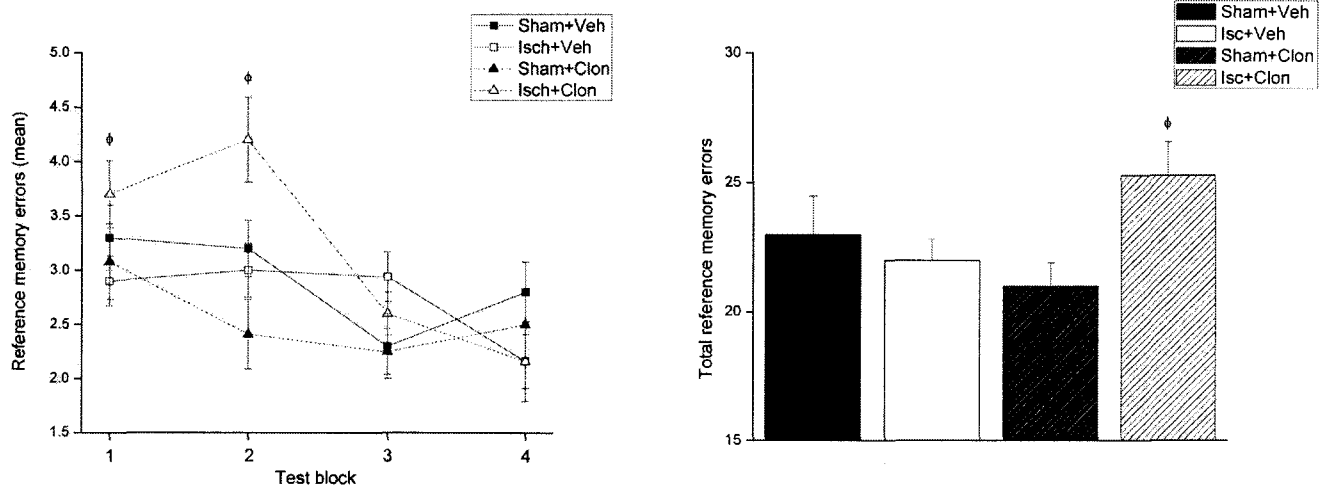
**Figure 1.** The effect of ischemia and pre-training administration of yohimbine on working memory errors during within-trial entries 2-5, 6-9, and 2-9. Symbols indicate significant differences between vehicle-treated ischemic and sham-operated groups (\*), between vehicle and yohimbine treated ischemic rats ( $\phi$ ), and between vehicle and yohimbine (or clonidine) treated sham-operated animals (#). Reported differences were significant at  $p < .05$ . Values are expressed as mean  $\pm$  SEM.



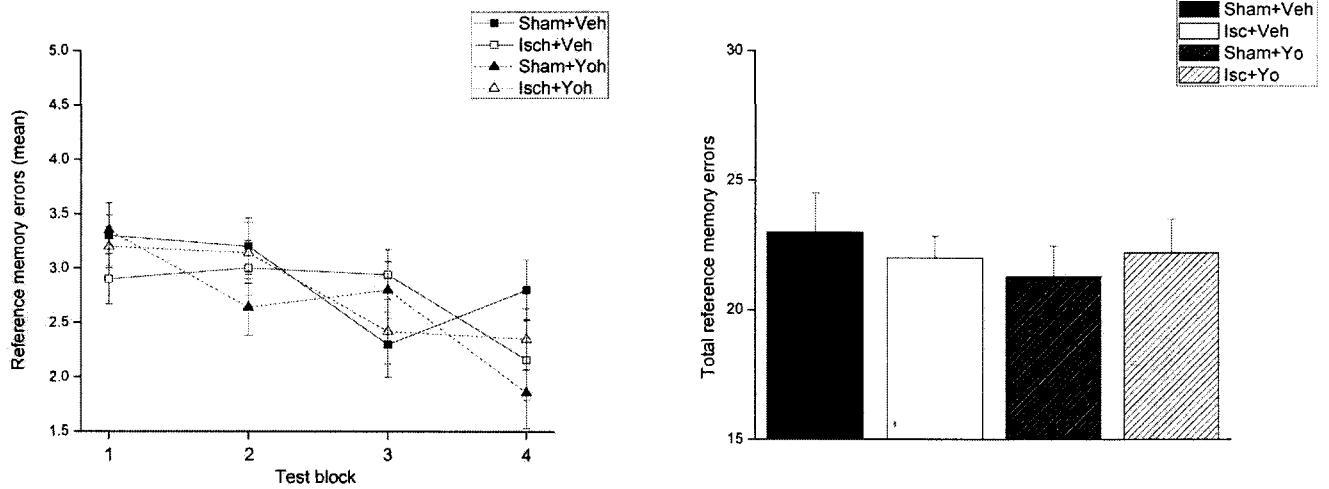
**Figure 2.** The effect of ischemia and pre-training administration of clonidine on working memory errors during within-trial entries 2-5, 6-9, and 2-9. Symbols indicate significant differences between vehicle-treated ischemic and sham-operated groups (\*), between vehicle and clonidine treated ischemic rats ( $\phi$ ), and between vehicle and clonidine treated sham-operated animals (#). Reported differences were significant at  $p < .05$ . Values are expressed as mean  $\pm$  SEM.



**Figure 3.** The effect of ischemia and pre-training administration of yohimbine or clonidine on radial maze arm entry rate during working memory assessment. No differences were observed between any of the groups. Reported differences are significant at  $p < 0.05$ . Values are expressed as mean  $\pm$  SEM.



**Figure 4.** The effect of ischemia and post-training administration of clonidine on reference memory errors. Symbols indicate significant differences between vehicle-treated ischemic and sham-operated groups (\*), between vehicle and clonidine treated ischemic rats ( $\phi$ ), and between vehicle and clonidine treated sham-operated animals (#). Reported differences were significant at  $p < 0.05$ . Values are expressed as mean  $\pm$  SEM.



**Figure 5.** The effect of ischemia and post-training administration of yohimbine on reference memory errors. Symbols indicate significant differences between vehicle-treated ischemic and sham-operated groups (\*), between vehicle and yohimbine treated ischemic rats ( $\phi$ ), and between vehicle and clonidine treated sham-operated animals (#). Reported differences were significant at  $p < 0.05$ . Values are expressed as mean  $\pm$  SEM.

## **General Discussion**

The present thesis investigated the effect of forebrain ischemia on emotional reactivity and arousal, and the possible contribution of such alterations to ischemia-induced behavioural changes and cognitive impairment during the post-reperfusion period. The series of behavioural studies aimed to document the impact of forebrain ischemia on behavioural and neuroendocrine/neurochemical measures of emotionality and arousal at different post-reperfusion intervals. Experiments 1 and 2 examined the impact of modulation of the environmental emotional tone on post-ischemic behavioural performance by manipulating illumination level during testing. Experiment 3 further examined behavioural measures of emotionality at a variety of post-reperfusion time intervals (1 day to 1 month). Experiments 4 and 5 examined the effects of ischemia on the HPA axis and the noradrenergic system and their relationship to cognitive-behavioural impairments observed in ischemic rats.

### **1. The effect of ischemia on behavioural and emotionality reactivity**

#### *1.1. Experiments 1 and 2*

The general objective of these initial experiments (1 and 2) was to characterize the effects of 10 min forebrain ischemia on behavioural reactivity/activation, and determine if differential regulation of emotionality could influence behaviour. The overall findings of Experiments 1 and 2 supported our hypothesis that ischemia leads to alterations in behavioural arousal or reactivity to stressful conditions (e.g., during testing), effects which might be implicated in certain post-ischemic behaviour. The majority of the effects of illumination were limited to open-field exploration, having no

impact on object recognition and spatial memory tests. The observation of hypoexploration under dim illumination versus hyperexploration under bright illumination in ischemic rats showed that exploratory alterations commonly observed after forebrain ischemia are not necessarily indicative of an underlying memory deficit related to hippocampal cell death and disrupted habituation capabilities. All ischemic groups displayed normal habituation (both within and between sessions) at all tested intervals despite changes in exploration rate. Had a habituation deficit been the sole mediator of post-ischemic open-field performance, no differences between ischemic and sham-operated animals would have been expected under the different illumination conditions. Instead, the opposite behavioural pattern observed in ischemic relative to sham animals suggested that these animals had unique appraisal and behavioural reactivity to the two testing contexts, given they were different only in their degree of illumination, possibly affecting their arousing/anxiogenic properties.

As discussed in greater detail in the general introduction of this thesis, open-field testing assesses reactivity of the subjects to a stressful event rather than limited effects on exploration (Prut & Belzung, 2003). The open-field hyperactivity commonly reported following ischemia in rodents (Colbourne, Auer, & Sutherland, 1998; Colbourne & Corbett, 1994; E. J. Green et al., 1995; Plamondon & Khan, 2005; X. B. Yan, Wang, Hou, Ji, & Zhou, 2007) may consequently be a representation of increased behavioural activation/arousal. This hypothesis is consistent with the observation that open-field alterations were elicited in the initial minutes of open-field exposure (Experiment 1). If the ischemia-induced hyperactivity were more related to impaired habituation memory, differences in locomotor activity might not be expected in the initial minutes of testing,

but only after the passage of time when the spatial context would presumably become more familiar for sham-operated animals (thereby selectively reducing activity in this group). Although the impact of forebrain ischemia on reactivity has not been tested in other paradigms, the behavioural findings of these experiments are consistent with alterations in reactivity to novel and/or stressful stimuli reported in rats following 7 min asphyxial cardiac arrest reproducing forebrain ischemia (in this model asphyxiation leads to CA and rats are resuscitated with epinephrine) and demonstrating increased acoustic startle one week after ischemia, indicating increased arousal in these animals (Hickey, Akino, Strausbaugh, & De Courten-Myers, 1996). The ischemia-induced hyperactivity we observed under bright illumination is also consistent with the report of increased open-field activity (locomotion and rearing) observed after 5 consecutive days of physical (shocks) or emotional stress (observing other rats getting shocked)(Pijlman, Wolterink, & van Ree, 2002), behavioural activation that was observed in the first few (3) minutes of testing, similar to our results in Experiment 1. The stressor of ischemia, as demonstrated by increased plasma CORT levels observed for up to four days post-reperfusion (Hwang et al., 2006)(Experiment 4), might consequently prime ischemics for increased behavioural activation in response to testing in a novel (and stressful) environment such as the open-field. Finally, the observation that ischemic rats did not display habituation memory impairment under any of the testing conditions further support the idea that alterations in behavioural reactivity/activation might represent a plausible explanation for ischemia-induced changes in in open-field exploration.

The open-field represents a behavioural measure mainly used to assess emotional reactivity (Prut & Belzung, 2003). Its use for the measurement of open-field habituation

memory capabilities was initially reported by one research group studying ischemic gerbils (Wang & Corbett, 1990). This research group concluded that ischemic gerbils had a deficient ability to form spatial maps due to neuronal degeneration in the CA1 subfield of the hippocampus, leading to habituation deficit (failure to familiarize to the spatial context) in the open-field manifested as increased exploration (e.g., thus displaying “hyperactivity” relative to sham controls). Since then, this same research group published the majority of studies (60%) using the open-field to measure habituation after cerebral ischemia, many of which directly associated the ischemia-induced CA1 cell death to open-field hyperactivity (Colbourne & Corbett, 1994, , 1995). However, this research group later found that ischemic preconditioning, which entailed subjecting gerbil to a short and non-damaging forebrain ischemia the day before the damaging ischemia of longer duration, protected CA1 neurons against degeneration without attenuating the ischemia-induced open-field hyperactivity (Corbett & Crooks, 1997), a phenomenon replicated in rats (Plamondon, Davignon, Khan, & Charron, 2008). These findings suggest that CA1 neurons are not implicated in open-field alterations after ischemia, and that increased locomotor activity reported over days in gerbils tested in the open-field for short periods of time over consecutive days (Corbett, Giles, Evans, McLean, & Biernaskie, 2006; Corbett, Nurse, & Colbourne, 1997; Dowden & Corbett, 1999) may be linked to neuronal injury in other brain areas but also related to neurochemical dysfunction. However, the fact that animals experienced comparable reduction of activity as time elapsed over the initial exposure does suggest that upon longer observation period, these animals do habituate normally. As Experiment 1 showed, habituation deficits were not elicited both within (30 min session) and between-session (over days;

short 10 min sessions), contrary to the habituation memory deficit observed in ischemic gerbils, considered to occur both within-session and between-session open-field exposure.

One possible explanation for the interpretation of habituation memory deficits in ischemic gerbils is possibly linked to the failure of these studies to examine within-subject alterations in exploration. Indeed, examining exploratory activity changes after ischemia in the open-field from a within-subject analysis would more accurately measure habituation capabilities. Open-field habituation is defined as a decrease in exploratory activity as the environment becomes more familiar, and individual differences in initial locomotor activity (for example, on the first block of time) necessarily impacts the level of activity on any subsequent blocks, thus will have a significant effect on group mean scores. In this context, statistical analyses like repeated-measures ANOVA can actually fail to detect important within-subject effects because they consider only changes (e.g., over time) in the mean scores. Calculating a habituation index which considers within-subject changes in addition to the group differences (like performed in the present thesis) might therefore be more sensitive in the detection of habituation capabilities, and perhaps lead to a failure to detect actual habituation impairments in ischemic gerbils. Analyzing open-field behaviour in this manner makes it possible for ischemic rats to be hyperactive without actually having habituation memory impairment.

The impairing effect of forebrain ischemia on object recognition memory (Mumby et al., 1996; Plamondon, Morin, & Charron, 2006) and working memory assessed by spontaneous alteration measurement (X. B. Yan, Wang, Hou, Ji, & Zhou, 2007) was replicated in Experiment 2, but illumination level had no impact on their

manifestation. It may be that two distinct neurophysiological mechanisms mediated the illumination-dependent ischemia-induced alterations in open-field activity while having comparable impairing effects on the memory tasks. Consequently, it cannot be concluded based on these findings that underlying alterations in emotional reactivity did not mediate the observed ischemia-induced cognitive impairments in Experiment 2.

### *1.2. Experiment 3*

The findings of Experiment 3 helped reconcile discrepant observations in the literature; specifically those of ischemia-induced anxiety (Dhooper, Young, & Reid, 1997; Nakashima, Ajiki, Nakashima, & Takahashi, 2003), anxiolysis (Nelson, Lebessi, Sowinski, & Hodges, 1997; Plamondon & Khan, 2005; B. Yan et al., 2007) or of no effect (Bantsiele et al., 2004; Roberge, Hotte-Bernard, Messier, & Plamondon, 2008) in the EPM which was hypothesized the result of assessment at different post-reperfusion intervals. Indeed, examining the timelines in these rat studies, increased anxiety was observed shortly after reperfusion (2 days) (Dhooper, Young, & Reid, 1997), with decreased anxiety a few days later (e.g., 4-7 days) (Plamondon & Khan, 2005; X. B. Yan, Wang, Hou, Ji, & Zhou, 2007), and no differences three weeks post-reperfusion (20 days) (Bantsiele et al., 2004). These findings are concordant with the findings of Experiment 3, despite some of these studies not using test-naïve animals. More importantly, the results showed that open-field alterations were elicited even after delayed post-reperfusion intervals (up to 30 days) when cognitive assessment often occurs. The changes in open-field exploratory activity were not consistently associated to anxiety or memory alterations level, particularly at longer post-ischemic intervals when hyperactivity was

observed despite no alterations in anxiety (e.g., in the EPM and/or center exploration). There were also no significant correlations between open-field activity and EPM scores (unreported analysis from Experiment 3). These findings therefore demonstrate that the activating effect of ischemia on open-field locomotor activity (under bright illumination) cannot be considered the result of a global reduction in anxiety or habituation memory deficits.

### *1.3 Concluding remarks on Experiments 1, 2 and 3.*

Overall, the findings of Experiments 1, 2 and 3 support the notion of differential behavioural reactivity in ischemic rats, observed after short and longer post-reperfusion intervals. Specifically, the most salient (and repeatedly observed) effect of ischemia was hyperactivity in a bright novel open-field (day 1-10, 30). One relevant observation by this author (although not a reported thesis finding) was that while ischemic rats showed hyperexploration in a bright novel open-field, these same rats displayed locomotor activity comparable to sham animals when placed in a semi-novel cage (different from their home cage only in size; 2 times larger) located in a familiar environment (testing was performed in their home room with the cage placed on a table in the middle of the room). This finding is important as it further suggests that locomotor hyperactivity after ischemia is an index of behavioural reactivity to stressful situations rather than an indicator of global differences in motoric activity (e.g., a neurological deficit), or as determined by the other thesis behavioural studies, largely independent of alterations in anxiety or memory. Consequently, the ischemia-induced open-field hyperactivity likely represents behavioural activation/disinhibition under relatively stressful conditions (such

as behavioural testing in novel environments, typical of most animal tasks). In our studies, increased behavioural activation in ischemic rats was solely observed under bright and novel testing contexts, with hypoarousal under novel but dimly lit (and less stressful) conditions, and no alterations in activity when tested under fairly familiar conditions. Consequently, ischemic rats may not show greater arousal in resting conditions (e.g., in their familiar homecage), but only in response to significant psychogenic stressors. Although animal testing is typically conducted under some degree of illumination (and not dimly lit as in Experiments 1 and 2), illumination level during testing should be taken into greater consideration or at least more consistently reported in order to better compare studies and understand the impact of cerebral ischemia on post-reperfusion behaviour.

## **2. Altered neuroendocrine regulation following ischemia: relationship to functional outcome**

### *2.1. Experiment 4: HPA axis alterations after ischemia*

Following up on the above described observations suggesting ischemia-induced behavioural activation, a second main thesis goal was to explore whether learning and memory impairments post-reperfusion were associated to alterations in neuroendocrine reactivity in response to testing. The results from Experiment 4 were consistent with previous reports of increased CORT observed up to four days after reperfusion (Hwang et al., 2006). Our 10 min ischemia produced significant elevations in circulating ACTH and CORT in the hours following ischemia, with continued elevations of CORT for a period of 3 days. This effect translated into increased post-testing levels of CORT associated to

memory impairment in both spatial working memory in the Y-maze, and spatial memory retrieval in the Barnes Maze. Metyrapone treatment aggravated hippocampal damage in ischemic rats, yet improved spatial memory relative to the saline-treated group simultaneous with attenuated neuroendocrine reactivity (as measured by post-training CORT levels). Findings from this study are the first to show an association between altered neuroendocrine reactivity and post-ischemia functional outcome.

We observed ischemia-induced memory retrieval impairment elicited in the Barnes maze (when considering performance across all trials) largely on the second trial, and the increased percentage in traveled distance in ischemic rats on this trial relative to first suggested ischemia-induced behavioural reactivity alterations. The relationship between enhanced neuroendocrine/behavioural reactivity and impaired functional outcome was supported by the exclusive significant correlation between post-training CORT levels and spatial performance on the second daily trial, which indicated that memory retrieval/spatial navigation impairment was elicited after the initial potentiation of arousal/HPA reactivity following Barnes maze exposure. Part of the analysis, similar to the open-field habituation index (Experiments 1-3), relied on within-subject analysis once again drawing on the importance of analyzing changes in performance relative to some baseline (in this case, the first daily trial) in each rat. In this context, this was the first study to test the effect of ischemia on the Barnes maze, considered a “land” version of the MWM similarly assessing the animals’ ability to find an escape area on multiple daily trials across many days. Given disruption of spatial memory (e.g., acquisition) in the MWM following 8-10 min cerebral ischemia (Nelson, Lebessi, Sowinski, & Hodges, 1997; Nunn et al., 1994; Olsen, Scheel-Kruger, Moller, & Jensen, 1994a, , 1994b), it is

possible that a within-trial/within-subject analysis would reveal similar alterations in reactivity to the testing conditions leading to impairments not on the first daily trials (which can be considered the most robust measure of reference memory in this task) but on subsequent trials typically occurring minutes later. This analysis could actually be rapidly conducted on already acquired (and published) data to confirm whether this phenomenon can be replicated.

Our findings confirmed our hypothesis that acute exposure to a physiological stressor such as ischemia might sensitize the HPA axis to the subsequent stressor of behavioural assessment in novel testing paradigms, having negative impact on memory performance, indirect of impairment associated to discrete hippocampal damage per se. The impact of cerebral ischemia was consequently similar to the effects of other significant single stressors (both psychogenic and physical) altering behaviour and physiological functions many weeks post-stressor (Belda et al., 2008), including spatial memory impairments in the radial maze weeks after a single exposure to a cat/predator (El Hage, Griebel, & Belzung, 2006). Although we did not assess open-field performance in this study, it might be reliable to assume that given its similarity to the BM (e.g., bright, open area), comparable elevations in CORT might be expected in response to open-field testing at a similar post-reperfusion interval, as well as hyperactivity. Indeed, although velocity was not measured in the Barnes maze, ischemic rats did not spend more time exploring the maze compared to sham despite showing increased traveled distance to complete the task. This observation provides indirect evidence that ischemic rats were hyperactive (or had increased velocity) relative to sham-operated animals during Barnes maze exploration, a behaviour associated to increased HPA activation. Specifically open-

field hyperactivity under bright illumination (1000 lux for the first reference) was elicited by low doses of CRH (i.c.v.) (Sutton, Koob, Le Moal, Rivier, & Vale, 1982; Veldhuis & De Wied, 1984), contrary to the effects of CRH deficiency which can suppress open-field activity (Swain & Maric, 1995).

Together, these findings demonstrated a significant effect of cerebral ischemia to increase activation of the HPA axis in response to testing at delayed time points, an effect associated to spatial memory impairment. Given that the effects of CORT on cognitive processes are elicited only following a significant delay (e.g., non-genomic mechanisms), the mechanisms through which increased post-training CORT in ischemic rats was associated to memory retrieval impairment could not be determined. However, because elevations in CORT can be an indication of increased noradrenergic stimulation (Pacak et al., 1993; Ziegler, Cass, & Herman, 1999) the rapid effects of NE on cognition and behaviour (McEwen & Sapolsky, 1995) might have been associated to the observed post-reperfusion behavioural alterations.

## 2.2. Experiment 5: Noradrenergic alterations after ischemia

This study is the first to specifically investigate the impact of global cerebral ischemia on central noradrenergic reactivity in the context of functional outcome. It was also the first study to attempt to pharmacologically prevent ischemia-induced cognitive impairment by administering drugs prior to behavioural testing (versus prior, during, or shortly after ischemia). In this sense, animals were tested and administered the pharmacological compound after the time-window during which neuroprotective agents can exert their effects on neuronal survival. In this experiment, the worsening versus

attenuation of working memory capabilities in ischemic animals by pre-training yohimbine and clonidine administration suggested an overactive noradrenergic system at the tested post-reperfusion time intervals (day 10-25), associated to the spatial working memory alterations observed in these animals in a radial maze. These effects were not observed in sham-operated animals, indicating a selected sensitivity to their administration by rats subjected to forebrain ischemia. These findings were in agreement with previous demonstrations of ischemia-induced noradrenergic hyper-reactivity at short (Iwasaki, Kitamura, Ohgami, Mishima, & Fujiwara, 1996) and delayed post-ischemic intervals (Pich et al., 1993), and concordant with the role of NE on stress-induced cognitive impairments (Birnbaum, Gobeske, Auerbach, Taylor, & Arnsten, 1999), given that Experiment 4 demonstrated that ischemia was a potent stressor. Further, enhanced NE synthesis (in mPFC) and working memory impairment have been observed in animal models of traumatic brain injury (head trauma), supporting a role for noradrenergic dysfunction in functional outcome following different types of cerebral injury (Kobori, Clifton, & Dash, 2006).

The findings of this experiment are in agreement with the premise that working memory depends on optimal noradrenergic stimulation of frontal structures (Lapiz & Morilak, 2006), and not hippocampal sites damaged by ischemia (more details later). Indeed, saline-treated ischemic rats, which presumably had significant CA1 damage during testing (e.g., as observed in Experiment 3 at similar post-reperfusion interval day 15), did not display impairments when treated with clonidine prior to testing, supporting the idea that ischemia-induced working memory deficits are associated to excessive central NE activation. A mechanism for the disruption of working memory ( by ischemia

and yohimbine) by excessive noradrenergic stimulation with yohimbine in humans is increased impulsivity and distractibility (Swann, Birnbaum, Jagar, Dougherty, & Moeller, 2005) and in rodents, short-term changes in brain function also consistent with increased impulsivity (Arnsten, Mathew, Ubriani, Taylor, & Li, 1999) and correlated to impairments in spatial working memory (Zhang & Cai, 2005). Consequently, the ischemia-induced working memory impairment observed early within a test trial may have been due to impairments in selective attention and increased distractibility elicited by excessive NE stimulation.

During reference memory testing, post-training clonidine administration increased the number of errors in our ischemic rats, supporting the hypothesis that increased arousal in these animals might mask underlying reference memory by means of increased consolidation. Increased arousal, thus increased NE and CORT post-testing, is associated to improvement in retention in spatial memory and object recognition tests in normal animals (Okuda, Roozendaal, & McGaugh, 2004; Roozendaal, 2002; Roozendaal, Okuda, Van der Zee, & McGaugh, 2006). This finding is also interesting because it may help clarify why spatial reference memory is sometimes less impacted by cerebral ischemia compared to working memory.

Although ischemic animals displayed improved working memory (in the later entries within a trial), as discussed in greater detail elsewhere, this effect may also have been due to increased (but beneficial levels) of noradrenergic stimulation at those times (e.g., entries 6-9). In this context, ischemia might lead to excessive noradrenergic activation enough to disrupt working memory performance in the early moments of test exposure (when stress/arousal may be the highest), but actually improve performance at

longer delays (e.g., by entries 6-9) when the testing context is more familiar and NE levels attenuated (and/or having reached optimal levels). These results are not counter-intuitive if ischemia-induced spatial working memory impairments are considered as associated to differential arousal in these animals. The observation of ischemia-induced improvements in working memory can actually be interpreted as indirect evidence for alterations in arousal (and other neurochemical systems) given the “dynamic” non-linear behaviour observed in the tested animals; a discrete brain lesion would likely not improve performance in the manner observed (and as we shall discuss, discrete CA1 lesions do not actually produce working and/or spatial memory deficits).

It remains to be determined what precisely led to the increased noradrenergic sensitivity in ischemics. One possibility is that cerebral ischemia caused an upregulation of noradrenergic receptors without affecting NE synthesis. Thus, following exposure to the stressor of testing, stimulation of a greater number of NE receptors could have altered performance in rats subjected to ischemia independent of increases in outflow from terminal areas. In this same context, the selective effect of yohimbine on ischemics may not have been due to increased outflow of NE (due to increased existing stores) but because of alterations in receptor density/stimulation. Alternatively, ischemia may have led to a sensitization of structures such as the amygdala and LC leading to potentiation of arousal and effects of a stressor. These hypotheses remain to be investigated, and would help explain/confirm the role of the noradrenergic system in post-ischemia behaviour.

### *2.3 Concluding remarks on Experiments 4 and 5*

The effects of ischemia on the HPA axis and noradrenergic reactivity in Experiments 4 and 5 are in agreement with the open-field behavioural reactivity deficits observed in the early thesis experiments which predicted increased CORT and noradrenergic activity in response to testing in relatively novel environments. In addition, these findings are also concordant (as discussed in greater details in the thesis introduction) with previously observed stimulatory effects of noradrenergic system on the HPA axis (Feldman, Conforti, & Siegel, 1982; Ziegler, Cass, & Herman, 1999), and the increased NE sensitivity after ischemia (observed in Experiment 5) provides a plausible explanation for the post-training CORT elevations observed in these animals (Experiment 4). Conversely, the results of Experiment 4 which showed long lasting stress-induced cognitive impairments in ischemic rats are concordant with the increased NE sensitivity observed in Experiment 5, in that this neurochemical has previously been shown to aggravate stress-induced (in this case, pre-testing infusions of the pharmacological stressor, FG7142) cognitive impairments (Birnbaum, Gobeske, Auerbach, Taylor, & Arnsten, 1999). The effects of cerebral ischemia on neurophysiological indices of reactivity/arousal consequently appear to be long-lasting and implicated in post-reperfusion cognitive performance at a variety of time intervals, not only in neuronal outcome.

Notably, it may be the novelty of the testing situations leading to behavioural manifestation of the effects of ischemia on neuroendocrine reactivity, given that the novelty of testing paradigms can induce noradrenergic and HPA axis activation impacting learning and memory (Rooszendaal, Okuda, de Quervain, & McGaugh, 2006;

Roozendaal, Okuda, Van der Zee, & McGaugh, 2006). In Experiment 4, deficits in spatial reference memory (e.g., the reactivity deficit as displayed by ischemic rats in the second trial) were not observed at the last test day when post-training CORT levels had stabilized and the Barnes maze context presumably become more familiar. In a similar manner, ischemia-induced working memory impairments in Experiment 5 were restricted to the first few blocks of testing when the radial maze was relatively novel. Although such improvements over time might have been manifestations of normal learning, this is not a possibility supported by the observation of memory deficits selectively displayed in ischemic rats on the second daily trial of test days 2 and 3 (Barnes maze; Experiment 4), given the normal performance on the first daily trials of these test days actually indicated intact day to day learning in these animals.

### **3. Recontextualization: Ecological and clinical relevance of the thesis findings**

Combined, the thesis experiments showed long-lasting effects of forebrain ischemia on behavioural and neurophysiological measures of arousal. Interestingly, these effects (and cognitive impairments) were not necessarily detected in resting levels or in conditions that were not stressful (and more familiar), but elicited during early stages of testing in relatively novel and stressful context and associated to impairments/alterations in performance. Consequently, it may not be reliable to presume that the cognitive impairments/behavioural alterations observed after 10 min ischemia are solely the result of discrete brain damage so much as manifestations of alterations in emotional reactivity (at least in the employed tasks). Overall these findings strongly suggest that ischemia-induced alternations in behavioural and cognitive performance do not always resemble

those that would be expected with acute memory impairment related to discrete neuronal damage (e.g., such as a chemical lesion), but appear to have the dynamic properties of neurochemical and/or neuroendocrine systems.

The notion that ischemia-induced behavioural impairments are associated to increased reactivity and arousal during testing (and assuming further support for this hypothesis in future studies) might limit the generalizability of findings to the cognitive impairments observed in human survivors of CA. As much of the research in this thesis demonstrated, differential reactivity to stressful conditions of testing was observed in ischemic rats despite pre-testing equivalent resting levels in CORT activity. It is difficult to envision whether similar to rats experiencing relatively mild stressors such as Y-maze exposure, humans would show 10-15 fold increases in resting levels of cortisol in response to memory assessment, thus display a similar degree of acute emotional arousal. In humans having survived an ischemic event, the appraisal linked to realization of the cognitive losses in itself might influence the resting cortisol level, rather than cognitive assessment. This illustrates the contrast between the conditions during which assessment of cognitive capabilities are made in animals and humans, and the inherent special nature of rodent research in that their rearing and testing conditions may not actively promote generalizable findings. Further, deficits in spatial navigation (e.g., those demonstrated in ischemic rats in the present study) have yet to be reported in CA survivors. Consequently, while the effect of 10 min ischemia might lead to comparable forebrain damage (if not a bit more) than that observed in human survivors of CA, ischemic rats remain to be tested in tasks more analogous to those used to assess cognitive impairments in humans, and conversely, humans have yet to be tested for “emotional reactivity” or “behavioural

reactivity” *in response* to stressful conditions, and whether such reactivity deficits might also alter cognitive functioning during their assessment (or everyday life experiences). It similarly remains to be determined whether CA survivors have altered central noradrenergic/HPA reactivity, or whether rats with ischemic damage will display working or reference memory impairments in a task not influenced by or measuring emotional reactivity.

It is crucial to determine the cause of cognitive impairments following cerebral ischemia in rodents given that behavioural assessments used to determine the effects of potentially neuroprotective treatments on functional recovery are likely utilized to determine potential treatments for use in clinical trials. However, since neuronal and functional outcome are not consistently associated in current rodent paradigms, determinations of potential treatments for clinical use may not be based on valid data/interpretations. For example, in unreported studies performed over the course of this author’s doctoral training, the piracetam derivative aniracetam failed to protect against neuronal degeneration, yet improved functional recovery in terms of working memory in a Y-maze spontaneous alternation task (Milot & Plamondon, 2005). Conversely, the 5-HT<sub>3</sub> receptor antagonist ondansetron provided significant protection against neuronal degeneration but did not alter functional impairment (unpublished data). From a clinical perspective, it would be easy not to consider either of these drugs for use in human survivors of cerebral ischemia. However, due to less than optimal testing/rearing conditions in rodent research and arousal-related effects, a positive effect of ondansetron on behaviour might have gone undetected, and conversely, the absence of an effect of aniracetam might have similarly gone undetected. In order to better generalize findings to

humans it would be important to determine precisely the causes of impairment in human survivors (there is actually very little empirical evidence for the specific causes, beyond the common hippocampal-based interpretation) and to ensure that rodent paradigms are capable of inducing similar alterations to the brain/body and to assess similar types of cognitive impairments, not just spatial capabilities; doing so would favour a greater degree of generalizability.

The notion that “performance deficits in rats following forebrain ischemic injury may be similar to some of the cognitive deficits found in human survivors” of cerebral hypoxia-ischemia and cardiac arrest has its roots in early studies by one research group (H. P. Davis, Baranowski, Pulsinelli, & Volpe, 1987; Ordy, Thomas, Volpe, Dunlap, & Colombo, 1988; Volpe, Davis, Towle, & Dunlap, 1992; Volpe & Hirst, 1983; Volpe, Holtzman, & Hirst, 1986; Volpe, Pulsinelli, & Davis, 1985; Volpe, Pulsinelli, Tribuna, & Davis, 1984). The problem with this interpretation is that their rats were subjected to 30 min of forebrain ischemia, thus would have suffered significant hippocampal and extra-hippocampal damage (Nelson, Lebessi, Sowinski, & Hodges, 1997; Nelson, Sowinski, & Hodges, 1997; Nunn et al., 1994), while human survivors would presumably have a maximum of 7 min of global ischemia (Herlitz et al., 1994), and not suffer as severe damage to the CA1 or extrahippocampal damage. Indeed, while there is significant hippocampal damage in CA survivors (Horn & Schlote, 1992; Ng, Graham, Adams, & Ford, 1989), in one study of human survivors of CA eventually succumbing to their injury at a variety of time interval, cell death appeared only after 7 days (Horn & Schlote, 1992), which is not comparable to insults of greater duration (e.g., 20-30 min) leading to detectable damage within a few days or even hours (Schurr et al., 1995; Urban, Neill,

Crain, Nadler, & Somjen, 1989). Therefore, findings of behavioural studies using 10-15 min forebrain ischemia might have greater ecological/clinical validity.

#### **4. Memory impairments after ischemia: The role of CA1 degeneration.**

In order to better support the existence of alternative mediators of post-ischemia performance (such as arousal deficits), this section will provide more precise arguments supporting the view that CA1 damage may not represent an essential mediator of behavioural impairment after 10 min ischemia. As discussed elsewhere in this document, there is much evidence in the field of ischemia challenging the notion of a primary effect of CA1 on cognitive impairment related to spatial memory. Interestingly though, outside the field of ischemia, there too is actually very little support for an involvement of the CA1 subfield of the hippocampus in the performance of spatial memory tasks or working memory, despite the findings of place cells in this structure (O'Keefe, 1976). Of all the studies investigating the effects of selective CA1 lesion in rats, there is only one report of impaired acquisition of spatial memory, as assessed in the of MWM (Nunn, Gray, & Hodges, 1998). The remainder of the other studies do not report impaired spatial memory (Goodrich-Hunsaker, Hunsaker, & Kesner, 2008; Hunsaker, Fieldsted, Rosenberg, & Kesner, 2008; Hunsaker & Kesner, 2008; Hunsaker, Lee, & Kesner, 2008), including working spatial memory (Gilbert, Kesner, & Lee, 2001; Lee, Jerman, & Kesner, 2005), but rather these experiments showed impaired processing of temporal order in these animals (Gilbert, Kesner, & Lee, 2001; Hoge & Kesner, 2007; Hunsaker, Fieldsted, Rosenberg, & Kesner, 2008; Hunsaker & Kesner, 2008; Lee, Jerman, & Kesner, 2005). Other studies have similarly concluded that spatial working memory is not CA1

dependent (Kikusui, Aoyagi, & Kaneko, 2000). Another interesting finding against the contribution of CA1 damage to memory impairments relates to the acknowledged ischemia-induced object recognition deficits (Mumby et al., 1996), despite intact performance in animals receiving more complete lesions to the dorsal hippocampus or hippocampal ablation (which includes the CA1 in its entirety and other subfields including CA2 and CA3)(Mumby, 2001). Together, these findings suggest that hippocampal damage (primarily restricted to CA1) cannot explain the various memory deficits observed after 10 min ischemia.

Although the present thesis did not endeavour to directly examine the relationship between hippocampal/extra-hippocampal damage and behavioural impairment, there was experimental support for the lack of a linear relationship between CA1 neuronal degeneration and post-reperfusion behavioural impairments. In none of the thesis studies were there significant correlations between the degree of neuronal death and behavioural performance observed. It is important however to consider that small samples of ischemic rats were used for these analyses and that neuronal degeneration in many instances reflected final histopathological outcome rather than cell viability at the time of testing, effects which may have impacted the sensitivity of the analysis. Indirect evidence (for lack of a relationship) was obtained in Experiments 1 and 2 where open-field behaviour appeared mediated by illumination, and in Experiment 3 where rats displayed the greatest degree of hyperactivity on day 1 when neuronal degeneration was not significant, compared to reduced activity at times when neuronal degeneration was increased, indicating that this post-ischemic behaviour was not associated to deficits in habituation memory to a spatial context or CA1 cell injury. In Experiment 4, metyrapone-treated rats

simultaneously displayed aggravated neuronal degeneration and attenuated spatial memory deficits, and in Experiment 5 acute doses of clonidine reduced the ischemia-induced deficits in spatial working memory despite tested at a post-reperfusion time interval when neuronal degeneration was presumably significant. Had neuronal degeneration in the CA1 (and other areas) been directly associated to working memory impairment in this experiment, selective improvements observed in ischemic groups receiving clonidine (or in saline-treated ischemic rats in the later entries) might not be expected. Therefore, the notion that behavioural performance after cerebral ischemia is predictive of final histopathological outcome in CA1 was not supported by the thesis findings. Consequently, the interpretation of the prevention or attenuation of cognitive impairment in the context of diet or enriched environment despite the presence of ischemia-induced CA1 neuronal degeneration (Gobbo & O'Mara, 2004; Roberge, Hotte-Bernard, Messier, & Plamondon, 2008) as related to enhanced functioning of remaining CA1 neurons is unsatisfactory, given it implies this structure as mediating spatial performance, an assumption not supported by lesion studies. However, this is not to say that CA1 injury cannot influence the activity/functioning of other hippocampal subfields such as the CA3 layer which pyramidal neurons are not significantly (if so, only mildly) affected by 10 min global ischemia and that is shown to play a role in spatial memory (Kesner, 2007). Indeed, CA3 axons via the Schaffer collateral input onto the more proximal dendrites of CA1, representing an important excitatory input shown to influence hippocampal output and learning and memory (Speed & Dobrunz, 2009) that would be affected by CA1 injury.

Given the findings of the thesis, and the tenuous relationship between neuronal degeneration in the CA1 after 10 min ischemia and behavioural impairment, an alternative mediator of performance such as an ischemia-induced arousal deficit may represent one plausible alternative explanation for the behavioural changes observed following cerebral ischemia, particularly given the relationship between arousal/emotionality and cognitive functioning. In this context, the intact performance of metyrapone-treated (Experiment 4) and clonidine-treated (Experiment 5) was probably mediated by structures such as the DG, CA3, and frontal cortex, brain regions which show little to no damage following 10 minute ischemia, and which have been implicated in spatial memory/learning and memory (e.g., via lesion studies) much more so than the CA1 subfield. It is important to note that the same conclusions may not apply to more severe forebrain ischemia (e.g., 15-30 min duration), given the expected significant extra-hippocampal (and frontal) damage.

##### **5. Limitations of the thesis experiments and future directions**

This thesis work represents pioneer research assessing the role of changes in emotionality or arousal post ischemia as possibly mediating behavioural and memory deficits. There are certain limitations characterizing the present thesis, the main one being related to the lack of a more comprehensive assessment of cell injury and/or functioning of extra-hippocampal brain areas (using Nissl stain, immunohistochemical detection or *in vivo* characterization of NE release profile), which could have provided greater support for underlying ischemia-induced alterations in HPA axis and noradrenergic systems. One such analysis is underway with regards to immunohistochemical characterization of NE

receptor densities or neurochemical detection of NE expression in brain tissue post-ischemia. Indeed, although the findings from Experiments 4 and 5 demonstrated ischemia-induced neuroendocrine and noradrenergic alterations, they did not directly investigate possible sources of their mediation. Immunohistochemistry would, for example, allow for the detection of noradrenergic receptor density alterations or for the quantification of NE-containing neurons in structures such as the amygdala, locus coeruleus and frontal areas; in vivo microdialysis of these areas could further quantify release of NE during exposure to stressors/testing. Further, the tasks used were relatively simple, not requiring the learning of rules (e.g., delayed matching or non-matching to sample task) or of trial unique locations, which may explain the mild effects of ischemia on performance. In this context, the effects of forebrain ischemia have been easier to detect using tasks of greater difficulty placing higher memory demands on the rats (Hartman, Lee, Zipfel, & Wozniak, 2005). However, using more “difficult” tasks (which often have a significant learning/acquisition component) makes less obvious the interpretation of behavioural findings since factors beyond spatial memory might be impaired. For Experiments 4 and 5, the objective was to examine relatively spontaneous spatial navigational ability previously determined as associated to CA1 functioning (O'Keefe, 1976) while minimizing components such as rule-based learning often typifying more difficult tasks, and solved independent of spatial memory per se (for example, learning that an escape box/platform location changes every day, or is located in the opposite side of its last position). Doing so allowed the thesis studies to specifically determine the effects of forebrain ischemia on basic spatial capabilities, thereby ensuring conclusions made with respect to the CA1 as being more relevant.

Taking into consideration these limitations (and others), future studies could entail: 1) examining the effect of global ischemia on NE receptors or availability (e.g., synthesis/cellular content) in a variety of brain regions (ex: such as frontal areas implicated in working memory) by means of immunohistochemistry, or measurements NE or CRH release in a variety of brain areas during cognitive assessment (via in vivo microdialysis). This last technique is more difficult than post-mortem assessment but has the advantage of within-individual comparisons of dynamic assessment of neurochemical release, 2) assessment of post-reperfusion capabilities in the attentional set shifting task, measuring selective attention and the ability to learn and then switch contingencies made with respect to discrimination of different odours (Lapiz & Morilak, 2006). Such testing might reveal important effects of ischemia on attention (perhaps also related to arousal), which might impact behaviour by indirectly impacting cognitive functioning, 3) testing whether the impairing effect of 10 min forebrain ischemia on spatial reference memory in the MWM (Nelson, Sowinski, & Hodges, 1997; Nunn et al., 1994; Olsen, Scheel-Kruger, Moller, & Jensen, 1994b) is similarly related to alterations in behavioural and HPA reactivity in BM, given it is considered a “land” version of the MWM, ensuring to analyze the daily trials separately (in addition to the average). Indeed, the MWM can be considered more stressful than the BM, thus perhaps more prone to elicit arousal-related cognitive deficits, 4) determining whether the absence of behavioural impairments in ischemic rats reared in enriched environments (Gobbo & O'Mara, 2004) or fed alternate diets (Plamondon & Roberge, 2008; Roberge, Messier, Staines, & Plamondon, 2008) might be due to alterations in emotional reactivity; even the impact of handling on performance of ischemic animals would be important to determine, 5) testing animals in

memory tasks more analogous to humans, such as those forcing reliance on recollection (Eacott, Easton, & Zinkivskay, 2005; Easton, Zinkivskay, & Eacott, 2009), given that recollection/recall in humans is very much associated to emotions (LeDoux, 1994, 2000; Phelps & LeDoux, 2005) and the most impaired type of memory in human survivors of CA (Moulaert, Verbunt, van Heugten, & Wade, 2009). Additionally, testing ischemic animals in an episodic-like memory task, such as one designed by this author which involves assessing the ability of rats to recall what happened, where and when, in a radial maze task simulating cache recovery and food degradation (Milot & Plamondon, 2008b), which are thought to measure memory content more analogous to humans (Clayton, Bussey, & Dickinson, 2003; Clayton & Dickinson, 1998). Ischemic and sham-operated rats were tested by this author (in an unreported study conducted during doctoral training) in an episodic-like object recognition tasks (assessing what-where-when memory) similar to (Dere, Huston, & De Souza Silva, 2005) and may reveal (analysis not yet complete) interesting effects of ischemia on cognitive capabilities, 6) studying in greater detail the impact of ischemia on arousal and consolidation, which might be necessary to better uncover underlying memory deficits associated directly to neuronal degeneration, and not to arousal deficits. For example, attenuating arousal (by environmental or pharmacological means) during testing in a reference memory test might lead to greater impairments in reference memory (as observed in Experiment 5). Such procedure may help discriminate the type of impairments more closely associated to discrete neuronal degeneration; correlations between neuronal damage and performance might consequently be more readily discovered. 7) including a stressor immediately prior to testing ischemic rats in a well-learned working memory or reference memory task to

determine whether deficits can be re-instated. For example, when performance was similar between ischemic and sham animals on the last testing day (BM, Experiment 4), memory deficits selectively re-instated in ischemic animals by a pre-test stressor (such as restraint stress) in a subsequent trial would support the notion that stressors (such as the initial novelty of a testing paradigm) mediate impairment, beyond what would be expected in by a normal learning curve. In fact, a trend for such an effect was observed in small groups of animals (unreported data from a pilot study conducted during course of doctoral training), thus might represent an interesting avenue of research, 8) testing ischemic rats for spatial memory performance in extremely familiar environments (such as home room) might attenuate the so-called deficits in spatial memory, 9) finally, many other neurochemical systems impacted by ischemia such as the glutamatergic and dopaminergic ones (Lipton, 1999; Nakane et al., 1995) are associated to cognitive functioning/spatial memory (Murphy, Arnsten, Jentsch, & Roth, 1996; Ungerer, Mathis, & Melan, 1998) and investigation of their contribution is also important. Together such studies would help determine more precisely the impact of cerebral ischemia on neurophysiological measures and their relationship to post-reperfusion behaviours. The above stated suggestions however do not take into consideration the impoverished/non-ethological rearing conditions of rodent research or the importance of circadian schedules in behaviour (e.g., tests were conducted during the sleep cycle of rats), factors which might be expected to greatly affect the results of these suggested studies (and those presented in the thesis).

## **6. Generalizability of four-vessel occlusion to cardiac arrest**

The 4VO model is considered one of the most useful and reproducible animal model to study the physiological cascade of events leading to neuronal injury and death (Small and Buchan, 2000). However, the fact that it is not a real world event may also limit the generalizability of the findings to human survivors of CA. During 4VO, the lower brainstem receives adequate cerebral blood flow (Furlow, 1982; Small & Buchan, 2000) and thus experiences only a moderate hindbrain ischemia (Pulsinelli, Waldman, Rawlinson, & Plum, 1982) compared to CA patients having suffered real global ischemia to the entire body and brain. Since hindbrain structures such as the locus coeruleus are implicated in cognitive functioning and arousal (Khakpour-Taleghani, Lashgari, Motamedi, & Naghdi, 2009; Tanaka, Yoshida, Emoto, & Ishii, 2000), their hypoperfusion during cardiac arrest might lead to behavioural alterations different from those observed after forebrain ischemia by means of 4VO. Surprisingly, although there are many models of cardiac arrest/then resuscitation (also referred to as cardiopulmonary resuscitation) in rats, they are rarely used to study neuroprotection, and no study (to this author's knowledge) has yet examined the impact of rodent CA on cognitive performance at delayed post-reperfusion intervals. This may be due to the very striking differences between animal models of 4VO and CA in mortality rate. While most rats subjected to 10 min ischemia survive the insult, most animals subjected to 7 min CA followed by resuscitation do not survive longer than a week. In one study, all animals subjected to 7 min CA via transoesophageal electrical fibrillation, then resuscitated via chest compression and defibrillation later died (Popp, Vogel, Teschendorf, & Bottiger, 2007). The increased death is thought due to difficulties in reinstating blood perfusion to

the brain after CA, which is not problematic using 4VO models. Due to these intrinsic factors, there is no compelling reason to assume forebrain ischemia replicates the effects of cardiac arrest on brain functioning and behavioural outcome, and the development of a CA/behavioural paradigm would be necessary to investigate this problematic. Further, many complications arise using 2VO + hypotension models (also leading to selective forebrain ischemia). For example, hypotension induced via vasoactive anesthetics such as halothane (Bendel, Alkass, Bueters, von Euler, & von Euler, 2005) may modify brain damage, thus have effects independent of treatment/ischemia (Warner, Ludwig, Pearlstein, & Brinkhous, 1995). Hypotension via exsanguination is complicated by the blood transfer procedure, and usage of anticoagulants may lead to cerebral haemorrhage (Zhao et al., 2001) as well as the modification of brain damage (Quartermain, Li, & Jonas, 2000). Consequently, global ischemia models although practical may not provide the most generalizable behavioural findings with respect to what would be expected in rats subjected to CA (and surviving), let alone to human clinical populations. Although this animal model remains useful in terms of studying endogenous mechanisms related to cell death, issues that are related to functional recovery post-insult and the impact of pharmacotherapy needs to be carefully addressed, taking into consideration the accumulating evidence suggesting that altered behavioural and cognitive functions involved complex physiological and biochemical pathways and appear more and more disconnected from CA1 cell death per se.

## 7. Final word

After 30+ years of research there are currently no robust treatment strategies available to protect the human brain against ischemia-induced neuronal degeneration, despite a staggering amount of research entailing over 1000 experimental papers and 400 clinical papers published just in the last six years (Ginsberg, 2008), indicating that the current approach to neuroprotection research in cerebral ischemia might require recontextualization and new approaches. One alternative approach (within the context of the thesis topic) might be the development of treatments aiming to reduce the cognitive/psychological impairments observed in CA/stroke patients by acting on mechanisms that are not tied to reduction of cellular damage per se, and aiming to improve quality of life and cognitive functioning despite the presence of brain damage. It remains to be determined whether altered emotionality/arousal observed in ischemic rats and associated to cognitive impairments in the present thesis can play a role in other stroke models and similarly be elicited in animals subjected to CA. However, from the current findings, it appears equally important for future research to aim at better controlling emotional reactivity during cognitive assessment after animal ischemia, therefore providing a paradigm more prone to elicit generalizable findings. Studies placing greater emphasis on the means by which reduction of cognitive impairment can be observed after neuronal degeneration is complete might provide novel avenues for the treatment of cognitive impairment after cerebral injury or CA in humans.

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