Global cerebral ischemia in male Long Evans rats impairs dopaminergic/ΔFosB signalling in the mesocorticolimbic pathway without altering delay discounting rates

By

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The clouds never expect it when it rains
But the sea changes colours
But the sea does not change

Stevie Nicks

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List of Abbreviations

Abbreviations	**Definitions**
4VO	Four-vessel occlusion
5-CSRTT	Five-choice serial-reaction time task
5-HT_{1B}	Serotonin 1B receptor subtype
A1-4	Adjacent zones one through four, respectively
ADHD	Attention-deficit-hyperactivity disorder
ADP	Adenosine diphosphate
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Analysis of variance
ANT	Antalarmin
ARC	Arcuate nucleus
AS	Autoshaping
ATP	Adenosine triphosphate
AUC	Area under the curve
BBB	Blood-brain-barrier
BDNF	Brain-derived neurotrophic factor
BLA	Basolateral amygdala
BS	Blood sampling
Bup HCl	Buprenorphine hydrochloride
CA	Cardiac arrest
CA1	*Cornu ammonis* 1 of the hippocampus
Ca^{2+}	Calcium ion

CA3	*Cornu ammonis* 3 of the hippocampus
CBF	Cerebral blood flow
CNS	Central nervous system
CORT	Corticosterone
CRH	Corticotropin-releasing hormone
D1-4	Distance zones one through four, respectively
DA	Dopamine
DAT	Dopamine transporter
DD	Delay discounting
DNA	Deoxyribonucleic acid
DRD_2	Dopamine receptor D_2 subtype
E	Epinephrine
ELISA	Enzyme-linked immunosorbent assay
EPM	Elevated-Plus Maze
FR	Food restriction
GABA	γ-Aminobutyric acid
GCI	Global cerebral ischemia
GCI-Post	Post-surgery delay discounting performance for ischemic rats
GCI-Pre	Pre-surgery delay discounting performance for ischemic rats
Glu	Glutamate
HPA	Hypothalamic-pituitary-adrenal
HRA	High reward arm
Iba-1	Ionized calcium-binding adapter molecule 1

IL-1β	Interleukin 1 beta
IR	Immunoreactivity
ITI	Inter-trial interval
LL	Larger, later lever
LRA	Low reward arm
LTP	Long-term potentiation
M1	M1 microglia subtype
M2	M2 microglia subtype
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
Na^+	Sodium ion
NAc	Nucleus accumbens
NAcC	Nucleus accumbens core
NAcS	Nucleus accumbens shell
NE	Norepinephrine
NMDA	*N*-methyl-D-aspartic acid
NO	Nitric oxide
OFT	Open Field Test
oPFC	Orbitofrontal cortex
PBS	Phosphate-buffered saline
PFC	Prefrontal cortex
PND	Postnatal day
PO	Predator Odour Test

ROS	Reactive oxygen species
RT	Room temperature
SEM	Standard error of the mean
Sh	Sham surgery
Sh-Post	Post-surgery delay discounting performance for sham-operated rats
Sh-Pre	Pre-surgery delay discounting performance for sham-operated rats
SS	Smaller, sooner lever
TMT	Trimethylthiazoline
TNF-α	Tumor necrosis factor alpha
TrkB	Tropomyosin receptor kinase B
vmPFC	Ventromedial prefrontal cortex
ΔFos	Delta fos
ΔFosB	Delta fos B

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Abstract

Global cerebral ischemia (GCI) in rats has been shown to promote exploration of anxiogenic zones of the Elevated-Plus Maze (EPM) and Open Field Test (OFT). This study investigated changes in impulsive choice and/or defensive responses as possible contributors of heightened anxiogenic exploration observed after ischemia. Impulsivity was assessed using delay discounting (DD) paradigms, while the Predator Odour Test (PO) served to assess changes in defensive responses towards a naturally aversive stimulus. Male Long Evans rats underwent 9 days of autoshaping training and 24 days of DD training prior to GCI or sham surgery (n= 9/group). Post-surgery, rats completed the OFT, EPM, and PO, followed by 6 days of DD sessions. Blood droplets served to evaluate corticosterone secretion associated with PO exposure. With impulsivity being regulated through mesocorticolimbic monoaminergic pathways, we also characterized post-ischemic changes in the expression of dopamine D2 receptors (DRD_2), dopamine transporters (DAT), and ΔFosB in the basolateral amygdala (BLA), nucleus accumbens core (NAcC) and shell (NAcS), and ventromedial prefrontal cortex (vmPFC) using immunohistofluorescence. Our findings revealed no impact of GCI on delay discounting rates, while PO approach behaviours were minimally affected. Nonetheless, GCI significantly reduced DRD_2 and ΔFosB-ir in the NAcS and NAcC, respectively, while DAT-ir was diminished in both NAc subregions. Collectively, our findings refine the understanding of cognitive-behavioural and biochemical responses following stroke or cardiac arrest. They support significant alterations to the dopaminergic mesocorticolimbic pathway after ischemia, which are not associated with altered impulsive choice in a DD task but may influence locomotor exploration of the OFT and EPM.

Introduction

Background Research Question

Global cerebral ischemia (GCI) in rats, which mimics cardiac arrest in humans, is associated with neurological damage, notably in the selectively vulnerable hippocampus, and leads to functional impairments including lasting deficits in visuospatial memory (Neumann et al., 2013). Interestingly, through years of research, many studies have found a reduction in what is typically referred to as anxiety-like behaviour in the Elevated-Plus Maze (EPM) following global ischemia, with rats spending more time in anxiogenic areas of the maze and doing more exploratory behaviours such as risk assessment behaviours from the closed arms and head dips over the open arms compared to sham-operated counterparts (Knowles et al., 2016; Lu et al., 2016; Morin et al., 2021; Plamondon & Khan, 2005; Yan et al., 2007). These findings are intriguing considering the heightened reactivity and dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis noted following global ischemia, which leads to accentuated corticosterone (CORT) expression upon exposure to stressors (de la Tremblaye et al., 2014; Girbovan & Plamondon, 2015; Roberge et al., 2008). As such, abnormalities in the ischemic rats' responses observed in the EPM have raised the question as to a possibility that these behaviours may result from a failure to properly appraise fear-inducing stimuli or rather a result of reduced post-ischemic inhibitory control. From Bari et Robbins' (2013) research, inhibitory control represents the ability (behavioural and cognitive) to prevent a behaviour or cognitive process from taking place. Behavioural inhibition happens when an action is being stopped, cancelled, or withheld. A dysfunction in that process leads to impulsive *action*. A specific aim of this study was to determine if anxiety-like behaviours in the EPM could be linked to behavioural disinhibition occurring after ischemia, thus leading to impulsive action. Second, delayed gratification happens when a subject chooses to withhold an immediate response known to lead to a smaller gratification without delay in order to obtain a larger gratification when the response is executed after an increasing amount of time. Greater preference for the immediate, but smaller gratification may be considered impulsive *choice* (Bari & Robbins, 2013; Winstanley, Theobald, et al., 2006). Impulsive choice in rodents is often studied using a delay discounting (DD) paradigm, and as such appeared to be an interesting tool to test such premise. Interestingly, a study by Déziel and Tasker (2017) found a significant decrease in discounting rates following endothelin-induced focal ischemic lesions to the orbitofrontal cortex (oPFC) in rats. Impairments in the ability to withstand an action have also been noted in humans, with a majority of cardiac arrest (CA) survivors showing some degree of behavioural disinhibition and impulsivity (Keijzer et al., 2020). Our study therefore aimed to examine, using a DD task, whether impaired response inhibition could be observed after ischemia and possibly contribute to increased exploration of the anxiogenic arms in the EPM. Importantly, knowing that impulsivity, and in a broader sense reward processing, is regulated by dopamine signalling in the mesocorticolimbic pathway (Beaulieu & Gainetdinov, 2011), this research also assessed dopamine (DA)-ergic alterations in the connected structures, which could be influenced by GCI-induced hippocampal injury.

Thus, this thesis aimed to uncover a possible contribution of impaired inhibitory control, more specifically behavioural disinhibition, in ischemia-induced extended exploration of the EPM open arms. This study also represented the first attempt to characterize possible alterations in the ability to delay reward gratification in GCI rats. In addition, in line with observed reduced anxiety-like behaviour observed in the EPM after ischemia, our study also examined the rats' responses to a fear-inducing stimulus (i.e., using the Predator Odour Test) as an alternative to impulsive action in the EPM. Finally, an in-depth assessment of post-ischemic DA and ΔFosB signalling, all markers of reward-processing mesocorticolimbic pathway activity, is provided.

To summarize, with an ageing population and with advancements in modern medicine resulting in higher likelihood of survival from cardiac arrest, there is an ever-increasing pool of humans having overcome a global ischemic event possibly living with impairments. It is therefore critical to better define how executive functions like inhibitory control may be affected after ischemia and what biochemical changes may lead to such impairments. The following sections will go in further detail about the theoretical basis supporting the thesis objectives.

Theoretical Background

Global Cerebral Ischemia

A state of cerebral ischemia is achieved when an interruption of cerebral blood flow (CBF) occurs in the brain, resulting in a failure to supply oxygen and nutrients (e.g., glucose, lipids, proteins) necessary to sustain proper neuronal metabolism (Traystman, 2003). This triggers an instant chain reaction called the ischemic cascade which ultimately culminates in neuronal death and other biochemical alterations, often leading to behavioural and cognitive impairments (Neumann et al., 2013). The brain regions affected by blood flow interruptions will be contingent on the type of ischemia taking place. Thus, focal ischemia in humans most commonly stem from a thrombus, or blood clot, getting trapped in the middle cerebral artery, which obstructs blood flow in a specific, defined part of the brain, leading to altered function of brain structures found downstream and irrigated by the targeted brain artery. Generally, the brain regions that underwent the most dramatic reduction in cerebral blood flow (i.e., ischemic core) will show the highest degree of histological damage, while the surrounding areas (i.e., ischemic penumbra), having been spared from a complete cessation of CBF irrigation can be preserved and recover functionality, as they are responsive to rehabilitation and behaviour-driven plasticity and reorganization initiated in the months after ischemia (Sokolowski et al., 2023). A second type of cerebral ischemia, more relevant to the subject of this thesis, is global cerebral ischemia, which occurs when the whole brain is temporarily deprived of blood flow, an event most often stemming from cardiac arrest or asphyxia (Neumann et al., 2013). In such case, neuronal damage is more diffuse and depends on the duration of CBF attrition to brain structures. GCI usually impacts the most vulnerable neurons such as the pyramidal neurons of the *cornu ammonis* 1 (CA1) region of the hippocampus (Neumann et al., 2013). For instance, the most frequently used preclinical paradigm to induce GCI in rodents, the four-vessel occlusion (4VO; Pulsinelli & Brierley, 1979; Pulsinelli & Buchan, 1988; Traystman, 2003) has been shown the reliably and reproducibly reduce CBF from 100 to 7% in the hippocampus, the striatum, and the neocortex (Ginsberg & Busto, 1989). Furthermore, while many studies have repeatedly indicated significant reductions in post-ischemic pyramidal neuron density in the CA1 layer of the hippocampus (Barra de la Tremblaye & Plamondon, 2016; Bueters et al., 2008; Lebesgue et al., 2009; Morin et al., 2021; Neumann et al., 2013; Schmidt-Kastner, 2015), others have also reported similar damage in the *cornu ammonis 3* (CA3; de la Tremblaye et al., 2017; Honkaniemi et al., 1996; Morin et al., in preparation; Olsson et al., 2003; Roberge et al., 2008) and dentate gyrus layers of the hippocampus (Honkaniemi et al., 1996; Khodanovich et al., 2016; Montes et al., 2019; Olsson et al., 2003) or in the basolateral amygdala (de la Tremblaye et al., 2017), and cerebral cortex (García-Chávez et al., 2008; Horstmann et al., 2010; Petito et al., 1987). Of note, GCI is known to induce delayed neuronal death with damage developing for a period of up to approximately one week after cardiac infarction. As such, CA1 pyramidal cells have been characterized as most vulnerable to global ischemic insult than the larger striatal neurons, which require a longer hypoperfusion duration to be similarly affected (Harukuni & Bhardwaj, 2006; Pulsinelli & Brierley, 1979; Pulsinelli & Buchan, 1988). The CA1 pyramidal neurons will undergo apoptotic and necrotic death from 48 h to seven days after GCI, a period after which the brain becomes more receptive to activity-dependant remodelling (Livingston-Thomas et al., 2015; Murphy & Corbett, 2009). Before discussing functional impairments mostly observed following GCI, it is necessary to present some of the molecular and cellular events (i.e., the ischemic cascade) occurring during the immediate and delayed post-ischemic period.

The Ischemic Cascade

Upon the onset of GCI, reserves in adenosine triphosphate (ATP), a major source of energy for neurons and glia, are rapidly depleted as insufficient CBF makes energetic demands far outpace ATP synthesis (Annoni et al., 2021; Neumann et al., 2013). Neurons then shift from aerobic to anaerobic metabolism to compensate for energy production and an accumulation of lactic acid induces a state of lactic acidosis (Annoni et al., 2021). Shortly after, a failure in the ATP-dependent ion transport pumps increases the intracellular positive charge, triggering a depolarization, leading to the massive entry of Ca^{2+} into the cell. Contrary to healthy conditions, these excessively engulfed cations are no longer able to exit the cell due to the ATP-dependent ion transport pump malfunction (Lau & Tymianski, 2010; Neumann et al., 2013). These elevated levels of Ca^{2+} prompt the synthesis and release of the central nervous system (CNS)'s primary excitatory amino acid, glutamate (Glu), which by binding to its two main ionotropic receptors [α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and Ca^{2+}-permeable *N*-methyl-D-aspartic acid (NMDA)], further maintains Ca^{2+} intracellular entry. This unsustainable rise in Ca^{2+} and adenosine diphosphate (ADP) induces excitotoxicity (Maida et al., 2020).

Through such massive depolarization, a plurality of deleterious agents to cell integrity are released. Among the commonly cited are Ca^{2+}-dependent agents like calpain and phospholipases, free radicals such as mitochondrial transition pore formation, nitric oxide (NO), and reactive oxygen species (ROS; Maida et al., 2020; Wang et al., 2022). The latter induces damage to lipids and proteins by a process of oxidation, which fails to be offset by a lagging synthesis of antioxidant enzymes (Granger & Kvietys, 2015; Yang, 2019). When culminating, the biochemical alterations participate in establishing ischemia-induced necrotic and apoptotic cell death. During the former, the cell will enlarge due to increased membrane permeability and entry of cerebrospinal fluid. These factors combined with Glu-ergic excitotoxicity, massive releases in intracellular Na^{+}, and Ca^{2+}-related lipase activity together lead to cellular lysis and the spreading of toxins and Glu in the extracellular matrix, therefore propagating neuronal injury vectors to neighbouring cells (Sabet Sarvestani & Azarpira, 2022). Concurrently, deoxyribonucleic acid (DNA) and mitochondria start to break down and engage a caspase-dependant apoptotic cascade (i.e., cellular suicide). Under such conditions, the cell is separated into several apoptotic bodies, which are then phagocytosed (Sanderson et al., 2013).

Such a cascade of events in maintained throughout the cessation of CBF, leading to death if the course of action is not reversed. However, if cardiac function resumes either naturally or medically, CBF is restored, and the reperfusion process takes place. While rapid reperfusion permits to keep the patient alive, sudden reperfusion in a compromised CNS undergoing massive necrotic and apoptotic trauma can be harmful. Specifically, reperfusion injury after ischemia has been shown to be associated with increased oxidative stress through a surge in ROS (Granger & Kvietys, 2015) and an infiltration of leucocytes that will bind to endothelial cells of the blood-brain-barrier (BBB) and secrete BBB-degrading matrix metalloproteinases (MMPs) and neutrophil-derived oxidants. Leucocytes will also release pro-inflammatory cytokines into the infarct areas (Liu et al., 2016). The resulting increased BBB permeability will in turn accentuate the extent of the ongoing cerebral hemorrhage (Harukuni & Bhardwaj, 2006). Albumins and other large molecules will further migrate to the brain through the now-permeable BBB followed by liquids through osmotic processes, thus creating a cerebral edema and compression of brain tissue (Annoni et al., 2021).

In the weeks following GCI, a strong inflammatory response develops. Microglia, the resident CNS macrophages, are critical in triggering this reaction. In the healthy brain, microglia remain active as a form of surveillance and to assume housekeeping tasks like removing unused synaptic connections. Thus, microglia may be represented as the first line of defense during an ischemic event (Anrather & Iadecola, 2016; Shichita et al., 2014). In fact, within minutes of excitotoxic and oxidative stress reactions, they will activate and aggregate at the lesion site. M1 microglia subtype will engage in phagocytosis and release of many pro-inflammatory markers [e.g., tumor necrosis factor α (TNF-α), interleukin (IL)-1β, MMP-9, chemokines, ROS], further degrading the BBB (Anrather & Iadecola, 2016; Shichita et al., 2014). Astrocytes will also release pro-inflammatory chemicals in the affected area (Maida et al., 2020). These will promote higher concentrations of cell-adhesion molecules that bind to endothelial cells, enabling the infiltration of peripheral neutrophils from the bloodstream who release pro-inflammatory cytokines, leading to cell death (Anrather & Iadecola, 2016; Lakhan et al., 2009). Conversely, M2 microglia will begin releasing anti-inflammatory cytokines and neurotrophic factors in the following hours and days as the ischemia reaches a more chronic phase (Anrather & Iadecola, 2016; Yoshimura & Ito, 2020).

This section has summarized in a non-exhaustive manner complex pathophysiological phenomena involved in GCI. However, at the heart of this cerebral reorganization, other changes significantly affect important neurotransmission systems which are likely to contribute to the deficits observed following a global ischemia in humans and animals. Among these, modulations in DA transmission have been noted, and such changes could contribute to the impairments that motivated the research within this thesis. For this reason, the following section focusses on presenting scientific evidence indicating possible alterations in DA-ergic transmission following GCI.

Dopamine and the Mesocorticolimbic Pathway

Interestingly, some studies have indicated DA signalling pathways to be affected after global ischemia (Baker et al., 1991; Caldwell et al., 1997; Fan et al., 2022; Kahn et al., 1995; Knowles et al., 2016; Ojo et al., 2023; Rafałowska et al., 2000). This could have major implications knowing the participation of this neurotransmitter in the regulation of a wide range of functions from motility to visuospatial memory, attention, and, importantly, reward-directed behaviour and motivation (Klein et al., 2019). Catecholamines, including DA, norepinephrine (NE), and epinephrine (E) are by-products of tyrosine, with the tyrosine hydroxylase enzyme acting as the rate-limiting factor of their synthesis. Important concentrations in DA are found in the midbrain, with cells extending to far-reaching synaptic connections with various structures, known to form four main DA-ergic pathways. The first two are implicated in the regulation of motivation and reward processing and are sometimes theoretically integrated in one larger pathway known as the mesocorticolimbic, or reward, pathway. Taken separately, the mesolimbic pathway mainly comprises of DA fibers sent from the ventral tegmental area (VTA) to the striatal nucleus accumbens and olfactory tubercule while the mesocortical pathway arises from the VTA with post-synaptic connections in the prefrontal cortex (PFC). The latter has been suggested to preferentially coordinate the more executive aspects of reward-directed behaviours. In addition, the nigrostriatal pathway begins from the *zona compacta* of the substantia nigra to reach the caudate nucleus and putamen in the striatum, regulating motor function and learning. Finally, prolactin secretion is monitored by the tuberoinfundibular pathway from the arcuate nucleus (ARC) and paraventricular nucleus (PVN) of the hypothalamus to the pituitary gland (Klein et al., 2019).

Notably, catecholaminergic neurons have also been found to be more vulnerable to GCI than serotonergic, glutamatergic, and γ-aminobutyric acid (GABA)-ergic neurons. Upon global ischemia, the brain sees a massive increase in DA release after which DA, along with its presynaptic DA transporter (DAT) and postsynaptic DA receptor D2 subtype (DRD_2) availability are significantly reduced (Martín et al., 2013; Momosaki et al., 2017; Takagi et al., 1995; Uzdensky et al., 2017). While the specific mechanisms have yet to be discovered, researchers have proposed DA surges to enhance neuronal damage by triggering Glu release (Caldwell et al., 1997). DA breakdown by-products could also contribute to deleterious effects for neuronal survival (Harukuni & Bhardwaj, 2006; Kahn et al., 1995). Furthermore, studies have supported behavioural and emotional impairments including sensorimotor deficits as well as increased anxiety- and depression-like behaviours to partly result from the altered neurochemical balance related to excessive post-ischemic DA release (C. J. Chang et al., 1993; Kawano et al., 1988; Kronenberg et al., 2012; Ruscher et al., 2012; Toner & Stamford, 1996). Notably, preclinical studies have determined that pre-ischemic stimulation of DA receptors (acting as a preconditioning stimulus) can minimize GCI-induced striatal DA surges and consequently reduce post-ischemic Glu release. Indeed, DA receptor D2 (DRD_2) agonism up to 1 h before GCI blunts the ischemia-induced DA surge, improving functional outcomes (Caldwell et al., 1997). Specifically, pre-ischemic stimulation of as DRD_2 and DRD_3 receptor subtype agonism have been shown to prevent from visuospatial memory impairments following GCI (W. Wang et al., 2018).

This section presented a brief summary of the interplay between GCI and DA and highlighted a significant role for DA-ergic signalling during and following ischemia on neuronal and behavioural functions. Aside from post-ischemic alterations in the DA system, GCI is also marked by persistent dysregulations of the HPA axis.

Effects of Global Ischemia on the Hypothalamic-Pituitary-Adrenal Axis

The role of GCI as a profound disruptor of HPA axis activity in humans (Kim et al., 2011; Sahebnasagh et al., 2021; Yamaji et al., 2009; Yu et al., 2022) and rodents (Barra de la Tremblaye & Plamondon, 2016; Lai et al., 2007; Morin et al., 2021; Neigh et al., 2009; Zhao et al., 2021) has long been acknowledged. As such, delayed impairments associated with these intense perturbations are worth studying. The HPA axis plays a crucial role in the regulation of many bodily functions related to energy storage, digestion, and, importantly, stress and immune responses. In this capacity, the axis regulates the secretion of endocrine vectors, namely stress-induced corticosteroid secretion (Spencer & Deak, 2017). Upon facing a real or perceived stressor, the PVN will secrete corticotropin-releasing hormone (CRH) which, through efferent synaptic connections, binds to CRH receptors (primarily the CRHR1 receptor subtype) in the anterior lobe of the neighbouring pituitary gland, stimulating the release of the adrenocorticotropic hormone (ACTH) in the bloodstream. ACTH then binds to melanocortin 2 receptors in the *zona fasciculata* of the adrenal gland cortex, which induce the release of glucocorticoids such as cortisol in humans and corticosterone in rodents (Spencer & Deak, 2017). CORT then increases glucose bioavailability in part through glucogenesis (De Kloet et al., 2005; Kuo et al., 2015; Ulrich-Lai & Herman, 2009). As a major regulator of homeostasis, healthy HPA axis functioning usually subsides after the stressor. Thus, rapid non-genomic mineralocorticoid receptors (MR) and delayed genomic glucocorticoid receptors (GR) activation from BBB-permeable CORT will initiate negative feedback mechanisms, enabling the heightened CRH secretion to be normalized (Joëls et al., 2018; Zhou et al., 2022).

Of note, both GR and MR are densely expressed in the hippocampus CA1 (De Kloet et al., 2005) such that, after global brain infarct and accompanying CA1 cell death, HPA axis activity and homeostasis become dysregulated (Spencer & Deak, 2017). In fact, GCI has been shown to significantly reduce GR expression in this ischemia-vulnerable hippocampus sublayer (Barra de la Tremblaye & Plamondon, 2016; de la Tremblaye et al., 2014). Compromised GR integrity then renders the axis unable to maintain a functional negative feedback loop regulating CORT expression (Gjerstad et al., 2018). Furthermore, the HPA axis itself receives sensory afferences from other regions like the amygdala (Spencer & Deak, 2017), in which neuronal death is also notable, albeit to a reduced extent (de la Tremblaye et al., 2017). Under basal conditions, the BLA interprets salience of fear-related stimuli and transmits this information through synaptic connections to the HPA axis and ventral hippocampus (Felix-Ortiz et al., 2013). However, BLA GR activation results in increased synaptic excitatory responses in this region and thus a rise in anxiety-like behaviours mediated by amplified signalling through the BLA-ventral hippocampus and HPA axis pathways (Felix-Ortiz et al., 2013; Karst et al., 2010; Myers et al., 2014). Interestingly, BLA GR immunoreactivity (-ir) is upregulated in the post-GCI juvenile male rat BLA at least 13 days after infarct, despite reduced sensitivity of the juvenile brain to ischemic damage compared to the developed adult brain (Morin et al., 2021). In addition, at the anterior pituitary level, the CRHR1 receptor subtype does not receive direct afferences from the cortex, limiting the axis' ability to use environmental or biographical context or to dissect more complex stressors in order to mitigate ischemia-induced CORT increases (Spencer & Deak, 2017).

These factors mentioned above are among the key drivers of HPA axis dysregulation and heightened stress-induced CORT secretion after ischemia (Joëls et al., 2018). Dysregulation of the HPA axis can manifest itself through two accentuated supraphysiological conditions: hyperactivity and hypersensitivity. The former is manifested by way of increased CORT secretion in the days to weeks following GCI (Barra de la Tremblaye & Plamondon, 2016). For instance, basal CORT levels in ischemic rodents remain significantly elevated compared to their sham-operated counterparts 24 h (Milot et al., 2012), three days (Milot & Plamondon, 2011), and seven days (de la Tremblaye et al., 2014) following GCI. In addition, HPA axis hypersensitivity, which refers to heightened CORT secretion upon acute stress exposure, is observed as late as 15 to 16 days post infarct and is also positively correlated with CA1 neuronal damage (Svensson et al., 2016). This is clinically relevant as chronic hypercortisolemia or hypercorticosteronemia may act as pathophysiological substrates for anxiety and depression disorders (Dedovic & Ngiam, 2015; Staufenbiel et al., 2013; Timmermans et al., 2013). Of interest, although some studies show a reduction in basal CORT levels as delay increases after ischemia, the exposure of ischemic rats to an acute stressor like restraint stress drastically elevates CORT expression, leading to what has been described as long-term HPA axis hypersensitivity. In this context, studies have reported acute restraint stress to upregulate CORT expression for at least 120 min when administered 27 days post-infarct (de la Tremblaye et al., 2014), 45 min when administered 68-69 days after ischemia (Roberge et al., 2008), and for up to 60 min when exposed on post-operative day 84 (Girbovan & Plamondon, 2015) compared to sham-operated rats. Of note, behavioural testing performed 10 days after GCI has been shown to upregulate CORT levels up to 24 h after the test (Milot & Plamondon, 2011).

While time-limited activation of the axis and resulting increases in CORT secretion may serve adaptative purposes in order to cope with stressors, perduring activation or hyperactivation of the HPA axis exerts detrimental ramifications on the brain and behavioural responses. Indeed, chronic stress impairs learning and memory processes through a decrease in Glu activity in the PFC, leading to a deficit in long-term potentiation (LTP) and a decrease in hippocampal synaptic plasticity and restructuring (Spencer & Deak, 2017; Timmermans et al., 2013). Furthermore, as bilateral relations between the HPA axis and the immune system have long been established, long-term CORT elevation induced by chronic stress paradigms such as restraint stress over several days or unpredictable chronic mild stress, known to emulate low-intensity real-life stressors through stimuli like wet bedding, predator sounds, and alternating light-dark cycle schedules, leads to increases in pro-inflammatory signalling molecules (Farooq et al., 2012; Spencer & Deak, 2017). For example, CD11b, an integrin molecule important in leukocyte recruitment to the site inflammatory response and ionized calcium-binding adapter molecule 1 (Iba-1), a marker of microglial activity, are upregulated in the PFC, hippocampus, NAc, and amygdala after long-term stress exposure (Walker et al., 2013). Similarly, GCI activates a panoply of immune reactive molecules (for reviews, see Danton & Dietrich, 2003; Wang et al., 2023; Zacharia et al., 2009), the stimulation of which could partly be attributable to HPA axis dysfunction.

While the consequences of long-term elevations in CORT levels are primarily negative, acute elevated in CRH and CORT secretion during GCI also generate a cascade of events detrimental to post-ischemic recovery. This is supported by studies showing the selective blockade of CRHR1 receptors using Antalarmin (ANT) during ischemia onset to blunt the surge in CORT normally observed during and after GCI. Such blockade results in significantly decreased infarct size and reduced basal and stress-induced CORT hyperexpression and is associated with normalization of ischemia-induced changes in BDNF and TrkB expression, and with reduced expression in pro-inflammatory markers in the hippocampus, medial PFC, and NAc at least up to 30 days post-ischemia (de la Tremblaye et al., 2014, 2016, 2017). Behaviourally, CRHR1 blockade protocol during ischemia acts to normalize anxiety-like behaviours, minimize impairments in visuospatial memory, fear memory, sociability, and prevents from post-GCI anhedonia and behavioural despair (de la Tremblaye et al., 2016, 2017).

Global Cerebral Ischemia and Anxiety-Like Behaviours

Anxiety-related disorders are widely observed among CA survivors. Keijzer et al. (2020) have reported consequent rates of survivors experiencing anxiety (61%), depression (45%) or posttraumatic stress disorder (27%), with up to a third suffering of attention deficits. An advantage of assessing anxiety in humans is the verbal assessments that offer direct insight into a subject’s emotions and affect, especially when the verbalized emotions may not correlate with the observed behavioural responses. In animal research, internal affect and emotions can only be inferred from observable and quantifiable variables (i.e., behaviours). As such, paradigms will often make use of specific aversive stimuli known to mimic those found in natural settings, and that elicit genetically programmed behavioural responses that may be interpreted as anxiety. This is why, in this thesis, the term “anxiety”, when used, refers to anxiety-like behaviours.

Among existing measuring tools, the Open Field Test (OFT) is widely used to assess anxiety-like behaviours and exploration (Seibenhener & Wooten, 2015). Briefly, rats performing this test are placed inside a novel, large, open, and brightly lit arena and are left to freely explore the novel environment for a period usually reaching 10 min. The expected "innate" response to such exposure would be to escape the most aversive stimuli and take refuge through exploration of the safer, darker, closed in areas adjacent to the arena's walls (i.e., periphery). A higher percentage of time spent in the anxiogenic centerzone and more exploratory behaviours in this area are indicative of a rodent's decreased anxiety. In GCI rats, the OFT further enables ensuring that the ischemic event did not induce impairments in gross motor function or induced locomotor hyperactivity. Such abnormalities would respectively be characterized as significant reductions and increases in distance travelled and number of entries in the two zones (Chang et al., 2018; Colbourne et al., 1998; Milot & Plamondon, 2008). If present, it is important to consider a potential impact on behavioural data gathered from the other tests performed by the same rats. Other behavioural tests that may be used to assess anxiety-like behaviour in GCI rats with proper gross motor function include the Light-Dark Box Test as well as tests measuring context-related fear memory like the Y-Maze Passive Avoidance Task (de la Tremblaye et al., 2017; Livingston-Thomas et al., 2015; Yan et al., 2007).

Aside from using the OFT, the gold standard test to measure anxiety-like behaviour remains the EPM. In this test, rats are placed in the centerzone of an elevated, cross-shaped apparatus consisting of a small central, square-shaped platform from which emanate four arms, two of which are enclosed by tall walls (i.e., closed arms) and two of them having no such protection aside from a thin Plexiglas rim around the edge of the arms (i.e., open arms). As control rats freely explore the maze, they initially spend time exploring the secure, dark closed arms, with slow increases in open-arm exploration and exploratory behaviours as the 5-min testing period progresses (Kraeuter et al., 2019). Studies using compounds that are known to exert anxiolytic effects, such as benzodiazepines, have reported increased exploration in the open arms (Himanshu et al., 2020; Kraeuter et al., 2019).

Different studies using the EPM have reported interesting behavioural responses in GCI rats. Specifically, ischemic rats have shown a propensity to spend increased time in the open arms, shorter latency to the first open arm entry, and increased frequencies of exploratory behaviours such as head dips from open arms and risk assessments (defined as extending its head out from the closed arms with limbs remaining in the closed arm) from the closed arms than their sham-operated counterparts (Knowles et al., 2016; Lu et al., 2016; Morin et al., 2021; Plamondon & Khan, 2005; Yan et al., 2007). This anxiolytic response seen in the EPM after GCI appears counterintuitive considering the increased anxiety generally noted in human CA survivors. Nonetheless, as the EPM tests prescribes, the response noted in GCI rats is qualified as expressing reduced anxiety-like behaviours. It is in this context also important to mention that this anxiolytic response after GCI has been replicated irrespective of tested rat strain and developmental stage (Morin et al., 2021; Yan et al., 2007).

In the context of GCI, rats concomitantly express increased CORT secretion (Milot & Plamondon, 2011) due to impaired feedback mechanisms (Barra de la Tremblaye & Plamondon, 2016). Thus, it is worth positing that GCI rats' behavioural response in the EPM may not indicate reduced anxiety-like behaviour. Rather, it could indicate an impairment of the rats' inhibitory control processes resulting in increased behavioural disinhibition (impulsive action). Such hypothesis has never been tested in GCI rats. These impairments could also be related to prefrontal cortex dysfunction and/or biochemical alterations in mesocorticolimbic circuitries (Green et al., 2015). This specific hypothesis will be discussed later in this work.

An alternative hypothesis that this research also aims to evaluate is the possibility that post-ischemic EPM behaviour could be related to a failure of GCI rats to properly appraise fear-inducing stimuli. By definition, fear is an anticipatory emotional reaction experienced when faced with a potentially dangerous or anxiogenic stimulus (Garcia, 2017), which is regulated by a complex network of brain structures mainly involving the amygdala, with the BLA nucleus occupying a pivotal role as an afference point of many somatosensory and context-related inputs from cortical, hippocampal, and thalamic sources. The BLA then attributes salience to the stimuli and activates the central amygdala, which then sends efferences to various brain regions, triggering a response (Luchkina & Bolshakov, 2019). This is of specific interest as GCI is known to significantly impact neuronal counts in the BLA (de la Tremblaye et al., 2017) and in afferent brain regions providing sensory inputs such as the hippocampus which could lead to impairments in proper fear regulatory mechanisms.

In this thesis, the Predator Odour Test (PO) was used as a novel way to test this hypothesis. This test is most often used to measure the effects of compounds on fear and anxiety, along with paradigms such as the Fear Conditioning Test. Although variations in adopted protocols are observed throughout studies, the main objective of the PO is to expose a rodent to a naturally aversive predator's smell in an enclosed arena with a gridded floor, and to quantify avoidance behaviours (e.g., time spent and entries in the zones adjacent and distal to the odour, number of interactions with the odour container; Storsberg et al., 2018). The odour is largely contingent on the test subject. For instance, mice will generally be exposed to rat odour while rats are exposed to cat or fox odour. The odour sometimes originates from a small piece of towel that was brushed against a predator (most often a cat) or a small piece of absorbent paper sprayed with trimethylthiazoline (TMT; an odorant component of fox urine and feces) or urine (Fendt &

Endres, 2008). Research teams may also opt for the use of control absorbent paper placed in other regions of the apparatus for ratio analyses (i.e., ratio of time spent with the odorant paper compared with the control ones) and also for a habituation session in the arena prior to the test session with odours in order to acclimate the animal and to measure baseline behaviours (repeated measures analyses). In healthy control rodent samples, the exposure to predator odour leads to significantly reduced exploration with increased freezing, a fear response in prey animals, avoidance and circulatory CORT expression (Apfelbach et al., 2005; Staples, 2010; Takahashi et al., 2009; Thomas et al., 2006). To our knowledge, this paradigm has never been used in the context of global ischemia in rats, although impairments in a Fear Conditioning Test after GCI exemplified as decreased freezing in the event of naturally aversive foot shocks indicates possible impairments of fear-related perception (de la Tremblaye & Plamondon, 2011). Exploration patterns and interactions with this fear-inducing stimulus could allow further characterization of the ability to appraise anxiogenic stimuli and behavioural disinhibition in ischemic rodents.

Inhibitory Control and Impulsivity

Considered crucial in maintaining a functional life, Bari et Robbins' (2013) methodology posits inhibitory control as being subdivided into two largely distinct components: behavioural inhibition and cognitive inhibition. These are defined as observable behaviours and mental processes, respectively, to be halted completely or incompletely, voluntarily or not. Behavioural inhibition can further be deconstructed in response inhibition (impulsive action), deferred gratification (impulsive choice), and reversal learning (inflexibility). Of interest, response inhibition necessitates the inhibition of a behavioural response until a go signal is given (action postponing), the withholding of a behavioural response when a no-go signal is given (action withholding), or the cessation of a behavioural response upon the presentation of a stop signal as the action was to be executed (action cancellation). A failure to adopt these rules leads to an observed impulsive action. Furthermore, deferred gratification occurs when a subject withholds a response known to lead to a reward in order to obtain a larger reward that comes after a greater amount of time (delay discounting; DD) or effort (effort discounting) or has less probable chance of taking place (probability discounting).

In this thesis, the EPM will be utilized to assess whether reduced EPM anxiety-like behaviours after ischemia may be characteristic of impulsive action, a failure in response inhibition. The PO will serve to support an alternative hypothesis in which ischemic rats simply may not be able to properly perceive aversive stimuli. Of note, this thesis will also examine if a failure in a certain facet of inhibition (here, response inhibition or impulsive action) following ischemia may also contaminate other facets of inhibition (here, deferred gratification or impulsive choice).

As a way to quantify impulsive choice, DD is defined as attributing lower intrinsic perceived value to a larger reward as the delay to gratification increases (Frost & McNaughton, 2017) and is usually tested using operant boxes. Generally, rats are placed in separated boxes and are presented two levers: one offering a small reward without delay (smaller, sooner; SS) and one offering a larger reward with an increasing delay (larger, later; LL; Mar & Robbins, 2007). In each session, the delay to gratification when pressing the LL reward will grow incrementally from 0 to usually up to 60 s after a lever press. The paradigm generally consists of one daily session performed over many consecutive days before and after a certain treatment. Within each session, DD rates, or preference for the LL lever, will decrease over time as rats will switch to selecting the smaller, immediate reward, thus displaying choice impulsivity. The steeper the DD curve, the faster rats switch over to the SS lever. Of interest, impulsive choice, or the inability to defer gratification, is often observed in pathological conditions and addiction studies (Amlung et al., 2017; Noda et al., 2020; Scherrer et al., 2020).

As GCI can modulate DA expression within the mesocorticolimbic pathway and also putatively impairs response inhibition, it is reasonable to predict that GCI will impact DD. In a human sample of CA survivors, Keijzer et al. (2020) found 55% of subjects to show disinhibition and impulsive behaviour. To our knowledge, only one animal study has assessed impulsive choice using a DD paradigm in the context of a focally induced brain ischemia. In a 2017 study, Déziel & Tasker used endothelin-1 to induce a focal ischemic stroke in adult Sprague-Dawley rats either in the oPFC or in the medial PFC. This resulted in higher preference for the SS lever in rats with oPFC lesions as opposed to control rats and rats with lesions to the medial PFC. Of note, this increase in impulsive choice was present for several weeks after ischemia. Unfortunately, the authors did not investigate neural correlates that may underpin such preferences for the SS lever other than infarct size used to confirm induction of ischemia. As noted, these impairments were induced using a model of focal ischemia, which does not produce the exact same neuronal and functional impairments as global ischemia. However, taking into account the previously described GCI-induced neuronal and behavioural alterations, it appears reasonable to predict increased impulsive choice in rats exposed to a GCI event.

Notably, the mesocorticolimbic pathway has been identified as playing a critical role in regulating impulse control, largely through DA-ergic activity (Beaulieu & Gainetdinov, 2011). More specifically, postsynaptic DA receptor D2 (DRD_2) density within this pathway is associated with elevated impulsivity (Besson et al., 2013; Dalley et al., 2007; Mitchell & Potenza, 2014), while increased DA release mediated by dopamine transporters (DAT) has been linked with a rodent's willingness to exert more effort to receive gratification in reward discounting paradigms (Castrellon et al., 2021). Furthermore, mesolimbic overexpression of ΔFosB, a transcription factor for neuronal activation, is associated with sustained search for gratification following repeated reward-related tasks (Nestler et al., 2001). The relationship of this network with the PFC also appears critical as the medial PFC is an important regulator of impulse control (Yates et al., 2014). Considering the impairments in response inhibition in other contexts and the importance of DA signalling in modulating reward-directed behaviour, it is reasonable to propose that changes to post-ischemic DA-ergic disruptions could also lead to impaired impulse control.

Objectives and Hypotheses of the Current Study

Considering the existing evidence and supporting studies, the objectives of this study were four-fold: (1) to assess whether decreased anxiety-like behaviours in the EPM after GCI can be characterized as impulsive action (i.e., impaired response inhibition), (2) to determine if post-ischemic EPM data may rather be interpreted as a failure to appraise aversive stimuli by use of the PO test, (3) to examine if putative impulsive action may extend to other branches of behavioural disinhibition such as impulsive choice (i.e., deferred gratification) using a DD task, and (4) to investigate underlying molecular changes in the mesocorticolimbic pathway as they may support impaired impulse control. Specifically, DRD_2, DAT, and ΔFosB in the basolateral amygdala (BLA), nucleus accumbens (NAc), core (NAcC) and shell (NAcS), and ventromedial prefrontal cortex (vmPFC) were assessed as they are closely linked to DD and decision-making (Cardinal et al., 2001; Churchwell et al., 2009; Hiser & Koenigs, 2018; Valencia-Torres et al., 2012; Winstanley et al., 2004). We hypothesized that GCI would impair inhibitory control in both tasks mentioned above, thus leading to impulsive action in the EPM and impulsive choice in the DD task, and that these changes would be reflected by lower DRD_2 and DAT expression, and overexpression of ΔFosB in the mesocorticolimbic pathway.

Materials and Methods

Subjects

Eighteen male Long Evans rats were obtained from Charles River Laboratories (Rochefort, Québec, Canada) and arrived at the facility on postnatal day (PND) 21. Upon arrival, rats were housed individually in Plexiglas cages containing beta chip bedding and a 3.5-inch diameter black polycarbonate tube, which provided a secure area for them to rest and hide. Rats were kept on a 12 h light:dark cycle (lights on at 6 a.m.), with all experiments completed during the light photoperiod. Room temperature was maintained at 21–23°C, and relative humidity was held at 60%. Rats had *ad libitum* access to water throughout the experiment.

Ad libitum access to standard rat chow (2018 Teklad global 18% Protein Rodent Diet, Envigo, United States) was given during the seven-day acclimation period, after which rats were placed on a food restriction (FR) regimen (14 g of chow/day). Autoshaping (AS) training began on the 7th day of FR. After each AS/DD session, daily food allocation was adjusted by subtracting the total weight of sucrose pellets consumed during the session (0.045 g/pellet) from the total food allowance of 14 g. *Ad libitum* access to chow was reinstated five days prior to surgery and maintained until six days before the start of DD sessions (see Figure 1 for experimental timeline). The FR protocol was determined by a pilot study performed in our laboratory and aimed at maintaining subjects at 85% of normal body weight, serving as motivation for participation in the DD task. Rats were weighed daily to ensure that FR was not interfering with healthy growth. All procedures were performed in accordance with ARRIVE guidelines and the Canadian Council on Animal Care and were approved by the Animal Care Committee of the University of Ottawa.

Delay Discounting Paradigm

The DD paradigm used for this study was modelled after previous research (Cardinal et al., 2001; Winstanley, Eagle, et al., 2006; Zeeb et al., 2010), with some modifications to length of delays (optimization of which was validated by the previously mentioned pilot study). All sessions took place between 10 a.m. and 3 p.m., from Monday to Friday. Rats were placed in a quiet, enclosed room for 30 min prior to the start of each session to habituate to the testing area. The task was completed using eight operant conditioning chambers (25.5 cm × 30.5 cm × 32.5 cm; HABITEST Modular Behavioral Test System, Coulbourn Instruments LLC, United States) controlled by the Graphic State 3.03 software (Coulbourn Instruments LLC, United States). Each box consisted of two retractable levers placed on either side of a central food magazine, in which sucrose pellets (5TUT Sucrose Reward Tablets 45 mg – Chocolate, TestDiet, St. Louis, MO, United States) were delivered by a pellet dispenser. The chamber contained two light sources: one within the food magazine (tray light) and one attached to the chamber's ceiling (house light). Each chamber was kept in a ventilated, sound-attenuating box to mask any external noises or sources of light. Operant boxes were thoroughly cleaned with 70% ethanol between each use.

Autoshaping

First, rats learned to associate lever presses with food rewards using a fixed reward ratio of 1. Each AS session consisted of a 40-s "Start" period, followed by two blocks, each comprising 50 trials (30 s/trial). The house light remained illuminated for the duration of all AS sessions. Following the "Start" period during which both levers were retracted, Block 1 began with presentation of the first lever. A lever press within 10 s led to a flash of the tray light and an immediate delivery of a single sucrose pellet. After the pellet was delivered, or if rats failed to press the lever within 10 s, rats entered a 30-s intertrial interval (ITI), during which both levers were retracted. After 50 presentations of the first lever, Block 1 was completed, and Block 2 was immediately initiated with the presentation of the second lever. The order of lever presentation was counterbalanced between subjects. All rats completed nine days of AS training prior to initiation of the DD task.

Delay Discounting

Each DD session consisted of a 40-s "Start" period with the house light illuminated and both levers retracted, followed by five blocks of 12 trials (70 s/trial). Each block began with two forced-choice trials. During the first forced-choice trial, the house light was illuminated, and the first lever was presented. A lever press led to the immediate delivery of a small reward (one sucrose pellet), with the tray light illuminated and house light extinguished. After reward delivery, or if the lever was not pressed within 10 s, rats entered the ITI, during which both levers were retracted, and all lights were extinguished until the next trial. The second forced-choice trial was identical to the first, with the exception of the second lever being presented, leading to the immediate delivery of a large reward (four sucrose pellets) if pressed. Ten free-choice trials followed, during which the house light was illuminated and both levers were presented. A lever press led to the immediate delivery of the associated food reward (small or large) and the initiation of the ITI stage. Omissions after 10 s led to induction of the ITI stage until the end of trial. After 10 free-choice trials, Block 2 was initiated. All 12 trials were repeated identically, with the exception that the large reward delivery now occurred after a 5-s delay following a lever press (free-choice trials), with the tray light illuminated until the four pellets were presented. Each consecutive block followed the same pattern, with the LL reward being increasingly delayed after a lever press: 10, 20, and 30 s for Blocks 3, 4, and 5, respectively (see Figure 2 for visual representation of the DD task). Small and large reward lever placement was counterbalanced between subjects. All rats completed 24 days of DD training prior to GCI surgery and six days of DD post-surgery.

Global Cerebral Ischemia

Global cerebral ischemia (GCI; n = 9) was induced using the four-vessel occlusion (4VO) model as previously described (Morin et al., 2021; Pulsinelli & Brierley, 1979; Pulsinelli & Buchan, 1988). After the induction of anaesthesia by inhalation of 4% isoflurane dissolved in 1.5 L/min O_2 (maintained at 1.5% during surgery), rats were placed in prone position in a stereotaxic apparatus and a 1.5-cm incision was made on the back of the neck. Bilateral alar foramina were exposed and irreversibly electrocauterized (3–4 mV current; MV-8 Veterinary Electrosurgical Unit, Macan Manufacturing, United States). The incision was closed with surgical clips, and the rats were moved to a supine position. A 1.5-cm vertical incision made above the animal's thorax allowed for the isolation of the common carotid arteries using loose small-diameter silk thread. The incision was closed, and rats were allowed an overnight recovery. The following day, the ventral incision was reopened under light anaesthesia. Then, bilateral common carotid arteries were reversibly occluded with microvascular clamps for 10 min in spontaneously ventilating rats. The absence of a righting reflex and the lack of response to light and sound confirmed the achievement of a state of ischemia. Control sham animals (n = 9) underwent the same anaesthesia and surgical incisions as GCI rats, without the electrocautery of alar foramina or microvascular clamping of common carotid arteries.

The analgesia protocol began on the first day of surgery and consisted of two daily (6:30 a.m. and 2:30 p.m.) sublingual buprenorphine (Bup; 0.4 mg/kg) doses for three consecutive days. The Bup pellets were crushed and evenly dissolved in hazelnut paste (Nutella©). The appropriate dosage was spread onto a small piece of surgical tape and stuck to the cage wall until complete ingestion. This analgesia delivery method has been used previously and has the advantage of not interfering with the ischemic process (Kalliokoski et al., 2010; Morin et al., 2021). If the dose had not been consumed by the time the next dose was to be given, a subcutaneous Bup HCl injection (0.05 mg/ml; Vetergesic© Multidose, Ceva Animal Health, Canada) was administered instead. Animals were kept on surgical heating platforms during surgeries (Prostation Rodent Workstation, Patterson Scientific, United States) and were placed in a heated incubator (32°C) immediately after each procedure. A subcutaneous saline injection (5 ml) followed the first procedure and wet rat chow and hydrogel (DietGel© Recovery, Clear H2O, United States) were provided after both.

Behavioural Tests

Behavioural testing began after 11 days of recovery. The OFT, EPM, and PO were conducted between 7 a.m. and 2 p.m., whereas six post-operative DD sessions were performed at the same times as the pre-operative sessions (between 10 a.m. and 3 p.m., see Figure 1). Prior to testing, rats were placed in a secure, quiet room for 30 min to habituate to the testing area. An overhead analogue camera (WV-CP284, Panasonic, Canada) was used to record behaviours. As previous literature has shown that lighting conditions are known to play an important role in modulating behavioural responses (Milot & Plamondon, 2008), the testing area was brightly illuminated (300 lux). All testing apparatus were thoroughly cleaned between each use with 70% ethanol (EPM) or Quato (OFT/PO; 1.6 ml Quato/100 ml water; Swish® Quato™ 44 General Purpose Disinfectant, Swish®, Peterborough, ON, Canada).

Open Field Test

The OFT was used to measure locomotor activity and exploratory or anxiety-like behaviours (Seibenhener & Wooten, 2015) and served as the habituation phase for the PO (Storsberg et al., 2018). The testing area was surrounded by white walls, and the researcher was hidden behind a white curtain. The apparatus was kept on a table raised 75 cm above ground and consisted of a grey floor surrounded by walls (LWH: 75 cm × 75 cm × 46 cm). The testing area surface was a painted grid of 36 equally sized squares. The peripheral zone was defined as the 20 external squares, whereas the central, more anxiogenic zone represented the 16 innermost squares. Rats were placed in one corner of the OFT, facing the central zone, and left to explore the testing area for 10 min, after which they were returned to their home cage. Noldus© EthoVision XT 7.1 software (Noldus, Leesburg, VA, United States) was used to track distance travelled in central and peripheral zones (cm), percent of time spent in central and peripheral zones, and latency to the initial entry into the central zone (s). The number of rearing behaviours in the central and peripheral zones, and time spent grooming in central and peripheral zones (s) were scored by a blinded examiner.

Elevated-Plus Maze

One hour after completion of the OFT, the EPM was used as a measure of anxiety-like behaviours (Rico et al., 2016) and behavioural disinhibition. The apparatus consisted of a cross-shaped platform elevated 60 cm above ground. The center of the platform was a square (LW: 10 cm × 10 cm), to which were attached two open arms (LW: 50 cm × 10 cm) and two closed arms surrounded by walls (LWH: 50 cm × 10 cm × 40 cm). Rats were placed in the center square facing one of the open arms and were left to explore for 5 min, before being returned to their home cage. During testing, subjects were hidden from the researcher by an opaque white curtain.

The Noldus© EthoVision XT 7.1 software was used to track time spent in the center zone, open arms and closed arms (s) [(time in arm or zone/(time in open + closed arms + centre zone)) × 100], number of open and closed arms entries, crossings (defined as rats going from one open or closed arm directly into the other open or closed arm, respectively), and latency to the initial entry of an open arm (s). A blinded examiner scored the number of risk assessment behaviours (each time a rat placed both of its forepaws into the central zone while remaining in a closed arm) and head dips (each time a rat looked over the edge of an open arm).

Predator Odour Test

The PO protocol was performed over two consecutive days, the first being the 10-min OFT serving as a habituation session where baseline exploration data was collected. The following day, the PO session aimed to assess defensive behaviour and appraisal of fear-based stimuli by exposing rodents to a predator odour within the previously habituated OFT arena (Storsberg et al., 2018). Four clear containers (LW: 12.5 cm × 12.5 cm) were placed in each corner of the arena, each containing a small piece of filter paper (LW: 2 cm × 2 cm), one of which had been sprayed with 3 ml of bobcat urine (Maine Outdoor Solutions, United States). Rats were placed in the center of the arena facing the container with the urine-spayed filter paper and were left to explore for 10 min. All pieces of filter paper were replaced between subjects. For statistical analyses, the arena was divided into 4 quadrants, which were subdivided into two zones: the “Adjacent” zone containing the clear plexiglass containers, and the “Distant” zone, which bordered the Adjacent zone (Quadrant 1 containing the bobcat urine, Quadrants 2 and 3 being adjacent to Quadrant 1, and Quadrant 4 being opposite to Quadrant 1; see Figure 2 for visual representation). The Noldus© EthoVision XT 7.1 software was used to determine the distance travelled (cm) and the time spent (s) in each zone, along with latency to enter each

Adjacent zone (s). Avoidance behaviour was determined by comparing time spent in each zone during the habituation phase (OFT) and during the PO. A blinded examiner scored the number of entries into each Quadrant, the percent time in direct interaction with each plexiglass container (defined as the amount of s of physical contact with containers), and the direction of approach into the Adjacent zones [defined as approaching from the squares near the outer walls (peripheral approaches) or from the center of the arena (central approaches)]. Additionally, freezing behaviours (defined as 3 s without movement), time spent grooming (s), and rearing behaviours were measured to quantify more discreet displays of anxiety-like/defensive behaviours.

Blood Sampling

Blood samples were obtained on the day of PO testing to provide a measure of circulatory CORT concentrations before and after exposure to an anxiogenic stimulus. The collection was done by quickly nicking the tail vein (Milot et al., 2012) and depositing two drops of blood onto Whatman Bloodstain Cards (Whatman International Ltd., Maidstone, United Kingdom). Blood samples were taken 15 min before (baseline) as well as 0, 30, and 120 min after the PO. The specimen collection paper was left to dry overnight, then stored at −80°C until the assay. These specific sampling intervals were selected as previous research has shown glucocorticoid serum levels to peak approximately 15 to 30 min after exposure to a momentary stressor (Belda et al., 2015). These timepoints thus enable to capture more acute and chronic phases of the stress response.

Blood Corticosterone Immunoassay

Using the samples collected during the PO, blood drop CORT concentrations were analyzed via enzyme-linked immunosorbent assay (ELISA; Corticosterone ELISA Kit, ADI-900-097, Enzo Life Sciences, Inc., Farmingdale, United States) as validated by Milot et al.

(2012). The Whatman cards were removed from the −80°C freezer and were left to thaw at room temperature for 30 min before a Gem Hole Punch (McGill Inc., Marengo, IL, United States) was used to punch samples into glass tubes. Assay buffer (200 µl) was pipetted into every tube, which were then sealed with parafilm and left for 24 h on a Belly Dancer® (Structure Probe Inc., West Chester, PA, United States). The following day, 2.5 µl of steroid displacement reagent was added to 100 µl of sample solution and were left to rest for 5 min at room temperature. Then, 200 µl of assay buffer was added to the samples which were then centrifuged at 5,000 g for 30 s. Afterwards, 100 µl of each sample were pipetted in the appropriate plate wells followed by 200 µl of alkaline phosphatase conjugated with CORT and sheep polyclonal antibody to CORT. The plate was covered and left on a plate shaker for 2 h at 500 rpm. The wells were then rinsed three times with wash buffer, 200 µl of pNpp solution was added, and the plate was covered and left to incubate for 45 min at room temperature. A stop solution was added then the plate was placed on a plate shaker at 500 rpm for 30 s before being scanned using a PowerWave XS2 Microplate Spectrophotometer (BioTek, United States). Assay detection range was 32–20,000 pg/ml and intra and inter-assay consistency was confirmed by measuring the coefficient of variability and normalizing detection levels for all plates, respectively.

Brain Tissue Collection

Immediately following the last DD session, rats were injected with a lethal dose of sodium pentobarbital (60 mg/ml; Euthanyl Bimeda-MTC Canada, Canada) and were transcardially perfused (4% paraformaldehyde; 20% picric acid). Brains were extracted and incubated in paraformaldehyde for 1 h, 10% sucrose for 1 h, then incubated in 10% sucrose overnight. The next morning, brains were incubated in 10% sucrose for 1 h, after which they were frozen with CO_2 and stored at −80°C. Subsequently, coronal brain slices (14 µm) were obtained using a cryostat (Leica CM1900, Leica Microsystems, Germany) and mounted onto Superfrost Plus Slides (Fisher Scientific, Canada). In this study, the regions of interest were the BLA (Bregma −1.60 to −2.12 mm), hippocampus CA1, and CA3 (Bregma −2.80 to −4.16 mm), NAcC and NAcS (Bregma 1.60 to 0.70 mm), and vmPFC (Bregma 2.70 to 1.70 mm). Brain regions were determined using the Paxinos and Watson rat brain atlas (Paxinos & Watson, 2006).

Assessment of Neuronal Cell Death

Thionine staining was used to quantify pyramidal neuron density in the hippocampus CA1 and CA3. Briefly, slides were transported in containers filled with 0.01 M phosphate-buffered saline (PBS; pH = 7.4) and were then soaked in dH_2O (30 s), thionine (10 min), 50, 70, 95, and 2 × 100% ethanol (2 min each), and 2 × Citrisolv (2 min each; Decon Labs, Inc., United States). The slides were covered with Permount Mounting Medium, were cover-slipped, and were left to seal overnight. For each subject, six histologically representative pictures of the CA1 and CA3 hippocampal subregions were obtained using a Leica DAS microscope with an attached SONY digital camera and were recorded via the Norton Eclipse 6.0 software (Empix Imaging, Mississauga, ON, Canada). Pyramidal neuron density within 1 mm linear length was quantified with the ImageJ software (National Institutes of Health, United States) by two blinded examiners with achievement of inter-rater reliability. Intact neurons were defined as having a clear nuclear area enclosing a nucleolus and a cytoplasm contained within a rounded cell body.

Fluorescence Immunohistochemistry

For ΔFosB and DAT fluorescence immunochemistry, slides containing the regions of interest were transported in 0.01 M PBS and were incubated for 30 min in blocking solution (5% donkey serum–0.2% triton–PBS). The slides were incubated overnight at 4°C in blocking solution containing monoclonal mouse anti-ΔFosB (1:2000; ab11959, Abcam, Canada – this antibody stained for both FosB and ΔFos) and monoclonal rabbit anti-DAT (1:1000; ab128848, Abcam, Canada) primary antibodies. After incubation, the slides were washed in PBS (3 washes × 5 min) and incubated in a blocking solution containing fluorescence-conjugated anti-mouse donkey (1:1000; A-21202, Thermo Fisher Scientific, Waltham, MA, United States) and anti-rabbit donkey (1:1000; A-21207, Invitrogen Canada Inc., Canada) secondary antibody for 1 h at room temperature (RT) in a dark cabinet. The slides were washed in PBS (3 washes × 5 min), incubated in Hoechst adenine-thymine binding dye solution (1:20,000 in PBS; Hoechst 33342, Invitrogen Canada Inc., Canada) for 5 min at RT, and PBS-washed (3 washes × 5 min). Slides were coated with 30 µl anti-fading solution, cover slipped, and sealed with nail polish. Negative controls (without primary antibody) ensured secondary antibody specificity and pilot studies determined optimal primary antibody concentrations. The staining and expression reported was similar to previous studies (Larson & Ariano, 1995 for DRD_2; Sun et al., 2020 for ΔFosB; Threlfell et al., 2021 for DAT). For DRD_2, an antigen retrieval protocol was performed prior to blocking. Briefly, the slides were incubated in 0.05 M sodium citrate-PBS (30 min), rinsed in PBS (3 washes × 5 min), soaked in 0.1 M Glycine-PBS (30 min), then PBS-washed again (3 washes × 5 min) before the continuation of the previously described protocol. Of note, the incubation in monoclonal rabbit anti-DRD_2 primary antibody (1:500, ab1558, Millipore, United States) lasted 24 h at room temperature, followed by three, 10-min PBS washes. The secondary antibody was the same as that for DAT (1:1000).

All fluorescence was detected using an Olympus DX51 microscope (Center Valley, PA, United States) with a 20× magnifier. Six anatomically matched pictures were taken for each structure using the Progress Pro 2.7.6 software (Jenoptik, Jena, Germany). The ImageJ software

(National Institutes of Health, United States) was used to quantify the percentage of immunoreactivity area relative to the total photomicrograph area (percentage of area) and the ratio between the mean immunoreactive foreground and subthreshold background (mean grey value; Jensen, 2013). Higher percentage of area represents a larger area covered by staining, whereas mean grey value measures the signal intensity related to staining density. The auto-threshold algorithm Triangle was used for all structures and markers, except for DAT expression in the NAc and BLA, for which Moments was used. For all markers, regions of interest were the BLA (Bregma −1.60 to −2.12 mm), NAcC and NAcS (Bregma 1.60 to 0.70 mm), and vmPFC (Bregma 2.70 to 1.70 mm). As previously mentioned, brain regions were determined using the Paxinos and Watson rat brain atlas (Paxinos & Watson, 2006).

Statistical Analyses

All analyses were completed using IBM© SPSS Statistics 27 (IBM, Armonk, NY, United States). Outliers were identified by using box plots and were corrected by adding or subtracting a value of one to the second most extreme data point within that group (or a value of 0.01 when data points were ratios). Homogeneity of variance and normality were assessed with Levene's and Shapiro-Wilk's tests, respectively. Data for DD were combined in three-day clusters, with the last three preoperative and the last three postoperative sessions being used for statistical analyses. The DD area under the curve (AUC) was calculated for each subject using the following equation $(x2-x1)[(y1+y2)/2]$ (Myerson et al., 2001). AUC data was used in a two-way mixed analysis of variance (ANOVA) with surgical status (preoperative vs. postoperative) as within-subject factor and group (sham vs. GCI) as between-subject factor. The LL lever choice percent was calculated by dividing the number of LL lever presses by total lever presses for that block, which was then multiplied by 100. All missing data points ($n = 2$; Block 5, one sham, one

GCI) were computed by multiple imputation. The effect of delay on LL lever preference was assessed with a three-way mixed ANOVA with blocks (Blocks 1–4) and surgical status (preoperative vs. postoperative) as within-subject factors and group (sham vs. GCI) as between-subject factors. The nonparametric Friedman's test was used to analyze omissions in the DD task. All behavioural (OFT, EPM, PO), thionine, and fluorescence immunohistochemistry data were analyzed using independent samples t-tests or Welch's t-test if homogeneity was violated. PO avoidance data was analyzed using a two-way mixed ANOVA, with session (habituation vs. PO) as within subject-factor and surgery (sham vs. GCI) as between-subject factor while ELISA data was analyzed using a two-way mixed ANOVA, with time (baseline, 0, 30, 120 min post-test) as within-subject factor and surgery (sham vs. GCI) as between-subject factor. Multiple imputation was used for a few missing ELISA data points. Pairwise comparisons were analyzed with the Bonferroni correction. Statistical significance was set at $p < 0.05$.

Results

All rats included in the GCI group ($n = 9$) achieved a state of ischemia, as confirmed during the surgery and through a neuronal density assessment. One sham subject could not be included in the thionine and fluorescence immunohistochemistry analyses due to technical issues (sham: $n = 8$).

Delay Discounting

A two-way mixed ANOVA of AUC data revealed no significant difference in overall LL lever preference between groups or surgical status ($p > 0.05$; see Figure 3). A three-way mixed ANOVA revealed a significant main effect of blocks on choice ratio [$F(3,48) = 20.338$, $p < 0.0005$, $\eta^2_p = 0.560$]. Pairwise comparisons found choice ratio during Block 1 to be significantly higher than that of Blocks 2 ($p = 0.002$), 3 ($p = 0.001$), and 4 ($p < 0.0005$). During

Block 2, the preference for the LL lever was significantly higher than during Blocks 3 ($p = 0.011$) and 4 ($p = 0.005$). All rats preferred the LL reward when the delay was short but showed increased preference for the SS reward as LL delay to gratification increased. Due to low participation rate, the 5th block was excluded from choice ratio analysis, being nonrepresentative of the overall performance.

As no between-group differences were reported for the AUC and choice ratio, omissions data from sham and GCI rats were analyzed together. Friedman's test revealed that omissions differed significantly between blocks [$\chi^2(9) = 112.176$, $p < 0.001$] (see Figure 3). Prior to surgery, pairwise comparisons showed omissions during Block 1 to be significantly lower than during preoperative Blocks 3 ($p = 0.001$), 4 ($p < 0.0005$), and 5 ($p < 0.0005$), while omissions during Block 2 were significantly lower than during Block 4 ($p < 0.0005$) and 5 ($p < 0.0005$). Similarly, post-operatively, post hoc analyses showed significantly less omissions during Block 1 than Blocks 3 ($p = 0.004$), 4 ($p < 0.0005$), and 5 ($p < 0.0005$). Omissions during Block 2 post-surgery were also lower than postoperative Blocks 4 ($p = 0.004$) and 5 ($p < 0.0005$). No differences between equivalent pre and postoperative blocks were noted ($p > 0.05$).

Behavioural Tests

Open Field Test

As shown in Figure 4, no differences between sham-operated and GCI subjects were found in regard to distance travelled or percentage of time spent in central and peripheral zones of the OFT ($p > 0.05$). However, a Welch t-test revealed a trend for a shorter latency the initial entry in the anxiogenic center zone in GCI vs. sham-operated rats [$t(10.427) = 2.035$, $p = 0.068$, $d = 0.959$]. There were no significant differences in grooming or rearing in the central and peripheral zones, and in the number of central or peripheral zone crossings (all $p > 0.05$).

Elevated-Plus Maze

As shown in Figure 4, GCI rats spent a significantly smaller percentage of time in the closed arms of the EPM than sham-operated controls [$t(16) = 4.207$, $p = 0.001$, $d = 1.983$]. No differences were found in the percent time spent in open arms or center square, or in the number of open or closed arm entries ($p > 0.05$). Sham-operated subjects performed more open arm crossings than GCI rats [$t(16) = 2.694$, $p = 0.016$, $d = 1.270$], but did not differ in the number of closed arm crossings ($p > 0.05$). Ischemic rats tended to perform more head dips than their sham-operated counterparts [$t(16) = -1.812$, $p = 0.089$, $d = -0.854$]. There were no differences in the number of risk assessments or latency to first open arm entry (both $p > 0.05$).

Predator Odour Test

A filter paper containing predator bobcat urine was placed in the Adjacent 1 zone (Quadrant 1) of the arena (see Figure 2B). Avoidance behaviour was assessed using the time spent in zones during the habituation phase and the PO. A two-way mixed ANOVA showed that during the PO, rats tended to spend less time in Adjacent 1 [$F(1,11) = 3.998$, $p = 0.071$, $\eta^2_p = 0.267$] and spent significantly less time in Distant 1 [$F(1,11) = 37.840$, $p < 0.001$, $\eta^2_p = 0.775$] than they had during the OFT habituation (see Figure 5). Simple main effects showed that GCI rats spent less time in Distant 1 during the PO than the OFT ($p < 0.001$) and compared to sham subjects during the PO ($p = 0.016$). However, GCI rats spent more time in Distant 1 compared to sham rats during the OFT ($p = 0.018$). Notably, rats spent an increased amount of time in Distant 2 [$F(1,11) = 21.858$, $p < 0.001$, $\eta^2_p = 0.665$] and in Adjacent 4 [$F(1,11) = 52.502$, $p < 0.001$, $\eta^2_p = 0.827$], and tended to spend more time in Adjacent 2 [$F(1,11) = 4.735$, $p < 0.052$, $\eta^2_p = 0.301$] during the PO than the OFT. Conversely, rats spent less time in Distant 3 [$F(1,11) = 24.956, p < 0.001, \eta^2_p = 0.686$] and Distant 4 [$F(1,11) = 35.892, p < 0.001, \eta^2_p = 0.765$] during the PO than the OFT.

For the number of rearings, independent samples t-test revealed a significant effect of surgery [$t(11) = 2.810, p = 0.017, d = 1.563$] with ischemic rats showing reduced rearings in the Distant 1 zone. No significant differences between groups were found for distance travelled, number of entries, and approach patterns in the arena zones (all $p > 0.05$). Inconsistent grooming and freezing scores did not enable valid statistical analyses of those measures.

Blood Corticosterone Immunoassay

For circulatory CORT levels, a two-way mixed ANOVA revealed a significant main effect of time [$F(1.943,31.087) = 39.378, p < 0.0005, \eta^2_p = 0.711$] and surgery [$F(1,16) = 4.538, p = 0.049, \eta^2_p = 0.221$] (see Figure 5). The pairwise comparisons revealed serum CORT levels to be increased immediately following PO ($p < 0.0005$) and to remain elevated 30 min later ($p = 0.006$) when compared to baseline. After 120 min, CORT concentrations had recovered to levels below baseline ($p = 0.001$), possibly due to a novelty effect of the sampling procedure. Concentrations immediately after and 30 min after completion were both higher than at the 120-min mark (both $p < 0.0005$). Finally, CORT levels recovered gradually, with concentrations immediately after PO being higher than concentrations 30 min post-completion ($p = 0.02$). Overall, serum CORT concentrations were higher in GCI subjects than sham subjects ($p = 0.049$), without a time × surgery interaction ($p > 0.05$).

Hippocampal Neuronal Injury Assessment

Figure 6 shows the pyramidal cell density in the CA1 and CA3 for sham and GCI rats. A Welch independent samples t-test found significant differences in mean neuronal density in the CA1 between sham and GCI subjects [$t(10.260) = 2.989, p = 0.013, d = 1.384$]. Neuronal density was significantly lower in the CA1 of rats having undergone GCI than in their sham-operated counterparts. There were no significant differences in mean neuronal density in the CA3 ($p > 0.05$).

Fluorescence Immunohistochemistry

Dopamine Transporter Expression

As shown in Figure 7, a Welch independent samples t-test revealed significant differences for mean grey value in the NAcC [$t(8.016) = 3.050$, $p = 0.016$, $d = 1.563$] and NAcS [$t(7.982) = 2.843$, $p = 0.022$, $d = 1.458$]. Higher DAT mean grey values were observed in sham-operated vs. ischemic rats in both subfields. In contrast, no significant differences in mean grey values were found in the BLA and vmPFC ($p > 0.05$). No significant differences in the percentage of area were detected ($p > 0.05$).

Dopamine Receptor D2 Expression

Figure 8 presents DRD_2 immunoreactivity in the BLA, NAcC, NAcS, and vmPFC of sham and GCI rats. An independent samples t-test revealed significant differences in mean grey value in the NAcS [$t(15) = 2.242$, $p = 0.04$, $d = 1.090$]. Sham-operated subjects had significantly higher DRD_2 density than GCI subjects in the NAcS. There were no differences in percentage of area in the NAcS ($p > 0.05$). Additionally, there were no significant differences in both measures in the BLA, NAcC, or vmPFC ($p > 0.05$).

ΔFosB Expression

Figure 9 displays ΔFosB immunoreactivity in the BLA, NAcC, NAcS, and vmPFC of GCI and sham-operated subjects. An independent samples t-test showed a significant difference in mean grey value in the NAcC [$t(15) = 2.854$, $p = 0.012$, $d = 1.387$], attributable to significantly higher ΔFosB mean grey value in sham compared to GCI rats. Percentage of area did not differ between groups in the NAcC ($p > 0.05$). Independent samples t-test also found a trend in percentage of area in the vmPFC [$t(15) = 1.886$, $p = 0.079$, $d = 0.916$] with sham-operated rats trending towards an elevated ΔFosB percentage of area compared to GCI rats. Mean grey value did not differ between groups in the vmPFC ($p > 0.05$). There were no significant differences in both measures in the BLA or NAcS ($p > 0.05$).

Discussion

To our knowledge, this study is the first to investigate behavioural disinhibition following GCI. To do so, we aimed to (1) characterize a recurring pattern of post-ischemic EPM behaviours as reflective of impulsive action or rather indicative of a failure to appraise fear-inducing stimuli and to (2) outline the effects of GCI on impulsive choice by analyzing reward selection in a DD paradigm and underlying changes to the dopaminergic mesocorticolimbic system activity regulating such processes. Indeed, findings from this study support reduced anxiety-like behaviours in the EPM of ischemic rats compared to sham-operated controls, and in the OFT, albeit to a lesser extent, which could indicate behavioural disinhibition. Interestingly, findings in the PO only marginally support the effects of GCI on defensive behaviour when facing a naturalistic or environmental threat. We also observed no significant changes in impulsive choice in a DD task after infarct. Notably, GCI led to reduced DRD_2, DAT, and ΔFosB expression within the mesocorticolimbic pathway, concomitant with the well-acknowledged significant hippocampal CA1 injury. In sum, the propensity of ischemic rats to rapidly explore the anxiogenic zones of the EPM and OFT did not correlate with enhanced choice impulsivity tested during a DD paradigm although it could be marginally related to deficits in the ischemic rats' ability to appraise fearful or anxiogenic stimuli. As such, our study supports possible differential impact of GCI on response inhibition required in different contexts of life situations.

Reduced Anxiety-Like Behaviour in Ischemic Rats Is Not Accompanied by Changes in Defensive Behaviour in the Predator Odour Test

In this study, ischemic rats spent reduced time spent in the EPM's anxiolytic closed arms and showed tendencies to perform increased head dips into the open arms when compared to sham-operated counterparts. Such responses were paralleled in the OFT by a reduced latency to enter the arena's anxiogenic centerzone, supporting the reduced anxiety-like behaviours after ischemia to be measurable using two different validated testing paradigms. Although intriguing considering the elevated anxiety often reported by humans having experienced CA, these observations are consistent with multiple studies having demonstrated increased exploration of the anxiogenic OFT and EPM zones by ischemic rats, along with decreased latency in initiating the exploration of these anxiogenic zones (Knowles et al., 2016; Lu et al., 2016; Morin et al., 2021; Plamondon & Khan, 2005; Yan et al., 2007). Using the validated response profiles and behavioural codifications established for these tests, such impetuous responses from ischemic rats have been interpreted as reduced anxiety-like behaviours, although possible links of these responses with reduced or impaired behavioural inhibition being proposed by some authors as a contributing factor (Milot & Plamondon, 2008; Plamondon & Khan, 2005). Due to the growing amount of studies replicating such findings, it has been hypothesized in recent years that this could be indicative of decreased inhibitory control, possibly reflecting behavioural impulsivity (Knowles et al., 2016; Morin et al., 2021). Indeed, previous studies have interpreted increased open-arm time in the EPM and decreased latency to the center of the OFT as potential indicators of impulsivity (Binder et al., 2004) in experimental paradigms involving febrile seizure

(Remonde et al., 2023) or modelling schizophrenia, where increased open-arm exploration in the latter being interpreted as a display of positive schizophrenia-like symptom (Hur et al., 2021). Furthermore, post-surgical EPM behaviour in traumatic brain injury models resembles that of GCI rats, possibly due to elevated levels of arousal (Tucker & McCabe, 2021). In models of attention-deficit-hyperactivity disorder (ADHD), similar results are present especially during adolescence, which can be interpreted as reduced attention, appreciation or consideration of environmental anxiogenic stimuli (Laviola et al., 2003). Study paradigms involving exercise have shown positive impacts on such responses, in part by exercise-induced increases in DA production in the substantia nigra and the striatum (Cho et al., 2014). From observations of rats displaying idiosyncratic behaviours, Steimer et Driscoll (2003) have developed an interesting analytical grid enabling to differentially appreciate behavioural responses in experimental paradigms used to test anxiety in rodents. Seeing the significant increases in novelty-seeking in the Roman high-avoidance rat strain that was tested, which went as far as to jump from the EPM open arms, the authors have designed a novel chart presenting a spectrum of graduating intensity of observed responses (i.e., ranging from low to high on defined x and y axes). The x axis represented the level of emotional reactivity noted while the y axis quoted the level of coping. Thus, animals with low emotional reactivity and high levels of coping could be considered to display anxiety, while subjects with low levels of coping and high levels of emotional reactivity would fare high on displayed impulsivity. This proposition certainly merits further exploration through a battery of nuanced behavioural tests of various animal models and housing/environmental conditions, possibly using endocrine correlates helping to further characterize the different response styles and behavioural expressions. A benefit of developing nuanced paradigms is the ability to best decipher subtle behavioural manifestations, such as those that could help distinguish expressions of anxiety from impulsivity in animal models (Cryan & Holmes, 2005; Rico et al., 2019).

In this study, behavioural disinhibition appeared less pronounced than that reported in previous assessments. Although there could be many explanations, one could relate to the extensive handling of the animals related to the DD task, which extended over multiple days. Nonetheless, our demonstration of impulsive action, exhibited through anxiety-like measures, in the absence of changes in DD performance is consistent with Broos et al.'s (2012) comprehensive assessment, which showed impulsive choice and impulsive action to be uncorrelated in both humans and rats. Using the 5-CSRTT as a measure of impulsive action, they found no significant relation between DD rates (i.e., impulsive choice) and 5-CSRTT data (i.e., impulsive action) in their rat sample, even upon the administration of amphetamine and NE reuptake inhibitor atomoxetine. In their human sample, similar results were found. As such, no significant correlation was detected between measures of impulsive choice (i.e., a DD task) and impulsive action (i.e., immediate and delayed memory task, stop signal task, Barratt impulsiveness scale), supporting the non-unitary nature of impulsivity constructs. Furthermore, the behavioural testing in our study was conducted under bright light conditions, in which ischemic rats have previously shown increased open-field exploration when compared with sham-operated controls. Importantly, ischemic rats tested under dim light conditions show reduced exploration compared to controls, further support the importance of context (Milot & Plamondon, 2008).

Multiple studies have shown exposure to a predator's urine to act as a psychogenic stressor inducing fear-like and defensive behaviours (Staples, 2010). This study used bobcat urine, a stimulus shown to elicit the expression of avoidance and defensive behaviours in rats

(Templeton et al., 2023), associated with activation of the amygdalo-piriform transition area and subsequent rise in circulatory CORT secretion (Albrechet-Souza & Gilpin, 2019). Our results of reduced time spent in zones nearest to the urine (Adjacent 1 and Distant 1) with increases in zones further away from the target anxiogenic stimulus (Adjacent 2 and 4 and Distant 2) during the PO when compared to the habituation session validate the odour to have induced avoidance behaviours in rats. In support of the PO stimulus also having prompted a stress response, all rats showed a sustained HPA axis activation to the PO stressor, as demonstrated by significantly heightened CORT expression (immediate and delayed), irrespective of surgical status. While this present ischemic rat sample did not demonstrate HPA hypersensitivity to an acute stressor, we nonetheless noted hypercorticosteronemia in ischemic rats, showing higher CORT levels than sham-operated rats at every timepoint in the study. Consistent with earlier studies reporting increased basal CORT concentrations for up to seven days after global ischemia (de la Tremblaye et al., 2014), our study supported an elevation to remain present at a longer 13-day post-ischemic interval. Furthermore, considering that the PO and blood sampling were conducted following a relatively short delay after surgery, one cannot exclude that ischemic rats may had reached a saturation point in CORT secretion levels. Such phenomenon could have hampered the emergence of a significantly elevated post-PO increase in ischemic rats' CORT secretion, resulting in the absence of differences with sham-operated controls.

Another interesting finding was related to an increased time spent by GCI rats in the Distant 1 zone during the habituation period, an outcome that was opposite to behavioural responses observed during predatory odour exposure. During the PO session, ischemic rats also showed decreased frequencies of rearings when in the Distant 1 zone. Together the observations could support a propensity for ischemic rats to display anxiogenic, fear-based responses when confronted with *survival*-associated stimuli. Our findings otherwise indicated similar response profiles in sham-operated and ischemic rats on all other measures (i.e., number of entries, latency to odour, time in direct interactions, approach patterns). Consistent with our findings, locomotor responses to weasel odour have been shown to be unaffected following colchicine-induced hippocampal lesions in rats (Perrot-Sinal & Petersen, 1997). Therefore, the difference observed between ischemic and sham-operated rats in the PO, which relates to approach behaviours in the presence of a predator odour appears to support cognitive appraisal of fear-specific cues to be preserved after ischemia. Overall, findings of this study have helped to further address post-ischemic functional impairments. Importantly, they support the behavioural disinhibition witnessed in the EPM/OFT to be context specific. Future studies assessing the differences related to odour specimen and/or rodent strains are required to further understand the impact of GCI on adaptive coping strategies when facing environmental threats (Adduci et al., 2021).

Global Cerebral Ischemia Does Not Alter Impulsive Choice in a Delay Discounting Task

Using a classic DD paradigm, we showed ischemic and sham rats to both opt for the larger rewards when the delay to gratification was short, with a gradual shift towards a preference for the smaller rewards as the delay increased. Contrasting our hypothesis and the increased preference for the SS reward following focal ischemic lesions to the oPFC in Sprague-Dawley rats (Déziel & Tasker, 2017), GCI did not accelerate the shift towards a preference for the SS reward, which would have been indicative of behavioural disinhibition, more specifically impulsive choice. It is of note that the protocol used by this research team when testing focal ischemia in rats differed slightly from the one used in our study. Mainly, the selected DD apparatus did not involve the use of operant boxes but was rather performed using a T-maze comprising of a start arm (stem) from which emanated two choice arms, one dubbed the “low reward arm" (LRA) and the other, the "high reward arm" (HRA). Each trial started with a rat being placed in the start arm with the two choice arms open. As the rat entered either the LRA or HRA, the researcher closed a Plexiglas gate to trap the rat in this arm. If in the LRA, a second gate in front of the rat would immediately open to reveal one reward pellet in a food tray. Upon selection of the HRA, the second gate would then open only following a specific delay to then reveal multiple reward pellets in the food tray. In this protocol, each daily session consisted of seven trials, the first two being forced-choice followed by five being free-choice trials. Interestingly, while the chosen delay for reward gratification in the HRA increased from 0 to 15 s throughout daily sessions before focal ischemia and from 15 to 60 s after infarct, the delay did not change within each daily session. As such, each cluster of four consecutive days was associated with a specific delay to reward in the HRA arm that would increase in the next four-day cluster. Such method appeared to be learned quite efficiently by the rats and could be interesting to use as an alternate testing paradigm with GCI rats.

Our study supports damage to vulnerable brain areas such as the hippocampal CA1 layer (Auer, 2016; Traystman, 2003) following a 10-min GCI to have a negligible impact on choice impulsivity. This could indicate that despite significant alterations of limbic inputs, the functions of prefrontal circuitries may be largely preserved in the post-ischemic brain. Surprisingly, assessment of prefrontal dysfunction following GCI remains largely unexamined by preclinical studies, especially in relation to its precise impact on cognitive processes. Interestingly, García-Chávez et al. (2008) have found statistically significant reductions in pyramidal neuron soma size, apical and basilar dendrite length, number of dendritic bifurcations, and spine density in the prefrontal cortical layers III and V, 120 days after a 15-min GCI. However, to our knowledge, there are no studies that have assessed neuronal death and integrity of PFC subregions, especially the cytoarchitechturally defined parts of the rat's frontal cortex that include the prelimbic, infralimbic, and anterior cingulate areas, after global ischemia. Thus, it would be of great interest for future research to conduct such analyses. In this context, previous research from our laboratory has shown reduced BDNF and TrkB protein and mRNA expression in the medial PFC region 30 days following GCI in Wistar rats (de la Tremblaye et al., 2016), while other groups have demonstrated significant mitochondrial damage and apoptosis in prefrontal cortical tissue 96 h following 20-min global ischemia (Jangholi et al., 2020). Thus, post-ischemic delays and intensity of the insult appear to play a critical role in prefrontal cortical dysfunctions.

The well-documented extensive damage to the vulnerable CA1 pyramidal neurons of the hippocampus following GCI (Bueters et al., 2008; Pulsinelli & Brierley, 1979; Pulsinelli & Buchan, 1988) was observed in our study. Interestingly, excitotoxic lesions to the hippocampus in Lister hooded rats have been found to cause impulsive choice in a DD task (Cheung & Cardinal, 2005). However, the induced lesions in that study resulted in the destruction of the entire hippocampal structure. Albeit being heavily altered, the CA1 maintains some functional capabilities following GCI and the CA3 pyramidal neurons generally show more resistance to ischemic insult (Knowles et al., 2016). In such context, the less direct global ischemia damage to hippocampal structures appears insufficient to affect DD performance. This study being the first to assess DD performance in GCI rats, further research seems necessary to better characterize the alterations in decision-making processes post-ischemia and associated neuronal determinants.

Global Cerebral Ischemia and the Mesocorticolimbic Pathway

Activation of DA-ergic pathways has been shown to be greatly impaired in the weeks following brain infarction, and marked by a global decrease in DA bioavailability (Takagi et al., 1995), and selective attrition of DRD_2 (Gower & Tiberi, 2018) and DAT (Yanagisawa et al.,

2006) expression. These observations are concordant with the reduced DRD_2 and DAT immunoreactivity observed in our study. Furthermore, levels of tyrosine hydroxylase, the rate-limiting enzyme in DA synthesis, have also been shown to fluctuate post-ischemia, being significantly decreased nine days after GCI (Knowles et al., 2016) prior to being upregulated at a 30-day interval (de la Tremblaye et al., 2014). A delayed DA upregulation could underlie the DRD_2 downregulation observed in our study.

Interestingly, the alterations to DA-ergic function appeared selective and confined to the NAc. The reduced level of NAcC activation inferred from a significantly decreased ΔFosB expression supports the vulnerability of this brain locus to global ischemia. Considering the crucial role of the NAc in regulating impulsive choice (Cardinal et al., 2004; da Costa Araújo et al., 2009), the absence of changes in DD performance in GCI rats is intriguing. One explanation could be tied to uncovered functional inputs of the shell and core portions of the NAc. For example, whereas lesions to the NAcC usually increase DD rates (Cardinal et al., 2001), lesions to the NAcS had no effects on the same task (Pothuizen et al., 2005). Furthermore, injections of the DRD_2 antagonist eticlopride into the NAcC decreased self-administration of both cocaine and food, whereas injection into the NAcS did not influence food self-administration (Bari & Pierce, 2005). As such, lowered DRD_2-ir density in the NAcS alone may not be sufficient to induce impulsive choice. Similarly, reduced ΔFosB at the core in isolation appear to exert a limited impact, at least when using this DD paradigm.

One additional point to consider is related to the dual action of DAT in removing DA from the synapse and repackaging it for future release, which complexifies identifying a precise role for this endogenous signal in regulating impulsive choice. In a meta-analysis by Castrellon et al. (2021), the modulation of DAT activity has been associated with more impulsivity and avidity to select the short delay-associated reward (i.e., decrease in discounting behaviours), a response proposed to result from heightened DAT-mediated DA release. In contrast, Adriani et al. (2009) found that accumbal injections of a DAT gene enhancer or DAT silencer surprisingly showed a similar impact in a DD task, with both drugs reducing the preference for the LL reward, with a slightly more pronounced effect for DAT overexpression. The authors concluded that any modification to DAT function, whether enhanced or reduced, altered inhibitory self-control. As such, the observation in our study that reduced NAc DAT density in GCI rats failed to impact impulsive choice is unexpected. Although mechanisms remain to be determined, strong connections exist between the NAc and BLA (Winstanley, Theobald, et al., 2006) and DA-ergic function in the BLA was not altered by GCI in this study. The integrity of amygdalar activation may have contributed to counteract the effects of post-ischemic NAc dysregulations.

Furthermore, research has supported ΔFosB expression to be associated with sensitization to the motivational properties of drugs of abuse, thus acting as a “molecular switch” involved in maintaining addiction (Nestler et al., 2001). In a study on food addiction-like behaviours, high impulsivity rats had increased ΔFosB expression in the NAcS compared to low impulsivity groups, irrespective of having access to regular rat chow or a highly palatable diet. In contrast, ΔFosB expression in the NAcC was increased in both highly palatable diet groups but did not vary between impulsivity groups (Velázquez-Sánchez et al., 2014). These findings further support differential roles of the NAc subregions in modulating impulsive choice and are consistent with an absence of changes in post-GCI impulsive choice in the presence of reduced ΔFosB expression at the NAcC. To our knowledge, our study is the first to measure ΔFosB expression at a remote 26 days after GCI. Our observations of reduced ΔFosB immunoreactivity contrast with shorter-term assessments which found ΔFosB to be overexpressed 48 h after ischemia (Kurushima et al., 2005; McGahan et al., 1998) and to return to near basal levels after one week (Kurushima et al., 2005).

Notably, while discrete changes to DA-ergic function in the mesocorticolimbic pathway following GCI are not sufficient to increase impulsive choice, these changes may play a role in promoting disinhibited exploratory behaviours in the EPM and OFT. Previous research has found intraperitoneal injections of a DRD_2 antagonist to increase open-arm entries and number of head dips in the EPM (Rodgers et al., 1994). Similarly, DAT knockdown in the NAc of mice increases time spent and number of entries in the open arms of the maze (Bahi & Dreyer, 2019). The altered ability to inhibit exploratory responses post-GCI may thus be influenced by diminished DRD_2-ir in the NAcS and reduced DAT expression in the NAc observed in this study. Other biochemical signals may contribute to impulsivity and exploratory behaviour. For instance, the stimulation of 5-HT_{1B} receptors and administration of selective serotonin reuptake inhibitors fluoxetine and paroxetine are known to reduce EPM exploration (Drapier et al., 2007; Lin et al., 2019). More specific to global ischemia, CRH knockdown restricted to the hypothalamus has led to decreased plasma CORT concentration and more EPM, OFT, and light-dark box exploration (Zhang et al., 2017), while the opposite has been noted upon CRH infusion into the NAcS prior to EPM and OFT (Chen et al., 2012).

The trend for ischemic rats to show reduced ΔFosB expression in the vmPFC could be an indicator of decreased neuronal activation (Kormos et al., 2016; Vialou et al., 2015). In this context, our observations present an interesting parallel with that of Feja and Koch (2014), which showed muscimol inactivation of the rat vmPFC to result in impaired impulse control, as assessed in the 5-CSRTT, although it failed to affect lever preference in a DD task. Other groups have supported that lesions to the vmPFC alter inhibitory measures of control (Chudasama et al., 2003). This leads us to hypothesize that reduced vmPFC activation might contribute to foster behavioural impulsivity in our ischemic rodents, in the absence of changes to impulsive choice. Furthermore, the interaction between the hippocampus and the vmPFC could be essential in modulating response inhibition, knowing that dissociation of these brain regions led to a dysregulation of impulse control in the 5-CSRTT (Chudasama et al., 2012). In sum, the extensive CA1 neuronal injury combined with reduced vmPFC activation after ischemia could play a role as physiological mechanism underlying the behavioural disinhibition/impulsive action exhibited by GCI rats. It is important to note that the EPM is classically known to measure anxiety-like behaviours, and that the interpretation of behavioural disinhibition in ischemic rodents represents an alternate interpretation that needs validation. As such, the use of other measures and behavioural tests, such as the 5-CSRTT or the newly designed Elevated Gradient of Aversion (Rico et al., 2019), could allow better elucidation of the subtypes of impulsivity impacted by GCI.

Limitations and Future Directions

Overall, this study has limitations that must be considered. As previously mentioned, the repeated physical manipulations necessary for the DD task may have hampered more striking EPM behaviours often found in post-ischemic samples. For example, repeated handling has been previously demonstrated to result in higher propensity for open-arm exploration in the EPM (Ueno et al., 2020) while gentle handling can counter the anxiogenic effects of direct CRH administration in anxiety-related measures of the EPM (Adamec et al., 1991). Conversely, handling can also reverse the anxiolytics effects of prior exposure to environmental enrichment in the EPM (Pritchard et al., 2013). As such, physical manipulations may be interpreted as potential confounding factors, especially if done so repeatedly. As suggested by researchers, future studies may use small, opaque Plexiglas tubes to manipulate rats when possible.

Rat strain has also been known to play a role in behaviour studies. For instance, all rat strains do not have equal levels of sensitivity or tolerance towards the same delays to gratification (Mar & Robbins, 2007). For this present study, Long Evans rats were selected as previous pilots conducted in our laboratory revealed that Wistar rats committed significant amounts of omissions and showed a low level of interest in participating in the DD task, even when placed under a strict FR protocol. Moreover, research has shown that pigmented rats such as Long Evans rats fare better than albino rats (e.g., Wistar) in tasks demanding visual abilities and discrimination, such as touchscreen tasks (Kumar et al., 2015; Martis et al., 2018). Pigmented rats also learn to associate lever presses with food rewards in AS tasks at a more rapid pace than albino rats, who are also more sensitive than the former to handling prior to AS since increased handling may improve their performance (Andrews, 1996). It is nevertheless crucial to recognize that Wistar rats remain the gold standard for GCI models and thus comparisons of the current results are not done on the basis of the exact same biological system used by a vast proportion of the existing literature.

Finally, while the use of bobcat urine in the context of the PO test has been validated to elicit the desired behavioural and endocrine responses in rats (Albrechet-Souza & Gilpin, 2019), TMT remains a compound used very widely in research since it is a laboratory-controlled extract of specific molecules from fox feces and provides more standardization and uniformity as a stimulus through studies and research teams (Takahashi, 2014; Takahashi et al., 2008). However, TMT is very cost-prohibitive, forcing many to turn to alternate sources for acute predator stressors.

Importantly, the VTA acts as the origin point for DA secretion in the mesocorticolimbic pathway and is very rich in both DAT and DRD_2. Thus, it also acts as an important regulator of DA negative feedback (Adell & Artigas, 2004; Lammel et al., 2014; Storch et al., 2004). Unfortunately, technical issues prevented the sampling of VTA tissue for immunohistochemical analyses in this study. We have also shown a study establishing the role of the oPFC in impulsive choice in a model of focal ischemia (Déziel & Tasker, 2017). This structure would have also been of interest in histological protocols.

Due to logistical constraints, this study could not enable for the analysis of dynamic, *in vivo* measures of DA-ergic and ΔFosB signalling during the tasks. While, for example, detection of ΔFosB expression is possible for a few hours after stimulation (Nestler et al., 2001), as was the case here when samples were collected, it would have been pertinent to be able to measure live changes with either functional imaging or microdialysis during the tasks.

Future studies may also make use of tasks such as the 5-CSRTT and the Go/No-Go paradigm to better dissect discreet changes in impulsive action after GCI. Likewise, as increased arousal may be the underlying driving force of EPM behaviour after TBI in rats (Tucker & McCabe, 2021), tests measuring this concept should be used in GCI subjects. Finally, it would be of interest to investigate neuronal death and integrity in both the NAcC and NAcS after global infarct to better differentiate between the two substructures as they seem to regulate specific aspects of impulsivity.

Conclusion

Our study is the first to demonstrate that global cerebral ischemia does not impact impulsive choice in a delay discounting task, irrespective of alterations to impulsive action witnessed in the Elevated-Plus Maze and significant reductions of dopaminergic and ΔFosB signalling. Furthermore, mesocorticolimbic expression of dopamine receptor D2, dopamine transporters, and ΔFosB support distinctive roles of the nucleus accumbens core and shell, and the ventromedial prefrontal cortex in regulating impulsive action and impulsive choice in ischemic rodents. These novel findings highlight the selective nature of the behavioural impairments caused by global cerebral ischemia on specific subtypes of impulsivity and emphasize the need for future studies to further characterize post-ischemic behavioural disinhibition.

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Figures

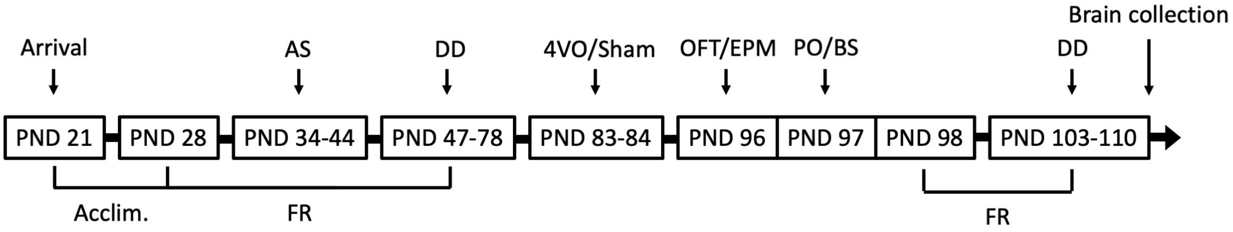

Figure 1.

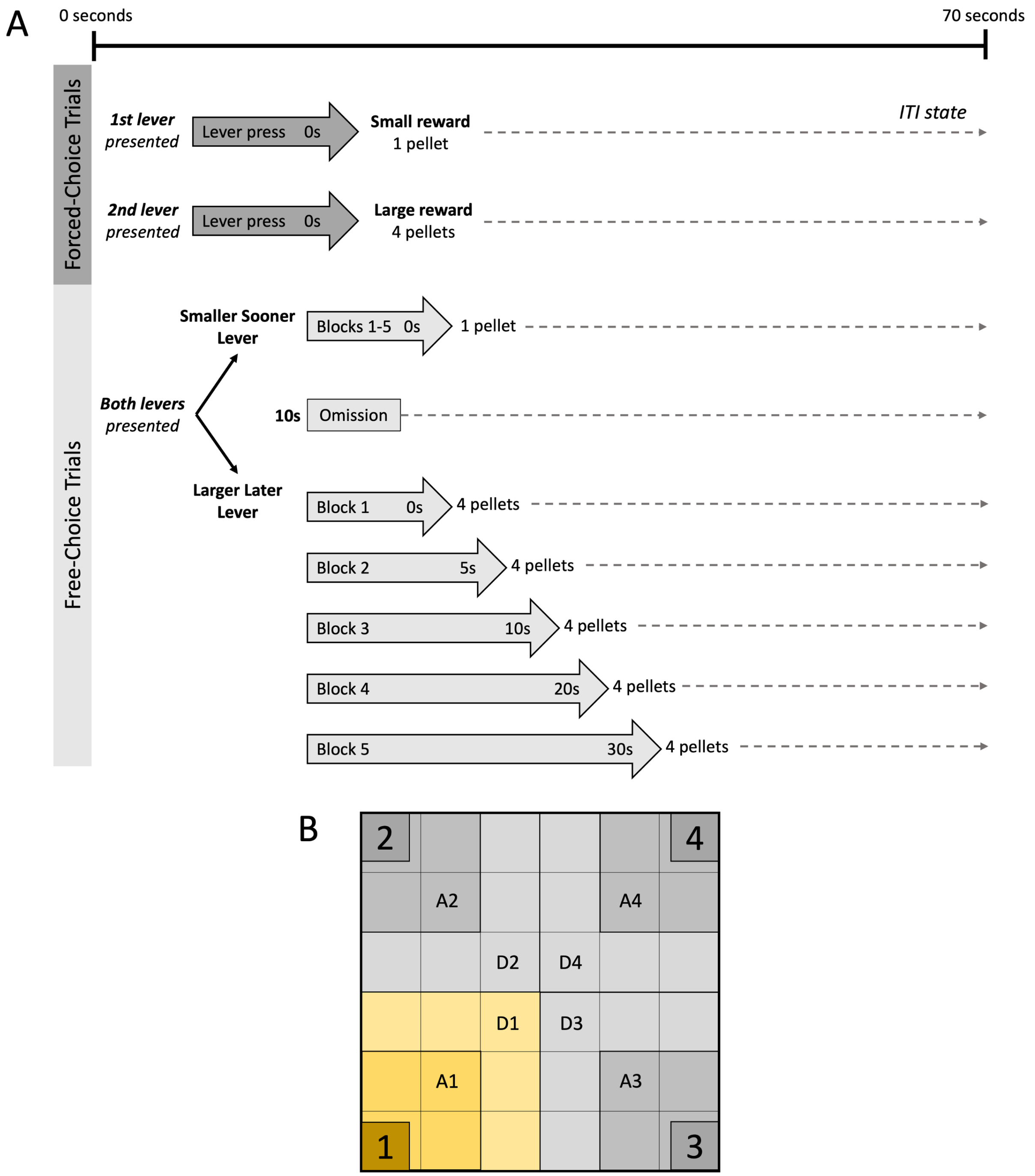
A
0 seconds
70 seconds
Forced-Choice Trials
1st lever presented
Lever press 0s
Small reward
1 pellet
ITI state
2nd lever presented
Lever press 0s
Large reward
4 pellets
Free-Choice Trials
Smaller Sooner Lever
Blocks 1-5 0s
1 pellet
Both levers presented
10s
Omission
Larger Later Lever
Block 1 0s
4 pellets
Block 2 5s
4 pellets
Block 3 10s
4 pellets
Block 4 20s
4 pellets
Block 5 30s
4 pellets
B
2
4
A2
A4
D2
D4
D1
D3
A1
A3
1
3

Figure 2.

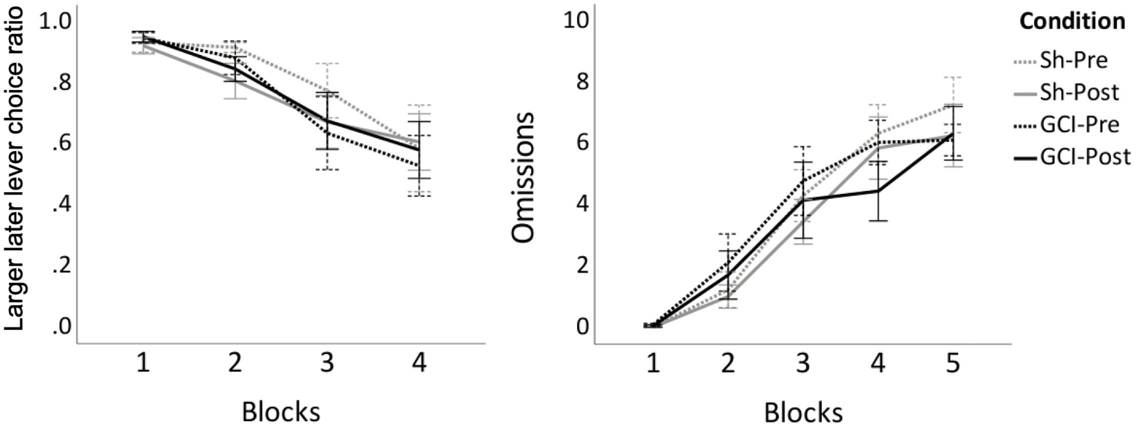
Larger later lever choice ratio
1.0
.8
.6
.4
.2
.0
1
2
3
4
Blocks
Omissions
10
8
6
4
2
0
1
2
3
4
5
Blocks
Condition
Sh-Pre
Sh-Post
GCI-Pre
GCI-Post

Figure 3.

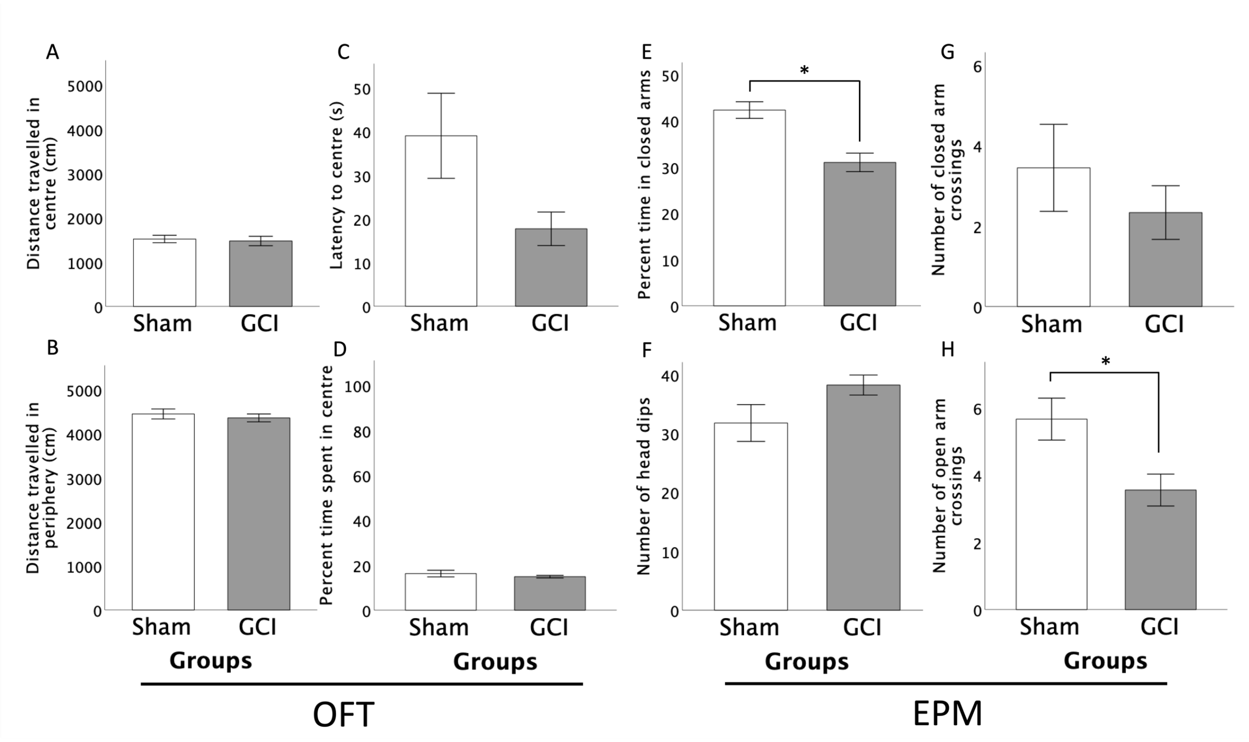
A
Distance travelled in centre (cm)
5000
4000
3000
2000
1000
0
Sham
GCI
B
Distance travelled in periphery (cm)
5000
4000
3000
2000
1000
0
Sham
GCI
Groups
C
Latency to centre (s)
50
40
30
20
10
0
Sham
GCI
D
Percent time spent in centre
100
80
60
40
20
0
Sham
GCI
Groups
OFT
E
Percent time in closed arms
*
50
40
30
20
10
0
Sham
GCI
F
Number of head dips
40
30
20
10
0
Sham
GCI
Groups
G
Number of closed arm crossings
6
4
2
0
Sham
GCI
H
Number of open arm crossings
*
6
4
2
0
Sham
GCI
Groups
EPM

Figure 4.

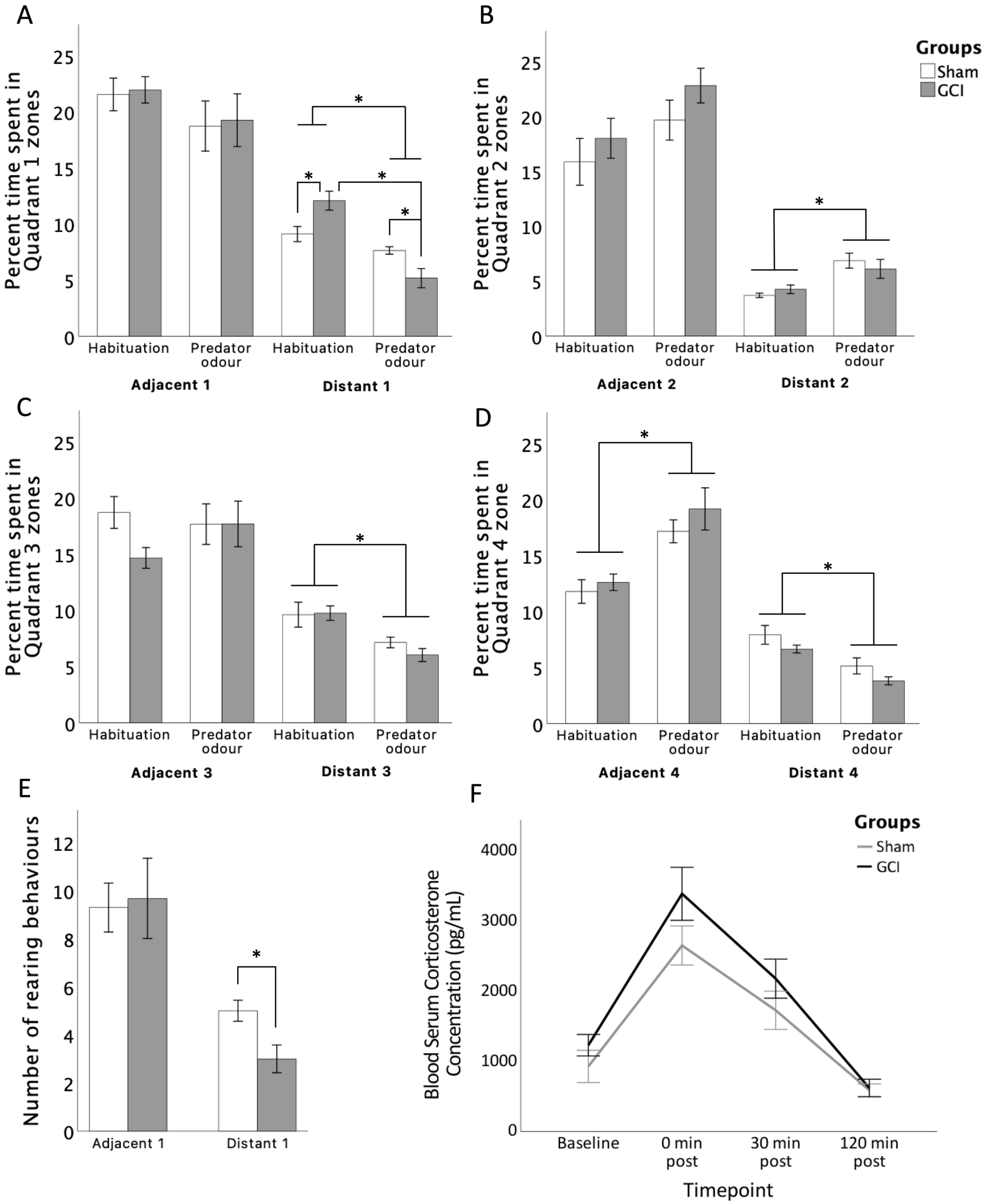
A
Percent time spent in Quadrant 1 zones
0
5
10
15
20
25
Habituation
Predator odour
Habituation
Predator odour
Adjacent 1
Distant 1
*
*
*
*
B
Percent time spent in Quadrant 2 zones
Groups
Sham
GCI
Habituation
Predator odour
Habituation
Predator odour
Adjacent 2
Distant 2
*
C
Percent time spent in Quadrant 3 zones
Habituation
Predator odour
Habituation
Predator odour
Adjacent 3
Distant 3
*
D
Percent time spent in Quadrant 4 zone
Habituation
Predator odour
Habituation
Predator odour
Adjacent 4
Distant 4
*
*
E
Number of rearing behaviours
0
2
4
6
8
10
12
Adjacent 1
Distant 1
*
F
Groups
Sham
GCI
Blood Serum Corticosterone Concentration (pg/mL)
0
1000
2000
3000
4000
Baseline
0 min post
30 min post
120 min post
Timepoint

Figure 5.

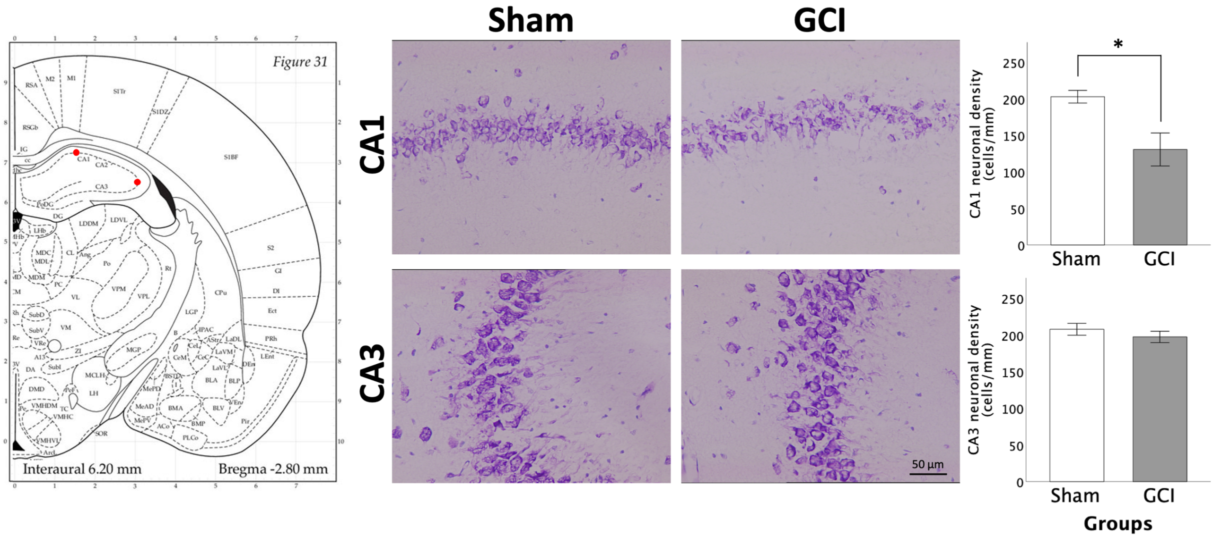
Figure 31
CA1
CA2
CA3
Interaural 6.20 mm
Bregma -2.80 mm
Sham
GCI
CA1
CA3
50 µm
*
CA1 neuronal density (cells/mm)
250
200
150
100
50
0
Sham
GCI
CA3 neuronal density (cells/mm)
250
200
150
100
50
0
Sham
GCI
Groups

Figure 6.

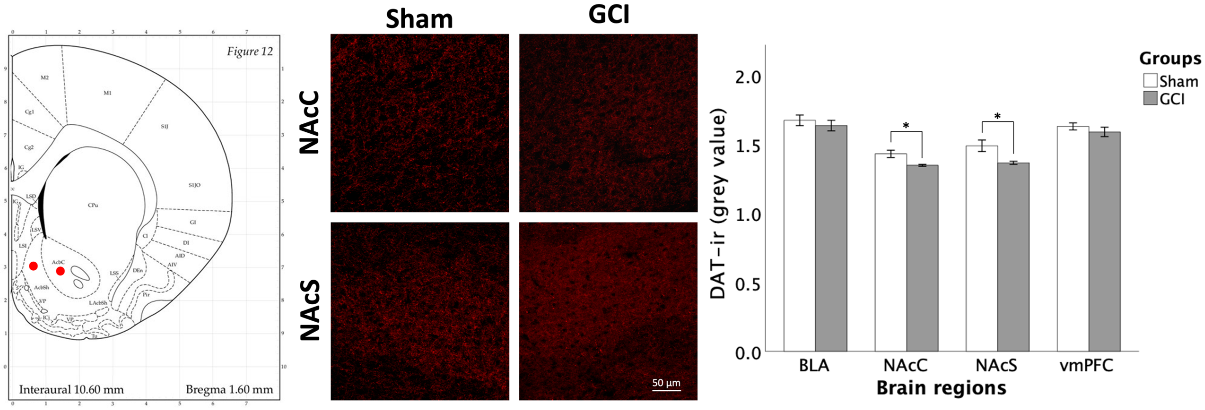
Figure 12
M2
M1
Cg1
Cg2
S1J
IG
S1JO
LSD
CPu
GI
LSV
Cl
DI
LSI
AcbC
AID
AIV
LSS
DEn
AcbSh
Pir
VP
LAcbSh
ICj
VP
Tu
Interaural 10.60 mm
Bregma 1.60 mm
Sham
GCI
NAcC
NAcS
50 µm
Groups
Sham
GCI
DAT-ir (grey value)
2.0
1.5
1.0
0.5
0.0
*
*
BLA
NAcC
NAcS
vmPFC
Brain regions

Figure 7.

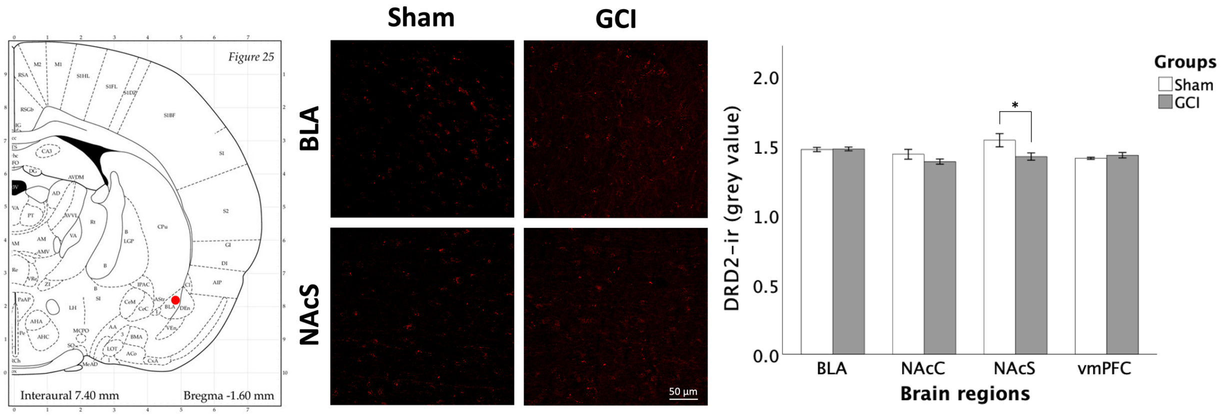
Figure 25
Interaural 7.40 mm
Bregma -1.60 mm
Sham
GCI
BLA
NAcS
50 µm
Groups
Sham
GCI
*
2.0
1.5
1.0
0.5
0.0
DRD2-ir (grey value)
BLA
NAcC
NAcS
vmPFC
Brain regions

Figure 8.

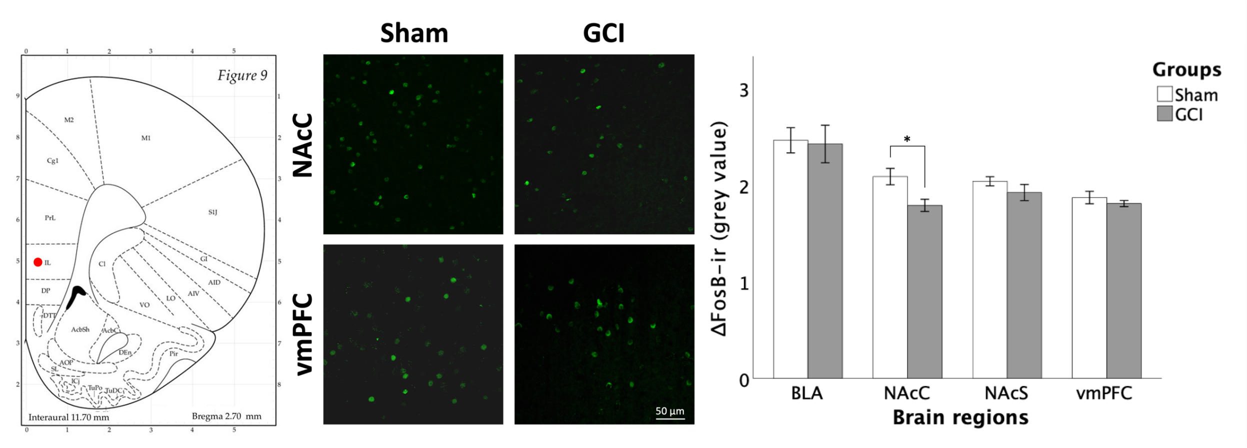
Figure 9
M2
M1
Cg1
PrL
IL
DP
DTT
S1J
GI
AID
AIV
Cl
LO
VO
AcbSh
AcbC
DEn
Pir
AOP
SL
ICj
TuPo
TuDC
Interaural 11.70 mm
Bregma 2.70 mm
Sham
GCI
NAcC
vmPFC
50 µm
ΔFosB-ir (grey value)
Groups
Sham
GCI
*
BLA
NAcC
NAcS
vmPFC
Brain regions
0
1
2
3

Figure 9.

Figure Captions

Figure 1. Experimental Timeline. Rats were acclimated for seven days prior to the start of food restriction (FR). Autoshaping (AS) and delay discounting (DD) occurred five consecutive days per week, for 9 and 24 days, respectively. The four-vessel occlusion (4VO) and sham (Sh) surgeries were performed when rats were 83-84 days old. Behavioural testing took place following 11 days of recovery and consisted of the Open Field Test (OFT), the Elevated-Plus Maze (EPM), and the Predator Odour Test (PO) with blood sampling (BS), after which FR was resumed. Rats completed six DD sessions and were perfused immediately after the last session. PND: Postnatal day.

Figure 2. Delay Discounting Task and Predator Odour Arena. (A) Delay discounting task: Each block consisted of two forced-choice trials, followed by ten free-choice trials. During the forced-choice trials, only one lever was presented at a time, and the corresponding food reward was dispensed if the lever was pressed within 10 s, followed by an intertrial interval (ITI), during which both levers were retracted. In the free-choice trials, both levers were presented. A lever press led to the delivery of the corresponding food reward (one or four sucrose pellets), after which the ITI was initiated for the remainder of the trial. The delays for large reward increased after each block (0, 5, 10, 20, and 30 s). A failure to press a lever within 10 s was considered an omission and led to an ITI. (B) Predator Odour Test arena: For analysis, each quadrant was divided into zones which were adjacent (A1-A4) and distant (D1-D4) to the clear plastic containers placed in each corner. Container #1 held the bobcat urine.

Figure 3. Delay Discounting Before and After Global Cerebral Ischemia. The figure shows the results of sham (Sh) and global cerebral ischemia (GCI) rats pre- and post-surgery for larger, later (LL) lever choice ratio (A) and number of omissions (B) during the 10 free-choice

trials of each block of the delay discounting task. The choice ratio for Block 5 was excluded due to a large number of omissions. The choice ratio for the LL lever significantly decreased over blocks ($p < 0.0005$), whereas omissions significantly increased over blocks ($p < 0.0005$). The values are presented as the means of last three preoperative sessions (Pre) and last three postoperative sessions (Post) for each group, ± S.E.M.

Figure 4. Effects of Global Cerebral Ischemia on Open Field Test and Elevated-Plus Maze Performance. The left side of the figure shows the distance travelled in the center (A) and periphery (B) of the Open Field Test (OFT), as well as the latency to the center (C) and percentage of time spent in the center zone (D). The right side of figure shows the percentage of time spent in the closed arms of the Elevated-Plus Maze (EPM) (E), the number of head dips (F), and the number of closed- (G) and open- (H) arm crossings. The values are presented as mean ± S.E.M. * indicates statistical significance at $p < 0.05$. GCI: Global cerebral ischemia.

Figure 5. Effects of Global Cerebral Ischemia on Predator Odour Test Performance and Circulatory Blood Corticosterone Levels. The bar graphs show the time spent in each zone (A-D) and the number of rearing behaviours in the Quadrant 1 (E). The line graph presents the blood serum corticosterone levels at baseline and at 0, 30, and 120 minutes after PO completion (F). The values are presented as mean ± S.E.M. * indicates statistical significance at $p < 0.05$. GCI: Global cerebral ischemia.

Figure 6. Thionine Neuronal Density in the CA1 and CA3 Regions of the Hippocampus. The figure shows representative photomicrographs of the CA1 and CA3 subregions of the hippocampus at 20× magnification (1 mm linear length). The histograms present the neuronal density in the CA1 and CA3 following sham surgery or global cerebral ischemia (GCI), assessed

in 14 µm thionine-stained coronal brain slices. The values are presented as mean ± S.E.M. * indicates statistical significance at $p < 0.05$.

Figure 7. Dopamine Receptor D_2 Fluorescence Immunohistochemistry Following Global Cerebral Ischemia. The figure shows representative fluorescence photomicrographs of dopamine receptor D_2 (DRD_2) expression at 20× magnification in the basolateral amygdala (BLA) and nucleus accumbens shell (NAcS) of rats having undergone sham surgery or global cerebral ischemia (GCI). The histogram presents the mean grey value for DRD_2 immunoreactivity in each brain region of interest. The values are presented as mean ± S.E.M. * indicates statistical significance at $p < 0.05$. NAcC: Nucleus accumbens core; vmPFC: Ventromedial prefrontal cortex.

Figure 8. Dopamine Transporter Fluorescence Immunohistochemistry Following Global Cerebral Ischemia. The figure shows representative fluorescence photomicrographs of dopamine transporter (DAT) expression at 20× magnification in the nucleus accumbens core (NAcC) and shell (NAcS) of rats having undergone sham surgery or global cerebral ischemia (GCI). The histogram presents the mean grey value for DAT immunoreactivity in each studied brain region. The values are presented as mean ± S.E.M. * indicates statistical significance at $p < 0.05$. BLA: Basolateral amygdala; vmPFC: Ventromedial prefrontal cortex.

Figure 9. ΔFos and FosB Fluorescence Immunohistochemistry Following Global Cerebral Ischemia. The figure shows representative fluorescence microphotographs of ΔFos and FosB expression at 20× magnification in the nucleus accumbens core (NAcC) and ventromedial prefrontal cortex (vmPFC) of rats having undergone sham surgery or global cerebral ischemia (GCI). The histogram presents the mean grey value for ΔFos and FosB immunoreactivity in each

studied brain region. The values are presented as mean ± S.E.M. * indicates statistical significance at $p < 0.05$. BLA: Basolateral amygdala; NAcS: Nucleus accumbens shell.